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Human Activin-A Is Expressed in the Atherosclerotic Lesion and Promotes the Contractile Phenotype of Smooth Muscle Cells

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Abstract—Activin is a member of the transforming growth factor-β superfamily, and it modulates the proliferation and differentiation of various target cells. In this study, we investigated the role of activin in the initiation and progression of human atherosclerosis. The expression of activin, its physiological inhibitor follistatin, and activin receptors were assayed in human vascular tissue specimens that represented various stages of atherogenesis. In situ hybridization experiments revealed activin mRNA in endothelial cells and macrophages and a strong induction of activin expression in neointimal smooth muscle cells from the early onset of atherogenesis. We developed an "in situ free-activin binding assay" by using biotinylated follistatin, which allowed us to detect bioactive activin at specific sites in atherosclerotic lesions. The mRNAs encoding the activin receptors are expressed similarly in normal and atherosclerotic tissue, which indicates that activin-A signaling in atherogenesis is most likely dependent on changes in growth factor concentrations rather than on receptor levels. In vitro, activin induces the contractile, nonproliferative phenotype in cultured smooth muscle cells, as is reflected by increased expression of smooth muscle-specific markers (SMα-actin and SM22α). Our data provide evidence that activin induces redifferentiation of neointimal smooth muscle cells, and we hypothesize that activin is involved in plaque stabilization. **(*Circ Res*. 1999;85:931-939.)**

Key Words: atherosclerosis ■ smooth muscle cell ■ follistatin ■ transforming growth factor-β

Activin belongs to the transforming growth factor-β (TGF-β) superfamily of growth factors, and it was initially identified as a protein that induces the release of follicle-stimulating hormone by pituitary cells.[1,2] Subsequently, it was demonstrated that activin is involved in the growth and differentiation of various cell types.[3–8] The glycoprotein activin consists of βA and βB chains, which are held in homodimeric or heterodimeric configurations by disulfide bonds. The distantly related inhibin-α subunit can dimerize with either of the activin-β subunits, resulting in inhibin A or inhibin B, which inhibit follicle-stimulating hormone release from pituitary cells.[9] Activin signaling is mediated by cell-surface type I and type II activin receptors (ActR). Two type I receptors have been described, ActR-I (ALK-2) and ActR-IB (ALK-4), as have 2 type II receptors, ActR-II and ActR-IIB.[10] The activity of activin is regulated by follistatin, a 34-kDa glycoprotein, which binds activin with high affinity in equimolar complexes that are unable to bind and activate the activin receptors.[11,12]

A potential role for activin in atherogenesis has been proposed on the basis of the observation that this factor modulates the proliferation and differentiation of several cell types that are involved in atherogenesis, notably endothelial cells (ECs), macrophages, and smooth muscle cells (SMCs). Activin inhibits the propagation of human ECs,[13] and it enhances the differentiation of monocytic cells into macrophages.[14] Most remarkably, with respect to activin function in atherogenesis, foam cell formation of THP-1–derived macrophages is inhibited.[15] The effect of activin on cultured SMCs is controversial; activin has induced DNA synthesis in rat SMCs in some studies,[16,17] whereas others reported that activin did not affect rat SMC growth.[18] In separate studies, activin[19] and follistatin[20] were expressed in atherosclerotic lesions of hyperlipidemic rabbits, and enhanced activin expression was also observed in the rat carotid artery after balloon injury.[17]

In the present study, we investigated the role of activin in human atherogenesis in detail. In situ hybridization of human tissue specimens demonstrated the expression of activin receptors in SMCs, ECs, and macrophages, as well as the distinct expression of activin and follistatin in atherosclerotic lesions. Immunohistochemical examinations revealed partial colocalization of activin and follistatin protein; this observation prompted us to design a method to detect free, bioactive

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From the Academic Medical Center (M.A.E., J.M.N., T.A.E.v.A., B.E.v.A., H.P., C.J.M.d.V.), University of Amsterdam, Department of Biochemistry, Amsterdam and Erasmus University (R.H.N.v.S.), Department of Endocrinology and Reproduction, Rotterdam, The Netherlands.

Correspondence to Carlie J.M. de Vries, PhD, Department of Biochemistry, Academic Medical Center K1-163, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. E-mail c.j.devries@amc.uva.nl

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activin in atherosclerotic lesions. We took advantage of the knowledge that free activin (not bound in complexes) can associate with follistatin, and we developed a new, highly specific "in situ free-activin binding assay" to show the presence of significant amounts of bioactive activin in advanced atherosclerotic lesions. To reveal the function of activin in human atherogenesis, we evaluated the expression of activin, activin receptors, and follistatin in cultured SMCs. Finally, we demonstrated that activin mediates the differentiation of proliferating SMCs toward a contractile phenotype, as was illustrated by the increased expression of smooth muscle (SM)–specific genes.

Materials and Methods

Tissue Specimens

Human tissue samples were obtained from organ donors or from patients undergoing vascular surgery with the informed consent of relatives or the patients, according to protocols approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam. The specimens were fixed in 3.8% vol/vol formaldehyde in PBS and embedded in paraffin or snap-frozen in liquid nitrogen within 5 minutes after resection. After sectioning (5 μm) and mounting on 3-aminopropyl-triethoxysilane–coated glass slides, the specimens were subjected to in situ hybridization, immunohistochemistry, or the in situ free-activin binding assay. Table 1 describes the specimens.

Immunohistochemistry

Antibody 1A4 (DAKO) recognizes SM α-actin and was used to detect SMCs, whereas antibody HAM56 (DAKO) was used to recognize macrophages in the sections. Activin was detected with monoclonal antibody E4 (Serotec), and follistatin with an anti-peptide antibody (provided by Prof S. Werner, ETH, Zurich, Switzerland). Immunohistochemical examinations were performed on 5-μm paraffin sections (1A4, HAM56) or acetone-fixed cryosections (E4, anti-follistatin) of tissue specimens. For pretreatment, the sections were rehydrated, incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity, and blocked with 10% vol/vol preimmune goat serum (DAKO) in Tris-buffered saline (TBS; 10 mmol/L Tris [pH 8.0] and 150 mmol/L NaCl). Subsequently, the sections were incubated with specific antibodies, followed by incubation with biotinylated secondary antibodies, which were detected with streptavidin-horseradish peroxidase conjugates (DAKO). ECs were specifically recognized with *Ulex europaeus* lectin, which was detected with an anti–Ulex lectin-horseradish peroxidase conjugate. Peroxidase activity was visualized with aminoethylcarbazole and hydrogen peroxide. After counterstaining with hematoxylin, the sections were embedded in glycergel (Sigma).

In Situ Hybridization/RNase Protection Assay

In vitro transcription of linearized plasmid DNA was performed to obtain radiolabeled anti-sense or sense riboprobes ([^{35}S]-UTP for in situ hybridization and [^{32}P]-UTP for RNase protection [Amersham]); see Table 2 for details on probes. The in situ hybridization assays and RNase protection assays were performed as described previously.[22] As a control for the specificity of the anti-sense riboprobes, matching sense riboprobes were assayed for each gene; the sense probes gave neither background nor an aspecific signal.

In Situ Free-Activin Binding Assay

Ten micrograms of purified follistatin (see Growth Factor Incubation and Total RNA Isolation) was biotinylated for 2 hours at 0°C with 3.25 μg Sulfo-*N*-hydroxysuccinimide-long chain (5 μg/μL in dimethylsulphoxide; Pierce) in PBS at a total volume of 20 μL. The reaction was stopped with 1 μL 1 mol/L Tris-HCl (pH 8.0). The biotinylated follistatin was purified on a Sephadex G25 column (Pharmacia). Subsequently, the preparation was incubated on cryosections that were pretreated as described in Immunohistochemistry. After 2 hours of incubation at room temperature in 250 nmol/L biotinylated follistatin and TBS containing 1% bovine serum albumin, the sections were washed 3 times for 2 minutes with TBS and incubated with avidin-horseradish peroxidase conjugate, which was visualized with aminoethylcarbazole (see Immunohistochemistry).

Cell Culture

SMCs were derived from human vessel explants and originated from the iliac artery and aorta of organ donors. The cultured SMCs were characterized by immunofluorescence with a murine monoclonal antibody directed against SM α-actin (1A4, DAKO), which was detected with a Cy3-conjugated goat anti-mouse antibody (Jackson Laboratories). With this method, the cells show uniform fibrillar staining. The cells were used at passage 5 to 7. Cell cultures were performed at 37°C in a humidified 5% CO_2 chamber in 40% Medium-199 with L-glutamine/L-amino acids, 40% RPMI 1640 with HEPES buffer/L-glutamine, and 20% vol/vol human serum supplemented with penicillin, streptomycin, and Fungizone (GIBCO BRL).

Growth Factor Incubation and Total RNA Isolation

Purified, recombinant human activin-A [lot 15365-36(1)] and follistatin (lot B3904) were obtained from Dr Pawson through the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Disease, the National Institute of Child Health and Human Development, and the US Department of Agriculture (Bethesda, Md). For growth factor incubations, SMCs were made quiescent for 72 hours in serum-free standard medium containing 10 mg/L bovine insulin, 5.5 mg/L human transferrin, and 6.7 μg/L sodium selenite (GIBCO BRL). Subsequently, recombinant human follistatin, activin-A, or human serum was added to the culture medium during the periods and at the concentrations indicated. After incubation, the cells were subjected to total RNA isolation with Trizol reagent (GIBCO BRL). After reprecipitation, the RNA samples were subjected to RNase protection analyses and Northern blotting procedures.

Northern Blotting

Northern blots were made as described previously[23] using Hybond N nylon membranes (Amersham). SM α-actin mRNA was detected by hybridization with the oligonucleotide 5′-AGTGCTGTCCTCT-TCTTCACACATA-3′ and radiolabeled with [^{32}P]-γATP (Amersham). After the removal of free nucleotides on a spin column, the probe was hybridized overnight at 50°C in 10 mmol/L sodium phosphate (pH 6.8), 6×SSC (20×SSC is 0.3 mol/L sodium citrate [pH 7.0] and 3 mol/L NaCl), 5×Denhardt's (50×Denhardt's contains 1% wt/vol Ficoll, 1% wt/vol polyvinylpyrrolidone, and 1% wt/vol bovine serum albumin), 1 mmol/L EDTA, 0.5% wt/vol SDS, and 0.1 mg/mL salmon sperm DNA. The blot was washed for 15 minutes at 65°C, increasing the stringency to 2×SSC and 0.1% wt/vol SDS. SM22α mRNA was detected with a 1027-bp, full-length rat cDNA (provided by Dr Shanahan, Cambridge, UK), radiolabeled with [^{32}P]-αATP (Amersham) using a random oligolabeling kit (Gibco BRL), and purified on a oligonucleotide-removal column (Qiagen). Probe hybridization in formamide was performed as described previously.[23] The blot was washed most stringently at 65°C with 0.1×SSC and 0.1% wt/vol SDS.

Results

Expression of mRNA Encoding Activin, Follistatin, and Activin Receptors in Human Vascular Tissue

We determined the expression of activin, follistatin, and activin receptor mRNA in human vascular tissue at different stages of atherosclerosis. The vascular specimens were characterized by immunohistochemistry on consecutive sections with specific antibodies to detect macrophages, SMCs, and ECs (Figures 1a and 1d, 1b, and 1c, respectively). To

TABLE 1. Pathological Specimens Used

In Situ Hybridization Assays									
Sex	Age, y	Class	Origin	βA	Fol	I	IB	II	IIB
Female	58	II	Aorta	x	x	x	x	x	x
Female	50	II	Art. iliaca	x	x	x	x	x	x
Male	12	0	Art. iliaca	x	x	x	x	x	x
Female	76	III	Art. iliaca	x	x	x	x	x	x
Female	76	II+V	Art. iliaca	x	x	x	x	x	x
Female	76	III	Art. iliaca	x	x	x	x	x	x
Female	34	III	Art. iliaca	x	x	x	x	x	x
Female	49	V	Aorta	x	x	x	x	x	x
Female	49	V	Aorta	x	x	x	x	x	x
Female	52	III	Aorta	x	x	x	x	x	x
Female	38	II	Aorta	x	x	x	x	x	x
Female	38	II	Aorta	x	x	x	x	x	x
Female	40	I	Aorta	x	x	x	x	x	x
Male	69	V	Art. femoralis	x	x	x	x	x	x
Female	27	IV	Art. iliaca	x	x			x	x
Female	40	II	Aorta	x	x			x	x
Female	38	II	Aorta	x	x			x	x
Male	63	VI	Aorta	x	x			x	x
Male	28	IV	Aorta	x	x			x	x
Female	58	II+IV	Aorta	x	x			x	x
Male	79	IV	Art. poplitea	x	x			x	x
Male	73	II	Art. thyroidia	x	x			x	x
Female	58	II	Aorta	x	x			x	x
Female	76	III	Art. iliaca	x	x			x	x
Male	12	0	Art. iliaca	x	x			x	x
Male	73	V	Art. femoralis	x	x			x	
Male	73	V	Art. tibialis	x	x			x	
Female	69	III	Art. carotis	x	x			x	
Male	43	III	Art. femoralis	x	x			x	
Female	50	I	Art. iliaca	x	x			x	
Female	50	I+III	Art. iliaca	x	x			x	
Female	50	II	Art. iliaca	x	x			x	

Activin and Follistatin Immunohistochemistry and Free-Activin Binding Assay						
Sex	Age, y	Class	Origin	αAct	αFol	AB
Male	66	III	Aorta	x	x	x
Female	69	VI	Aneurysm	x	x	x
Female	58	I	Aorta	x	x	x
Female	49	IV	Aorta	x	x	x
Female	58	II	Aorta	x	x	x
Male	78	V	Art. iliaca	x	x	x
Female	45	III	Aorta	x	x	x
Male	60	VI	Art. iliaca/aneurysm	x	x	x
Female	80	VI	Aorta	x	x	x
Female	76	II	Aorta	x	x	x
Male	49	VI	Aorta	x	x	x
Female	58	II	Aorta	x	x	x
Male	56	VI	Art. carotis	x	x	x
Male	70	VI	Art. carotis	x	x	x

Class indicates the degree of atherogenesis as classified according to the guidelines issued by the American Heart Association[21]; αAct, immunohistochemistry with antibodies directed against the activin βA subunit; αFol, immunohistochemistry with antibodies directed against follistatin; and AB, in situ free-activin binding assay. All specimens were subjected to immunohistochemistry specific for SMCs, macrophages, and ECs. Art. indicates arteria; βA, activin-A; Fol, follistatin; I, ActR-I; II, ActR-II; IB, ActR-IB; and IIB, ActR-IIB.

TABLE 2. Detailed Information on In Situ Hybridization and RNase Protection Riboprobes

Gene	GenBank Number	In Situ, bp	RP, bp
Activin βA	J03634	476–790	1215–1363
Activin βB	M31668/	1186–1214*	
	M31669	+80–640*	392–640
Inhibin α	M13981	125–411	125–411
Follistatin	M19480	786–4306†	3234–4306†
ActR-I	Z22534	419–601	419–601
ActR-IB	Z22536	105–255	105–255
ActR-II	M93415	597–792	597–792
ActR-IIB	X77533	258–517	258–517
GAPDH	M33197	...	480–545

RP indicates RNase protection assay; In situ, in situ hybridization; and bp, position of the probe in the GenBank sequence in base pairs.

*Activin βB in situ riboprobe spans part of exon 1 (M31668) and part of exon 2 (M31669).

†Intron sequences are not present in riboprobes.

illustrate the expression of the studied genes in lesion macrophages, we show the data from a radioactive in situ hybridization performed in an aorta lesion containing a substantial fibrotic core and, at the luminal side, a large infiltrate of macrophages in Figures 1a and 1d. Both activin-βA (Figure 1e) and follistatin (Figure 1f) are expressed by macrophages (Figure 1d); variations in expression levels between individual cells are observed.

Only a few macrophages expressed small amounts of ActR-I mRNA (Figure 1g), whereas ActR-IB (Figure 1h) was expressed more homogeneously. ActR-II and ActR-IIB were expressed by all macrophages present in the vessel wall (Figures 1i and 1j). The type I receptors are usually expressed at relatively low levels, which emphasizes the need to check for the absence of background signals with corresponding sense riboprobes. Each of the riboprobes applied in these hybridization experiments was designed to delimit nonspecific hybridization with complementary strand probes (data not shown).

In Figure 2, an example of the mRNA expression of activin-βA, follistatin, and activin receptors in neointimal SMCs and ECs is shown. Activin-βA (Figure 2e) and follistatin (Figure 2f) showed variable expression in the neointimal SMCs (Figure 2b): SMCs with high and low expression of these genes were randomly distributed throughout the early lesion. ActR-I and ActR-IB mRNAs (Figure 2g and 2h) were detected at relatively low levels in subsets of SMCs, and both type II receptors (Figure 2i and 2j) were expressed at high levels in all neointimal SMCs and in the medial SMCs. In addition, activin-βB and inhibin α mRNA expression were not detected in macrophages, SMCs, or ECs (data not shown).

Because our studies revealed that the activin receptors were not regulated in atherogenesis, we examined in more detail the expression of activin-βA and follistatin mRNAs, which encode soluble factors. In Figures 3a through 3d, cross-sections of an early aortic fibrotic lesion are shown. Follistatin mRNA (Figure 3c) was expressed at similar

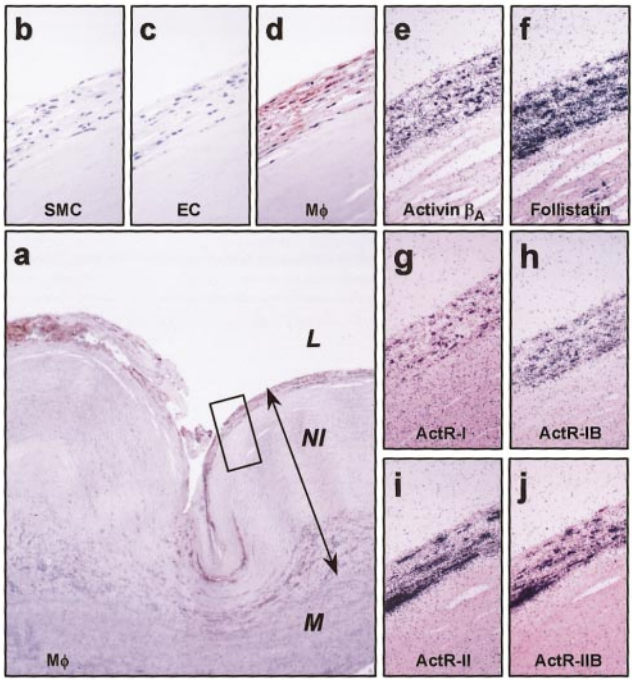

Figure 1. mRNA expression of activin, follistatin, and activin receptors in an aortic lesion containing macrophages. Serial sections of a specimen of the abdominal aorta from an organ donor were analyzed by immunohistochemistry (a through d) or radioactive in situ hybridization (e through j) (see Materials and Methods). Immunohistochemistry was performed to detect macrophages (a and d), SMCs (b), and ECs (c). Macrophages are identified in a subluminal layer of the lesion (d, enlargement of boxed area in a). SMCs are present in the media (not shown) but absent in the macrophage infiltrate (b). Expression of mRNA from activin, follistatin, and activin receptors was detected with in situ hybridization using [^{35}S]-radiolabeled anti-sense riboprobes on serial sections of this atherosclerotic lesion. mRNA expression of activin-βA (e), follistatin (f), ActR-I (g), ActR-IB (h), ActR-II (i), and ActR-IIB (j) was determined. Hybridization was visualized with bright-field microscopy, which resulted in black silver grains on a pink-red counterstain. The macrophages express activin, follistatin, and type II receptors at relatively high levels, whereas ActR-IB is expressed less abundantly, and ActR-I expression is hardly detectable. L indicates lumen; M, media; Mϕ, macrophage; and NI, neointima (double-headed arrow). Original magnification $\times$25 (a) or $\times$100 (b through j).

levels in the medial and in the neointimal SMCs (Figure 3a). Activin-βA mRNA expression (Figure 3d) was, however, higher in the neointimal SMCs than the medial SMCs. The lesion shown in Figures 3e through 3h represents an advanced aortic lesion with extensive neointima formation and a large macrophage infiltrate (Figure 3f). Activin-βA mRNA (Figure 3h) was detected in the media, and its expression was considerably higher in both neointimal cell types, ie, in neointimal SMCs and in macrophages (Figures 3e and 3f). Follistatin mRNA was readily detected in medial SMCs and, in contrast to activin-βA expression, was not increased in the neointima (Figures 3g and 3h). On the basis of these data, which showed increased mRNA expression of activin in the neointima and similar expression of follistatin in media and neointima, we hypothesized that free activin will only be available in the atherosclerotic lesion.

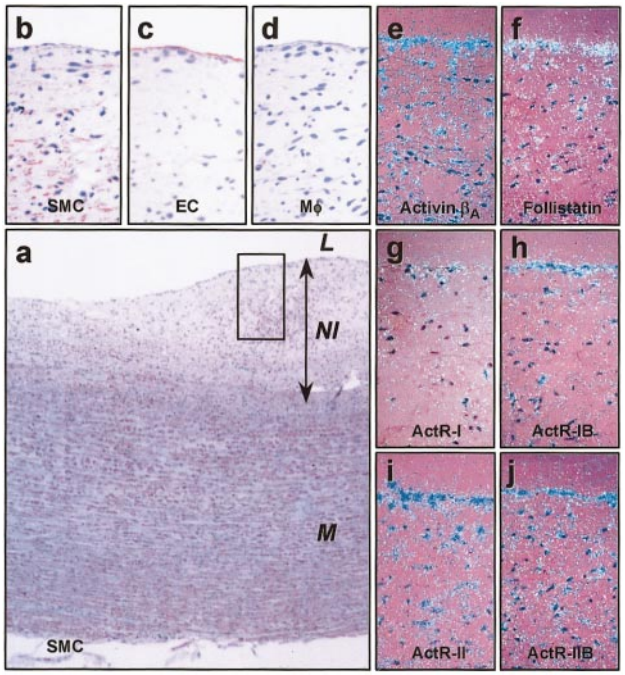

Figure 2. mRNA expression of activin, follistatin, and activin receptors in an early fibrotic aortic lesion containing ECs and SMCs. Serial sections of an aortic lesion were analyzed by immunohistochemistry (a through d) or radioactive in situ hybridization (e through j) (see Materials and Methods). The relatively small neointima on top of the media of the vessel wall is shown in a. The boxed area of the neointima is shown in more detail in b through j. The neointima is composed of SMCs (a and b) and is lined at the luminal side with ECs (c). No macrophages were observed (d). Consecutive sections were analyzed by in situ hybridization with specific probes for activin-βA (e), follistatin (f), ActR-I (g), ActR-IB (h), ActR-II (i), and ActR-IIB (j). Hybridization of the probes was visualized with epipolarization microscopy, which resulted in bright blue dots on a pink background. All genes are expressed in ECs on top of the lesion. Type I receptors show relatively low expression, whereas type II receptors are expressed abundantly and homogeneously. Activin, follistatin, and ActR-I show variable expression levels in neointimal SMCs. L indicates lumen; M, media; Mϕ, macrophage; and NI, neointima. Original magnification $\times$50 (a) or $\times$200 (b through j).

Immunohistochemistry and In Situ Free-Activin Binding Assay

In addition to the in situ hybridization experiments, the expression of activin and follistatin protein was assayed with specific antibodies. In agreement with the data obtained by the in situ hybridization experiments, activin and follistatin protein colocalized to some extent, as was shown in different specimens (Figures 4c, 4d, 4h, 4i, 4m, and 4n). To evaluate the presence of free activin, which is not inactivated in complex formation with follistatin, or other presently unknown activin-inhibiting proteins, we developed an in situ free-activin binding assay. This assay is based on the fact that only free, bioactive activin can bind exogenously added follistatin. We applied biotinylated follistatin in these experiments and, subsequently, bound molecules were detected with streptavidin-conjugates.

Dose-response experiments demonstrated that the signal of the free-activin binding assay was linear and saturable. Competition experiments, in which an incubation of the sections with excess unbiotinylated follistatin was performed, resulting in complete competition of the signal, confirmed the specificity of the assay (data not shown). In early lesions (Figures 4a through 4e), follistatin and activin were detected both in the media and the neointima (Figures 4c and 4d, respectively), and the in situ free-activin binding assay showed that no excess free activin was present (Figure 4e). In advanced lesions, however, activin and follistatin were relatively highly expressed in areas with high densities of both macrophages and neointimal SMCs, as is shown in Figures 4f through 4i and Figures 4k through 4n. Free activin (Figures 4j and 4o) is detected as a fraction of total activin protein (Figures 4i and 4n), specifically at those sites where SMCs and macrophages are in close proximity (Figures 4f and 4g and 4k and 4l, respectively). In conclusion, the immunohistochemical data, in combination with the data obtained by the in situ free-activin binding assay, show that bioactive activin is present in the neointimal region of the atherosclerotic vessel wall.

Expression of Activin, Follistatin, and Activin Receptor mRNA in In Vitro–Activated SMCs

Our data obtained from the analysis of vascular specimens demonstrated that the induction of activin in atherogenesis was most prominent in neointimal SMCs and macrophages. Medial SMCs present a contractile, fully differentiated phenotype, whereas the activated, atherosclerotic, neointimal SMCs are proliferating and/or migrating entities. To mimic this process in atherosclerosis in vitro, SMCs were subjected to growth stimulation, and the expression levels of activin, follistatin, and activin receptors were determined. SMCs were obtained as explant cultures derived either from the iliac artery (a muscular artery) or from the elastic aorta. Because of the different phenotypic properties of SMCs, we chose to culture the SMCs for 3 days in serum-free medium to induce a quiescent, contractile phenotype. Subsequently, SMC proliferation was stimulated by the addition of 20% human serum to the culture. Then, total RNA was isolated from the (un)stimulated SMCs and the RNA was subjected to RNase protection analyses (Figure 5). In addition, RNase protection analyses were performed using placenta RNA and tRNA as the positive and negative controls, respectively.

For each riboprobe, the hybridizations were performed simultaneously with a GAPDH riboprobe to verify the loading of equal amounts of RNA (Figures 5b, 5d, 5f, 5h, and 5k). Activin-βA mRNA expression (Figure 5a) in SMCs and serum stimulation had no effect on activin-βA mRNA levels in iliac artery SMCs, although in aortic SMCs, activin mRNA was induced 2.5-fold. Notably, we found that follistatin mRNA (Figure 5c) was downregulated 5-fold in serum-stimulated iliac artery SMCs. This observation was also made in aortic SMCs, although at relatively lower expression levels. Activin-βB and inhibin-α expression were assayed, but they could not be detected by RNase protection analysis. It is conceivable that on atherogenic stimulation of SMCs, the balance of follistatin and activin levels is shifted toward an excess of free, bioavailable activin.

The expression level of ActR-I mRNA (Figure 5e) was higher in iliac artery SMCs than in aortic SMCs; it was

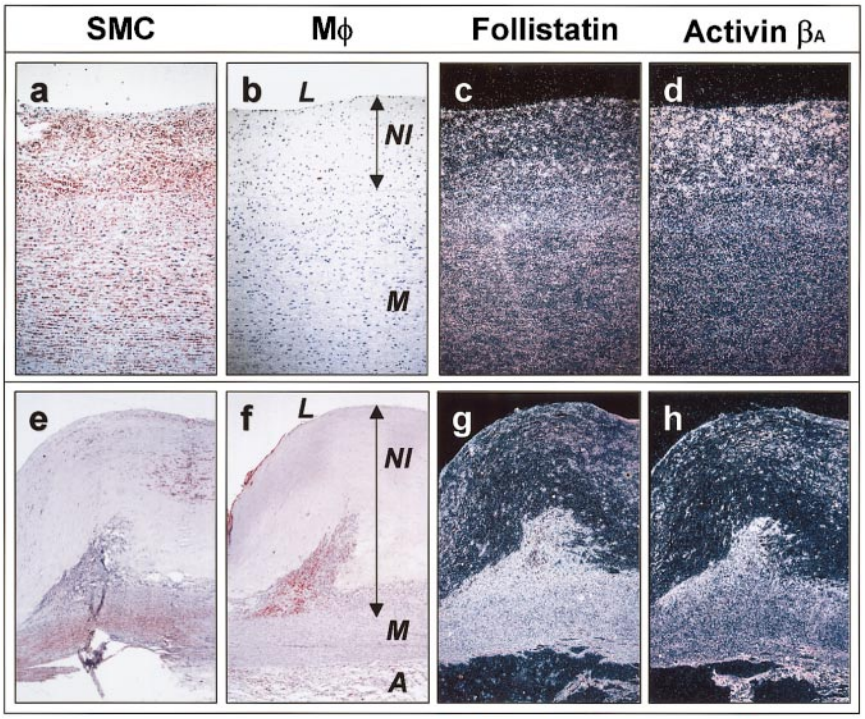

Figure 3. Differential mRNA expression of activin and follistatin in an early lesion (a through d) and an advanced lesion (e through h) of the aorta. SMCs (a and e) and macrophages (b and f) were detected by immunohistochemistry with specific antibodies (see Materials and Methods). Consecutive sections were analyzed for mRNA expression of activin-βA (d and h) and follistatin (c and g). Hybridization of probes was visualized by dark-field microscopy, which showed silver grains as white dots. In early (a through d) and advanced lesions (e through h), follistatin is expressed at similar levels in the media in comparison with the neointima (c and g), whereas activin-βA expression is relatively higher in the neointima than in the media (d and h). L indicates lumen; M, media; Mϕ, macrophage; and NI, neointima. Original magnification $\times$200 (a through d), $\times$50 (e through h).

decreased in serum-stimulated, iliac artery SMCs, but in aortic SMCs, serum had no effect. RNase protection analyses for ActR-IB (Figure 5g) showed an expression pattern similar to that of ActR-I, although overall ActR-IB mRNA expression was less pronounced. ActR-II mRNA (Figure 5i) was downregulated 5-fold in serum-stimulated, iliac artery SMCs; aortic SMCs displayed less abundant ActR-II mRNA expression, which is not affected by the stimulation of these cells. The expression of ActR-IIB mRNA (Figure 5j) was relatively low and not substantially influenced by the different culture conditions. These RNase protection analyses demonstrate that SMCs express at least one type I receptor and one type II receptor.

Activin Induces the Expression of SM α-Actin and SM22α mRNA in SMCs

Arrested proliferation and the induction of differentiation in SMCs coincides with a change from the activated and proliferative phenotype into the nonactivated, contractile phenotype of these cells. SMCs with a nonproliferative, contractile phenotype express both the SM-specific variant of α-actin (SM α-actin) and SM22α at high levels.[24–27] To assess the effect of activin on SMCs, we added purified activin-A for 0, 3, 6, 12, and 24 hours to cultured SMCs derived from the human aorta and iliac artery. In addition, the SMCs were incubated with follistatin to block the activity of endogenously synthesized activin. Subsequently, the expression of the differentiation markers SM α-actin and SM22α was assayed by Northern blot analysis (Figure 6). Follistatin had no effect on the expression of these markers in iliac artery SMCs, but the expression of SM22α was reduced 2-fold in aortic SMCs (Figure 6d), indicating that endogenous activin contributes to the induction of SM22α expression in these cells. Clearly, activin-A induces the expression of SMC-specific markers. After 24 hours of activin treatment, a 2-fold increase in SM α-actin expression was observed in aortic SMCs (Figure 6b), whereas SM22α expression did not change (Figure 6d). The activin treatment of iliac artery SMCs had more pronounced effects: after 24 hours of incubation, SM α-actin and SM22α mRNA levels increased 10-fold and 5-fold, respectively (Figures 6a and 6c). Collectively, these observations demonstrate that activin promotes the differentiation of proliferative SMCs into SMCs with a nonproliferative, contractile phenotype.

Discussion

In this study, we determined, for the first time, the mRNA and protein expression of both activin and follistatin in human vascular tissue at different stages of atherosclerosis. These experiments revealed the presence of activin-A in the normal media, with a higher level of expression in the lesion; follistatin was expressed at similar levels in the normal vessel wall and in the atherosclerotic plaque. Activin and follistatin colocalized to a large extent and, because follistatin can bind and neutralize the biological activity of this growth factor, it was essential to assess the presence of free, bioactive activin. A newly developed, in situ free-activin binding assay allowed us to selectively detect the presence of free activin in the advanced atherosclerotic lesion, especially in areas rich in both macrophages and neointimal SMCs. These results are consistent with data obtained by in vitro studies, showing that monocytic cells, upon differentiation into macrophages, and growth-stimulated aortic SMCs produce more activin-βA mRNA than nonactivated cells.[3,28] At this point, it should be added that neither in situ hybridization nor RNase protection analyses revealed any expression of activin-βB and inhibin-α transcripts, which excludes the possible presence of appreciable amounts of activin-AB, activin-B, and inhibin in the

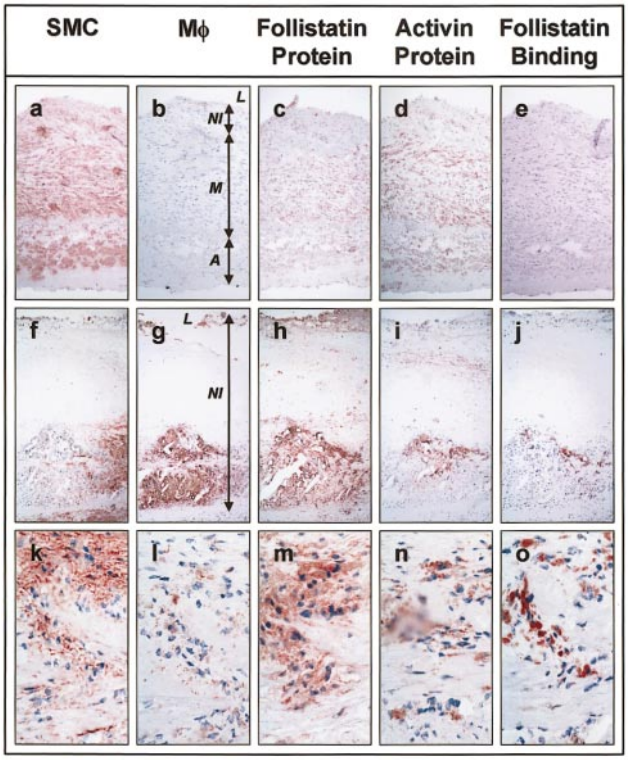

Figure 4. Immunohistochemical detection of activin-A and follistatin combined with in situ free-activin localization. SMCs (a, f, and k), macrophages (b, g, and l), follistatin protein (c, h, and m), and activin-A protein (d, i, and n) were detected by applying specific antibodies (see Materials and Methods). Activin that was not bound to its receptor or to follistatin was detected by the in situ free-activin binding assay using biotinylated follistatin (e, j, and o). The assays were performed on subsequent cryosections from an early aortic lesion (a through e; magnification ×50) and different advanced aortic fibrous lesions (f through j; magnification ×25; k through o, magnification: ×630). In neointimal areas containing both SMCs and macrophages, free, bioactive activin was detected as a subpopulation of total activin protein. L indicates lumen; A, adventitia; M, media; and NI, neointima.

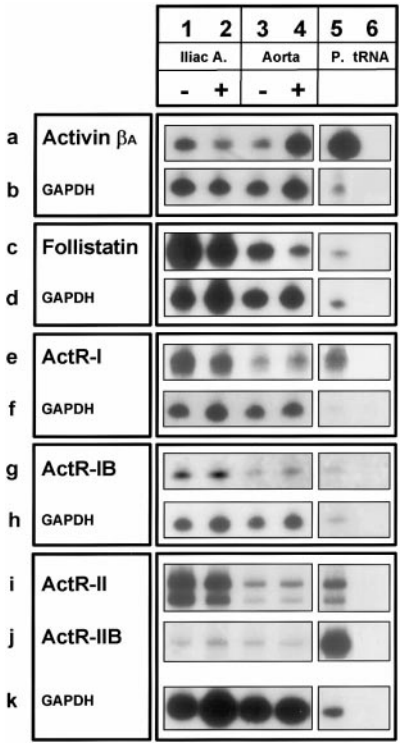

Figure 5. Expression of activin, follistatin, and activin receptor mRNA in SMCs, as determined by RNase protection assay. The mRNA expression level of these genes was assayed in quiescent iliac artery SMCs (lane 1), serum-stimulated iliac artery SMCs (lane 2), quiescent aortic SMCs (lane 3), and serum-stimulated aortic SMCs (lane 4). In lane 5, the mRNA expression in placenta is shown as a control. The probes were hybridized with yeast tRNA (lane 6) to detect aspecific background bands. The expression of the following genes was assayed in the above-mentioned resting and stimulated SMCs: activin-βA (row a), follistatin (row c), ActR-I (row e), ActR-IB (row g), ActR-II (row i), and ActR-IIB (row j). Simultaneous hybridization with a GAPDH riboprobe was performed for each of the gene-specific probes as a control for equal loading (rows b, d, f, and h, respectively). Because the expression of ActR-II, ActR-IIB, and GAPDH was determined simultaneously, the GAPDH expression data shown in row k were used to correct the quantitated data, both for ActR-II and ActR-IIB. The radioactivity in the bands was quantitated by PhosphorImager (Molecular Dynamics).

vessel wall or in cultured SMCs. The exclusive expression of the βA subunit in the human vasculature is reminiscent of the situation in rat vessels, where also only activin-A expression has been observed.[17,29]

We developed a free-activin binding assay to demonstrate the presence of bioactive activin in situ. This assay is based on the high-affinity interaction between follistatin and free activin, which results in almost irreversible complex formation.[30] Potential interactions of follistatin and bone morphogenetic proteins can be excluded because these interactions are highly reversible, and such complexes will dissociate during the procedure, which involves vigorous washing steps.[31] The mere presence of bioactive activin, especially in the advanced atherosclerotic lesion, suggests a role for this factor in the progression of the disease. A prerequisite for functional involvement of activin in atherogenesis is, however, the presence of type I and type II activin receptors, as well as downstream signaling components. Our RNase protection analyses on cultured SMCs and in situ hybridization studies on vascular specimens demonstrated that SMCs, ECs, and macrophages express at least one type I and one type II receptor, which indicates that these cell types can bind activin and probably also respond to activin. Because the expression levels of activin receptors are similar in the media and in the neointima, in contrast to a neointimal increase in bioactive activin, activin function in atherogenesis seems to be regulated by changes in expression levels of the soluble factor, rather than by altered expression levels of its receptors.

Limited information is available on the signaling pathways involved downstream of the activin receptors, except for the binding and phosphorylation of the transcription factors Smad2 and Smad3 by the activin-receptor complex. A complex of these phosphorylated Smads and Smad4 is subsequently translocated to the nucleus of the activin-stimulated cell to directly modulate the expression of, at present, mostly unknown genes.[10] Although the presence in vivo of activin signal transduction pathway components, including the Smads, needs to be confirmed, the demonstration of a

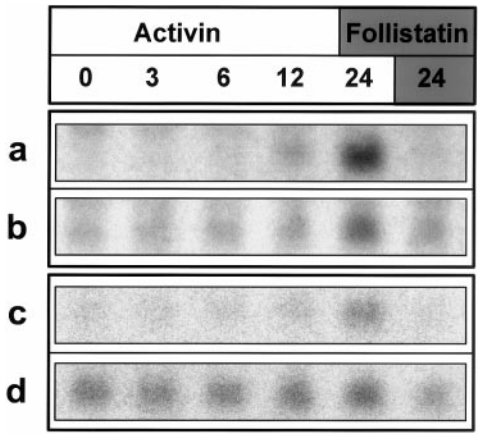

Figure 6. Expression of SMC differentiation markers after activin-A treatment. SMCs derived from iliac artery (a and c) and aorta (b and d) were treated with activin-A (100 ng/mL) for 0, 3, 6, 12, or 24 hours or with follistatin (200 ng/mL) for 24 hours. Total RNA was assayed by Northern blotting analysis for SM α-actin (a and b) and SM22α (c and d) gene expression (see Materials and Methods). Both SM-specific genes are induced after 24 hours of activin-A treatment. The radioactivity in the bands was quantitated by PhosphorImager.

physiologically relevant response of in vitro cultured cells on activin treatment supports the in vivo significance of activin in atherogenesis. Therefore, we will discuss the effect of activin on the most relevant cell types involved in atherogenesis: ECs, SMCs, and macrophages.

First, in human ECs, activin and TGF-β inhibit DNA synthesis,[13] and we confirmed these observations (data not shown). Second, in human SMCs derived from various vascular origins, we showed that activin does not affect DNA synthesis, whereas TGF-β does (data not shown). Also, in rat SMCs, one study showed that activin does not influence DNA synthesis,[18] although these data contrast with results obtained by other groups, which show increased DNA synthesis by activin.[16,17] To establish the physiological importance of activin receptors on human SMCs and the presence of functional signaling pathways, the effect of activin on SMC differentiation was analyzed. In vitro cultured SMCs exhibited a significant increase in mRNA expression of the SM-specific markers SM22α and SM α-actin in response to activin. The augmented expression of these genes is indicative of SMC differentiation toward a nonactivated, fully differentiated phenotype, which is commonly associated with SMCs residing in the normal media or in the fully differentiated fibrous cap of advanced lesions.[24–27] In the present study, we observed that iliac artery SMCs were more responsive to activin than aortic SMCs, which may be attributed to the relatively higher expression levels of ActR-I, ActR-IB, and ActR-II in the iliac artery SMCs (Figure 5).

To our knowledge, this is the first study showing that human SMCs originating from either the elastic aorta or the muscular iliac artery differ in their response to a growth factor. In this respect, it is of interest to note that chicken vascular SMCs reportedly exhibit opposite growth responses to TGF-β, depending on their embryonic origin (ectodermal or mesodermal).[32] These differences correlated with the extent of glycosylation of the TGF-β type II receptors. At present, it is not known whether activin receptors on human SMCs of distinct vascular origin would be differentially glycosylated, which could result in a different response toward activin in elastic and muscular SMCs. Finally, in human THP-1–derived macrophages, activin and TGF-β inhibit scavenger receptor expression, resulting in reduced lipid accumulation in these cells.[15,33] This would suggest that activin and TGF-β reduce atheroma foam cell formation during atherogenesis.

The distinct responses of these 3 cell types to activin illustrate the pleiotropic effects of this growth and differentiation factor and may give an indication of the functional involvement of activin in atherogenesis. It has been hypothesized that plaque stability is inversely related to macrophage and lipid content and is directly related to the number of SMCs present in the lesion.[34] By contrast, intimal SMCs are thought to suppress plaque rupture and subsequent local thrombosis, thereby preventing acute clinical problems. We propose, on the basis of our knowledge that activin inhibits foam cell formation in combination with our observations that it induces the redifferentiation of vascular SMCs, that activin, like TGF-β, may be beneficial for plaque stability.

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References

1. Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature*. 1986;321:776–779.
2. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin, R. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature*. 1986;321:779–782.
3. Murata M, Eto Y, Sakai M, Muramatsu M. EDF factor is encoded by the same mRNA as that of the inhibin βA chain. *Proc Natl Acad Sci U S A*. 1988;85:2434–2438.
4. Smith JC, Price BM, Van Nimmen K, Huylebroeck D. Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin-A. *Nature*. 1990;345:729–731.
5. van den Eijnden-Van Raaij AJ, van Zoelent EJ, van Nimmen K, Koster CH, Snoek GT, Durston AJ, Huylebroeck D. Activin-like factor from a Xenopus laevis cell line responsible for mesoderm induction. *Nature*. 1990;345:732–734.
6. Thomsen G, Woolf T, Whitman M, Sokol S, Vaughan J, Vale W, Melton DA. Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell*. 1990;63:485–493.
7. Hübner G, Hu Q, Smola H, Werner S. Strong induction of activin expression after injury suggests an important role of activin in wound repair. *Dev Biol*. 1996;173:490–498.
8. Matsuse T, Ikegami A, Ohga E, Hosoi T, Oka T, Kida K, Fukayama M, Inoue S, Nagase T, Ouchi Y, Fukuchi Y. Expression of immunoreactive activin A protein in remodeling lesions associated with interstitial pulmonary fibrosis. *Am J Pathol*. 1996;148:707–713.
9. Mayo KE, Cerelli GM, Spiess J, Rivier J, Rosenfeld MG, Evans RM, Vale W. Inhibin A-subunit cDNAs from porcine ovary and human placenta. *Proc Natl Acad Sci U S A*. 1986;83:5849–5853.
10. Heldin CH, Miyazono K, ten Dijke P. TGFβ signaling from cell membrane to nucleus through SMAD proteins. *Nature*. 1997;390:465–471.
11. Ueno N, Ling N, Ying SY, Esch F, Shimasaki S, Guillemin R. Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of FSH. *Proc Natl Acad Sci U S A*. 1987;84:8282–8286.
12. de Winter JP, ten Dijke P, de Vries CJ, van Achterberg TA, Sugino H, de Waele P, Huylebroeck D, Verschueren K, van den Eijnden-van Raaij AJ. Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. *Mol Cell Endocrinol*. 1996;116:105–114.

13. McCarthy SA, Bicknell R. Inhibition of vascular ECs growth by activin-A. *J Biol Chem*. 1993;268:23066–23071.
14. Yamada R, Suzuki T, Hashimoto M, Eto Y, Shiokawa K, Muramatsu M. Induction of differentiation of the human promyelocytic cell line HL-60 by activin/EDF. *Biochem Biophys Res Commun*. 1992;187:79–85.
15. Kozaki K, Akishita M, Eto M, Yoshizumi M, Toba K, Inoue S, Ishikawa M, Hashimoto M, Kodama T, Yamada N, Orimo H, Ouchi Y. Role of activin-A and follistatin in foam cell formation of THP-1 macrophages. *Arterioscler Thromb Vasc Biol*. 1997;17:2389–2394.
16. Kojima I, Mogami H, Kawamura N, Yasuda H, Shibata H. Modulation of growth of vascular SMC by activin A. *Exp Cell Res*. 1993;206:152–156.
17. Pawlowski JE, Taylor DS, Valentine M, Hail ME, Ferrer P, Kowala MC, Molloy CJ. Stimulation of activin A expression in rat aortic SMCs by thrombin and angiotensin II correlates with neointimal formation in vivo. *J Clin Invest*. 1997;100:639–648.
18. Nakaoka T, Gonda K, Ogita T, Otawara-Hamamoto Y, Okabe F, Kira Y, Harii K, Miyazono K, Takuwa Y, Fujita T. Inhibition of rat vascular SMC proliferation in vitro and in vivo by bone morphogenetic protein-2. *J Clin Invest*. 1997;100:2824–2832.
19. Inoue S, Orimo A, Hosoi T, Ikegami A, Kozaki K, Ouchi Y, Nomura S, Muramatsu M, Orimo H. Demonstration of activin-A in arteriosclerotic lesions. *Biochem Biophys Res Commun*. 1994;205:441–448.
20. Inoue S, Orimo A, Hosoi T, Matsuse T, Hashimoto M, Yamada R, Ouchi Y, Orimo H, Muramatsu M. Expression of follistatin, an activin-binding protein, in vascular SMCs and arteriosclerotic lesions. *Arterioscler Thromb*. 1993;13:1859–1864.
21. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis: a report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arterioscler Thromb Vasc Biol*. 1995;15:1512–1531.
22. Boot RG, van Achterberg TA, van Aken BE, Renkema GH, Jacobs MJ, Aerts JM, de Vries CJ. strong induction of members of the chitinase family of proteins in atherosclerosis: chitotriosidase and human cartilage gp-39 expressed in lesion macrophages. *Arterioscler Thromb Vasc Biol*. 1999;19:687–694.
23. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed, part 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
24. Mosse PR, Campbell GR, Wang ZL, Campbell JH. SMC phenotypic expression in human carotid arteries. *Lab Invest*. 1985;53:556–562.
25. Glukhova MA, Kabakov AE, Frid MG, Ornatsky OI, Belkin AM, Mukhin DN, Orekhov AN, Koteliansky VE, Smirnov VN. Modulation of human aorta SMC phenotype: a study of muscle-specific variants of vinculin, caldesmon, and actin expression. *Proc Natl Acad Sci U S A*. 1988;85:9542–9546.
26. Shanahan CM, Weissberg PL, Metcalfe JC. Isolation of gene markers of differentiated and proliferating vascular SMCs. *Circ Res*. 1993;73:193–204.
27. Owens GK. Regulation of differentiation of vascular SMC. *Phys Rev*. 1995;73:487–517.
28. Takahashi S, Yamashita T, Eto Y, Shibai H, Miyamoto K, Ogata E. Inducible gene expression of activin A/EDF in HL-60 cells. *Biochem Biophys Res Commun*. 1990;167:654–658.
29. Kanzaki M, Nobusawa R, Mogami H, Yasuda H, Kawamura N, Kojima I. Production of activin A and follistatin in cultured rat vascular SMC. *Mol Cell Endocrinol*. 1995;108:11–16.
30. Schneyer AL, Rzucidlo DA, Sluss PM, Crowley WF Jr. Characterization of unique binding kinetics of follistatin and activin or inhibin in serum. *Endocrinology*. 1994;135:667–674.
31. Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N. Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc Natl Acad Sci U S A*. 1998;95:9337–9342.
32. Topouzis S, Majesky MW. SMC lineage diversity in the chick embryo: two types of aortic SMC differ in growth and receptor-mediated transcriptional responses to TGFβ. *Dev Biol*. 1996;178:430–445.
33. Bottalico LA, Wager RE, Agellon LB, Assoian RK, Tabas I. TGFβ-1 inhibits scavenger receptor activity in THP-1 human macrophages. *J Biol Chem*. 1991;266:22866–22871.
34. Weissberg PL, Clesham GJ, Bennett MR. Is SMC proliferation beneficial? *Lancet*. 1996;347:305–307.