Statins Block Calcific Nodule Formation of Valvular Interstitial Cells by Inhibiting α-Smooth Muscle Actin Expression

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Supplemental Methods, Results, Captions, and Figures

Supplemental Methods

Cell Culture

VICs were isolated from porcine aortic valve leaflets by sequential collagenase digestion as previously described[1]. Cells were expanded in growth media (Medium 199, 15% fetal bovine serum (FBS), 2% penicillin/streptomycin (100 U/mL), 0.4% fungizone (0.5 μg/mL)) and passaged by trypsin digestion. For the experiments described here, VICs from passages two or three were plated at confluence and cultured in low serum media (1% FBS supplemented). TGF-β1 (R & D Systems) was added at 5 ng/mL, pravastatin (EMD Bioscience) at 100 μmol/L, mevalonate (Sigma) at 50 μmol/L, geranylgeranyl pyrophosphate (GGPP, Sigma) at 3.5 μmol/L, farnesyl pyrophosphate (FPP, Sigma) at 3.5 μmol/L, cell-permeable C3 Transferase (Cytoskeleton, Inc.) at 1 μg/mL, Rho kinase (ROCK) inhibitor Y-27632 at 20 μmol/L, and myosin light chain kinase (MLCK) inhibitor ML-7 at 20 μmol/L. Pravastatin was chosen as a statin inhibitor due to its water-soluble nature, thus eliminating the presence of solvent carriers required by other statins.

dsDNA Measurement

Total cellular dsDNA was measured using the Quant-It dsDNA PicoGreen Assay (Invitrogen) according to the manufacturer's instructions. Briefly, VICs were cultured in 96 well plates and treated with the indicated supplements. After four days of culture, cells were lysed with papainase (Worthington) and lysate supernatant dsDNA content was determined with the PicoGreen Assay.

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Supplemental Results

Statin Effects Specific to HMG-CoA Reductase Inhibition

To confirm that the effects of pravastatin were specific to HMG-CoA reductase inhibition, we supplemented pravastatin treated cells with downstream metabolites of HMG-CoA reductase activity. The addition of activated mevalonate, a direct downstream product of HMG-CoA reductase activity, prevented the ability of pravastatin to reduce calcific nodule formation and contractility (Supp. Fig. I). These results confirm that HMG-CoA activity plays an important role in myofibroblast activation of VICs.

To further elucidate the downstream pathways that are affected by statin inhibition of HMG-CoA reductase, we examined the isoprenoid intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). FPP is involved in cholesterol biosynthesis and plays an important role in prenylation of Ras[2]. GGPP activates downstream effectors such as RhoA GTPase, leading to activation of Rho Kinase and actin remodeling and contraction[3, 4]. The addition of GGPP, but not FPP, in combination with pravastatin prevented statin-mediated effects on VIC calcific nodule formation and contractility (Supp. Fig. I), suggesting that the cholesterol biosynthesis

pathway is not involved in myofibroblast activation. Instead, the inhibition of GGPP synthesis by pravastatin appears to be an important step in blocking nodule formation in VIC cultures.

RhoA Inhibition Reduces Nodule Formation and Contractility

In an effort to identify the target of GGPP activity, we investigated the role of RhoA GTPase, a known target of GGPP prenylation[2, 5], in nodule formation and contractility. By blocking the activity of RhoA with the specific enzyme inhibitor C3 Transferase[6], we observed that nodule formation was dramatically reduced in VIC cultures. In our studies, we utilized a specific enzyme inhibitor of RhoA, avoiding the use of small molecule inhibitors utilized by others that may have unknown pharmacological effects. As shown in Supplemental Figure IIA, C3 Transferase was a potent inhibitor of nodule formation, reducing the number of calcified nodules below the levels observed in pravastatin-treated samples. Consistent with our previous data demonstrating the link between contraction and nodule formation, VICs treated with the RhoA inhibitor showed a significant reduction in contractile activity in collagen gel assays (Supp. Fig. IIB).

The combination of C3 Transferase and pravastatin did not appear to have an additive effect on nodule numbers or contractility, suggesting that the two inhibitors act through the same pathway. The fact that RhoA inhibition was a more effective blocker of nodule formation suggests that C3 Transferase was a stronger inhibitor than pravastatin at the concentrations used. It is possible that the two inhibitors have different affinities for their targets, which could explain their difference in efficacy. Given that RhoA is likely a downstream target of statin-mediated HMG-CoA reductase

inhibition, it is also possible that specifically blocking RhoA activity prevents competing signals upstream of RhoA, but downstream of HMG-CoA reductase, from interfering with the ability of statins to inhibit RhoA.

Supplemental References

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Supplemental Figure Captions

Supplemental Figure I. Prenylation pathways mediate pravastatin effects on nodule formation and contractility. A. Mevalonate (50 μmol/L) and GGPP (3.5 μmol/L), but not FPP (3.5 μmol/L), repressed pravastatin effects on nodule formation. Downstream metabolites of HMG-CoA reductase were added into pravastatin-treated (100 μmol/L) VIC cultures to identify which pathways are involved in pravastatin-mediated inhibition of nodule formation. B. Mevalonate (50 μmol/L) and GGPP (3.5 μmol/L), but not FPP (3.5 μmol/L), repress pravastatin effects on contractility in collagen gel assays. Downstream metabolites of HMG-CoA reductase were added into pravastatin-treated (100 μmol/L) VICs that had been encapsulated in collagen gels and gel diameter was measured over 24 hours. *p<0.01

Supplemental Figure II. RhoA inhibition reduces nodule formation and contractility in VICs. A. Inhibition of RhoA with C3 Transferase reduced calcific nodule formation. The number of calcium-positive nodules formation per area of cell culture with indicated treatment and transfection conditions (n = 8). B. Inhibition of RhoA with C3 Transferase reduced VIC contractility. VICs were encapsulated in collagen gels, supplemented with combinations of 5 ng/mL TGF-β1, 100 μmol/L pravastatin, and 1 μg/mL C3 Transferase. Contraction is shown as reduction from original gel area with time (n = 8).

Supplemental Figure III. ROCK and MLCK are involved in the formation of calcific nodules. A, Inhibition of ROCK (Y27632) leads to significant reduction of calcific nodule formation. Calcific nodule formation per area of cell culture with indicated treatment and transfection conditions. Results have been normalized to control where n = 8, *p<0.01. B. Inhibition of MLCK (ML-7) leads to significant reduction of calcific nodule formation. Calcific nodule formation per area of cell culture with indicated treatment and transfection conditions. Results have been normalized to control where n = 8, *p<0.01.

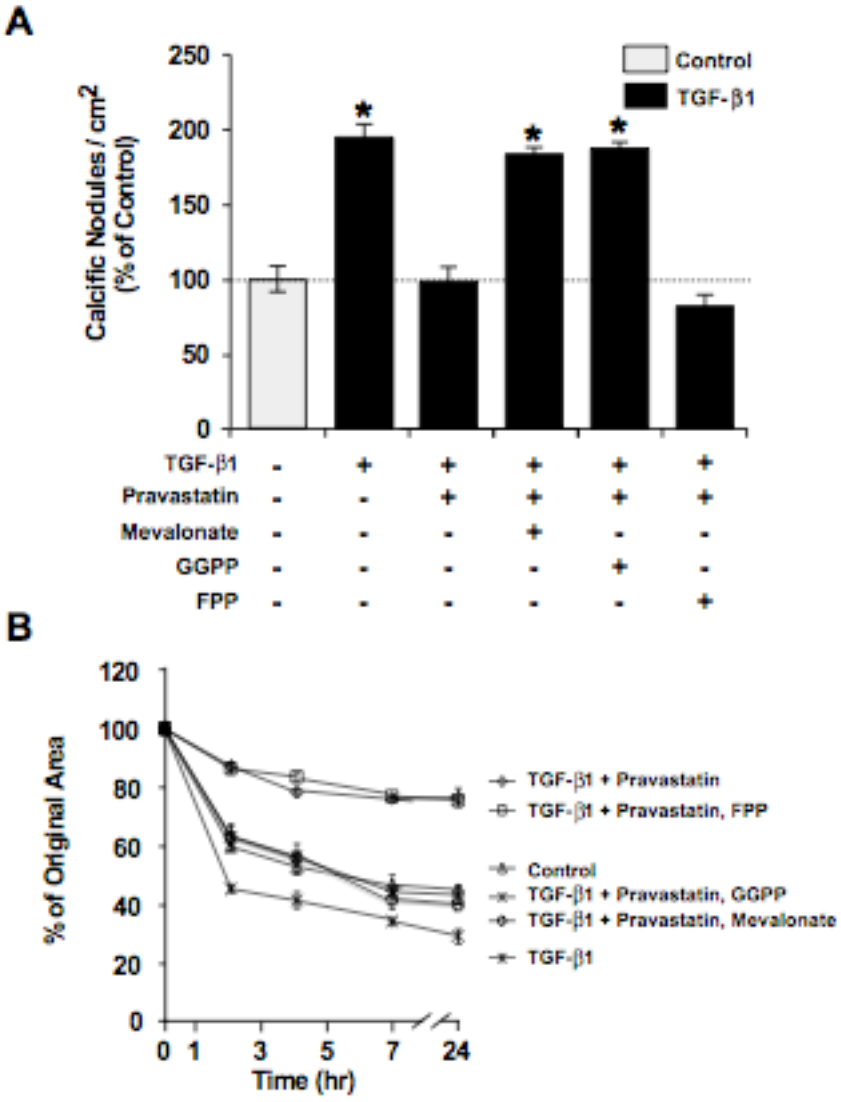
Supplemental Figure IV. Pravastatin reduced expression of Smad 2/3 driven luciferase. Results have been normalized to no pravastatin supplementation condition (control) and are from two experiments each with n = 24. *P<0.001 over control.

Supplemental Figure V. Representative calcium stained calcific nodules after eight days in culture where A. cells have been continuously cultured in the presence of 5 ng/mL TGF-β1 or B. with 5 ng/mL TGF-β1 but treated with 100 μmol/L pravastatin after four days of culture. Qualitatively, nodules appeared taller without pravastatin treatment and flatter with pravastatin treatment.

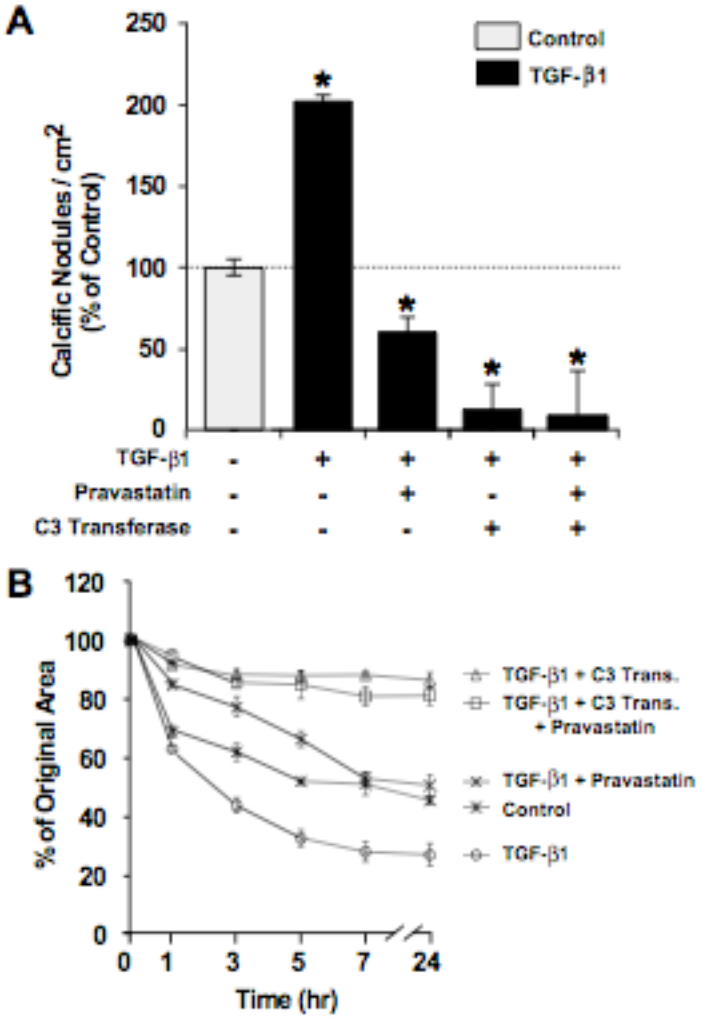
Supplemental Figure VI. Cellular dsDNA content was measured per well with the indicated treatment where cells were cultured in the presence of 5 ng/mL TGF-β1 and/or 100 μmol/L pravastatin. No significant change in dsDNA content was measured. n = 12. $p > 0.05$.

Supplemental Videos I. and II. VIC calcific nodule formation shown in real-time in the presence of 5 ng/mL TGF-β1 appears to be mediated through monolayer contraction. The time-stamp of each image is located in the black box as Day:Hour:Minute.

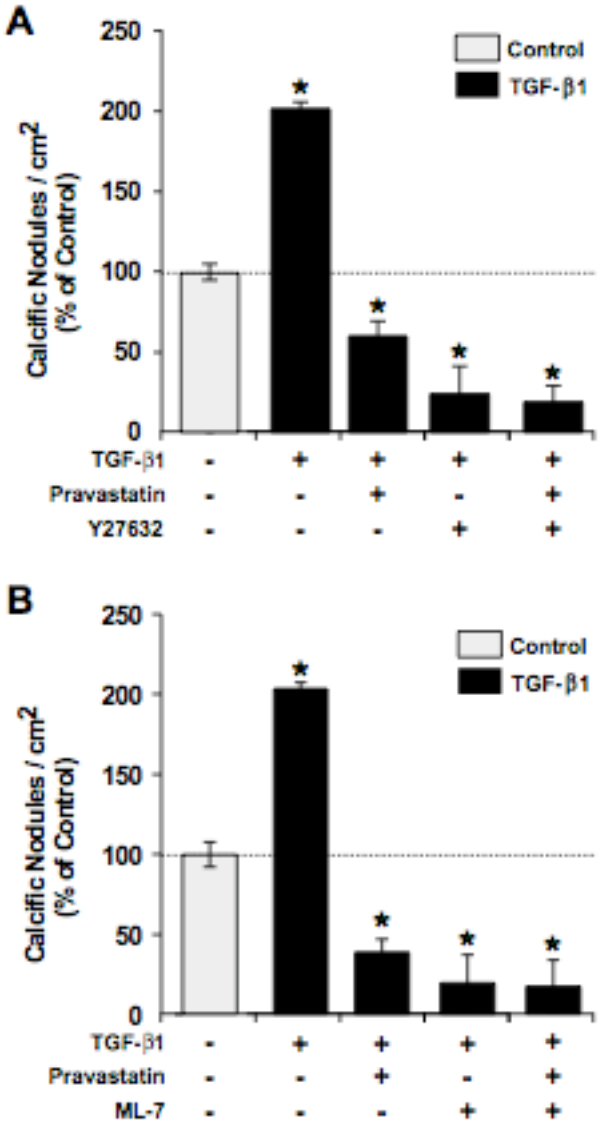
Supplemental Figure I



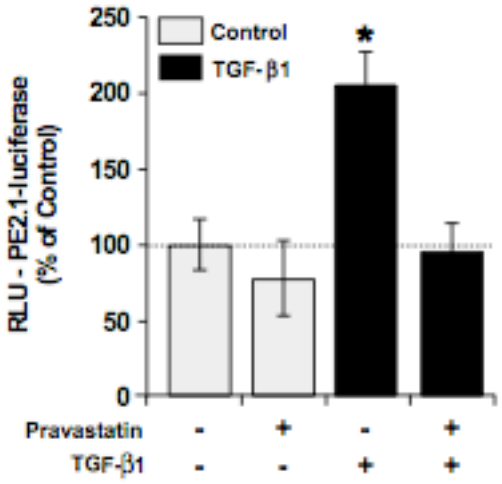
Supplemental Figure II



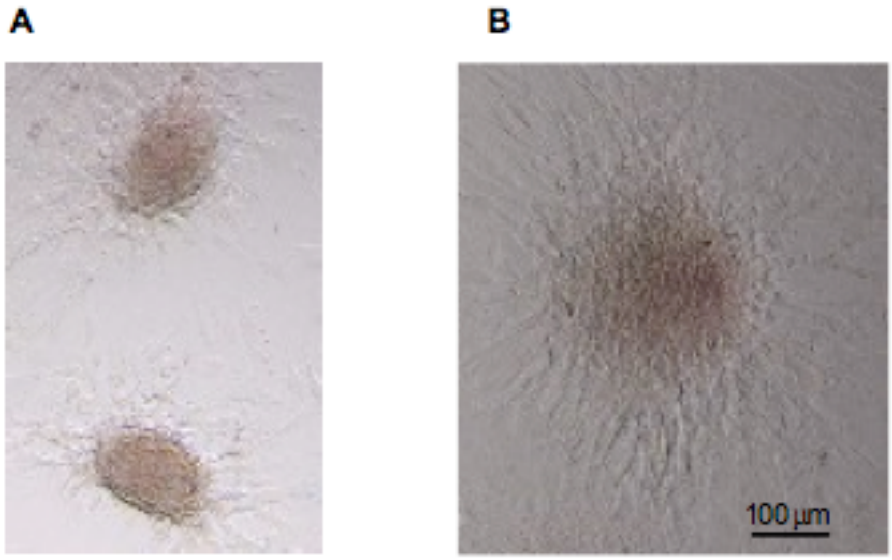
Supplemental Figure III



Supplemental Figure VI



Supplemental Figure V



Supplemental Figure VI