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RESEARCH PAPER

Statins inhibit the growth of variant human embryonic stem cells and cancer cells *in vitro* but not normal human embryonic stem cells

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Background and purpose: Statins inhibit proliferation of various human cancer cell lines *in vitro*. As human embryonic stem cells (hESCs) possess neoplastic-like properties we have evaluated the role of various statins on karyotypically normal hESCs (HES3 and BG01), abnormal hESCs (BG01V) and breast adenocarcinoma cells (MCF-7) to evaluate whether the mode of action of the statins was via a stemness pathway.
Experimental approach: All cell lines were treated with simvastatin, pravastatin, lovastatin and mevastatin (1 μmol·L^{-1} to 20 μmol·L^{-1}) up to 7 days and their effects on cell proliferation, cell cycle, apoptosis and pluripotency studied.
Key results: All four statins did not inhibit HES3 and BG01 proliferation, but BG01V and MCF-7 were inhibited by simvastatin, lovastatin and mevastatin. These inhibitory effects were reversed by the endogenous isoprenoids, farnesylpyrophosphate and geranylgeranylpyrophosphate. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling and cell cycle assay confirmed apoptosis in BG01V and MCF-7. Stem cell surface markers [stage-specific embryonic antigen-4, tumour rejection antigen-1-81, octamer-4 (OCT-4)] were expressed in HES3 and BG01, but not in BG01V cells, even after prolonged treatment with simvastatin. In BG01V and MCF-7, the pro-apoptotic Bcl-2-associated X protein genes were up-regulated, while the antiapoptotic BCL2 and SURVIVIN genes were down-regulated. Expression of the stemness-related genes namely, the growth differentiation factor-3, NANOG and OCT-4 was decreased in BG01V compared with BG01 and HES3.
Conclusions and implications: Normal hESCs were resistant to prolonged exposure to statins over a range of doses, compared with BG01V and MCF-7, probably because of genetic and behavioural differences. The statins not only have anti-cancer properties but can suppress abnormal hESCs thus promoting growth of normal hESCs *in vitro*.
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Keywords: statins; stem cells; cell proliferation; isoprenoids; apoptosis; pluripotency

Abbreviations: BAX, Bcl-2-associated X protein; BG01, karyotypically normal human embryonic stem cells; BG01V, karyotypically abnormal human embryonic stem cells; DAPI, 4′,6-diamidino-2-phenylindole; FPP, farnesylpyrophosphate; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGTI, geranylgeranyl transferase inhibitor; HES3, karyotypically normal human embryonic stem cells; hESCs, human embryonic stem cells; HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A; MCF-7, breast adenocarcinoma cells; mESCs, mouse embryonic stem cells; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OCT-4, octamer-4; PI, propidium iodide; RhoA, Ras homolog gene family member A; ROCK, Rho-associated coiled coil kinase; SSEA, stage-specific embryonic antigen; TRA, tumour rejection antigen; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling

Introduction

Cell division and proliferation is an essential function that contributes to the maintenance of tissue homeostasis. The continued supply of cells for normal physiological replacement and tissue repair is made possible by the presence of

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stem cells within special niches in certain tissues that are capable of self-replication and directed cell differentiation towards the mature phenotype. By asymmetric cell division such stem cells give rise to two daughter cells of which one enters the stem cell pool and the other enters the maturation process towards a particular phenotype (Clevers, 2005). Thus, a constant number of stem cells persist in the adult tissue and these have been referred to as mesenchymal stem cells that have limited differentiation potential.

In contrast, the human embryonic stem cell (hESC) is a highly pluripotent cell that is derived from the inner cell mass of *in vitro* fertilized blastocysts (Reubinoff *et al.*, 2000). As these cells are pluripotent, tissues differentiated from them offer great promise in regenerative medicine in the treatment of a variety of diseases by transplantation therapy. Currently, hESCs have been successfully differentiated into cardiomyocytes (Mummery *et al.*, 2002; Laflamme *et al.*, 2007), endothelial cells (Levenberg *et al.*, 2002), neuronal cells (Hornstein and Benvenisty, 2004; Yang *et al.*, 2008), bone cells (Sottile *et al.*, 2003) and pancreatic islets (Shim *et al.*, 2007). hESCs are 'social' cells and tight junctional complexes holding them together (Sathananthan *et al.*, 2002) seem to offer survival advantages while dissociated hESCs are vulnerable to apoptosis (Wong *et al.*, 2004).

Unlike other secondary adult and fetal cell lines, hESC lines are quite sensitive and any addition or deletion of important ingredients in the basic culture protocol leads to various degrees of differentiation and loss of pluripotency (Maitra *et al.*, 2005). Thus, knowledge of the effects of various commonly used drugs such as anti-hypertensives, anti-thrombotics, anti-cholesterol and anti-inflammatory agents on hESCs would provide essential information before hESC-derived tissues are used as transplants in patients who are already receiving these pharmacological agents and both undifferentiated and differentiated hESCs may serve as ideal screening systems for potential drugs in the pharmaceutical industry. Currently, pharmaceutical companies use animal cell lines as screening platforms for potential drugs but interesting examples of such hESC pharmacological assay systems were recently reported. Y-27632 [a selective inhibitor of p160 Rho-associated coiled coil kinase (ROCK)] was shown to inhibit apoptosis in dissociated hESCs (Watanabe *et al.*, 2007) and a group of statins was shown to inhibit the Ras homolog gene family member A (RhoA)/ROCK dependent signalling pathway by inhibition of geranylgeranylation (Rattan *et al.*, 2006). Furthermore, statins were shown to inhibit colorectal and ovarian cancer cells *in vitro* (Gauthaman *et al.*, 2007).

Statins are 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors which prevent the conversion of HMG-CoA to mevalonate, a precursor in the cholesterol biosynthetic pathway leading to reduction in cholesterol synthesis. Statins are widely prescribed for their potent cholesterol lowering properties and have high safety profiles. Cholesterol is a major structural component of cell membranes and therefore all biological functions associated with cell membranes such as cell integrity, receptor-ligand binding, pinocytosis, cell osmolarity and membrane pumps are dependent on normal cholesterol levels. In addition to reducing cholesterol synthesis, statins bring about the reduction of other downstream products of mevalonic acid, namely, farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), the isoprenoid precursors. These isoprenoids attach to several cellular proteins, such as oncoproteins (ras), nuclear proteins (laminins) and small guanosine triphosphate (GTP)-binding proteins (rho, rac, rab). This isoprenylation or post-translational modification is essential in signal transduction leading to cell proliferation, differentiation and cell death (Goldstein and Brown, 1990; Edwards and Ericsson, 1999). HMG-CoA reductase inhibition may affect several biochemical pathways and other beneficial effects of statins are fast emerging such as its potent anti-neoplastic properties *in vitro* and *in vivo* (Hindler *et al.*, 2006).

It was recently reported that statins suppress self-renewal in mouse embryonic stem cells (mESCs) by RhoA-mediated inhibition (Lee *et al.*, 2007). In contrast, short-term statin exposure did not inhibit the karyotypically normal human embryonic stem cells (HES3) while the karyotypically abnormal human embryonic stem cells (BG01V) and cancer cells (colorectal and ovarian) were inhibited (Gauthaman *et al.*, 2007). However, information on the effects of prolonged exposure of the statins on the self-renewal of aberrant hESCs (BG01V) in comparison with the parent cell line (BG01) and other normal hESC lines (HES3) are lacking to evaluate the specific role of statins on embryonic stem and cancer cells. As hESCs possess neoplastic-like properties, we wanted to compare the role of prolonged exposure (7 days of treatment) of various statins on the self-renewal properties of normal hESCs with abnormal hESCs and breast carcinoma cells so as to evaluate the usefulness of hESCs and cancer cells as an *in vitro* assay for their screening potential for anti-cancer drugs and evaluate the role of statins in the maintenance and scaling up of undifferentiated hESCs for hESC-derived tissue transplantation therapy. In the present study, we evaluated the *in vitro* effects of four different statins (pravastatin, simvastatin, lovastatin and mevastatin) every 48 h for 7 days on normal hESCs (HES3, BG01), variant hESC (BG01V) and the breast carcinoma cells (MCF-7) in terms of cell proliferation, cell cycle, apoptosis, pluripotency and related gene expression.

Methods

Cell culture

The genetically normal hESC line (HES3) was obtained from ES Cell International, Singapore. The normal parental hESC line (BG01), the karyotypically variant hESC line (BG01V) and breast adenocarcinoma cells (MCF-7) were obtained from the American Type Culture Collection (Rockville, MD, USA). Ethical approval for use of these commercial human cell lines was given by the National University of Singapore (NUS) Institutional Review Board.

BG01V carried duplications of chromosomes 12 and 17 while MCF-7 had multiple chromosomal abnormalities ranging from hypertriploidy to hypotetraploidy. HES3, BG01 and BG01V cells were maintained undifferentiated by culturing them on mouse embryonic fibroblasts in 80% Dulbecco's modified Eagle's medium culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 20% knockout serum replacement, 1% nonessential aminoacids, 2 $mmol \cdot L^{-1}$ L-glutamine, 0.1 $mmol \cdot L^{-1}$ β-mercaptoethanol,

16 ng·mL^{-1} basic fibroblast growth factor and 1% insulin-transferrin-selenium. MCF-7 cells were maintained in Eagle's minimal essential medium with Earle's balanced salt solution supplemented with 10% FBS, 2 mmol·L^{-1} L-glutamine, 0.1 mmol·L^{-1} non-essential aminoacids, 1 mmol·L^{-1} sodium pyruvate, 0.01 mg·mL^{-1} bovine insulin and 1.5 g·L^{-1} sodium bicarbonate. The population doubling time for the stem cell lines (HES3, BG01 and BG01V) were 48–96 h and for the cancer cell line (MCF-7) was 24–48 h.

Cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured in 24-well plates to achieve 50% confluence before the addition of simvastain, pravastatin, lovastatin and mevastatin (all from Calbiochem, San Diego, CA, USA) to separate wells and incubated at 37°C in a 5% CO_2 atmosphere for 3 days with fresh changes of culture medium and the statins after 48 h. Pravastatin, simvastatin, lovastatin and mevastatin were used in the concentrations of 1, 3, 5, 10 and 20 μmol·L^{-1} and respective vehicles were used as controls. Once we realized that simvastatin was the most potent, HES3, BG01, BG01V and MCF-7 cells were treated with simvastatin only at 5, 10 and 20 μmol·L^{-1} for a longer period of 7 days with fresh changes of culture medium and simvastatin every 48 h. Except for the hydrophilic pravastatin, all the other statins were used in their open acid forms (Li *et al.*, 1999).

Following the treatment period, the cytotoxicity assay was performed using MTT reagent [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Absorbance was spectrophotometrically measured using a microplate ELISA reader (μQuant, BioTek, Winooski, VT, USA). To confirm that the effects observed were specifically due to the statins, the reversal of statin effect was evaluated using 100 μmol·L^{-1} FPP (Sigma, St. Louis, MO, USA) or 10 μmol·L^{-1} geranylgeranylpyrophosphate (GGPP; Sigma, St. Louis, MO, USA) together with the different statins at their highest concentration tested (20 μmol·L^{-1}) using the MTT assay. The drugs were added at the same time as the statins and the cultures were incubated at 37°C in a 5% CO_2 atmosphere for 3 days with fresh changes of culture medium and the drugs after 48 h.

Additionally, the effect of farnesyl transferase inhibitor (FTI-277; 10 μmol·L^{-1}; Sigma, St. Louis, MO, USA) and geranylgeranyl transferase inhibitor (GGTI-298; 10 μmol·L^{-1}; Sigma, St. Louis, MO, USA) was also studied by incubating the cultures at 37°C in a 5% CO_2 atmosphere for 3 days with fresh changes of culture medium and the drugs after 48 h.

Phase contrast microscopy. HES3, BG01, BG01V and MCF-7 cells were plated in a 24-well plate and treated with pravastatin, simvastain, lovastatin and mevastatin at 20 μmol·L^{-1} concentrations and incubated at 37°C in a 5% CO_2 in air atmosphere for 3 days with fresh changes of culture medium and statins after 48 h. At the end of the treatment period, changes in cell morphology were photographed for the control and treatment groups. Additionally, cell morphology was also studied following treatment with simvastatin (20 μmol·L^{-1}) alone for an extended incubation period of up to 7 days with fresh changes of culture medium and simvastatin every 48 h.

Cell cycle analysis

HES3, BG01, BG01V and MCF-7 cells were cultured in 60 mm culture dishes and treated with simvastatin, lovastatin and mevastatin at 20 μmol·L^{-1} concentration for 48 h with untreated cells as controls. Briefly, the cells were trypsinized and fixed with ice-cold 70% ethanol. The fixed cells were stained with 50 μg·mL^{-1} propidium iodide (PI) in phosphate-buffered saline (PBS) containing 0.1% TritonX-100 and 50 μg·mL^{-1} RNAse-A and then analysed using a flow cytometer (Epics-Altra, Beckman Coulter, Fullerton, CA, USA).

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assay

The dead end fluorometric system (Promega) was used according to the manufacturer's instructions to detect TUNEL-positive cells. HES3, BG01, BG01V and MCF-7 cells were cultured in 24-well plates and treated separately with simvastatin, lovastatin and mevastatin at 20 μmol·L^{-1} concentration for 48 h with untreated cells as controls. Briefly, the cells were washed and fixed with 4% methanol-free formaldehyde solution for 25 min at 4°C followed by addition of TritonX-100 solution for 5 min at 4°C. Labelling of the DNA fragments was performed by treating the cells with fluorescin-12dUTP and terminal deoxynucleotidyltransferase for 1 h at 37°C in a humidified chamber in the dark. The reaction was stopped by the addition of sodium chloride-sodium citrate solution and the cells washed three times and stained with PI (1 μg·mL^{-1}) in PBS containing DNase-free RNase 250 μg·mL^{-1} for 15 min at room temperature in the dark. The TUNEL-positive cells were analysed under a fluorescence microscope.

Marker analysis of hESCs

Briefly, the HES3, BG01 and BG01V cells cultured in 24-well plates and treated with simvastatin (20 μmol·L^{-1}) for 72 h were fixed with 4% buffered paraformaldehyde solution, washed with PBS and treated with 10% normal goat serum. The cells were incubated with primary antibody [stage-specific embryonic antigen (SSEA-4, 5 μg·mL^{-1}); tumour rejection antigen (TRA-1-81, 5 μg·mL^{-1}); transcription factor octamer-4 (OCT-4, 4 μg·mL^{-1})] (Chemicon, Temecula, CA, USA) for 1 h and goat anti-mouse fluorescein isothiocyanate secondary antibody [at 5 μg·mL^{-1} (Sigma, IgM); 2 μg·mL^{-1} (Alexa Fluor 488)] for 1 h. Following PBS washes, the cells were treated with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg·mL^{-1}) (Molecular probes, Invitrogen Life Technologies, Carlsbad, CA, USA) and incubated for 5 min at room temperature, washed with PBS and analysed using fluorescence microscopy. The expression of alkaline phosphatase (AP) was studied using a Vector® Red substrate kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (PCR)

HES3, BG01, BG01V and MCF-7 cells were cultured in 60 mm culture dishes and treated with 5, 10 or 20 μmol·L^{-1} simvas-

Table 1 The genes and primer sequences used for quantitative real time PCR

Gene name	*Primer sequence*	*Product size (bp)*
BCL2	F: 5′-GGCTGGGATGCCTTTGTG-3′ R: 5′-CAGCCAGGAGAAATCAAACAGA-3′	66
BAX	F: 5′-TGGAGCTGCAGAGGATGATTG-3′ R: 5′-GCTGCCACTCGGAAAAAGAC-3′	70
SURVIVIN	F: 5′-ACCAGGTGAGAAGTGAGGGA-3′ R: 5′-AACAGTAGAGGAGCCAGGGA-3′	309
GDF3	F: 5′-AGACTTATGCTACGTAAAGGAGCT-3′ R: 5′-CTTTGATGGCAGACAGGTTAAAGTA-3′	150
NANOG	F: 5′-AGAACTCTCCAACATCCTGAACCT-3′ R: 5′-TTCTGCCACCTCTTAGATTTCATTC-3′	87
OCT-4	F: 5′-GGAGGAAGCTGACAACAATGAAA-3′ F: 5′-GGCCTGCACGAGGGTTT-3′	64
GAPDH	F: 5′-ACCACAGTCCATGCCATCAC-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′	500

BAX, Bcl-2 associated X protein; F, Forward primer; GDF3, growth differentiation factor-3; OCT-4, octamer-4; PCR, polymerase chain reaction; R, Reverse primer.

tatin for up to 7 days with untreated cells as controls. The culture medium containing the respective statin was replaced every 48 h. Total RNA was isolated using TRIzol™ reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and RNA quality and quantity was measured with a Nanodrop™ spectrophotometer (Nanodrop technologies, Wilmington, DE, USA). All samples were treated with DNase-I prior to first strand synthesis. First strand cDNA synthesis was carried out with random hexamers using the SuperScript™ first strand synthesis system (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Primer sequences (summarized in Table 1) were either taken from published studies or designed using the Primer Express Software v3.0 (ABI). qRT-PCR analysis was performed with the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR green as previously described (Richards *et al*., 2004) and relative quantification was performed using the comparative CT (2-ΔΔCT) method (Livak and Schmittgen, 2001).

Statistical analysis

The differences in treatment for each cell line for the different drugs at various concentrations were compared and analysed using either one-way ANOVA with Bonferroni's multiple comparisons *post hoc* analysis or Students *t*-test using the statistical package for Social Sciences (SPSS 13). All the results were expressed as mean ± SEM and the level of significance for comparisons set at $P < 0.05$.

Results

Cytotoxicity – MTT assay

Karyotypically normal HES3 and BG01 cells when treated with simvastatin, pravastatin, mevastatin or lovastatin (1 μmol·L^{-1}–20 μmol·L^{-1}) for 3 days did not exhibit any significant toxicity to cells for all concentrations tested (Figure 1A,B).

Unlike normal hESCs (HES3 and BG01), the variant hESC (BG01V) demonstrated a significant inhibition in cell proliferation following treatment with simvastatin, lovastatin and mevastatin in a dose-dependent manner compared with controls. However, statistically significant inhibitions were observed only at the higher doses for simvastatin (5, 10, 20 μmol·L^{-1}), lovastatin (10, 20 μmol·L^{-1}) and mevastatin (20 μmol·L^{-1}) (Figure 1C).

The MCF-7 cells also demonstrated significant inhibition in cell proliferation following treatment with simvastatin, lovastatin, mevastatin in a dose-dependent manner compared with control. For the MCF-7 cells statistically significant inhibitions were observed from lower concentrations upwards of 3, 5, 10 and 20 μmol·L^{-1} for simvastatin; 5, 10 and 20 μmol·L^{-1} for lovastatin and 10 and 20 μmol·L^{-1} for mevastatin (Figure 1D).

When HES3, BG01, BG01V and MCF-7 cells were treated with only simvastatin (5, 10 and 20 μmol·L^{-1}) for an extended period of up to 7 days, HES3 and BG01 did not show inhibition of proliferation (Table 2) but BG01V cells were inhibited in a dose dependent manner for all three doses tested at 3, 5 and 7 days with cell inhibition of 30–70% compared with controls (Table 2). MCF-7 cells were also inhibited in a dose dependent manner for all three doses tested at 3, 5 and 7 days with cell inhibition of 20–70% compared with controls (Table 2).

Effect of isoprenoids on cell inhibition induced by statins

In a separate experiment, treatment of the abnormal BG01V cells with simvastatin, lovastatin or mevastatin (20 μmol·L^{-1}) induced significant loss of cell viability, about 40%, compared with the controls. These effects of the various statins were not overcome by adding FPP (100 μmol·L^{-1}) but were reversed by incubation of BG01V cells with GGPP (10 μmol·L^{-1}) (Table 3).

Treatment of MCF-7 cells with simvastatin, lovastatin and mevastatin (20 μmol·L^{-1}) inhibited viability compared with controls (Table 3). Only the inhibitory effect of simvastatin was partly reversed by incubation with FPP (100 μmol·L^{-1}), whereas GGPP (10 μmol·L^{-1}) was able to completely reverse the inhibition induced by all three statins (Table 3).

Effect of inhibitors of isoprenoid-related enzymes on cell viability

The karyotypically normal HES3 and BG01 cells when treated with GGTI-298 (10 μmol·L^{-1}) or FTI-277 (10 μmol·L^{-1}) for 3 days did not show significant loss of viability (Table 4), in contrast to the variant hESCs (BG01V) that were clearly inhibited following treatment with GGTI-298 (10 μmol·L^{-1}) but unaffected by FTI-277 (10 μmol·L^{-1}). Similarly, MCF-7 cells also lost viability after treatment with GGTI-298 (10 μmol·L^{-1}) but not after FTI-277 (10 μmol·L^{-1}) (Table 4).

Cytotoxicity – Phase contrast microscopy

Karyotypically normal HES3 and BG01 cells did not show any evidence of cell toxicity in culture following treatment with simvastatin, lovastatin or mevastatin at 20 μmol·L^{-1} and the same culture pattern and normal cell morphology persisted even at 72 h (Figure 2A). In contrast, the abnormal BG01V and MCF-7 cells demonstrated varying levels of cytological changes associated with loss of normal culture characteristics

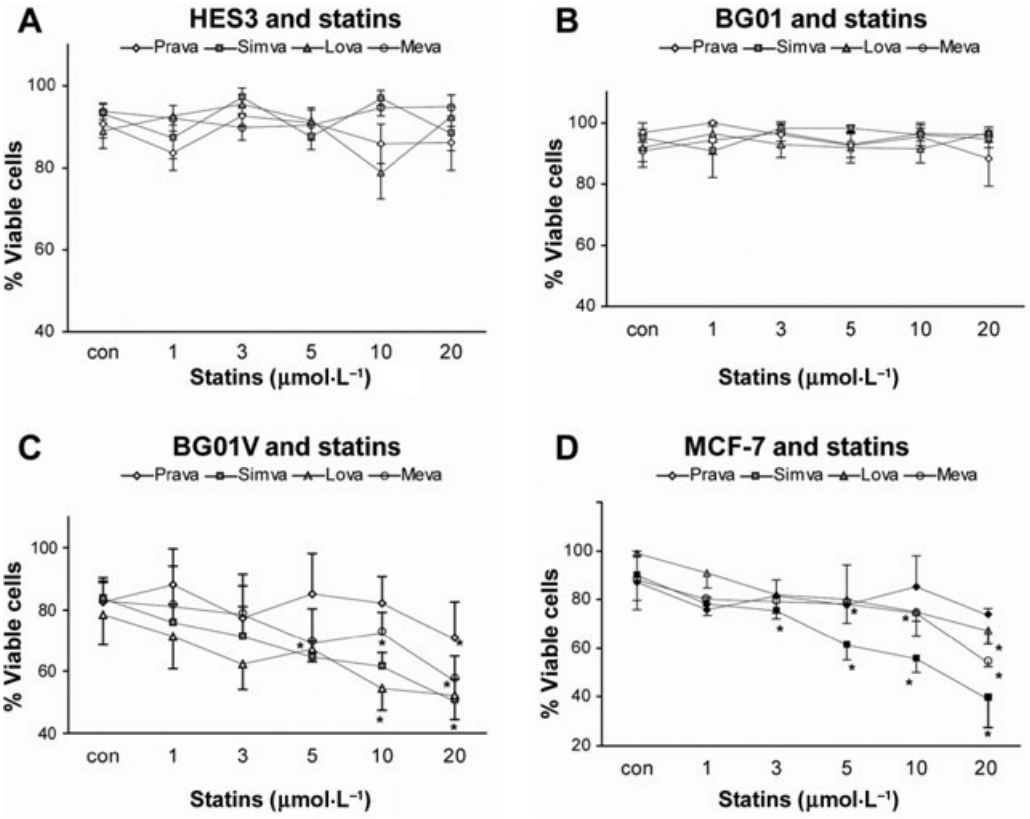

Figure 1 Effect of statins on cell viability: A – karyotypically normal human embryonic stem cells (HES3), B – normal human embryonic stem cells (BG01), C – human embryonic variant stem cells (C-BG01V) and D – breast adenocarcinoma cells (MCF-7) following treatment with simvastatin, pravastatin, lovastatin and mevastatin at different concentrations (1, 3, 5, 10 and 20 μmol·L^{-1}) for 3 days. *$P < 0.05$, significantly different from control: one-way ANOVA; Bonferroni's multiple comparisons; $n = 6$.

Table 2 Viability of cells cultured with simvastatin (5–20 μmol·L^{-1}) for up to 7 days

Cell line	*Days of culture*	*Concentration of simvastatin (μmol·L^{-1})*		
		5	*10*	*20*
HES3	3	111 ± 7%	103 ± 4%	96 ± 4%
	5	88 ± 6%	82 ± 5%	82 ± 3%
	7	96 ± 7%	92 ± 7%	96 ± 3%
BG01	3	87 ± 6%	90 ± 4%	84 ± 7%
	5	92 ± 7%	89 ± 6%	92 ± 6%
	7	91 ± 5%	86 ± 9%	83 ± 6%
BG01V	3	70 ± 8%*	65 ± 8%*	53 ± 4%*
	5	61 ± 6%*	44 ± 7%*	33 ± 8%*
	7	62 ± 4%*	38 ± 5%*	33 ± 3%*
MCF-7	3	77 ± 4%*	83 ± 7%*	71 ± 3%*
	5	57 ± 10%*	42 ± 12%*	37 ± 3%*
	7	42 ± 5%*	34 ± 8%*	26 ± 11%*

Values shown represent the viability in the cell cultures as measured by the MTT assay, relative to control cultures (no statin) set at 100%.
*$P < 0.05$, significantly different from 100%: one-way ANOVA; Bonferroni's multiple comparisons; $n = 6$.
BG01, karyotypically normal human embryonic stem cells; BG01V, karyotypically abnormal human embryonic stem cells; HES3, karyotypically normal human embryonic stem cells; MCF-7, breast adenocarcinoma cells; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

that eventually resulted in cell death. Those cells that survived were thin, elongated and had numerous vacuolations (Figure 2A).

When both karyotypically normal cells (HES3 and BG01) and karyotypically abnormal cells (BG01V and MCF-7) were treated with simvastatin (20 μmol·L^{-1}) for extended periods of up to 7 days, HES3 and BG01 continued to retain normal cell and culture characteristics with no evidence of cell toxicity (Figure 2B) while BG01V and MCF-7 demonstrated significant cytotoxicity changes at day 3 and more than 80% of cell death was evident by day 7 (Figure 2B).

Cell cycle analysis

Cell cycle analysis of BG01V and MCF-7 cells following treatment with simvastatin, lovastatin or mevastatin at 20 μmol·L^{-1} concentration for 48 h showed presence of peaks in the sub G1 phase, indicative of apoptosis. In contrast, HES3 and BG01 demonstrated maximum survival as seen by the peaks in 'S' phase and there were no significant changes both in the control and statin treated groups (Figure 4).

TUNEL assay

TUNEL staining with flourescin-12-dUTP and PI of BG01V and MCF-7 cells following incubation with simvastatin, lovastatin or mevastatin at 20 μmol·L^{-1} for 48 h demonstrated

Table 3 Effects of adding endogenous isoprenoids on loss of viability in BG01V or MCF-7 cells, induced by statins (20 μmol·L^{-1})

Cell line	*Statin*	*Isoprenoids*		
		None	*FPP (100 μmol·L^{-1})*	*GGPP (10 μmol·L^{-1})*
BG01V	Simvastatin	53 ± 4%	57 ± 7%	82 ± 5%*
	Lovastatin	48 ± 3%	59 ± 5%	80 ± 4%*
	Mevastatin	48 ± 5%	57 ± 4%	77 ± 5%*
MCF-7	Simvastatin	64 ± 3%	86 ± 6%*	81 ± 4%*
	Lovastatin	72 ± 6%	80 ± 8%	86 ± 7%*
	Mevastatin	64 ± 4%	80 ± 8%	87 ± 5%*

Data shown represent the viability in the cell cultures as measured by the MTT assay, relative to control cultures (no statin) set at 100%.
*$P < 0.05$, significantly different from values with statin alone: one-way ANOVA; Bonferroni's multiple comparisons; $n = 3$.
BG01V, karyotypically abnormal human embryonic stem cells; FPP, farnesylpyrophosphate; GGPP, geranylgeranyl pyrophosphate; MCF-7, breast adenocarcinoma cells; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 4 Effect of inhibiting isoprenoid biosynthesis on cell viability

Cell line	*Inhibitor*	
	GGTI (10 μmol·L^{-1})	*FTI (10 μmol·L^{-1})*
HES3	96 ± 7%	92 ± 5%
BG01	90 ± 4%	85 ± 10%
BG01V	55 ± 9%*	73 ± 8%
MCF-7	25 ± 5%*	78 ± 2%

Values shown represent the viability in the cell cultures as measured by the MTT assay, relative to control cultures (no inhibitor) set at 100%.
*$P < 0.05$, significantly different from 100%: one-way ANOVA; Bonferroni's multiple comparisons; $n = 3$.
BG01, karyotypically normal human embryonic stem cells; BG01V, karyotypically abnormal human embryonic stem cells; FTI, farnesyl transferase inhibitor; GGTI, geranylgeranyl transferase inhibitor; HES3, karyotypically normal human embryonic stem cells; MCF-7, breast adenocarcinoma cells; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

TUNEL-positive cells while HES3 and BG01 cells were negative (Figure 3). More cell death was evident in the karyotypically abnormal hESC (BG01V) and the MCF-7 line following treatment with various statins at 20 μmol·L^{-1} and this was reflected by sparse colony morphology in BG01V and by the presence of very few cells in MCF-7 that were TUNEL-positive. In contrast, both karyotypically normal HES3 and BG01 retained their colony morphology following treatment with statins and were also negative for TUNEL staining. This clearly demonstrates that the statins did not affect karyotypically normal hESCs (HES3 and BG01).

Marker analysis of hESCs

Immunophenotyping of statin treated HES3, BG01 and BG01V cells with various antibodies to detect cell surface markers namely SSEA-4 and TRA-1-81 and OCT-4 the nuclear transcription marker, demonstrated strong positive staining with these antibodies in HES3 and BG01 cells but weak to negative staining in BG01V cells. As the karyotypically normal hESCs (HES3 and BG01) were viable following treatment with simvastatin at 20 μmol·L^{-1} for 72 h and maintained their colony morphology, intense DAPI staining was observed. However, only sparse nuclear staining was visible with karyotypically variant BG01V as more cell death was observed following simvastatin treatment. Similarly, BG01V also demonstrated weak alkaline phosphatase activity compared with intense staining in HES3 and BG01 cells (Figure 5).

Quantitative real-time PCR

Following simvastatin (20 μmol·L^{-1}) treatment for 3 days, the karyotypically normal hESCs (HES3) had a 1.7-fold increase in anti-apoptotic *BCL2* gene expression compared with the controls (Figure 6A). In BG01 cells, the pro-apototic Bcl-2-associated X protein (BAX) was decreased (fourfold), while no difference was seen in both BCL2 and SURVIVIN gene expression, compared with the controls (Figure 6B). The BG01V and MCF-7 cells showed increased pro-apoptotic BAX gene expression and decreased SURVIVIN expression (Figure 6C,D).

The expression levels of the stemness-related genes growth differentiation factor-3, NANOG and OCT-4 were significantly decreased in HES3 and BG01 cells compared with the control. In BG01V cells, all these three stemness genes were significantly decreased at day 5 following treatment with simvastatin (5 μmol·L^{-1}) (Figure 6E), and these decreases were more marked than those in HES3 and BG01 cells.

Discussion

Statins are very safe drugs that have been in use for more than two decades primarily for control of high lipidaemic states and associated cardiovascular diseases (Collins *et al*., 2003; Topol, 2004). The therapeutic spectrum of statins has considerably widened in recent years with more studies of their beneficial effects on various physiological functions such as cell signalling (Werner *et al*., 2002), immunomodulation (Leung *et al*., 2003) and anti-cancer mechanisms (Hawk and Viner, 2005; Poynter *et al*., 2005; Sleijfer *et al*., 2005).

In the present study, the parental normal hESC line BG01 was used for more reliable and accurate comparisons of statin effects on its variant daughter cell line, BG01V. BG01V is the same hESC line as BG01 but contains duplications of chromosomes 12 and 17 brought about by serial passaging. Additionally, a completely different, non-parental, normal proprietary hESC line from a different source was also studied to make effective comparisons and confirm any specific effects of the statins on hESCs in general. As normal and variant hESC have neoplastic-like properties and the statins are

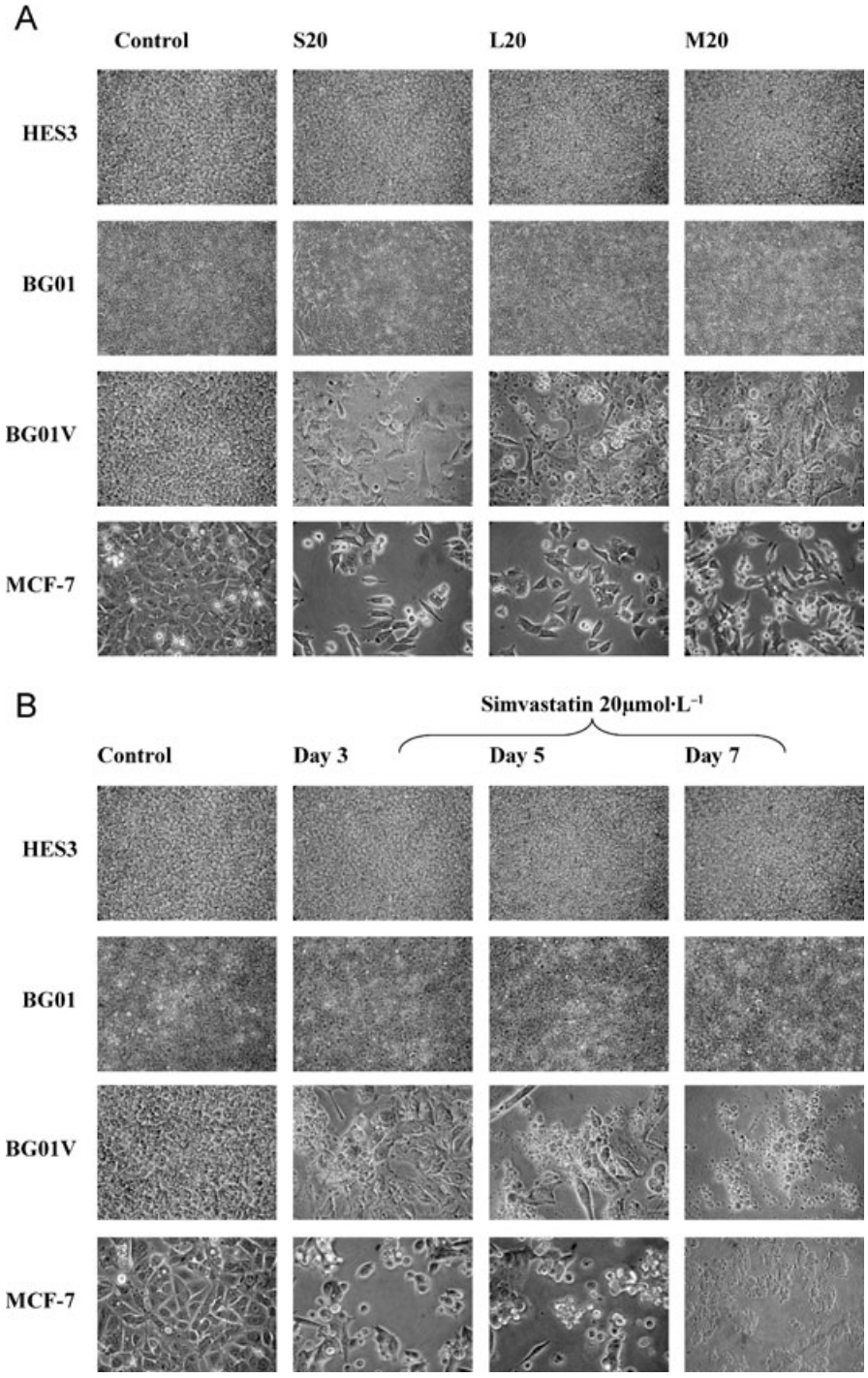

Figure 2 A – Cytological changes in normal human embryonic stem cells (HES3, BG01), human embryonic stem cell variant (BG01V) and breast adenocarcinoma cells (MCF-7) following treatment with simvastatin (20 μmol·L^{-1}) (S20), lovastatin (20 μmol·L^{-1}) (L20) and mevastatin (20 μmol·L^{-1}) (M20) for 3 days. B – Cytological changes in HES3, BG01, BG01V and MCF-7 cells following treatment with simvastatin (20 μmol·L^{-1}) for 3, 5 and 7 days (40× magnification).

known anti-cancer agents, breast cancer cells were included in this study to standardize and make more meaningful comparisons.

We found that exposure for 3 days to various statins at different concentrations did not affect proliferation of the karyotypically normal HES3 and BG01 cells. This lack of effect of the statins over a period of 3 days observed with hESCs in the present study is in contrast to those reported with mESCs, where brief exposure to statins inhibited cell proliferation (Lee *et al.*, 2007). Furthermore, even extended treatment of up to 7 days with simvastatin at higher concentrations did not produce any inhibition of normal hESC proliferation (both HES3 and the parent BG01 cell line), whereas the hESC variant (BG01V) and MCF-7 cells exhibited statistically

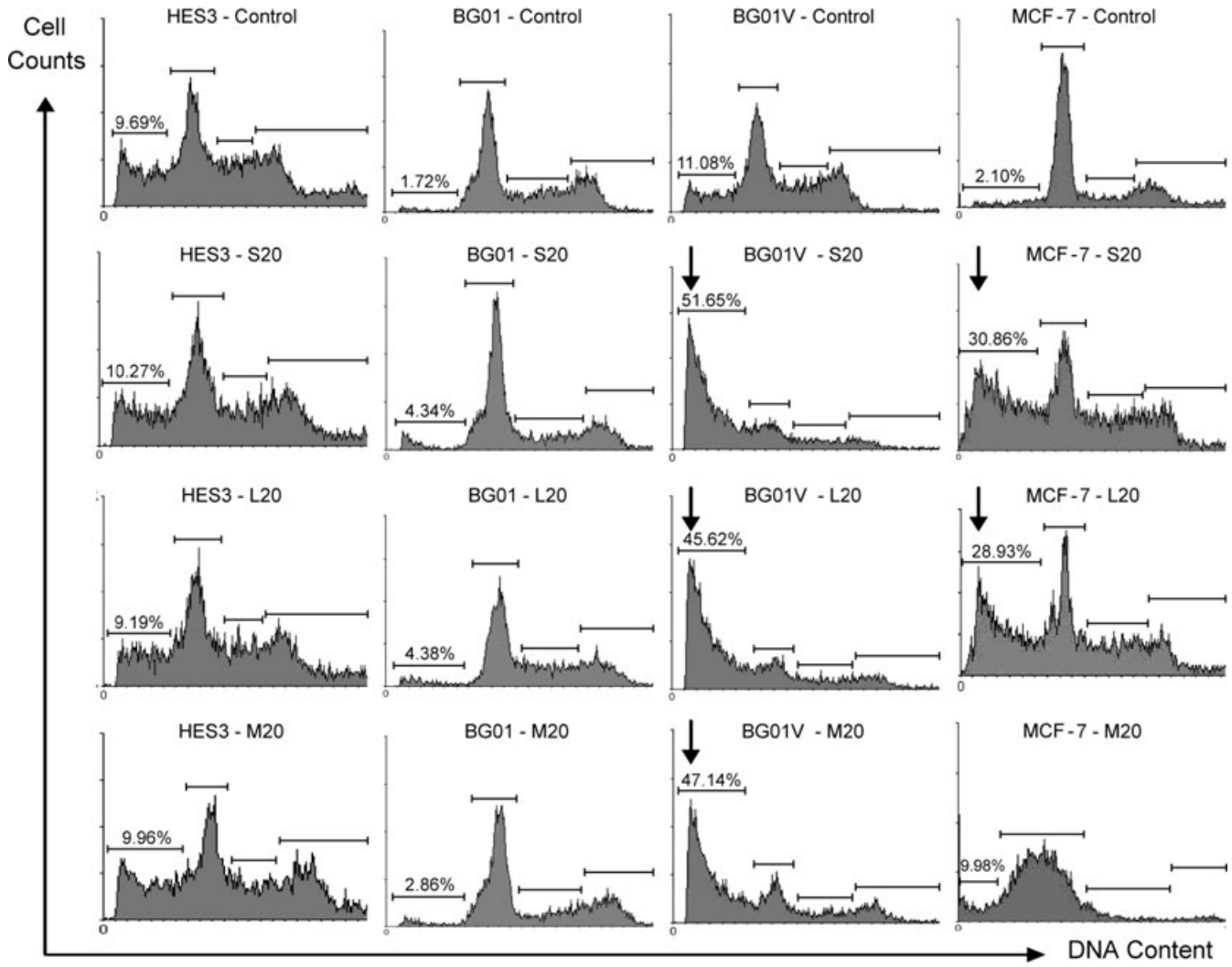

Figure 3 Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling images of normal human embryonic stem cells (HES3, BG01), human embryonic stem cell variant (BG01V) and breast adenocarcinoma cells (MCF-7) following treatment with simvastatin (20 μmol·L^{-1}), lovastatin (20 μmol·L^{-1}) or mevastatin (20 μmol·L^{-1}) for 48 h. The images are represented from top to bottom as A – positive control, B – negative control, C – simvastatin, D – lovastatin and E – mevastatin treated (20× magnification).

significant inhibition. Various differences exist between mESCs and hESCs both in culture characteristics and cellular properties and these could have led to the differential statin effects. Although, the mechanism behind the HES3 and BG01 cell resilience to statins is not clearly understood, probably its normal intrinsic cellular status and strong cell to cell interaction is an advantage as statins are reported to cause cell death in cells where the presence of small GTP-binding proteins namely 'rho' and 'rac' are altered as this leads to disturbances in various cellular functions (Green and Evan, 2002). This was confirmed in the present study where the statins (simvastatin, lovastatin, mevastatin) inhibited the karyotypically abnormal BG01V and MCF-7 cells at high concentrations suggesting that the inhibitory effects are specifically targeted at abnormal cells which might have different cellular functions due to their inherited gene mutations. The results of this study showed clearly that there were differences in the efficacy of various statins, as simvastatin was the most potent, followed by lovastatin and mevastatin. Similar potency for simvastatin was also identified in the study involving mESCs (Lee *et al.*, 2007). The lack of effect observed with pravastatin was possibly because of its hydrophilic properties and lack of efficient transporters thereby limiting its ability to enter cells (van Vliet *et al.*, 1995).

In the present study, in addition to the cytotoxicity effects demonstrated by the MTT assay we have also clearly demonstrated the morphological changes in BG01V and MCF-7 cells following statin therapy. HES3 and BG01 cells were not affected by the various statins studied and they continued to grow in colonies with preservation of undifferentiated culture characteristics. However, BG01V and MCF-7 cells demonstrated various degrees of cytological damage from thinning of cytoplasm to vacuolations and eventual cell death. Furthermore, the normal hESCs (HES3 and BG01) were not affected even after extended treatment of up to 7 days with simvastatin at the highest concentration (20 μmol·L^{-1}) studied, whereas maximum cell death was seen with both BG01V and MCF-7 cells. The cell death observed with both BG01V and

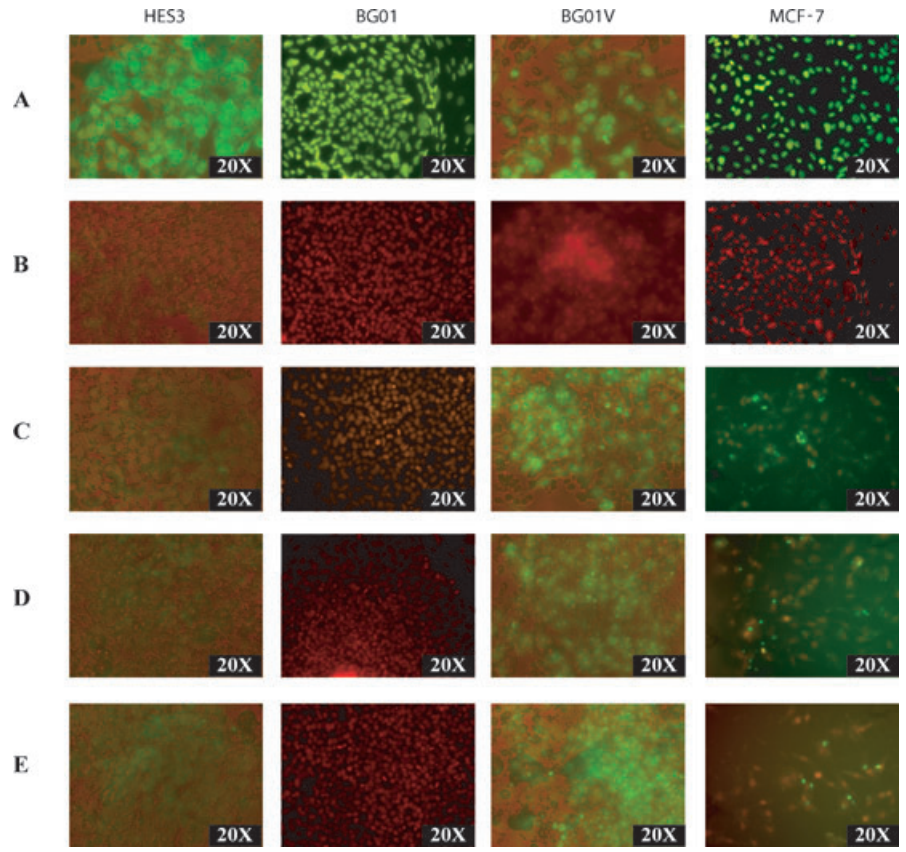

Figure 4 Flow cytometry representative images of cell cycle assays with normal human embryonic stem cells (HES3, BG01), human embryonic stem cell variant (BG01V) and breast adenocarcinoma cells (MCF-7) following propidium iodide staining. The cells were treated earlier with simvastatin (20 μmol·L^{-1}), lovastatin (20 μmol·L^{-1}) and mevastatin (20 μmol·L^{-1}) for 48 h. Peaks in the sub G1 phase, indicative of apoptosis were evident in BG01V cells following simvastatin, lovastatin and mevastatin as indicated by short arrows.

MCF-7 cells could be attributed to statins altering the membrane properties of these cells with loss of focal adhesion points, leading to cellular detachment as well as induction of apoptosis. Pravastatin did not cause cytological damage and the cells continued to survive even after the maximum treatment period studied (data not shown). As stated previously, pravastatin is hydrophilic and lacks efficient transporters thereby limiting its ability to enter cells (van Vliet *et al.*, 1995). These findings correlate with the results of the MTT assay and demonstrate differences in action between statins.

Protein-protein interaction and membrane association depend upon isoprenylation which are downstream products of the mevalonate pathway. In the present study, the isoprenoids FPP and GGPP demonstrated significant reversal of the inhibitory effects of cell proliferation observed with BG01V and MCF-7 cells following simvastatin, lovastatin and mevastatin treatment. With the BG01V cells, GGPP, but not FPP, was effective in reversing all the statins and it may be that geranylgeranylation is mainly inhibited following statin therapy in BG01V cells. Consistent with our findings, inhibition of RhoA geranylgeranylation was also reported to be the mechanism observed with mESCs following treatment with statins (Lee *et al.*, 2007). In addition, downstream inhibition of FPP and GGPP is the mechanism by which statins inhibit squamous cell cancer (Dimitroulakos *et al.*, 2001) and prostate cancer cells (Moyad and Merrick, 2005) *in vitro*.

Geranylgeranylpyrophosphate transferase and FPP transferase (FT) are enzymes involved in the production of GGPP and FPP, collectively known as isoprenoids, in the mevalonate pathway of cholesterol synthesis. In the present study, the enzyme inhibitors, GGTI-298 but not FTI-277, inhibited BG01V and MCF-7 cell proliferation, whereas both were ineffective in HES3 and BG01 cells. The inhibition observed with GGTI correlated with the results of adding the isoprenoid to reverse the statin effect, where GGPP was found to be more effective. Therefore geranylgeranylation appears to be the main mechanism behind BG01V and MCF-7 cell inhibition. Isoprenoids bind to several important cellular proteins including Ras and other GTP-binding proteins by a reaction called isoprenylation. Attachment of these proteins to cell membranes and their downstream functions involving GTP-binding proteins leading to alteration of gene transcription involved in cell proliferation, differentiation and apoptosis is due to isoprenylation (Maltese, 1990).

The observed reduction in the S phase and increase in G1 phase of the cell cycle assay with BG01V and MCF-7 cells may either be due to down-regulation of cell cycle promoters (Wachtershauser *et al.*, 2001) or up-regulation of cell cycle inhibitors such as P21 or P27 (Rao *et al.*, 1999; Ukomadu and Dutta, 2003). TUNEL-positive cells indicative of apoptosis were observed following treatment with simvastatin, lovastatin and mevastatin in BG01V cells. Statins are known to affect

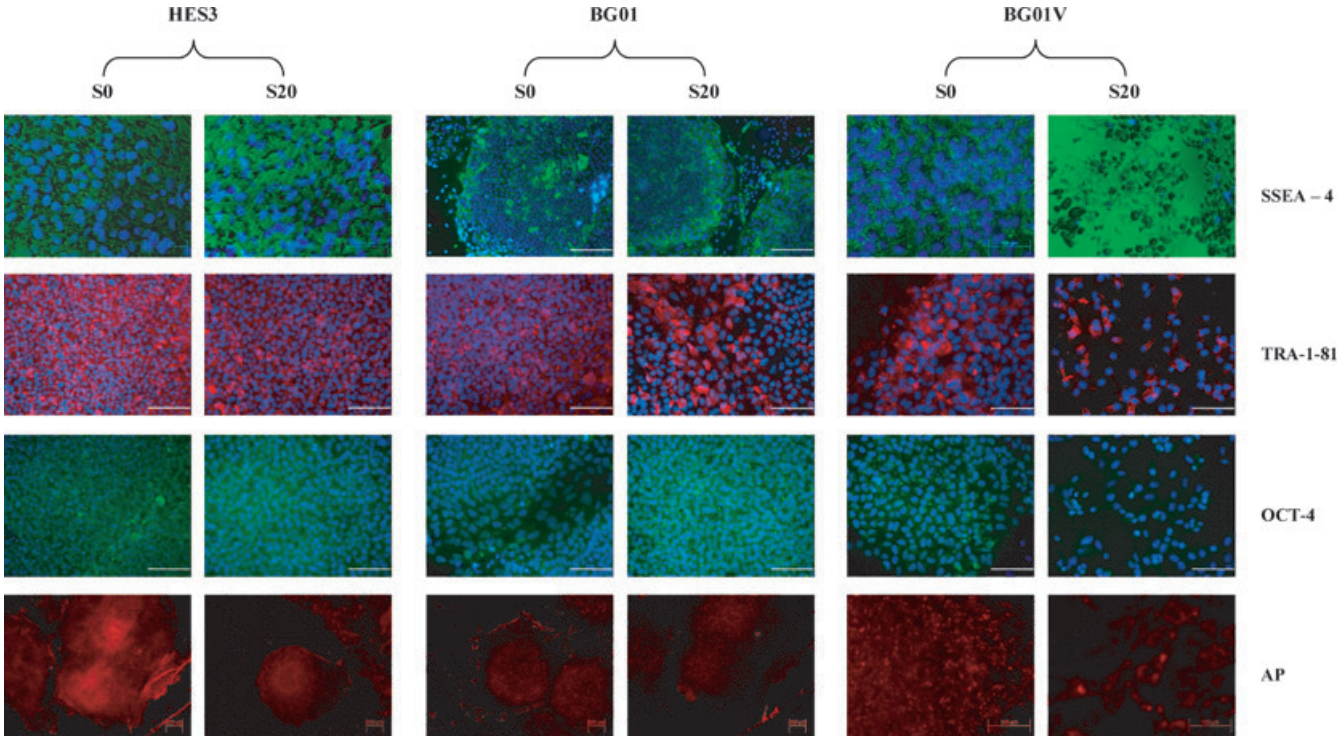

Figure 5 Immunohistochemistry images of normal human embryonic stem cells (HES3, BG01; shown as S0) and human embryonic stem cell variant (BG01V) for the stem cell surface markers antigens [stage-specific embryonic antigen (SSEA-4), tumour-related antigen (TRA-1-81), octamer-4 (OCT-4) and alkaline phosphatase (AP) following treatment with simvastatin (20 μmol·L^{-1}) (S20) for 48 h]. Goat anti-mouse FITC and DAPI nucleic acid stain was used (20× magnification). DAPI, 4′,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

both the intrinsic and extrinsic apoptotic pathways (Gniadecki, 2004). The expression of apoptotic related genes, namely BAX which is pro-apoptotic (Marsden *et al.*, 2002), BCL2 which is antiapoptotic (Oltvai *et al.*, 1993) and SURVIVIN (Li *et al.*, 1998) which is an inhibitor of apoptosis, were all assayed by qRT-PCR. The increase in BAX and a decrease in SURVIVIN expression observed after simvastatin treatment may be indicative of an increase in the activity of apoptotic signalling pathways in BG01V and MCF-7 cells leading to an inhibition of cell proliferation. In the normal HES3 and BG01 cells, simvastatin did not have any significant effect on the expression of SURVIVIN gene, but increased expression of the anti-apoptotic BCL2 in HES3 and decreased pro-apoptotic BAX in BG01 cells.

Stage-specific embryonic antigen-4 is a glycoprotein specifically expressed in early embryonic development and by undifferentiated hESCs and TRA-1-81 is a tumour-related antigen that is normally synthesized by undifferentiated hESCs. OCT-4 is a transcription factor that is essential for establishment and maintenance of undifferentiated hESCs. Immunophenotyping for SSEA-4, TRA-1-81 and OCT-4 following exposure of BG0IV to simvastatin showed only a few positive cells as most BG01V cells were dead following treatment with simvastatin, unlike HES3 and BG01 cells that were strongly positive confirming that the normal hESCs survived and remained undifferentiated even after simvastatin treatment. The results from this study correlated well with that of Plaia *et al.* (2006) who showed BG0IV cells remain undifferentiated and do not produce teratomas in immunodeficient mice even after 12 weeks of injection. Additionally, increased alkaline phosphatase expression which is a marker for undifferentiated hESCs (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000) was higher in HES3 and BG01 compared with BG01V cells after statin exposure. It therefore appears that abnormal hECSs may be quite sensitive to the statins *in vitro* at the doses in the present study and this sensitivity may be used to eliminate such abnormal cells if they were to be generated in a normal, undifferentiated hESC population in culture.

Growth differentiation factor-3, NANOG and OCT-4 are associated with the maintenance of pluripotency in hESCs (Richards *et al.*, 2004; Ezeh *et al.*, 2005). All three stemness-related genes were found to be considerably down-regulated in BG01V cells compared with HES3 and BG01 even upon treatment with lower concentrations of simvastatin (5 μmol·L^{-1}) for 5 days. With higher concentrations and extended periods of incubation, maximum cell death was encountered with BG01V cells and therefore it was difficult to compare between the cell lines studied. The observed decreases in stemness-related gene expression with HES3 cells is in line with the culture characteristics of hESCs where normal hESC colonies in bulk culture tend to undergo differentiation after 5 days of culture making it mandatory to passage after 5 days.

In the present study, statins were found to inhibit proliferation of karyotypically abnormal hESCs (BG01V) and MCF-7 but not karyotypically normal hESCs (HES3 and BG01). But statins were recently reported to suppress self-renewal and also inhibit cell proliferation in normal mESCs (Lee *et al.*, 2007). Many of the effects of statins are not clearly related to the reduction of cholesterol, and therefore HMG-CoA

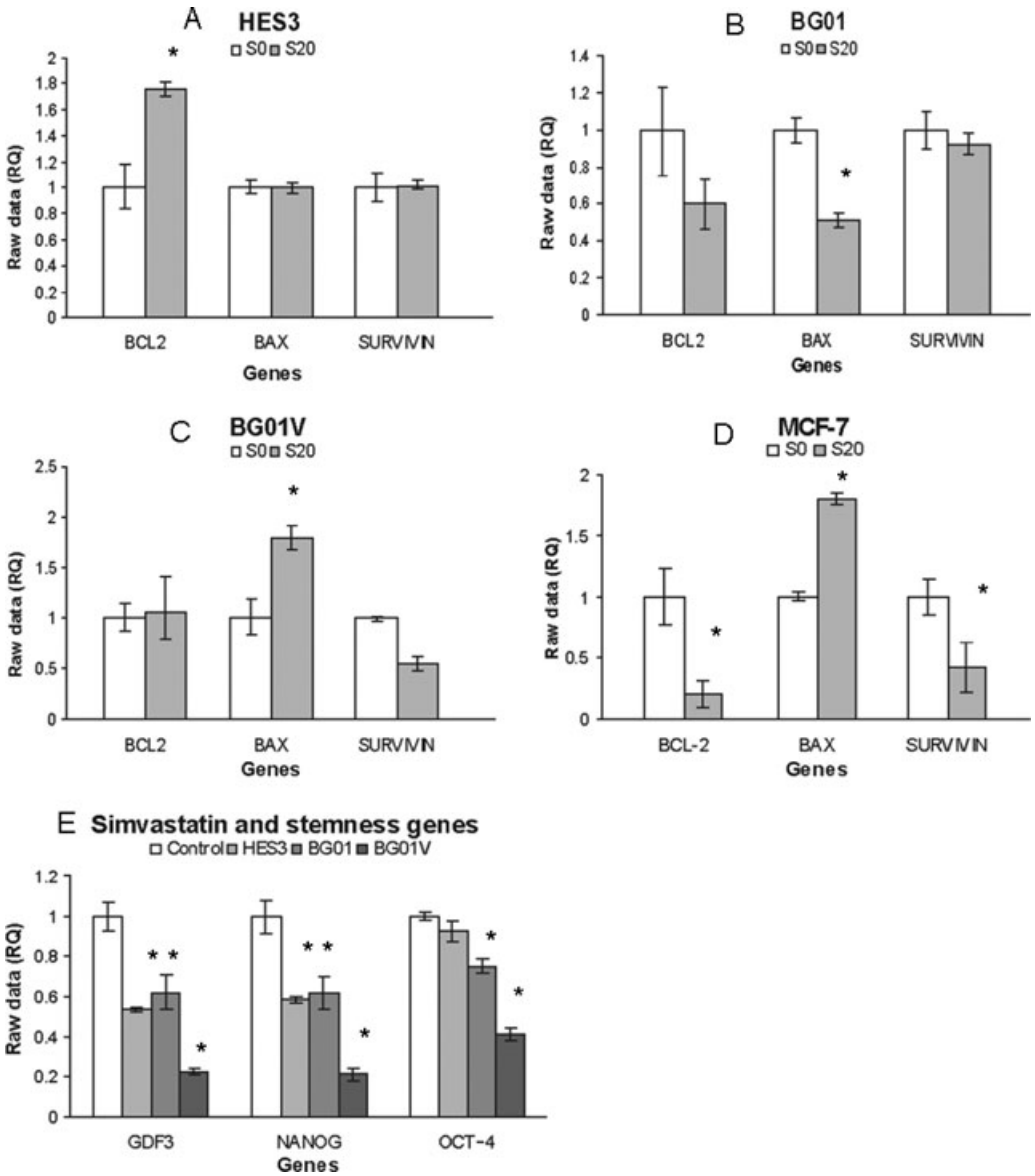

Figure 6 Gene expression profile (qRT-PCR) of *BCL2*, *BAX* and *SURVIVIN* under control conditins (S0) and following treatment with simvastatin (20 μmol·L^{-1}; S20) for 72 h in normal human embryonic stem cells (A – HES3, B – BG01), human embryonic stem cell variant (C – BG01V) and breast adenocarcinoma cells (D – MCF-7). E – Gene expression profile of *GDF3*, *NANOG* and *OCT-4* following treatment with simvastatin (5 μmol·L^{-1}) for 5 days in HES3, BG01 and BG01V cells. Data analysis and relative quantitation were performed using the comparative Ct (ΔΔCT) method. Values shown are means ± SD from two experiments with triplicate samples for each. *$P < 0.05$, significantly different from the untreated control (S0): one-way ANOVA; with Bonferroni's multiple comparisons (E) and Students *t*-test (A–D). PCR, polymerase chain reaction.

reductase-independent effects of statins must also be given due consideration as differential effects of statins are observed with different stem cell types. Studies are in progress to investigate whether statins could enhance significantly the prolonged undifferentiated growth of hESCs in culture following dissociation into single cells like that observed with Y-27632 (a ROCK inhibitor), as statins also act through the Rho/ROCK pathway.

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Conflict of interest

All authors have no conflict of interests and also no competing financial interests exist.

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