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The impact of statins on biological characteristics of stem cells provides a novel explanation for their pleiotropic beneficial and adverse clinical effects

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Izadpanah R, Schächtele DJ, Pfnür AB, Lin D, Slakey DP, Kadowitz PJ, Alt EU. The impact of statins on biological characteristics of stem cells provides a novel explanation for their pleiotropic beneficial and adverse clinical effects. *Am J Physiol Cell Physiol* 309: C522–C531, 2015. First published July 29, 2015; doi:10.1152/ajpcell.00406.2014.—Statins reduce atherosclerotic events and cardiovascular mortality. Their side effects include memory loss, myopathy, cataract formation, and increased risk of diabetes. As cardiovascular mortality relates to plaque instability, which depends on the integrity of the fibrous cap, we hypothesize that the inhibition of the potential of mesenchymal stem cells (MSCs) to differentiate into macrophages would help to explain the long known, but less understood "non-lipid-associated" or pleiotropic benefit of statins on cardiovascular mortality. In the present investigation, MSCs were treated with atorvastatin or pravastatin at clinically relevant concentrations and their proliferation, differentiation potential, and gene expression profile were assessed. Both types of statins reduced the overall growth rate of MSCs. Especially, statins reduced the potential of MSCs to differentiate into macrophages while they exhibited no direct effect on macrophage function. These findings suggest that the limited capacity of MSCs to differentiate into macrophages could possibly result in decreased macrophage density within the arterial plaque, reduced inflammation, and subsequently enhance plaque stability. This would explain the non-lipid-associated reduction in cardiovascular events. On a negative side, statins impaired the osteogenic and chondrogenic differentiation potential of MSCs and increased cell senescence and apoptosis, as indicated by upregulation of p16, p53 and Caspase 3, 8, and 9. Statins also impaired the expression of DNA repair genes, including *XRCC4*, *XRCC6*, and *Apex1*. While the effect on macrophage differentiation explains the beneficial side of statins, their impact on other biologic properties of stem cells provides a novel explanation for their adverse clinical effects.

stem cells; statin drugs; atherosclerotic plaque; macrophages; cardiovascular events

SINCE THEIR INTRODUCTION over 20 years ago, statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) have become one of the most widely prescribed medications for treatment of hypercholesteremia and cardiovascular diseases. The enzyme inhibition in the liver, the major site for cholesterol biosynthesis, results in reduction of plasma cholesterol, causing increased synthesis of hepatic cell surface low-density lipoprotein (LDL) receptors. This induces an increased hepatic uptake of plasma LDL with reduced circulating levels (1). In addition to cholesterol lowering, statins are known to modulate cellular functions such as cell proliferation and apoptosis through the inhibition of the formation downstream of intermediates in cholesterol synthesis. Statins also have been associated with pleiotropic effects unrelated to the lowering of LDL, including immunosuppressive and immunomodulatory actions (23). In addition to their highly beneficial clinical effects, long-term use of statins has been associated with adverse effects including myopathy, neurological side effects, and an increased risk of diabetes (22, 34, 44, 50).

Atherosclerosis is a chronic disease that can remain asymptomatic through decades of life. It is associated with accumulation of LDL, macrophages, T cells, smooth muscle cells, proteoglycans, collagen, calcium, and necrotic debris in the vessel wall. This accumulation is called a fatty streak and constitutes the earliest histopathologic stage of atherosclerosis (18). Low endothelial shear stress also contributes to atherosclerotic plaque formation, vulnerability, and rupture (55). The typical atherosclerotic plaque has a lipid core and a fibrous cap. Following initial vascular injury, monocytes infiltrate beneath the endothelium, differentiate into macrophages, phagocytose oxidized LDL and are transformed into foam cells. The cellular components within the plaque, mainly endothelial cells and monocytes/macrophages, express adhesion molecules [e.g., vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and P selectin] and chemokines [e.g., monocyte chemoattractant peptide (MCP)-1]. This promotes the transmigration of leukocytes into the intima (55). In addition, the extent of oxidized LDL accumulation in the subendothelial space is a major stimulus for an ongoing inflammatory process. This inflammation is also clinically evidenced by an increased temperature in the vicinity of a vulnerable plaque (53). The inflammation cascade promotes a phenotypic change of vascular smooth muscle cells (VSMCs) from a "contractile" phenotype to an active "synthetic" state. These VSMCs in the synthetic state also migrate and proliferate from the media to the intima, where they produce excessive amounts of extracellular matrix that transforms the lesion into a fibrous plaque (17, 42). This enhances the pathologic intimal thickening which results in arterial remodeling (43).

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Mesenchymal stem cells (MSCs) are tissue-resident multipotent stem cells that have shown the ability to proliferate and differentiate into different cell lineages including the mesodermal adipogenic, osteogenic, chondrogenic, and myogenic lines, as well as into mesodermal, hepatogenic, and ectodermal neurogenic lineages (2, 4, 27, 29, 49). MSCs have been identified from a variety of tissues including bone marrow, adipose tissue, muscle, and heart (31, 32, 40, 56).

We, as well as others, have demonstrated that pluripotent MSCs are primarily located within vascular structures lining the abluminal side of blood vessels (13, 14, 25, 35). It has been shown that precursor cells in the stroma-vascular fraction from adipose tissue can develop into macrophages (11). MSCs are distributed ubiquitously throughout all tissue and are responsible for tissue repair and homeostasis. These early MSCs are mainly found in a quiescent state, and it is believed that upon stimulation by either internal or external stimuli, they reenter the cell cycle and progress towards proliferation and differentiation (47). This direct interaction of MSCs with other cell types within tissues is crucial for normal tissue homeostasis. For example, it has been shown that a combination of endothelial cells with MSCs is involved in vascular network formation (51). Previously, and for the first time, our group reported that tissue-resident stem cells are able to differentiate into macrophages as evidenced by gene expression, cell surface marker characteristics, cytokine production, and functional behavior (20). In addition, MSCs have been shown to have the potential to migrate in response to inflammatory cytokines (28, 46).

Since statins are used for an extended period of time, it is important to understand the long-term consequences of statin usage and how it might affect the biological properties of MSCs. In the present study, we investigated the effects of statins on the proliferation and the differentiation potential of MSCs, especially their effects on the potential of MSCs to differentiate into macrophages. The effect of statin treatment on macrophage function was assessed in U937 cells.

The results of these studies show that the ability of MSCs to differentiate into macrophages is reduced by statins and suggest that this novel pleiotropic effect may contribute to decreased inflammation and improved plaque stability in patients with cardiovascular disease.

MATERIALS AND METHODS

Isolation and Expansion of MSCs

Adipose tissue specimens were obtained under a protocol approved by the Institutional Review Board of the Tulane University Health Sciences Center; all subjects have been informed and written consent was obtained. MSCs were isolated from adipose tissue of healthy donors between the ages of 20 and 65 yr using previously described methods (6, 27) and for later analysis were grouped into cells from young donors (mean 38 yr old) and old donors (mean 56 yr old). Briefly, 50 g of tissue were minced and processed with enzyme (InGeneron, Houston, TX) at 37°C. Following this, cells were subjected to RBC lysis buffer (BioWhittaker, Walkersville, MD). The cells were then plated at a fixed density in α-MEM medium, supplemented with 20% fetal bovine serum (Atlanta Biological, Atlanta, GA), 1% L-glutamine, and 1% penicillin-streptomycin (Cellgro, Herndon, VA) at 37°C with a 5% CO_2 atmosphere. Upon reaching 70% confluency, cells were passaged further. Then MSCs were treated with either reported serum concentration of pravastatin (55 ng/ml) and atorvastatin (65 ng/ml)(8) or 10 times the reported serum concentration of pravastatin and atorvastatin (550 and 650 ng/ml, respectively) for two passages.

Colony-Forming Unit

MSCs from a younger and older age group were plated at densities of 1,000, 500, 250, 100, 50, and 25 cells/cm^2 in 12-well dishes and were treated with pravastatin and atorvastatin. Untreated MSCs served as controls. Cells were cultured for 10 days before they were fixed and stained with 1% crystal violet in methanol. Colonies with diameters >3 mm were considered for counting.

Population Doubling Time and Cell Senescence

For the doubling time experiments, MSCs treated with the reported serum concentration and 10 times the reported serum concentration of pravastatin and atorvastatin were cultured at a density of 1,000 cells/cm^2. The cell numbers were counted at 48, 72, 96, and 120 h time points and were compared with untreated MSCs. At each time point, the population doubling time was calculated using the following equation: [log10 (N/N_0) × 3.33], where N is the total number of cells and N_0 is the number of seeded cells (48). Also, in a similar experiment the doubling time was assessed in atorvastatin and pravastatin serum concentration-treated cells and untreated cells from the two age groups. For the cell senescence assay, pravastatin- and atorvastatin-treated MSCs were cultured for 120 h before the β-galactosidase-reactive cells were counted (Sigma) (16).

Apoptosis assay. Flow cytometry analysis was performed on U937 cells treated with either serum concentration of pravastatin (55 ng/ml) and atorvastatin (65 ng/ml) or 10 times greater than serum concentration of pravastatin and atorvastatin (550 and 650 ng/ml, respectively) for two passages. Treated cells and untreated control cells were stained with Annexin V (BD Pharmingen) according to the protocol described by the manufacturer.

Cell cycle analysis. For analysis of cellular DNA content, U937 cells were fixed in 70% ethanol, rehydrated in PBS, treated for 30 min with RNase A (1 mg/ml), and stained with 1 μg/ml of propidium iodide (PI) for 5 min. The fluorescence intensity was determined using a fluorescence-activated cell sorter (FACS), and the percentage of cells in different phases of the cell cycle was assessed.

Quantitative Real-Time PCR Analysis

Total cellular RNA was isolated from pravastatin- and atorvastatin-treated MSCs and untreated cells using an RNeasy mini kit (Qiagen, Valencia, CA). cDNA was obtained by using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Real-time PCR assay was performed with 100 ng of target DNA. The following primers were used: GAPDH 5′-CGAGATCCCTCCAAAATCAA-3′ and 5′-GGTGCTAAGCAGTTGGTGGT-3′; CHEK1 5′-AGCG GTTGGT-CAAAAGAATG-3′ and 5′-CCCTTAGAAAGCCGGAAGTC-3′; E2F4 5′-GAGCCCATCTGCTGTTTCTA-3′ and 5′-CTGAGCT-CACCACTGTCCTT-3′; APEX1 5′-TGTGTGGA GACCTCAAT-GTG-3′ and 5′-GTAGGCATAGGGTGTGTTGG-3′; Caspase3 5′-CCCCTGGATCTACCAGCATA-3′ and 5′-TGTCTCTGCTCAG-GCTCAAA-3′; Caspase8 5′-AACCTCG GGGATACTGTCTG-3′ and 5′-CCTGTCCATCAGTGCCATAG-3′; p53 5′-TCTACCTC-CCGCCATAAAA-3′ and 5′-CTCCTCCCCACAA CAAAAC-3′; BMP-6 5′-AACCTGGTGGAGTACGACAA-3′ and 5′- CGGGT-GTCCAACAAAAATAG-3′; COL2A1 5′-TCACGTACAC-TGC CCTGAAG-3′ and 5′-TGCAACGGATTGTGTTGTTT-3′; COL10A1 5′-CTGGGACCCCTCTTGTTAGT-3′ and 5′-TTC-CAGTCCTTGGGT CATAA-3′; *CD4*: 5′-GTA GTA GCC CCT CAG TGC AA-3′, 5′-AAA GCT AGC ACC ACG ATG TC-3′; *CD14*: 5′-ACA GGA CTT GCA CTT TCC AG-3′, 5′-TCC AGG ATT GTC AGA CAG GT-3′; *CD68*: 5′-CAA CTG CCA CTC ACA

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GTC CT-3′, 5′-CAA TGG TCT CCT TGG AGG TT-3′; *MRC1*: 5′-GGC GGT GAC CTC ACA AGT AT-3′, 5′-ACG AAG CCA TTT GGT AAA CG-3′; (Realtimeprimers.com, Elkins Park, PA). All reactions were run at 58°C using a Bio-Rad iCycler (Bio-Rad, Hercules, CA).

Multilineage Differentiation

Osteogenic differentiation was induced as previously described (27). Differentiated cells were either fixed and stained with Alizarin Red (Diagnostic BioSystems), quantified for alkaline phosphatase activity (ALP) using the SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, San Jose, CA), or evaluated by real-time PCR analyses for the expression of lineage-specific genes. All analyses were carried out in triplicate. Adipogenic differentiation was determined in pravastatin-treated and untreated cultures of MSCs using previously described methods (2, 33). The adipogenic potential was evaluated by Oil Red O staining and real-time PCR analyses of lineage-specific genes (Diagnostic BioSystems, Pleasanton, CA). Chondrogenic differentiation was accomplished by using the Stempro chondrogenesis differentiation kit (Invitrogen, Carlsbad, CA). About 1×10^5 cells were spun in a 15 ml conical tube and grown in chondrogenic media for 21 days. Chondrogenic potential was evaluated by real-time PCR analyses for the expression of lineage-specific genes. Hematopoetic macrophage differentiation was induced on untreated and treated MSCs as previously described (20).

Immunohistochemistry

The pravastatin-treated and untreated macrophage differentiated cells were fixed, permeabilized, and incubated with human-specific primary antibodies for CD68 and NOS2 at a final concentration of 0.02–0.04 mg/ml, then incubated with 0.002 mg/ml of the matching secondary antibody. The signal was detected with a Leica TCS SP-2 confocal microscope equipped with Argon (457–477 nm; 488 nm, 514 nm) and HeNe lasers (543 nm; 633 nm) at a magnification of

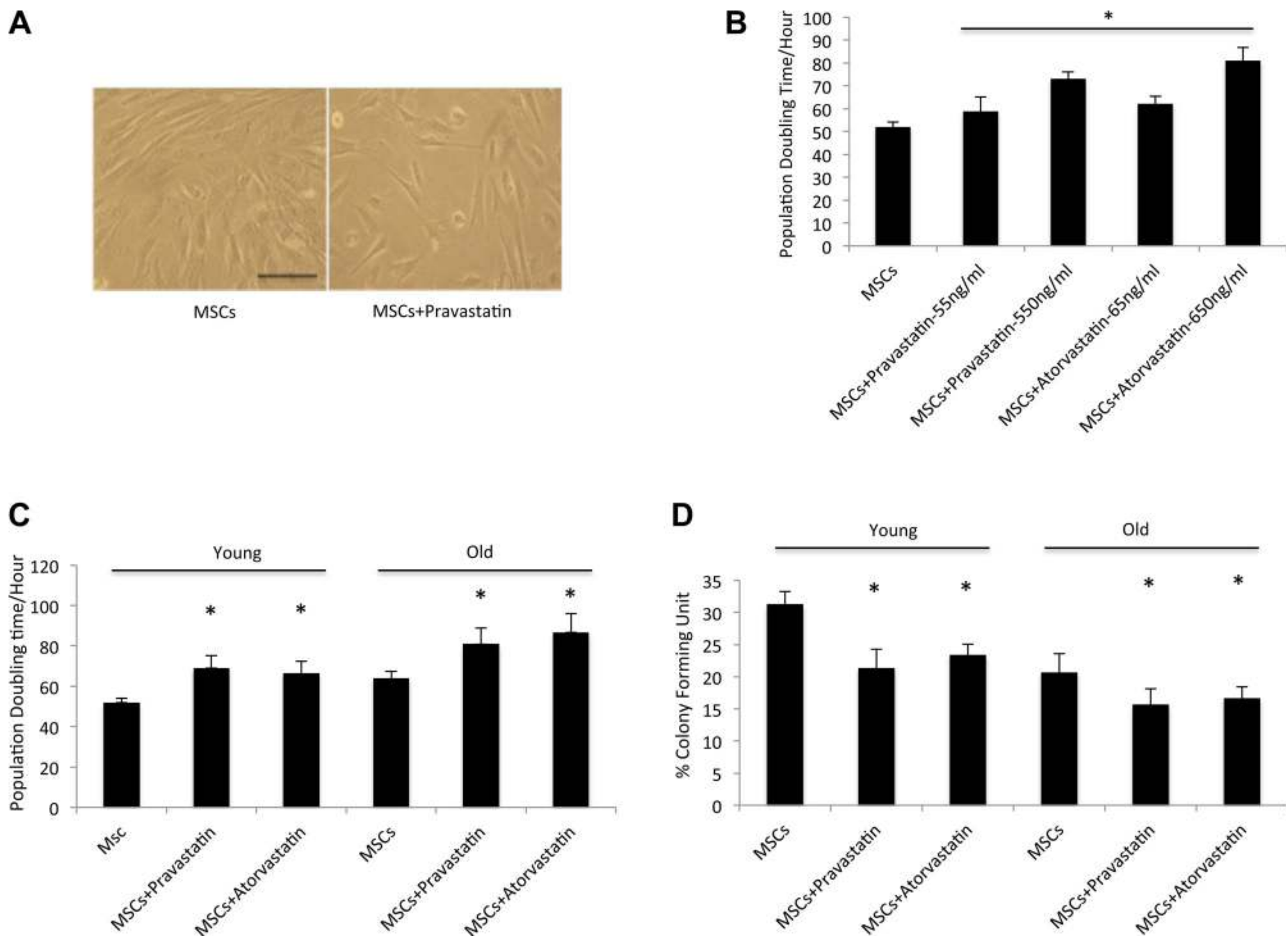

Fig. 1. Effect of statins on mesenchymal stem cell (MSC) growth. *A*: morphologic characteristics of pravastatin-treated MSCs. Pravastatin-treated MSC cultures demonstrate a flattened morphology with larger cytoplasmic areas compared with spindle-shaped fibroblastic morphology in control cultures (MSC+DMSO). Scale bar, 100 μm. *B*: dose-dependent effect of atorvastatin and pravastatin treatment on the proliferation of MSCs. MSCs were treated with pravastatin and atorvastatin in either serum concentration (55 ng/ml and 65 ng/ml) or 10 times the serum concentration (550 and 650 ng/ml, respectively) for two passages. Then the population doubling time of MSCs was assessed up to 120 h. ANOVA analysis indicates that treatment with pravastatin and atorvastatin has a significant effect on population doubling time. Group *t*-test indicates that each doubling time value is significant compared with untreated MSCs (n = 5/age group; $^*P < 0.05$). *C*: age-dependent effect of pravastatin and atorvastatin on MSC proliferation. A total of 1,000 cells/cm^2 were plated, and the population doubling time of MSCs from two young and old age groups (mean age for young age group is 38 yr and for old age group is 56 yr) was calculated up to 120 h. The graph presents the mean population doubling time. The mean doubling time was significantly increased in both atorvastatin- and pravastatin-treated MSC cultures. In young donors, pravastatin and atorvastatin produced significant differences in doubling time (group *t*-test; $^*P < 0.05$). *D*: colony counts from atorvastatin- and pravastatin-treated MSCs from two young and old age groups after 10 days, stained with 1% crystal violet. The data indicate a significantly smaller number of colonies in atorvastatin and pravastatin MSCs. Group *t*-test indicated that pravastatin and atorvastatin treatment significantly reduced the percentage of colony-forming units (CFU) in both young and old age groups (n = 5/age group; $^*P < 0.05$).

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HCX PL APO ×63/1.4 at 21°C. The data were processed with Leica confocal software.

Statistical Analysis

The data are expressed as means ± SE and were analyzed using a one-way ANOVA with unpaired *t*-tests. The criterion used for statistical significance was $P < 0.05$.

RESULTS

Effect of Statins on the Proliferation of MSCs

MSCs cultures were treated with pravastatin (55 ng/ml) and atorvastatin (65 ng/ml). These amounts correlate to clinically relevant concentrations of the respective statin in the serum of patients (8). Both types of statins changed MSC morphology from fibroblastic spindle-shaped cells into large and flattened cells with extended cytoplasmic areas (Fig. 1*A*). MSCs treated with pravastatin and atorvastatin showed an increased population doubling time compared with untreated MSCs. The effect of higher concentration of the statins on the population doubling time was investigated and when 10-fold higher than the reported concentrations of pravastatin and atorvastatin were used the population doubling time was increased when compared with the effective reported therapeutic plasma concentration of the statins (Fig. 1*B*).

We included MSCs from donors of a younger and an older age group to study a potential age-associated effect of statins. At least a 10-yr difference between the two age groups was selected to produce a more distinct data separation between cohorts. As depicted in Fig. 1*C*, treatment with either pravastatin or atorvastatin increased the population doubling time in MSCs from the younger age group, and this effect was more pronounced in the older age group (Fig. 1*C*). The assessment of the colony-forming unit potential (CFU) indicates a significant decrease in the CFU ability in both pravastatin- and atorvastatin-treated MSCs in both age groups compared with untreated MSCs from the same donor; however, the decrease was more pronounced in the older age group (Fig. 1*D*). This is important because as cholesterol levels constitute a significant risk factor for cardiovascular disease, a higher percentage of elderly patients are treated with statins for a prolonged period of time (52, 54).

In addition, we found statin treatment enhanced cell senescence by ~25%. This was further confirmed by gene expression analysis, which indicated a significant upregulation of different cell cycle regulators and genes associated with apo-

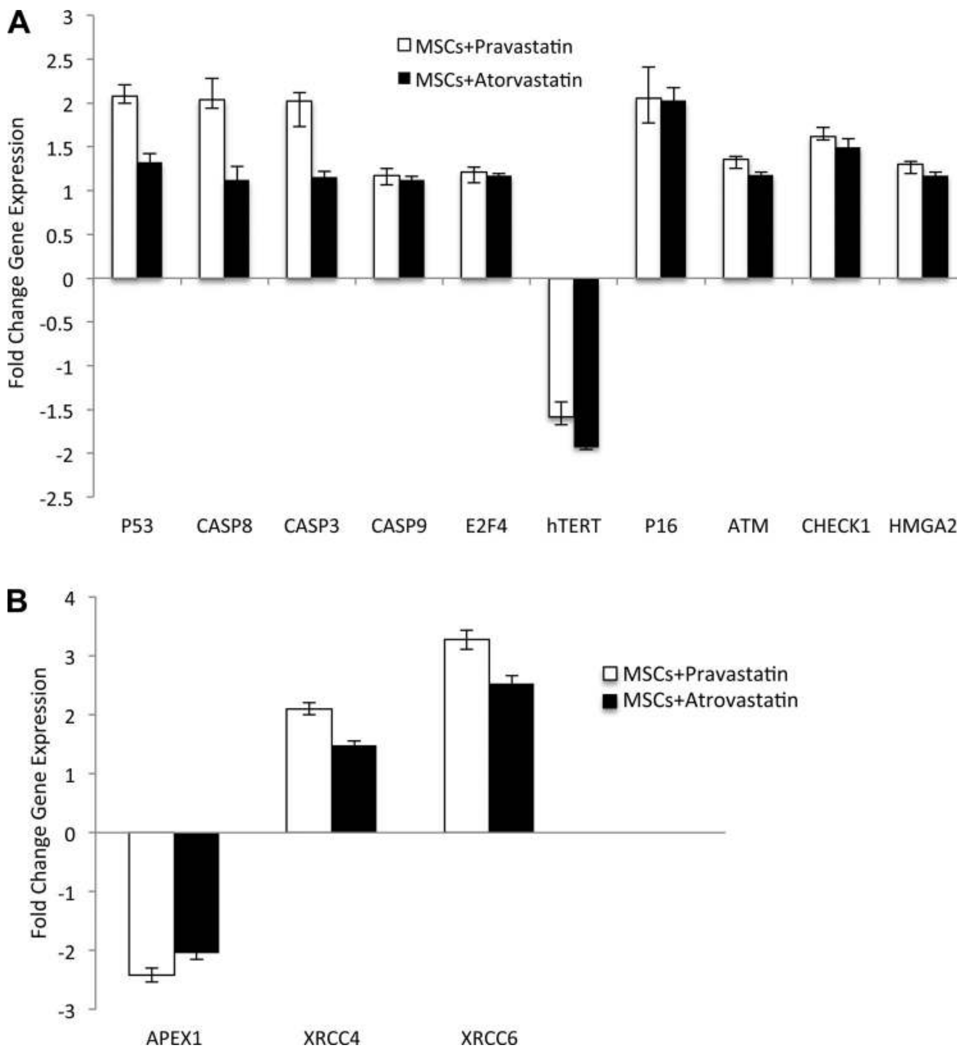

Fig. 2. Pravastatin and atorvastatin treatment alters gene expression. *A* and *B*: real-time RT-PCR analysis of genes associated with cell cycle, apoptosis (*A*) and DNA repair (B). All changes in gene expression are significant (group *t*-test; $P < 0.05$). The effect of pravastatin treatment on the expression of p53, CASP8, CASP3, XRCC4, and XRCC6 genes was significantly greater than the effect of atorvastatin treatment on MSCs. All values were normalized to untreated MSCs in three independent experiments (n = 9 donors/experiment).

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ptosis, including *chek1*, *HMGA2*, *ATM*, *E2F4*, *p16*, *p53*, *Caspase 3*, *8*, and *9*. Interestingly, the expression of *p53*, *Caspase 3* and *8*, and also *p16* was highly upregulated in the statin-treated MSCs. However, atorvastatin-treated cells showed less upregulation of *p53*, *Caspase 3* and *8* (Fig. 2*A*). The expression of DNA break repair genes was also significantly influenced by statin treatment, and *XRCC4* and *XRCC6* were upregulated, whereas the expression of *Apex1* was downregulated (Fig. 2*B*). These findings suggest that not only increased cell senescence but also an upregulation of DNA break repair genes can occur as a possible consequence of statin treatment.

Effect of Statins on the Differentiation Potential of MSCs

We investigated the effect of pravastatin on macrophage differentiation potential of MSCs. Cells were cultured in serum containing pravastatin and subsequently differentiated into macrophages according to the protocol described earlier (20). The pravastatin-treated MSCs displayed significantly lower morphologic changes compared with untreated MSCs, within the course of differentiation (Fig. 3*A*). Real-time PCR analysis of markers for the specific hematopoetic lineages showed that pravastatin treatment decreases the expression of *CD4*, *CD14*, *CD68*, *PROM1*, *HLA-DRB1*, and *MRC1* genes compared with the untreated cells (Fig. 3*B*). Immunohistochemistry analysis of the MSCs, which were differentiated in the presence of pravastatin, also indicated a decreased expression of specific markers of macrophages, CD68 and NOS2 (Fig. 3*C*). These results indicate an important and relevant inhibitory effect of pravastatin on the potential of MSCs to differentiate into macrophages.

Subsequently, we studied the effect of pravastatin on the osteogenic, chondrogenic, and adipogenic differentiation potential of MSCs. Pravastatin treatment reduced the osteogenic

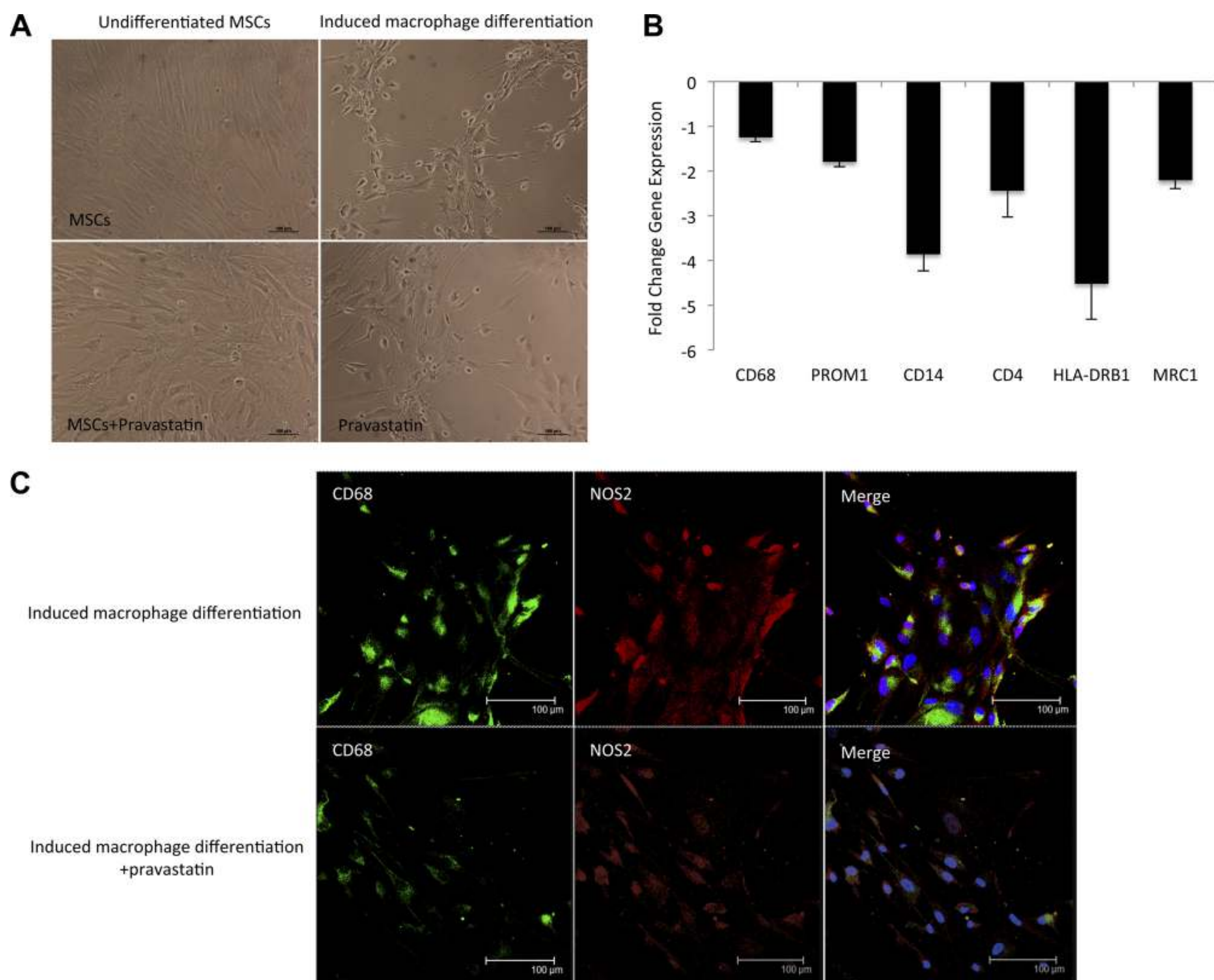

Fig. 3. Effect of pravastatin treatment on the potential of MSCs to be induced to differentiate into macrophages. *A*: pravastatin-treated MSCs were incubated for 9 to 12 days in macrophage differentiation media. Treated cells show only minimal change in morphology. *B*: real-time RT-PCR analysis of genes in pravastatin-treated macrophage-differentiated cells. The fold change in the expression of CD68, PROM1, CD14, CD4, HLA-DRB1, and MRC1 genes was significant compared with the expression of those genes in untreated differentiated MSCs. The graph presents the mean gene expression normalized to untreated MSCs in three independent experiments (n = 4 donors/experiment; group t-test, $P < 0.05$). *C*: cells were fixed for immunohistochemistry. Compared with untreated control, pravastatin-treated MSCs showed less immunoreactivity for CD68 and NOS2. Images (representative of triplicate experiments) were taken with a LeicaTCS SP-2 confocal microscope at an original magnification of ×63/1.4 oil. Scale bar, 100 μm.

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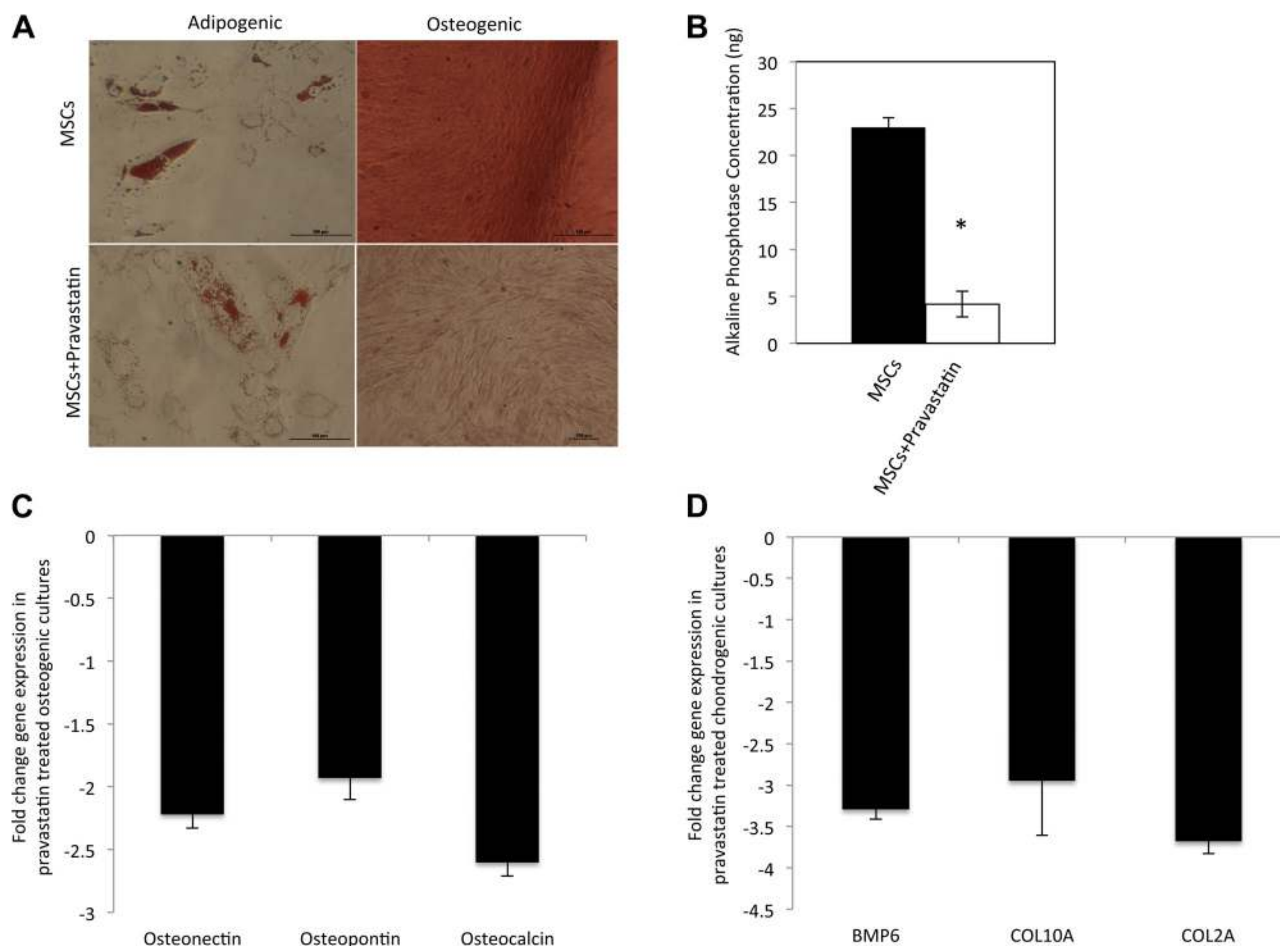

Fig. 4. Effect of pravastatin treatment on the potential of MSCs to differentiate into cells of mesodermal lineage. *A*: adipogenic and osteogenic potential of pravastatin-treated MSCs. Primary cultures of MSCs treated with pravastatin were induced for 6 to 9 days for adipogenic (*left*) or osteogenic differentiation (*right*). Individual cells were fixed and stained for adipocytes (Oil Red O), or mineralization (Alizarin Red). Scale bar, 100 μm. *B*: levels of alkaline phosphatase in pravastatin-treated MSCs following incubation of cells in the osteogenesis induction medium for 12 days ($n = 5$; group *t*-text; $P < 0.05$; $^*P < 0.001$). *C* and *D*: real-time RT-PCR analysis indicates significant fold change of genes in pravastatin-treated osteogenic (*C*) and chondrogenic (*D*) differentiated cells compared with untreated differentiated MSCs ($n = 5$; group *t*-test; $P < 0.05$).

potential while the adipogenic potential of MSCs did not change significantly (Fig. 4*A*). Compared with untreated controls, pravastatin-treated osteogenic differentiated MSCs expressed a significantly lower amount of alkaline phosphatase (Fig. 4*B*). RT-PCR analysis showed a remarkable decrease of osteoblast specific genes including osteonectin, osteopontin, and osteocalcin in pravastatin-treated and differentiated MSCs (Fig. 4*C*). In addition, MSCs treated with pravastatin and differentiated into chondrocytes showed a marked downregulation of chondrogenic lineage-specific genes including *BMP6*, *Col10A*, and *Col2A* (Fig. 4*D*). Although the present data show that pravastatin has an important inhibitory effect on the potential of MSCs to differentiate into macrophages, it also could be possible that statins enhance plaque stability by a direct effect on existing macrophages i.e., suppressing the proliferative activity or induction of apoptosis of existing macrophages. To provide information on the effect of statins on macrophage proliferation, the effect of atorvastatin and pravastatin on the cell cycle profile of U937 cells (a macrophage/monocyte cell line) was investigated. The results of our evaluation demonstrate that treatment with concentrations of statins similar to clinically relevant plasma levels and concentrations even 10 times greater than a therapeutic plasma level had little if any effect on the G1, S, and G2 phases of the cell cycle in U937 cells (Fig. 5*A*). Our studies further demonstrate that the therapeutic plasma level and 10 times greater than the plasma therapeutic levels of atorvastatin and pravastatin did not change the level of Annexin V, indicating no effect on apoptosis in U937 cells (Fig. 5*B*).

DISCUSSION

This study investigated the effect of statins on MSC characteristics including proliferation and differentiation potential and shows that these widely used lipid-lowering drugs impact the differentiation potential of MSCs.

Pravastatin and atorvastatin are type 1 and type 2 statins and are among the most widely used statins. One of the main differences between the type 1 and type 2 statins is the replacement of the butyryl group of type 1 statins by a fluorophenyl group in type 2 statins. Type 2 statins have higher polar interactions that cause tighter binding to the 3-hydroxy-3-methylglutaryl-CoA reductase (26). Prolonged administration of statins has been associated with a number of side effects

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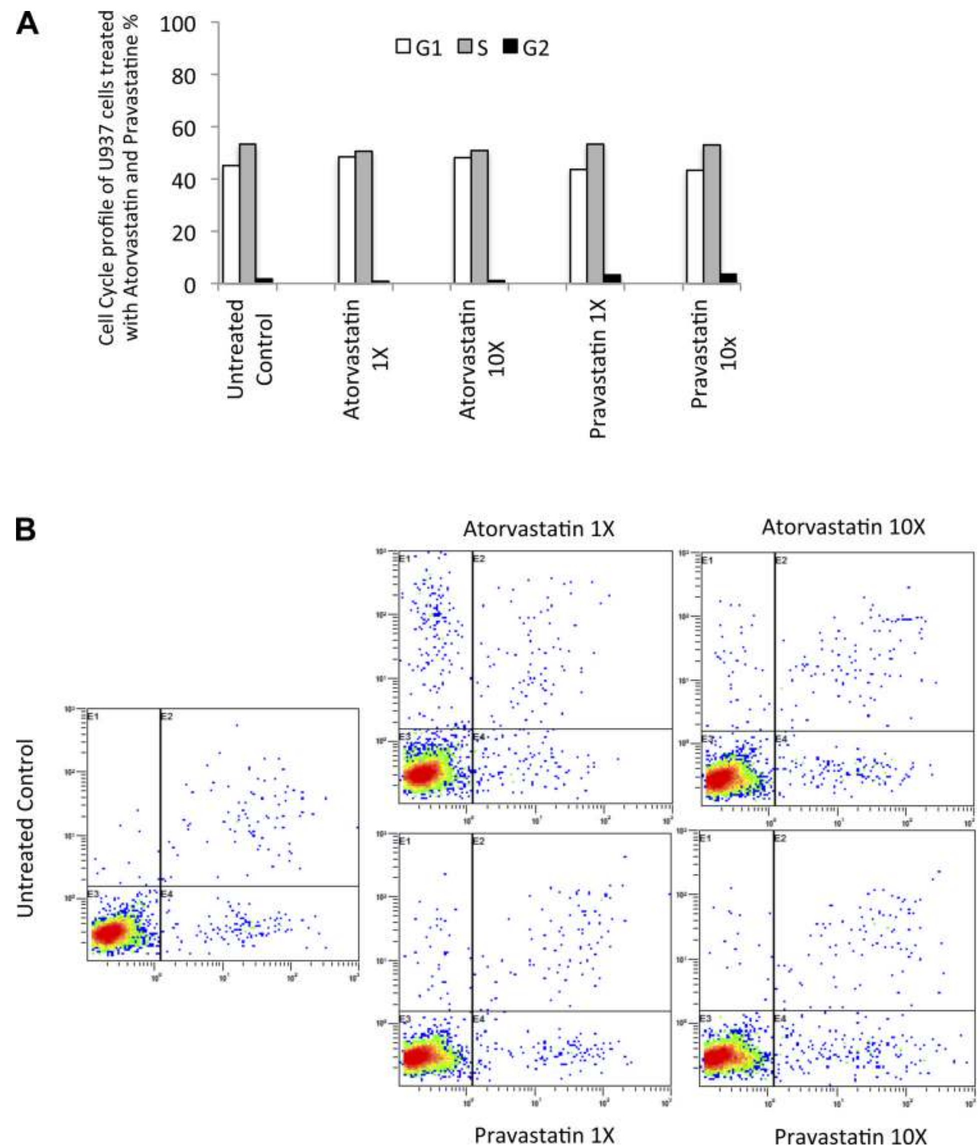

Fig. 5. Effect of statin treatment on U937 cells. *A*: dose-dependent effect of atorvastatin and pravastatin treatment on the cell cycle profile of U937 cells. U937 cells were treated with pravastatin and atorvastatin in either serum concentration (55 ng/ml and 65 ng/ml) or 10 times greater than the serum concentration (550 and 650 ng/ml, respectively) for two passages. Then the cell cycle profile was assessed. *B*: U937 cells treated with pravastatin and atorvastatin in either serum concentration (55 ng/ml and 65 ng/ml) or 10 times greater than the serum concentration (550 and 650 ng/ml, respectively) for two passages were then subjected to Annexin V level analysis using flow cytometry.

including myopathy, neuropathy, and increased risk of diabetes (22, 34, 44, 50).

The most important finding of our study is the demonstration that statins have a significant inhibitory effect on both MSC differentiation potential and their ability to differentiate into macrophages. It has been shown that inflammation in arterial plaque is dominated by macrophages. Specifically, the macrophage density in arterial plaque is associated with plasma cholesterol, LDL cholesterol, and plaque lipid content (21). Macrophages and macrophage-derived foam cells enhance the local inflammatory response and neointimal growth by increased expression of inflammatory cytokines and growth factors that significantly contribute to the neointimal tissue composition. Additionally, active inflammation enhances further migration and infiltration of macrophages and T cells. Macrophages promote plaque instability by weakening the fibrous cap through the release of metalloproteinases 2 and 9, thereby facilitating plaque disruption, thrombosis, and finally cardiovascular mortality (15, 21, 45). Our group has previously shown that MSCs migrate in response to inflammatory cytokines (28, 46). Also, we have shown that inflammatory cytokines enhance the differentiation of MSCs into macrophages (20). Based on these findings, we conclude that the inhibition of differentiation of tissue- and vascular-resident MSCs into macrophages could have a beneficial effect on local vascular inflammation, stabilize the thickness of the fibrous cap, help prevent plaque rupture and subsequent thrombotic events, and thereby reduce cardiovascular mortality. It is also possible that statin treatment may have an inhibitory effect on already existing macrophages. We investigated the effect of statin treatment on macrophage proliferation and apoptosis function by assessing the effect of pravastatin and atorvastatin on cell cycle and apoptosis in a monocyte/macrophage cell line (U937). The results of our evaluation indicate that statin treatment had little if any effect on the cell cycle or on apoptosis in the monocyte/macrophage cell line, suggesting that statins have no direct effect on macrophage proliferation.

Proliferation analysis revealed that both type 1 and type 2 statins slow the growth of stem cells by increasing the population doubling time in a dose-dependent manner and reduce

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CFU potential in MSCs. Cell senescence was enhanced in MSC cultures treated with statins. In the mechanism studies, the gene expression analysis showed altered expression of genes associated with cell cycle, apoptosis, and DNA break repair as both pravastatin and atorvastatin markedly increase the expression of p16, a marker of cell senescence known to prevent the S-phase entry and to arrest the cell cycle. Unlike MSCs, statins have been shown to reduce cellular senescence and apoptosis in different vascular cell types, including circulating bone marrow-derived endothelial progenitor cells, mature endothelial cells, and vascular smooth muscle cells (5, 10, 36). Previously, we have shown that similar cell replication changes occur in MSCs as an effect of aging (3). Our previous study showed that a combination of an increase in the MSCs doubling time, a decrease in their ability to form colonies (CFU potential) and increase in expression of P16 and apoptosis markers are associated with "aging". Interestingly, Fig. 1, *B* and *C*, indicates that the doubling time and the ability to form colonies as a marker of stemness in MSCs from young donors treated with pravastatin are comparable to those of untreated cells from the old age group, indicating that statins may advance the process of aging.

The expression of *p53*, *Caspase 3*, *8*, and *9* genes, which play an important role in the induction and execution of apoptosis, are more upregulated in both pravastatin- and atorvastatin-treated MSCs than in untreated cells from the same donor. In addition, the expression of DNA break repair genes including *Chek1* (a G2 arrest gene) and *HMGA2* (a DNA damage checkpoint between the G2 phase and mitosis) are also altered similar to aged MSCs (3). These results explain the mechanism of the increased doubling time and decreased CFU potential. The suppressive effect of statins on MSC differentiation potential and macrophage function will need to be studied further with cells isolated from human atherosclerotic plaques in the future.

We further found that statins reduce the osteogenic and chondrogenic differentiation potential of MSCs. A significantly lower level of alkaline phosphatase as a marker of osteogenic differentiation was detected in pravastatin-treated osteogenic MSC cultures. Pravastatin treatment also reduced the expression of osteoblast specific genes including osteonectin, osteopontin, and osteocalcin. While pravastatin exhibited only minimal effects on the adipogenic differentiation potential of MSCs, the chondrogenic potential of MSCs was negatively affected by pravastatin. RT-PCR analysis confirmed the downregulation of genes associated with chondrogenesis such as *BMP6*, *COL2A*, *COL10A* following treatment.

Statins are used for their beneficial effect in the treatment of patients with known cardiovascular disease. In fact, statin use has proven to significantly decrease hospital admissions, improve surrogate endpoint outcomes, and reduce overall mortality (19, 24, 41). However, several studies have shown that statins, when used for primary prevention, have little effect on cardiovascular disease (7, 9, 37–39). In addition, considering their possible side effects, long-term adherence to statin treatment poses a potential risk, especially among individuals without cardiovascular disease. The primary use of statins should be critically assessed, especially when considering the potential risks associated with statin use. These are, based on our findings, associated with a negative effect of statins on stem cell properties. In this context, the recently published findings of an increased risk of cataract formation with long-term statin therapy might represent a further clinical side effect potentially attributable to the impact of statins on stem cells and their reduced ability to differentiate into mature differentiated cells (7, 9, 30, 37–39). As a complementary explanation, initial unpublished data from our group indicate that stem cells have the ability to reverse the severity of cataract development.

We have previously shown that aging and chronic metabolic diseases such as diabetes reduce the differentiation and proliferation potential of MSCs (3, 12). The novel results of this study indicate that statins impair the differentiation potential of MSCs in a similar fashion to the process of aging and diabetes. Moreover, since there is no direct effect of statins on macrophage function, we believe that the known beneficial pleiotropic effect of statin treatment on cardiovascular mortality is based on the impact on MSCs to differentiate into macrophages and thereby effectively increase plaque stability. In light of our findings it is important to critically balance a possible benefit of statin therapy against the less favorable negative effects of statins. While here we present novel in vitro findings, future in vitro and in vivo studies should aim to better understand the underlying molecular mechanisms as well as to assess a possible reversibility of long-term statin treatment on stem cell function.

Limitations

Statins inhibit HMG-CoA reductase and reduce blood cholesterol levels. The beneficial effects of statins are usually attributed to reduced hepatic cholesterol synthesis and reduced cholesterol accumulation in the vessel wall, resulting in reduced inflammation and enhanced plaque stability. However, since mevalonate is the precursor for many other nonsteroidal isoprenoids, the inhibition of HMG-CoA reductase can result in numerous pleiotropic effects including the inhibition of MSC differentiation into macrophages. It is unknown whether this inhibition of macrophage differentiation is associated with a change in macrophage function. The effect of statin treatment on HMG-CoA reductase activity in MSCs is uncertain. Our data, however, demonstrate that treatment with statins did not alter the cell cycle profile or change the apoptosis level in a monocyte/macrophage cell line, suggesting that these agents have no direct effect on macrophage function.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.I., D.J.S., D.P.S., P.J.K., and E.U.A. conception and design of the research; R.I., D.J.S., A.B.P., D.L., and E.U.A. performed the experiments; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. analyzed the data; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. interpreted the results of the experiments; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. prepared the figures; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. drafted the manuscript; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. edited and revised the manuscript; R.I., D.J.S., D.P.S., P.J.K., and E.U.A. approved the final version of this manuscript.

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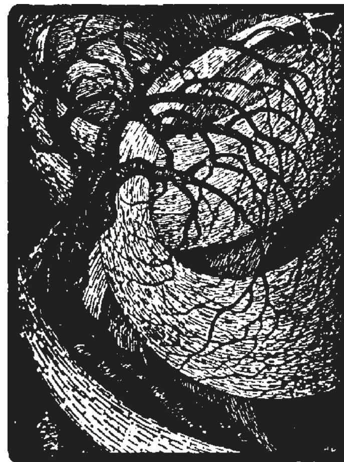

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