Perlecan Regulates Oct-1 Gene Expression in Vascular Smooth Muscle Cells

Mary C.M. Weiser,* Nicole A. Grieshaber, Phillip E. Schwartz,
and Richard A. Majack

Departments of Pediatrics and Cell and Structural Biology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Vascular smooth muscle cells (SMCs) are very quiescent in the mature vessel and exhibit a remarkable phenotype-dependent diversity in gene expression that may reflect the growth responsiveness of these cells under a variety of normal and pathological conditions. In this report, we describe the expression pattern of Oct-1, a member of a family of transcription factors involved in cell growth processes, in cultured and in vivo SMCs. Oct-1 mRNA was undetectable in the contractile-state in vivo SMCs; was induced upon disruption of in vivo SMC–extracellular matrix interactions; and was constitutively expressed by cultured SMCs. Oct-1 transcripts were repressed when cultured SMCs were plated on Engelbreth-Holm-Swarm tumor-derived basement membranes (EHS-BM) but were rapidly induced after disruption of SMC–EHS-BM contacts; reexpression was regulated at the transcriptional level. To identify the EHS-BM component involved in the active repression of Oct-1 mRNA expression, SMCs were plated on laminin, type IV collagen, fibronectin, or perlecan matrices. Oct-1 mRNA levels were readily detectable when SMCs were cultured on matrices composed of laminin, type IV collagen, or fibronectin but were repressed when SMCs were cultured on perlecan matrices. Finally, the Oct-1-suppressing activity of EHS-BM was sensitive to heparinase digestion but not to chondroitinase ABC or hyaluronidase digestion, suggesting that the heparan sulfate side chains of perlecan play a biologically important role in negatively regulating the expression of Oct-1 transcripts.

INTRODUCTION

Smooth muscle cell (SMC) replication plays a significant role in vascular pathologies such as arteriosclerosis, the restenosis of vascular grafts and angioplasties, and the maintenance of the hypertensive state (Ross, 1993; Schwartz *et al.*, 1995). In vitro and in vivo studies have provided an abundance of information regarding the factors that contribute to SMC growth control. For instance, a number of mitogens, including platelet-derived growth factors and fibroblast growth factors, are known to be important positive regulators of SMC migration and replication in culture and after vascular injury in vivo (Mosse *et al.*, 1985; Lindner *et al.*, 1990;

Ferns *et al.*, 1991). Other factors, such as transforming growth factor- β (Assoian and Sporn, 1986), heparin-like molecules (Clowes and Karnovsky, 1977; Castellot *et al.*, 1981), and endogenous heparan sulfate proteoglycans (HSPGs; Marcum and Rosenberg, 1984; Fritze *et al.*, 1985; Benitz *et al.*, 1990), appear to be potent inhibitors of SMC growth. In addition to known growth-regulatory molecules, other factors, such as hemodynamics (Kohler and Jawien, 1992), SMC phenotype (Chamley-Campbell *et al.*, 1981; Owens 1995), extracellular matrix (ECM) components (Thyberg, 1996), and apparent developmental age (Cook *et al.*, 1994), also appear to play important roles in determining the growth potential of SMCs. SMCs exhibit a remarkable phenotype-dependent diversity in gene expression, and the response of SMC to specific stimuli may vary according to the phenotypic state of the

* Corresponding author: Department of Pediatrics, Box B131, 4200 East Ninth Avenue, University of Colorado Health Sciences Center, Denver, CO 80262.

cells. Although extensive data describing the SMC growth-promoting signaling pathways induced by growth factors exist, little is known about the molecular changes underlying the SMC response to mitogenic stimulation, the potential role of SMC-ECM contacts in regulating these changes, or the molecular mechanisms that maintain SMC quiescence in the uninjured vessel wall.

Determining the mechanisms underlying the suppression of SMC replication during vascular development is important for our understanding of normal vascular morphogenesis and may well provide the basis for the development of therapeutic agents to control vascular diseases characterized by abnormal SMC growth. However, the factors that suppress SMC replication once morphogenesis is complete are almost completely unknown. Extracellular factors that may contribute to developmental growth suppression in vascular SMCs include the loss of expression of growth-promoting agents and/or the gradual deposition of a growth-inhibitory ECM. One approach to elucidating these mechanisms is to study the alterations in gene expression that occur when fully differentiated (adult *in vivo*) SMCs modulate from quiescent to replication-competent cells. Because a number of transcription factors are known to be essential for cell cycle progression, we hypothesized that SMCs may express specific growth-essential transcription factors as a function of injury to the ECM. Determining the molecular basis for the suppression of expression of these factors in the mature vessel wall may, therefore, lead to a more thorough understanding of the mechanisms maintaining SMC quiescence in adult blood vessels.

In this study, we describe the regulated expression of the mRNA encoding the transcription factor Oct-1 in vascular SMCs, with particular emphasis on its regulation by the ECM. Oct-1 is the prototype member of the POU domain family of transcriptional regulators; one of its functions involves the S-phase-specific transcriptional regulation of the histone *H2b* gene (Fletcher *et al.*, 1987; Sturm *et al.*, 1988; Rosenfeld, 1991; Ruvkan and Finney, 1991; Scholer, 1991). Oct-1 is, therefore, considered essential, but not sufficient, for cellular replication. We show that Oct-1 mRNA is not expressed by vascular SMCs in the uninjured, adult vessel wall and that Oct-1 transcripts are rapidly induced upon disruption of SMC-ECM interactions. Finally, we provide evidence that the heparan sulfate side chains of perlecan, the predominant basement membrane proteoglycan, specifically inhibit Oct-1 gene expression. The data suggest a model in which injury to the SMC basement membrane "activates" SMCs, in part by the derepressed expression of transcription factors, such as Oct-1, through the removal of inhibitory influences to allow subsequent replication.

MATERIALS AND METHODS

Cell Culture and Supplies

The aortic media of adult Sprague Dawley rats were aseptically dissected, and SMCs were obtained by digestion in DMEM containing 1 mg/ml collagenase (type IV; Sigma Chemical, St. Louis, MO), 0.125 mg/ml elastase (type IV; Sigma Chemical), and 0.375 mg/ml soybean trypsin inhibitor (type 1-S; Sigma Chemical) for 4 h at 37°C. Cells were maintained in 10% calf serum (CS) in DMEM and were used for experiments in the 6th to 10th passages. Growth-arrested SMCs were obtained by maintaining cultures in serum-free DMEM (SFM) for 96 h. To study the effect of basement membrane components, cell culture flasks were coated with 50 μ l/cm² Engelbreth-Holm-Swarm mouse tumor-derived basement membrane extract (EHS-BM; commercially available as Matrigel) before cell plating following the instructions provided by Collaborative Biomedical Products (Bedford, MA). To investigate the effect of heparan sulfate components of EHS-BM on gene expression, basement membranes were made and were left untreated or were treated with 10 U of heparinase I, 1 U of chondroitinase ABC, or 0.1% hyaluronidase before use. To examine the effects of individual basement membrane components, bacteriological plastic was coated with 10 μ g/ml laminin, 10 μ g/ml type IV collagen, 5 μ g/ml fibronectin, or 2 μ g/ml perlecan overnight at room temperature. Matrix components were diluted in SFM for coating. After coating, the plates were rinsed with SFM and treated with 0.5% bovine serum albumin in SFM for 4 h before plating of SMC. Matrix components were from Collaborative Biomedical Products. Gels composed of types I and III collagens (commercially available as Vitrogen) were made following the instructions provided by Celtrix Laboratories (Palo Alto, CA). Tissue culture reagents were from Life Technologies (Gaithersburg, MD). Transforming growth factor- β_1 (TGF- β_1) from porcine platelets was purchased from R&D Systems (Minneapolis, MN), and heparin, heparinase I, chondroitinase ABC, and hyaluronidase were purchased from Sigma Chemical.

RNA Preparation

Total RNA was isolated from SMC cultures using the acid-guanidinium isothiocyanate method. Briefly, cultures were washed twice with ice-cold SFM and lysed in 4 M guanidinium isothiocyanate/0.2 M NaOAc (pH 4.0)/0.5% Sarcosyl/50% phenol at 4°C. For the *in vivo* SMC experiments, tunica medias of adult uninjured rat aortas were isolated, immediately frozen in liquid N₂, and crushed into a fine powder before acid-guanidinium isothiocyanate extraction of total RNA. "Dispersed" total RNA was isolated after enzymatic dispersion of the aortic media into single cells in SFM containing 1 mg/ml collagenase and 0.125 mg/ml elastase. The quantity and purity of the RNA samples were determined by spectrophotometric analysis.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Southern Blot Hybridization Analysis

Equal amounts of total RNA were reverse transcribed into first-strand cDNA using random oligonucleotides and reverse transcriptase (Superscript Preamplification System, Life Technologies). PCR amplification was for 40 cycles using the following parameters: denaturing step, 4 min at 95°C; amplification steps, 1 min at 95°C, 1.5 min at 65°C (Oct-1, G3PDH), 60°C (p53), or 50°C (Rb), and 2 min at 72°C; elongation step, 10 min at 72°C. Ethidium bromide-stained amplification products were resolved by agarose gel electrophoresis, and sizes were determined by comparison to known molecular weight standards. Because the Oct-1 PCR product was barely detectable by ethidium bromide gel electrophoresis, Southern blots were prepared from the agarose gels. Gels were sequentially denatured in 1.5 M NaCl/0.5 N NaOH and neutralized in 1.5 M NaCl/

0.5 M Tris-HCl (pH 7.4), and the DNA was transferred to Durolon-UV hybridization membranes (Stratagene, La Jolla, CA) overnight in $10\times$ SSC and then was cross-linked to the filters by UV exposure. The filters were prehybridized for 2 h at 42°C in $5\times$ SSPE, $3\times$ Denhardt's solution, 50% formamide, 0.5% sodium dodecyl sulfate, and 200 $\mu\text{g}/\text{ml}$ fragmented, denatured DNA, and then the filters were hybridized overnight at 42°C in the same buffer solution containing the labeled probe. Filters were washed in two changes of $0.1\times$ SSC/1% sodium dodecyl sulfate at 65°C for 60 min and then were exposed to autoradiography film (Dupont Reflection NEF-496, NEN Research Products, Boston, MA) at -70°C .

PCR Primers and DNA Probes

Gene-specific oligonucleotide primers were designed for RT-PCR based on the published human Oct-1 sequence (5'-TTGAGGAGCT-TGAGCAGTTTGCCAA-3' and 5'-TAGGTGAGCTGCTGGTCCCA-CCACT-3'; Sturm *et al.*, 1988), the published rat p53 sequence (5'-TCAGTCTGAGTCAGGCCCCAC-3' and 5'-CACATGACTGAGGT-CGTGAGA-3'; Soussi *et al.*, 1988), the published mouse Rb sequence (5'-TCTATAACTCCGTTTTTCATG-3' and 5'-TTCAGTTTCTGCTTT-TGCAT-3'; Bernards *et al.*, 1989), and the published rat G3PDH sequence (5'-ATGGTGAAGGTCGGGTGTCAG-3' and 5'-TCA-CACCCATCACAAACATGGG-3'; Tso *et al.*, 1985). Oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). The cDNA probe for Oct-1 was a 509-bp *Pf*MI/*Hinc*II fragment of a human Oct-1 cDNA (a kind gift from W. Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and was labeled with [α - ^{32}P]dCTP and random primers (Megaprime System, Amersham, Buckinghamshire, United Kingdom) to a specific activity of 10^8 – 10^9 cpm/ μg .

DNA Sequencing

The PCR products for Oct-1, p53, Rb, and G3PDH were extracted from agarose and subcloned into the pCRII vector (TA cloning system, Invitrogen, San Diego, CA) according to the procedures supplied by the manufacturer. Subcloned products were sequenced using double-stranded DNA sequencing techniques (Sequenase, version 2.0, United States Biochemical, Cleveland, OH).

Nuclear Extract Preparation

Nuclear proteins were prepared from cultured adult aortic SMCs according to the method described by Zerivitz and Akusjarvi (1989). Briefly, SMCs were harvested into phosphate-buffered saline, pelleted, washed twice in phosphate-buffered saline, and suspended in 2 packed cell volumes of buffer A [0.25 M sucrose, 20 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol (DTT), 0.5 mM spermidine, 0.15 mM spermine]. Cells were centrifuged and resuspended in buffer A for 5 min. Cells were then lysed by the addition of lysolecithin (400 $\mu\text{g}/\text{ml}$; Sigma Chemical); the lysis reaction was stopped by the addition of ice-cold buffer B (buffer A plus 3% bovine serum albumin). Nuclei were collected by centrifugation, were extracted with buffer C (20 mM HEPES, pH 7.9, 25% vol/vol glycerol, 0.6 M KCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride), and were disrupted by passage through a 23-gauge syringe. The nuclear extract was then dialyzed against 100 volumes of buffer D (20 mM HEPES, pH 7.9, 20% vol/vol glycerol, 0.1 M KCl, 2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride). To prepare nuclear extract from *in vivo* SMCs, the media from adult uninjured aortas were homogenized in buffer A and were then processed as described above.

Electrophoretic Mobility-Shift Assay (EMSA)

EMSAs were conducted on nuclear extract preparations from cultured and *in vivo* SMCs according to the procedures provided by Promega (Madison, WI). Double-stranded oligonucleotides contain-

ing the consensus octamer-binding site were end labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase and were used as protein-specific probes in the EMSA reactions. Oligonucleotides containing the consensus TFIID-binding site were used as controls. Binding reactions contained 20 μg of nuclear extract, 3.5 pmol of labeled probe, 4% glycerol, 1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.05 mg/ml poly(dI-dC). Specificity of Oct-1-binding activity was determined by the addition of an Oct-1-specific polyclonal antibody (kindly provided by W. Herr, Cold Spring Harbor Laboratory) to the binding reaction.

Growth Assays

Bromodeoxyuridine Immunocytochemistry. Adult aortic SMCs were maintained in culture at confluent ($>90\%$ confluent) or sparse ($<50\%$ confluent) density in SFM, DMEM containing 10% CS, SFM containing TGF- β_1 (10 ng/ml), or SFM containing heparin (100 $\mu\text{g}/\text{ml}$) for 96 h. All cultures were exposed to 10 mM 5-bromodeoxyuridine (BrdUrd) for the final 24 h of culture, fixed in 100% MeOH/0.3% H_2O_2 , and immunocytochemically stained using a monoclonal antibody against BrdUrd (Cook *et al.*, 1994). Labeling indices (percentage of BrdUrd-labeled nuclei) were determined by cell counting; a minimum of 500 total cells were counted per condition.

Cell Number Assays. SMCs were plated at sparse density (2×10^4 cells/24-well plate) in DMEM containing 10% CS on tissue culture plastic, plastic coated with Vitrogen gel (type I and type III collagens), or plastic coated with Matrigel and were allowed to attach and spread overnight. The media were changed and cell numbers were determined in triplicate by hemocytometer at 0, 24, 48, and 96 h.

RESULTS

Our research goal is to identify factors in the arterial tunica media that contribute to the inhibition of SMC replication. Our approach to this question is to identify specific replication-essential transcription factors that are induced upon "injury" to the vascular wall and then to determine the factors that negatively regulate their expression. In this study, we examined the regulated expression of the gene encoding Oct-1, a transcription factor known to play an integral role in cellular replication. Because detection of Oct-1 transcripts in cultured SMCs by Northern blot analysis required 20 μg of poly(A) $^+$ -selected RNA and an exposure time of 2 wk, we used a combined RT-PCR/Southern blot hybridization technique for the experiments presented in this study. Using this technique, we could readily detect Oct-1 mRNA in SMCs cultured in 10% CS (Figure 1A). No reaction product was detected when total RNA or genomic DNA was used as template for the PCR (Figure 1A), eliminating the possibility that our signals result from contaminating DNA in the RNA preparation. To verify that the PCR product was Oct-1, the 494-bp PCR product was sequenced and was shown to be $>98\%$ homologous to the published human Oct-1 sequence (our unpublished results; Sturm *et al.*, 1988).

We first examined the expression of Oct-1 mRNA *in vivo* to determine whether Oct-1 is expressed in fully differentiated, contractile SMCs. Whereas Oct-1 mRNA was readily detectable in cultured, exponen-

Figure 1. Oct-1 mRNA is expressed by cultured rat aortic SMCs but is undetectable in adult *in vivo* SMCs. (A) Total RNA was extracted from aortic SMCs maintained in culture in 10% CS for 72 h and then was reverse transcribed into first-strand cDNA. Expression of Oct-1 mRNA was assessed using gene-specific primers and RT-PCR coupled to Southern blot hybridization analysis (+RT). As controls, total RNA (-RT) and genomic DNA (G) were used as templates for the PCR. Cultured aortic SMCs express readily detectable levels of Oct-1 transcripts, and no message is detected using this technique when total RNA or genomic DNA is used in the PCR, eliminating the possibility of contaminating DNA present in the RNA preparation. (B) Total RNA was extracted from cultured exponentially growing SMCs (culture) or from the tunica medias of adult uninjured aortas (*in vivo*), reverse transcribed into first-strand cDNA, and analyzed for expression of Oct-1 using gene-specific primers and RT-PCR coupled to Southern blot hybridization. G3PDH-specific primers were used to amplify a 402-bp PCR fragment as a control for the RT and PCR reactions. Compared with cultured SMCs, adult *in vivo* SMCs do not express detectable levels of Oct-1 mRNA.

tially growing SMCs, using identical methods, Oct-1 mRNA was undetectable in *in vivo* SMCs (Figure 1B). Two separate sets of primers were used in RT-PCR to eliminate the possibility that Oct-1 may be present, but alternatively spliced, in *in vivo* SMCs. Both sets amplified a specific PCR product in cultured SMCs but failed to detect Oct-1 in *in vivo* SMC (our unpublished results).

EMSAs using oligonucleotide probes containing the consensus octamer-binding site were conducted on cultured SMC nuclear extracts and on nuclear extracts prepared from the media of adult uninjured aortas to further show that Oct-1 was not expressed by *in vivo* SMCs. We consistently see two distinct binding activities in cultured SMCs using the octamer-binding site as probe (Figure 2). The upper activity is completely

Figure 2. Oct-1-binding activity is present in cultured SMCs but is undetectable in adult *in vivo* SMCs. Nuclear proteins were extracted from cultured exponentially growing SMCs (*in vitro*; upper left panel) or from the tunica medias of adult uninjured aortas (*in vivo*; upper right panel) and analyzed for Oct-1-binding activity using ³²P-labeled oligonucleotides containing the consensus octamer-binding site and standard EMSA reactions. The position of retarded DNA-protein complexes containing Oct-1 is indicated by the arrowhead (as determined by the addition of an Oct-1-specific antibody to the binding reaction). ³²P-labeled oligonucleotides containing the consensus TFIID-binding site were used as controls to demonstrate active nuclear proteins in the *in vivo* extract (lower left panel). Consistent with the mRNA data, compared with cultured SMCs, active Oct-1 protein is not detectable in *in vivo* SMCs.

inhibited, whereas the lower activity is only partially inhibited, by preincubation with a cold competitor probe. The upper binding activity was identified as Oct-1 by preincubation with an Oct-1-specific antibody; this antibody inhibits binding of Oct-1 to the octamer-binding site (Figure 2). Consistent with the mRNA data, although Oct-1-specific binding activities were readily detected in cultured SMCs, we were unable to detect Oct-1-binding activities in *in vivo* SMCs (Figure 2). Oligonucleotide probes containing the TFIID-binding site were used in the binding reactions as a control to demonstrate active nuclear pro-

teins in the in vivo extract; one distinct binding activity was detected in both the in vivo (Figure 2) and the cultured (our unpublished results) extract preparations. These data (Figures 1B and 2) suggested that Oct-1 is regulated at one of three potential points in the transition from quiescent, in vivo SMCs to replicative, cultured SMCs: 1) after removal of the in vivo ECM; 2) after attachment and spreading of SMCs onto tissue culture plastic; or 3) after growth stimulation.

Oct-1 Is Constitutively Expressed by Cultured Aortic SMCs, Independently of the Growth State

To investigate whether Oct-1 mRNA expression is regulated by SMC growth state, total RNA was extracted from confluent growth-stimulated (10% CS) and growth-arrested (SFM, 96 h) SMC cultures, sparse growth-stimulated and growth-arrested SMC cultures, sparse SMC growth-arrested with TGF- β_1 (10 ng/ml, 96 h), and sparse SMC growth-arrested with heparin (100 μ g/ml, 96 h). RNA was then analyzed for Oct-1 expression using Oct-1-specific oligonucleotide primers and RT-PCR/Southern blot hybridization analysis as described in MATERIALS AND METHODS. Duplicate SMC cultures were labeled with BrdUrd for the final 24 h of culture and were immunocytochemically stained using a monoclonal antibody against BrdUrd. SMCs were effectively growth arrested when sparse and confluent cultures were maintained in SFM and when sparse SMCs were treated with heparin or TGF- β_1 , and demonstrated high replication indices when sparse and confluent cultures were stimulated with 10% CS (Figure 3A). Under this variety of conditions, Oct-1 mRNA was readily detectable in all SMC cultures (Figure 3B). The data suggest that Oct-1 mRNA is constitutively expressed by cultured SMCs, independently of growth state, mitogen stimulation, cell density, or exposure to growth inhibitors.

Oct-1 mRNA Is Induced upon Removal of SMCs from Their In Vivo ECM

Because Oct-1 is not expressed by in vivo SMCs (Figure 1B) and is constitutively expressed by cultured SMCs (Figure 3), we next determined whether removal of SMCs from their in vivo environment per se was sufficient to induce Oct-1 message accumulation. To test this possibility, the aortic media were enzymatically digested into single cells in SFM containing collagenase and elastase. After isolation, SMCs were immediately processed for RNA extraction and analyzed for Oct-1 mRNA expression. These cells were not exposed to serum or plated onto plastic. Removal of the in vivo ECM induced expression of Oct-1 mRNA in SMCs (Figure 4), suggesting that the ECM or components of the SMC basement membrane may negatively regulate Oct-1 expression. For controls, we compared the expression of the *p53* tumor suppressor gene and the retinoblastoma (*Rb*) susceptibility gene, two low abundance transcription factor

Figure 3. Oct-1 mRNA is constitutively expressed by cultured SMCs. (A) Sparse and confluent SMCs were cultured in 10% CS or growth arrested by serum deprivation (SFM, 96 h) or treatment with TGF- β_1 (10 ng/ml in SFM, 96 h) or heparin (100 μ g/ml in SFM, 96 h), pulse labeled with BrdUrd for the final 24 h of culture, fixed, and immunocytochemically stained using a monoclonal antibody against BrdUrd. BrdUrd-labeling indices (expressed as percentage of BrdUrd-positive cells) were determined by cell counting. Data are presented as means $\pm$ SE. (B) Total RNA was extracted from duplicate SMC cultures and reverse transcribed into first-strand cDNA. Oct-1-specific oligonucleotide primers were used in RT-PCR/Southern blot hybridization analysis. G3PDH-specific primers were used as a control for the cDNA synthesis and PCR. Oct-1 mRNA was expressed by all SMC cultures, independently of growth state, cell density, or exposure to growth inhibitors. Sp SFM, sparse density (<50% confluent) in SFM; Sp 10%, sparse density in 10% CS; Cfl SFM, confluent density (>90% confluent) in SFM; Cfl 10%, confluent density in 10% CS; Sp TGF- β_1 , sparse density plus TGF- β_1 ; Sp Hep, sparse density plus heparin.

transcripts, in in vivo and freshly dispersed SMCs. Unlike Oct-1, *p53* and *Rb* were readily detectable in in vivo SMCs (Figure 4). In data not shown, we also found that serum-response factor and *c-myc*, two transcription factors important in mitogen-activated growth promotion, are abundantly expressed by in vivo SMCs. These results suggest that Oct-1 mRNA is selectively suppressed in vivo and that disruption of cell-matrix interactions may induce its expression.

Aortic SMCs Cultured on EHS-BM Fail to Replicate in 10% CS and Do Not Express Oct-1 mRNA

Because Oct-1 mRNA was shown to be induced after enzymatic dispersion of the arterial media into single

Figure 4. Oct-1 mRNA is induced upon removal of SMCs from their *in vivo* environment. Total RNA was extracted from the tunica medias of adult uninjured aortas (*in vivo*) and from the media of aortas that were enzyme dispersed, under mitogen-free conditions, into single cells (*dispersed*). RT-PCR/Southern blot hybridization was used to analyze the expression of Oct-1 mRNA. The *p53* tumor suppressor gene, the retinoblastoma susceptibility gene (*Rb*), and G3PDH were used as controls. Disruption of SMC-ECM interactions is sufficient to induce expression of Oct-1 mRNA.

cells, we investigated the potential of ECM or basement membrane components to negatively regulate its expression. SMCs plated on a basement membrane-rich matrix form a highly ordered, cord-like network, quite different from the typical "hill-and-valley" morphology described for SMCs cultured on plastic, and appear to reexpress a more differentiated phenotype (Stadler *et al.*, 1989; Pauly *et al.*, 1992; Li *et al.*, 1994). EHS-BM have been shown to contain most, if not all, of the basement membrane components normally surrounding vascular SMCs *in vivo*, including laminin, type IV collagen, perlecan, and entactin/nidogen (Kleinman *et al.*, 1986). SMCs were sparsely plated on plastic, on EHS-BM, and on gels composed of types I and III collagens in 10% CS, and cell numbers were determined at 0, 24, 48, and 96 h. Control SMCs were sparsely plated on plastic and were growth arrested in SFM; cell numbers were determined at the same time points. When SMCs were plated onto basement membranes, the cells did not replicate even in the presence of 10% CS (Figure 5A), in marked contrast to SMCs plated on tissue culture plastic. Growth inhibition by basement membranes did not appear to be due to the physical characteristics of the gel itself as SMCs cultured on collagen gels in 10% CS showed rates of replication similar to those of SMCs cultured on plastic (Figure 5A).

Parallel SMC cultures were prepared, lysed, and analyzed for Oct-1 transcripts as described in MATERIALS AND METHODS. SMCs cultured on plastic, when growth arrested in SFM or stimulated with 10% CS, and cultured on a collagen gel continue to express Oct-1 mRNA, again confirming that growth state per

Figure 5. EHS-BM repress SMC replication and Oct-1 gene expression. (A) SMCs were plated at sparse density in 10% CS on tissue culture plastic, plastic coated with a gel composed of type I and type III collagen (Vitrogen), or plastic coated with EHS-BM (Matrigel) and were allowed to attach and spread overnight. The media were changed, and cells were cultured in SFM or 10% CS. Cell numbers were determined in triplicate at 0, 24, 48, and 96 h. SD, <10% of the means. □, Matrigel/10% CS; ◇, plastic/10% CS; ○, plastic/SFM; △, Vitrogen/10% CS. (B) SMCs were plated on plastic (PI 10%), collagen gels (Vit 10%), or EHS-BM (Mat 10%) and maintained for 48 h in media containing 10% CS. Control SMCs were either cultured on plastic and growth arrested for 96 h in SFM (PI SFM) or plated on basement membranes, trypsinized, and replated onto plastic (Mat → PI). Total RNA was isolated and reverse transcribed, and expression of Oct-1 was analyzed with use of gene-specific primers and RT-PCR/Southern blot hybridization. G3PDH-specific primers were used as controls. Compared with growth-stimulated and growth-arrested SMCs plated on plastic, and SMCs plated on a collagen gel, Oct-1 mRNA expression was inhibited when cells were cultured on basement membranes.

se does not influence Oct-1 expression (Figures 3B and 5B). In SMCs cultured on EHS-BM for 48 h, however, Oct-1 mRNA expression was repressed (Figure 5B), suggesting that basement membrane components are capable of specifically regulating expression of Oct-1. As a further control, SMCs were plated on basement membranes, trypsinized after 48 h on the membranes, and replated onto plastic; these cells recovered their ability to replicate in the presence of 10% CS (our unpublished data) and, within 24 h, reexpressed Oct-1 mRNA (Figure 5B).

To determine the time course of down-regulation of Oct-1 mRNA expression by basement membranes, SMCs were harvested at various times after plating on

Figure 6. Oct-1 mRNA expression is repressed within 24 h of culturing SMCs on basement membranes. SMCs were plated on EHS-BM (Matrigel) in 10% CS and were allowed to attach for the indicated times. SMCs were harvested at 30 min, 1, 2, 4, 8, 12, 24, and 48 h after plating, and total RNA was extracted and analyzed for Oct-1 mRNA. Oct-1 mRNA is down-regulated within 24 h when SMCs are plated on basement membranes, whereas under identical conditions, SMCs plated on plastic continue to express Oct-1.

EHS-BM. RT-PCR/Southern blot analysis showed that Oct-1 mRNA was completely repressed within 24 h of plating SMCs on EHS-BM (Figure 6). In contrast, SMCs cultured on plastic and growth arrested in SFM retained an Oct-1-positive, replication-competent phenotype (Figure 6). Removal of SMCs from basement membranes by digestion in SFM containing collagenase resulted in the rapid accumulation (within 60 min) of Oct-1 transcripts (Figure 7), in agreement with the above data (Figure 4) demonstrating that disruption of cell-matrix contacts is sufficient to induce Oct-1 expression in SMCs. Reexpression of Oct-1 mRNA was inhibited when SMC cultures were pretreated with actinomycin D (an inhibitor of RNA transcription) but was not altered when SMCs were pretreated with cycloheximide (an inhibitor of protein synthesis), suggesting that the matrix injury-mediated expression

Figure 7. Oct-1 mRNA is reexpressed after removal of SMCs from basement membranes. SMCs were cultured on EHS-BM in 10% CS for 48 h and then enzymatically removed in SFM containing collagenase for 15, 30, 60, and 120 min. Total RNA was immediately extracted after digestion for the indicated time periods, and the presence of Oct-1 mRNA was determined. G3PDH-specific primers were used as controls for the cDNA synthesis and PCR. Oct-1 mRNA levels were rapidly (within 60 min) reexpressed after enzymatic disruption of SMC-basement membrane interactions.

Figure 8. Reexpression of Oct-1 mRNA is regulated at the level of gene transcription. SMCs were cultured on EHS-BM for 48 h in 10% CS (Mat 10%) and then treated with collagenase for 60 min (60' digest). Cultures were pretreated with 50 μ g/ml cycloheximide (+CHX) or 40 μ g/ml actinomycin D (+ActD) as indicated for 30 min before digestion. Total RNA was analyzed for the presence of Oct-1 transcripts as described. The induced expression of Oct-1 mRNA is regulated at the transcriptional level and does not appear to require the synthesis of an intermediary protein.

of Oct-1 is regulated at the level of gene transcription and that synthesis of an intermediary protein is not essential for Oct-1 gene induction (Figure 8). The data suggest a model in which Oct-1 gene transcription is repressed by signals from the ECM.

The Heparan Sulfate Side Chains of Perlecan Appear to Be the Active Components Suppressing Oct-1 mRNA Expression

To identify the matrix component(s) responsible for the regulation of SMC Oct-1 mRNA expression, we plated SMCs on individual basement membrane components, including laminin, type IV collagen, fibronectin, and perlecan (the predominant HSPG in both EHS-BM and SMC basement membranes). Bacteriological plastic was coated with individual basement membrane proteins (diluted in SFM) overnight at room temperature. The plates were then treated with 0.5% bovine serum albumin in SFM for 4 h to block nonspecific binding and rinsed with SFM before plating. This coating procedure ensured that SMCs did not attach to the plastic surface unless it was pretreated with a matrix protein (Figure 9a). SMCs plated on EHS-BM demonstrated a cord-like morphology as previously shown by other investigators (Figure 9b; Stadler *et al.*, 1989; Pauly *et al.*, 1992; Li *et al.*, 1994). SMCs plated on laminin (Figure 9c), type IV collagen (Figure 9d), and fibronectin (Figure 9e) attached and spread as normally observed on tissue culture plastic. However, SMCs plated on perlecan demonstrated a morphology similar to that observed on EHS-BM, including the formation of cord-like structures (Figure 9f). Analysis of Oct-1 mRNA expression revealed that, in contrast to SMCs plated on plastic, Oct-1 transcript levels were repressed when SMCs were plated di-

Figure 9. SMCs cultured on perlecan display a morphology similar to that observed on intact basement membranes. SMCs were plated on bacteriological plastic coated with EHS-BM (50 μ l/ml), laminin (10 μ g/ml), type IV collagen (10 μ g/ml), fibronectin (5 μ g/ml), or perlecan (2 μ g/ml). SMCs did not attach to the plastic surface unless it was precoated with a matrix protein (a). SMCs plated on basement membranes demonstrated a cord-like appearance (b). Although SMCs plated on laminin (c), type IV collagen (d), and fibronectin (e) attached and spread normally, SMCs plated on perlecan (f) demonstrated a morphology very similar to SMCs plated on basement membranes, including the formation of cord-like structures. Bar for a–f, 250 μ m.

rectly on perlecan matrices, similar to SMCs plated on intact basement membranes (Figure 10). SMCs plated on laminin, type IV collagen, and fibronectin matrices continued to express detectable levels of Oct-1 mRNA (our unpublished data), suggesting that perlecan is the basement membrane component primarily responsi-

Figure 10. Oct-1 mRNA expression is suppressed in SMCs cultured on perlecan matrices. SMCs were plated on tissue culture plastic (Plastic), perlecan-coated bacteriological plastic (Perlecan), or EHS-BM (Matrigel) and were maintained in culture for 36 h. Total RNA was extracted and was analyzed for the presence of Oct-1 mRNA with use of RT-PCR/Southern blot hybridization analysis. G3PDH-specific primers were used as controls. Perlecan specifically regulates the expression of Oct-1 transcripts in SMCs.

ble for the suppression of *Oct-1* gene expression by SMCs.

Because perlecan in EHS-BM contains both heparan sulfate and chondroitin sulfate side chains (Hassell *et al.*, 1980; Iozzo *et al.*, 1994), we next sought to determine whether glycosaminoglycan side chains were involved in the regulation of *Oct-1* gene expression. For these experiments, EHS-derived membranes were left untreated or were digested with heparinase, chondroitinase, or hyaluronidase at 37°C for 4 h. SMCs were then plated on the membranes and maintained in culture for 48 h. In contrast to chondroitinase-treated and hyaluronidase-treated basement membranes, heparinase-treated basement membranes lost their ability to repress Oct-1 transcripts (Figure 11). To control for the purity of the heparinase, similar experiments were repeated in the presence of protease inhibitors; no differences in the expression of Oct-1 mRNA were noted (our unpublished data). Taken together, the data suggest that the heparan sulfate side chains of perlecan are responsible for suppressing Oct-1 mRNA expression by SMCs.

DISCUSSION

The main function of SMCs in the uninjured blood vessel is contraction, and as such, these cells are extremely

Figure 11. The Oct-1-suppressing activity of EHS-BM is heparinase sensitive. SMCs were plated and maintained in 10% CS for 48 h on intact basement membranes (Matrigel), membranes treated with heparinase (Heparinase), membranes treated with chondroitinase ABC (Chondroitinase), or membranes treated with hyaluronidase (Hyaluronidase). Total RNA was extracted and analyzed for Oct-1 mRNA as described. Removal of heparan sulfate side chains from intact basement membranes results in the loss of Oct-1-suppressing activity, suggesting that the heparan sulfate side chains of perlecan are the active components inhibiting *Oct-1* gene expression by SMCs.

quiescent, respond poorly to exogenous mitogens, and produce minimal ECM proteins. However, during vascular development, under pathological conditions such as atherosclerosis, during vascular repair, and in culture, SMCs are capable of numerous functions, including proliferation, matrix protein synthesis, migration, and contraction. An increasing body of information has shown that multiple, phenotypically distinct SMC subpopulations comprise the normal arterial media (Glukhova *et al.*, 1990; Majesky *et al.*, 1992; Frid *et al.*, 1994; Topouzis and Majesky, 1996), suggesting that specific functions may require specific SMC phenotypes. Despite this apparent diversity in SMC phenotype, in the absence of vascular trauma or injury, the adult blood vessel remains a highly quiescent tissue (Lindner *et al.*, 1990; Weiser *et al.*, 1995), suggesting the existence of active growth suppressive mechanisms operative in the mature vessel wall. Removal or inhibition of these putative SMC growth suppressors may result in changes in SMC gene expression and replicative potential thus activating SMC to respond appropriately to vascular injury. However, virtually nothing is known concerning the molecular changes that occur during the activation process or about the factors that regulate this process. The importance of this phenomenon has recently been demonstrated by studies showing that the extent of SMC activation in atherectomy specimens strongly correlates with the degree of SMC replication contributing to arterial restenosis after balloon catheter angioplasty (Simons *et al.*, 1993). Our research goal is to identify factors endogenous to the uninjured arterial media that suppress SMC replication and, possibly, SMC activation. Our approach to this

problem was first to identify genes induced by "injury" to the vascular wall and then to determine the molecular mechanism by which these genes are normally suppressed. In this study, we show that *Oct-1* gene expression is undetectable in the contractile-state in vivo SMCs but is rapidly induced after injury to the in vivo ECM or disruption of cell-matrix contacts in culture. We also show that SMC Oct-1 transcripts are specifically and actively repressed by the heparan sulfate components of perlecan, the predominant basement membrane proteoglycan. The data suggest possible important roles for Oct-1, perlecan, and other components of the SMC basement membrane in regulating and defining SMC activation. We propose a model in which the matrix injury-induced expression of growth-essential transcription factors, such as Oct-1, may be one of the earliest responses to vascular trauma and may render SMC competent to respond to subsequent mitogenic stimulation.

Potential Role for Oct-1 in Vascular SMC Biology

Oct-1, a DNA-binding protein widely expressed in adult tissues and ubiquitously expressed in cultured cell lines, is the prototype member of the POU domain family of transcriptional regulators (Fletcher *et al.*, 1987; Sturm *et al.*, 1988). Members of this family are known to play integral roles in the regulation of cell growth and/or differentiation (Rosenfeld, 1991; Ruvkan and Finney, 1991; Scholer, 1991). Although all of its functions remain unknown, Oct-1 positively regulates the expression of the histone *H2b* gene and is, therefore, considered essential for cellular replication (Fletcher *et al.*, 1987; LaBella *et al.*, 1988). Furthermore, Lakin *et al.* (1995) showed that Oct-1 mRNA and protein levels are undetectable in mature neuronal cells and suggested that the absence of Oct-1 expression by these cells is associated with the morphological differentiation to a nonreplicating phenotype. We show here that transcripts for Oct-1 are not present in the tunica media of adult uninjured vessels but are constitutively expressed by cultured SMCs, mitogenically responsive cells that display, in general, a less differentiated phenotype. These data suggest that cultured SMCs (in the absence of specific growth-inhibitory matrix contacts) may express the molecular machinery required for replication. Although the function of Oct-1 in SMC biology is not yet known, the pattern of *Oct-1* gene expression clearly parallels that of SMC "activation" and replicative potential, suggesting a role for Oct-1 in SMC activation or growth responsiveness.

Role of Basement Membrane Components in Regulating SMC Function

Ample evidence that ECM and basement membrane components are involved in the control of cellular differentiation and in the activation of tissue-specific genes exists (Schmidhauser *et al.*, 1992; Adams and Watt, 1993; Lin and Bissell, 1993; Streuli *et al.*, 1995). In

the vascular system, several matrix components are known to influence the phenotypic and replicative state of SMCs. Fibronectin, for example, has been shown to facilitate the expression of a more "modulated," highly replicative phenotype, whereas other matrix proteins, such as laminin, type IV collagen, and HSPGs, appear to promote a more differentiated, contractile-state, quiescent phenotype (Hedin *et al.*, 1988; Campbell *et al.*, 1992; Thyberg and Hultgardh-Nilsson, 1994). Previous studies have shown that culturing synthetic-state replicative SMCs on reconstituted basement membranes inhibits replication (even in the presence of known SMC mitogens), promotes a more differentiated phenotype, and inhibits mitogen-induced activation of mitogen-activated protein kinases (Stadler *et al.*, 1989; Pauly *et al.*, 1992; Li *et al.*, 1994). However, the individual basement membrane components actively involved in the regulation of these processes were not identified. Our studies show that culturing SMCs on reconstituted basement membranes inhibits *Oct-1* gene expression and cell growth. Moreover, we have found that these effects are sensitive to heparinase treatment and can be mimicked by the basement membrane HSPG perlecan.

Perlecan, first isolated from EHS-derived tumor extracts, is a large (467 kDa) heparan sulfate/chondroitin sulfate hybrid proteoglycan (Hassell *et al.*, 1980; Noonan *et al.*, 1991; Noonan and Hassell, 1993; Iozzo *et al.*, 1994). The protein core consists of five distinct molecular domains, including a perlecan-specific domain containing heparan sulfate attachment sites and domains homologous with the low-density lipoprotein receptor, the laminin A globular region, immunoglobulin repeats, and epidermal growth factor-like repeats (Noonan *et al.*, 1991; Noonan and Hassell, 1993; Iozzo *et al.*, 1994). Perlecan is widely expressed in the basement membranes of adult tissues and in all vascular structures (Couchman, 1987; Murdoch *et al.*, 1994) and has been directly implicated as a potent endothelial cell-derived inhibitor of SMC replication (Benitz *et al.*, 1990). Our data indicate that at least some of the gene-regulating activity of perlecan resides in its heparan sulfate side chains, consistent with a large body of evidence implicating heparin-like molecules in the regulation of various SMC functions (Clowes and Karnovsky, 1977; Castellot *et al.*, 1981; Majack and Bornstein, 1984; Majack and Clowes, 1984; Marcum and Rosenberg, 1984; Majack *et al.*, 1985; Fritze *et al.*, 1985; Benitz *et al.*, 1990; Campbell *et al.*, 1992; Pukac *et al.*, 1992; Au *et al.*, 1993).

Heparin-like molecules appear to be among the most important physiological regulators of SMC function. Endogenous HSPGs of several types are produced by both endothelial cells and SMCs (Castellot *et al.*, 1981; Marcum and Rosenberg, 1984; Fritze *et al.*, 1985; Benitz *et al.*, 1990; Jarvelainen *et al.*, 1991; Cizmeci-Smith *et al.*, 1992; Bernfield *et al.*, 1993), and

administration of soluble heparin after experimental balloon catheter injury to the rat carotid artery *in vivo* inhibits SMC replication, migration, and neointimal formation and alters the composition of the ECM surrounding SMCs (Clowes and Karnovsky, 1977; Snow *et al.*, 1990; Au *et al.*, 1993). In addition, culturing SMCs on a heparan sulfate-rich glycosaminoglycan extract inhibited SMC phenotypic modulation in a heparinase-sensitive manner (Campbell *et al.*, 1992). Finally, a wide variety of *in vitro* studies have demonstrated that heparin/heparan sulfates can regulate SMC replication (Clowes and Karnovsky, 1977; Castellot *et al.*, 1981; Fritze *et al.*, 1985; Benitz *et al.*, 1990; Pukac *et al.*, 1992; Au *et al.*, 1993; Ottlinger *et al.*, 1993), differentiation (Campbell *et al.*, 1992), and gene expression (Majack and Bornstein, 1984; Snow *et al.*, 1990; Pukac *et al.*, 1992; Au *et al.*, 1993).

The mechanism underlying the observed cellular and molecular effects of soluble heparin on SMCs remains unidentified, although a number of potential mechanisms have been proposed (Majack *et al.*, 1985; Snow *et al.*, 1990; Pukac *et al.*, 1992; Au *et al.*, 1993; Ottlinger *et al.*, 1993). Interestingly, the addition of soluble heparin to cultured SMCs does not elicit the changes in cell morphology (our unpublished results) and gene expression (Figure 3B) observed with perlecan matrices (Figures 9 and 10). Given that several cell types specifically bind the RGD sequence of perlecan in an integrin-dependent manner (Hayashi *et al.*, 1992; Chakravarti *et al.*, 1995), it is possible that the efficacy of perlecan in altering SMC functions may derive from the coordinate binding of the core protein and heparan sulfate side chains to the SMC cell surface. Alternatively, the failure of soluble heparin to inhibit *Oct-1* gene expression by SMCs cultured on plastic suggests that the focal contacts created between SMCs and tissue culture plastic may result in growth-promoting intracellular signals that heparin is unable to override. Unpublished data from our laboratory demonstrate that pretreatment of SMCs with soluble heparin before plating on plastic or fibronectin matrices (both growth promoting) reduces the adherence of SMCs and thereby inhibits the growth-promoting potential of these matrices. Identification of a perlecan receptor and/or elucidation of the signaling pathway(s) involved in perlecan-mediated growth inhibition and changes in gene expression will provide important information regarding how SMC growth and function are regulated by ECM components; these studies are ongoing in our laboratory.

Potential Role of a Growth-suppressive, Perlecan-rich Basement Membrane in Vascular Homeostasis

Based on the data presented herein, we propose that adult SMCs are surrounded by a perlecan-rich, growth-inhibitory ECM that may largely prevent SMC

Figure 12. Hypothetical role for SMC basement membrane heparan sulfate proteoglycans in the control of SMC growth. During development, inhibitory components (e.g., HSPGs) of the SMC basement membrane may accumulate and repress the expression of specific growth-essential transcription factors such as Oct-1. Physical, proteolytic, or compositional "injury" to the vascular wall may induce Oct-1 expression (and possibly other growth-essential transcription factors) through disruption of this inhibitory basement membrane. The acquired, matrix injury-dependent expression of growth-essential transcription factors after vascular injury may serve to activate SMCs to allow rapid and immediate responses to mitogens.

replication in the absence of matrix injury, and that one primary function of this growth-suppressive matrix may be to negatively regulate the expression of transcription factors, such as Oct-1, required for cell replication. In a separate study (Weiser *et al.*, 1996), we have shown that expression of the perlecan gene is developmentally regulated in rat aortic SMCs and is first detectable in SMCs at embryonic day 18, a point in development when SMC replication begins to dramatically decrease (Cook *et al.*, 1994). Perlecan was preferentially expressed by nonreplicating SMCs, suggesting that the developmentally timed accumulation of a perlecan-rich matrix may play an integral role in SMC growth suppression. We propose that in the adult blood vessel, disruption of this growth-suppressive basement membrane by vascular injury may lead to the expression of transcription factors essential for SMC replication, thereby activating SMCs and facilitating subsequent responses to mitogens (Figure 12). In this context, vascular injury may involve physical, proteolytic, or compositional changes in the SMC basal lamina. Of these, proteolytic injury to the SMC basement membrane, such as might occur after secretion of matrix-degrading enzymes from macrophages, endothelial cells, or SMCs (Galis *et al.*, 1994), may be the most physiologically important. Compositional alterations in the basement membrane, such as would follow the deposition of fibronectin, tenascin, or thrombospondin by replicating cells within the injured vessel wall, may also act to disrupt inhibitory cell:matrix interactions (Sage and Bornstein, 1991). The matrix injury-dependent expression of Oct-1 and other growth-related genes may, therefore, represent a pivotal and essential step in the vascular response to injury and may define, at least in part, the process of SMC activation. Identification of the molecular mechanisms underlying the perlecan-mediated inhibition

of Oct-1 gene expression may provide the basis for the development of therapeutic agents capable of treating a variety of vascular diseases involving excessive SMC replication.

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REFERENCES

- Adams, J.C., and F.M. Watt. (1993). Regulation of development and differentiation by the extracellular matrix. *Development* 117, 1183–1198.
- Assoian, R.K., and Sporn, M.B. (1986). Type beta transforming growth factor (TGF-beta) in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* 102, 1217–1223.
- Au, Y.P., Kenagy, R.D., Clowes, M.M., and Clowes, A.W. (1993). Mechanisms of inhibition by heparin of vascular smooth muscle cell proliferation and migration. *Haemostasis* 23(suppl 1), 177–182.
- Benitz, W.E., Kelley, R.T., Anderson, C.M., Lorant, D.E., and Bernfield, M. (1990). Endothelial heparan sulfate proteoglycan. I. Inhibitory effects on smooth muscle cell proliferation. *Am. J. Respir. Cell Mol. Biol.* 2, 13–24.
- Bernards, R., Schackelford, G.M., Gerber, M.R., Horowitz, J.M., Friend, S.H., Schartl, M., Bogenmann, E., Rapaport, J.M., T. McGee, and Dryja, T.P. (1989). Structure and expression of the murine retinoblastoma gene and characterization of its encoded protein. *Proc. Natl. Acad. Sci. USA* 86, 6474–6478.
- Bernfield, M., Hinkes, M.T., and Gallo, R.L. (1993). Developmental expression of the syndecans: possible function and regulation. *Dev. Suppl.*, 205–212.

- Campbell, J.H., Rennick, R.E., Kalevitch, S.G., and Campbell, G.R. (1992). Heparan sulfate-degrading enzymes induce modulation of smooth muscle phenotype. *Exp. Cell Res.* 200, 156–167.
- Castellot, J.J., Addonizio, M.L., Rosenberg, R.D., and Karnovsky, M.J. (1981). Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. *J. Cell Biol.* 90, 372–379.
- Chakravarti, S., Horchar, T., Jefferson, B., Laurie, G.W., and Hassell, J.R. (1995). Recombinant domain III of perlecan promotes cell attachment through its RGDS sequence. *J. Biol. Chem.* 270, 404–409.
- Chamley-Campbell, J.H., Campbell, G.R., and Ross, R. (1981). Phenotype-dependent response of cultured aortic smooth muscle cells to serum mitogens. *J. Cell Biol.* 89, 379–383.
- Cizmeci-Smith, G., Asundi, V., Stahl, R.C., Teichman, L.J., Chernousov, M., Cowan, K., and Carey, D.J. (1992). Regulated expression of syndecan in vascular smooth muscle cells and cloning of rat syndecan core protein cDNA. *J. Biol. Chem.* 267, 15729–15736.
- Clowes, A.W., and Karnovsky, M.J. (1977). Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature* 265, 625–626.
- Cook, C.L., Weiser, M.C.M., Schwartz, P.E., Jones, C.L., and Majack, R.A. (1994). Developmentally timed expression of an embryonic growth phenotype in vascular smooth muscle cells. *Circ. Res.* 74, 189–196.
- Couchman, J.R. (1987). Heterogenous distribution of a basement membrane heparan sulfate proteoglycan in rat tissues. *J. Cell Biol.* 105, 1901–1916.
- Ferns, G.A., Raines, E.W., Sprugel, K.H., Motani, A.S., Reidy, M.A., and Ross, R. (1991). Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 253, 1129–1132.
- Fletcher, C., Heintz, N., and Roeder, R.G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. *Cell* 51, 773–781.
- Frid, M., Moiseeva, E., and Stenmark, K.R. (1994). Multiple phenotypically distinct smooth muscle cell populations exist in the adult and developing bovine pulmonary arterial media in vivo. *Circ. Res.* 75, 669–681.
- Fritze, L.M., Reilly, C.F., and Rosenberg, R.D. (1985). An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. *J. Cell Biol.* 100, 1041–1049.
- Galis, Z.S., Muszynski, M., Sukhova, G.K., Simon-Morrissey, E., Unemori, E.N., Lark, M.W., Amento, E., and Libby, P. (1994). Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ. Res.* 75, 181–189.
- Glukhova, M., Frid, M., and Kotliansky, V. (1990). Developmental changes in expression of contractile and cytoskeletal proteins in human aortic smooth muscle. *J. Biol. Chem.* 265, 13042–13046.
- Hassell, J.R., Robey, P.G., Barrach, H.J., Wilczek, J., Rennard, S.I., and Martin, G.R. (1980). Isolation of a heparan sulfate-containing proteoglycan from basement membrane. *Proc. Natl. Acad. Sci. USA* 77, 4494–4498.
- Hayashi, K., Madri, J.A., and Yurchenco, P.D. (1992). Endothelial cells interact with the core protein of basement membrane perlecan through beta 1 and beta 3 integrins: an adhesion modulated by glycosaminoglycan. *J. Cell Biol.* 119, 945–959.
- Hedin, U., Bottger, B.A., Forsberg, E., Johansson, S., and Thyberg, J. (1988). Diverse effects of fibronectin and laminin on phenotypic modulation of cultured arterial smooth muscle cells. *J. Cell Biol.* 107, 307–319.
- Iozzo, R.V., Cohen, I.R., Grassel, S., and Murdoch, A.D. (1994). The biology of perlecan: the multifaceted heparan sulfate proteoglycan of basement membranes and pericellular matrices. *Biochem. J.* 302, 625–639.
- Jarvelainen, H.T., Kinsella, M.G., Wight, T.N., and Sandell, L.J. (1991). Differential expression of small chondroitin/dermatan sulfate proteoglycans, PG-I/biglycan and PG-II/decorin, by vascular smooth muscle and endothelial cells in culture. *J. Biol. Chem.* 266, 23274–23281.
- Kleinman, H.K., McGarvey, M.L., Hassell, J.R., Star, V.L., Cannon, F.B., Laurie, G.W., and Martin, G.R. (1986). Basement membrane complexes with biological activity. *Biochemistry* 25, 312–318.
- Kohler, T.R., and Jawien, A. (1992). Flow affects development of intimal hyperplasia after arterial injury in rats. *Arterioscler. Thromb.* 12, 963–971.
- LaBella, F., Sive, H.L., Roeder, R.G., and Heintz, N. (1988). Cell-cycle regulation of a human histone H2b gene is mediated by the H2b subtype-specific consensus element. *Genes Dev.* 2, 32–39.
- Lakin, N.D., Palmer, R., Lillycrop, K.A., Howard, M.K., Burke, L.C., Thomas, N.S.B., and Latchman, D.S. (1995). Down regulation of the octamer binding protein Oct-1 during growth arrest and differentiation of a neuronal cell line. *Mol. Brain Res.* 28, 47–54.
- Li, X., Tsai, P., Weider, E.D., Kribben, A., Van Putten, V., Schrier, R.W., and Nemenoff, R.A. (1994). Vascular smooth muscle cell grown on Matrigel, a model of the contractile phenotype with decreased activation of mitogen-activated protein kinase. *J. Biol. Chem.* 269, 19653–19658.
- Lin, C.Q., and Bissell, M.J. (1993). Multi-faceted regulation of cell differentiation by extracellular matrix. *FASEB J.* 7, 737–743.
- Lindner, V., Majack, R.A., and Reidy, M.A. (1990). Basic fibroblast growth factor stimulates endothelial regrowth in denuded arteries in vivo. *J. Clin. Invest.* 85, 2004–2008.
- Majack, R.A., and Bornstein, P. (1984). Heparin and related glycosaminoglycans regulate the secretory phenotype of vascular smooth muscle cells. *J. Cell Biol.* 99, 1688–1695.
- Majack, R.A., and Clowes, A.W. (1984). Inhibition of vascular smooth muscle cell migration by heparin-like glycosaminoglycans. *J. Cell. Physiol.* 118, 253–256.
- Majack, R.A., Cook, S.C., and Bornstein, P. (1985). Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. *J. Cell Biol.* 101, 1059–1070.
- Majesky, M.W., Giachelli, C., Reidy, M., and Schwartz, S.M. (1992). Rat carotid neointimal smooth muscle cells reexpress a developmentally regulated phenotype during repair of arterial injury. *Circ. Res.* 71, 759–768.
- Marcum, J.A., and Rosenberg, R.D. (1984). Anticoagulant active heparin-like molecules from vascular tissue. *Biochemistry* 23, 1730–1737.
- Mosse, P.R., Campbell, G.R., Wang, Z.L., and Campbell, J.H. (1985). Smooth muscle phenotypic expression in human carotid arteries. I. Comparison of cells from diffuse intimal thickenings adjacent to atheromatous plaques with those of the media. *Lab. Invest.* 53, 556–562.
- Murdoch, A.D., Liu, B., Schwarting, R., Tuan, R.S., and Iozzo, R.V. (1994). Widespread expression of perlecan proteoglycan in basement membranes and extracellular matrices of human tissues as detected by a novel monoclonal antibody against domain III and by in situ hybridization. *J. Histochem. Cytochem.* 42, 239–249.
- Noonan, D.M., Fulle, A., Valente, P., Cai, S., Horigan, Sasaki, M., Yamada, Y., and Hassell, J.R. (1991). The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density li-

- poprotein receptor, and the neural cell adhesion molecule. *J. Biol. Chem.* 266, 22939–22947.
- Noonan, D.M., and Hassell, J.R. (1993). Perlecan, the large low-density proteoglycan of basement membranes: structure and variant forms. *Kidney Int.* 43, 53–60.
- Ottlinger, M.E., Pukac, L.A., and Karnovsky, M.J. (1993). Heparin inhibits mitogen-activated protein kinase activation in intact rat vascular smooth muscle cells. *J. Biol. Chem.* 268, 19173–19176.
- Owens, G.K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* 75, 487–517.
- Pauly, R.R., Passaniti, A., Crow, M., Kinsella, J.L., Papadopoulos, N., Monticone, R., Ladatta, E.G., and Martin, G.R. (1992). Experimental models that mimic the differentiation of vascular cells. *Circulation* 86(suppl III), 68–73.
- Pukac, L.A., Ottlinger, M.E., and Karnovsky, M.J. (1992). Heparin suppresses specific second messenger pathways for protooncogene expression in rat vascular smooth muscle cells. *J. Biol. Chem.* 267, 3707–3711.
- Rosenfeld, M.G. (1991). POU-domain transcription factors: powerful developmental regulators. *Genes Dev.* 5, 897–907.
- Ross, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990's. *Nature* 362, 801–809.
- Ruvkan, G., and Finney, M. (1991). Regulation of transcription and cell identity by POU domain proteins. *Cell* 64, 475–478.
- Sage, E.H., and Bornstein, P. (1991). Extracellular proteins that modulate cell-matrix interactions: SPARC, tenascin, and thrombospondin. *J. Biol. Chem.* 266, 14831–14834.
- Schmidhauser, C., Casperson, G.F., Myers, C.A., Sanzo, K.T., Bolten, S., and Bissell, M.J. (1992). A novel transcriptional enhancer is involved in the prolactin- and extracellular matrix-dependent regulation of β -casein gene expression. *Mol. Biol. Cell* 3, 699–709.
- Scholer, H.R. (1991). Octamania: the POU factors in murine development. *Trends Genet.* 7, 323–329.
- Schwartz, S.M., deBlois, D., and O'Brien, E.R.M. (1995). The intima: soil for atherosclerosis and restenosis. *Circ. Res.* 77, 445–465.
- Simons, M., Leclerc, G.L., Safian, R.D., Isner, J.M., Weir, L., and Baim, D.S. (1993). Relation between activated smooth muscle cells in coronary-artery lesions and restenosis after atherectomy. *N. Engl. J. Med.* 328, 608–613.
- Snow, A.D., Bolender, R.P., Wight, T.N., and Clowes, A.W. (1990). Heparin modulates the composition of the extracellular matrix domain surrounding arterial smooth muscle cells. *Am. J. Pathol.* 137, 313–330.
- Soussi, T., Caron de Fromental, C., Breugnot, C., and May, E. (1988). Nucleotide sequence of a cDNA encoding the rat p53 nuclear oncoprotein. *Nucleic Acids Res.* 16, 11384.
- Stadler, E., Campbell, J.H., and Campbell, G.R. (1989). Do cultured vascular smooth muscle cells resemble those of the artery wall? If not, why not? *J. Cardiovasc. Pharmacol.* 14(S6), S1–S8.
- Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A.P.N., Roskelley, C., and Bissell, M.J. (1995). Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* 129, 591–603.
- Sturm, R.A., Das, G., and Herr, W. (1988). The ubiquitous octamer-binding protein Oct-1 contains a POU-domain with a homeobox subdomain. *Genes Dev.* 2, 1582–1599.
- Thyberg, J. (1996). Differentiated properties and proliferation of arterial smooth muscle cells in culture. *Int. Rev. Cytol.* 169, 183–265.
- Thyberg, J., and Hultgardh-Nilsson, A. (1994). Fibronectin and the basement membrane components laminin and collagen type IV influence the phenotypic properties of subcultured rat aortic smooth muscle cells differently. *Cell Tissue Res.* 276, 263–271.
- Topouzis, S., and Majesky, M.W. (1996). Smooth muscle lineage diversity in the chick embryo. *Dev. Biol.* 178, 430–445.
- Tso, J.Y., Sun, X.H., Kao, T.H., Reece, K.S., and Wu, R. (1985). Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.* 13, 2485–2502.
- Weiser, M.C.M., Majack, R.A., Tucker, A., and Orton, E.C. (1995). Static tension is associated with increased smooth muscle cell DNA synthesis in rat pulmonary arteries. *Am. J. Physiol.* 268, H1133–H1138.
- Weiser, M.C.M., Grieshaber, S.S., Belknap, J.K., Kinsella, M.G., and Majack, R.A. (1996). Developmental regulation of perlecan gene expression in aortic smooth muscle cells. *Matrix Biol.* 15, 331–340.
- Zerivitz, K., and Akusjarvi, G. (1989). An improved nuclear extract preparation method. *Gene Anal. Tech.* 6, 101–109.