

**Title Page**

**Anti-cancer efficacy of simvastatin on prostate cancer cells and tumor xenografts is associated with inhibition of Akt and reduced PSA expression**

Samith T. Kochuparambil, Belal Al-Husein, Anna Goc, Sahar Soliman and Payaningal R. Somanath<sup>\*</sup>

Department of Medicine, Medical College of Georgia, Augusta, GA (STK); Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA (BA, AG, SS, PRS); VA Medical Center, Augusta, GA (STK, AG, PRS); Clinical Pharmacy, Medical College of Georgia, Augusta, GA (PRS)

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**Corresponding Author:** Payaningal R. Somanath Ph.D., Assistant Professor, Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, HM1200 Medical College of Georgia, Augusta, Georgia 30912, USA, Phone: 706-721-4250; Fax: 706-721-4250; E-mail: [sshenoy@mail.mcg.edu](mailto:sshenoy@mail.mcg.edu)

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**List of non-standard abbreviations:** BrDU, *5-Bromo-2-deoxyuridine*; FBS, Fetal bovine serum; DMSO, Dimethyl sulfoxide; PI3K, PI3 Kinase; PAGE, Polyacrylamide gel electrophoresis; TRAMP, Transgenic adenocarcinoma of the mouse prostate; LNCaP, Lymph node carcinoma of the prostate; RTK, Receptor tyrosine kinase; GSK, Glycogen synthase kinase; DMEM, Dulbecco's modified eagle medium; ELISA, Enzyme-Linked immunosorbent assay; BrDU, Bromodeoxyuridine; ACAT, Acyl-CoA cholesterol acyltransferase; EGF, Epidermal growth factor; PSA, Prostate specific antigen, SREBP-2, Sterol-responsive Element-binding Protein; PTEN, Phosphatase and tensin homolog; VCaP, Vertebral cancer of the prostate; myrAkt, Myristoylated Akt.

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## Abstract

Prostate cancer is the second leading cause for cancer-associated death among men in the United States. More recently, there has been a renewed interest in the potential therapeutic benefits of statins for cancer. Simvastatin, a widely used generic drug for preventing cardiovascular events, is well known for its effects on cellular proliferation and inflammation, two key processes that also determine the rate of tumor growth. While a growing body of evidence suggests that statins have the potential to reduce the risk of many cancers, there are discrepancies over the pro- and anti-cancer effects of statins on cancers. In the current study, we sought to investigate the effects of simvastatin on the Akt pathway in prostate cancer cells with respect to the regulation of various cell functions *in vitro* and tumor growth *in vivo*. Time- and dose-effects of simvastatin on LNCaP (androgen-dependent) and PC3 (androgen-independent) cells indicated that treatment with as low as 25  $\mu$ M simvastatin was sufficient to inhibit serum-stimulated Akt activity. Akin to this, treatment with simvastatin significantly inhibited serum-induced cell migration, invasion, colony formation and proliferation. Simvastatin-mediated effects on colony formation was rescued by Adenovirus-mediated expression of constitutively active Akt (myristoylated Akt) in PC3 cell lines. A PC3 xenograft model performed in nude mice exhibited reduced tumor growth with simvastatin treatment associated with decreased Akt activity and reduced PSA levels. Our findings demonstrate the therapeutic benefits of simvastatin for prostate cancer and suggest a link between simvastatin, regulation of Akt activity and PSA expression in prostate tumors.

**Introduction**

Statins [3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors], the second most prescribed drugs following analgesics, are also considered to be among the safest drugs. Despite the long-term nature of the treatments, use of statins have not been shown to inflict any serious side effects, but have shown to yield additional benefits, particularly in the management of cancer. A recent meta-analysis performed using the information retrieved from QResearch database indicated that use of statins is not associated with risk for diseases such as Parkinson's disease, rheumatoid arthritis, venous thromboembolism, dementia, osteoporosis or cancers of the gastric, colon, lung, melanoma, renal, breast or prostate (Hippisley-Cox and Coupland, 2010). However, moderate increases in the risk for liver or kidney dysfunction, myopathy and cataract were associated with statin use. In humans, reports on the effects of statins on cancer have yielded varied results ranging from increased risk, to no net effect, to decreased risk of cancer (Jakobisiak and Golab, 2010). Many believe that these differences could be either due to variations in the doses used for the treatment of many cardiovascular conditions (Elewa et al., 2010) or due to the hydrophobic nature of some, but not all statins (Murtola et al., 2008). A number of pre-clinical studies have implicated that statins can modulate the efficacy of many anti-tumor therapeutic modalities (Jakobisiak and Golab, 2010).

Hydrophobic statins (simvastatin, lovastatin and fluvastatin) have been shown to inhibit cancer growth. In cell based experiments *in vitro* and in experimental animal models, these statins have displayed inhibitory effects on many cancers, including head and neck, prostate, lung, breast, colon, pancreas, skin (melanoma), renal cell, bladder, liver and multiple myeloma (Jakobisiak and Golab, 2010). Information from patient-based studies on the effects of statins on prostate cancer has only started to trickle down, and the reports have been highly contradictory. Initial case-controlled study showed that use of statins is associated with 50% reduction in the risk of prostate cancer (Shannon et al., 2005), which was supported by another study on atorvastatin and prostate cancer clinical outcome (Moyad et al., 2005). However, a study performed in Finnish population on statin use and incidence of prostate cancer did not

show any significant correlation between them (Haukka et al., 2010). In contrast, another study performed in Finnish population showed decreased overall relative risk of prostate cancer and reduced serum PSA levels among current statin users with proportional changes corresponding to the amount and duration of use (Murtola et al., 2010). Also, a number of reports published in the recent months demonstrate that statin use is associated with decreased chances of undergoing prostate biopsy and receiving a Gleason score of 7 or greater (Breau et al., 2010; Katz et al., 2010). Reduction in serum-PSA and total testosterone levels among statin users compared to non-statin users has also been reported by other groups (Mondul et al., 2010). A very recent study focused on characterizing the association between statin use and PSA recurrence after prostatectomy demonstrated a dose dependent reduction in the risk of biochemical recurrence (Hamilton et al., 2010). Although controversial, together these studies suggest that long-term statin use can prevent or delay prostate cancer onset in men.

Simvastatin, a generic drug, is the most widely used statin for the prevention and treatment of cardiovascular events. In a recent study that established a strong correlation of statin use with decreased serum-PSA levels and risk of biochemical recurrence of prostate cancer after radical prostatectomy, simvastatin was used by most of the subjects (171 out of 236) involved in the study (Hamilton et al., 2010). In the current study, we focused on studying the effects of simvastatin on prostate cancer cell functions *in vitro*, growth and prostate cancer xenograft in nude mice *in vivo* and characterizing the major molecular mechanisms regulating the process. Our findings indicated that simvastatin has direct effects on prostate cancer cells in the regulation of multiple cellular functions such as cell migration, invasion, proliferation, cell survival/apoptosis and colony formation *in vitro* as well as growth of prostate tumor xenograft *in vivo*. Simvastatin treatment inhibited Akt activity in prostate cancer cells in a dose- and time-dependent manner. More importantly, our results indicated that prostate cancer cells stably expressing constitutively active Akt (myr-Akt) were resistant to simvastatin-mediated inhibition of prostate cancer cell functions. We conclude that simvastatin can be developed as a potential therapeutic agent for the management of prostate cancer. In addition, changes in Akt phosphorylation, in addition to reduced

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serum-PSA levels, can be an important surrogate marker to determine the patient response to simvastatin therapy.

## Methods

**Cell lines, reagents, and antibodies:** Human PC3 and LNCaP cell lines were obtained from ATCC (Manassas, VA). All cell lines were maintained in DMEM (HyClone) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO<sub>2</sub> atmosphere at 37°C. DilC<sub>12</sub> fluorescence dye was purchased from BD Biosciences (San Jose, CA). Primary antibodies such as: anti-Akt, anti-phospho-Akt<sup>S473</sup> and anti-phospho-GSK3<sup>S9/21</sup> were purchased from Cell Signaling (Boston, MA). Primary antibodies against β-actin were purchased from Sigma (St Louis, MO) and anti-PSA antibody was purchased from Pierce Biotechnology Inc. (Rockford, IL). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from BioRad (Hercules, CA). Docetaxel and simvastatin were purchased from Sigma (St Louis, MO). Simvastatin was activated in the lab using the manufacturer's instructions.

**Migration assay:** PC3 cells were grown to confluence, and a scratch was made in the monolayer followed by treatment with simvastatin (control buffer, 25, 50 and 100 µM). Scratch recovery was determined at 16h and 24h. Microscopic pictures were analyzed using Image J software and recovery was calculated using the equation:  $[100X (1-T_f/T_0)] \%$ , where T<sub>f</sub> is the area at the end-point and T<sub>0</sub> is the area at the time zero. The data are presented as mean ± SD.

**Invasion assay:** The invasion of PC3 cell lines was measured using BD BioCoat Tumor Invasion Assay Kit (BD Biosciences) coated with BD Matrigel Matrix according to the manufacturer's protocol. PC3 cells were labeled with BD DilC<sub>12</sub> fluorescence dye and seeded onto the upper chamber of a 96-well Transwell plate at a density of 1X10<sup>4</sup> cells per well in 400 µL medium. DMEM containing 10% FBS was then added to the lower chamber. After 24h, the cells were treated with control buffer, 25 and 100 µM of simvastatin in DMEM medium. The fluorescence from the stained cells was measured after 12h and 24h on an ELISA plate reader at 549/565 nm (Ex/Em). The data are presented as mean ± SD.

**Cell doubling time assessment:** In each experiment, cell doubling time was determined according to direct cell count and in consideration of logarithmic growth of cancer cells ([www.doubling-time.com](http://www.doubling-time.com)). For direct cell count, approximately 100 cells per well were seeded in 400  $\mu$ L medium on a 48-well plate, in quadruplicates. After 24h, medium was replaced and cells were counted. The cells were treated with control buffer, 25  $\mu$ M and 100  $\mu$ M of simvastatin in DMEM. At 24h, cell counts were repeated. The cell doubling time was calculated as the mean  $\pm$  SD.

**Trypan blue viability assessment:** In the trypan blue method, cells were grown to confluence in DMEM medium with 10% FBS. The cells were treated with simvastatin 25  $\mu$ M and 100  $\mu$ M in DMEM. After 24h, cells were collected and re-suspended in PBS with 0.4% Trypan blue solution. Total cells and Trypan blue stained (i.e., non-viable) cells were counted and percentage of non-viable cells was calculated.

**Apoptosis assessment:** Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA<sup>PLUS</sup> Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 cell lines were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well. After 24h, the cells were incubated in DMEM containing 25 and 100  $\mu$ M simvastatin for 16h. Control cells were treated with 0.1% DMSO (vehicle control). Cells were lysed, centrifuged (200g for 10min) and the collected supernatant was subjected to ELISA. The absorbance was measured at 405nm (reference wavelength at 492nm). The data are presented as mean  $\pm$  SD.

**Cell proliferation assay:** The effect of simvastatin on proliferation of PC3 cell lines was determined using the nonradioactive BrDU-based cell proliferation assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Briefly, PC3 cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells per well. After 24h, the cells were incubated in DMEM containing 25 and 100  $\mu$ M simvastatin for 16h. Control cells were treated with 0.1% DMSO (vehicle control). Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were subjected to a 5-



bromo-2-deoxyuridine assay using the BrDU Labeling and Detection Kit III (Roche Applied Science), according to the manufacturer's protocol. BrDU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690nm on an ELISA plate reader. The data are presented as mean  $\pm$  SD

**Colony formation assay:** Colony formation assay was performed using standard protocol (3872166). In this approach, PC3 cells were cultured on 6-well plates till monolayer was reached. The wells were treated with DMEM containing 25 and 100  $\mu$ M simvastatin. Control cells were grown in DMEM media. At 5 days post treatment, each of the wells was counted for the number of colonies and simvastatin-treated wells were compared to the vehicle treated control. Plates were fixed using 2% paraformaldehyde, briefly stained with crystal violet and counted visually or using Image J software. The data are presented as mean  $\pm$  SD.

**Western analysis:** Cells/Tissue PC3 and LNCaP cell lines were cultured to reach a monolayer in DMEM in 6 well plates. The wells were treated with DMEM containing 25  $\mu$ M and 100  $\mu$ M simvastatin. Control cells were grown in DMEM alone. Whole cell lysates were prepared using lysis buffer [50 mM Tris-HCl (pH=7.4), 1 % TritonX-100, 150mM NaCl, 1mM EDTA, 2mM Na<sub>3</sub>VO<sub>4</sub>, and 1X Complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. Tissue obtained from mice was snap frozen with liquid nitrogen. Tissue was pulverized with mortar and piston. Tissue lysates were prepared using lysis buffer. The protein concentration was measured by the D<sub>L</sub> protein assay (Bio-Rad, Hercules, CA). Western analyses were performed using standard Laemmli's method as done previously (17562714).

***In vivo* nude mouse tumor xenograft model:** All animal procedures listed in the manuscript were performed as per the protocol approved by the IACUC at the Charlie Norwood VA medical Center, Augusta (protocol # 09-07-011 dated July 10, 2009). PC 3 cells were grown to confluence in 250cc flasks. Cells were re-suspended in PBS to a concentration of  $1 \times 10^6$ /ml. 1ml of cell suspension was

injected subcutaneously (SC) in 6-8 weeks old nude mice (Athymic nude mice, Harlan Laboratories, Indianapolis, IN). Mice were divided into two groups. The groups were subjected to intraperitoneal (IP) injections of simvastatin at the dose of 2mg/kg body weight/every 12h (or 24h in a second set of experiments) for 2 weeks. The respective controls were injected IP with 0.9% saline every 12 or 24h. Tumor sizes were measured on day 7 and day 11 respectively. Mice were sacrificed on day 11 and tumors were dissected and weighed.

**Statistical Analysis:** Mean activities were calculated from 3-5 independent experiments done at least in triplicates. The Student's two-tailed t test was used to determine significant differences between treatment and control values.

## Results

### **Simvastatin treatment inhibits phosphorylation and activity of Akt in prostate cancer cells in a dose- and time-dependent manner.**

Since Akt is central to many signaling pathways and is a known mediator of many functions of cancer cells, we sought to determine whether treatment with simvastatin will have any effect on phosphorylation and activity of Akt. Our results indicate that treatment with simvastatin had a robust effect on inhibition of Akt phosphorylation in LNCaP and PC3 cells. Effects were seen from 25  $\mu$ M and maximum inhibition was observed when 75  $\mu$ M simvastatin was used (Figure 1A). A time-course study of simvastatin effects on LNCaP cells indicated that while 100  $\mu$ M simvastatin inhibited phosphorylation of Akt in 4h, a maximum reduction in Akt phosphorylation by 25  $\mu$ M simvastatin was observed at 16h (Figure 1B). Similar effects of simvastatin were observed in metastatic human PC3 prostate cancer cell lines (Figure 1C). In order to determine whether reduction in phosphorylated Akt levels in PC3 and LNCaP cells had any effect on its activity, we determined levels of phosphorylated GSK3, a well-known substrate of Akt. Our analyses indicated that, similar to its effects on Akt phosphorylation, simvastatin inhibited phosphorylation of GSK3 in PC3 and LNCaP cells in a time- and dose-dependent manner. Together, our results indicate that simvastatin inhibits Akt activity in prostate cancer cells.

### **Simvastatin inhibits migration and invasion of PC3 cells.**

Since simvastatin treatment inhibited Akt activity in prostate cancer cells, we determined whether simvastatin has any effect on prostate cancer cell migration and invasion. Our data indicated that treatment with 25  $\mu$ M simvastatin on PC3 cells maintained in serum containing medium significantly impaired their ability to migrate (Figures 2A and 2B) as analyzed at 16h ( $p < 0.04$  for 25  $\mu$ M and  $p < 0.05$  for 100  $\mu$ M) and 24h ( $p < 0.0002$  for 25  $\mu$ M and  $p < 0.001$  for 100  $\mu$ M) post treatment (~2 and 3 fold decrease). At 24h, treatment with 100  $\mu$ M simvastatin almost completely inhibited (~90% inhibition compared to control) PC3 cell migration. Similarly, treatment of PC3 cells with 25  $\mu$ M simvastatin

significantly inhibited invasion in response to EGF ( $p < 0.005$  for 12h and  $p < 0.01$  for 24h) and 10% FBS ( $p < 0.03$  for 12h and  $p < 0.0002$  for 24h) (Figure 2C). At 12h and 24h post treatment with 25  $\mu\text{M}$  simvastatin, we observed ~12% and ~15% inhibition in EGF-stimulated PC3 cell invasion, respectively. Treatment with 100  $\mu\text{M}$  simvastatin further enhanced the inhibition of PC3 cell invasion up to 26% compared to the EGF-treated control ( $p < 0.01$ ). Effects of simvastatin on EGF-stimulated prostate cancer cell invasion was significantly higher compared to cells that were maintained in 10% FBS containing medium (Figure 2D). In the presence of 10% FBS, 25  $\mu\text{M}$  simvastatin treatment resulted only in a modest inhibition of PC3 cell invasion (1.5-3% inhibition, compared to control). Inhibition of PC3 cell invasion was slightly higher upon treatment with 100  $\mu\text{M}$  simvastatin, compared to the control (3.5-7.5%) ( $p < 0.005$  for 12h and  $p < 0.003$  for 24h). Overall, our data indicates that simvastatin treatment significantly inhibits PC3 cell migration and invasion.

#### **Simvastatin inhibits proliferation and induces apoptosis of PC3 cells.**

We next determined whether simvastatin treatment has any effect on prostate cancer cell proliferation. Normally, metastatic PC3 cells have a doubling time of 10-14 hours. Our study showed that treatment with 25  $\mu\text{M}$  simvastatin resulted in a 6-8 fold increase in doubling time for PC3 cells ( $p < 0.0003$ ) (Figure 3A). This effect was even greater when cells were treated with 100  $\mu\text{M}$  simvastatin reaching well above 300h (~20 fold) ( $p < 0.001$ ) (Figure 3A). Data from the proliferation assay revealed that treatment with simvastatin resulted in significant inhibition of PC3 cell proliferation by 25-35% for 25  $\mu\text{M}$  ( $p < 0.0001$ ) and 100  $\mu\text{M}$  ( $p < 0.00001$ ) simvastatin, respectively (Figure 3B). The effect of 25  $\mu\text{M}$  simvastatin on proliferation was similar to the effects of a low dose treatment with Docetaxel/Taxotere (10 nM) ( $p < 0.0001$ ), a currently used chemotherapy drug for the management of prostate cancer in patients (Figure 3B). Thus, our data indicates that simvastatin significantly inhibits prostate cancer cell proliferation *in vitro*.

Agents that can induce apoptosis in cancer cells have been an excellent choice for cancer treatment. Our study indicated that treatment with 25  $\mu$ M simvastatin increased cell death by ~30% over a 12h period in PC3 cells ( $p<0.0001$ ) (Figure 4A). This effect was further enhanced by 100  $\mu$ M simvastatin, which exhibited more than 2 fold increase in cell death ( $p<0.0001$ ). Similarly, treatment of PC3 cells with 25 and 100  $\mu$ M simvastatin resulted in 1.5 fold ( $p<0.00005$ ) and 1.75 fold ( $p<0.00005$ ) increases in apoptosis, respectively (Figure 4B). Our studies demonstrate that simvastatin induces apoptosis and cell death in prostate cancer cells.

**Simvastatin-inhibited colony formation by PC3 cells can be partially rescued by adenovirus-mediated expression of constitutively active Akt.**

An important feature of the tumor cells is that they are resistant to contact inhibition and form colonies or foci. We determined whether inhibition of Akt activity by simvastatin has any effect on colony formation by prostate cancer cell lines. Our experiments show that PC3 cell lines develop colonies once they are allowed to form a monolayer and left for additional 5 days. Treatment with 25  $\mu$ M simvastatin significantly inhibited (~25%) colony formation by PC3 cells ( $p<0.04$ ) (Figure 5A), suggesting that simvastatin inhibits prostate cancer foci formation, possibly via Akt inhibition. Unlike the effects of simvastatin on proliferation, its effects on colony formation were lower compared to the effects of low dose Docetaxel (10 nM), which inhibited colony formation by PC3 cells by ~60% ( $p<0.01$ ) (Figure 5B). In order to investigate whether inhibition of colony formation by simvastatin was mediated through Akt inhibition, we next determined if prostate cancer cells expressing constitutively active Akt (myrAkt) can resist inhibition of colony formation by simvastatin. Our initial studies comparing PC3 cells expressing GFP (control) with those expressing myrAkt (both the transfections were performed via adeno-virus infections) showed that cells expressing myrAkt exhibit a significantly higher number of colonies compared to cells expressing GFP ( $p<0.03$ ) (Figure 5C). As we hypothesized, our data indicated that PC3 cells expressing myrAkt were partially resistant to simvastatin-mediated inhibition of colony formation by PC3 cells. There was no significant difference between simvastatin-treated and non-treated

PC3 cells expressing ad-myrAkt ( $p<0.5$ ) (Figure 5D). In sum, these results demonstrate that treatment with simvastatin inhibits colony formation by PC3 cells and that the Akt pathway is one of the major pathways modulated by simvastatin in prostate cancer cells.

**Simvastatin inhibited growth of PC3 tumor xenograft in male nude mice is associated with an inhibition of Akt activity and a reduced expression of prostate specific antigen (PSA).**

Inhibition of colony formation by simvastatin provided the essential message that treatment with simvastatin may be an effective strategy to either prevent or manage prostate cancer *in vivo*. In order to test this, we performed tumor xenograft study in nude mice. In an initial study, PC3 cells were administered in nude mice and were treated with simvastatin (2mg/kg body wt/day), administered intra-peritoneally as performed previously (Shinozaki et al., 2010). Analyses of tumor size on a daily basis for 14 days and the tumor weight on day 14 after tumor cell injections were made. Data did not show a significant difference in tumor size on any day except day 14 ( $p<0.04$ ) (Figure 6A). However, no significant changes in tumor weight on day 14 ( $p<0.9$ ) between control and simvastatin-treated mice (Figure 6B) were observed. Next, we also determined if simvastatin would affect the growth rate of prostate tumors once they have already grown. Hence, tumor sizes measured on days 7 and 11 were used to determine the change in tumor growth in simvastatin-treated mice compared to saline control. Mice treated with simvastatin did not exhibit any differences on changes in tumor size between days 7 and 11 ( $p<0.2$ ) (Figure 6C and 6D) or between days 11 and 14 (data not shown).

Although the effect of simvastatin on the growth of tumor xenografts was not significant compared to saline administered controls, a trend towards reduced growth of tumor xenografts in simvastatin-treated mice compared to control mice was apparent. Hence, in the next step, we modified the protocol to study the effects of simvastatin in mice based on its dose and frequency of administration. The time of simvastatin administration was increased to twice a day (2mg/kg body wt/12h). These changes showed significant differences in prostate tumor growth between saline and simvastatin administered

mice (Figure 6). Overall tumor weight determined on day 14 post-tumor injection showed reduced growth of prostate tumor xenograft in simvastatin administered mice compared to saline control ( $p<0.03$ ) (Figure 6E). A significant reduction in tumor size was also observed in simvastatin-treated mice compared to saline control ( $p<0.03$ ) (Figure 6F). Next, we sought to analyze the percentage change in tumor growth between day 7 and day 11 post-tumor injection. Our data indicated that simvastatin inhibited growth of tumors from day 7 to day 11 when compared to its original size on day 7 ( $p<0.02$ ) (Figures 6F, 6G and 7A).

In order to determine whether the effect of simvastatin on the growth of PC3 tumor xenograft involves inhibition of Akt and/or changes in the expression levels of prostate specific antigen (PSA), we prepared tumor lysates and subjected for western analyses using antibodies specific for phospho-Akt and PSA. Our data indicated that simvastatin treatment in PC3 cells resulted in a significant reduction in phospho-Akt (~70% reduction) and PSA levels (~95% reduction) ( $p<0.0001$  and  $p<0.002$ , respectively) (Figure 7B and 7C). Overall, our studies on the effects of simvastatin on tumor xenograft in male nude mice demonstrates that simvastatin inhibit prostate tumor growth *in vivo* involving inhibition of Akt activity and a reduction in PSA expression.

## Discussion

Although controversial, many recent analyses of patient samples conducted by different groups have revealed the potential benefits of statins in the management of prostate cancer (Jakobisiak and Golab, 2010). In the current study, we report the potential benefits of simvastatin in the management of prostate cancer. In LNCaP and PC3 prostate cancer cell lines, simvastatin exhibited a dose- and time-dependent inhibition of Akt activity. Simvastatin treatment resulted in significant inhibition of cell migration, invasion, survival, doubling time, proliferation and colony formation as well as enhanced apoptosis in PC3 cells. The effect of simvastatin on colony formation was partially rescued in PC3 cells stably expressing constitutively active Akt. Intra-peritoneal administration of simvastatin in nude mice bearing PC3 tumor xenografts exhibited significant reduction in tumor size and weight associated with a reduction in PSA expression, compared to saline administered controls. Apart from this, we also observed significant reduction in the rate of tumor growth (from day 7 to day 11) in simvastatin-treated mice, compared to control. In sum, our data clearly demonstrates the ability of simvastatin to inhibit pro-tumorigenic functions of prostate cancer cells, to induce apoptosis and to inhibit tumor growth *in vivo*.

A number of characteristic effects of statins on cells provide the necessary clues for its potential benefits in cancer therapy. First, statins inhibit synthesis of mevalonate, which is necessary for the synthesis of isoprenoid compounds. Isoprenoid compounds are the precursors of cholesterol, licocholic acid and ubiquinone and are the substrates for post-translational modifications of many proteins (Liao and Laufs, 2005). Second, statins are known to inhibit proliferation of smooth muscle cells in the vasculature leading to primary and secondary prevention of cardiovascular events (Porter et al., 2002). In addition, they are known to induce apoptosis in smooth muscle cells (Porter et al., 2002) and many cancer cell types (Jakobisiak and Golab, 2010). These properties of statins can be very promising for their prospective use in inhibiting proliferation and survival of cancer cells. The dose at which statins enhance Akt activation and survival in endothelial cells (Laufs et al., 1998; Rikitake et al., 2001) is the same dose that inhibits Akt activity, cell proliferation and induces apoptosis in malignant smooth muscle cells in atherosclerotic



lesions (Guijarro et al., 1998) and cancer cells. This property of statins will be extremely important in avoiding side effects when statins are used for cancer therapy. An earlier study performed in hormone-responsive LNCaP cells showed that lovastatin specifically activated caspase-7 via enhanced expression of caspase-7 mRNA (Marcelli et al., 1998), which was prevented by pre-treatment with mevalonate. Our results further support the existing hypothesis that statin can be developed into a potential therapeutic drug for the long-term management of prostate cancer without inflicting any major side-effects.

Molecular mechanisms regulating statin-mediated responses in cancer cells have been a recent focus of investigation. Cholesterol lowering effects of statins are believed to be a very important factor in the regulation of prostate cancer cell functions. Androgens are known to mediate cholesterol metabolism in LNCaP cells involving Acyl-CoA cholesterol acyltransferase (ACAT) facilitating tumor progression (Locke et al., 2008). Previous studies have shown that prostate cancer cells lack sterol mediated feedback regulation of sterol regulatory element binding protein 2 (SREBP-2) in LNCaP and PC3 cells, a transcription factor regulating cholesterol homeostasis (Krycer et al., 2009). Samples collected from prostate cancer patients have revealed accumulation of cholesterol (Swyer, 1942). Cholesterol rich lipid rafts have been implicated in tumor progression and metastasis (Di Vizio et al., 2008). Cholesterol depleting agents are known to induce apoptosis via decreased production of cholesterol-rich lipid rafts in normal prostatic epithelium, human epidermoid carcinoma (A431) and breast cancer (MCF-7 and MDA-MB-231) cell lines (Li et al., 2006). At the same time, products of the mevalonate pathway also include dolichol, ubiquinol and isoprenoids such as farnesol and geranylgeraniol, which serve as lipid anchoring units for a number of signaling molecules such as small GTPases, Ras and Rho. These are known to mediate oncogenic transformations (Karreth and Tuveson, 2009) and might account for the non-cholesterol-mediated regulation of prostate cancer by statins.

Akt (protein kinase B), a serine-threonine kinase, is central to multiple pro-survival and anti-apoptotic cellular pathways (Somanath et al., 2006). Akt has been shown to be among the most frequently activated signaling molecule in cancers (Engelman, 2009), and activation of the PI3 kinase-Akt pathway

due to PTEN deficiency is a very common cause of prostate cancer (Li et al., 1997; Blanco-Aparicio et al., 2007). Enhanced apoptosis in response to cholesterol lowering drugs on prostatic epithelial cells as well as breast cancer and human epidermoid carcinoma cell lines was reported to be due to inhibition of pro-survival kinase Akt, reduced expression of anti-apoptotic molecule Bcl-xL and activation of pro-apoptotic caspase-3 dependent pathway (Li et al., 2006). Reconstituting rafts by the addition of cholesterol restored Akt activity resulting in inhibition of apoptosis. A very recent study performed in a different cancer type supports these findings and reports that simvastatin induces apoptosis, inhibits Akt phosphorylation and Bcl-xL expression in breast cancer cells via inhibition of NF $\kappa$ B, de-repression of PTEN and subsequent inhibition of PI3 kinase (Ghosh-Choudhury et al., 2010). Statins, in general, have also shown to inhibit Akt-mTOR signaling in p53-deficient hepatocellular carcinoma (Roudier et al., 2006). A previous study performed on PC3 and LNCaP cell lines shows that simvastatin, fluvastatin and lovastatin have profound effects on inducing a cell cycle arrest at G1 phase via inhibition of cyclin E/cdk2 kinase (Sivaprasad et al., 2006), possibly via inhibition of Akt (Murtola et al., 2008). Our study indicated that simvastatin inhibits Akt activity in LNCaP and PC3 cells in a dose- and time-dependent manner. However, until today, a causal relationship between decreased Akt activity and reduced tumor growth by any statins in any type of cancer is not established. Our finding that PC3 cells expressing myrAkt (constitutively active) is resistant to the effects of simvastatin on colony formation demonstrates a causative relationship between inhibition of Akt activity and impaired prostate cancer cell function. Furthermore, tumor xenografts collected from nude mice treated with simvastatin exhibited significant reduction in phosphorylated Akt levels associated with its reduced tumor size and weight, compared to saline treated mice. Together, our results indicate that there is a causal relationship between Akt inhibition and inhibition of tumor growth by simvastatin.

A number of recent meta-analyses from medical databases and epidemiological studies indicate the effect of statins in reducing serum PSA levels (Hamilton et al., 2010; Murtola et al., 2010). Among them, a very recent survey show that decreases in PSA levels are correlative in subjects who are on statin

treatment and might influence the risk assessment for prostate cancer (Chang et al., 2010). Another recent study indicates that statins have the ability to reduce expression of PSA mRNA via inhibition of androgen receptor (AR) protein expression in hormone responsive LNCaP cells (Yokomizo et al., 2010). Our results demonstrate that the effect of statins on PSA expression is not just correlative, but is a true reflection of the ability of statins to inhibit prostate cancer growth. Further, our observations extend this information and demonstrate that effects of statins are not limited to the hormone responsive stage of prostate cancer. Metastatic and hormone-insensitive PC3 cell tumor xenografts also exhibited reduced expression of PSA levels upon simvastatin treatment compared to saline treated controls, demonstrating the potential benefits of simvastatin in the management of prostate cancer. However, an important concern in our study is the dose at which simvastatin is found to exert effects on prostate cancer cells. A proper conversion of the therapeutic dose to the working concentration at a cellular level is not well-defined for statins in the existing literature. In our study, we utilized 20-50 times the prescribed therapeutic dose of simvastatin. This concentration of simvastatin has also been shown by others to be the right dose to work at a cellular level (Habib et al., 2007), and appears to be the dose necessary to inhibit isoprenylation of the proteins in cultured cells (Finder et al., 1997; Grosser et al., 2004). However, we have also shown that at doses very close to therapeutic concentrations (less than 10 times), simvastatin inhibits growth of PC3 tumor xenograft *in vivo*. Moreover, it should be noted that simvastatin effects that we studied on prostate cancer cells is on a short-term basis. At very low doses, close to therapeutic concentrations, simvastatin has been shown to enhance the inhibitory effects of acetylsalicylic acid and rosiglitazone on proliferation of normal prostatic epithelial cells and LNCaP and VCaP prostate cancer cells (Murtola et al., 2009). In summary, we show that treatment of prostate cancer cells with simvastatin significantly inhibit Akt activity, prostate cancer cell functions *in vitro* and tumor growth *in vivo* associated with a significant reduction in PSA expression. Our results suggest that long-term simvastatin medication may have beneficial effects in the management of prostate cancer.

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**Authorship contributions**

*Participated in research design:* STA, BA, PRS

*Conducted experiments:* STA, BA, AG, SS, PRS

*Performed data analysis:* STA, BA, AG, SS, PRS

*Wrote or contributed to the writing and review of the manuscript:* STA, BA, PRS

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**Footnotes:**

Samith T Kochuparambil and Belal Al-Husein contributed equally to this manuscript.

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**Legends for Figures:**

**Figure 1: Simvastatin treatment inhibits Akt pathway in human prostate cancer cells:** **A**, A dose-dependent (10, 25, 50, 75 and 100  $\mu\text{M}$ ) study on the effects of simvastatin (16h) on phosphorylation of Akt and its downstream substrate GSK-3  $\alpha$  and  $\beta$  in LNCaP cells. Densitometry of the corresponding bands normalized to  $\beta$ -actin is shown below. **B**, Time-course effect of 25  $\mu\text{M}$  simvastatin (4 and 16h) on Akt and GSK-3 phosphorylation in LNCaP cells. **C**, Time-course effect of 25  $\mu\text{M}$  simvastatin (4, 8, 16 and 24h) on Akt phosphorylation in PC3 cells. Corresponding densitometry values normalized to  $\beta$ -actin are shown below.

**Figure 2: Simvastatin significantly inhibits PC3 cell migration and invasion:** **A and B**, PC3 cells were grown to confluence, and a scratch was made in the monolayer followed by treatment with control PBS and simvastatin (25 and 100  $\mu\text{M}$ ). **A**, Scratch recovery as determined at 16h post simvastatin treatment. **B**, Scratch recovery as determined at 24h post simvastatin treatment. **C**, Invasion assay data after treatment of EGF-stimulated PC3 cells with 25 and 100  $\mu\text{M}$  simvastatin for 12 and 24h. **D**, Invasion assay data after treatment of serum-stimulated PC3 cells with 25 and 100  $\mu\text{M}$  simvastatin for 12 and 24h. Bar graph shows the percentage inhibition of invasion in simvastatin-treated PC3 cells normalized to saline control.

**Figure 3: Simvastatin inhibits PC3 cell proliferation:** **A**, Actively growing PC3 cells were plated in 96-well plates at a density of  $1 \times 10^4$  cells/well in triplicates. After 24h incubation in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ , cells were treated with 25 and 100  $\mu\text{M}$  simvastatin for 16h. Cell counts were performed at 0 and 24h time points and doubling time was calculated. **B**, Actively growing PC3 cells were plated in 96-well plates at a density of  $1 \times 10^4$  cells/well in triplicates. After 24h incubation in a  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ , cells were treated with the indicated concentrations of 25 and 100  $\mu\text{M}$  Simvastatin or 10 nM docetaxel for 16h. In Control cell DMSO was used. Cell proliferation was determined by the BrDU exclusion assay. Bar

graph shows the percentage inhibition of proliferation in simvastatin-treated PC3 cells normalized to saline control.

**Figure 4: Simvastatin induces apoptosis in PC3 cells:** **A**, Cell viability was measured using trypan blue exclusion method. PC3 and LNCaP cells were grown to confluence and treated with simvastatin (25, 50 and 100  $\mu$ M) for 24h. Cells then were collected, re-suspended in PBS with 0.4% Trypan blue solution. Total cells and Trypan Blue stained cells were counted separately and percentage of non viable cells were calculated. **B**, PC3 cells were treated with 25 and 100  $\mu$ M simvastatin for 16h and subjected for apoptosis assay. Bar graph shows the fold increase in apoptosis in simvastatin-treated PC3 cells compared to saline control.

**Figure 5: Simvastatin inhibited colony formation by PC3 cells is rescued by expression with constitutively active Akt (myrAkt):** **A**, Cells were allowed to form a monolayer and were subjected to treatment with saline or DMSO (controls for simvastatin and docetaxel, respectively), 25  $\mu$ M simvastatin or 10 nM docetaxel. On day 5, cells were fixed, stained and counted for colonies. Bar graph showing reduced number of colonies compared to control with simvastatin treatment. **B**, Bar graph showing reduced number of colonies compared to control with docetaxel treatment. **C**, Bar graph showing PC3 cells stably expressing myrAkt (constitutively active) develop significantly higher number of colonies compared to cells stably expressing GFP (control). **D**, Bar graph showing PC3 cells stably expressing myrAkt are resistant to inhibition of colony formation by simvastatin.

**Figure 6: Simvastatin inhibits growth of PC3 tumor xenograft in nude mice:** **A and E**, Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the weight of 2 week old tumor xenografts. **B and F**, Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the size ( $\text{mm}^2$ ) of 2 week old tumor xenografts. **C and G**, Bar graph showing the effect of simvastatin administered every 24h and 12h, respectively, on the changes in tumor size ( $\text{mm}^2$ ), compared to control (saline), between day 7 and day 11 tumor sizes. **D and H**, Bar graph showing the

effect of simvastatin administered every 24h and 12h, respectively, on the percentage changes in tumor size ( $\text{mm}^2$ ), compared to control (saline), between day 7 and day 11 tumor sizes.

**Figure 7: Simvastatin effects on PC3 tumor growth is associated with an inhibition of Akt activity and reduced expression of PSA: A,** Pictures showing tumor xenografts isolated from nude mice treated with saline (control) and simvastatin on day 14. **B,** Western blot picture and bar graph of densitometry analyses for the phospho-Akt levels in tumor xenograft lysates collected from nude mice treated with saline (control) and simvastatin. **C,** Western blot picture and bar graph of densitometry analyses for the prostate specific antigen (PSA) levels in tumor xenograft lysates collected from nude mice treated with saline (control) and simvastatin.

Figure 1

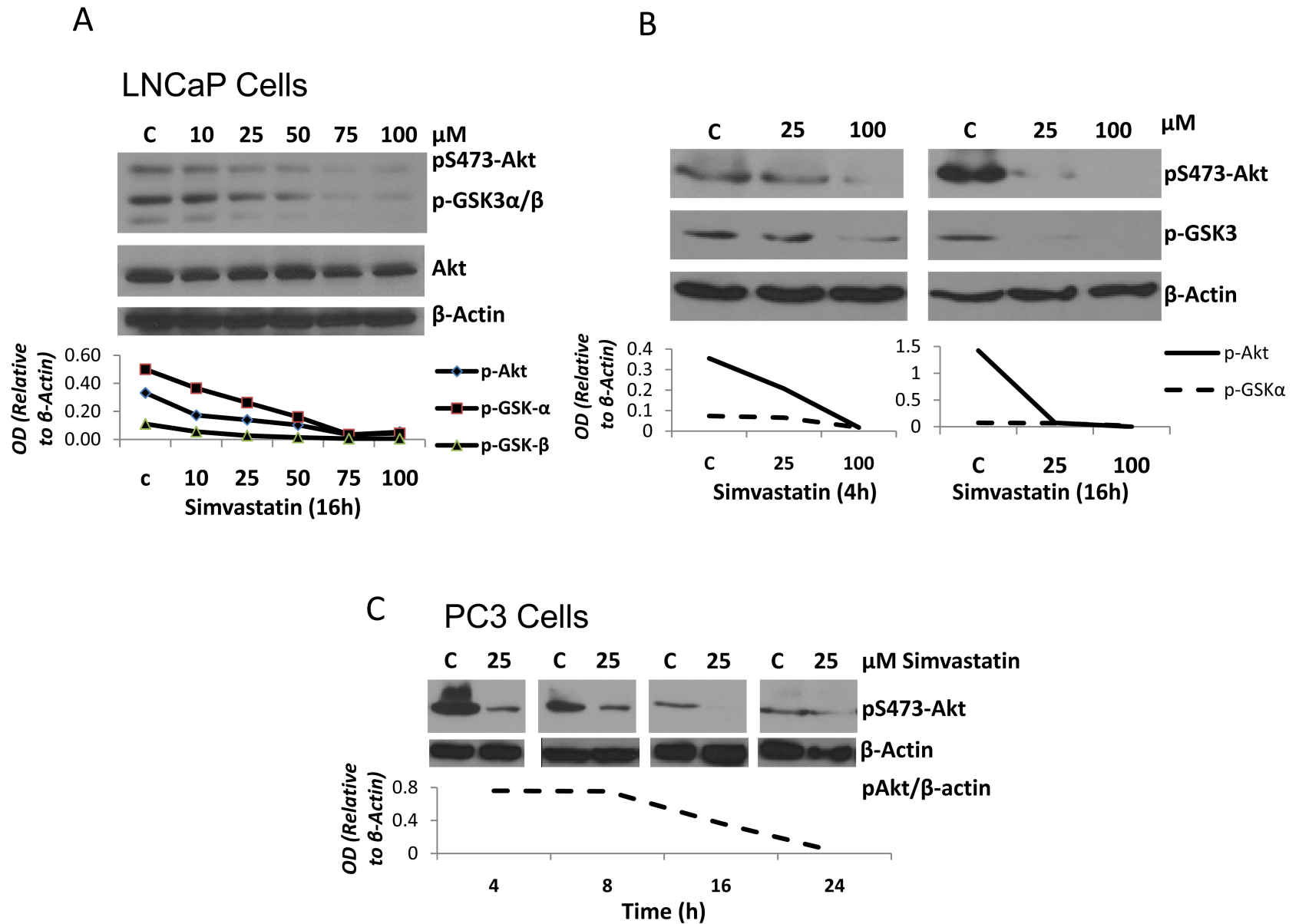
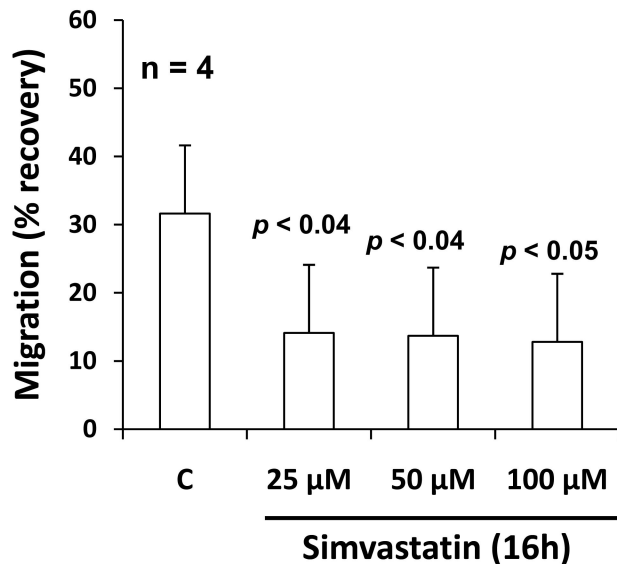


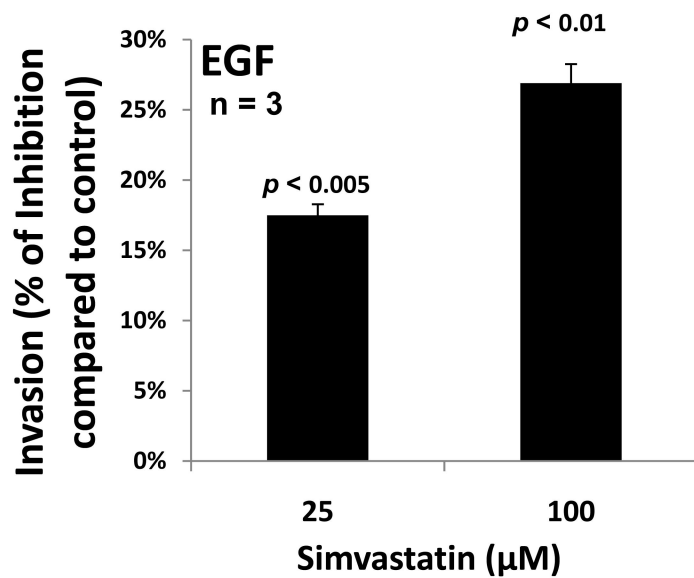
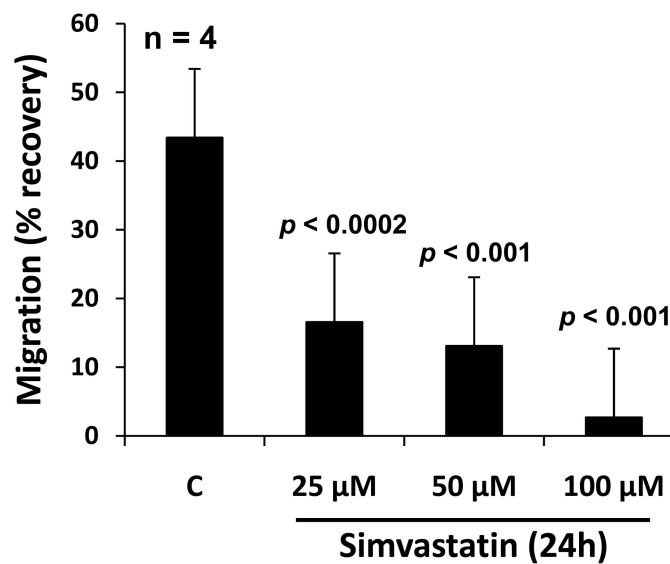


Figure 2

A



B



D

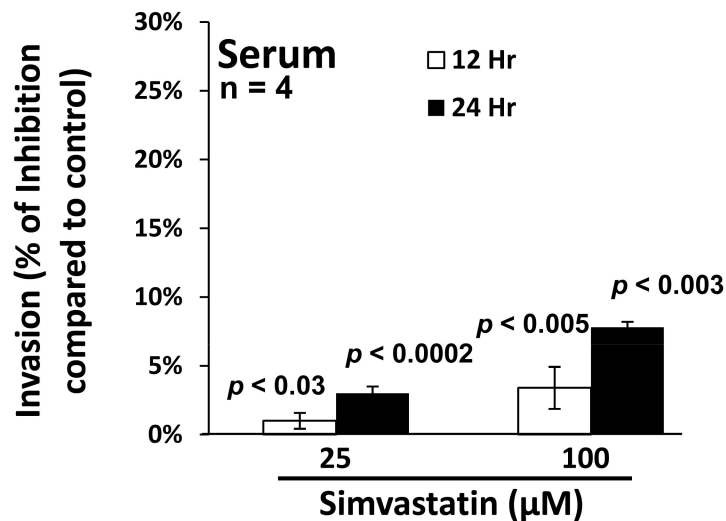


Figure 3

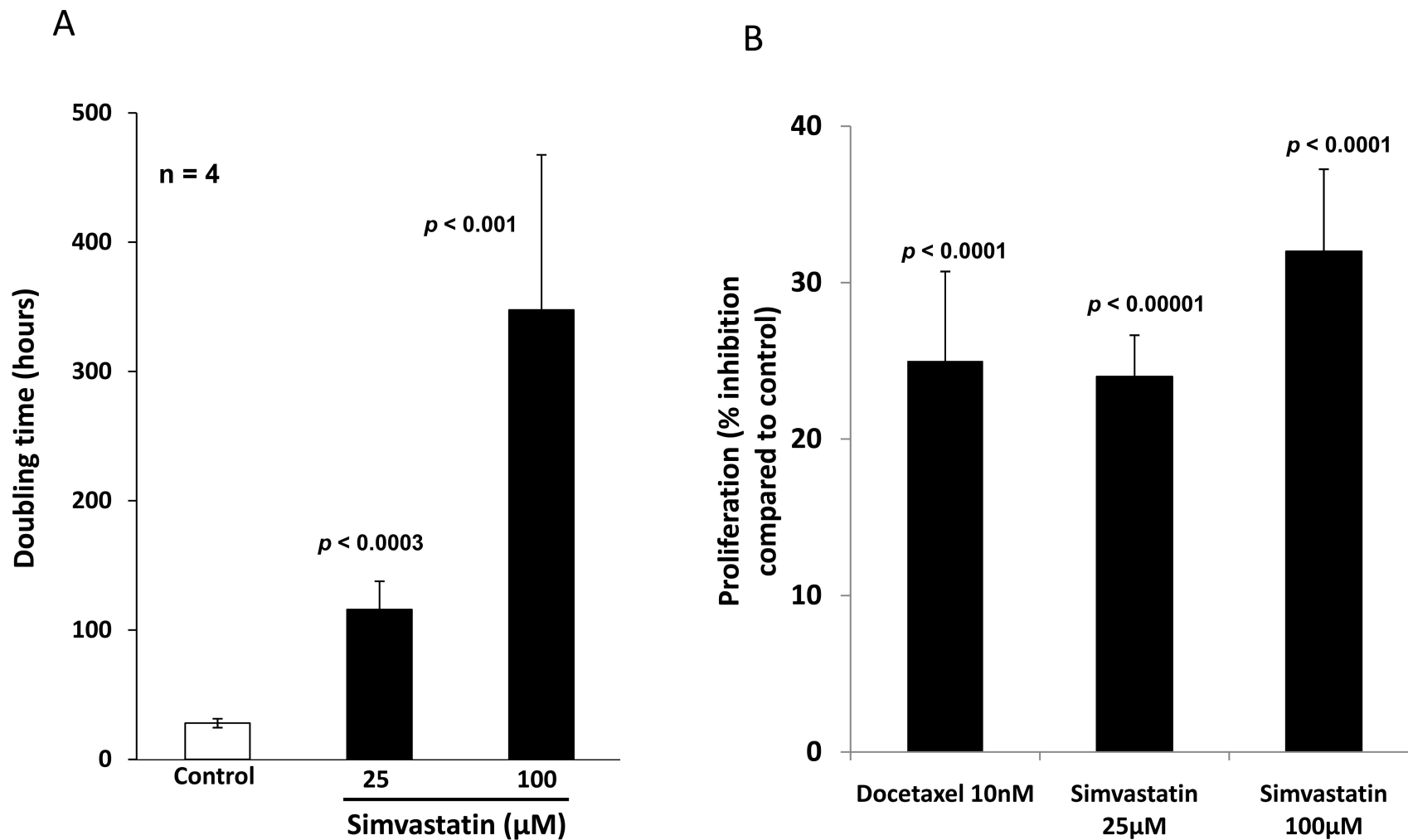
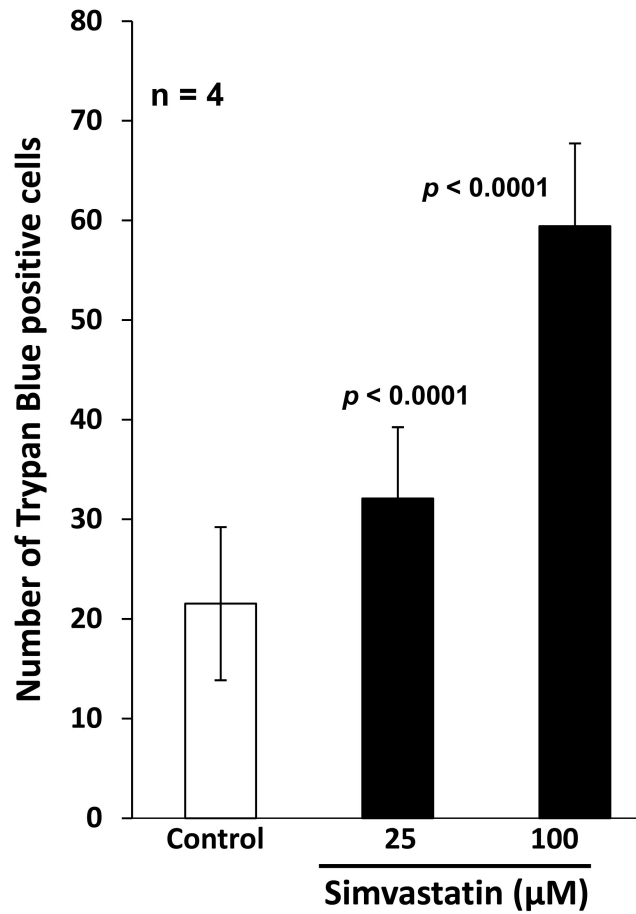


Figure 4

A



B

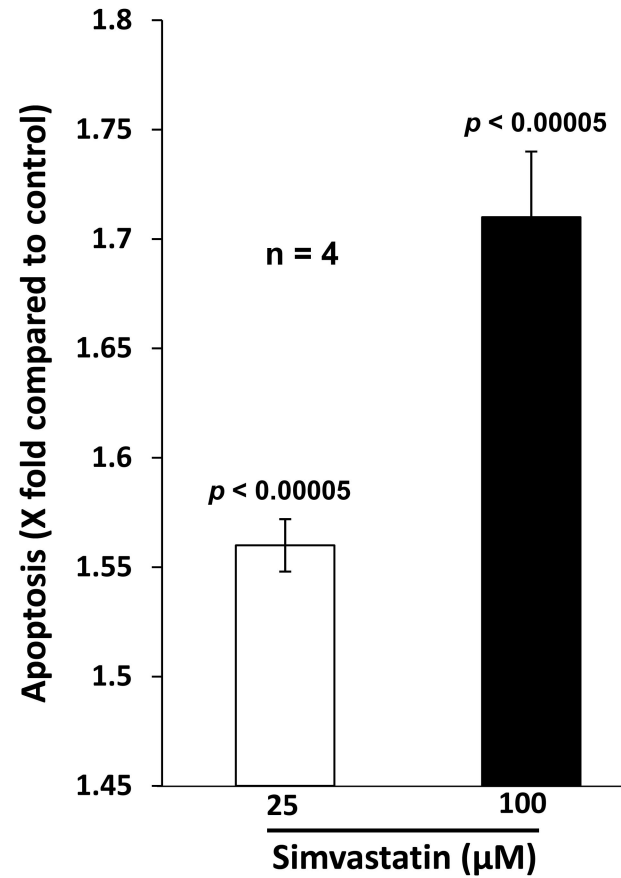
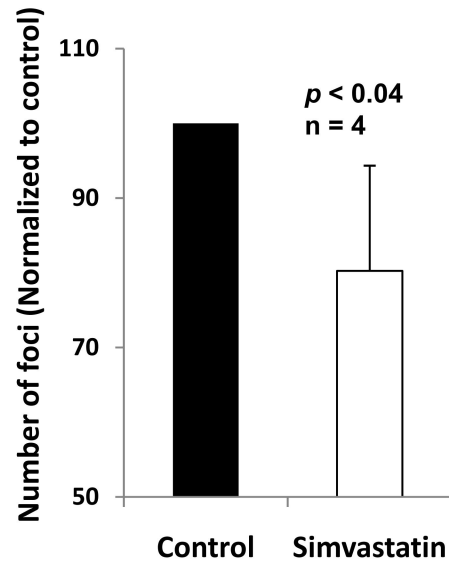
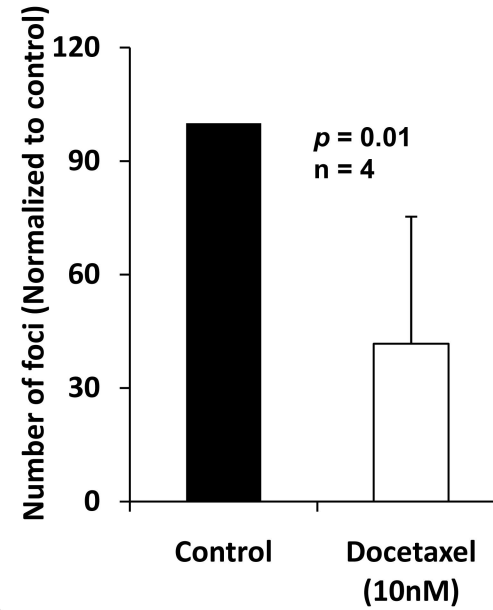


Figure 5

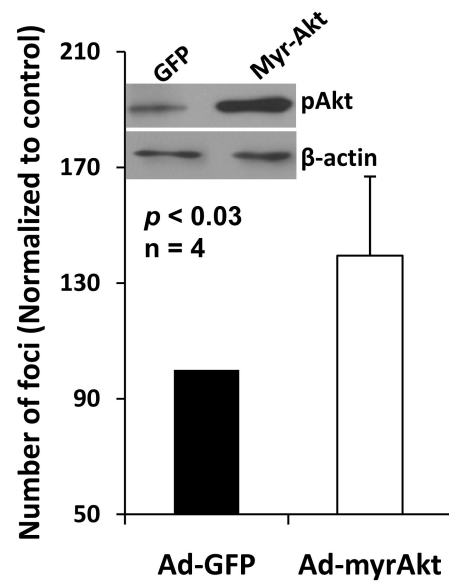
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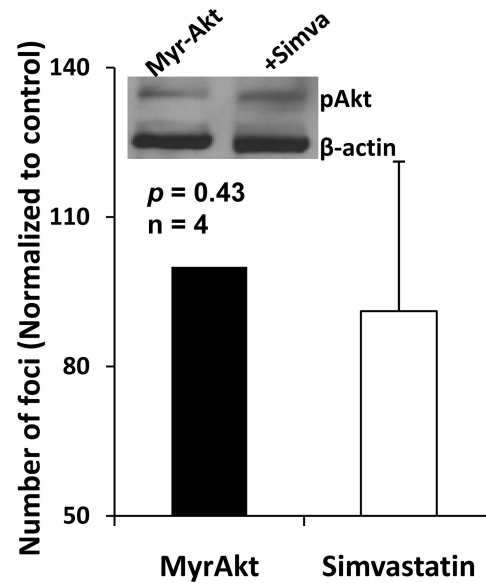
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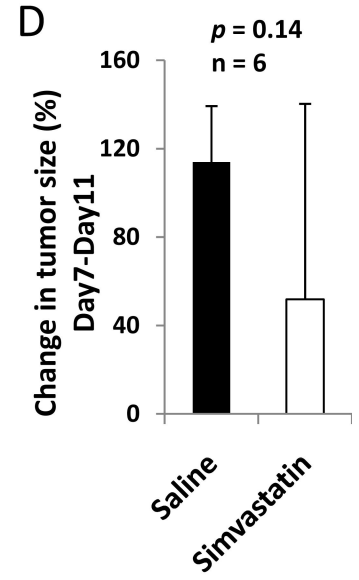
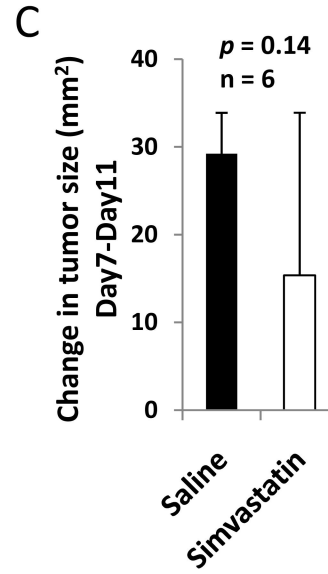
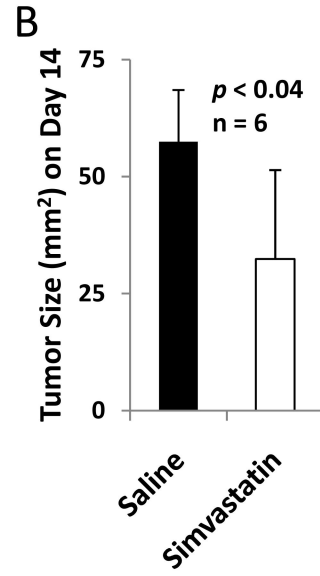
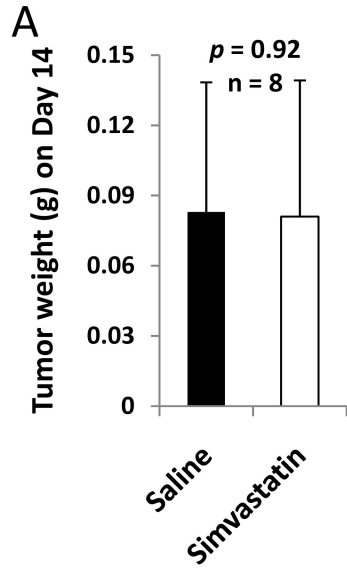


D



# Figure 6

## (i) 2mg/kg body weight/day



## (ii) 2mg/kg body weight/12h

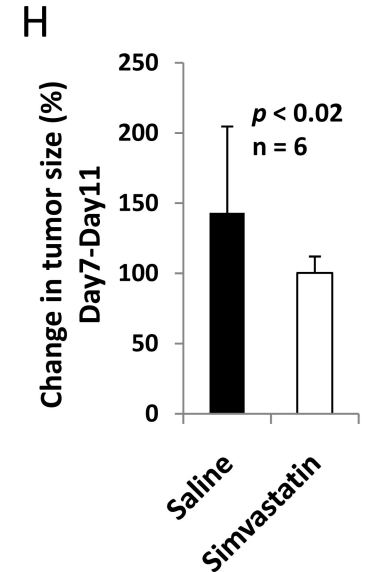
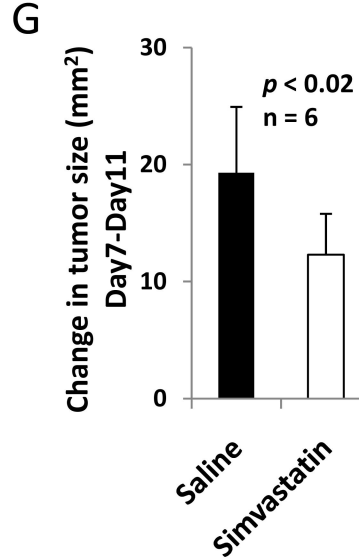
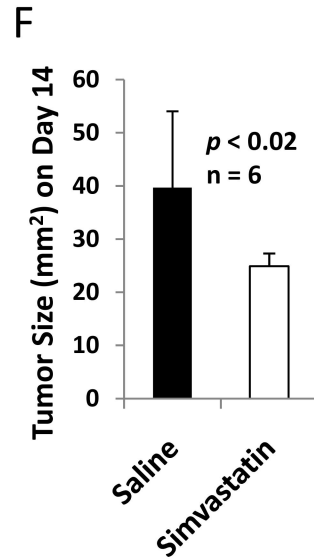
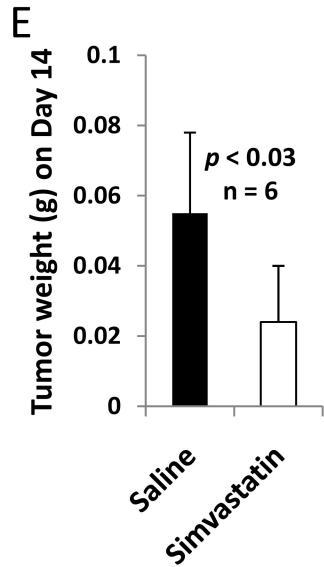
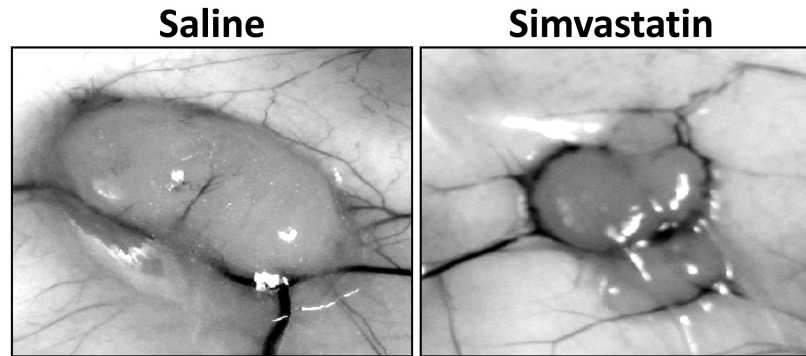
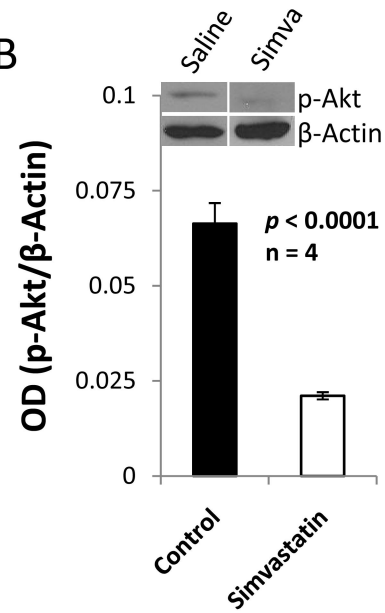


Figure 7

A



B



C

