

Cofilin is a marker of myofibroblast differentiation in cells from porcine aortic cardiac valves

M. Pho,¹ W. Lee,¹ D. R. Watt,² C. Laschinger,¹ C. A. Simmons,² and C. A. McCulloch¹

¹Canadian Institutes of Health Research Group in Matrix Dynamics and ²Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Canada

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Pho M, Lee W, Watt DR, Laschinger C, Simmons CA, McCulloch CA. Cofilin is a marker of myofibroblast differentiation in cells from porcine aortic cardiac valves. *Am J Physiol Heart Circ Physiol* 294: H1767–H1778, 2008. First published February 8, 2008; doi:10.1152/ajpheart.01305.2007.—The formation of myofibroblasts in valve interstitial cell (VIC) populations contributes to fibrotic valvular disease. We examined myofibroblast differentiation in VICs from porcine aortic valves. In normal valves, cells immunostained for α -smooth muscle actin (α -SMA, a myofibroblast marker) were rare ($0.69 \pm 0.48\%$), but in sclerotic valves of animals fed an atherogenic diet, myofibroblasts were spatially clustered and abundant ($31.2 \pm 6.3\%$). In cultured VIC populations from normal valves, SMA-positive myofibroblasts were also spatially clustered, abundant (21% positive cells after 1 passage), and stained for collagen type I and vimentin but not desmin. For an analysis of stem cells, two-color flow cytometry of isolated cells stained with Hoechst 33342 demonstrated that 0.5% of VICs were side population cells; none stained for SMA. Upon culture, sorted side population cells generated $\sim 85\%$ SMA-positive cells, indicating that some myofibroblasts originate from a rare population with stem cell characteristics. Plating cells on rigid collagen substrates enabled the formation of myofibroblasts after 5 days in culture, which was completely blocked by culture of cells on compliant collagen substrates. Exogenous tensile force also significantly increased SMA expression in VICs. Isotope-coded affinity tags and mass spectrometry were used to identify differentially expressed proteins in myofibroblast differentiation of VICs. Of the nine proteins that were identified, cofilin expression and phospho-cofilin were strongly increased by conditions favoring myofibroblast differentiation. Knockdown of cofilin with small-interfering RNA inhibited collagen gel contraction and reduced myofibroblast differentiation as assessed by the SMA incorporation into stress fibers. When compared with normal valves, diseased valves showed strong immunostaining for cofilin that colocalized with SMA in clustered cells. We conclude that in VICs, cofilin is a marker for myofibroblasts *in vivo* and *in vitro* that arise from a rare population of stem cells and require a rigid matrix for formation.

actin; fibroblasts; isotope-coded affinity tags; α -smooth muscle actin

VALVULAR HEART DISEASE IS responsible for >19,800 deaths per year, is a contributing factor to over 42,500 deaths, and accounts for $\sim 95,000$ hospital discharges and $\sim 50,000$ valve replacement surgeries each year in the United States alone (49). Of the four heart valves, the aortic valve is most commonly affected by disease. Aortic valve sclerosis affects 20–30% of the population 65–74 yr of age (36, 45), and aortic stenosis affects 2–4% of individuals >84 yr (27, 36, 45). In diseased states, the structural integrity of the aortic valve is disrupted by the combined effects of increased cellularity, lipid accumulation, and the deposition of a disorganized extracellu-

lar matrix (ECM) (35, 56). These changes lead to imperfect coaptation, stiffening of the leaflets, and disturbed hemodynamic flow.

The phenotype and metabolism of valve interstitial cells (VICs) are critical determinants of valve morphology and function in health and disease (26, 40). VICs are the most abundant cells in valves and are responsible for synthesizing and remodeling the ECM (40) that ultimately determines the mechanical properties and function of the valve. During development, VICs may arise in part from endocardial endothelial cells that have undergone epithelial-to-mesenchymal transformation (15, 30). Transitions in early embryonic atrioventricular valvular function are related to changes of endocardial cushion biomechanics that, in turn, are dependent on tissue composition (7). In normal valves, VICs exhibit the morphological appearance of fibroblasts, but when activated by disease, excessive mechanical forces, or transforming growth factor (TGF)- β 1, VICs can mediate alterations of ECM biomechanics that can alter valve function (37, 40, 53).

VIC populations are not well defined in adult mammals but likely comprise a heterogeneous population consisting of fibroblasts, myofibroblasts, and smooth muscle cells (3, 12, 32, 33, 47). In healthy valves, the majority of interstitial cells are probably quiescent fibroblasts; only a small percentage (2.5%) are considered to be myofibroblasts (8, 37). If present at all, myofibroblasts appear to be restricted to the ventricular side of the leaflet interstitium (39) or are found in clusters in the spongiosa (5). In diseased valves, the appearance and localization of myofibroblasts are correlated with ECM disorganization, increased levels of matrix metalloproteinase expression, the formation of degenerative lesions, and fibrosis (34, 35, 36a, 44). The activation of valve myofibroblasts by profibrotic cytokines like TGF- β may play an important role in the pathogenesis of valve stenosis and sclerosis (53).

In cells cultured from valves, some reports estimate that a substantial percentage of myofibroblasts comprise the VIC population (25), whereas others have found few myofibroblasts in fresh isolated ovine aortic and porcine pulmonary VICs (39). The reported prevalence range of myofibroblasts is wide (7–100%) with a mean of $\sim 50\%$ (47), similar to that of other studies (48, 53). In one report of continuously cultured VICs, the expression of α -smooth muscle actin (α -SMA), a myofibroblast marker, was initially high and then decreased with a higher passage number. In contrast, another report described a relatively constant expression of SMA, regardless of the passage number (47), whereas others have found increased percentages of myofibroblasts at higher passage numbers (57).

Address for reprint requests and other correspondence: C. A. McCulloch, Univ. of Toronto, 150 College St., Rm. 244, Fitzgerald Bldg., Toronto, ON, Canada M5S 3E2 (e-mail: christopher.mcculloch@utoronto.ca).

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Currently, the origins and the biological factors that regulate differentiation of myofibroblasts from VICs are not defined. We used a pig aortic valve model and an analysis of side population cells by flow cytometry to examine the growth and differentiation of myofibroblasts from VICs. We employed isotope-coded affinity tag (ICAT) methods to screen for proteins that are differentially expressed in myofibroblast differentiation of cultured VICs. The data show that 1) some myofibroblasts from VICs originate from a small precursor population that is enriched in side population cells, 2) myofibroblast differentiation of VICs *in vitro* is regulated by the stiffness of the ECM, and 3) the actin-severing protein cofilin is expressed during myofibroblast differentiation of VICs and is required for the expression of SMA in stress fibers and for collagen gel contraction.

MATERIALS AND METHODS

Animal model. Animal protocols were approved by the Animal Care Committee of the University of Toronto and conducted according to their guidelines. Aortic valves were obtained from freshly euthanized 8-mo-old pigs (Quality Meat Packers, Toronto, ON, Canada). Valves were immediately placed on ice or were fixed in 10%-buffered formalin for paraffin embedding and preparation of transverse sections ($\sim 5 \mu\text{m}$) for immunohistochemistry. In some experiments, hypercholesterolemia was induced by ad libitum administration of an atherogenic diet (0.5% cholesterol, 10% lard, and 1.5% sodium cholate) as described (17), and the aortic valves were removed for immunohistochemistry. This hypercholesterolemic diet induces the formation of lesions in the fibrosa of the aortic valves that are rich in lipids and are focally calcified, similar to human lesions (43).

Cell culture. VICs were isolated by collagenase digestion of aortic valves, incubated at 37°C in complete DMEM containing 10% fetal bovine serum and a 1:10 dilution of an antibiotic solution (0.17% wt/vol penicillin V, 0.1% gentamycin sulfate, and 0.01% $\mu\text{g/ml}$ amphotericin), allowed to attach for 2 h, and then washed to remove nonadherent cells. Cultures were maintained in a humidified incubator gassed with 95% compressed air-5% CO_2 and were passaged with 0.01% trypsin. The cells were passaged at $\sim 75\%$ confluence. In some experiments, TGF- $\beta 1$ (R&D, Minneapolis, MN) was added to cultures at 10 ng/ml, a treatment that has been reported to strongly stimulate SMA expression in porcine VICs (53). To assess the effect of substrate compliance on myofibroblast differentiation, VICs were cultured on soft or rigid collagen substrates as characterized earlier (2) in which the soft substrate is ~ 10 -fold more compliant than the rigid substrate. We examined the ability of cells to contract collagen gels using the stress-relaxed gel contraction assay described earlier (23). Passage numbers were assigned consecutively after the initial plating.

ICAT. The production and analysis of ICAT-labeled peptides were performed according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Myofibroblast-differentiated (*day 7* primary) and undifferentiated (*day 3* primary) cultures were lysed in 0.5% SDS and 0.25 M tris(hydroxymethyl)aminomethane (Tris), and protein quantification was performed using the Pierce bicinchoninic acid (BCA) protein assay. ICAT-labeled samples were prepared with cleavable ICAT reagent, which reacts specifically with cysteine sulphhydryls and leaves an attached biotin group. To equal amounts of myofibroblast and undifferentiated culture protein samples (100 μg each), in denaturing buffer containing 50 mM Tris and 0.1% SDS (pH 8.5), Tris[2-carboxyethyl]phosphine hydrochloride was added to enable a disulfide reduction for 15 min at 37°C, followed by an addition of the appropriate ICAT reagent in acetonitrile at 37°C. Light- and heavy-labeled samples were combined before digestion with porcine trypsin for 18 h at 37°C in ammonium bicarbonate buffer (pH 8.5). Excess reagents were removed with a cation exchange cartridge (Applied Biosystems), and biotin-labeled ICAT peptides were isolated

by avidin affinity column chromatography. The biotin group was cleaved from eluted peptides using trifluoroacetic acid. Samples were lyophilized and taken up in acetonitrile and trifluoroacetic acid for analysis by nanoHPLC (Agilent HPLC 100 series) and by an Applied Biosystems/MDS Sciex API QSTAR XL Pulsar mass spectrometer. The data were analyzed with ProICAT SP2 (version 1.1; Applied Biosystems). The tandem mass spectrometry data were submitted for database searching to Mascot, and the nonredundant National Center for Biotechnology Information protein database was used. For quantifying the relative abundance of a protein, the peak height of the monoisotopic peak of the light ICAT-labeled peptide was divided by the peak height of the monoisotopic peak of the heavy-labeled form of the peptide.

Flow cytometry, side population analysis, and terminal deoxynucleotide transferase-mediated dUTP nick-end labeling. Analyses and sorting were conducted on a Beckman-Coulter Altra flow cytometer (Beckman-Coulter, Mississauga, ON, Canada). For an analysis of the percentage of S-phase cells, cell suspensions were stained with propidium iodide containing RNase and the DNA histograms were analyzed with Beckman-Coulter cell kinetics best-fit software ($n = 10,000$ cells/analysis). For side population analysis (22), single cell VIC suspensions (10^6 cells/ml) in prewarmed DMEM containing 20% fetal calf serum with 1 mM HEPES, penicillin, streptomycin, and 5 $\mu\text{g/ml}$ of Hoechst 33342 (Sigma) were incubated for 90 min at 37°C. For an assessment of staining specificity, cells were incubated with 50 μM verapamil (Sigma). Hoechst-labeled cells were excited at 350 nm, and emitted blue and red fluorescences were measured with a 450/20-nm band-pass filter and a 675-nm long-pass edge filter. A 610-nm short-pass dichroic mirror was used to separate the emission wavelengths. Blue and red fluorescences were analyzed with linear amplifiers. The cells were sorted into tubes containing 20% fetal calf serum, and an aliquot was removed at the end of the sort and reanalyzed to establish the purity of the sort. Side population cells, main population cells, and unsorted cells were cultured in separate dishes to assess myofibroblast phenotype by immunostaining for SMA. For an assessment of cell death in cultured VICs, adherent and floating cells were prepared as cytopins, stained with terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) reagents (Roche, Laval, QC, Canada), and immunostained for SMA. The percentage of TUNEL-positive cells that were positively stained for SMA was estimated by fluorescence microscopy.

Immunocytochemistry. Paraffin sections, transverse to the valve long axis, were stained for SMA (clone 1A4; mouse monoclonal antibody; Sigma-Aldrich, Oakville, ON, Canada) or for Ki-67 using the Mib-1 antibody (DakoCytomation, Carpinteria, CA) to estimate the percentages of SMA-positive cells and proliferating cells, respectively. For VICs cultured on collagen-coated glass coverslips, cells were fixed with 2% paraformaldehyde, permeabilized, and incubated with antibodies to collagen type I (1:200; sheep anti-pig; from J. Sodek, University of Toronto) or mouse monoclonal antibodies to SMA (1:100; clone 1A4), vimentin (1:100; clone VIM-13.2), smooth muscle myosin (1:200; clone HSM-V), desmin (1:100; clone DE-U-10; all from Sigma-Aldrich), or smoothelin (1:100; Chemicon). Staining was done with either a FITC-conjugated goat anti-mouse antibody or with a rhodamine-conjugated rabbit anti-sheep antibody. The percentage of positively stained cells was estimated by a visual inspection of staining intensity and by counting the total number of cells and the number of positive cells in 10×10 interocular counting grids with a $\times 25$ power objective lens.

Immunoblotting. The protein concentrations of cell lysates were determined by Pierce BCA protein assay. Equal amounts of protein were loaded onto SDS-polyacrylamide gels (10% or 12% acrylamide), resolved by electrophoresis, transferred to nitrocellulose membranes, and immunoblotted for SMA, GAPDH, or cofilin (Cell Signaling), and blot density was quantified as described (54).

Small-interfering RNA. The specific inhibition of cofilin was conducted with Silencer small-interfering RNAs (siRNAs; Ambion) with

the following human sequences, which were used simultaneously: CCAGUAAGGGACCUUCGAUtt, GGAUCAAGCAUGAAUUGCtt, and GGAGAGCAAGAAGGAGGAUtt. *Day 1* primary culture cells were transfected with 200 nM of cofilin siRNA or a green fluorescent protein (GFP)-negative control siRNA (Ambion) using oligofectamine (Invitrogen) for 5 h. After transfection, the cells were washed with PBS, incubated in DMEM containing 10% serum, and allowed to proliferate for 48 h. The cells were washed in PBS, lysed with Laemmli buffer, and immunoblotted to assess the efficacy of the siRNA knockdown. In a subset of cells, after 48 h, transfections were repeated with cofilin siRNA or a GFP-negative control siRNA (both, 200 nM). The cells were allowed to proliferate for 48 h, and the efficacy of siRNA knockdown was assessed. *Day 1* primary culture cells incubated in DMEM containing 10% serum served as a positive control for the duration of the transfection experiment.

Tensile force application. Exogenous tensile forces were applied *in vitro* as described previously (55) to examine the inducibility of SMA in VICs. Briefly, magnetite beads (400 mg; Sigma) were incubated for 30 min with 1 ml of an acidic bovine collagen solution (3 mg/ml; >95% collagen type I) at 37°C and neutralized to pH 7.4 with NaOH. Under these conditions, collagen polymerizes and forms fibrils around the beads within 30 min. The beads were sonicated to eliminate clumps and were then dispersed. Before incubation with cells, the beads were rinsed in PBS, washed, resuspended in Ca²⁺-free buffer, and added to attached cells in complete medium. The cells were washed three times to remove unbound beads before exposure to force. A ceramic permanent magnet was used to apply perpendicular forces to the beads attached to the dorsal surface of the cells. For all experiments, the pole face was parallel with and 2 cm from the surface of the cell culture dish. Since the surface area of the magnet was larger than the culture dishes and since the bead covering was relatively uniform for all cells, the forces applied to cells across the width of the culture dish were relatively uniform (21). Constant forces (0.65 pN/μm², projected cell area) were used for all experiments.

Statistical analyses. For quantitative data, means ± SE were computed. When appropriate, comparisons between groups were evaluated by Student's *t*-test or with ANOVA. Post hoc testing was carried out with Tukey's test. Statistical significance was set at *P* < 0.05. For all analyses, data from *n* ≥ 3 independent experiments were used. In each experiment, three replicates were used. For ICAT, discriminant analysis was used. Statistical significance for positive protein identification was set at >99% and with acceptable protein scores of 2.0 considered reliable.

RESULTS

In normal valves, interstitial cells immunostained for SMA (0.69 ± 0.48%; Fig. 1A) were rare. In sclerotic valves from hypercholesterolemic pigs, there were abundant SMA-positive cells (31.2 ± 6.3% of interstitial cells; *P* < 0.001). These SMA-stained cells from diseased valves were often spatially clustered (Fig. 1B), suggesting an apparent clonal distribution. In normal valves immunostained for Ki-67 with Mib-1 to estimate the percentage of proliferating cells, very few cells (0.35 ± 0.3% of interstitial cells) were stained. In cytopins of cell suspensions prepared from VICs of normal valves, 1.0 ± 0.4% of cells were immunostained for SMA, a marginally higher percentage of SMA cells than were enumerated in valves *in situ*.

When VIC suspensions were plated in medium containing serum, cultured for 4 days, passaged once, and cultured for at least 4 days, for all samples examined (*n* = 10 samples), VICs stained for vimentin (100% of 2,767 cells examined; Fig. 1C), but none of the cells stained for desmin (0 out of >2,500 cells), smoothelin (0 out of 1,535 cells), or smooth muscle myosin (0

out of 1,332 cells). This inability to detect desmin, smoothelin, and smooth muscle myosin staining was not because of a lack of immunoreactivity of the antibody since smooth muscle cells were prominently stained for these proteins with the same antibodies (data not shown). In first-passage cultures, there were abundant SMA-positive cells (~21.7 ± 5.0% at 5 days; from 10 different isolates) that were usually spatially clustered in the culture dishes, suggesting clonal expansion, and that also stained for collagen type I (98.2 ± 2.2% of SMA-stained cells were also positive for collagen type I; Fig. 1D). The cells that were not stained for SMA were not usually stained for collagen type I (85 ± 5% of SMA-negative cells were unstained for collagen type I). If primary cultures were grown for >6 days, SMA-positive cells were also commonly observed, but not if cultured for <3 days (Fig. 1E).

We examined the induction of SMA in cultured VICs in more detail. Freshly isolated VICs were plated and split 4:1 at passaging. The population doubling time of the cultures up to *passage 5* was ~30 h. The percentage of SMA-positive cells at the end of each passage ranged between 7% and 21%, depending on the passage number. At each passage, an aliquot was immunoblotted for SMA and GAPDH. For all isolates (*n* = 25), there was a strong increase of SMA expression between cells at early and late stages of primary culture (the fresh isolate). In some instances, there was no detectable SMA up to *day 3* in first-passage cultures (Fig. 2A). An analysis of a larger number of cultures by quantification of the ratio of immunoblot densities of SMA normalized to GAPDH showed that SMA increased fourfold between isolation and *day 7* of culture (*n* = 10 cultures). When SMA was quantified over different passages of culture, there was somewhat more abundant SMA at *passage 1*, which often fluctuated up to *passage 8*, but there were no significant differences of SMA content over passaging (Fig. 2B). In cells derived from some single parental cultures (3 out of 25), we found wide variations of SMA expression between *passages 1* and *7* (Fig. 2C) that occasionally resulted in a complete loss of SMA-expressing cells in later passage cultures.

Since the loss of SMA expression in later cultures could be a result of a deletion of SMA-positive cells, we estimated the percentage of SMA-positive cells that were attached and were TUNEL positive, as well as those that had detached from the culture dish (i.e., floaters) and were TUNEL positive. There were more SMA-positive apoptotic cells than SMA-negative cells (61 ± 9% of TUNEL-positive cells were immunostained for SMA, data from 12 different passages; and ~38 ± 5% of TUNEL-positive cells were unstained for SMA; *P* < 0.05). The percentage of SMA-positive apoptotic cells varied with passage number, and there were higher percentages of SMA-positive apoptotic cells at earlier than at later passages (*passages 1-4*, 81 ± 6.9%; and *passages 7-10*, 62 ± 4.7%; *P* < 0.05).

A measurement of the proliferation of cultured cells by staining cells with propidium iodide and estimating the percentage of S-phase cells by flow cytometry showed that for *passages 1-3* the percentage of S-phase cells was 23 ± 5% and that for late passage (*passages 9-12*) cells the percentage of S-phase cells was 16 ± 2% (*P* > 0.2). These data and the rapid population doubling times indicated that a substantial proportion of the cultured cell population proliferated in culture, accounting for the 4- to 10-fold increase of the cell number that

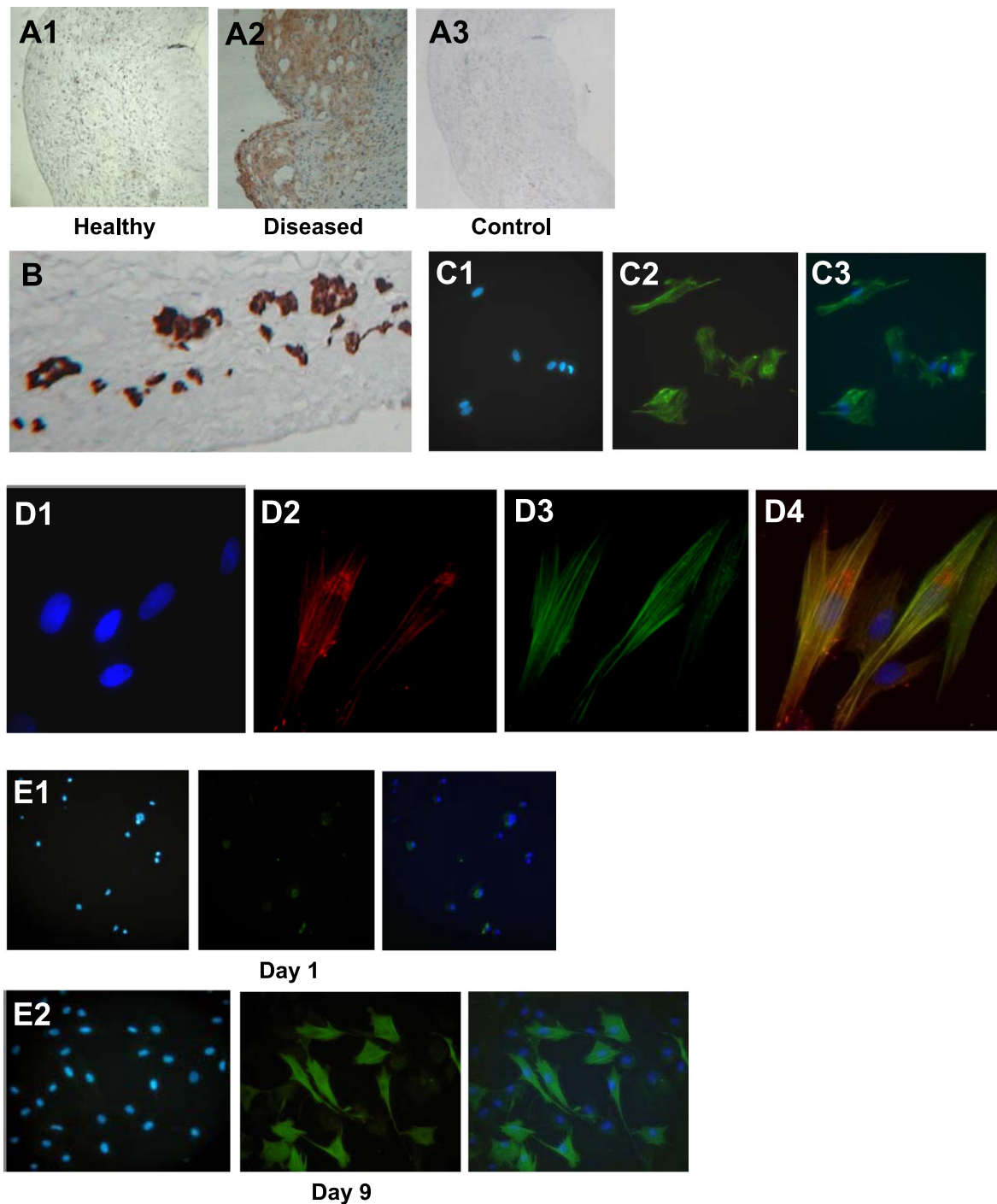


Fig. 1. *A*: valves immunostained for smooth muscle actin (SMA) from pigs fed normal (*A1*) and atherogenic (*A2*) diet. A section immunostained with an irrelevant antibody is shown in *A3* as a control. *B*: note the clustered distribution of SMA-stained cells in a high-power view of valve from atherogenic diet. *C1* and *C2*: valve interstitial cells (VICs) first-passage culture at 4 days stained for vimentin. *C3*: overlay. *D*: cluster of VICs in a single, first-passage culture at 4 days stained for 4,6-diamidino-2-phenylindole (DAPI; *D1*), collagen type I (*D2*), SMA (*D3*), and overlay (*D4*). *E*: primary culture of VICs stained for DAPI, SMA, and overlay at 1 day (*E1*) and 9 days (*E2*) of culture.

occurred in early passages and possibly the generation of SMA-positive cells. The very low percentages of SMA-positive cells at the time of plating, the rapid increase of SMA expression over the first two culture passages, and the distinctly clustered spatial localization of SMA-positive cells in diseased valves and in cultures suggested that a relatively small proportion of progenitor VICs may, under appropriate condi-

tions, proliferate, differentiate, and contribute in part to the formation of myofibroblasts both in vivo and in culture.

Since a more defined population of progenitor cells can be isolated from various tissues based on the efflux of the fluorescent DNA dye Hoechst 33342 (22), we sorted VIC suspensions into progenitor/stem cells (side population) and normal population cells (Fig. 2*D*). The authenticity of the flow cytom-

etry method for the isolation of the side population was verified by the coincubation of VIC suspensions with verapamil during staining with Hoechst 33342, which blocked dye efflux (Fig. 2E). Aliquots of sorted cells from the side and normal populations were plated at the same density in chamber slides (10^2 cells/cm²), allowed to proliferate for 7 days, stained with 4,6-diamidino-2-phenylindole to facilitate total cell counts, and immunostained for SMA. Of those cells that attached, approximately three population doublings occurred, although in many of the wells, the cell growth was limited, possibly because of the low plating density. Of the side population cells, 0 of 535 cells (from 5 separate isolates) stained for SMA, whereas 6 of 590 cells (from 5 separate isolates) of the nonside population cells stained for SMA. When the sorted cells were cultured in medium containing 10% serum for 5 and 10 days, a larger percentage of the cells from the side population ($45 \pm 4\%$, 5 days; and $85 \pm 8\%$, 10 days; from 3 separate cell isolations each) stained for SMA than that from the unsorted population ($25 \pm 3\%$, 5 days; and $35 \pm 6\%$, 10 days; from 3 separate cell isolations each; $P < 0.01$; Fig. 2, F and G). None of the cells in the side or the normal population stained for smoothelin, smooth muscle myosin, or desmin (smoothelin side/normal, 0 out of 150/135 cells; smooth muscle myosin side/normal, 0 out of 165/158 cells; and desmin side/normal, 0 out of 135/162 cells).

To determine whether SMA expression in porcine VICs could be enhanced by TGF- β as has been reported earlier (53), *passage 1, day 2* cells on rigid collagen substrates were transferred to serum-reduced medium (0.5% serum overnight) and treated with TGF- β 1 (10 ng/ml for 2 days) or with vehicle (for 2 days). There was a slight ($\sim 20\%$ by densitometry) reduction of SMA content in TGF- β 1- compared with vehicle-treated cultures (Fig. 3A). Similar results were found in cells plated on tissue culture plastic (data not shown). We also examined SMA expression in *passage 12* cells, but TGF- β did not increase SMA expression in these cells either. This lack of effect of TGF- β was not due to ineffective reagents since SMA was strongly increased by TGF- β in human cardiac fibroblasts that were used as a positive control (2). Furthermore, this lack of effect was also not due to the cells already being fully differentiated since we purposefully used cells at the beginning of the experiment that did not express SMA.

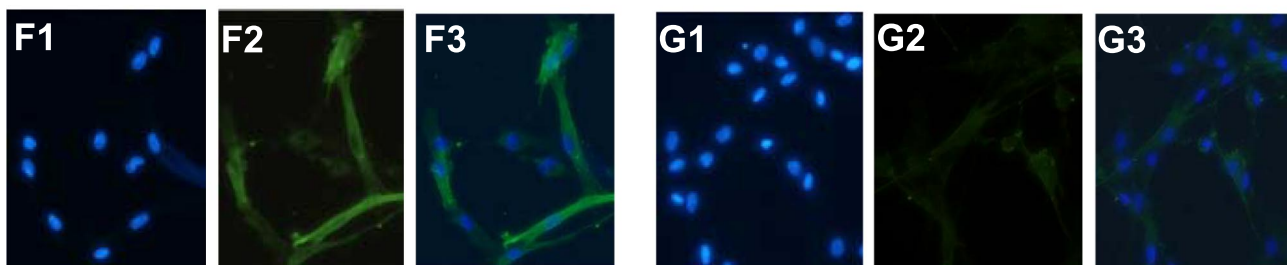
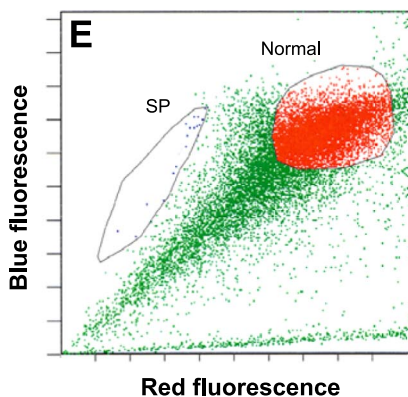
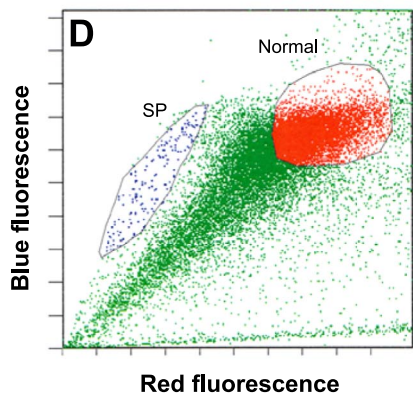
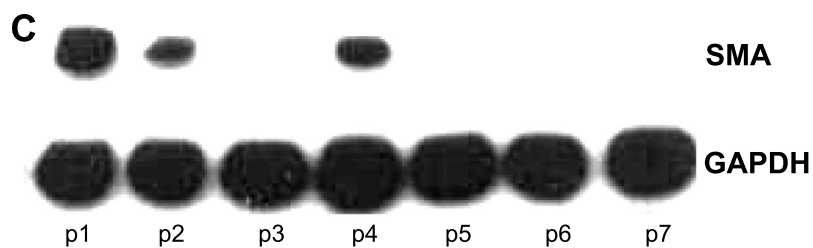
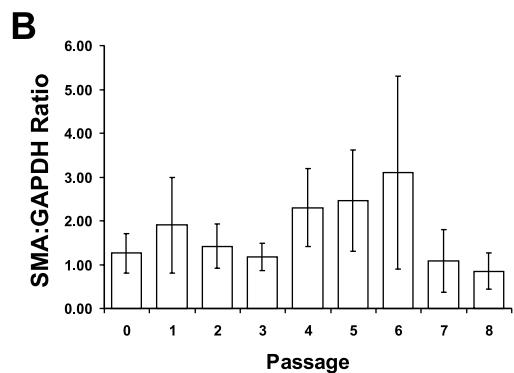
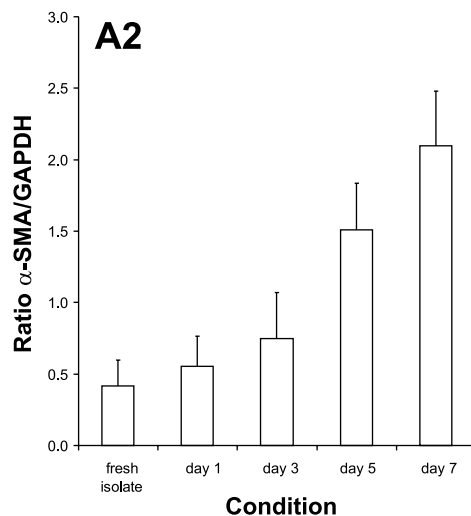
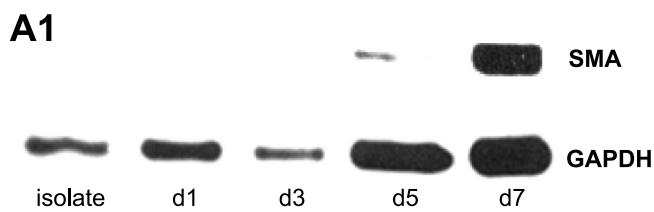
Previous data have shown that in gingival fibroblasts, TGF- β -induced SMA expression is dependent on the compliance of the collagen substrate, which in turn regulates the ability of cells to generate endogenous forces (2). In this experimental approach, cell-generated forces are developed in cultures on rigid but not compliant collagen gels. We plated primary culture cells and allowed them to proliferate for 7 days on rigid or soft collagen substrates as previously characterized (2) and stained cells with FITC-phalloidin. Although cells on rigid substrates formed well-defined actin stress fibers, cells on soft substrates did not (Fig. 3B), indicating that they generated low levels of intracellular tension. When cells under these same conditions were immunostained for SMA, only cells plated on rigid collagen gels expressed SMA (Fig. 3, C and D). We next determined whether treatment with TGF- β could overcome this reliance on substrate rigidity for SMA expression. After TGF- β (10 μ g/ml) treatment, primary culture cells at *day 7* grown on soft collagen did not express SMA (Fig. 3E), whereas cells on rigid collagen substrates showed abundant

SMA staining (Fig. 3F). Consistent with the immunoblotting data above, TGF- β treatment did not enhance staining for SMA (compare Fig. 3, D and F). These data indicated that myofibroblast differentiation of VICs *in vitro* is regulated by the stiffness of the ECM and that cell-generated tensile forces may be particularly important for regulating this process.

Since exogenous tensile force can rapidly enhance SMA expression in fibroblasts (54), we determined whether tensile forces, without simultaneous TGF- β treatment, could increase SMA expression in VICs. *Passage 2, day 3* cells were incubated with collagen-coated magnetite beads and subjected to vertically directed tensile forces [$0.5 \text{ pN}/\mu\text{m}^2$ of cell surface (20)] for 8 h. Under these experimental conditions, when SMA content was adjusted for cell protein by blotting for GAPDH and analyzed by densitometry, SMA was significantly increased (SMA, GAPDH blot density ratios, no force = 1.9 ± 0.3 ; and force = 3.2 ± 0.4 ; $P < 0.05$; Fig. 3G). Therefore, both exogenous and endogenous (cell generated) forces can enhance SMA expression.

Since myofibroblast differentiation, as assessed by pronounced increases of SMA expression, consistently occurred in late-stage primary cultures or *passage 1* and *2* cultures plated on rigid substrates, we screened for differential protein expression by ICAT in cultures from these same conditions. Lysates (100 μ g) were pooled from primary cultures at *day 3* or *7* and immunoblotted for SMA. First, we established culture conditions where there was very low SMA expression in the *day 3* cultures but abundant SMA in *day 7* cultures (Fig. 4A). These culture conditions promoted the formation of cells from VICs with the phenotypes of premyofibroblasts and myofibroblasts, respectively. The lysates from these same cultures were denatured, reduced, labeled with either light or heavy (+9 Da) ICAT biotin-coupled reagents, combined, digested with trypsin, fractionated by cationic exchange, purified with avidin columns, cleaved, and analyzed by HPLC and tandem mass spectrometry. In two separate analyses, with the use of discriminant analysis (with positive protein identification set at $>99\%$) and protein scores of 2.0 or greater, six and seven different novel proteins (not including SMA) from the two analyses were identified as being differentially expressed under the two experimentally different conditions. From the 88 peptides that were identified with $>99\%$ certainty, nine different proteins were predicted to be differentially expressed in the two analyses (4 proteins were common to the 2 analyses). The proteins that were identified in the first analysis included fetuin, enolase 1, cyclophilin A, vimentin, and cofilin (with 2 high-probability peptides identified). In the second analysis, enolase 1, cyclophilin A, nonmuscle myosin II-A, vimentin, and cofilin (with 4 high-probability peptides identified) were found. Of these proteins, since cofilin was found in both analyses and was of particular interest because of its role in mediating the formation of stable actin filaments and stress fibers (1), we investigated it in more detail.

To determine whether the ICAT analyses were consistent with the independent immunochemical examination, we immunoblotted VIC primary cultures for cofilin under conditions that would prevent or enable myofibroblast differentiation, respectively. In three separate cultures each, cofilin expression was increased by greater than threefold in myofibroblast-differentiated (7 days growth) compared with premyofibroblast primary (3 days growth; Fig. 4B) cultures. We next determined



whether knockdown of cofilin by siRNA was feasible in VIC cultures. Cofilin knockdown (2 days treatment) in primary cultures reduced cofilin content by >80% compared with irrelevant GFP siRNA but only slightly reduced the expression of SMA as measured by immunoblotting (Fig. 4C). Notably, however, cofilin knockdown substantially reduced the ability of myofibroblasts derived from VICs to form brightly staining SMA-containing stress fibers (Fig. 4, D1 and D2), which are thought to be important in cell contractility and are a hallmark of myofibroblast differentiation (50). Cofilin knockdown, compared with irrelevant siRNA controls, also reduced by twofold the contraction of stress-relaxed collagen gels (Fig. 4E; $P < 0.001$ at all sample times). The agreement between the data of stress fiber formation and stress-relaxed collagen gel contraction indicates that in VICs, cofilin plays an important role in mediating cell contraction, further underlining its utility as a marker of myofibroblasts, which are contractile cells.

Since cofilin is associated with the formation of stress fibers in certain cell types (1) and the phosphorylation of cofilin stabilizes actin filaments (10), we measured the ratios of phospho-cofilin to cofilin in cells from premyofibroblast and myofibroblast-differentiated cultures. Cells were serum starved overnight, and serum was then added 1 h before preparation of cell lysates for immunoblotting (Fig. 4F). We anticipated that the serum stimulation would activate cofilin. As before, we found much more cofilin in the myofibroblasts than in the premyofibroblasts, consistent with the notion that cofilin is a myofibroblast marker. After adjustment for the much-reduced cofilin in premyofibroblasts, we found that there was >2.5-fold greater phospho-cofilin in myofibroblasts than premyofibroblasts (phospho-cofilin-to-cofilin immunoblot densities, premyofibroblasts, 0.97 ± 0.2 ; and myofibroblasts, 2.6 ± 0.2 ; $P < 0.01$).

In porcine valves immunostained for cofilin, normal valves displayed low intensity staining throughout the valve interstitium, whereas diseased valves exhibited strong staining for cofilin of cells in focal clusters that colocalized with SMA (Fig. 4G), consistent with the notion that myofibroblasts are clonally distributed in diseased valves.

DISCUSSION

The principal findings of this study are that 1) myofibroblasts in VIC populations arise by clonal expansion, in part from a relatively small number of progenitor cells; 2) the mechanical stiffness of the ECM and exogenous tensile force can regulate myofibroblast differentiation of VICs in culture; and 3) cofilin is a marker for myofibroblast differentiation in VICs and plays an important role in the ability of VICs to form stress fibers and to contract collagen gels. These data point to the possible existence of rare stem cells in VICs both in vivo and in vitro that contribute to the formation of myofibroblasts and that may contribute to the development of sclerotic valves. Furthermore, cell-generated forces are evidently a critical de-

terminant of myofibroblast differentiation since rigid substrates are required for SMA expression in vitro, which may provide an in vitro model for the stiffening of the sclerotic valve. Finally, cofilin appears to be a marker of, and may be required for, myofibroblast differentiation. This finding has important implications since matrix remodeling by VICs is reliant on the development of cell-generated forces, which likely involve cofilin.

Myofibroblasts have been found in cardiac valves (33, 37) and are likely important in the pathogenesis of valve sclerosis and stenosis (40), but the factors that regulate their formation are not defined. Myofibroblasts were first described over 30 years ago in granulation tissue (19) and were named because they exhibited characteristics of both fibroblasts and smooth muscle cells (11, 28, 41). Myofibroblasts are currently defined based on their generation of actin stress fibers in vitro, the expression of collagen, SMA, and, in certain situations, extra domain A of fibronectin (50). The cells identified as myofibroblasts here are not apparently related to smooth muscle cells since they did not express smooth muscle myosin, smoothelin, or desmin. Myofibroblasts are thought to arise from activated fibroblasts in the surrounding connective tissue (18, 50), and indeed our immunostaining of diseased porcine aortic valves for SMA showed that myofibroblasts are apparently clonally distributed within the valve stroma, indicating that they may arise from sparsely distributed progenitor cells. Recent evidence indicates that circulating pluripotent stem cells may also contribute to VIC subpopulations (52).

For in situ proliferation analyses, we stained normal valves for Mib-1 and found that cells immunostained for this protein were very rare in vivo. But upon isolation in culture, a much larger proportion of the VIC population was in the S phase of the cell cycle, as determined by propidium iodide staining. The large size of this S-phase population (16–23% of VICs) and the rapid doubling times indicated that the VIC cultures contained mainly proliferating cells, some of which might be able to differentiate into myofibroblasts. When cell populations are vitally stained with the DNA-binding dye Hoechst 33342 and sorted by flow cytometry on the basis of red and blue Hoechst 33342 emissions, active efflux of the dye by the ATP-binding cassette G2 transporter causes certain cells to appear as a segregated cohort, known as a side population (22). Stem cells from several tissues have been shown to possess the side population phenotype. For example, previous utilization of side population analyses by two-color Hoechst 33342 flow sorting has been applied to the isolation of progenitor cells from blood-forming cells (22), bone cells (58), and ocular cells (6). However, the lack of specific markers for VIC progenitor and differentiated cell populations has hindered isolation and the characterization of VIC populations. Although our findings of the enrichment of myofibroblast progenitor cells in side population isolates do not indicate either a stromal or hematogenous origin for these cells, they do demonstrate that some

Fig. 2. A1: VIC primary cultures at various stages of maturation were immunoblotted for SMA and GAPDH. d, Time of culture in days. A2: means \pm SE of ratios of immunoblot densities of SMA to GAPDH at each indicated day of culture obtained from 10 different cultures. B: means \pm SE of ratios of immunoblot densities of SMA to GAPDH at end of indicated passage numbers. There were no significant differences between passages ($P > 0.2$). C: immunoblotting for GAPDH and SMA in serially cultured VICs at passages (p) 1–7. Note the strong SMA expression at early passages and the substantial reduction of SMA expression in later passages. D and E: side population (SP) analyses using Hoechst 33342 staining of VICs. Note that in presence of verapamil (E), the separate staining pattern of the SP-specific cells disappears. Immunostaining for SMA and nuclear staining with DAPI of SP cells [F1 and F2 and F3 (overlay)] and unsorted population cells [G1 and G2 and G3 (overlay)]. Cells were prepared, cultured for 7 days, immunostained for SMA, and counterstained with DAPI.

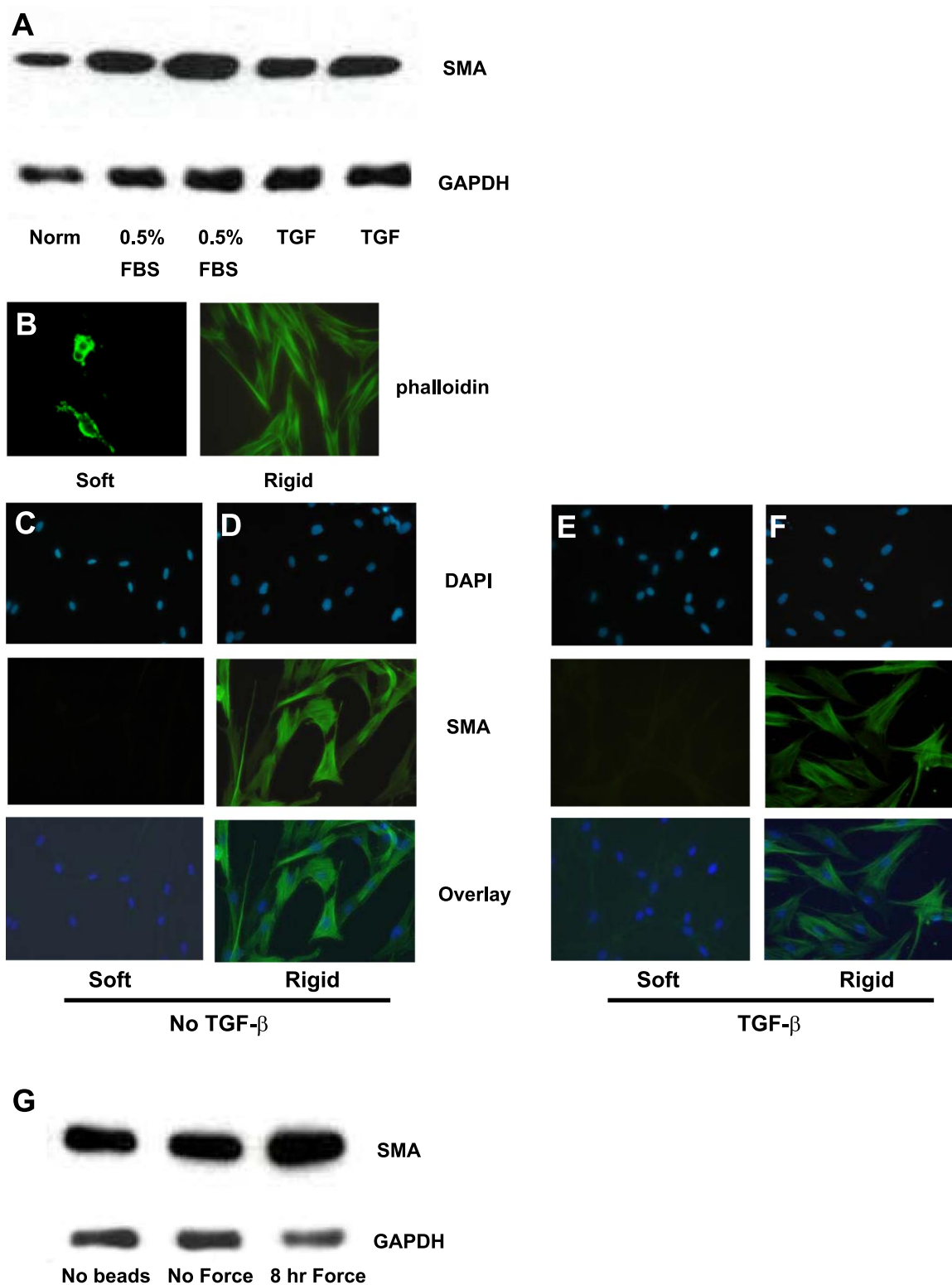


Fig. 3. *A*: passage 1 VICs were cultured with serum (Norm) or with reduced serum (0.5% FBS) or with reduced serum plus 10 ng/ml transforming growth factor (TGF)- β for 2 days, lysed, and immunoblotted for SMA and GAPDH. *B*: primary culture VICs (7 days) were plated on soft or rigid collagen substrates, grown in low serum (0.5%), and stained for actin filaments with phalloidin. *C* and *D*: cultures grown as in *B* were stained for SMA and counterstained with DAPI. *E* and *F*: cultures grown as in *B* but with 10 ng/ml TGF- β . *G*: passage 2 VICs were untreated (no collagen beads), incubated with collagen beads but without magnetically induced force (No force), or with collagen beads and force (8 h). Cell lysates were immunoblotted for SMA and GAPDH. Densitometry and computation of ratios of the blot densities showed that force did not significantly ($P > 0.2$) increase the relative amount of SMA.

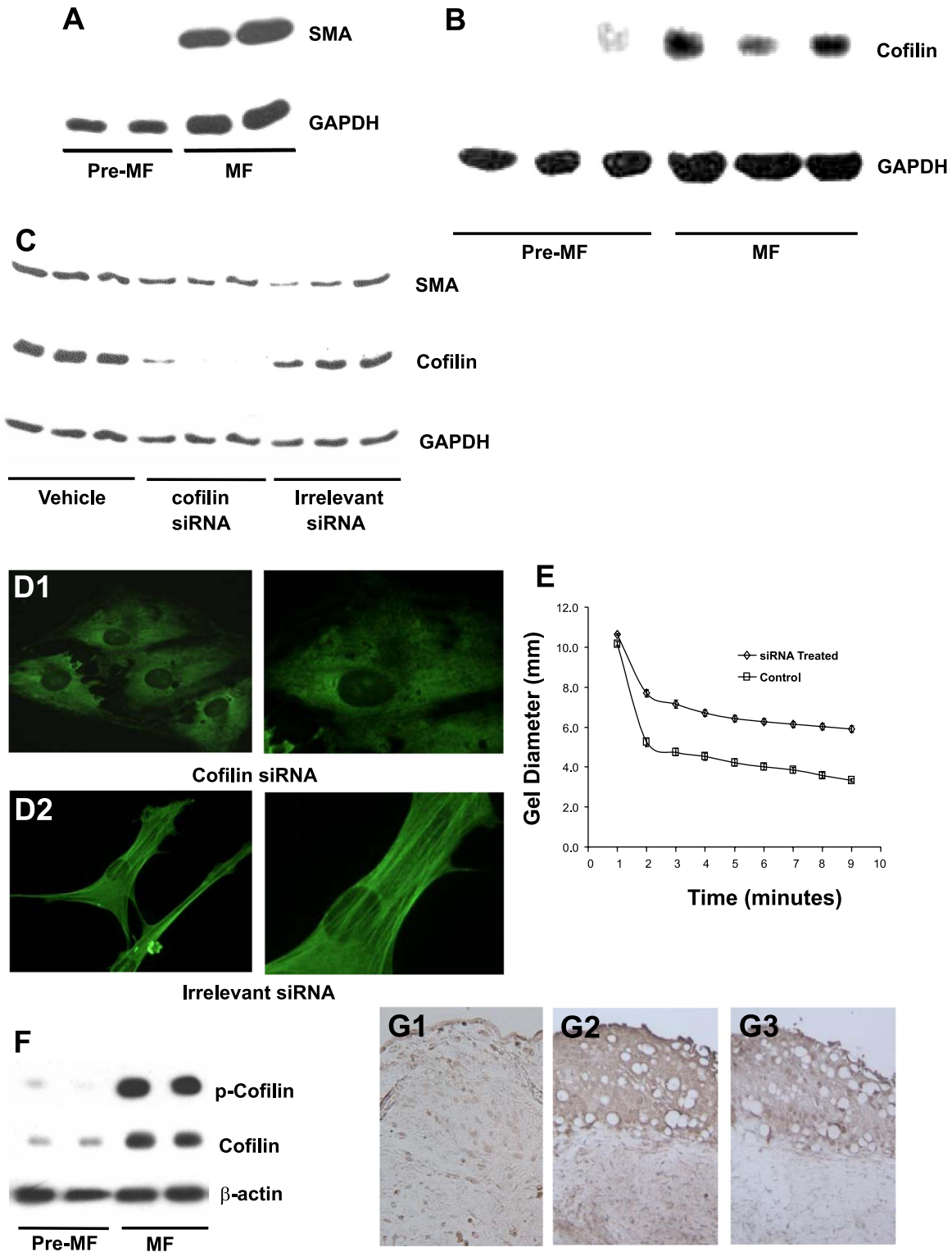


Fig. 4. *A*: premyofibroblasts (preMFs; 3-day cultures) or myofibroblasts (MFs; 7-day cultures) were immunoblotted for SMA and GAPDH. *B*: triplicate samples each of either preMFs or MFs similar to *A* were immunoblotted for cofilin and GAPDH. *C*: small-interfering RNA (siRNA) for cofilin of first-passage VICs. Three separate cultures each were treated with either vehicle, siRNA for cofilin, or siRNA for green fluorescent protein (GFP; irrelevant siRNA) for 2 days. *D*: immunostaining of VIC cultures treated with siRNA for cofilin (*D1*) or with siRNA for GFP (irrelevant siRNA; *D2*) for 2 days and then immunostained for SMA. Note the loss of SMA-stained stress fibers after knockdown of cofilin. *E*: contraction of stress-relaxed collagen gels in cells treated with siRNA for cofilin or with irrelevant GFP siRNA. The data are means \pm SE for gel diameter over time after release of the gel from the dish. *F*: immunoblotting of 2 separate cultures each of preMFs (3 days) or MFs (7 days) for β -actin, cofilin, and phospho-cofilin (p-cofilin). Cells were serum starved and then exposed to serum for 1 h to induce p-cofilin before preparation of lysates. *G*: immunoperoxidase staining for cofilin in paraffin sections through healthy (*G1*) and diseased (*G2*) porcine aortic valves. Note the strong, focal staining for cofilin in the diseased valve. An adjacent serial section was stained for SMA (*G3*), showing colocalization with cofilin in the diseased valve.

SMA-expressing myofibroblasts arise from a relatively rare cell population in VICs. To our knowledge, this is the first application of side population analyses to VICs. Upon culture, these low-abundance side population cells gave rise to greater than threefold more SMA-stained cells than did unfractionated cells. Accordingly, these data indicate that, at least in part, the VIC side population cells are enriched for myofibroblast progenitor cells and possibly other, as yet uncharacterized, VIC populations.

Previous studies have shown that almost all VICs stain positively for vimentin (5, 25, 39, 47, 53, 57), a marker of mesenchymal cells (16), consistent with our own findings here that all VICs stained for vimentin. Collagen type I is expressed by almost all cultured VICs and is present at a constant level from fresh isolates to late passage cells (57), again consistent with our data reported here. These findings and our use of culture methods to eliminate poorly adherent cells indicate that the cells examined in our studies were relatively pure populations of VICs and were not contaminated by endothelial or other cell types. Notably, none of the cultured cells including the progeny of the side population cells expressed the smooth muscle markers smooth muscle myosin, smoothelin, or desmin. Our data on the presence of a relatively rare population of myofibroblast progenitor cells also suggest that the marked published variations of myofibroblast abundance in VIC cultures (47, 48, 53, 57) may arise in part from the relative abundance of VIC progenitor cells that are included in the culture. Similarly, the occasional observation of the loss of SMA expression could be due to the overgrowth of myofibroblast by nonmyofibroblast progenitor cells that numerically overwhelm the cultures at later time periods or by a differential loss of the myofibroblast by apoptosis, as was observed here.

We found that SMA expression in cultured VICs was markedly regulated by matrix compliance, as has been reported earlier for gingival fibroblasts (2), and that exogenous tensile forces could increase SMA expression, as has been reported earlier for Rat-2 fibroblasts (55). The stiffening of valves that occurs in disease may be mediated by the responses of VICs to local tissue stress by altering cellular stiffness (possibly by increasing SMA content) and by the increased synthesis of collagen fibrils (31). Our finding independent of the matrix ligand itself (i.e., collagen) is that the stiffness of the matrix was a determining factor for VICs differentiating into myofibroblasts. These *in vitro* data point to an important role for the increased stiffness of sclerotic valves and local deforming forces as contributing factors to subsequent myofibroblast formation and further inhibition of valve function.

In addition to mechanical force, TGF- β 1 is one of the primary direct inducers of fibroblast-to-myofibroblast differentiation (13, 42, 51) and exhibits a strong, dose-dependent induction of SMA (51, 53). In contrast to a previous report (53), our data showed no significant enhancement of SMA expression in cultured VICs, even when the cells were grown on rigid substrates, a condition that is necessary for myofibroblast differentiation (2), and when cells with very low SMA content were treated. Conceivably, the VICs that were examined here expressed some form of TGF- β resistance, possibly because of the reduction of TGF- β receptors as has been observed earlier in fibroblasts that express less SMA in response to matrix effects (24).

We found that cofilin is coexpressed with SMA in the culture-induced conversion of premyofibroblasts to myofibroblasts. The observation that cofilin is a potential marker of myofibroblast differentiation in VICs is consistent with earlier findings in fibroblasts (4, 9, 29) and supports the notion that stress fiber assembly and the formation of stable actin filament arrays in cells are mediated by cofilin (1). Although SMA expression was not significantly altered after siRNA cofilin knockdown, the incorporation of SMA into stress fibers of cultured myofibroblasts was almost completely absent. Since the formation of SMA-containing stress fibers is a defining criterion for myofibroblasts (14), the knockdown of an actin-binding protein such as cofilin, which is apparently required for stress fiber assembly, indicates that cofilin may be both a marker and a required protein for myofibroblast differentiation in diseased heart valves. Furthermore, cofilin knockdown strongly inhibited the contraction of stress-relaxed collagen gels. These data indicate that cofilin is not just a marker of myofibroblast differentiation but may contribute to the determination of the differentiated state. Furthermore, since the phosphorylation of cofilin blocks actin severing and promotes actin filament stabilization (46), our finding that myofibroblasts have a much greater ratio of phosphorylated cofilin to cofilin than do premyofibroblasts indicates that the expression level and phosphorylation status of cofilin in VICs have important effects on stress fiber formation and, presumably, collagen contraction.

In summary, our data point to the presence of rare progenitor/stem cells in VIC populations that are clonally distributed, both *in vivo* and *in vitro*. These cells contribute to the formation of myofibroblasts and, therefore, may have an impact on the stiffening of sclerotic valves. Our data also show that cofilin is a novel myofibroblast marker and functionally contributes to the development of cell-generated forces, which are critical for matrix remodeling by VICs.

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GRANTS

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