Changes in the Balance of Phosphoinositide 3-Kinase/Protein Kinase B (Akt) and the Mitogen-activated Protein Kinases (ERK/p38MAPK) Determine a Phenotype of Visceral and Vascular Smooth Muscle Cells

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Abstract. The molecular mechanisms behind phenotypic modulation of smooth muscle cells (SMCs) remain unclear. In our recent paper, we reported the establishment of a novel culture system of gizzard SMCs (Hayashi, K., H. Saga, Y. Chimori, K. Kimura, Y. Yamanaka, and K. Sobue. 1998. J. Biol. Chem. 273: 28860–28867), in which insulin-like growth factor-I (IGF-I) was the most potent for maintaining the differentiated SMC phenotype, and IGF-I triggered the phosphoinositide 3-kinase (PI3-K) and protein kinase B (PKB(Akt)) pathway. Here, we investigated the signaling pathways involved in de-differentiation of gizzard SMCs induced by PDGF-BB, bFGF, and EGF. In contrast to the IGF-I–triggered pathway, PDGF-BB, bFGF, and EGF coordinately activated the ERK and p38MAPK pathways. Further, the forced expression of active forms of MEK1 and MKK6, which are the upstream kinases of ERK and p38MAPK, respectively, induced de-differentiation even when SMCs were stimulated with IGF-I. Among three growth factors, PDGF-BB only triggered the PI3-K/PKB(Akt) pathway in addition to the ERK and p38MAPK pathways. When the ERK and p38MAPK pathways were simultaneously blocked by their specific inhibitors or an active form of either PI3-K or PKB(Akt) was transfected, PDGF-BB in turn initiated to maintain the differentiated SMC phenotype. We applied these findings to vascular SMCs, and demonstrated the possibility that the same signaling pathways might be involved in regulating the vascular SMC phenotype. These results suggest that changes in the balance between the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways would determine phenotypes of visceral and vascular SMCs. We further reported that SMCs cotransfected with active forms of MEK1 and MKK6 secreted a nondialyzable, heat-labile protein factor(s) which induced de-differentiation of surrounding normal SMCs.

Key words: smooth muscle cells • phosphoinositide 3-kinase • mitogen-activated protein kinases • ERK • p38MAPK

Phenotypic modulation of smooth muscle cells (SMCs) is critical in the onset of serious diseases such as atherosclerosis, hypertension, and leiomyogenic tumorigenicity. In the progression of these diseases, SMCs change from a differentiated state to a de-differentiated one (reviewed by Ross, 1993). Differentiated SMCs show a spindle-like shape and organize their unique intracellular structures including well-developed dense membranes, dense bodies, and myofibrils. They also display ligand-induced contraction. In contrast, de-differentiated SMCs lose these characteristic properties. In addition to these morphological and functional alterations, the expression levels and/or the isoforms of several proteins change in the two phenotypes. Therefore, these proteins...
are used as SM C-specific molecular markers (Owens, 1995; Sobue et al., 1998). For example, the expressions of caldesmon (Yano et al., 1995; Kashiwada et al., 1997), calponin (Gimona et al., 1992; Shanahan et al., 1993), SM 22α (Gimona et al., 1992; Shanahan et al., 1993), β-tropomyosin (Kashiwada et al., 1997), and α1 integrin (Glukhova et al., 1993; O bata et al., 1997) at the mRNA and protein levels are upregulated in differentiated SMCs, but downregulated in de-differentiated SMCs. Isoform changes of caldesmon (Ueki et al., 1987), calponin (Yano et al., 1995), and smooth muscle myosin heavy chain (Nagai et al., 1999; Kuro-o et al., 1989) are also controlled by SM C phenotype-dependent alternative splicings. Recently, the transcription machineries of caldesmon (Yano et al., 1995), smooth muscle myosin heavy chain (Kato et al., 1994; Madsen et al., 1997), SM 22α (Solway et al., 1995; Kim et al., 1997) and α1 integrin (Obata et al., 1997) have been partially characterized. However, the molecular mechanisms behind phenotypic modulation of SM Cs remain unclear. The slow progress in this area may be due to the plasticity of SM Cs. Under conventional culture conditions, SM Cs in primary culture display a rapid change in their phenotype (reviewed by Chamley-Campbell et al., 1979). And, SM C-derived clonal cell lines that maintain a fully differentiated phenotype have not yet been established. We have recently established a novel culture system for gizzard SM Cs in which they maintain a differentiated phenotype for a long time. Extracellular matrices partially affect the SM C phenotype. Of these, laminin is the most potent for delaying the progression of SM C de-differentiation, but can not maintain a differentiated phenotype for a long culture period, suggesting a requirement for additional factor(s). Among several growth factors and cytokines examined, insulin-like growth factor-I (IGF-I) is the most potent for maintaining the differentiated SM C. In the IGF-I-stimulated culture system, phosphoinositide 3-kinase (PI3-K) and its downstream target, protein kinase B (PK B(Akt)), but not mitogen-activated protein kinases (MAPKs), mediate the critical signaling pathways (Hayashi et al., 1998). M APKs have been implicated in the signaling cascades involved in the proliferation and hypertrophy of SM Cs (reviewed by Force and Bonventre, 1998). These include extracellular signal-regulated kinase (ERK) and the stress-activated MAPKs, c-jun NH2-terminal protein kinase (JNK) and p38 MAP K. ERK is activated in response to growth factors, cytokines, and cellular stresses (Ray and Turgill, 1988; D enhardt, 1996), and is involved in a variety of biological processes (Force and Bonventre, 1998). Treatment with growth factors such as PDGF, EGF, and basic fibroblast growth factor (bFGF), which induce the proliferation or migration of cultured SM Cs, activates ERK. JNK and p38 MAPK are also activated by cellular stresses including inflammatory cytokines, heat shock, osmolar stress, ultraviolet irradiation, and inhibition of protein synthesis (Derijard et al., 1994; K yrian and A vruch, 1996). The ER K and J NK activities are increased in aortic, carotid, and femoral arteries by hypertensive agents, angiotensin II and phenylephrine (Xu et al., 1996). In cultured airway SM Cs, endothelin activates both the ER K and J NK signaling pathways, resulting in cell proliferation (Shapiro et al., 1996). ER K (Pyles et al., 1997), ER K/J NK (Hu et al., 1997), or p38 MAP K (Larrivee et al., 1998) are enhanced in rat carotid arteries after balloon injury. p38 MAP K is also elicited in airway SM Cs by PDGF stimulation (Pyne and Pyne, 1997). These findings suggest that some smooth muscle disorders are closely associated with the activation of M APKs. However, the direct involvement of M APK pathways in regulating the SM C phenotype has not yet been demonstrated.

We investigated the signaling pathways involved in SM C de-differentiation induced by PDGF-BB, bFGF and EGF, and compared them with the IGF-I–triggered signaling pathway in maintaining a differentiated phenotype of gizzard SM C in culture. Here, we demonstrated the first direct evidence for a mechanism by which the distinctly different signaling pathways regulate the SM C phenotype. Both the ER K and p38 MAP K pathways triggered by PDGF-BB, bFGF, and EGF were found to play an essential role in inducing SM C de-differentiation, whereas the PI3-K/PKB(Akt) pathway was critical in maintaining a differentiated state. Interestingly, PDGF-BB only triggered both types of signaling pathways. When the ER K and p38 MAP K pathways were blocked by their specific inhibitors, PDGF-BB in turn initiated to maintain a differentiated phenotype of gizzard SM Cs. The same signaling pathways involved in the phenotypic determination were observed in vascular SM Cs. Thus, changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ER K and p38 MAP K pathways would determine phenotypes of visceral and vascular SM Cs. We further demonstrated a de-differentiation–inducing factor(s) secreted from SM Cs in which both the M APK pathways were activated by cotransfection with active forms of M EK 1 and M KK 6.

Materials and Methods

Antibodies

A nti–PI3-K p85 subunit antiserum was purchased from Upstate Biotechnology. Polyclonal antibodies against PKB(Akt), ER K, J NK, p38 MAP K, M EK 1, and M KK 6 were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against c-Myc and Flag were purchased from Santa Cruz Biotechnology and Sigma Chemical Co., respectively.

Plasmids

Construction of the caldesmon promoter plasmid, GP3CAT, was described previously (Yano et al., 1995). The expression vector containing the constitutively active form of the c-Myc–tagged PI3-K p110α (pCMV5p110α) was kindly provided by Drs. H. Kurosu and T. Katada (Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo). This cDNA was constructed by Hu et al. (1995) and was inserted downstream of the cytokemavirus promoter, pCMV5. Expression vectors of constitutively active and dominant-negative forms of M EK 1 and M KK 6, and Flag-tagged ER K2 and Flag-tagged p38 MAP K were kindly provided by Dr. K. Sugiyama (Boehringer Ingelheim), Drs. M. Hibi and T. Hirano (O saka University, Medical School), and Dr. E. Nishida (Graduate School of Science, Kyoto University). M u tant cDNAs of M EK 1 and M KK 6 were constructed as described elsewhere (Man sourn et al., 1994; R aingeaud et al., 1996), and were inserted downstream of the cytokemavirus promoter of pCMV5– or the SRα promoter of pCDLSRα296. In this study, we used expression vectors constructed in pCS2+ for active and dominant-negative forms of M EK 1 (pCS2+–M EK 1act and pCS2+–M EK 1DN), respectively) and M KK 6 (pCS2+–M KK 6act and pCS2+–M KK 6DN, respectively). A PKB(Akt) cDNA was amplified by reverse transcriptase PCR using human placental...

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mRNA as a template, and the accuracy of its sequence was verified. A PK B(A kti) cDNA was obtained by RT-PCR using primers complementary to the PVH region of the PKB(Akt) cDNA thus obtained was inserted downstream of the cytomegalovirus promoter of pCMV5 (+1 cm y-cadherin-targeted (MT) (pCS2-MT-PAK A 101, for expression of c-Myc-y-cadherin-targeted type PK B(A kti). The expression plasmid of c-Myc-y-cadherin constitutively active form of PK B(A kti), pCS2-MT-PK B(A kti) act, was constructed as described previously (Dario et al. 1996).

Cell Culture
Isolated gizzard SMCs were prepared from a 15-d-old chick embryo gizzards as described elsewhere (Hayashi et al., 1998), and cultured on laminin-coated six-well plates with the indicated growth factors under kinase inhibited or stimulated conditions. Vascular SMCs were isolated from 5–7-d-old rat aorta by enzyme-dispense methods as follows. Aorta were dissected under sterile conditions, minced well with scissors, and incubated at 37°C in 0.1% collagenase for 30 min, followed by incubation in the mixtures of 0.07% collagenase and 0.03% elastase for 90 min. 25 TLC cells were isolated after digestion by tissue enzymes. Cells obtained were plated in dishes coated with growth factor-free basic medium (DME supplemented with 0.2% BSA) and were cultured in the medium containing IGF-I or PDGF-BB on laminin-coated culture plates. Treatment with specific inhibitors was performed as follows: gizzard or vascular SMCs were preincubated for 1 h with inhibitors, and then stimulated with 1 mM carbachol treatment were photographed. Cells obtained were observed with an Olympus microscope, and the same fields before and after carbachol treatment were photographed.

Northern Blotting
2 μg of total RNA from precultured or cultured SMCS under the indicated conditions were separated on 1.0% agarose-formaldehyde denaturing gels, and then transferred to nylon membranes. A caldesmon cDNA (GenBank M 28417) fragment (nucleotides 286 to 810) and a calponin cDNA (GenBank M 63559) fragment (nucleotides 1 to 867) were used as probes to monitor the expression of respective mRNAs. This caldesmon cDNA fragment, which contains parts of exons 2 and 3α is a common probe for the h- and l-caldesmons (Hayashi et al., 1993; Hayashi et al., 1992). In our previous studies using specific probes for h- or l-caldesmon, we demonstrated that the full length of h- and l-caldesmon mRNAs are 4.8 and 4.1 kb, respectively (Kashiwada et al., 1997; Obata et al., 1997). Probes were labeled with 32P on the antisense strands and used for hybridization under the following conditions: 42°C for 16 h in 50% formamide, 6× SSC, 10°C. Other conditions were as described previously (Kashiwada et al., 1997; Obata et al., 1997).

Immunoblotting
Total protein of the cell lysates from SM cultures was separated by SDS-PAGE and transferred to nitrocellulose membranes. Detection of target proteins was performed using an ECL Western blotting detection kit (A mansham Pharmacia Biotech) with the indicated polyclonal antibodies.

PI3-K Assay
Phospholipid mixtures (2 mg/ml) containing phosphatidylinositol (PI) and phosphatidylserine (PS) were dried under a stream of nitrogen, and sonicated in 10 mM Heps (pH 7.4) in a bath sonicator at 0°C for 15 min. 10 μl of the resulting vesicles (PI/PS) were used as a substrate for PI3-K. The preparation of cell extracts and immunoprecipitation for PI3-K were performed at 4°C. The cultured cells were washed three times with ice-cold PBS, and then lysed in 550 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 1% NP-40, 50 mM NaF, 1 mM Na2VO4, 50 μg/ml PM SF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). A lter

Promoter Analysis and Transfection
The caldesmon promoter activity was analyzed using the chloramphenicol acetyltransferase (CAT) construct, GP3CAT, according to the method described previously (Yano et al., 1995; Obata et al., 1997). The SM cells prepared as described above were seeded onto laminin-coated six-well plates, and cultured in the indicated medium for 1 or 3 d. Transfection was carried out using Trans IT™-LT1, polyamine transfection reagents (Pan Vera Corporation). Complex mixtures composed of 10 μg of trans IT™-LT1 reagent and 2 μg of GP3CAT, 1 μg of control plasmid carrying the luciferase gene under the Rous sarcoma virus (RSV) promoter (RSV- luciferase), and 1 μg of each control expression plasmid (pCMV5, pCS2+, or pCS2-MT), expression plasmid carrying a c-Myc-y-cadherin constitutively active form of PI3-K (p120x subunit, a c-Myc-y-cadherin wild-type or a constitutively active form of PKB(Akt) (p53P530A), pCS2+MT-PK B(Akt) act, or pCS2+MT-PK B(Akt) act), or either or both of expression vectors carrying constitutively active and dominant-negative forms of MEK1 (pCS2+MEKLa ct and pCS2+MEK1D N) and MKK 6 (pCS2+MK K6 ac and pCS2+MK K6 D N), were added to the cells in Opti minimum Eagle’s medium (GIBCO BRL). A lter a further 4-h incubation, these cells were transfected with IME supplemented with 0.25% BSA plus 2 mg/ml 1G1-F or 20 ng/ml PGDF-BB, and the transfected cells were harvested 48 h later. Standardization of transfection efficiency was performed by measuring luciferase activity as described previously (Yano et al., 1995; Obata et al., 1997). The cell extracts containing equal amounts of luciferase activity were used for the CAT assay. The transfection experiments were repeated at least three times on duplicate cultures with two or
three different plasmid preparations. The CAT activities were quantified by Scanning Imager (Molecular Dynamics).

The effects of forced expression of MEK1 and MKK6 in cultured SMCs were analyzed as follows. The indicated amounts of control expression plasmid and either or both of expression plasmids carrying active or dominant-negative MEK1 and MKK6 were transfected into cultured SM Cs together with 1 μg of a reporter plasmid carrying the β-galactosidase gene downstream from the SV-40 early promoter. Total RNA was isolated from the transfected cells and the expression levels of caldesmon and calponin mRNAs were analyzed by Northern blotting as described above. Transfection efficiencies were determined by staining for β-galactosidase activity from the reporter plasmid using 5-bromo-4-chloro-3-indolyli-β-D-galactoside (X-gal) as a substrate.

Expression of Epitope-tagged Kinases and Kinase Assays

Transfection was carried out as described above in Promoter Analysis and Transfection. In the cases of PI3-K and PKB(Akt) assays, SM Cs were transfected with 3 μg of respective expression plasmids of constitutively active form of c-Myc-tagged PI3-K p110α subunit, pcMV Sp110α, or wild-type or constitutive active form of c-Myc-tagged PKB(Akt), pCS2+MT-PKB(Akt)wt or pCS2+MT-PKB(Akt)act. Two micromgs of expression plasmid of each Flag-tagged ERK2 or Flag-tagged p88MAPK PK was cotransfected with 2 μg of either expression plasmid of active or dominant-negative MEK1 or MKK6, or control plasmid. In both cases, SM Cs were cultured under nonstimulated conditions after transfection. 2 d later, SM Cs were stimulated under indicated conditions. The cell extracts containing the equal amounts of proteins were precleaned with control mouse IgG coupled protein G-Sepharose for 30 min and immunoprecipitated with monoclonal antibody against c-Myc or Flag followed by protein G-Sepharose. The kinase activities were determined as described above in PI3-K assay and other protein kinase assays.

Characterization of Conditioned Medium

Conditioned medium obtained from SM Cs transfected with both expression plasmids carrying active MEK1 and MKK6 was filtered through a 0.22–μm membrane. The conditioned medium was heated to 100°C for 15 min or treated with trypsin (30 μg/ml) for 3 h at 30°C, followed by the addition of trypsin inhibitor at a 10-fold excess. The heat- or trypsin-treated conditioned medium was dialyzed against DMEM supplemented with 0.2% BSA at 4°C for 16 h, and adjusted to the concentration of IGF-I to 2 ng/ml. The SM Cs were cultured in these medium for 3 d. Heparin-Sepharose affinity chromatography was carried out as follows. One ml of 50% slurry of heparin-Sepharose (Amersham Pharmacia Biotech) equilibrated with PBS was added to 20 ml of the conditioned medium and gently agitated for 5 h at 4°C. The mixture was poured over a 1-ml Prep Column (Bio-Rad Labs), and the follow through fraction (non-heparin-binding) was collected. The column was rinsed with 10 vol of PBS and eluted stepwise with 1 ml of PBC containing NaCl (0.5, 1.0, and 1.5 M). Each fraction was collected and desalted by dialysis against DMEM supplemented with 0.2% BSA through a membrane of 3-kD cutoff (Spectrum). Aliquots of each fraction were diluted (1:4) with DMEM supplemented with 0.2% BSA and centrifuged at 100,000 g for 20 min.

Results

Different Downstream Signaling Pathways Triggered by PDGF-BB, bFGF, EGF, and IGF-I

We have recently established a novel culture system of gizzard SM Cs in which they maintain a differentiated phenotype for a long culture period. Of growth factors and cytokines examined, IGF-I is the most potent for maintaining the differentiated SM C phenotype as defined by the expression of SM C-specific molecular markers, cell morphology, and function (Hayashi et al., 1998). On the other hand, PDGF-BB, bFGF, and EGF potently induce SM C de-differentiation (Fig. 1). In 3- and 5-d-cultured SM Cs stimulated with PDGF-BB, bFGF, or EGF, h-caldesmon mRNA converts to l-caldesmon mRNA, and total h- and l-caldesmon mRNA s decrease to 20% of the levels...
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seen in precultured SMCs. Calponin mRNA is also downregulated to a negligible level (Fig. 1 A). However, the levels of h-caldesmon and calponin mRNAs in cultured SMCs under IGF-I–stimulated conditions are identical with those seen in precultured cells (Fig. 1 A). Similar results are obtained using α- and β-tropomyosins (Kashiwada et al., 1997) and α1 integrin (Obata et al., 1997), which are also SMC-specific molecular markers (data not shown). With regard to cell morphology and function, cultured SMCs under IGF-I–stimulated conditions showed a spindle-like shape, formed a meshwork structure, and displayed carbachol-induced contraction. In contrast, SMCs stimulated with PDGF-BB, bFGF, or EGF showed a fibroblast-like shape and lost the contractility (Figs. 1 B and 4), indicating that PDGF-BB, bFGF, and EGF are potent factors for inducing SMC de-differentiation.

Our previous studies revealed that the PI3-K/PKB(Akt) pathway triggered by IGF-I plays a critical role in maintaining the differentiated SMC phenotype (Hayashi et al., 1998). To investigate the downstream signaling pathways involving in SMC de-differentiation triggered by PDGF-BB, bFGF, or EGF, we analyzed several kinases including ERK, JNK, p38MAPK, PI3-K, and PKB(Akt). PDGF-BB, bFGF, and EGF all activated ERK (Fig. 2 A) and p38MAPK (Fig. 2 C). Their maximum activations were found at 10 min after stimulation. bFGF and EGF also activated JNK, whereas PDGF-BB did not (Fig. 2 B). IGF-I had no effect on ERK (Fig. 2 A), JNK (Fig. 2 B), and p38MAPK (Fig. 2 C). These data suggest that growth factors inducing SMC de-differentiation coordinately activate the ERK and p38MAPK pathways.

As demonstrated previously (Hayashi et al., 1998), IGF-I potently activated PI3-K (Fig. 2 D) and PKB(Akt) (Fig. 2 E); the maximum activation of PI3-K was achieved at 10 min after IGF-I stimulation and this activation reduced thereafter, while the activation of PKB(Akt) by IGF-I (2 ng/ml) lasted for more than 180 min. The activation of PI3-K and PKB(Akt) by IGF-I was suppressed by specific PI3-K inhibitors, wortmannin or LY249002 (data not shown), indicating that the PKB(Akt) activation exclusively depends on the PI3-K activity. No significant activation of PI3-K and PKB(Akt) was observed in SMCs stimulated by either bFGF or EGF (Fig. 2, D and E). Among the three growth factors inducing SMC de-differentiation, PDGF-BB was the only one that could activate PI3-K and PKB(Akt) (Fig. 2, D and E). The PKB(Akt) activation by PDGF-BB was more potent than that by IGF-I at 15 min after growth factor stimulation, whereas this activation was transient, but retained at a substantial level for 180 min. By contrast, the PKB(Akt) activation by IGF-I was sustained at a high level for more than 180 min. These results suggest the possibility that in addition to PI3-K,
The results are shown from four independent experiments.

**Dual Function of PDGF-BB on Gizzard SMC Phenotype**

It is curious that PDGF-BB, which is a potent factor inducing SMC de-differentiation (Fig. 1), triggered the dual signaling pathways mediated through both PI3-K/PKB(Akt) and two MAPKs, ERK and p38MAPK (Fig. 2). To simplify the PDGF-BB–triggered signaling pathways, we examined the effects of specific MAPK inhibitors, PD 98059 for ERK kinase (MEK1) and SB 203580 for p38MA PK, on the PDGF-BB–stimulated SMC phenotype. Either PD 98059 or SB 203580 specifically inhibited the PDGF-BB–induced activation of ERK or p38MAPK, respectively, to near basal levels (Fig. 3 A), but had no effect on PI3-K and PKB(Akt) (data not shown). Treatment with either PD 98059 or SB 203580 only slightly suppressed the PDGF-BB–induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNA s (Fig. 3 B). However, simultaneous treatment with both drugs strongly suppressed the PDGF-BB–induced SMC de-differentiation as monitored by the expression of caldesmon and calponin mRNAs (Fig. 3 B). In addition to these molecular events, both drugs could also rescue the morphological alteration from a spindle-like shape to a fibroblast-like shape change and a loss of contractility induced by PDGF-BB (Fig. 4). As a control, treatment with individual drug resulted in less significant effect on cell morphology and function. Table I shows the effects of PD 98059 and/or SB 203580 on carbachol-stimulated contractility of SMCs under various culture conditions. Further, both drugs only slightly delayed the induction of SMC de-differentiation by bFGF or EGF, but did not prevent SMC de-differentiation (data not shown).

Promoter analyses of the caldesmon gene further support these findings (Fig. 5). We used the caldesmon promoter/CAT construct, GP3CAT, which produces the high promoter activity in differentiated SMCs (Yano et al., 1995). The promoter activity in SMCs stimulated by PDGF-BB reduced to 30% of that by IGF-I (Fig. 5 C). Even under PDGF-BB–stimulated conditions, inhibition of both the ERK and p38MAPK pathways by their specific inhibitors or the forced expression of active PI3-K (Fig. 5 A) or active PKB(Akt) (Fig. 5 B) could protect such reduction (Fig. 5 C). These results suggest that PDGF-BB displays the dual function in maintaining the differentiated SMC phenotype mediated through the PI3-K/PKB(Akt) pathway and inducing SMC de-differentiation by the ERK and p38MAPK pathways. Thus, changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways would determine the SMC phenotype.

**Direct Involvement of ERK and p38MAPK Inducing Gizzard SMC De-differentiation**

The MAPK signaling cascades are involved in a variety of cell functions (Force and Bonventre, 1998). Dual phosphorylation on Thr and Tyr within the Thr-Xaa-Tyr motif catalyzed by MAPK kinases is essential for MAPK activation (Davis, 1994). M EK 1 and MKK 6 are specific upstream kinases for ERK and p38MAPK, respectively (Cohen, 1997). To investigate the direct involvement of ERK and p38MAPK in SMC de-differentiation, we examined the effects of active or dominant-negative MEK 1 and/or MKK 6 on the caldesmon promoter activity in SMCs under IGF-I–stimulated conditions. We determined the respective MAPK kinase activity by in vitro kinase assay (Fig. 6 A). In this experiment, expression plasmids carrying active or dominant-negative MAPK kinases were cotransfected with expression plasmid carrying Flag-tagged ERK or Flag-tagged p38MAPK into cultured SMCs, and Flag-tagged proteins were immunoprecipitated from the cell lysates with anti-Flag monoclonal antibody. The ERK or p38MAPK activities were potently enhanced in SMCs cotransfected with active MEK 1 or MKK 6 under nonstimulated or PDGF-BB–stimulated conditions (Fig. 6 A, a
and b). Even when SMCs were stimulated by PDGF-BB, their enhancements were strongly abolished by the forced expression of dominant-negative MEK1 or MKK6 (Fig. 6 A, a and b). The expressions of active and dominant-negative MEK1 or MKK6 proteins were confirmed by immunoblotting (data not shown). The caldesmon promoter activity in differentiated SMCs under IGF-I–stimulated conditions was analyzed by the forced expression of active PI3-K and p38MAPK pathways. Gizzard SMCs plated on laminin were stimulated with 2 ng/ml IGF-I (a and d), 20 ng/ml PDGF-BB (b and e), or 20 ng/ml PDGF-BB in the presence of both PD98059 (30 μM) and SB203580 (20 μM) (c and f) for 3 d. Ligand-induced contractility was monitored by the addition of carbachol (1 mM) for 1 min. Photographs show cultured SMCs before (a, b, and c) and after (d, e, and f) carbachol treatment. The data are presented from five independent experiments.

**Figure 5.** Regulation of the caldesmon promoter activity mediated through the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways. (A) The PI3-K activity in nonstimulated SMCs transfected with control plasmid (pCMV5) and expression plasmid of c-Myc–tagged active PI3-K p110α subunit, pCMV5p110αact (PI3-Kact). Gizzard SMCs transfected with indicated plasmids were cultured under nonstimulated conditions, and the PI3-K activity was determined by immunoprecipitation with anti-c-Myc monoclonal antibody, followed by in vitro kinase assays as described in Materials and Methods. (B) The PKB(Akt) activity in SMCs transfected with control plasmid, pCS2+MT, expression plasmid of c-Myc–tagged wild-type PKB(Akt), pCS2+MT-PKB(Akt)wt, or expression plasmid of c-Myc–tagged active PKB(Akt), pCS2+MT-PKB(Akt)act. The SMCs were cultured under nonstimulated conditions for 2 d after transfection, and then half of the cultures were stimulated by 2 ng/ml IGF-I with or without treatment of 20 μM LY294002. PKB(Akt) assays were carried out as described above. In A and B, the top and bottom panels are the results of kinase assay and immunoblotting (IB) to determine the amounts of kinase proteins in the cell lysates, respectively. The kinase activities are shown from three independent experiments. (C) Effects of PDGF-BB– or IGF-I–triggered signalings on the caldesmon promoter activity. The promoter construct of caldesmon, GP3CAT, was transfected into 3-d–cultured SMCs under the following conditions: DME supplemented with 0.2% BSA alone or BSA plus 20 ng/ml PDGF-BB. The GP3CAT (2 μg) was cotransfected with RSV–luciferase (1 μg) and control plasmid (1 μg), expression plasmid of c-Myc–tagged active PI3-K p110α (PI3-Kact, 1 μg), or expression plasmid of c-Myc–tagged active PKB(Akt), pCS2+MT-PKB(Akt)act. After transfection, SMCs were stimulated with 2 ng/ml IGF-I, 2 ng/ml IGF-I plus 20 μM LY294002, 20 ng/ml PDGF-BB, or 20 ng/ml PDGF-BB plus PD98059 (30 μM) and SB203580 (20 μM). The promoter activity was assayed at 48 h after transfection as described in Materials and Methods. The relative promoter (CAT) activities were normalized to the activity in culture SMCs under IGF-I–stimulated conditions, which was defined as 100%. Each value represents the average ± SD of three independent experiments. A promoterless control CAT plasmid (pUC0CAT) did not show detectable CAT activity under the same conditions (data not shown).
or dominant-negative MAPK kinases (Fig. 6 B). The promoter activity was not affected by either or both dominant-negative MEK1 and/or MKK6. Transfection with either active MEK1 or MKK6 significantly reduced the caldesmon promoter activity, while cotransfection with both active kinases further suppressed the promoter activity. Since the SV-40 promoter was not affected by the forced expression of MEK1 and/or MKK6 (data not shown), suppression of the caldesmon promoter activity by active MEK1 and MKK6 was specific. These results indicate that the ERK and p38MAPK pathways are directly involved in the induction of SMC de-differentiation.

Detection of De-differentiation-inducing Factor(s) from Gizzard SMCs Cotransfected with Active MEK1 and MKK6

We further examined the effects of active and dominant-negative MEK1 or MKK6 on the endogenous expression of caldesmon and calponin mRNAs and on cell morphology. Transfection with either active kinase alone or cotransfection with dominant-negative kinases had less significant effects on caldesmon and calponin mRNAs than on the calponin promoter activity. The SMCs were cotransfected with 2 μg each of expression plasmid of active or dominant-negative MEK1 and/or MKK6 and control plasmid together with 2 μg Flag-tagged ERK2 or Flag-tagged p38MAPK. The SMCs were cultured under nonstimulated conditions for 2 d, and half of the cultures were stimulated with 20 ng/ml PDGF-BB for 10 min. The cells were lysed and subjected to kinase assays after immunoprecipitation with anti-Flag monoclonal antibody. The top and bottom panels are the results of kinase assay and immunoblotting (IB) to determine the amounts of Flag-tagged kinase proteins in the cell lysates. The representative data are from two independent experiments. (B) Effects of the forced expression of active or dominant-negative MEK1 and/or MKK6 on the caldesmon promoter activity. The SMCs were cotransfected with G3P3CAT (2 μg), RSV-luciferase (1 μg), and either or both expression vectors carrying active or dominant-negative MEK1 and/or MKK6 (1 μg). The total amounts of transfected plasmids were adjusted to 5 μg by the addition of control vector, pCS2+. The promoter activities were determined as described in the legend of Fig. 5. The relative promoter activities were normalized to the activity in cultured SMCs under IGF-I-stimulated conditions without expression vectors carrying MEK1 or MKK6, which was defined as 100%. Each value represents the average ± SD of three independent experiments.

4-d-cultured SMCs stimulated by PDGF-BB (Fig. 7 A, lanes 3, 9, and 13). These results indicate that after cotransfection with active MEK1 and MKK6, almost all of cultured SMCs alter their phenotype to de-differentiation. To compare transfection efficiency and cell morphology, cultured SMCs stimulated by IGF-I were transfected with control plasmid (Fig. 7 B, a) or both expression plasmids carrying active MEK1 and MKK6 (Fig. 7 B, b) together with β-galactosidase expression plasmid. As revealed by β-galactosidase staining, transfection efficiencies were ~25%. The SMCs transfected with control vector remained to show a spindle-like shape (Fig. 7 B, a). In the case of SMCs cotransfected with active MEK1 and MKK6, all of the β-galactosidase-stained and -unstained cells converted from a spindle-like shape to a fibroblast-like shape (Fig. 7 B, b). The transfection efficiency of both active MEK1 and MKK6 was correlated with SMC-specific marker gene expression and cell morphology (Fig. 7, C and D). These data suggest that SMCs cotransfected with active MEK1 and MKK6 secrete some factor(s) which induces de-differentiation of surrounding normal SMCs.

To further characterize such a factor(s), conditioned medium (CM1) obtained from SMCs cotransfected with expression plasmids carrying active MEK1 and MKK6 was prepared. Fig. 8 A shows the expression of caldesmon and calponin mRNAs in 3-d-cultured SMCs stimulated with the conditioned medium. Even when SMCs were stimulated by IGF-I, the conditioned medium potently induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNAs (Fig. 8 A, lane 1), whereas control medium (CM2) obtained from SMCs...
Figure 7. Detection of de-differentiation-inducing factor(s) from SMCs cotransfected with active MEK1 and MKK6. (A) Effects of the forced expression of active or dominant-negative MEK1 and/or MKK6 on the endogenous expression of caldesmon and calponin mRNAs. Gizzard SMCs were transfected with 1 μg of indicated expression plasmids, and then cultured under IGF-I–stimulated conditions for 2 d (lanes 1–5) and for 4 d (lanes 7–11). The total amounts of transfected plasmids were adjusted to 2 μg by the addition of pCS2+. The SMCs were also cultured under IGF-I–stimulated conditions (2 ng/ml) without transfection for 2 d (lane 6) and 4 d (lane 12), or under PDGF-BB–stimulated conditions (20 ng/ml) without transfection for 4 d (lane 13). Caldesmon and calponin mRNAs in cultured SMCs were analyzed by Northern blotting as shown in Fig. 1A. (B) Transfection efficiency and comparison of cell morphology between SMCs transfected with control plasmid (a) and expression plasmids carrying active MEK1 and MKK6 (b). The SMCs were transfected with pCS2+ (2 μg) and pSV β-galactosidase (1 μg) (a) or with pCS2+MEK1act (1 μg), pCS2+MKK6act (1 μg), and pSV (β-galactosidase (1 μg) (b), and then cultured under IGF-I–stimulated conditions. At 4 d after transfection, β-galactosidase activity was visualized using X-gal as a substrate. (C and D) Dose-dependent effect of transfection with active MEK1 and MKK6 on the SMC phenotype. The SMCs were transfected with the indicated amounts of pCS2+MEK1act and pCS2+MKK6act together with pSV β-galactosidase (1 μg), and then cultured for 4 d under IGF-I–stimulated conditions. Transfection efficiency was increased in increasing amounts of transfected plasmids as follows: lane 1 in C and top panel in D, <1%; lane 2 in C and middle panel in D, 5%; and lane 3 in C and bottom panel in D, 25%. The expression of caldesmon and calponin mRNAs (C) and cell morphology (D) of transfected SMCs are shown. The representative data are shown from four (A and B) or three (C and D) independent experiments.
transfected with control plasmid alone did not (Fig. 8 A, lane 2). Further, the conditioned medium enhanced the ERK, JNK, and p38 MAPK activities (Fig. 8 B). These results exclude the possibility of retransfection with residual expression vectors, because MEK1 and MKK6 proteins derived from expression plasmids were not detected in cultured SMCS by immunoblotting (data not shown). Thus, this study revealed that the forced activation of both ERK and p38 MAPK in SMCS induces the secretion of some factor(s) which initiates de-differentiation of surrounding normal SMCS in a paracrine manner. Heat or trypsin treatment of the conditioned medium completely abolished the activity inducing SM C de-differentiation (Fig. 8, lanes 3 and 4), suggesting that the factor(s) is a protein in nature. The conditioned medium was further fractionated using a heparin-Sepharose column. The flow through (non-heparin-binding) fraction retained the potential de-differentiation activity, while the eluted fraction by 0.5, 1.0, and 1.5 M NaCl did not (Fig. 8 A, lanes 5-8). PDGFs and bFGF show heparin-binding abilities; the former was eluted with 0.5 M NaCl and the latter with 1.5 M NaCl (data not shown). By contrast, EGF is known as a non-heparin-binding growth factor, suggesting a candidate for the active protein factor. A specific inhibitor for EGF receptor kinase, AG1478, only slightly inhibited the conditioned medium-induced de-differentiation, but this effect was less significant (Fig. 8 A, lane 9). Therefore, the active protein factor(s), which induces SM C de-differentiation, in the conditioned medium is considered to be different from PDGFs, bFGF, and EGF.

**Signaling Pathways in Regulating the Vascular SMC Phenotype**

To examine whether vascular SMCS could be regulated by the same signaling pathways as revealed in gizzard SMCS, we first applied our culture system of gizzard SMCS to vascular SMCS. We isolated rat vascular SMCS by the enzyme-disperse method, and cultured them on laminin-coated plates under IGF-I-stimulated conditions. Because of difficulty to obtain a lot of cell numbers from rat aortae, we observed cell morphology and ligand-induced contractility to determine the vascular SM C phenotype (Fig. 9). Vascular SMCS could also maintain a spindle shape for more than 2 weeks under IGF-I-stimulated conditions, and showed ligand-induced contractility (Fig. 9, a and f). A blockade of the P13K pathway by LY 294002 resulted in a morphological change from a spindle shape to a fibroblast-like shape and a loss of contractility (Fig. 9, b and g). Inconsistent with the case of gizzard SMCS, PDGF-BB rapidly induced de-differentiation of vascular SMCS as monitored by cell morphology and ligand-induced contractility (Fig. 9, c and h). Even under PDGF-BB-stimulated conditions, simultaneous treatment with PD98059 and SB203580 could retain a spindle shape and contractility (Fig. 9, d and i). A specific control treatment with each drug individually was less significant effect on the PDGF-BB-induced de-differentiation (data not shown). Furthermore, the conditioned medium obtained from gizzard SMCS transfected with both active MEK1 and MKK6 remarkably induced de-differentiation of vascular SMCS (Fig. 9, e and j). Therefore, these data also suggest that the PI3-

**Discussion**

Under pathological conditions, phenotype of SMCS can change from a differentiated state to a de-differentiated state in vivo and in vitro. During de-differentiation, SMCS show dramatic and irreversible alterations in their cell shape, function, and expression of SM C-specific molecular markers. Long spindle-shaped cells change to fibroblast-like cells, accompanied by losses in a ligand-induced contractility and SM C-specific molecular marker expression. Since there has not been a primary culture system available for SMCS or SM C-derived cell lines in which they can maintain a fully differentiated phenotype, the intracellular signaling pathways regulating the SM C phenotype have not been well characterized. Recently, we established a novel culture system in which gizzard SMCS can maintain a differentiated phenotype for a long culture time (Hayashi et al., 1998). In this culture system, IGF-I is the most potent for maintaining the differentiated SM C phenotype,
and the IGF-I–triggered signaling pathway, PI3-K/PKB-(Akt), plays a critical role in this maintenance. Here, we investigated the signaling pathways inducing SMC dedifferentiation and compared them with the PI3-K/PKB-(Akt) pathway.

It has been reported that PDGF, EGF, bFGF, or angiotensin II enhance cell proliferation or hypertrophy through the activation of the ERK signaling cascade in passaged vascular SMCs (Force and Bonventre, 1998). MAPKs such as JNK and p38MAPK are also activated in response to various cellular stresses (Derijard et al., 1994; Kyriyan and Avruch, 1996). Angiotensin II and phenylephrine, which induce acute hypertension, enhance the ERK and JNK activities in aortic, carotid and femoral arteries (Xu et al., 1996), and endothelin activates both of these kinases in proliferative airway SMCs (Shapiro et al., 1996). p38MAPK, ERMK, and/or JNK are also activated by balloon injury of carotid arteries (Hu et al., 1997; Pyles et al., 1997; Larrivee et al., 1998). These findings suggest a close association of MAPK cascades with smooth muscle disorders. However, the direct involvement of these signaling cascades in regulating the SMC phenotype has been unknown. As a first step, we used a novel culture system of gizzard SMCs and demonstrated that activations of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways are directly involved in maintaining SMC differentiation and inducing SMC de-differentiation, respectively. This conclusion is based on the following findings.

First, the signaling pathways in regulating the phenotypic determination of gizzard SMCs were distinctly different; the PI3-K/PKB(Akt) pathway played a critical role in maintaining the differentiated SMC phenotype (Figs. 2 and 5) and the ERK and p38MAPK pathways triggered by PDGF-BB, bFGF, and EGF were closely associated with SMC de-differentiation (Figs. 1 and 2). Second, among the three growth factors inducing SMC de-differentiation, PDGF-BB only triggered both the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways. When both the MAPK pathways were blocked by their specific inhibitors, PD98059 and SB203580, or when SMCs were transfected with active PI3-K or PKB(Akt), PDGF-BB in turn initiated to maintain the differentiated SMC phenotype (Figs. 3–5 and Table I). Third, even when SMCs were cultured under IGF-I–stimulated conditions, the forced activation of both the MAPK pathways by the coexpression of active MEK1 and MKK6 potently induced SMC de-differentiation (Fig. 6). Fourth, SMCs cotransfected with active MEK1 and MKK6 secreted a nondialyzable and heat-labile protein factor(s), which induced de-differentiation of surrounding normal SMCs (Figs. 7 and 8). Finally, the same signaling pathways as described above were observed to be involved in regulating the vascular SMC phenotype (Fig. 9).

Since IGF-I enhances the proliferation and migration of cultured vascular SMCs (Clemmons 1985; Bornfeldt et al., 1990; Cercek et al., 1990; Delafontaine et al., 1991; Born-
under IGF-I–stimulated conditions (Hayashi et al., 1998), notype for more than 2 wk in primary culture. Since rapamycin had no effect on the differentiated SMC phenotype under IGF-I–stimulated conditions (H ayashi et al., 1998), p70 ribosomal S6 kinase (p70S6K) is unlikely to be a down-stream target of PI3-K/PKB (Akt). Further study is required to identify the downstream targets of PI3-K/PKB (Akt) in SMCs. It has been reported that IGF-I and its downstream signaling, PI3-K/PKB (Akt), play a role in protection against programmed cell death (A lessi and Cohen 1998). We observed neither a significant decrease in cell numbers nor DNA fragmentation in our SMC cultures even under nonstimulated conditions or in the presence of IGF-I neutralizing antibodies (data not shown). Therefore, cultured SMCs might secrete anti-apoptotic factor(s) in the absence of IGF-I, and the maintenance of the differentiated SMC phenotype by IGF-I would be distinct from an anti-apoptotic action. In passaged SMCs, IGF-I enhanced the JNK and p38 MAPK activities (data not shown). These results suggest that the downstream signalings of IGF-I might be modulated during cell passage. Actually, differentiated SMCs rapidly change their phenotype under serum-stimulated conditions (K ashiwada et al., 1997; H ayashi et al., 1998). Since passaged SMCs do not represent a stable differentiated state, studies reported previously might not be able to reveal the IGF-I’s function and signaling in differentiated SMCs. In this study, we used SM Cs that showed well-characterized and stable phenotypes. We analyzed the relationship between the modulation of SM C phenotype and cell proliferation. Although serum-induced SM C de-differentiation was concomitant with cell proliferation, other growth factors that trigger SM C de-differentiation, such as PDGF-BB, bFGF, and EGF, did not significantly induce SM C proliferation (data not shown). This result also suggests that SM C de-differentiation is not essentially associated with cell prolifera-tion.

It has been reported that de-differentiated SM Cs produce and secrete PDGF which further promotes cell prolifera-tion and migration in an autocrine/paracrine manner (Sjolund et al., 1988). PDGF is also known to promote the activation of ERK and p38 MAPK in passaged SM Cs (Bornfeldt et al., 1994; Pyne and Pyne, 1997). In our culture system, PDGF-BB triggered the dual signaling pathways mediated by PI3-K/PKB (Akt) and two MAPKs, ERK and p38 MAPK. Under these culture conditions, PDGF-BB stimulation resulted in SM C de-differentiation. When the two MAPK pathways were blocked by their specific inhibitors, PD 98059 and SB 203580, PDGF-BB stimulation, in turn, initiated to maintain SM C differentiation (Figs. 3 and 4). bFGF and EGF are known to activate ERK and also to induce proliferation of SM Cs (Berrou et al., 1996; Jones et al., 1997; Y u et al., 1997; Miyamoto et al., 1998). In our culture system, both growth factors activated ERK and p38 MAPK and potently induced SMC de-differentiation (Figs. 1 and 2). However, PD 98059 and SB 203580 could not prevent such de-differentiation (data not shown). This is because the signaling pathway mediated by PI3-K/PKB (Akt) was not activated by bFGF or EGF (Fig. 2). In the present culture system, bFGF and EGF also activated JNK, but IGF-I and PDGF-BB did not (Fig. 2). It is, therefore, unlikely that JNK is involved in regulating the SMC phenotype. PDGF-BB reduced the caldesmon promoter activity and this reduction could be overcome by the forced expression of active PI3-K or PKB (Akt), or by treatment with both PD 98059 and SB 203580 (Figs. 3–5 and Table I). Further, the activation of ERK and p38 MAPK by the forced expression of both active MEK1 and MKK6 could overcome the PI3-K/ PKB (Akt) pathway triggered by IGF-I, resulting in the induction of SMC de-differentiation (Fig. 6). These data support our hypothesis that the SMC phenotype would be determined by the balance between the strengths of the signaling pathways mediated by PI3-K/PKB (Akt) and by ERK and p38 MAPK.

Curiously, even though the transfection efficiencies of both expression vectors carrying active MEK1 and MKK6 were only 25%, almost all of SM Cs came to de-differentiate as monitored by cell morphology and endogenous expression of SM C-specific molecular markers (Fig. 7). These findings indicate that the production and secretion of some de-differentiation-inducing factor(s) occur in SM Cs in which both the ERK and p38 MAPK pathways are constitutively activated. We have not yet identified such a factor(s), but the conditioned medium from these cells activated three MAPK pathways (ERK, p38 MAPK, and JNK). The activating factor(s) was a heat-labile, non-heparin-binding protein factor (Fig. 8). From these biochemical properties (Fig. 8), we excluded the possibility that PDGF-BB, bFGF, and EGF would be the main factor inducing SM C de-differentiation in the conditioned medium. Further study is required to identify such a factor(s). Anyway, this study provides a evidence that only a small portion of de-differentiated SM Cs secretes a protein factor(s) for the surrounding normal SM Cs to be de-differentiated. These findings could be helpful to understand the progression of smooth muscle disorders such as atherosclerosis.

We then applied a culture system of gizzard SM Cs to that of vascular SM Cs and investigated the signaling pathways in regulating the vascular SM C phenotype (Fig. 9). Like gizzard SM Cs, IGF-I potently maintained a differentiated phenotype of vascular SM Cs and a specific PI3-K inhibitors, LY 24002, prevented this IGF-I’s action. Treatment with two MAPK pathway inhibitors, PD 98059 and SB 203580, could rescue the PDGF-BB–induced de-differen-tiation of vascular SM Cs. Further, the conditioned medium obtained from gizzard SM Cs also induce de-differentiation of vascular SM Cs. These results strongly suggest that a culture system of gizzard SM Cs is applicable for that of vascular SM Cs and that the distinct signaling pathways mediated by PI3-K/PKB (Akt) and two MAPKs are also involved in the phenotypic determination of vascular SM Cs. Our results presented here are summarized in Fig. 10. The signaling pathway mediated by PI3-K/PKB (Akt) is...
required to maintain a differentiated phenotype of visceral and vascular SMCs, while the activation of ERK and p38MAPK leads to SM C de-differentiation of both types of SMCs. Thus, the signaling pathways in regulating the phenotypic determination are considered to be essentially the same in visceral and vascular SMCs, and the SM C phenotype would be determined by the balance between the strengths of these signaling pathways. Although visceral and vascular SM Cs originate from different precursors, our present findings provide a further insight in the common molecular mechanism of phenotypic determination of two types of SM Cs. This is because visceral and vascular SM Cs have common characteristics with respect to cell structure, function, and expression of molecular markers as follows. The main function of both types of SM Cs is contraction, which is Ca^{2+}-dependent and controlled by a myosin-linked, actin-linked dual regulation (Sobue et al., 1988, 1991). Both types of SM Cs are rich in myofibrils and are organized in a three-dimensional direction with two prominent electron dense structures such as the dense body in the cytoplasm and the dense membrane (dense plaque) in cell–cell contact. Further, contractile and cytoskeletal proteins are also specifically expressed in and serve as specific molecular markers for differentiated SM Cs. The expression patterns of these molecular markers are identical in both visceral and vascular SM Cs and their expression mechanisms, including transcription and splicing, are also regulated in common ways (Owens 1995; Sobue et al., 1998). Further studies are required to understand the detailed signaling pathways in regulating the vascular SM C phenotype and the functional linkage between such signaling pathways and SM C-specific gene regulation machineries, and to apply these findings to SM C disorders.

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