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A role for serum response factor in coronary smooth muscle differentiation from proepicardial cells

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SUMMARY

Coronary artery smooth muscle (SM) cells originate from proepicardial cells that migrate over the surface of the heart, undergo epithelial to mesenchymal transformation and invade the subepicardial and cardiac matrix. Prior to contact with the heart, proepicardial cells exhibit no expression of smooth muscle markers including SMαactin, SM22α, calponin, SMγactin or SM-myosin heavy chain detectable by RT-PCR or by immunostaining. To identify factors required for coronary smooth muscle differentiation, we excised proepicardial cells from Hamburger-Hamilton stage-17 quail embryos and examined them ex vivo. Proepicardial cells initially formed an epithelial colony that was uniformly positive for cytokeratin, an epicardial marker. Transcripts for flk-1, Nkx 2.5, GATA4 or smooth muscle markers were undetectable, indicating an absence of endothelial, myocardial or preformed smooth muscle cells. By 24 hours, cytokeratin-positive cells became SMαactin-positive. Moreover, serum response factor, undetectable in freshly isolated proepicardial cells, became strongly expressed in virtually all epicardial cells. By 72 hours, a subset of epicardial cells exhibited a rearrangement of cytoskeletal actin, focal adhesion formation and acquisition of a motile phenotype. Coordinately with mesenchymal transformation, calponin, SM22α and SMγactin became expressed. By 5-10 days, SM-myosin heavy chain mRNA was found, by which time nearly all cells had become mesenchymal. RT-PCR showed that large increases in serum response factor expression coincide with smooth muscle differentiation in vitro. Two different dominant-negative serum response factor constructs prevented the appearance of calponin-, SM22α- and SMγactin-positive cells. By contrast, dominant-negative serum response factor did not block mesenchymal transformation nor significantly reduce the number of cytokeratin-positive cells. These results indicate that the stepwise differentiation of coronary smooth muscle cells from proepicardial cells requires transcriptionally active serum response factor.

Key words: Epicardium, Coronary artery, Calponin, Cardiac development

INTRODUCTION

Coronary artery smooth muscle cells (SMCs) originate from progenitors in the proepicardial organ (PEO), a transient structure formed by mesothelial cells overlying the sinus venosus (Mikawa and Gourdie, 1996; Gittenberger-de Groot et al., 1998). In the avian embryo, the PEO first appears at Hamburger-Hamilton stage (HH) 14 as a small cluster of finger-like projections that extend across the coelomic cavity toward the external surface of the looped heart tube (Manasek, 1971; Manner, 1992). Around HH18, the PEO makes contact with the heart in the region of the atrioventricular (AV) sulcus. From HH20 to HH24, epicardial cells extend over the surface of the myocardium to cover the heart (Himura and Hirakow, 1989). Failure of the epicardial covering to form in mice deficient in VCAM-1 or its counter-receptor α4-integrin results in a thin myocardial wall and a failure of coronary vessels to develop (Kwee et al., 1995; Yang et al., 1995). Coronary endothelial cells are thought to arise from preexisting vessels in the sinus venosus plexus via migration across the tissue bridge formed when the PEO contacts the heart (Vrancken Peeters et al., 1997). During stages HH19-23, the subepicardial matrix in the region of the AV canal and interventricular septum thickens and becomes populated by mesenchymal cells. The principle source of subepicardial mesenchymal cells has recently been shown to be epicardial cells that have undergone epithelial to mesenchymal transformation (EMT) (Dettman et al., 1998). These subepicardial mesenchymal cells subsequently give rise to cardiac fibroblasts and coronary SMCs and contribute to subendocardial cushion tissue cells involved in valvulogenesis (Gittenberger-de Groot et al., 1998). Around HH32, the nascent coronary plexus makes connections with the systemic circulation at the level of the aortic root, and coronary blood flow is established (Bogers et al., 1989; Waldo et al., 1990; Poelman et al., 1993). At the same time,

endothelial cells in the proximal coronary segments begin to recruit SMCs to establish a tunica media (Hood and Rosenquist, 1992; Poelman et al., 1993; Vrancken Peeters et al., 1997). While these cellular events have been described in considerable detail, the extracellular factors and signaling pathways that direct mesenchymal cells to express a SMC phenotype remain largely unknown.

Serum response factor (SRF), a highly conserved member of the MADS box family of DNA binding proteins (Shore and Sharrocks, 1994), is composed of a central 60-amino-acid MADS domain, responsible for DNA binding and dimerization, followed by a C-terminal transactivation domain (Triesman, 1994). Previous studies have demonstrated that dimeric SRF recognizes a DNA motif termed the serum response element (SRE), composed of a central CArG box [$CC(A/T)_6GG$] and its immediate flanking sequences (Pellegrini et al., 1995). SRF plays a key role in transcriptional activation of immediate early genes in response to growth factors and other extracellular stimuli that activate MAP kinase or Rho kinase-dependent signaling pathways (Triesman, 1994). Growth factor signaling through the *c-fos* SRE is mediated by formation of ternary complexes between SRF and an accessory factor, $p62^{TCF}$. The ETS domain proteins Elk-1, SAP-1 and SAP-2/NET and the paired-like homeodomain protein Phox-1 can potentiate the ability of SRF to transcriptionally activate the *c-fos* promoter in response to growth factor signaling (Grueneberg et al., 1992; Hill et al., 1994; Johansen and Prywes, 1995).

A variety of studies have shown that MADS box transcription factors also play important roles in muscle-specific gene transcription in vertebrates. A null mutation in the *Drosophila* MADS box factor D-Mef-2 resulted in failure of somatic, cardiac and visceral muscle differentiation (Lilly et al., 1995). Microinjection of antibodies to SRF blocked myogenic differentiation in two skeletal myoblast cell lines, mouse C2 and rat L6 (Soulez et al., 1996; Vandromme et al., 1992). Multiple CArG elements are found in a number of muscle-specific promoters, including those that direct cell-specific transcription in vertebrate smooth muscle. A dependence upon upstream CArGs for SMC-specific transcription has been reported for SMαA (Carroll et al., 1986; Blank et al., 1992; Shimizu et al., 1995; McNamara et al., 1995), SMγA (Szucsik and Lessard, 1995; Browning et al., 1998), SM-MHC (Madsen et al., 1997; Zilberman et al., 1998), caldesmon (Yano et al., 1995), α1-integrin (Obata et al., 1997), telokin (Herring and Smith, 1996) and SM22α (Li et al., 1997; Kim et al., 1997; Moessler et al., 1996). Transcription of the SM22α promoter in arterial SMCs in transgenic mice was also found to be dependent upon an upstream CArG box found within the first 280 bp of 5′ promoter sequence (Kim et al., 1997). Thus, factors that mediate CArG box-dependent transcription are likely to be important for maintenance of ongoing smooth muscle-specific gene expression in cells that are already expressing an SMC phenotype (Kim et al., 1997; Li et al., 1997; Madsen et al., 1997). What role SRF, in particular, plays in transcriptional activation of SMC-specific genes during the process of SMC differentiation from committed progenitor cells has not been tested. In the experiments reported here we show that differentiation of proepicardial cells into coronary SMCs occurs in a stepwise fashion and that transcriptionally active SRF is required for expression of an SMC phenotype in these cells.

MATERIALS AND METHODS

Quail eggs and embryos

Fertilized Japanese quail (*Coturnix coturnix japonica*) eggs were purchased from GQF Manufacturing (Savannah, GA). Embryos were staged (Hamburger and Hamilton, 1951) and either prepared for primary explant cultures or fixed in 4% paraformaldehyde (PFA) or methacarn (MCN) and then paraffin-embedded for immunolocalization studies.

Primary culture

HH16-17 embryos (54-64 hours of incubation) were microdissected into ice-cold PBS treated with 2× antibiotic/antimycotic solution (αB/M, Gibco-BRL, Gaithersburg, MD) with the aid of paper rings. Adherent yolk was rinsed free and the embryo was transferred to a clean Petri dish in a drop of complete medium consisting of M199 supplemented with αB/M, 30 mM glucose, 5 mM glutamine, 1.25 mM putrescine and either 10% fetal bovine serum (FBS) or 0.1% bovine serum albumin (BSA). Membranes covering the heart were gently opened and the heart tube carefully displaced with tungsten needles etched to a fine edge in NaOH. The PEO was determined by visual inspection to be either ‘early’, defined by small volume and few, if any, villous processes, ‘optimal’, defined by large volume, extensive villous processes but no attachments to the heart tube, or ‘late’, defined as firmly attached to the heart tube. ‘Optimal’ PEOs were drawn into the tip of a finely pulled glass pipet, carefully cut at their base to avoid inclusion of liver primordium or sinus venosus tissues and removed from the embryo. Isolated PEOs were placed into prewarmed medium in 6-well or 24-well Primaria-coated plates or into 8-well chamber slides (Becton-Dickenson, Lincoln Park, NJ) and incubated at 37°C in 95% air/5%CO_2.

Antibodies

Primary antibodies against avian calponin (clone CP-93) and SMαA (clone 1A4) were from Sigma (St Louis, MO). Monoclonal anti-SM22α (clone E11) and polyclonal anti-SM22α were generous gifts from Dr Mario Gimona (University of Padova, Padova, Italy). Monoclonal anti-SMγA was from ICN (clone B4, Costa Mesa, CA). Polyclonal anti-SM-MHC was a generous gift from Dr Robert Adelstein (NIH, Bethesda, MD). Monoclonal antibodies to SM-MHC were generously provided by Dr R. Nagai (University of Tokyo) and Dr U. Groschel-Stewart (Institut fur Zoologie, Darmstatd, Germany) or purchased from Sigma (clone hSM-V). Anti-SRF polyclonal antibody was obtained from Dr Ron Prywes (Columbia University). Anti-cytokeratin (Z0622) was from DAKO. The QH1 monoclonal antibody was from the Developmental Studies Hybridoma Bank (Johns Hopkins University, Baltimore, MD (Pardanaud et al., 1987). Anti-β-galactosidase monoclonal antibody was from Boehringer-Mannheim. IgG subtype-specific fluorescent and biotinylated secondary antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, AL). Phalloidin-Oregon green conjugate, fluorescent anti-rabbit, anti-mouse and streptavidin-conjugated antibodies were from Molecular Probes (Eugene, OR).

Immunohistochemistry

For paraffin-embedded embryos and heart tissues, 5-7 μm sections were dewaxed and rehydrated through xylene, ethanol and PBS solutions. Non-specific binding was blocked with 5% milk, 5% normal goat serum in PBS followed by incubation with primary antibodies overnight at 4°C. Biotinylated secondary antibodies and ABC-alkaline phosphatase tertiary amplification reagent (Vector

Labs, Burlingame, CA) were used and the staining visualized with NBT-BCIP (Boehringer-Mannheim, Indianapolis, IN). Slides were then dehydrated, coverslips placed on top and photographed. For PEO explant cultures, cells were fixed in 4% PFA and permeabilized with methanol:acetone (1:1). Non-specific binding was blocked with normal goat serum (1:50) in PBS and primary antibody was applied overnight at 4°C. After extensive washing with 0.1% BSA in PBS, cultures were incubated with fluorescent secondary antibodies (1:250) in 0.1% BSA/PBS for 1 hour at room temperature and again washed extensively. Coverslips were placed on the cultures, which were then photographed using a Nikon Optiphot 2 microscope equipped for epifluorescence.

RT-PCR analysis of single PEOs

RT-PCR assays were carried out on RNA prepared from single, freshly dissected PEOs or cultures derived from single PEOs, as described (Schultheiss et al., 1995). Intact PEOs or explant cultures were washed once with ice-cold PBS, incubated for 20 minutes on ice in 200 μl guanidinium isothiocyanate-containing solution D (Chomczynski and Sacchi, 1987), harvested, placed into microfuge tubes and frozen. Tubes were thawed on ice and RNA extracted with phenol-chloroform. The aqueous layer was removed to fresh tubes, 20 μg of oyster glycogen (Calbiochem, La Jolla, CA) was added and RNA precipitated overnight with isopropanol. After centrifugation, the RNA-glycogen pellet was resuspended in 20 μl DEPC-treated water, digested with DNase (1 unit/tube, 30 minutes, 37°C) followed by a second phenol-chloroform extraction and ethanol-glycogen precipitation. RNA was resuspended in TE (10 mM Tris HCl/1 mM EDTA, pH 7.4) and single-stranded cDNA was synthesized by reverse transcriptase (Perkin-Elmer, Norwalk, CT) using either specific antisense downstream primer (Table 1) or random primers. PCR amplification was carried out using the specific upstream and downstream primers listed in Table 1. To ensure linear amplification, samples were withdrawn from each tube after 15, 20, 25 and 30 cycles of PCR and DNA products were separated on 1.5% agarose gels, stained with 0.5 μg/ml ethidium bromide for 2 hours at room temperature, photographed on Polaroid type 667 film under standardized UV light intensity and camera exposure times. RT-PCR product bands were subjected to scanning densitometry and quantitated with Bio Image Whole Band Analyzer software (Bio Image Corp., Ann Arbor, MI). The identity of each RT-PCR product was confirmed by complete DNA sequencing (Sequenase II, Amersham). Control reactions in which reverse transcriptase was omitted or the sample was preincubated with RNAse (10 units, 30 minutes, 37°C) resulted in no detectable amplified products.

Transfection of dominant-negative SRF and analysis of single cells

Two different dominant negative SRF constructs (dnSRF) were used: (1) pSRFpm1 has three point mutations engineered into the MADS box that disable the DNA binding capacity of SRF (Johansen and Prywes, 1993), and (2) pSRFΔ5 has the C-terminal transactivation domain deleted so that it retains DNA binding capacity but is unable to activate transcription (Belaguli et al., 1997). dnSRF constructs, along with intact, wild-type SRF, were subcloned into CMV-expression vectors that contain an encephalomyocarditis virus internal ribosome entry sequence (IRES), allowing for linked expression of a *lacZ* reporter gene (pm1-IRES and Δ5-IRES). dnSRF or wild-type SRF plasmid DNAs (800 ng/well) were transfected into cultured PEOs 8 hours after explant, incubated in transfection medium for 5 hours, washed and allowed to grow for 4 days. Cells were then examined by double-label immunofluorescence for β-galactosidase and for SMC marker proteins. Areas of the outgrowth where single cells were well spread and readily visualized were selected for analysis. β-galactosidase-positive cells in randomly selected microscopic fields were identified and counted. The number of β-gal-positive cells that also expressed the particular SMC marker being tested was determined by viewing with a triple fluorochrome filter cube. When potential interference of fluorescence signals from neighboring cells was encountered, individual cells were reexamined under high magnification using the appropriate monochrome filters. When fluorescence signals from one cell were compromised by overlapping portions of another cell, neither cell was included in the analysis. Results were obtained for 70-100 β-galactosidase-positive cells per SMC marker protein examined and were expressed as the percentage of β-gal-positive cells that also expressed the test SMC marker protein.

RESULTS

Explant outgrowth from HH17 proepicardial organ (PEO)

PEOs were removed from HH17 quail embryos, placed into explant culture and examined over 5 days in vitro. During the first 24 hours, a monolayer of epicardial cells formed around the explant that displayed extensive cell-cell contacts and prominent subcortical actin bundles (Fig. 1A,D). By 3 days, a marked expansion of the epicardial cell outgrowth was

Table 1. RT-PCR primer sequences

Target	Upstream	Downstream	Product (bp)
cCalponin	CTG GCA CAG AAA TAC GAC CC	CTG CTG CTT CTC TGC GTA CT	383
cSM22α	CCA GTC CAA GAT CGA GAA GA	CTT GTT GGT CCC CAT CTG TA	507
cSMαActin	ACT GCT GCC TCT TCC TCC TCT	CAC CGT ATC CAA TTA ACC AGC C	563
cSM-MHC	GTC TAC TCT TAG TGT CAA GGT C	GCA CTT CCA CCT TGT GAA TGG T	371
qFlk-1	GCT CTT TTG TTT TTC CCG GCT	TCG GAT AAC CCA TCA AGA CCA	507
cNkx2.5	CCT TCC CCG GCC CCT ACT AC	CTG CTG CTT GAA CCT TCT CT	221
cTroponin-T	ATC TAT AAG GCG GCG GTT GA	CAG TGA TCG TCT CTC CAG TT	379
cSRF	AAC GGG ACA GTG CTG AAG AC	GGC CTC TCA GTC ACT CTT GG	578
cMEF-2	GAT TTC CAC TCT CCT GT	TTA CCA TGG GAC ATC T	304
cGATA-4	ATG ATT ATT CAG AAG GGC GAG	GGA TGA ATT GAA GAT CCA TGT C	562
cGATA-5	GTA TTT GGA GGA GTT CCC CG	TGA CCG TGC TGT CTG AGT TC	440
cGATA-6	GAG CTG CTG GAA GAC CTC TC	CAG CCC ATC TTG ACC TGA AT	573
cE-Hand	AGG AGA ACG GAG AGC ATC AA	TCA GGG GTT CAG TTC CAG AG	330
cCrp2/SLim	GCA GAG AAA TGT TCT CGG	CTA GCA TCT GTG TCA CAG T	327
cGAPDH	ACG CCA TCA CTA TCT TCC AG	CAG CCT TCA CTA CCC TCT TG	578
cEF1α	CAG CAA GAA TGA TCC TCC AAT G	CTG CCA CGA AAC AAA TGG TCC	624

Oligonucleotide sequences (5′ to 3′) obtained from either chick (c) or quail (q) cDNAs that were used as RT-PCR primers are listed. The sizes of the amplified products are given in bp.

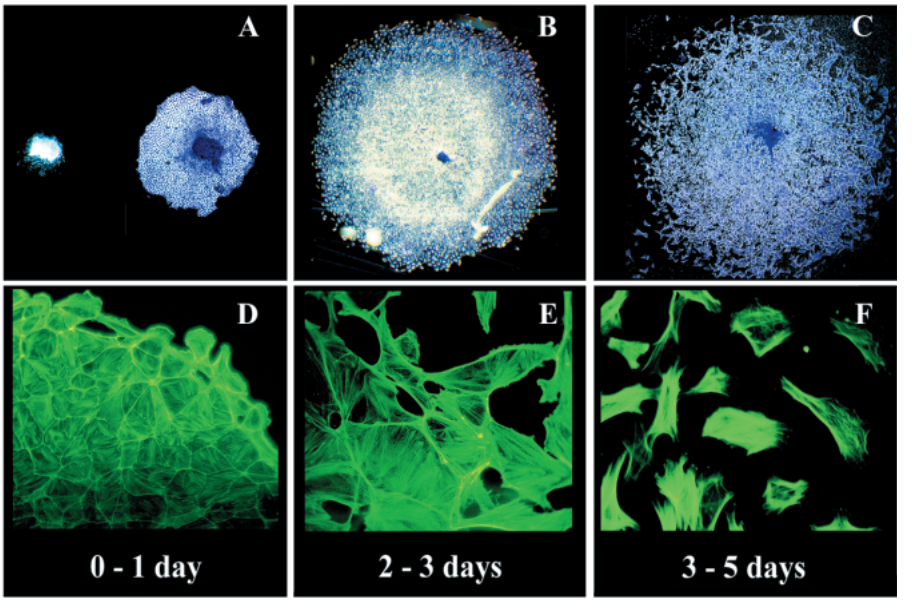

Fig. 1. Outgrowth of explanted PEO. Primary explant cultures of PEOs from HH17 quail embryos. (A-C) Coomassie Blue staining at 20× magnification. (D-F) Phalloidin-FITC staining at 200×. Note that outgrowth in the first 24 hours is entirely epithelial (A) and is characterized by prominent subcortical actin bundles and extensive cell-cell contacts (D). By 3 days, cells at the periphery of the culture begin to exhibit mesenchymal transformation. By 5 days nearly the entire culture is composed of independent mesenchymal cells containing prominent actin stress fibers (F).

observed (Fig. 1B). Cells at the periphery of the outgrowth had become detached from their neighbors, and developed vinculin-containing focal adhesions and actin cytoskeletal rearrangements characteristic of mesenchymal transformation (Figs 1E, 2F). By 5 days, the vast majority of epicardial cells had become mesenchymal, appeared to be motile, displayed few, if any, direct cell-cell contacts and exhibited cytoplasmic actin filaments organized into stress fibers (Fig. 1C,F).

Expression of smooth muscle markers during epicardial cell outgrowth

To determine whether PEO-derived cells that had become mesenchymal also expressed an SMC phenotype, we examined a panel of cell-type-specific markers by immuno-fluorescence. Cells in the monolayer that appeared around the explant at 1 day were uniformly positive for cytokeratin (Fig. 2A), an epicardial cell marker (Vrancken Peeters et al., 1995). These cells were also uniformly positive for SMαA (Fig. 2B), which was colocalized in subcortical actin bundles also labeled by FITC-phalloidin (compare Figs 2B and 1D). By contrast, no specific immunostaining was found for either calponin (Fig. 2D), SMγA (Fig. 2E) or SM22α (Fig. 2F).

By 5 days, PEO-derived cells had become mesenchymal in appearance, developed vinculin-containing focal adhesions (Fig. 2F) and reorganized subcortical actin bundles into stress fibers (Fig. 1B,E). Mesenchymal cells remained SMαA-positive (Fig. 2G) and cytokeratin-positive (not shown), the former being redistributed from subcortical bundles (Fig. 2B) into stress fibers (Fig. 2G). Epicardial-derived mesenchymal cells now exhibited strong immunoreactivity for the SMC

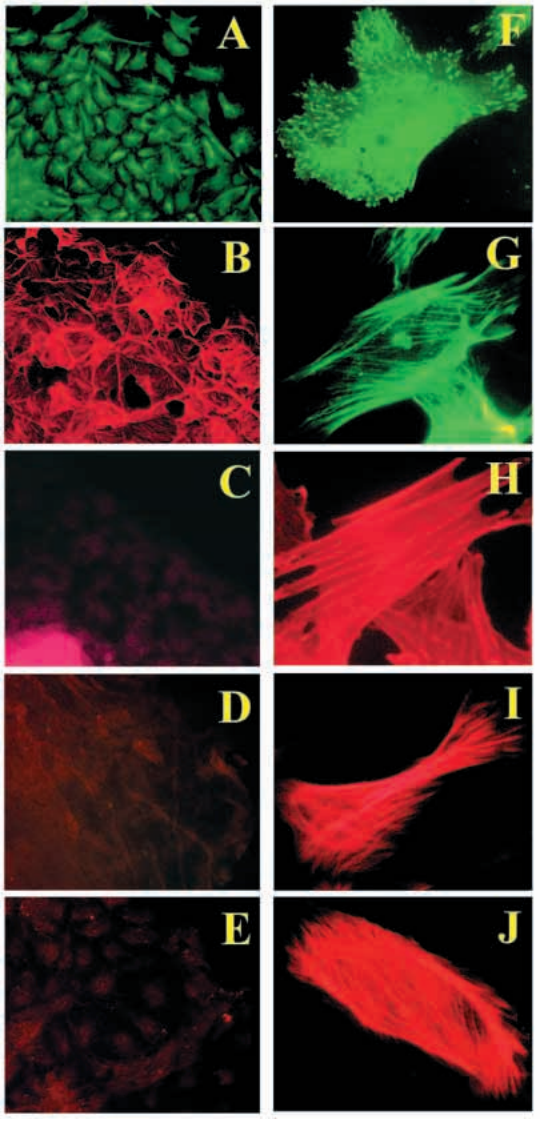

Fig. 2. SMC marker expression in explanted PEO cultures. Single or double immunofluorescence at 1 day (left) and 5 days (right). PEO-derived epicardial cells at 1 day are positive for cytokeratin (A) and SMαA (B) but not for calponin (C), SMγA (D) nor SM22α (E). Mesenchymal cells at 5 days remain positive for cytokeratin (not shown), develop vinculin-containing focal adhesions (F) and express all SMC markers tested: SMαA (G), calponin (H), SMγA (I) and SM22α (J).

marker proteins calponin (Fig. 2H), SMγA (Fig. 2I) and SM22α (Fig. 2J) in a filament pattern similar to SMαA. Therefore, expression of SMC marker proteins in PEO-derived cells could be divided into three stages: (1) a proepicardial stage in which none of the SMC markers were expressed, (2) an epicardial stage in which SMαA was strongly expressed in essentially all cells while calponin, SMγA and SM22α remained undetectable and (3) a mesenchymal stage in which all four SMC markers were coexpressed in the same cells.

RT-PCR analysis of smooth muscle marker gene expression

To determine whether changes in SMC marker proteins observed by immunofluorescence were due to activation of SMC-specific gene expression, we employed a RT-PCR assay designed for small quantities of starting material (Schultheiss et al., 1995). When carefully dissected from the embryo, freshly isolated HH17 PEOs exhibited no detectable expression of any of the SMC marker genes examined, either by RT-PCR (Fig. 3, day 0) or by immunostaining (data not shown). Likewise, transcripts for myocardial markers Nkx 2.5, GATA4 or cardiac troponin T (cTnT) could not be detected (data not shown). By contrast, if the PEO had made contact with the heart tube at the time of dissection (HH18 or later), then variable levels of transcripts from the myocardial genes together with SMαA and SM22α (known to be expressed in cardiac myocytes at these stages) and flk-1 (an endothelial marker) were usually found (data not shown).

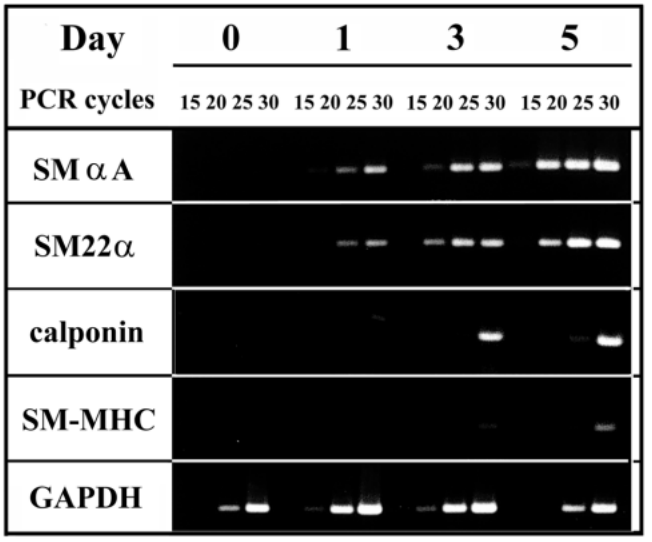

Fig. 3. Time course of SMC marker expression by RT-PCR. Individual HH17 PEOs or explant-derived colonies were analyzed by RT-PCR for expression of SMαA, SM22α, calponin, smooth muscle myosin heavy chain (SM-MHC) and GAPDH at the indicated time points. Day 0 refers to freshly dissected HH17 PEOs that were not cultured. To monitor linearity of amplification, samples were removed from individual PCR reaction tubes at 15, 20, 25 and 30 cycles. Note that no SMC markers are detectable at 0 days, SMαA and SM22α mRNA become detectable at 1 day (epithelial stage) and that calponin and SM-MHC mRNAs become detectable at 3 days or later (mesenchymal stage). The size of each PCR product is given in Table 1. The identity of each band was confirmed by complete sequencing of the PCR product. The results shown are representative of at least five independent experiments for each marker.

By 24 hours after explant of HH17 PEOs, SMαA transcripts had become readily detectable (Fig. 3), in agreement with the immunofluorescence results shown in Fig. 2. Between days 1 and 5, the abundance of SMαA mRNA continued to increase relative to GAPDH. To ensure that changes in SMαA mRNA levels shown in Fig. 3 represented true increases in gene expression and not artifacts due to normalization, we compared GAPDH to another housekeeping gene, ribosomal elongation factor 1α (EF-1α). The ratio of GAPDH to EF-1α mRNA in PEO-derived cells did not vary more than twofold over the 5-day period studied here (data not shown). SM22α gene expression closely paralleled that of SMαA (Fig. 3). By contrast, neither calponin nor SM-MHC mRNA were detectable at day 1. While RT-PCR products for both calponin and SM-MHC became evident by day 3 and continued to increase by day 5, the rate of accumulation of these gene products was clearly slower than that for SMαA and SM22α. By day 16, PEO-derived mesenchymal cells had become organized in a hill and valley pattern typical for SMCs in culture (Owens, 1995) and expression of the four SMC marker genes was two- to eightfold greater than that observed at day 5 (data not shown). Direct sequencing of the RT-PCR product for SM-MHC indicated that the SM1, but not SM2, isoform was expressed by PEO-derived cells on day 5.

RT-PCR analysis of smooth muscle regulatory gene expression

To gain insight into factors that mediate the activation of SMC marker gene expression in proepicardial cells, we next examined the expression of a set of regulatory genes that have been implicated in the control of SMC-specific gene expression. SRF transcripts were undetectable in freshly isolated HH17 PEOs whereas low but detectable levels of Mef-2b, GATA5 and Crp2/SM-Lim transcripts were found (Fig. 4, day 0). The expression of all four genes, relative to GAPDH

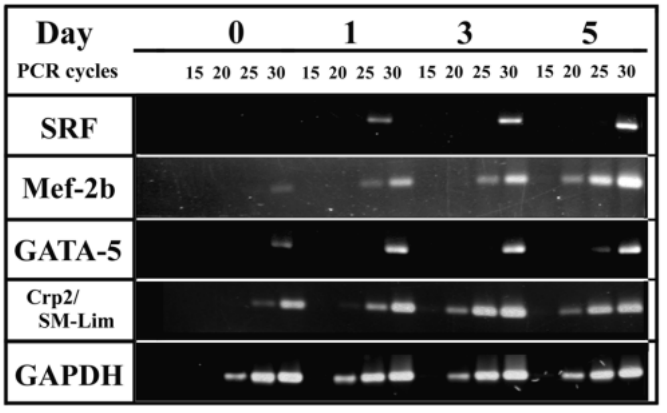

Fig. 4. Time course of SMC regulatory gene expression by RT-PCR. Individual PEOs or explant-derived colonies were obtained at the times indicated and transcript levels for SRF, Mef-2b, GATA-5, Crp2/SM-Lim and GAPDH were examined by RT-PCR analysis. Note that SRF transcripts are undetectable at 0 days and increase in abundance relative to GAPDH over the 5 days in culture. Mef2b, GATA5 and Crp2/SM-Lim mRNAs are detectable at low levels at 0 days and also increase over the 5 days in culture. The size of each product shown is given in Table 1. The identity of each band was confirmed by complete sequencing of the PCR product. Results shown are representative of at least four independent experiments.

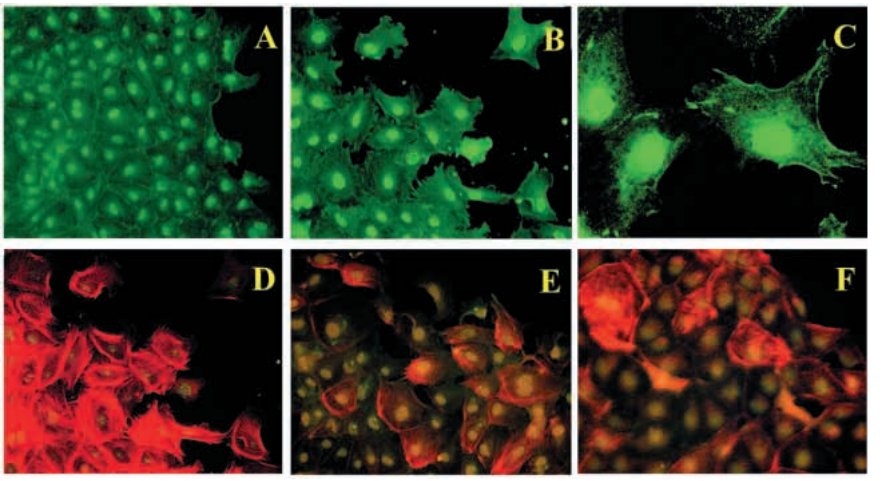

Fig. 5. Expression of SMC markers during epicardial to mesenchymal transformation. (A-C) Uniformly positive nuclear staining for SRF in epicardial cells prior to (A, 2 days), during (B, 3 days) and after (C, 5 days) mesenchymal transformation. (D) Double-labeling for SRF (green) and SMαA (red) shows that all SMαA-positive cells are also SRF-positive. (E) (calponin, red) and (F) (SMγA, red) show that the onset of expression of these two SM marker proteins occurs only in those cells that are both SRF-positive (green) and appear to have initiated mesenchymal transformation at the edges of the epicardial colony.

or EF-1α, increased strongly by day 1 and continued to increase, albeit at a slower rate, between days 1 and 5 (Fig. 4). GATA6 and eHAND transcripts were present at day 0 and decreased in abundance over time in culture to become undetectable by day 5 (data not shown).

Expression of SRF during epicardial to SMC differentiation

We next examined the distribution of SRF protein by immunofluorescence. Strong nuclear immunostaining for SRF was found in essentially all PEO-derived cells, either epicardial or mesenchymal, from day 1 to day 5 (Fig. 5A-C), consistent with the RT-PCR results shown in Fig. 3. Double-label immunofluorescence revealed that virtually all SMαA-positive cells exhibited nuclear immunostaining for SRF (Fig. 5D). Likewise, all calponin-positive or SMγA-positive cells displayed nuclei that were positive for SRF expression (Fig. 5E,F). Characteristically, calponin- or SMγA-positive cells were found only within the zone of mesenchymal transformation. Therefore, even though SMγA was found to be colocalized in the same filaments with SMαA, the temporal and spatial kinetics of SMγA expression in PEO-derived cells were identical to those of calponin rather than SMαA (Fig. 5).

These results show that SRF expression is coordinated with SMαA and preceeds the appearance of calponin and SMγA in PEO-derived cells in vitro.

Localization of SRF during coronary artery development in vivo

To determine if SRF is expressed during coronary SMC development in vivo, hearts from quail embryos were sectioned and examined by immunostaining. At HH18, myocardial cells were strongly positive for SRF, as previously reported (Croissant et al., 1996; Arsenian et al., 1998) while proepicardial cells in the PEO itself as well as the epicardial cells overlying the myocardium were negative (Fig. 6C). By

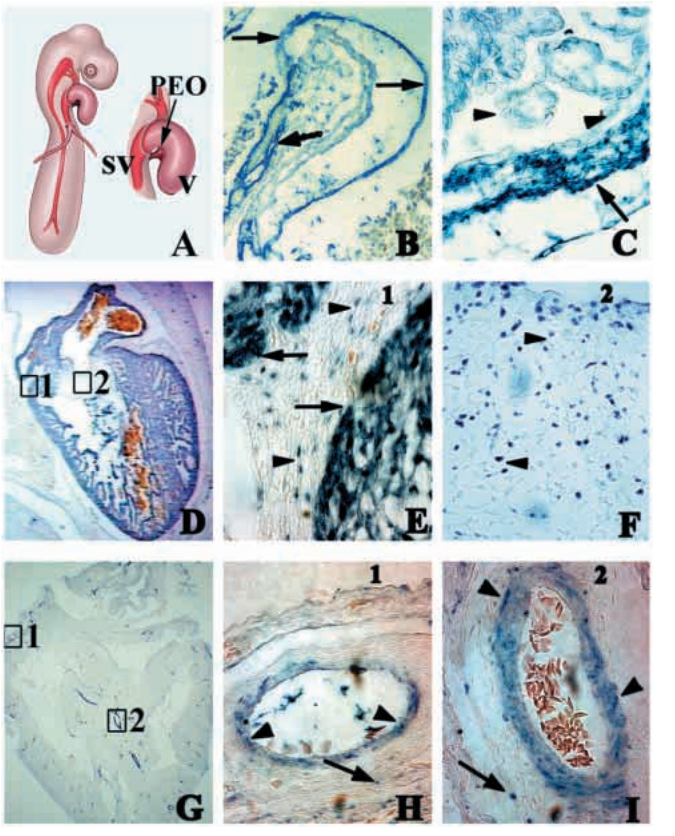

Fig. 6. Localization of SRF during coronary artery development. Sections from quail embryos at HH18 (approx. 2.5 days, A-C), HH26 (approx. 5.5 days, D-F) and HH40 (14 days, G-I) were analyzed by immunostaining. The cartoon in (A) shows the position of the PEO (arrow) between the sinus venosus (SV) and ventricular heart tube (V) at HH17-18. (B) QH-1 stains endothelial cells within the PEO (thick arrow) and endocardium (arrow), which is continuous with the endothelium of the sinus venosus (arrow). (C) SRF is found in 2.5-day myocardium (arrow) but is absent from PEO cells (arrowheads). (D) 5.5-day heart and outflow vessels. Boxed areas (1 and 2) are magnified in E and F. (E) SRF is found in subepicardial mesenchymal cells in the AV canal (arrowheads) as well as in the myocardium itself (arrows). (F) Anti-SRF stains nuclei of mesenchymal cells in the subendocardial cushion tissue matrix (arrowheads). (G) A 14-day heart cross section stained for SMαA, showing profiles of well-developed coronary vessels. Boxed areas 1 and 2 are magnified in H and I. SRF is present in SMCs of subepicardial vessels in the region of the AV canal (arrowheads) (H), and in SMCs within a coronay artery wall in the interventricular septum (I). Some adventitial fibroblasts are also positive for SRF staining in both regions (H,I, arrows).

HH26, a well-developed subepicardium had formed in the region of the atrioventricular canal consisting of mesenchymal cells dispersed within a loose extracellular matrix which were strongly positive for SRF (Fig. 6E). Cushion tissue mesenchymal cells involved in valvuloseptal tissue formation were also SRF-positive (Fig. 6F). By HH40, SMαA-positive coronary artery profiles were evident throughout the heart (Fig. 6G). SRF was detected in SMCs present within the walls of subepicardial as well as penetrating coronary vessels (Fig. 6H,I). These results indicate that SRF is expressed in coronary SMCs and their progenitors throughout the period of SMC differentiation in vivo.

Dominant-negative SRF inhibits SMC differentiation in PEO-derived mesenchymal cells

To determine if SRF activity is required for coronary SMC differentiation from proepicardial cells, two different dominant negative constructs (dnSRF) were employed to inhibit SRF function (Fig. 7A). pSRF-pm1 has three point mutations engineered into the MADS box, which disable binding to DNA (Johanson and Prywes, 1995). pSRF-Δ5 is a naturally occurring variant that lacks the C-terminal transactivation domain encoded by exon 5 while retaining the ability to dimerize and bind to DNA. dnSRF as well as wild-type SRF (wtSRF) constructs were cloned upstream of an internal ribosome entry sequence (IRES) that permits a β-galactosidase reporter to be coexpressed in cells that transcribe the form of SRF cloned upstream of the reporter gene (Fig. 7A).

dnSRF constructs were transfected into PEO cells 8 hours after explant and the expression of cell type-specific marker proteins in β-galactosidase-positive cells was determined 4 days later by immunofluorescence. Neither form of dnSRF had any effect on mesenchymal transformation (Fig. 7B,C) or on the number of cytokeratin-positive cells (Fig. 8). Similarly, there were only minor effects (pSRF-pm1) or none at all (pSRF-Δ5) of dnSRF on SMαA expression (Fig. 8). By contrast, both forms of dnSRF inhibited the appearance of calponin-positive cells by 60-80% (P<0.001) when compared to either pEmpty (a control for the effects of CMV promoter sequences) or pSRF-wt (expressing wtSRF) (Fig. 8; Table 2). Likewise, both forms of dnSRF also inhibited the appearance of SMγA-positive cells and SM22α-positive cells to the same extent as that observed for calponin-expressing cells (60-80%, P<0.001). Moreover, cotransfection of wtSRF together with either form of dnSRF rescued SMC marker gene expression (Table 2), confirming the specificity of dnSRF for inhibition of SRF function in PEO-derived cells in vitro. These results strongly suggest that differentiation of SMCs from PEO-progenitor cells requires functional SRF.

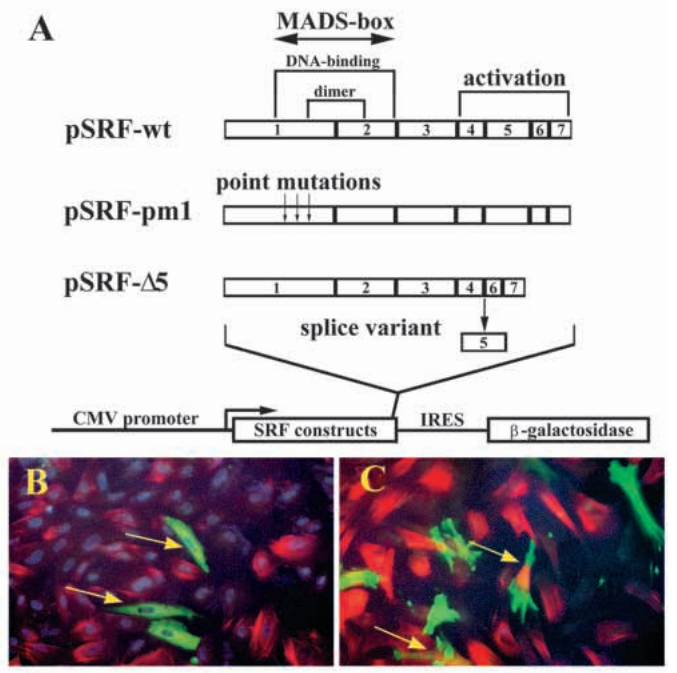

Fig. 7. (A) Construction of dominant-negative SRF expression vectors. Wild-type SRF (pSRF-wt) consists of seven exons with a central MADS box and a C-terminal transactivation domain. The MADS box contains residues essential for DNA binding and dimerization. pSRF-pm1 has three point mutations engineered into the MADS box that disrupt SRF binding to DNA. pSRF-Δ5 is a naturally occuring variant that lacks the C-terminal transactivation domain while retaining both DNA binding and dimerization activities. Wild-type and dominant-negative SRF constructs were subcloned into CMV promoter-driven expression vectors upstream of encephalomyocarditis internal ribosome entry sequence (IRES), allowing for linked expression of a β-galactosidase reporter gene. Thus all β-galactosidase-positive cells will also express the particular SRF construct that was cloned upstream of the IRES. (B,C) Co-labeling experiments for β-gal and the SMC marker calponin. (B) β-gal (green) and calponin (red) do not colocalize after transfection of PEO cultures with pSRF-pm1 (arrows). (C) β-gal (green) and calponin (red) are found colocalized in the same cells after transfection with pSRF-wt (arrows).

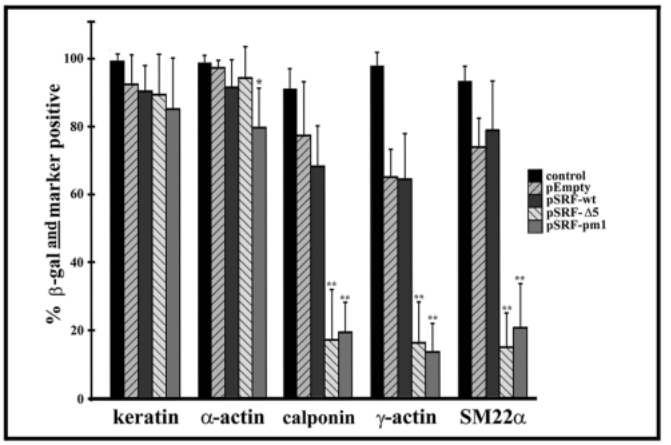

Fig. 8. Dominant-negative SRF inhibits SMC marker expression. Proteins evaluated for dependency upon functional SRF included cytokeratin (keratin), SMαA (α-actin), calponin, SMγA (γ-actin) and SM22α. Control values are the average percentage of cells expressing the particular SMC marker protein at 4 days after explanting the PEO. All other values are means ± s.d. of the percentage of β-gal-positive cells that are also SMC marker-positive (see Fig. 7). pEmpty was tested to control for possible effects of CMV promoter and IRES sequences as well as cryptic vector or reporter sequences acting independently of the test SRF constructs. Wild-type SRF and two different dominant-negative (dn) forms of SRF (pSRF-Δ5 and pSRF-pm1) were examined (800 ng/well each). Statistical evaluations were made based on comparisons between pEmpty and dn or wtSRF constructs: *P<0.05 and **P<0.001 by Student's t-test.

Table 2. Effects of dominant negative SRF (dnSRF) on SMC marker expression

	Calponin	SM-γ-actin	SM22α	SM-α-actin	Cytokeratin
Treatment					
control	90.7±5.75	97.3±3.93	92.7±4.32	98.3±2.14	99.0±2.00
pEmpty	76.8±15.8	64.1±8.52	73.2±8.49	96.7±1.96	92.0±8.65
pSRF-wt	67.6±12.1	63.7±13.6	77.9±14.6	90.7±8.12	89.7±7.72
pSRF-Δ5	16.5±15.0**	15.5±12.2**	14.3±10.1**	93.5±9.38	88.9±11.9
pSRF-pm1	18.6±9.07**	12.9±8.46**	20.0±13.0**	78.8±11.5*	84.3±15.2
Rescue experiment					
pm1+Empty	17.1±9.84**	16.9±8.74**			
pm1+wtSRF	69.1±7.29	78.6±13.8			
Δ5+Empty	17.1±5.27**	20.0±6.90**			
Δ5+wtSRF	67.9±2.98	66.7±9.42			

Control values are the means ± s.d. for the percentage of cells examined that express the marker protein indicated (usually >90%). All other values are means ± s.d. for percentages of β-galactosidase-positive cells that also express the indicated SMC marker protein (see Fig. 8).

pEmpty is the pCGN vector without an insert. Wild-type and dnSRF constructs are described in Fig. 7.

All plasmids contained an IRES sequence that allowed SRF constructs to be expressed upstream of a β-galactosidase reporter gene (Fig. 7).

Rescue experiment

Cells were cotransfected with dnSRF constructs (600 ng/well) and either wild-type SRF or empty vector (also 600 ng/well) to assess the specificity of action of the dnSRF constructs. *P<0.05 and **P<0.001 compared to pEmpty values for the specific marker indicated.

DISCUSSION

In the studies described here, we report that proepicardial cells obtained from HH17 quail PEOs exhibit a sequence of changes in cell morphology, cytoskeletal organization and gene expression patterns consistent with differentiation of committed progenitor cells into vascular SMCs in vitro. Moreover, expression of three well-characterized markers of the smooth muscle phenotype, calponin, SM22α and SMγA, was found to depend upon functionally active SRF being available during the period of transcriptional activation of these SMC marker genes. The time-dependent sequence of cellular events we observed in vitro is strongly reminiscent of the phenotypic changes that proepicardial cells exhibit as they form the epicardial covering of the heart, transform into mesenchymal cells in the AV canal and interventricular septum and differentiate into coronary SMCs in vivo. We conclude that coronary SMC differentiation from progenitor cells in the PEO is a multistep process that depends upon transcriptionally active SRF to activate and maintain SMC-specific gene transcription.

A variety of approaches, including retroviral-based lineage analysis, chimeric avian embryos and gene deletion studies, have shown that the embryological origins of coronary artery SMCs are in lateral plate mesoderm-derived mesothelial cells that line the pericardial coelom (Kwee et al., 1995; Mikawa and Gourdie, 1996; Vrancken Peeters et al., 1997; Gittenberger-de Groot et al., 1998). The factors that cause mesothelial cells at the junction between the sinus venosus and vitelline veins to begin to produce large quantities of a proteoglycan-rich extracellular matrix (Kalman et al., 1995) and extend villous projections into the cardiac coelom while their neighboring mesothelial cells do not undergo these changes are not known. Likewise, it is not clear if the potential to form SMCs is restricted only to mesothelial cells within the PEO or if neighboring coelomic mesothelial cells also have this potential. Unlike skeletal or cardiac myoblasts, SMCs have multiple origins within the embryo and can arise from progenitors in both ectoderm and mesoderm-derived mesenchyme (LeLievre and Le Douarin, 1975; Jain et al., 1998). Given the widespread distribution of blood vessels throughout the embryo, it would follow that the potential to form vascular smooth muscle is not likely to be restricted to a small precursor population but may be a common property of most mesenchymal cells. Therefore, it may not be surprising to find that transcription factors distributed throughout the mesoderm (e.g. SRF) play critical roles in activation of SMC-specific gene expression in vascular development. At the same time, 441 bp of the SM22α promoter has been shown to direct reporter gene expression in arterial SMCs while no reporter expression was found in venous, gut or coronary SMCs in vivo, sites where the endogenous gene is expressed at levels equal to those in arterial SMCs (Li et al., 1997). In addition, our data suggest that SRF is necessary, but not sufficient, for SMC differentiation since SRF is expressed and localized to the nucleus in epicardial cells, yet these cells do not express calponin, SMγA and SM-MHC until they undergo mesenchymal transformation. While post-translational modification of SRF during SMC differentiation remains to be investigated, increasing evidence suggests that interactions between SRF and other DNA binding proteins including Nkx factors, GATA factors or Mhox can act cooperatively to stimulate target gene transcription (Chen and Schwartz, 1996; Grueneberg et al., 1992). Therefore, while each smooth muscle lineage may exhibit a common requirement for SRF, it is likely that SMC type-specific transcription depends on combinatorial interactions of different CArG elements embedded in different contextual sequences with accessory factors unique to that SMC subtype.

Expression of SMαA is the earliest marker currently known for SMC differentiation (Owens, 1995). The first cells to express SMαA during vascular development appear at the ventral surface of the descending aorta in apposition to the foregut endoderm (Hungerford et al., 1996). As development of the aortic wall proceeds, additional markers of the mature SMC phenotype appear. Duband et al. (1993) showed that vascular SMC differentiation in vivo occurs in stages and that SMαA expression marks an early stage whereas calponin and SM22α are representative late-stage markers (Duband et al., 1993). Similar to developing vessels in vivo, we found that SMαA was the first SMC marker to appear in PEO-derived cells in vitro. Since SMαA was expressed in cells that retained

the epicardial phenotype, including extensive cell-cell contacts and epithelial-like organization, SMαA expression seemed to identify cells with the potential to differentiate into SMCs, i.e. presumptive SMCs, rather than SMCs per se. This is supported by the findings that SMαA-positive epicardial cells failed to express calponin, SMγA or SM-MHC. A consistent finding in these studies was that expression of the SM marker proteins calponin, SM22α and SMγA was first observed only in those epicardial cells within the zone of mesenchymal transformation that exhibited a downregulation of cell-cell contacts, a reorganization of cytoplasmic actin and formation of vinculin-containing focal adhesions. An important role for mesenchymal transformation in the formation of coronary SMCs was recently emphasized by Dettman et al. (1998), who tagged surface epicardial cells with the fluorescent dye DiI or with an adenovirus carrying CMV-lacZ and examined their fates 3-16 days later. Labeled cells were found in the subepicardial mesenchyme, dispersed deep within the myocardial layer and incorporated into the walls of the subepicardial and penetrating coronary arteries. Dettman et al. suggested that the most likely origin of coronary SMCs was from epicardial cells that had undergone mesenchymal transformation rather than from a population of preformed SMCs that had migrated in with epicardial cells upon contact of the PEO with the heart, as had been previously believed. Although our findings are based on PEOs isolated prior to contact with the myocardium whereas Dettman et al. obtained epicardial cells from explanted beating hearts, our data strongly support the model of Dettman et al. (1998) since we could directly observe the conversion of cytokeratin-positive, calponin-negative epicardial cells into cytokeratin-positive, calponin-positive mesenchymal cells within 48-72 hours ex vivo. Moreover, the first cells to express SM22α or SMγA were also cytokeratin-positive cells that exhibited mesenchymal transformation. By 5 days in culture, nearly all epicardial cells had become mesenchymal and expressed SMαA, SMγA, calponin and SM22α, indicating that the majority (perhaps all) of proepicardial cells at HH17 have the potential to become SMCs. Since not all of the epicardial-derived mesenchymal cells in the heart express an SMC phenotype (Dettman et al., 1998; Gittenberger-de Groot et al., 1998), additional controls must exist in the developing heart that restrict expression of the SMC phenotype to those cells in close association with coronary endothelial cells.

The findings reported here with two different dnSRF constructs that produce loss of function by two different mechanisms reveal an essential and specific role for SRF in SMC differentiation from committed progenitor cells. Moreover, wild-type SRF completely rescued the inhibitory effects of dnSRF on calponin and SMγA expression. Furthermore, SRF is expressed in vivo in a pattern that is consistent with a role in SMC differentiation during normal coronary artery formation. These results support a role for SRF in the activation and maintenance of SMC-specific gene expression during coronary development. They are consistent with promoter-analysis studies in vitro and in vivo, which suggest that CArG-box factors are important for the maintenance of SM-specific gene expression in cells that are already expressing an SMC phenotype. They are also suggestive of a more general role for SRF in muscle development. Once the primordial germ layers are formed, high levels of SRF expression are restricted to developing skeletal, cardiac and smooth muscle tissues (Croissant et al., 1996; Arsenian et al., 1998). Moreover, dnSRF has been shown to block myogenic differentiation in both skeletal and smooth muscle lineages (Vandromme et al., 1992; this report). It was recently shown that SRF –/– embryos arrest shortly after gastrulation and are severely deficient in mesoderm formation (Arsenian et al., 1998). The necessity to pursue tissue- and developmental stage-specific knockouts for SRF highlights the need for a better understanding of the mechanisms that generate different types of SMCs during development.

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