Isolation of Gene Markers of Differentiated and Proliferating Vascular Smooth Muscle Cells

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To isolate specific markers of both differentiated and proliferating vascular smooth muscle cells (VSMCs), we used the technique of differential cDNA screening using RNA from cultured rat aortic VSMCs. The tissue specificity of expression of all of the cDNAs isolated was determined by Northern analysis. We isolated seven distinct cDNAs that were more strongly expressed in freshly dispersed, differentiated, aortic VSMCs compared with dedifferentiated late-passage cells. These were the cDNAs for tropoelastin, a matrix protein; α-smooth muscle (SM) actin, γ-SM actin, calponin, and phospholamban, which are all proteins associated with the contractile function of differentiated VSMCs; SM22α, a smooth muscle–specific protein of unknown function, and CHIP28, a putative membrane channel protein that is not highly expressed in other SM tissues and may therefore be a new VSMC marker. Two cDNAs that were expressed preferentially in late-passage dedifferentiated VSMCs were also isolated. These were the cDNAs for osteopontin and matrix Gla protein (MGP). Like CHIP28, MGP was strongly expressed in aortic VSMCs but not in other types of tissues containing SM cells, suggesting that both have specific functions in vascular tissue. Osteopontin and MGP have both previously been isolated from developing bone. Their expression in proliferating VSMCs suggests that they may be involved in regulating the calcification that commonly occurs in vascular lesions. The set of cDNAs obtained extends the range of DNA probes that are available for identifying VSMCs and characterizing their phenotype in vivo by in situ hybridization. Therefore, they should aid in the analysis of gene expression during the development of vessel lesions.

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KEY WORDS • vascular smooth muscle cells • gene expression • differentiation • proliferation

Vascular smooth muscle cells (VSMCs) are usually confined to the tunica media of the vessel wall, where their contractile function serves to maintain vascular tone. However, inappropriate proliferation of VSMCs in the vessel intima is a major component of vascular diseases including atherosclerosis, vascular rejection, and restenosis following angioplasty. It has been suggested that the cells that are contractile in the vessel wall are differentiated, whereas those undergoing proliferation become dedifferentiated. This dedifferentiation is characterized by a reduction in the volume fraction of myofilaments, an increase in the proportion of synthetic organelles, and the loss of protein markers associated with the contractile phenotype, such as α-smooth muscle (SM) actin. Vascular lesions are composed of several cell types, including VSMCs, macrophages, T cells, and endothelial cells, most of which can be readily identified using specific antibodies. However, the loss of conventional markers from VSMCs complicates identification of these cells in lesions. Furthermore, there is evidence that the phenotypes of VSMCs in restenosis and atheromatous plaques are not equivalent, although both appear to share properties with neonatal cells. Indeed, there is evidence that a small percentage of cells in the normal vessel media contain neonatal markers, which has led to speculation that the cells that proliferate are these remnant neonatal-like cells rather than VSMCs that have undergone phenotypic modulation. Thus, heterogeneity of VSMCs and their involvement in particular pathologies can only be studied if markers of specific VSMC types can be isolated.

Phenotypic modulation of VSMCs has been studied extensively in cell culture, and a number of changes similar to those observed in vivo have been observed as cultured VSMCs undergo proliferation. VSMCs in vivo or freshly plated in vitro have a high volume fraction of myofilaments and few biosynthetic organelles and retain the ability to contract in response to vasoconstrictors, whereas VSMCs grown in vitro have a low volume fraction of myofibrils and a high proportion of biosynthetic organelles and may lose their ability to contract. These two phenotypes were originally defined as “contractile” (differentiated) or “synthetic” (dedifferentiated). The loss of contractility is reflected in the changes in protein content that occur when cells proliferate. SM isoforms of proteins involved in contraction, including SM myosin heavy chain (SM-MHC) isoforms SM1 and SM2, caldesmon, α-SM actin, and calponin, are found predominantly in the contractile phenotype but are progressively lost during proliferation. Similarly, other proteins not specific to SM cells, including myosin regulatory light chain and gelsolin, are downregulated. There is also evidence that proteins...
not directly involved in contraction, such as the adhesion protein integrin\textsuperscript{17} and the cytoskeletal protein vinculin,\textsuperscript{18} may be downregulated.

It is uncertain to what extent the phenotypic changes that occur in culture reflect the phenotypic modulation of VSMCs that proliferate in vivo. However, proliferative capacity and the gain and loss of protein markers is not only dependent on the method and duration of culture\textsuperscript{19} but also on the origin of the cells within the vessel wall and the age of the animal from which the culture was established.\textsuperscript{20,21} Thus, it is at least plausible that the heterogeneity observed in vivo is reflected in VSMCs in culture.

With the aim of isolating specific gene markers of both differentiated and proliferating VSMCs, we have used differential screening of a cDNA library from a well-defined rat aortic VSMC culture\textsuperscript{22} that contains cells with characteristics of both states. The screening protocols were designed to isolate genes in three categories: (1) genes present in intact aorta but not in cells that have proliferated, (2) genes repressed in cells that have proliferated and redifferentiated in culture, and (3) genes present in cells that have proliferated but have lost the ability to redifferentiate in culture.

**Materials and Methods**

**Cell Culture**

Primary cell cultures were established from thoracic and abdominal aortas excised from 12-week-old (adult) Wistar rats. The tunica adventitia and endothelium were removed by collagenase treatment, and the resultant tunica media was dispersed enzymatically.\textsuperscript{22} This enzymatic dispersion of the cells does not cause detectable dedifferentiation or proliferation.\textsuperscript{19}

Dispersed cells were plated at a density of 8x10\textsuperscript{4} cells/cm\textsuperscript{2} in Dulbecco's modified Eagle's medium and 10% fetal calf serum. After 7 days in culture, the cells (termed D7) reached confluence, and RNA was harvested 30 minutes after serum removal. Cells to be passaged were incubated in trypsin/EDTA solution for 4 to 5 minutes and then diluted 1:2 (vol/vol) before replating. These subcultured cells were given fresh medium at 48-hour intervals and split every 4 days. RNA was harvested from confluent cells 30 minutes after serum withdrawal. Passaged cells used for Northern analyses were harvested after being subcultured 4, 10, 12, 16, and 24 times (S4, S10, S12, S16, and S24). The RNA transcribed to produce the cDNA probe for differential screening was derived from S12 cells.

**RNA Isolation**

After trypsinization of cultured cells (or enzymatic dispersion of tunica media), total cytoplasmic RNA was isolated from VSMCs by lysis in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM MgCl\textsubscript{2}, and 0.5% (vol/vol) Nonidet P-40. Nuclei were pelleted, and the supernatant was made 1.5% with sodium dodecyl sulfate and extracted twice with Tris-equilibrated phenol. The RNA was precipitated with 0.1 vol sodium acetate (pH 5.2) and 2.5 vol ethanol, and the RNA pellet was resuspended in water. RNA from a variety of rat tissues was prepared as previously described.\textsuperscript{23}

**cDNA Library Construction and Differential Screening**

A cDNA library from D7 VSMC poly(A\textsuperscript{+}) RNA was constructed in λ Zap (Stratagene Inc, La Jolla, Calif) using a cDNA cloning kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Poly(A\textsuperscript{+}) RNA was isolated using streptavidin magnetic particles (Promega Corp, Madison, Wis) according to the manufacturer's instructions.

Forty thousand plaques of the library were differentially screened by hybridization to \textsuperscript{32P}cDNA generated from avian myoblastosis virus reverse-transcribed D7 RNA versus S12 RNA. Plaques hybridizing more strongly to cDNA from the D7 or the S12 cells were selected for grid replating and were rescreened using the same probes. Plaques exhibiting differential hybridization after rescreening were rescued using the λ Zap system into Bluescript SK+. Inserts were amplified using the polymerase chain reaction\textsuperscript{24} and used as probes in Northern and cross-hybridization analyses.

**Northern Analysis**

Total cytoplasmic RNA (10 μg) was electrophoresed in 1.5% agarose gels containing 2.2 M formaldehyde in a buffer containing 20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, and 0.5 μg/mL ethidium bromide. The integrity of the RNA was visualized by UV illumination of gels before and after transfer to Hybond-N (Amersham International, UK) as specified by the manufacturer. Filters were hybridized as described previously.\textsuperscript{25} \textsuperscript{32P}-labeled cDNA probes were generated from purified insert DNA using a oligolabeling kit (Pharmacia). Filters were washed twice at 65°C for 30 minutes in 0.1× standard saline citrate (1× standard saline citrate contains 150 mM NaCl and 15 mM sodium citrate)/0.1% sodium dodecyl sulfate before exposure to Fuji RX x-ray film.

**Sequence Analysis**

cDNA clones rescued into Bluescript or subcloned into M13p18 were sequenced using the dyeoxy chain-termination method with Sequenase (United States Biochemical Corp, Cleveland, Ohio). Initially, both ends of each clone were sequenced while full-length sequences were generated using random sonicated clones.\textsuperscript{26} Complete cDNA sequences were assembled using the Staden\textsuperscript{27} sequence assembly program. Protein and nucleic acid alignments were made using the FASTA algorithm.\textsuperscript{28}

**Results**

**Selection and Characterization of cDNA Clones**

The VSMC preparations and the differential screening procedures used are shown in Fig 1. When freshly dispersed aortic VSMCs are placed in culture at the appropriate density (as described in "Materials and Methods"), they lose SM-MHC and α-SM actin (ie, two standard markers of differentiated VSMCs) as they start to proliferate.\textsuperscript{22} After 7 days in culture (D7), the cells reach confluence; when serum is removed, approximately 40% of the cells reexpress SM-MHC (D.J. Grainger, C.M. Mitchell, C.M. Shanahan, J.C. McCall, and P.L. Weissberg, submitted manuscript) and
expressed in D7 VSMCs or in S12 cells, the library was differentially screened against $^{32}$P-labeled cDNAs from D7 cells and S12 cells. Northern analysis of the cDNAs isolated from this differential screen also revealed genes that were strongly expressed in the freshly dispersed aortic cells but only weakly expressed in D7 or S12 cells.

Of the 40,000 plaques screened, 300 showed greater hybridization to D7 cDNA than S12 cDNA, whereas 60 plaques showed greater hybridization to S12 than D7 (Fig 1). After one further round of screening, 120 plaques remained of the 300 that exhibited strong hybridization to D7 cell cDNA, whereas 20 of the 60 plaques hybridized strongly to S12 cDNA. The cDNA clones from these 140 remaining plaques were analyzed by hybridization to Northern blots of RNA from freshly dispersed aortic cells, D7 and S12 cells, and rat fibroblasts (Rat-2).

Northern analysis revealed five classes of expression pattern for the cDNA clones selected (numbered as in Table 1; see also Fig 1): (1) Fifty of the cDNA clones were expressed to a similar level in all of the RNAs. Two of these clones were identified by partial sequence analysis as rat insulinoma gene (RIG)$^{32}$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)$^{33}$ genes known to be expressed at similar levels in a variety of tissues and cell lines. These cDNAs and the remaining clones in this group were not analyzed further. (2) Four cDNA clones were highly expressed in RNA from freshly dispersed aortic cells but were barely detectable in the D7 or S12 cells and were not detectable in Rat-2 RNA. Sequence analysis of these partial cDNA clones and searching of the EMBL/GENBANK data bases did not reveal any homologies. Therefore, isolation of full-length cDNAs and further sequence analysis was required to identify and characterize these cDNAs in aortic cells. (3) Two clones were expressed at similar levels in freshly dispersed aortic cells, D7, and S12 cells. One cDNA clone (3RAS) was identified as calmodulin (CaM)$^{34}$ and expression was barely detectable in Rat-2 fibroblasts. The second clone (4A6) was identified as a-tropomyosin.$^{35}$ Although levels of expression did not change in VSMCs throughout culture, there was a change in transcript number with only the SM-specific transcript expressed in freshly dispersed aortic cells but additional transcripts expressed in D7, S12, and Rat-2 fibroblasts (see Fig 2). This is due to alternate splicing and has been described previously$^{36}$ (see Table 1 and Fig 2). (4) Seventy-three cDNA clones showed higher expression in freshly dispersed aortic cells and D7 cells than in S12 cells and Rat-2 fibroblasts. (5) Eleven cDNA clones were expressed more strongly in S12 cells than in freshly dispersed aortic cells, D7 cells, and Rat-2 fibroblasts.

The cDNA clones corresponding to classes 4 and 5 were analyzed further. Cross-hybridization analysis was used to resolve the clones into groups corresponding to separate cDNAs. This analysis resolved the 73 clones in class 4 into seven distinct cDNAs and the 11 cDNA clones in class 5 into two distinct cDNAs (see Table 1). Initially, the ends of each clone were sequenced, and the sequence data searched against the EMBL data base. One or more representative cDNA clones corresponding to each gene in classes 4 and 5 were sequenced, of which five represented previously sequenced rat cDNAs. Full-
### Table 1. Characterization of Clones Selected by Differential Screening

<table>
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<tr>
<th>Clone</th>
<th>N</th>
<th>Gene</th>
<th>RNA size (kb)</th>
<th>Expression pattern</th>
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<th>D7</th>
<th>LP</th>
<th>Rat-2</th>
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<td>+ +</td>
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<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
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<td>+ +</td>
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<td>+ +</td>
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<td>4</td>
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</table>

N, Number of clones isolated in the differential screen; A, freshly dispersed aortic cells; D7, cells 7 days in culture; LP, late-passage cells; Rat-2, rat fibroblasts; RIG, rat insulinoma gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ND, not determined; SM, smooth muscle; MHC, myosin heavy chain; RAMHC15, clone described in Reference 29; RAT1-12, clone described in Reference 30; VH2, clone described in Reference 31.

Expression results were derived from a number of different Northern blots loaded with RNA from at least five different sources for each SM cell type. When more than one clone of a particular cDNA was isolated, multiple clones were tested by Northern analysis. Relative RNA levels estimated from Northern blots are indicated by plus and minus symbols. The levels indicated by plus and minus symbols are not numerically quantitative but represent the relative levels of expression for each cDNA during culture and between the different cDNAs tested; e.g., α-SM actin was the most highly expressed gene in A cells, and this maximum level is indicated by + + + + + + + ++. Genes less highly expressed have fewer plus signs. Some genes such as calponin have very low levels of expression in late-passage cells (+ /−); in comparison, others such as α-SM actin and SM22α are still highly expressed in late passage cells (+++). Some genes have barely detectable expression (+ /−), and some genes have no detectable expression (−).

*CDNs that exhibited variable expression at D7, with an average pattern of expression indicated.

‡Change in transcript size for matrix Gla protein occurred in cultured vascular smooth muscle cells.

‡Clones that were not selected from the differential screen but were included as reference clones for the expression pattern of genes associated with the differentiated phenotype (i.e., class 4) in cell culture.

Length coding sequences were obtained for the remaining four cDNA clones (2RB7, 3RF10, 4A9, and 3F3 shown in Fig 3). Northern blots illustrating the expression patterns and relative expression levels of the nine cDNAs in classes 4 and 5, calmodulin, and α-tropomyosin are shown in Fig 2.

**Genes Expressed Preferentially in the Differentiated Cell Phenotype**

Seven distinct genes (class 4 in Table 1) were more highly expressed in D7 cells than S12 cells. To determine whether this expression pattern was consistent for all passaged cell and D7 cell RNA preparations, Northern analyses were performed using RNA derived from a number of different passage numbers (S4, S10, S12, S16, and S24) and additionally from S12 and S16 cells from a number of separate cultures. Furthermore, expression of each clone was tested on five separate preparations of D7 cell RNA and between two and five separate preparations of freshly dispersed aortic cell RNA. In all experiments, expression in freshly dispersed aortic cells and D7 cells was higher than in passaged cells; thus, although the cDNA in the differential screen was derived from S12 cells, the expression data are applicable to all passaged cells tested.

Expression of class 4 genes was generally higher in freshly dispersed aortic cells than in D7 cells, although some genes did exhibit variable expression in D7 cells. There was no detectable downregulation and possibly some upregulation of γ-SM actin, phospholamban, and CHIP28 RNA in D7 cells compared with freshly dispersed aortic cells. However, for phospholamban, only the smaller transcripts were upregulated when D7 cells were compared with freshly dispersed aortic cells (Fig 2). Expression in D7 cells was always higher than expression in any passaged cell RNA preparation. However, there was some variation in the levels of expression of class 4 genes in passaged cells that did not correlate with passage number. Generally, after four passages, expression had dropped to a basal low level and was maintained at this level. Variation in expression in later passages was probably associated with the state of
confluence of the culture when RNA was isolated. This was verified when an experiment was carried out to determine whether high expression of the genes in D7 cells was due to withdrawal of cells from the cell cycle or to redifferentiation.

Expression of all class 4 genes was examined in passaged cells deprived of serum for 72 hours. This has been shown previously to induce quiescence in late-passage VSMCs. A small increase in expression for some of the genes was detected when passaged cells were made quiescent, but the expression levels were not enhanced to levels equivalent to those in D7 cells. These genes are therefore expressed more strongly in primary unpassaged cells than in passaged cells, irrespective of whether the cells are in the cell cycle. Other previously defined markers of the differentiated phenotype (SM-MHC, α-SM actin, integrin, and vinculin) were also strongly expressed in the D7 cells but downregulated in passaged cells (Table 1).

Of the seven cDNAs identified, the most abundant cDNA isolated showed total homology to rat vascular α-SM actin, whereas a less abundant sequence was identified as rat enteric γ-SM actin. Only the SM-specific mRNAs of α- and γ-actin were detected in freshly dispersed aortic cells, whereas both SM-specific and nonmuscle actin mRNAs were present in D7 and passaged cells. Another abundant cDNA showed total homology to rat tropoelastin. The aortic cells expressed one major tropoelastin mRNA transcript at 3.5 kb.

Clone 2RB7 showed significant DNA homology to human, rabbit, and canine cardiac phospholamban, with the predicted protein exhibiting 100% homology to the rabbit protein. Thus, 2RB7 is a 702-nucleotide clone of rat cardiac phospholamban.

Clone 4A9 showed 76.2% homology at the DNA level to chicken α-calponin, and the predicted protein from full-length sequence analysis suggested that it was the rat equivalent of this gene, with the proteins showing 81.9% identity over a 287-amino acid overlap. However, the rat calponin protein is 297 amino acids with an Mr of 33,330 compared with the chicken protein, which is 292 amino acids with an Mr of 32,308. This difference is due to divergence in amino acid content and number at the carboxy terminus of the protein (Fig 4). To confirm that the predicted rat sequence was correct, the divergent region of the rat gene was amplified from total cDNA using polymerase chain reaction primers to conserved regions and the 3' polyadenylated region (see Fig 3), cloned, and subsequently sequenced.
FIG 3. Sequence and amino acid composition of the four clones identified by full-length coding sequence analysis. 2RF7, rat cardiac phospholamban; 3RF10, rat SM22a; 4A9, rat calponin; 3F3, rat CHIP28. Underlined sequences F1, F2, and R1 indicate forward and reverse polymerase chain reaction primers used to amplify and verify rat sequence. Underlined amino acids indicate regions of divergence from chicken protein. Polyadenylation signals are underlined. These sequence data are available from EMBL/GenBank under accession numbers X71068 for 2RF7, X71070 for 3RF10, X71071 for 4A9, and X71069 for 3F3.
Clone 3RF10, the second most abundant cDNA isolated from the library, hybridized to two bands on Northern blots: a major band at 1.1 kb and a weaker band at 1.3 kb. The 3RF10 cDNA was 1027 nucleotides in length with an open reading frame of 201 amino acids encoding a putative protein with an M, of 22,603. Analysis of the derived protein sequence of 3RF10 revealed 86% homology over a 200-amino acid overlap to the chicken SM22a protein, suggesting that 3RF10 was the rat homologue. Rat SM22a is 38% identical to rat calponin over a 144-amino acid overlap (the present study) and 41% identical to chicken a1-calponin protein (144-amino acid overlap) and shows 38% identity (192-amino acid overlap) to a Drosophila muscle protein, mp20 (Fig 5).4 Southern analysis (results not shown) suggested that a single gene coded for SM22a. The origin of the 1.3-kb transcript was unknown because screening the library failed to identify a full-length sequence.

Clone 3F3 was a 2.3-kb clone that hybridized on Northern analysis to an mRNA of approximately 3.0 kb. Fig 3 shows the amino acid composition of a single open reading frame of approximately 1200 nucleotides. If translated, the sequence encodes a putative protein of 269 amino acids with an M, of 28,843. This amino acid sequence is 93% homologous over the entire 269 amino acids to a human channel protein, CHIP28, identified from fetal liver, erythrocytes, and renal tubules.46 The sequences differ by only 19 conservative and 3 nonconservative substitutions; therefore, it is likely that 3F3 is the rat homologue of CHIP28. 3F3 also shows significant homology (64%) at the DNA level and 46% identity over a 266-amino acid overlap at the protein level (Fig 6) with the rat major intrinsic protein (MIP26) of the eye lens.47 CHIP28 and MIP26 consist of six transmembrane domains with both the N- and C-terminal regions of the protein being intracellular.48,49 The hydropathicity plot of 3F3 (not shown) suggested that it was also a transmembrane protein of similar structure. 3F3 shares significant homology with a number of other channel proteins, including the Drosophila bib (big brain) gene product,50 tobacco root-specific channel protein AtR97 (36% over 234-amino acid overlap),51 NOD26 (25% over a 179-amino acid overlap),52 and the glyceral facilitator of Escherichia coli (28% over 127-amino acid overlap).52

**Genes Expressed Preferentially in the Dedifferentiated Cell Phenotype**

Two cDNAs (class 5 on Table 1) showed greater expression in passaged cells compared with freshly dispersed aortic cells, D7 cells, and Rat-2 fibroblasts (tested on numerous cell preparations of each as described for class 4 cDNAs). These were sequenced and identified as encoding rat osteopontin53 and rat matrix Gla protein (MGP).54 The MGP RNA was detectable in freshly dispersed aortic cells at a high level. This expression was downregulated more than twofold in D7 cells but upregulated more than fivefold in passaged cells (Fig 2). However, the size of the MGP transcript was approximately 150 bp smaller in freshly dispersed aortic cells than in both D7 and passaged cells (Fig 2). The origin of the smaller aortic transcript of MGP is unknown because the larger transcript in the cultured cells corresponds to the published sequence. Therefore, MGP is expressed in both differentiated and dedifferentiated cells, but there is a change in transcript size and upregulation of the larger transcript associated with

**Fig 4. Comparison of rat and chicken amino acid sequences for the protein calponin.** The proteins share 81.9% identity in a 287-amino acid overlap. Divergence occurs at the carboxy terminus with the rat protein containing five additional amino acids and with very little homology over the last 20 amino acids (underlined).

**Fig 5. Comparison of amino acid sequences of rat and chicken SM22a, Drosophila mp20, and chicken and rat calponin.** EF hands in mp20 are underlined. Homologous regions are boxed.
proliferation in culture. Osteopontin was barely detectable in freshly dispersed cells by Northern analysis but was highly expressed in both D7 and passage cultured cells. Osteopontin expression was only slightly higher in passaged cells than in D7 cells (Fig 2).

To determine whether the expression of osteopontin and MGP mRNA was high because the cells were proliferating, passaged cells were made quiescent by removal of serum. The levels of both osteopontin and MGP did not change significantly (results not shown), suggesting that the high expression of these two genes occurs in VSMCs that have undergone proliferation but does not depend on the cells being in the cell cycle.

Tissue Specificity of Gene Expression

The expression in adult rat tissues of each distinct cDNA identified by differential screening was compared by Northern analysis (shown in Table 2 and Fig 7). It can be seen from Table 2 that, with the exception of phospholamban and γ-SM actin, all of the cDNAs isolated showed their highest level of expression in the aorta. The RNA preparations from most tissues in which SM cells are found will be derived from a variety of cell types. However, α-SM actin, SM22α, and calponin were highly specific to SM, with high expression in aorta, bladder, vas deferens, and uterus. Two other genes, tropoelastin and γ-SM actin, were found only in tissues with some SM content. In contrast, phospholamban is the major phosphorylated protein in heart sarcoplasmic reticulum, and expression was at least 10-fold higher in heart than in aorta. Phospholamban has previously been isolated from pig stomach SM, although expression in other rat SM tissues was too low to be detected.

CHIP28 expression was highest in the aorta, with lung, kidney, and heart showing some expression (Fig 7). However, expression was not detected in other SM tissues. MGP was detected in aorta, kidney, heart, and

![Table 2. Tissue-Specific Expression Patterns in Adult Rats](http://circres.ahajournals.org/)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SM tissues</th>
<th>Other muscle</th>
<th>Non-SM tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SM actin*</td>
<td>A, Vas, St, U, SmI</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>γ-SM actin*</td>
<td>St, Lgl, SmI, A, U</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>SM22α</td>
<td>A, Vas, Bl, U, Lgl, SmI, Ov, Lu, St</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Calponin</td>
<td>A, Bl, U, Lgl, Ov, Ov</td>
<td>H</td>
<td>...</td>
</tr>
<tr>
<td>Tropoelastin</td>
<td>A, Lu, Ov, Vas, U</td>
<td>H</td>
<td>...</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>A</td>
<td>H</td>
<td>...</td>
</tr>
<tr>
<td>CHIP28</td>
<td>A, Lu, Bl†</td>
<td>H, SM†</td>
<td>K</td>
</tr>
<tr>
<td>Osteopontin‡</td>
<td>A, St, U</td>
<td>H</td>
<td>K</td>
</tr>
<tr>
<td>Matrix GlA</td>
<td>A, Ov, Lu, Vas</td>
<td>H</td>
<td>K, Bl†</td>
</tr>
</tbody>
</table>

SM, smooth muscle; A, aorta; Vas, vas deferens; St, stomach; U, uterus; SmI, small intestine; Lgl, large intestine; Bl, bladder; Ov, ovary; Lu, lung; H, heart; Sm, skeletal muscle; K, kidney; B, brain. Other tissues tested in which no expression was detected included liver, thymus, and spleen.

Tissues are listed in each group according to level of expression, with the highest expressing tissue first. The tissue exhibiting the highest expression is shown in bold.

*Data are from the present study and Reference 40. Expression in bladder was not tested.
†Expression was barely detectable in these tissues.
‡Data are from the present study and Reference 55.
l lung with aortic expression higher by at least twofold than that in kidney. Like CHIP28, MGP was low or undetectable in vas deferens, bladder, and uterus, all tissues with high SM content and showing a similar level of expression to aorta of SM-MHC, α-SM actin, SM22α, and calponin. This suggests that both MGP and CHIP28 have specific functions in VSMCs. Osteopontin was expressed at a low level in several tissues, with the highest level of expression in the aorta. The same expression pattern for osteopontin has been previously reported.55

Discussion

Differential screening has enabled us to isolate cDNAs from rat aortic VSMCs whose expression is modulated in culture and whose tissue specificity is shown by Northern analysis. Some of these cDNAs (MGP and CHIP28) are new markers of VSMCs in that they have not previously been shown to be expressed in VSMCs. Furthermore, they are not highly expressed in other SM tissues. Other markers (SM22α and calponin) have previously been characterized only as proteins and can now be used as DNA probes. Some of the cDNAs isolated are specific for SM cells (α- and γ-actin, SM-22α, and calponin). These four genes, together with phospholamban, tropoelastin, and CHIP28, were expressed preferentially in differentiated VSMCs, whereas osteopontin and MGP were expressed strongly in dedifferentiated VSMCs. The set of cDNAs obtained extends the range of DNA probes that are available for identifying VSMCs and characterizing their phenotype in vivo by in situ hybridization.

Properties of the cDNAs Isolated

The protein products of several of the genes identified as expressed in VSMCs are associated with the contractile function of the cells. They include those that have previously been described as SM-specific (α-SM actin and γ-SM actin) and that form part of the muscle filaments. Other previously characterized proteins include phospholamban, a membrane protein of the sarcoplasmic reticulum, which plays a role in calcium regulation and hence contraction, and elastin, which is a major component of the extracellular matrix of elastic tissue. The α-calponin isolated was of interest in that it had a highly modified carboxy terminus compared with previously characterized chicken α-calponin. The functional role and cellular location of SM22α is not known, nor is the significance of the sequence homologies between SM22α, calponin, and mp20 (see Fig 6). SM22α and mp20 are easily extracted from the contractile apparatus and are not therefore tightly bound components, whereas calponin is closely associated with troponin T, actin, and caldesmon.43 The molecular mechanisms regulating SM contraction have not been fully elucidated, but they differ greatly from those of skeletal muscle, and it is likely that both SM22α and calponin have specific roles in SM function. The Drosophila protein mp20 is found specifically in the syncytial muscles and is absent from the nonsynchronous oscillatory flight muscles. The protein has two potential calcium binding sites (EF hand sequences), one of which is reasonably conserved in SM22α. The calcium binding capacity of mp20 has lead to the suggestion that it is involved in excitation-contraction coupling, which involves different mechanisms in synchronous and asynchronous muscle.

Two genes, MGP and CHIP28, were expressed at high levels in aortic SM cells but were either not detectable or were expressed at a very low level in other SM tissues. This suggests that expression of these genes may have a specific role in VSMC function. CHIP28 is a membrane channel protein that probably allows bidirectional passage of small molecules and may not be involved in the contraction mechanism of VSMCs. It was initially isolated from human erythrocytes and later found to be expressed in neonatal liver and kidney tubules. Its sequence homology to channel proteins in plants and the tissue specificity suggested that it was a water channel.46 CHIP28 shares sequence homology with the Drosophila gene bib. In flies carrying a mutant bib gene, loss of channel function results in incorrect differentiation of a subset of cells in the brain. This is thought to be due to failure of small messenger molecules to pass between different cell layers in the embryo. Consequently, ectodermal cells fail to develop into epidermobilasts and follow a default pathway to neuroblast development.48 This evidence and the expression of CHIP28 in a wide variety of tissues during fetal and neonatal development (Reference 46; C.M. Shanahan, unpublished data) suggest that it may have a more complex role than that of a water channel. Furthermore, its continued high expression in adult aorta compared with other tissues containing smooth muscle suggests that it may have a specialized function in intercellular communication specific to VSMCs.

MGP is a small, insoluble, vitamin K-dependent protein containing five residues of Ca2+ binding γ-carboxyglutamic acid. Treatment of rats with the vitamin K inhibitor warfarin results in excessive mineralization of bone and cartilage, suggesting that MGP plays a role as an inhibitor of mineralization.57 Immunohistochemistry
has demonstrated the presence of MGP within the nucleus, the cytoplasm, and the pericellular matrix of bone tissue in monkeys, and electron microscope studies have shown that MGP in the matrix is associated with vesicles. The exact origin of these vesicles is unknown, although they may represent cell or lipid debris from dying cells. In pathological conditions (e.g., atheroma), mineralization is often associated with cellular lipid debris.

Two genes, osteopontin and MGP, were highly expressed in the VSMCs that had proliferated. Both proteins were originally isolated from fetal bone, and both genes are highly expressed during mineralization. Subsequently, the proteins have been detected in several soft tissues, suggesting that both may have roles in tissues that do not normally undergo mineralization.

Osteopontin is an Arg-Gly-Asp-Ser-containing protein that binds calcium; it is also phosphorylated and rich in sialic acid. It has fibronectin, vitronectin, and thrombospondin binding capacity and a high affinity for hydroxypatite. Expression of osteopontin has previously been reported in VSMCs, and consistent with the present study, expression was higher in cultured cells than in intact aorta or freshly dispersed aortic cells. There was an increase in osteopontin expression in the neonatal cells of rat carotid arteries injured by balloon catheter.

It is interesting to speculate that osteopontin and MGP may have a role in regulating the calcification, which can occur rapidly in vascular lesions. Osteopontin may cause accumulation of calcium, whereas MGP expression may regulate the extent of calcification. Both osteopontin and MGP are regulated by 1,25-dihydroxyvitamin D$_3$ in bone, consistent with involvement of the genes in calcification. VSMCs are known to express 1,25-dihydroxyvitamin D$_3$ receptors and to respond to 1,25-dihydroxyvitamin D$_3$ in vitro and in vivo in a variety of ways. It is a growth factor in VSMC culture, and it can delay the loss of contractility in cultures of whole vessels. Furthermore, the administration of high doses of vitamin D$_3$ has been shown to cause calcinosis or atheroma in dogs, rabbits, and pigs. In situ hybridization of diseased vessels with osteopontin and MGP should reveal the pattern of expression of these genes in the disease process.

**Phenotypic Modulation of VSMCs in Culture**

This study of changes of gene expression in VSMCs in culture has provided insight into the processes of dedifferentiation that occur during proliferation of the cells. The genes highly expressed in dispersed aortic cells and D7 cells (all 4 genes in Table 1) are downregulated in passaged cells. However, this process of dedifferentiation is not complete in D7 cells. The upregulation of osteopontin, the change in transcript size of MGP, and the presence of both SM and nonmuscle actin and o-tropomyosin transcripts at D7 compared with freshly dispersed aortic cells all suggest that D7 cells have properties characteristic of both the differentiated and proliferating phenotype. Furthermore, the markers of the differentiated phenotype are still detectable at a low level in S12 cells. However, the analysis of phenotypic modulation in culture is complicated by the heterogeneity of the rat aortic VSMCs. At stationary phase, only approximately 40% of the D7 cells stain positively for SM-MHC or SM-α-actin proteins, and the shift in pattern of gene expression may reflect this heterogeneity.

Preliminary experiments have been performed to examine the expression in vivo of some of the cDNAs isolated. For example, in situ hybridization analysis of balloon-injured rat carotid arteries suggests that dividing intimal cells present 7 days after injury express high levels of both osteopontin and MGP RNA. In contrast, osteopontin in only weakly expressed in the media of intact rat aorta and carotid arteries (C. Shanahan, unpublished data). These preliminary data suggest that the set of genes now available for in situ hybridization studies will enable heterogeneity of VSMCs in culture to be defined and compared with the phenotypes of VSMCs in intact tissues and lesions to determine (1) the specific pattern of gene expression during lesion formation and (2) whether specific subpopulations of VSMCs contribute to lesion formation.

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