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mTOR Regulates Vascular Smooth Muscle Cell Differentiation From Human Bone Marrow–Derived Mesenchymal Progenitors

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Objective—Vascular smooth muscle cells (VSMCs) and circulating mesenchymal progenitor cells (MSCs) with a VSMC phenotype contribute to neointima formation and lumen loss after angioplasty and during allograft arteriosclerosis. We hypothesized that phosphoinositol-Akt-mammalian target of rapamycin-p70S6 kinase (PI3K/Akt/mTOR/p70S6K) pathway activation regulates VSMC differentiation from MSCs.

Methods and Results—We studied effects of PI3K/Akt/mTOR signaling on phenotypic modulation of MSC and VSMC marker expression, including L-type Ca(2+) channels. Phosphorylation of Akt and p70S6K featured downregulation of VSMC markers in dedifferentiated MSCs. mTOR inhibition with rapamycin at below pharmacological concentrations blocked p70S6K phosphorylation and induced a differentiated contractile phenotype with smooth muscle (sm)-calponin, sm-α-actin, and SM protein 22-alpha (SM22α) expression. The PI3K inhibitor Ly294002 abolished Akt and p70S6K phosphorylation and reversed the dedifferentiated phenotype via induction of sm-calponin, sm-α-actin, SM22α, and myosin light chain kinase. Rapamycin acted antiproliferative without impairing MSC viability. In VSMCs, rapamycin increased a homing chemokine for MSCs, stromal cell–derived factor-1–alpha, at mRNA and protein levels. The CXCR4-mediated MSC migration toward conditioned medium of rapamycin-treated VSMCs was enhanced.

Conclusions—We describe novel pleiotropic effects of rapamycin at very low concentrations that stabilized differentiated contractile VSMCs from MSCs in addition to exerting antiproliferative and enhanced homing effects. **(*Arterioscler Thromb Vasc Biol*. 2009;29:232-238.)**

Extensive neointima formation is a primary pathophysiologic mechanism of postintervention restenosis and transplant arteriosclerosis.[1] Until recently, the neointima formation was attributed to uncontrolled proliferation of vascular smooth muscle cells (VSMCs) that migrate from the media and replace the intima of affected arteries.[2] A phenotypic switch from a contractile to a proliferative and less differentiated synthetic phenotype accompanies the migration process.[3] Animal and human studies have indicated that neointimal cells may not only originate from the vascular media or adventitia, but also from bone marrow– and nonmarrow-derived progenitors. The cells migrate on injury to affected vasculature and actively become part of neointima formation by differentiating into VSMCs.[4,5] Mesenchymal stem cells (MSCs) in particular seem to have a high potential for neointimal growth.[6] The precise mechanisms responsible for differentiation and homing of VSMC precursors are unknown. The phosphoinositol-Akt-mammalian target of rapamycin-p70S6 kinase (PI3K/Akt/mTOR/p70S6K) pathway regulates cell growth and cell differentiation in response to nutrients, growth factors, and cytokines.[7] Pharmacological rapamycin inhibition of mTOR markedly reduces transplant vasculopathy progression in recipients of cardiac[8,9] and renal transplants.[10] Comparison of rapamycin-eluting stents with paclitaxel-eluting stents documented the superiority of rapamycin-eluting stents in terms of preventing late in-stent lumen loss and a lower incidence of in-stent restenosis.[11] Based on our previous findings on VSMC differentiation of human bone marrow MSCs,[12] we investigated activation and inhibition of the PI3K/Akt/mTOR/p70S6K pathway in the phenotypic modulation and expression of VSMC markers in MSCs. We also sought to determine how low-dose mTOR inhibition influences MSC proliferation and migration.

Methods

Cell Culture

Human MSCs were isolated by plastic adherence from aspirated iliac crest marrow from consenting normal adult donors and cultured as described previously.[12] Passages 2 to 6 were used for experiments.

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Under these culture conditions MSCs maintain multilineage differentiation capacity,[12] are positive for CD73, CD90, and CD105 and negative for CD11b, CD14, CD19, CD34, CD45, CD79alpha, and HLA-DR (supplemental Figure I, available online at http://atvb.ahajournals.org), thereby meeting the criteria for MSCs.[13] Human coronary artery VSMCs were obtained from Lonza (Cambiex Bio Science, Walkersville, Md).

Ca^{2+} Imaging

Cells were seeded onto glass coverslips and loaded with the Ca^{2+} indicator fluo-4-AM (Invitrogen). Changes in intracellular calcium were expressed as relative fluorescence changes, ie, F/Fo (with Fo indicating the fluorescence before stimulation and F the time-dependent fluorescence signal after stimulation) as described previously.[14]

Western Blotting

Western blots were performed according to standard procedures as described previously.[12]

BrdU Incorporation and MTT Assays

Cell proliferation was measured as BrdU incorporation (BrdU cell proliferation kit, Roche) in MSCs grown in presence of 10% FCS and rapamycin for 24 hours. Cytotoxicity was tested in MSCs incubated with rapamycin for 24 hours and addition of 1.5 mmol/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) during the last 4 hours. Absorption was measured at 570 nm in an ELISA reader.

Quantitative Real-Time PCR

VSMCs and MSCs were grown serum-free for 24 hours before incubation with rapamycin for 8 hours. Total RNA was extracted using the phenol/chloroform method and transcribed into cDNA with the PCR core kit (Roche). Quantitative real-time PCR was performed using a Roche Light Cycler system.

SDF-1α ELISA

VSMCs grown with serum-free DMEM were incubated with control or rapamycin for 72 hours. SDF-1α in supernatants was measured with a human SDF-1α ELISA (R&D Systems) and normalized to total protein content.

MSC Migration on Conditioned Medium of Rapamycin-Treated VSMCs

Supernatants of VSMCs cultured for 72 hours with or without rapamycin were applied to the lower chamber of a micro chemotaxis chamber (Neuro Probe). MSCs in serum-free DMEM in presence of control IgG or anti-CXCR4 (R&D Systems) were seeded in the upper chamber and allowed to migrate for 24 hours. Migrated cells on the bottom side were stained with crystal violet. Absorbance was measured with an ELISA reader at 570 nm. Results were normalized to total protein content of the supernatants.

Statistical Analysis

Values for calcium influx are given as mean±SEM and were compared by Student *t* test. Band intensities of smooth muscle markers were normalized to α-tubulin. Control without treatment was set 1.00. Analysis of multiple blots is expressed as mean±SD. The Kruskal—Wallis test was used for multiple comparisons, whereas pairwise comparison with control was done using Mann–Whitney *U* test. $P<0.05$ was considered statistically significant. Please see supplemental materials for expanded methods section and for detailed figure legends.

Results

Resting MSCs Have Functional VSMC-Like L-Type Calcium Channels

Voltage-dependent dihydropyridine-sensitive ($Ca_v1.2$) L-type Ca^{2+} channels represent a specific marker of differentiated

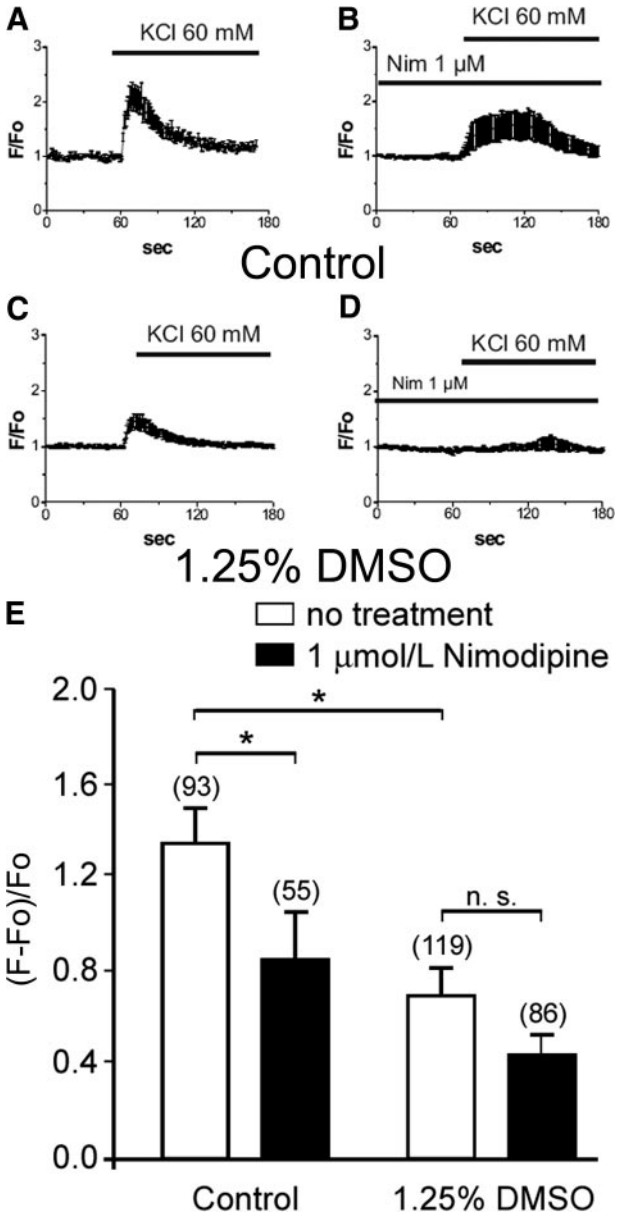

Figure 1. MSCs express functional VSMC like L-type Ca^{2+} channels. Calcium transients without (A) and after (B) nimodipine pretreatment. Calcium transients after treatment with DMSO without (C) and after (D) nimodipine pretreatment. E, Peak amplitudes of calcium transients. Numbers above bars indicate number of cells. Mean values±SEM, *$P<0.05$.

VSMCs[15,16] and play a key role in excitation-contraction coupling in terminally differentiated contractile VSMCs.[17] Based on our findings that resting MSCs with multilineage differentiation potential express smooth muscle specific contractile proteins,[12] we investigated whether or not they would display functional L-type $Ca_v1.2$ Ca^{2+} channels. We studied how L-type Ca^{2+} channel activation relates to the expression of contractile proteins during MSC differentiation and dedifferentiation. We applied culture conditions in which MSCs maintain their capability to differentiate into several cell types such as adipocytes and osteoblasts, as previously established.[12] We observed an increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) when MSCs loaded with the calcium

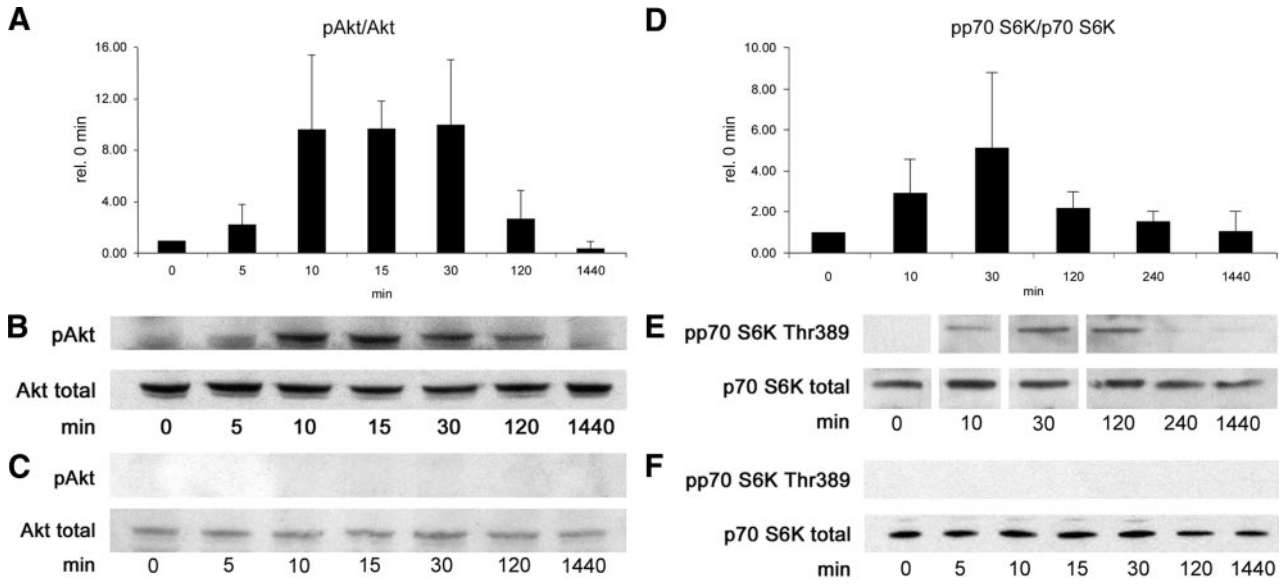

Figure 2. Activation of the PI3K/Akt/mTOR-pathway in MSCs by 1.25% DMSO. A, Densitometric analysis of pAkt normalized to total Akt. B, Representative Western blots. C, Ly294002 preincubation abolished Akt phosphorylation. D, Densitometric analysis of phosphorylated p70S6K normalized to total p70S6K. E, Representative Western blots. F, Rapamycin preincubation abolished p70S6K phosphorylation.

sensitive fluorescent dye fluo-4 were depolarized with isoosmotic 60 mmol/L KCl (Figure 1A and 1E). This increase in $[Ca^{2+}]_i$ was largely blocked by preincubation of the cells with 1 μmol/L nimodipine (Figure 1B and 1E). MSC dedifferentiation can be reproducibly achieved by incubation with nontoxic DMSO concentrations.[12] After incubation of MSCs with 1.25% DMSO for 8 days, the Ca^{2+} responses to KCl were significantly reduced and nimodipine did not have a significant effect on KCl-induced Ca^{2+} influx (Figure 1C, 1D, and 1E). The results suggest that multipotent MSCs share important features of VSMCs by expressing functional L-type $Ca_v1.2$ Ca^{2+} channels, which represent a specific marker of differentiated VSMCs and are required for excitation-contraction coupling.[14]

The PI3K/Akt/mTOR Pathway Is Activated in Dedifferentiated MSCs

Akt links signals downstream from PI3K-coupled growth factor receptors to mTOR, whereas p70S6K, which is downstream from mTOR, governs protein synthesis.[18] We tested the activation status of Akt and p70S6K in differentiated and dedifferentiated MSCs by immunoblotting with phospho-specific antibodies. Treatment with 1.25% DMSO induced a strong (10-fold) increase in phosphorylated Akt, the downstream target of PI3K (Figure 2A and 2B) without change in total Akt. The maximal phosphorylation occurred rapidly after 10 minutes and persisted for 30 minutes. Preincubation with Ly294002, a specific PI3K inhibitor, for 30 minutes completely blocked Akt phosphorylation (Figure 2C). DMSO also induced a strong (5-fold) increase in p70S6K phosphorylation at the mTOR-dependent Thr389 phosphorylation site (Figure 2D and 2E). Compared to Akt, the maximal increase in p70S6K phosphorylation occurred after 30 minutes, consistent with the more downstream location of p70S6K in the pathway. Preincubation with pharmacological 6 nmol/L rapamycin concentration for 30 minutes entirely abrogated p70S6K phosphorylation at Thr389 (Figure 2F). As expected, Akt phosphorylation was not influenced by rapamycin preincubation (data not shown). Preincubation with Ly294002, targeting PI3K upstream from mTOR, achieved the same inhibitory effect on p70S6K phosphorylation at Thr389 (data not shown).

PI3K/Akt/mTOR-Pathway Blockade Preserves the Differentiated MSC Phenotype and Induces Expression of VSMC Antigens

To further establish the notion that VSMC differentiation and the phenotypic switch from differentiated and contractile to a less differentiated MSC phenotype is regulated via the PI3K/Akt/mTOR pathway, we performed coincubation studies with specific inhibitors of different signaling elements. Under culture conditions in which MSCs retain their pluripotent differentiation potential, VSMC-like progenitors change from large cells with prominent stress fibers to spindle-shaped cells without stress fibers after 8 days of 1.25% DMSO incubation (supplemental Figure IIA and IIB). This morphological transformation was accompanied by marked downregulation of the smooth muscle marker proteins sm-calponin, MLCK, and sm-α-actin, indicating dedifferentiation of MSCs.[12] When we coincubated cells for 8 days with 1.25% DMSO together with the PI3K inhibitor Ly294002 at 15 μmol/L concentration or with 6 nmol/L rapamycin, MSCs preserved their VSMC-like phenotype (supplemental Figure IIC and IID).

In additional experiments, we tested whether or not PI3K inhibition mediated the switch toward differentiated contractile phenotype in MSCs and induces the expression of VSMC antigens. Quiescent MSCs with the contractile phenotype express the VSMC markers sm-calponin (Figure 3A), sm-α-actin (Figure 3B), SM22α (Figure 3C), and the short MLCK isoform (Figure 3D). Incubation with 1.25% DMSO down-

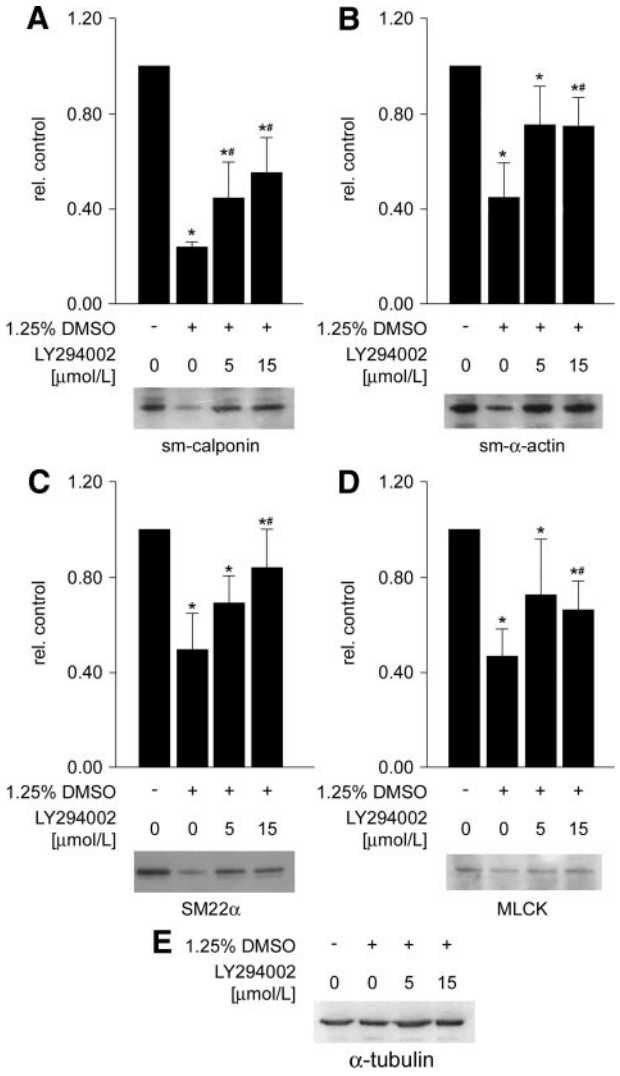

Figure 3. PI3K inhibitor Ly294002 induced upregulation of VSMC marker proteins in MSCs treated with 1.25% DMSO for 8 days. A, sm-calponin. B, sm-α-actin. C, SM22α. D, Short MLCK. E, α-tubulin. Representative Western blots and densitometric analysis normalized to α-tubulin. *$P<0.05$ for comparison with control, #$P<0.05$ for comparison with DMSO.

regulated all 4 markers. In contrast, blockade of PI3K with 5 and 15 μmol/L Ly294002 reversed DMSO-mediated downregulation of all smooth muscle markers, as shown by Western blot analysis.

Rapamycin Promotes VSMC Marker Expression in Quiescent MSCs

Our data supported the hypothesis that the PI3K/Akt/mTOR pathway regulates VSMC differentiation from MSCs and that activation of this pathway induces dedifferentiation and loss of the contractile phenotype. We next studied whether or not modulation of the downstream element, mTOR, would also enhance spontaneous VSMC differentiation from quiescent MSCs in culture. Incubation of MSCs with rapamycin for 8 days at concentrations even below the recommended levels used in transplant patients, as low as 1 nmol/L, resulted in upregulation of VSMC antigens sm-calponin (supplemental Figure IIIA), α-smooth muscle actin (supplemental Figure IIIB), SM22α (supplemental Figure IIIC), but had no influence on the short MLCK isoform (supplemental Figure IIID). The observed effects on VSMC antigen expression were not further enhanced by the recommended low pharmacological 6 nmol/L rapamycin concentration or at suprapharmacologic 20 and 100 nmol/L concentrations.

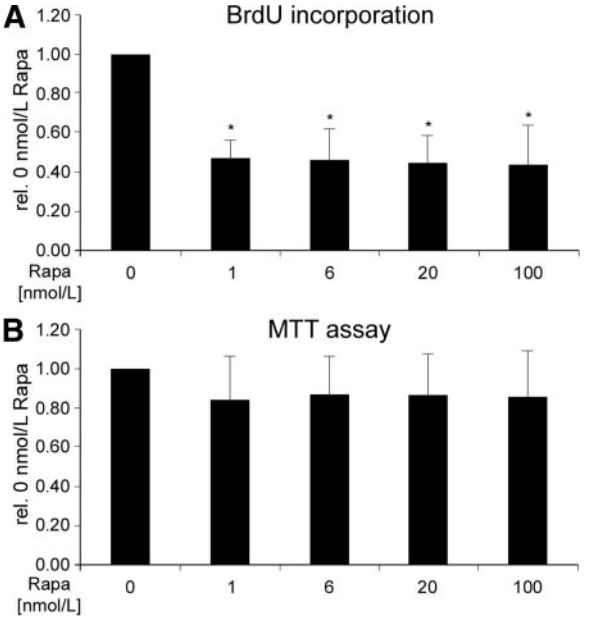

Figure 4. Rapamycin acts antiproliferative but not cytotoxic on MSCs. A, Measurement of incorporated BrdU during 24-hour incubation with rapamycin. B, MTT assay after 24 hours of treatment with rapamycin. n=3, mean values±SD, *$P<0.05$.

Rapamycin Acts Antiproliferative on MSCs Without Impairment of Viability

Beneficial effects of rapamycin and its derivatives on prevention of neointima formation during transplant vasculopathy or after angioplastic interventions were attributed to antiproliferative effects via prevention of cell cycle progression from G1 to S phase.[19] We tested the antiproliferative effects in MSCs cultured in medium containing increasing rapamycin concentrations for 24 hours by measuring BrdU incorporation into newly synthesized DNA. Rapamycin exerted a potent antiproliferative effect (−50%) at below pharmacological concentrations as low as 1 nmol/L (Figure 4A). Therapeutic concentrations of rapamycin and the derivative everolimus in transplant patients are in the 4 to 10 nmol/L range.[20,21] There was no additional proliferation inhibition at above pharmacological concentrations up to 100 nmol/L. To exclude the possibility that the observed decrease in DNA synthesis was related to cytotoxic effects, we analyzed cell viability by the MTT assay. MSCs were exposed to the same increasing rapamycin concentrations for 24 hours. We observed no toxic effects of rapamycin on MSCs, even at suprapharmacological concentrations (Figure 4B).

Rapamycin Induces SDF-1 Expression in VSMCs and Enhances MSC Migration

VSMCs are target cells of injury during allograft rejection or after angioplastic interventions. Vascular cells secrete the

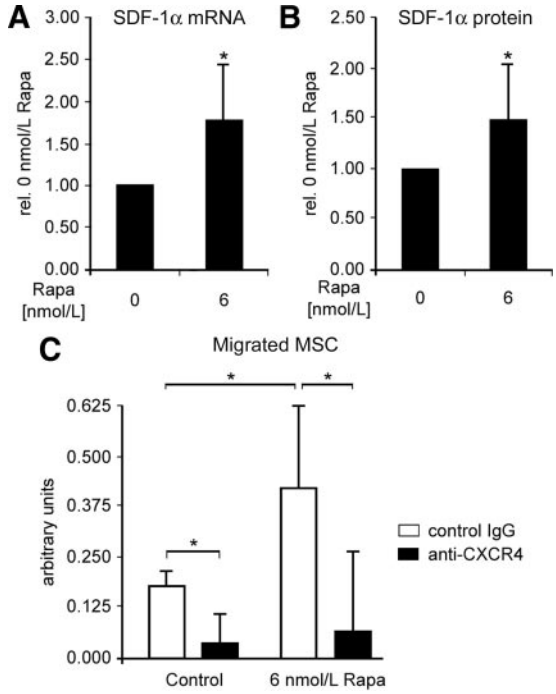

Figure 5. Rapamycin induces the homing chemokine SDF-1α and enhances migration of MSCs toward VSMCs. A, SDF-1α mRNA in rapamycin-treated VSMCs. B, Secreted SDF-1α in supernatant of VSMCs. C, Enhanced CXCR4-dependent migration of MSCs to conditioned medium of VSMCs treated with rapamycin. n=4, mean values±SD, *$P<0.05$.

chemokine SDF-1α as part of the response to injury and interact with target receptor CXCR4 expressed on MSCs.[22] SDF-1α stimulates MSC migration. Furthermore, there is evidence that MSCs are mobilized by and directed to sites of tissue injury by a SDF-1α gradient.[22] We tested how mTOR inhibition could affect VSMC SDF-1α secretion and influence mobilization and homing of MSCs. Human coronary VSMCs were incubated with pharmacological 6 nmol/L rapamycin concentrations and analyzed for SDF-1α mRNA expression and secretion of SDF-1α protein. SDF-1α mRNA was significantly upregulated after eight hours (Figure 5A), whereas secretion of SDF-1α protein (Figure 5B) increased after 72 hours of rapamycin incubation. We also documented enhanced CXCR4-mediated migration of MSCs toward conditioned medium of rapamycin-stimulated VSMCs in Boyden chamber model (Figure 5C).

Discussion

Recipient-derived hematopoietic and mesenchymal progenitor cells contribute to neointima formation after extensive injury to the vascular media. We identified a critical role for PI3K/Akt/mTOR pathway activation in VSMC differentiation in mesenchymal progenitors. Pharmacological PI3K inhibition by Ly294002, as well as mTOR inhibition by rapamycin, modulated several aspects of MSC physiology including induction of a VSMC-like differentiated state and induction of a protective contractile phenotype in MSCs. In parallel, rapamycin exerted a potent antiproliferative effect on MSCs without signs of toxicity. Finally, rapamycin also interfered with mobilization and homing of MSCs by induction of SDF-1α in VSMCs and MSC migration. We describe novel effects, even at very low doses of mTOR inhibition on MSCs, and propose protective phenotypic switching together with MSC homing modulation. We believe our findings serve to explain the unique potential of mTOR inhibitors that extend beyond antiproliferatiion and T-cell suppression.

The finding that rapamycin promotes a VSMC-like differentiated contractile phenotype in MSCs is compelling and may facilitate the understanding of several unexplained beneficial and adverse effects observed in transplant recipients treated with mTOR inhibitors or in patients with rapamycin-coated stents. Both rapamycin[23] and the derivative everolimus[8] are superior to other immunosuppressants in prevention and treatment of transplant vasculopathy in patients with cardiac allografts. Comparison of rapamycin-eluting stents with paclitaxel-eluting stents documented the superiority of rapamycin-eluting stents in terms of preventing late in-stent lumen loss and a lower incidence of in-stent restenosis.[11] Similar results were reported in high-risk patients with diabetes mellitus.[24] Stronger beneficial effects of rapamycin-eluting stents were thus far attributed to more prominent effects of rapamycin on VSMC proliferation and migration, lesser cytotoxicity, and stronger effect on Akt activation compared to paclitaxel.[25] Similar to local drug delivery, oral rapamycin for 10 days beginning 2 days before angioplastic reintervention resulted in a significantly reduced restenosis rate in rapamycin-treated patients compared to control patients.[26]

Some of the rapamycin-induced effects on MSCs with the VSMC-like phenotype in terms of potent antiproliferative and lack of cytotoxic effects are consistent with studies performed in VSMCs.[27] However, proliferation inhibition alone is not sufficient to promote VSMC differentiation.[28] Subtle changes in signaling mechanisms may be responsible for injury-specific phenotypic changes. Loss of the contractile phenotype is faster and more prominent in transplant vasculopathy compared to atherosclerosis of native vessels.[29] Transition of the contractile to the synthetic phenotype is also regulated by mTOR in VSMCs.[30] Rapamycin-mediated effects on VSMC differentiation were attributed to mTOR/PI3K feedback in VSMCs.[31] The authors proposed that a unique signaling pattern where S6K1, downstream from mTOR, is inhibited and Akt, upstream from mTOR but downstream from PI3K, is activated results in optimal differentiation signals and induction of contractile phenotypic markers.[31] We did not focus on mTOR/PI3K feedback. Nevertheless, Akt phosphorylation was not influenced by rapamycin in quiescent MSCs. Furthermore, we documented not only reprogramming toward the differentiated state in dedifferentiated cells, but also increased VSMC marker protein expression in resting MSCs after mTOR Inhibition. Interestingly, VSMCs were described as "multifunctional mesenchymal cells" forty years ago,[32] anticipating the later findings of MSCs as subpopulations of VSMCs within the vascular wall. Location and type of injury may determine the importance of MSC accumulation.[33]

Severe injury of the vascular media that occurs after angioplasty or during allograft vascular rejection is prerequisite for progenitor cell homing and lesion repair.[33] Peritransplant ischemic injury is associated with significant SDF-1α

upregulation in cardiac allografts.[34] SDF-1α probably serves to recruit stem cells in response to hypoxic injury.[35] Rapamycin inhibits VSMC migration.[36] Rapamycin increased expression and secretion of SDF-1α in VSMCs, an effect that has not been previously described. CXCR4-dependent MSC migration to conditioned medium of rapamycin-treated VSMCs was also enhanced. Thus, rapamycin probably enhances, or at least does not impair, MSC migration and homing to target cells after vascular injury. Most studies on progenitor cell homing and differentiation in vitro and in vivo refer to cells of hematopoietic origin.[22] Hematopoietic cells isolated from buffy coats cultured in platelet-derived growth factor (PDGF)-BB–enriched medium can also acquire the VSMC-like phenotype and express α-smooth muscle actin and calponin.[37] In mouse model of severe wire-induced femoral artery injury, locally delivered rapamycin decreased the number of bone marrow–derived CD45-positive hematopoietic cells and attenuated neointima formation.[38] Induction of cell death in endothelial progenitors after rapamycin treatment may offer an explanation for this observation. Our data add to the complexity of rapamycin-mediated actions that also may help explain effects observed in other lineages. Rapamycin may influence differentiation process through opposing cell-type specific effects. For example, rapamycin induces hematopoietic differentiation,[39] yet inhibits adipocyte differentiation[40] and chondrogenesis.[41] The PI3K/Akt/mTOR pathway is also critically involved in tumor biology. Simultaneous promotion of beneficial phenotypic switch together with antiproliferative and antiangiogenic effects may explain the reduced incidence of tumors in patients treated with mTOR inhibitors.[42] Bone marrow–derived MSCs are also implicated in the initiation and progression of cancer metastasis.[43]

Dose-related side effects limit the use of mTOR inhibitors in transplant patients. Our finding that the pleiotropic effects were already operative at the 1 nmol/L concentration, 8-fold below the recommended trough rapamycin or everolimus levels, inspires confidence in the relevance of our findings.[20,21] Poor wound healing that is associated with higher trough levels is well documented for mTOR inhibitors.[44] Control of amount of drug release in eluting stents is becoming an increasingly important issue that enables application of different target drug concentrations.[45] We suggest that our data may well fit to these concepts. We believe that mTOR inhibition at very low concentrations may open new treatment options with minimal side effects to suppress the development of transplant arteriosclerosis and postintervention restenosis.

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Disclosures

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Methods

Cell Culture

We obtained human MSC from aspirated iliac crest marrow from consenting normal adult donors and cultured the cells as described previously.[12] Briefly, following Ficoll density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Freiburg, Germany), 7-12x10^6 mononuclear cells per 100-mm dish were seeded in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% pre-tested fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L glutamine. Non-adherent cells were removed by washing with PBS after 24 hours. When cultures reached about 90% confluence, cells were re-plated at 3x10^5 cells per 100-mm dish. Passages 2 to 6 were used for experiments. Under these culture conditions MSC maintain multilineage differentiation capacity,[12] are positive for CD73, CD90, and CD105 and negative for CD11b, CD14, CD19, CD34, CD45, CD79alpha, and HLA-DR (supplemental Figure I), thereby meeting the criteria for MSC.[13] We obtained human VSMC from coronary arteries from Lonza (Bio Science Walkersville, USA), cultured the cells with SmGM2 medium (Lonza) and used them between passages 4 and 6. Cultures with 70-80% confluence were used for experiments. For signal transduction analyses, MSC were incubated over night in serum free medium prior to stimulation with DMSO (cell culture grade, Sigma-Aldrich, Munich, Germany). For blocking experiments, cells were preincubated for 30 minutes with the PI3K inhibitor Ly294002 (15 µmol/L, Sigma-Aldrich) or the mTOR inhibitor rapamycin (6 nmol/L, Sigma-Aldrich). We have elected to use the term rapamycin rather than sirolimus, the generic synonym for rapamycin used in humans.

Ca2+ Imaging

Cells were seeded onto glass coverslips and loaded with the Ca^{2+} indicator fluo-4-AM (Invitrogen, Karlsruhe, Germany) (10 µM) and pluronic acid (Merck, Darmstadt,

Germany) (0.01%; w/v) for 30 min at room temperature in PSS (NaCl 134 mM, KCl 6 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, pH 7.4 with NaOH). Before taking records, the cells were washed with PSS and further incubated for 20 min to allow de-esterification of the dye. Pretreatment with 1 μmol/L nimodipine (Sigma-Aldrich) was carried out for 5 minutes before adding KCl. Fluo-4 loaded cells were imaged using a BioRad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 300 inverted microscope. Excitation was achieved at 488 nm and the emission wavelength was 500 nm. Images were collected at a rate of 1/second. Image processing was done using imageJ 1.41i (National Institutes of Health, USA, http://rsbweb.nih.gov/ij/). Background fluorescence was subtracted and changes in intracellular calcium were expressed as relative fluorescence changes, i.e. F/Fo (with Fo indicating the fluorescence before stimulation and F the time-dependent fluorescence signal after stimulation). Stock solutions of fluo-4 AM (2.5 mM) and of nimodipine (1 mM) were made using DMSO as solvent. High external potassium solutions were made by iso-osmotic substitution of NaCl with KCl in the PSS.

Western blotting

Western blots were performed as previously described.[12] Total protein content was determined with the DC protein assay (Bio-Rad, Munich, Germany). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Freiburg, Germany) using the NuPage system (Invitrogen). Membranes were blocked with 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBST). Primary antibodies were diluted in blocking solution (sm-calponin (hCP) 1:5000, MLCK (K36) 1:7500, sm-α-actin (1A4) 1:7500, and α-tubulin (DM1A) 1:10000, Sigma-Aldrich; SM22α goat polyclonal 1:5000, Abcam, Cambridge, UK; Akt, pAkt (Thr308), p70S6K, and

pp70S6K (Thr389) 1:1000, Cell Signaling Technology, Danvers, USA) and allowed to bind for 2 h or over night at room temperature. After incubation with the appropriate horseradish peroxydase-conjugated secondary antibodies (1:5000 in TBST) immunoreactive proteins were detected by chemiluminescence (ECL kit, Amersham). Band intensities were quantified using ImageJ 1.41i software.

BrdU incorporation and MTT assays

Cell proliferation was measured as BrdU incorporation following the manufacturer's instructions (BrdU cell proliferation kit, Roche) in MSC grown in presence of 10% FCS, BrdU, and the indicated rapamycin concentrations for 24 hours. Cytotoxicity was tested in MSC incubated with indicated rapamycin concentration for 24 hours and addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, final concentration 1.5 mmol/L) during the last 4 h. Formazan crystals formed by viable cells were solubalized with isopropanol/0.4 mol/L HCl. Absorption was measured at 570 nm in an ELISA reader.

Quantitative real time PCR

VSMC and MSC were grown serum free for 24 h before incubation with rapamycin for 8 hours. Total RNA was extracted using the phenol/chloroform method and transcribed into cDNA with the PCR core kit (Roche). Quantitative real time PCR was performed using a Roche Light Cycler system. GAPDH served as housekeeper gene. The following primer sequences were used: SDF-1: 115-bp amplicon, sense TGCCAGAGCCAACGTCAAG, anti-sense CTTTAGCTTCGGGTCAATGCAC; GAPDH: sense CCATGGAGAAGGCTGGGG, anti-sense CAAAGTTGTCATGGATGACC.

SDF-1α ELISA

For analysis of SDF-1α secretion, $2x10^5$ VSMC grown in 6-well plates with serum-free DMEM without phenol red were incubated with control or 6 nmol/L rapamycin for

72 hours. Measurement of SDF-1α in supernatants was performed with a human SDF-1α ELISA (R&D Systems, Wiesbaden, Germany) following the manufacturer's instructions. Results were normalized to total protein content.

MSC migration on conditioned medium of rapamycin treated VSMC

Supernatants of VSMC cultured for 72 h with or without rapamycin were applied to the lower chamber of a micro chemotaxis chamber (Neuro Probe, Gaithersburg, USA). Membranes with 8 µm pore diameter were coated with 1 µg/cm^2 fibronectin on both sides. MSC (32500 cells per well) in serum free DMEM without phenol red in presence of either 20 µg/ml control IgG or anti-CXCR4 (clone 44717, R&D Systems) were seeded in the upper chamber and allowed to migrate for 24 h. Cells on the top side of the membrane were carefully removed with a cell scraper and rinsed off with PBS. Migrated cells on the bottom side of the membrane were fixed (10 min with 4% paraformaldehyde in PBS), rinsed, and stained for 5 sec with 0.25% crystal violet in 20% methanol. Absorbance was measured with an ELISA reader at a 570 nm wavelength. Results were normalized to total protein content of the supernatants.

Statistical analysis

Values for calcium influx are given as mean ± SEM and were compared with Student's t-test. Band intensities of smooth muscle markers were normalized to α-tubulin. Control without treatment was set 1.00. Analysis of multiple blots was expressed as mean ± SD. At least three independent experiments were performed. The Kruskal-Wallis-Test was employed for multiple comparisons while pairwise comparison with control was done using Mann-Whitney-U-test. $P<0.05$ was considered statistically significant.

Detailed figure legends

Figure 1. MSC express functional VSMC like L-type Ca^{2+} channels. A, time-courses of intracellular calcium transients evoked by 60 mmol/L KCl in MSC without pretreatment and B, after pretreatment with 1 µmol/L nimodipine. C, KCl-induced calcium transients after treatment of MSC for 8 days with 1.25% DMSO without pretreatment and D, after pretreatment with nimodipine 1 µmol/L. Application time of substances is indicated by bars. Each trace represents mean ± SEM of 20 – 30 single cell recordings. Data are expressed as fractional fluorescence (F/Fo). E, mean peak amplitudes of KCl-evoked calcium transients in MSC. Numbers above bars indicate number of cells tested. Mean values ± SEM are shown. *P<0.05, n. s.: not significant.

Figure 2. Activation of the PI3-kinase/Akt/mTOR-pathway in dedifferentiated MSC with synthetic phenotype. A, densitometric analysis of western blots for pAkt normalized to total Akt (n=3, mean values ± SD). Incubation of MSC with 1.25% DMSO resulted in a rapid (starting at 5 to 10 minutes) and strong (tenfold) increase in Akt phosphorylation without change in total Akt. B, representative western blots for pAkt and total Akt. C, preincubation with the PI3-kinase inhibitor Ly294002 abolished Akt phosphorylation without change in total Akt. D, densitometric analysis of western blots for phosphorylated p70S6K normalized to total p70S6K (n=3, mean values ± SD). When MSC were incubated with 1.25% DMSO phosphorylation of p70S6-kinase increased five-fold after 30 minutes. E, representative western blots for pp70S6K and total p70S6K. F, preincubation with 6 nmol/L rapamycin abolished p70S6K phosphorylation without change in total p70S6K.

Figure 3. PI3K inhibitor Ly294002 induced up-regulation of VSMC marker proteins in

MSC. Incubation with 1.25% DMSO induced down-regulation of A, sm-calponin, B, sm-α-actin, C, SM22α, and D, the short isoform of MLCK. The PI3-kinase inhibitor Ly294002 reversed DMSO mediated effects and up-regulated all four markers at 5 and 15 μmol/L (A-D). E, expression of α-tubulin. Representative western blots and statistic analysis of densitometry (n=3, mean values ± SD) are shown. Bands were normalized to α-tubulin. Control with 0 μmol/L Ly294002 was set 1. *P<0.05 for comparison with control, #P<0.05 for comparison with 1.25% DMSO alone.

Figure 4. Rapamycin acts antiproliferative but not cytotoxic on MSC. A, rapamycin induced a 50% reduction in proliferation at 1 nmol/L without additional effects at higher concentrations up to 100 nmol/L during 24h incubation, as assessed by measurement of incorporated BrdU. B, increasing rapamycin concentrations up to 100 nmol/L were not cytotoxic on MSC after 24 h as assessed by the MTT assay. n=3, control with 0 nmol/L rapamycin was set 1, mean values ± SD, *P<0.05.

Figure 5. Rapamycin induces the homing chemokine SDF-1α and enhances migration of MSC towards VSMC. A, increased SDF-1α mRNA expression in VSMC after 8 h of treatment with pharmacologic 6 nmol/L rapamycin. B, secreted SDF-1α in supernatant of VSMC after 72 h. C, enhanced CXCR4 dependent migration of MSC to conditioned medium of VSMC treated with 6 nmol/L rapamycin. n=4, mean values ± SD, *P<0.05.

Legends to supplemental figures

Figure I. FACS analysis of MSC demonstrating expression of typical MSC surface proteins and lack of hematopoietic markers. Isotype controls are shown in blue, specific antibodies in green. Cultured cells used in the experiments were uniformly positive for CD73, CD90, and CD105 and negative for CD11b, CD14, CD19, CD34, CD45, CD79alpha, and HLA-DR.

Figure II. Influence of PI3K/mTOR pathway on phenotypic modulation of MSC. A, resting cells showed a differentiated contractile phenotype characterized by an irregular shape and prominent stress fibers. B, incubation with 1.25% DMSO for 8 days induced a phenotypic change to spindle-like cells without stress fibers. C, PI3-kinase inhibitor Ly294002 at 15 μmol/L and D, pharmacologic (6 nmol/L) rapamycin reversed these phenotypic changes.

Figure III. Rapamycin induced up-regulation of VSMC marker proteins in MSC. A, expression of sm-calponin. B, expression of sm-α-actin. C, expression of SM22α. Markers were up-regulated after 8 days already at 1 nmol/L rapamycin without additional effects at supra-pharmacologic concentrations up to 100 nmol/L. D, expression of the short MLCK isoform remained unchanged. Representative western blots and statistic analysis of densitometry of three independent experiments (mean values ± SD) are shown. Control with 0 nmol/L rapamycin was set 1.

Figure I

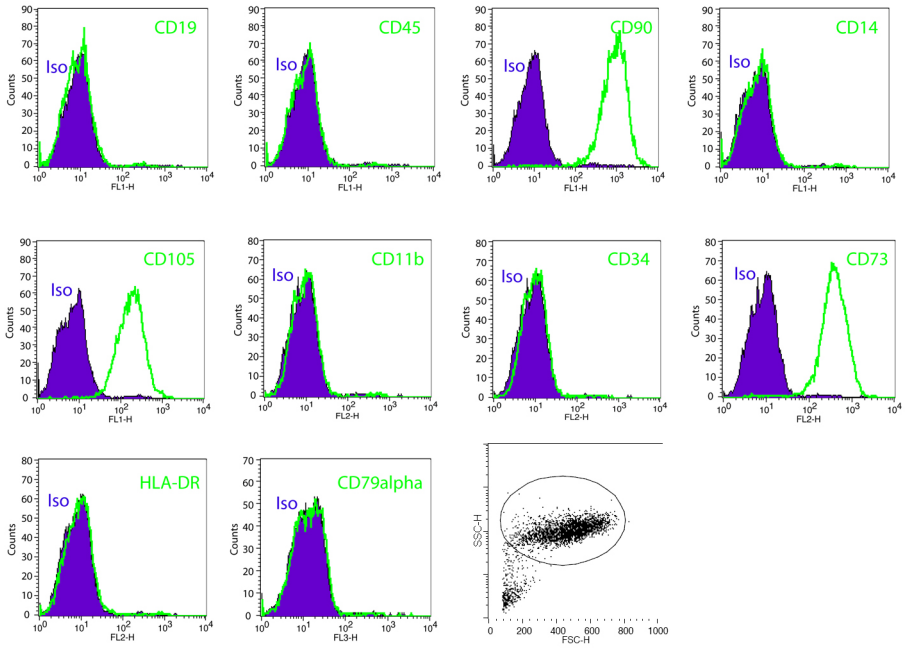

Figure II

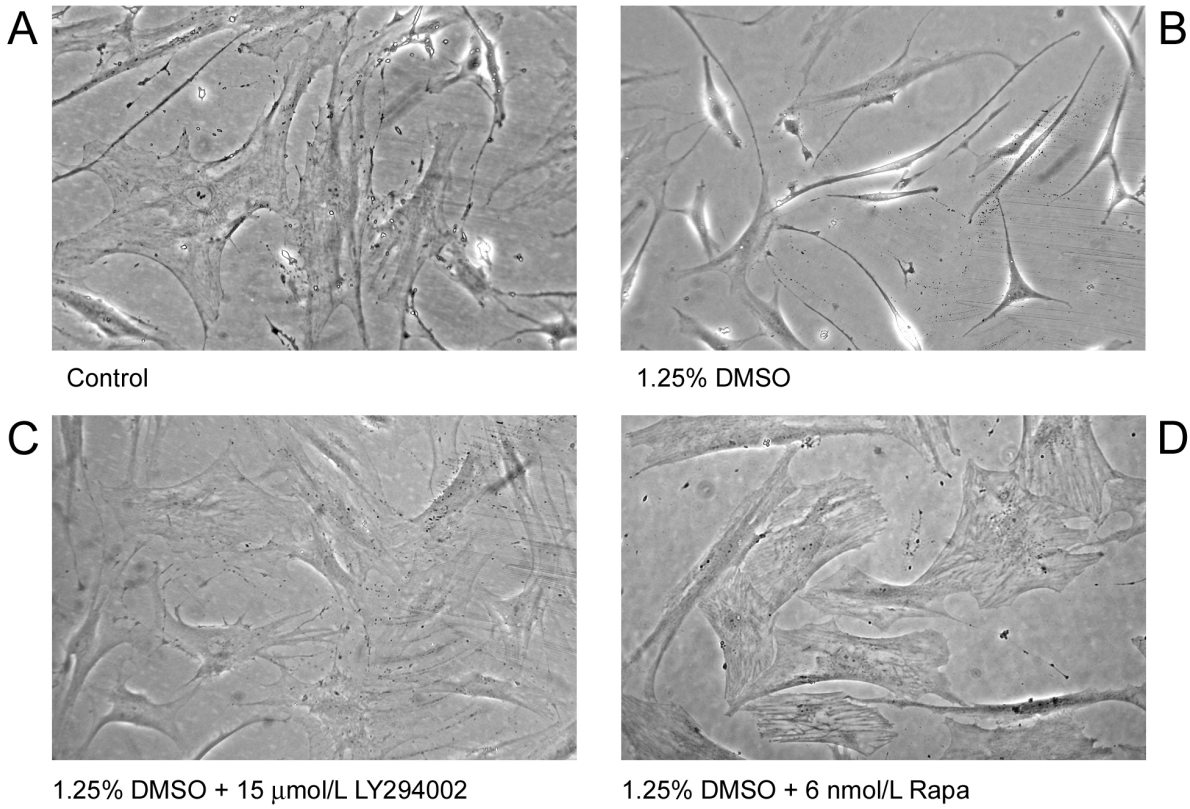

Figure III