Gene Expression Programs of Human Smooth Muscle Cells: Tissue-Specific Differentiation and Prognostic Significance in Breast Cancers

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Smooth muscle is present in a wide variety of anatomical locations, such as blood vessels, various visceral organs, and hair follicles. Contraction of smooth muscle is central to functions as diverse as peristalsis, urination, respiration, and the maintenance of vascular tone. Despite the varied physiological roles of smooth muscle cells (SMCs), we possess only a limited knowledge of the heterogeneity underlying their functional and anatomic specializations. As a step toward understanding the intrinsic differences between SMCs from different anatomical locations, we used DNA microarrays to profile global gene expression patterns in 36 SMC samples from various tissues after propagation under defined conditions in cell culture. Significant variations were found between the cells isolated from blood vessels, bronchi, and visceral organs. Furthermore, pervasive differences were noted within the visceral organ subgroups that appear to reflect the distinct molecular pathways essential for organogenesis as well as those involved in organ-specific contractile and physiological properties. Finally, we sought to understand how this diversity may contribute to SMC-involving pathology. We found that a gene expression signature of the responses of vascular SMCs to serum exposure is associated with a significantly poorer prognosis in human cancers, potentially linking vascular injury response to tumor progression.

Introduction

Smooth muscle (SM) is a morphologically distinct tissue that mediates the contraction of hollow organs in the circulatory, respiratory, gastrointestinal, and urogenital systems. Beyond the viscera, SM is also present in a variety of anatomical locations, such as the hair follicles, irises, and lacrimal ducts. Smooth muscle cells (SMCs) are the main cell type in SM tissue and have a distinct "smooth" appearance because their sarcomeres, the units of contraction and force generation, are arranged with no specific banding pattern. SMCs share many lineage-specific markers, such as smooth muscle α-actin (SM-α-actin), SM myosin heavy chain (SM-MHC), SM22α, calponin, and caldesmon [1].

The differentiation of SMCs is marked by the expression of SMC-specific lineage markers and the acquisition of the contractile function. The discovery of common *cis*-acting elements in the promoter regions of these lineage markers has provided some insights into how SMC differentiation is initiated and maintained (reviewed in [2,3]). The promoters of many SMC-specific genes contain CArG [$CC(A/T)_6GG$] boxes or CArG box–like sequences; expression of these SMC-specific genes is triggered by cooperative binding of the ubiquitous serum responsive factor (SRF) and the SMC-specific coactivator myocardin to CArG sequences [2–4].

Other transcription factors, such as MEF2B, P311 (also known as C5orf13), MRF2 (also known as ARID5B), and GATA4 also possess the ability to trigger SMC differentiation programs when ectopically expressed [2,5–9], but their relative contributions to the process of SMC differentiation, in comparison to SRF/myocardin, are not known. Also unclear are the temporal and spatial patterns in which these transcription

Abbreviations: BMP4, bone morphogenic protein 4; ECM, extracellular matrix; FDR, false discovery rate; HGF, hepatocyte growth factor; LIF, leukemia inhibitory factor; MITF, microphthalmia-associated transcription factor; NKI, Netherlands Cancer Institute; OSM, oncostatin M; RT-PCR, reverse transcriptase PCR; SAM, Significance Analysis of Microarrays; SM, smooth muscle; SM-α-actin, smooth muscle α-actin; SMC, smooth muscle cell; SMD, Stanford Microarray Database; SRF, serum responsive factor; TGF-β, transforming growth factor-β; VCAM1, vascular cell adhesion molecule-1 (VCAM1)

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Author Summary

It has been estimated that the human body contains approximately 200–400 distinct cell types. These estimates are largely based on the morphological characteristics of cells and have yielded, among many others, the category of smooth muscle cells, which have a distinct appearance and are present in a wide variety of tissues. By using DNA microarrays to interrogate the gene expression of anatomically varying smooth muscle cells, we were able to accurately tease apart many of the distinct cell subtypes that are classically categorized as smooth muscle cells. Remarkably, genes expressed by these newly identified, distinct subtypes corroborate many of their known biological properties and give clues about their susceptibility to specific disease states, retained developmental programs, and potential drugable targets. Additionally, from a smooth muscle cell model of vascular injury, we were able to extract a gene expression signature that provides prognostic information for human breast cancers. Of particular interest for modeling tumor progression was the finding that this gene expression signature was associated with tumor hypoxia. This study adds much to our ever-growing depth of understanding of cellular diversity and the contributions of this diversity to normal physiology and disease.

factors act to initiate or maintain SMC differentiation programs.

Although all SMCs share many morphological and molecular features, they carry out distinct functions in different organs and tissues and are, therefore, likely to vary significantly in their contractile and mechanical properties, hormonal control, physiological regulation, and pathological alterations. For example, arterial SMCs must maintain proper vascular tone to ensure adequate tissue perfusion in response to rapid fluctuations in blood volume, pressure, and tissue oxygen demands, as well as hormonal and nervous inputs [10]. Gastrointestinal tract SMCs, in contrast, participate in the periodic peristalsis that facilitates food passage and, therefore, operate at an autonomous and slower pace [10]. These functional differences imply that significant variations may exist in the epigenetic programs of different SMCs. Indeed, several studies have found heterogeneity among SMCs in the visceral organs [11,12], within the blood vessel wall, and in atherosclerotic lesions of the vascular wall [13–15]. But our knowledge about the nature, extent, and molecular details of these differences is limited.

Unlike skeletal and cardiac muscle cells, which are terminally differentiated, mature SMCs retain their ability to undergo large-scale, reversible phenotypic modulations in response to various genetic and environmental influences [16,17]. For example, the transition of vascular SMCs from the physiological quiescent (contractile) to the pathological activated (synthetic) state is associated with vascular injuries, which cause migration of SMCs into the intima, where they proliferate and produce matrix proteins [16,17]. This phenotypic plasticity plays an important role in the pathogenesis of human diseases, including atherosclerosis, hypertension, asthma, and human cancers.

SMCs are commonly found in cancers as components of blood vessel walls. Blood vessels in tumors are often abnormal—they can be greatly enlarged, tortuous, and "leaky"—and the component SMCs often have abnormal morphology and sometimes fail to express the appropriate SMC differentiation marker genes [18]. These structural abnormalities contribute to the spatial and temporal heterogeneity in tumor blood flow, resulting in elevation of tumor interstitial pressure, hypoxia, and acidosis [19,20]. These hostile tumor microenvironments play a major role in tumor progression and treatment failure, but it has been challenging to quantify and dissect these factors [19].

In this study, we investigated molecular details of SMC heterogeneity by systemically examining the global expression profiles of purified, cultured SMCs isolated from various organs and anatomical structures. We found pervasive differences among SMCs from different organs and tissues. An investigation of the gene expression program induced in vascular SMCs by serum exposure, an ex vivo model of vascular injury, identified a signature that proved to be significantly associated with prognosis in various human cancers. This observation suggests a possible link between blood vessel injury and tumor progression and treatment response in cancer patients.

Materials and Methods

Cell Culture

SMCs isolated from bronchus, uterus, cervix, different blood vessels, urinary bladder, ureters, urethra, and pulmonary artery were obtained from Cambrex. The cells were thawed and propagated in SmGM-2 media (Cambrex), according to the manufacturer's instructions, and cultured under standard conditions (5% FCS with fibroblast growth factors, steroids, and epidermal growth factors) or low-growth conditions (1% FCS with no growth factors). Once the cells reached 60%–70% confluency, mRNA was harvested using the FastTrack mRNA Isolation Kit (Invitrogen). The cells were harvested between the third and fifth passages, after approximately 10–15 generations in culture. We confirmed that each cultured cell population consisted of SMCs, free of epithelial, endothelial, or Schwann cells, by immunofluorescent staining, using antibodies against cytokeratins (C-11, Sigma), desmin (Ab-1, NeoMarkers), glial fibrillary acid protein (ab-7, NeoMarkers), vimentin (V9, Sigma), and CD31 (Ab-2, Neomarkers or Pharmingen).

cDNA Microarray Procedure and Data Analysis

Human cDNA microarray production (from Stanford Functional Genomic Facility) and hybridization were performed as previously described [21]. mRNA was purified using the FastTrack mRNA Isolation Kit (Invitrogen), according to the manufacturer's instructions. Human common RNA reference (Strategene) was used in all experiments as the standard reference. Two micrograms of all RNA samples were fluorescently labeled with amine-reactive dyes after reverse transcription. SMC samples were labeled with Cy5, and common reference samples were labeled with Cy3. The Cy5- and Cy3-labeled samples were mixed together and heated at 95 °C for 3 min before hybridizing with printed cDNA microarrays for 12–16 h in a 65 °C water bath in sealed cassettes. Following hybridization, microarrays were washed and dried prior to high-resolution scan on a GenePix 4000B Array Scanner (Axon). Each element was located and analyzed using the GenePix Pro 5.0 software package (Axon). These data were submitted to the Stanford Microarray Database (SMD) for further analysis. Data were normalized globally per array, such that the average LogRatio was 0 after

normalization. Hierarchical clustering with weighted average linkage clustering was performed after indicated data filtering based on spot quality and variations in signal intensity as described [22].

Results

Genome-Wide Analysis of Smooth Muscle Cell Heterogeneity

We established 39 primary SMC cultures from 18 different anatomic locations under identical culture conditions (see Materials and Methods). The 36 primary SMCs included cells purified from five different arteries (aorta, coronary artery, pulmonary artery, iliac artery, and umbilical artery) and four different veins (hepatic, renal, saphenous, and popliteal veins) as well as SMCs isolated from the bronchus, gastrointestinal tract (colon), female genital tract (uterus and cervix), and urinary tract (urinary bladder, ureter, and urethra) (detailed information on all SMC samples is available on the supplemental Web site). All SMCs displayed a uniform spindle shape in culture and were positive for SMC-α-actin. All SMC samples were also stained for endothelial cell and epithelial cell markers (CD31 and pan-keratins, respectively) to rule out contamination from other cell lineages. Three SMC samples (one from bronchus, one from colon, and one from saphenous vein) were excluded from further analyses due to significant contamination (>10%) from other cell types. To investigate how the gene expression patterns in diverse SMCs respond to a common physiological stimulus, 24 of the 36 remaining SMC samples were also cultured in low-growth media (basal media [SMBM] supplemented with only 1% serum and no additional growth factors).

The global expression patterns of all 60 samples (36 SMCs grown in standard-growth and 24 SMCs grown in low-growth conditions) were analyzed using cDNA microarrays, containing approximately 42,000 elements, representing 27,291 unique Unigene clusters (Build number 173, released on 28 July 2004) to generate a total of 2.6 million gene expression measurements; these measurements cover a significant portion of known genes. The expression data were submitted to the SMD and globally normalized so that the average LogRatio was 0 after normalization [23]. We further analyzed the 16,352 gene elements that were expressed in at least 80% of the SMC samples. These elements were identified by looking for elements with Cy5 signal more than 2.5-fold above local background. Unsupervised hierarchical clustering of the gene expression patterns of all 60 samples produced consistent groupings of most SMCs according to their sites of origin (Figure 1A), suggesting that SMCs from different anatomic locations have distinct expression patterns that persist with serial passage in vitro. The 60 SMC samples were clustered into two large, distinct branches: a "vascular" branch contained all the cells isolated from blood vessels and airways, and a "visceral" branch contained all the cells isolated from visceral organs (colon, urinary tract, uterus, and cervix) (Figure 1A). Within the visceral branch, there were three distinct subbranches; one containing all the SMCs from the urinary tract (including ureter, urinary bladder, and urethra), one with female reproductive tract SMCs (uterus and cervix), and one subbranch of colon SMCs (Figure 1A). This grouping pattern is not related to the age of the donor since there was no statistically significant difference in the

donor ages between the "vascular" and "visceral" SMC groups (Table S2). To rule out the contribution of gender in sample clustering, we performed an unsupervised analysis on the 24 SMC samples from female donors (under high-growth conditions) and were able to generate a similar grouping pattern (detailed in the Web supplement). Interestingly, culturing SMCs in low-growth conditions had only a relatively modest effect on the clustering pattern (Figure 1A). The clustering of all SMC samples into either the vascular or visceral SMC groups was driven mainly by two large clusters of genes, to which we will refer as the vascular and visceral gene clusters (Figure 1B).

The *HOX* gene family encodes a family of evolutionarily conserved transcription factors known to be involved in determining positional identity and tissue specialization in animals [24]. Previously, we found that fibroblasts could be clustered by anatomic location based on their patterns of expression of a small number of *HOX* genes [25]. To assess the possible role of *HOX* genes in SMC topographic differentiation, we identified 63 genes encoding homeodomain transcription factors that had well-measured expression in these experiments. Hierarchical clustering of the cultured SMCs based solely on their patterns of expression of these 63 homeodomain genes recapitulated grouping of the SMCs according to their site of origin (Figure 1C, the dendrogram labeled as "all" is identical to the dendrogram from Figure 1B); the vascular and visceral groupings were also retained. This homeodomain gene set also successfully separated the visceral SMCs into the three previously defined subbranches: urinary tract, female reproductive tract, and colon, suggesting that this family of transcription factors may play an important role in specifying the distinct developmental programs of the SMCs.

Genes Expressed Preferentially in Vascular and Bronchial Smooth Muscle

To determine the differences in molecular features between vascular and visceral SMCs, we used a supervised method, Significance Analysis of Microarrays (SAM) [26], to identify 3,276 unique genes (represented by 4,870 array elements), whose expression varied consistently between the 31 vascular SMCs and the 29 visceral SMCs, with a false discovery rate (FDR) of less than 0.001%. All SMC samples were then arranged by hierarchical clustering, based on expression of these 3,276 unique genes (Figure 2A), to yield a clustering pattern almost identical to the unsupervised sample groupings (Figure 1A).

Among genes preferentially expressed in vascular SMCs were many that encode proteins in the transforming growth factor-β (TGF-β) pathway, which affects differentiation, proliferation, migration, and the induction of extracellular matrix (ECM) production, as well as genes responsible for ECM biosynthesis and modification [27–30]. The high expression of these genes in the vascular SMCs contributes to their ability to maintain the tensile strength of blood vessels through the synthesis and deposition of connective tissue proteins [31]. Vascular SMCs also expressed many genes involved in inflammatory responses, suggesting an intrinsic ability of vascular SMCs to communicate with inflammatory cells to initiate and modulate the chronic inflammatory and fibroproliferative processes underlying atherosclerosis and other vascular diseases [32].
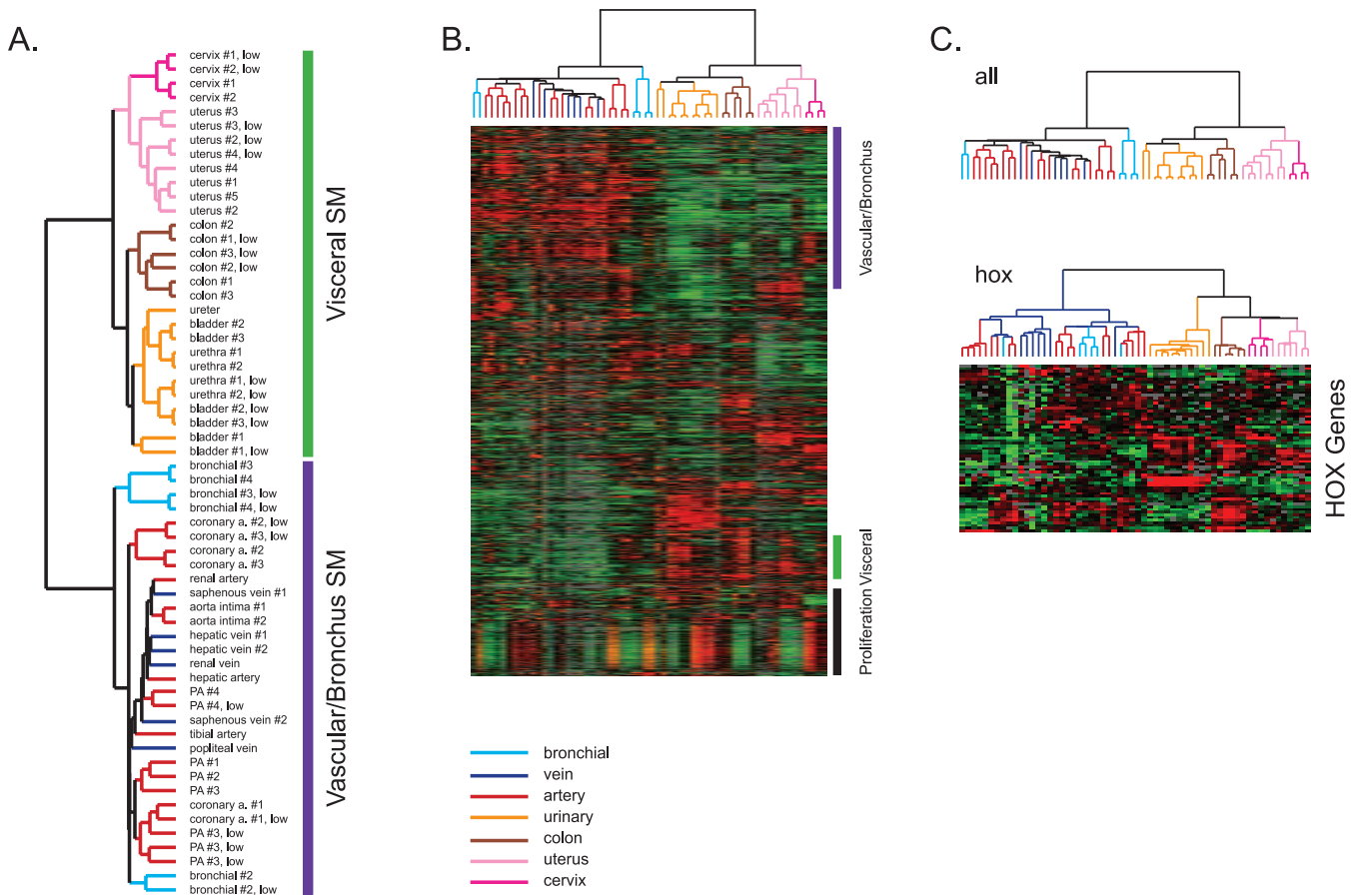
Figure 1. Diversity of SMC Gene Expression Patterns

(A) Gene expression patterns of cultured SMCs organized by unsupervised hierarchical clustering. The global gene expression patterns of 60 cultured SMCs were sorted based on similarity by hierarchical clustering. Approximately 6,166 gene elements (representing 5,236 distinct genes) were selected from the total dataset, based on variations in expression relative to the mean expression level across all samples greater than 3-fold in at least two cell samples. The anatomic origins of each SMC culture are indicated and color coded. The apparent order in the grouping of SMC gene expression patterns is indicated in this dendrogram.

(B) Overview of gene expression patterns of all SMC samples. The variations in gene expression described in Figure1A are shown in matrix format. The scale extends from 0.25- to 4-fold variation from the mean across all samples (-2 to $+2$ in log2 scale). Gray represents missing data. The gene clusters characteristic of vascular/bronchial, visceral SMCs, and proliferation are indicated on the right. Complete data can be found on the supplementary Web site and SMD.

(C) Gene expression patterns of cultured SMCs organized by unsupervised hierarchical clustering based on expression of 63 homeodomain genes. The dendrogram generated by 63 hox genes (labeled as "hox") is compared with the dendrogram generated by all genes (labeled as "all").

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Vascular SMCs also expressed high levels of transcripts encoding proteins known to be involved in reciprocal signaling with adjacent endothelial cells and in the eicosanoid/prostaglandin signaling pathways that regulate vascular tone (Figure 2B, gene names shown in purple). A more extensive discussion of these genes appears in the supplemental text in the supplemental Web site.

To further search for systematic differences in molecular pathways between the two broad divisions of SMCs (vascular and visceral), we employed a gene-set enrichment analysis [33] that evaluated differential expression of predefined sets of functionally related genes within our dataset. A running statistic (the Kolmogorov-Smirnof or KS statistic) determines how highly the coordinate expression of each gene set ranks. This statistical tool provides a systematic approach to objectively identify gene sets with functional themes that can be correlated with biological phenotypes. We tested 410 gene sets (compiled from previously published expression

studies of common cellular pathways and pathological states and curated by Biocarta [34] and KEGG [35]) for their enrichment in vascular or visceral SMCs. Gene sets that achieved enrichment greater than expected by chance alone were identified by permuting the vascular and visceral SMC sample labels 1,000 times. Of the ten pathway-specific gene sets with the highest normalized enrichment scores (the normalized enrichment scores for all 410 pathways are detailed in the web supplement), eight were enriched in vascular SMCs and two were enriched in visceral SMCs (Figure 2C). The gene sets showing enrichment in vascular SMCs were associated with inflammation, tumor necrosis factor (TNF), TGF-β, interleukin 1 receptor (IL1R), and chemokine receptor pathways. Interestingly, genes of the HIF pathway [such as *VEGFA*, endothelin-1 (*EDN1*), lactate dehydrogenase A (*LDHA*), and *HIF1A*] were also enriched among genes differentially expressed in vascular SMCs. To test whether an expression signature of the hypoxia response
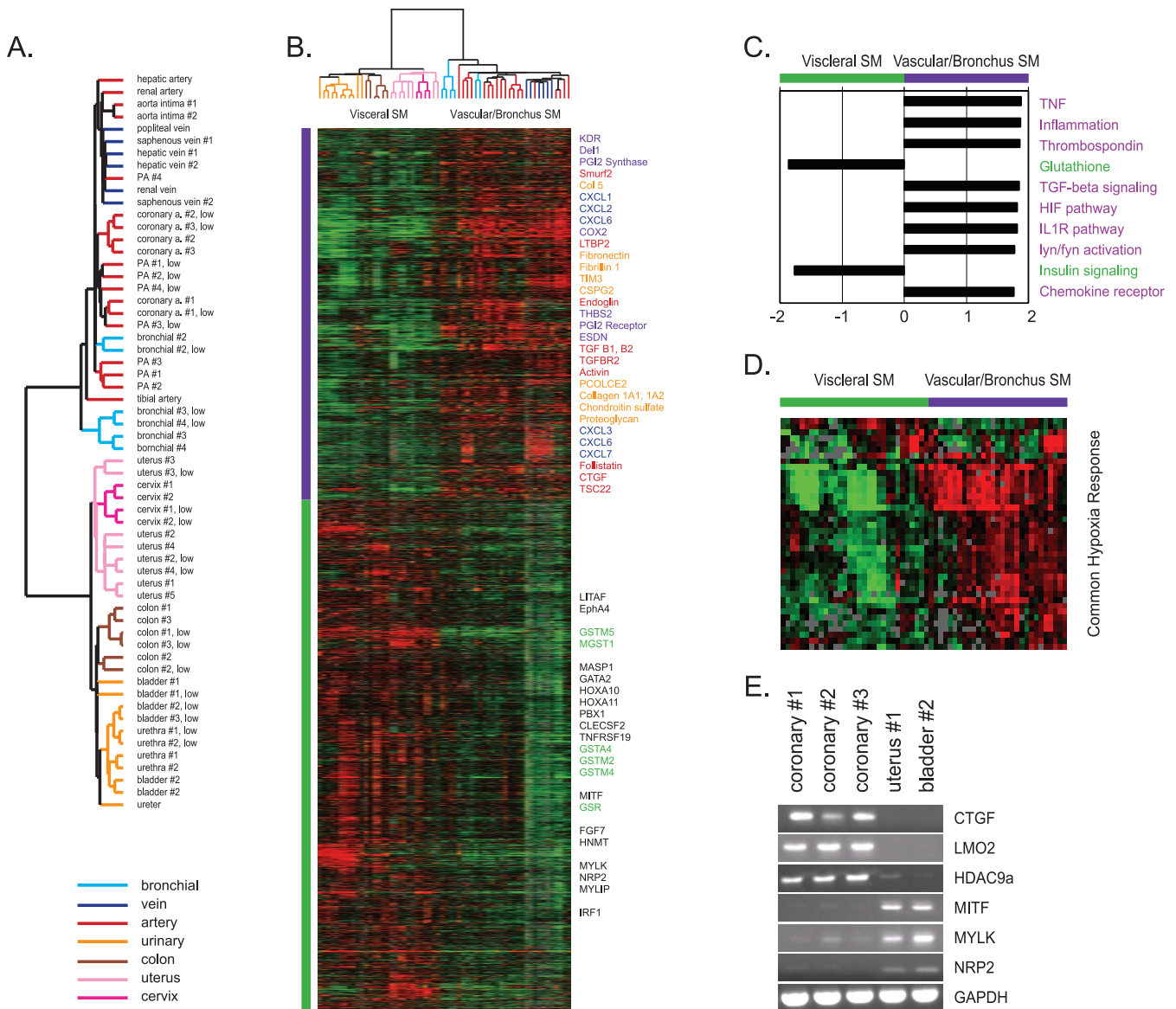
Figure 2. Vascular and Visceral SMC Gene Expression Programs

(A) Identification of vascular and visceral SMC gene expression programs. Dendrogram representing the result of hierarchical clustering of SMCs, based on the similarities in their pattern of expression of the genes selected by a two class SAM test.

(B) Features of vascular and visceral SMC gene expression programs. The expression of 2,338 vascular SMC-specific genes and 2,462 visceral SMC-specific genes selected by SAM analysis is shown here. Genes involved in TGF-β signaling (red), ECM components and biosynthesis (orange), inflammatory response (blue), and endothelial cell interaction (purple) are labeled by the indicated colors. Complete data can be found on the supplementary Web site and SMD.

(C) Comparison of pathway activity in vascular and visceral SMCs by Gene Set Enrichment Analysis. The top ten molecular pathways ranked by normalized enrichment scores enriched specifically in vascular (purple, positive) or visceral (green, negative) SMC samples are shown.

(D) The expression of a common hypoxia gene signature among all SMCs. The expression values of 71 genes constituting the common hypoxia response in the vascular versus visceral SMCs are shown.

(E) Confirmation of specific expression of CTGF, LMO2, and HDAC9 in vascular SMCs; of specific expression of MITF, MYLK, and NRP2 in visceral SMCs; and of control gene GAPDH in all SMCs with RT-PCR.

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was indeed overrepresented in vascular SMCs, we used a previously obtained hypoxia-response gene signature [36] to analyze the SMC expression dataset. When all 60 SMC samples were arranged by hierarchical clustering based on the expression levels of all 71 genes in the common hypoxia-response gene list [36], most vascular SMCs clustered separately from visceral SMCs as a result of their relatively high expression of *HIF1A* and the common hypoxia-response

genes (Figure 2D). This concordant expression of *HIF1A* and the other hypoxia-response genes was also observed in renal proximal tubule epithelial cells and ovarian cancers, and high *HIF1A* transcript levels were associated with a vigorous hypoxia response [36]. Interestingly, hypoxia of the blood vessel wall has been linked to the development of athero-sclerosis [37,38], so the differential expression of this hypoxia-response gene set and its influence on the transcriptional

program under low oxygen tension may have implications for the pathogenesis of atherosclerosis.

Genes Expressed Preferentially in All Visceral Smooth Muscle

All SMCs isolated from visceral organs share features of the gene expression programs that distinguish them from vascular SMCs (Figure 2B, gene names shown in black), notably including several transcription factors involved in the establishment and maintenance of SMC differentiation (MRF2 (also known as ARID5B) [9], PBX1, HoxA10, and HoxA11 [6,39,40]). Disruption of the PBX1 gene in mice leads to a general hypotrophy of many visceral organs, yet no vascular abnormalities are noted [40], in accordance with its visceral SMC-specific expression. Visceral SMCs also preferentially expressed microphthalmia-associated transcription factor (MITF). In addition to its expression in melanocytes and mast cells [41], MITF has been shown to be present in uterus and other tissues [42]. MITF can inhibit the activities of transforming growth factors (TGF-β) by binding to Smad3, a key signaling component of the TGF-β pathways [43]. High levels of MITF may play a role in modulating the activity of the TGF-β pathway in visceral SMCs (Figure 2B), thereby promoting a functional partitioning of visceral from vascular SMCs.

Visceral SMCs also preferentially expressed Histamine N-methyltransferase (HNMT), an essential enzyme that regulates histamine levels in tissues by catalyzing its inactivation by N-methylation [44] (Figure 2B). Histamine is a strong agonist of smooth muscle contraction [45] with an important role in maintenance of contractile tone. The importance of HNMT in regulating histamine signaling is illustrated by the increased incidence of bronchial asthma that is seen in patients with certain HNMT polymorphisms [46]. Preferential expression of HNMT in all visceral SMCs suggests a strong, intrinsic ability of these cells to catalyze the local degradation of histamine. The difference in HNMT levels between visceral and bronchial/vascular SMCs may be at the root of the clinical observation that the respiratory and vascular system responses (hypotension, tachycardia, and bronchoconstriction) are more prominent than those of the visceral organs (urinary or digestive tract) when circulating histamine levels are high as a result of systemic anaphylaxes [47]. We have further confirmed the vascular- and visceral-specific expression in these SMC cells with reverse transcriptase PCR (RT-PCR) (Fig 2E).

Expression Profiles of Bronchial, Arterial, and Venous SMCs

In an unsupervised analysis (Figure 1A), SMCs isolated from arteries, veins, and bronchi did not segregate according to their tissue types of origin, unlike their endothelial cell counterparts, whose clustering pattern based on global gene expression closely reflected their arterial or venous origins [21]. Taken together, these results show that endothelial cells have stronger intrinsic arterial versus venous gene expression programs than their SMC counterparts, suggesting that endothelial cells, instead of SMCs, are the main cellular determinants for the arterial or venous identity [48]. To further explore this concept, we used a supervised analysis to determine whether and how the diversity in developmental origins, structures, and functions among bronchi, arteries, and veins were associated with identifiable, tissue-specific

molecular features. Using a multi-class SAM analysis, at an FDR of 0.1%, we identified a set of 1,037 genes differentially expressed among SMCs isolated from these three distinct kinds of tissues. The distinct patterns of expression of these genes allow the hierarchical clustering of vascular SMC samples into three separate groups largely based on tissue of origin (bronchus, vein, or artery) (Figure 3A). Gene clusters showing arterial- (red), venous- (blue), and bronchial-specific (light blue) expression are expanded and shown (Figure 3B). Several arterial SMCs were grouped in the venous branch, even with this set of selected genes (Figure 3A and 3B).

Interactions between the immune system and vascular SMCs play important roles in the pathogenesis of many vascular diseases. Arterial SMCs, in particular, express a large set of genes that mediate these interactions—such as cytokines/chemokines (IL6, CCL8, CCL7, CCL2, CXCL1, CXCL2, CXCL3, and CXCL6), complement pathway (complement factor B [CFB]), and surface receptor (ICAM1) (Figure 3B)—suggesting that vascular SMCs may themselves mediate recruitment of immune cells to the vascular walls. Perhaps these specific molecular interactions between the immune system and arterial SMCs may contribute to the preferential occurrence of atherosclerosis in arteries.

SMC samples derived from lung tissue (pulmonary artery and bronchus) expressed a common set of genes (Figure 3C), notably including the genes encoding FOXP1, a transcriptional repressor expressed in lung mesenchyme that modulates gene expression in lung tissue [49], and endothelin receptor A (EDNRA), the high-affinity receptor for EDN1, a peptide hormone that stimulates vasoconstriction and proliferation of SMCs [50].

Gene Expression Patterns of Visceral Organ SMCs

When the global gene expression patterns of cultured SMCs isolated from urinary tract (ureter, urinary bladder, and urethra), colon, and the female reproductive tract (uterus and cervix) were hierarchically clustered, they consistently grouped into three distinct subbranches according to their anatomic origin (Figure 1A). To identify genes expressed differentially among the three subgroups of visceral SMCs, a multi-class SAM was performed. The analysis identified 3,879 genes (represented by 4,889 array elements) with an FDR of 1.5%. We then performed a hierarchical cluster analysis of all visceral SMCs based on expression of these genes (Figure 4A). The distinct gene expression patterns of SMCs from different visceral organs are likely to be related to the characteristic differences in the developmental fates and physiological functions unique to visceral organs.

For example, colonic SMCs expressed fibrillin-2 (Figure 4B), a component of connective tissue microfibrils that is involved in elastic fiber assembly, organization of the ECM (which allows it to influence the physical properties of connective tissue), and regulation of growth factor signaling (which allows it to also direct a broad spectrum of cellular activities) [51]. The urinary tract gene cluster contained several genes that are known to be essential for the development of the renal and urinary tract systems, including the leukemia inhibitory factor (LIF) and oncostatin M (OSM) receptors (LIF receptor, OSM receptor, and IL6ST [52]) and their downstream signaling molecule SOCS3 (Figure 4C). The activation of these receptors by their respective ligands (LIF and OSM, two members of the IL6 family) induces the
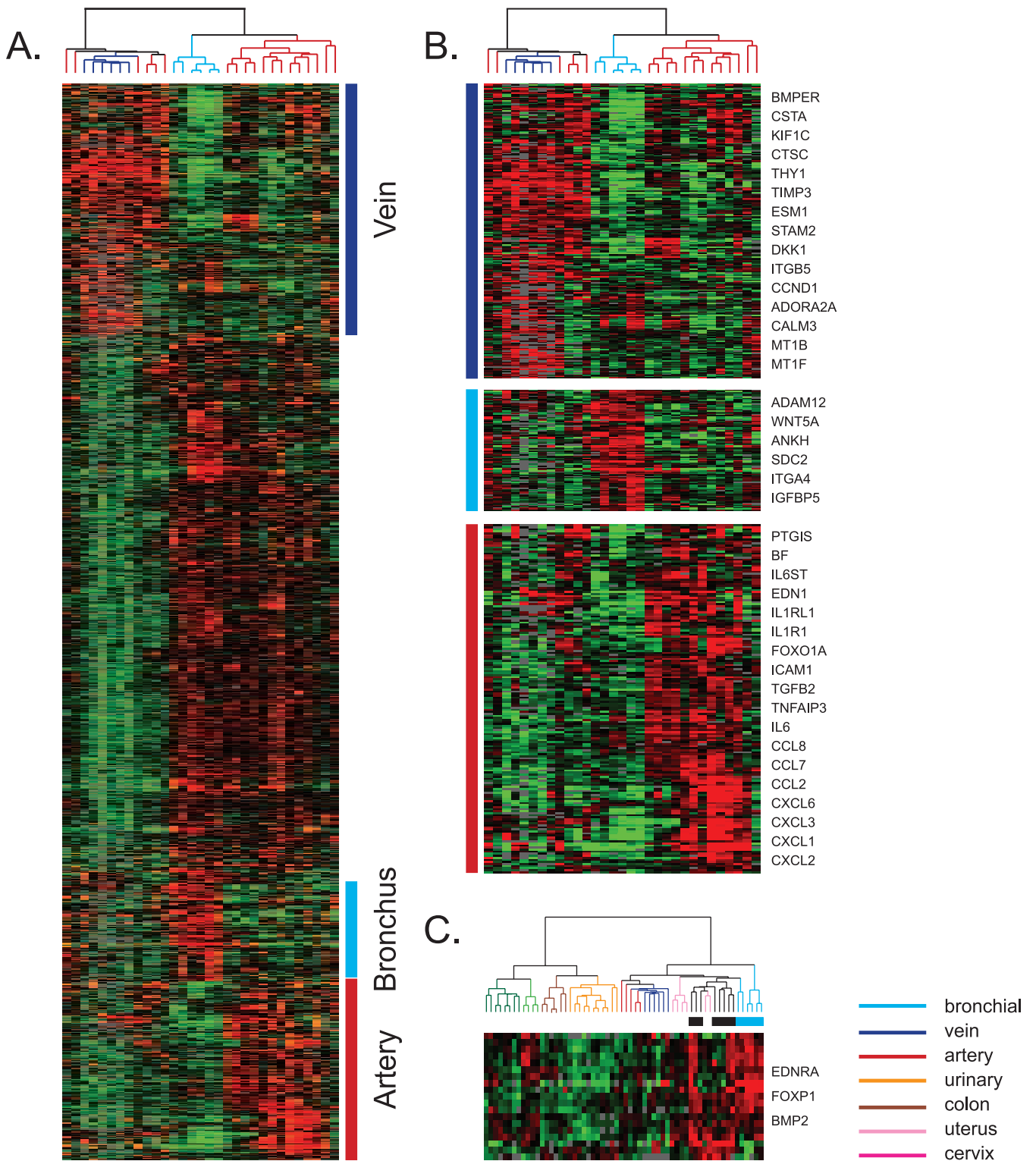
Figure 3. Artery, Vein, and Bronchial SMC-Specific Gene Expression Program

(A, B) Artery-, vein-, and bronchus-specific genes were identified by a multi-class SAM analysis. The names of select vein-specific genes (blue bar), bronchus-specific genes (light blue bar), and artery-specific genes (red bar) are shown and expanded in (B). Complete data can be found on the supplementary Web site and SMD.

(C) Features of a gene cluster with expression in lung tissues. Pulmonary artery clusters are marked by the black horizontal bar and bronchus clusters are marked by the light blue horizontal bar. Selected genes are shown.

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interconversion between metanephros mesoderm and epithelium in the urinary tract [53,54]. Inactivation of IL6ST, a component of the functional receptors for LIF and OSM, leads to developmental defects in the kidney [53,54]. Bone morphogenic protein 4 (BMP4) was also selectively expressed by SMCs isolated from urinary tract structures (Figure 4C). This protein has an essential role in the inductive signal between the endodermal epithelium and mesenchyme derived from splanchnic mesoderm [55], and BMP4 haplodeficiency can lead to renal defects [56]. It appears, therefore, that expression of several of the genes involved in key inductive signals during urinary tract development persists beyond embryogenesis and remains a feature of locally specialized SMCs, allowing these cells to selectively detect and respond to local induction cues.

We have also found components of a regulatory network consisting of FOXF1, vascular cell adhesion molecule-1 (VCAM1), and hepatocyte growth factor (HGF) selectively expressed in colon and urinary tract SMCs (Figure 4D). FOXF1 is a transcription factor essential for development of visceral splanchnic mesenchyme [57,58]. Haplodeficiency of FOXF1 abolishes the expression of VCAM1 and HGF and leads to structural defects in the gallbladder and other visceral organs [59]. The simultaneous presence of FOXF1, VCAM1, and HGF in colon and urinary tract SMC gene clusters (Figure 4D) suggests that their regulatory relationship may be preserved in these SMCs.

For hormones that reach their distant target tissues through systemic circulation, an important determinant of the specificity of their biological effects is the differential expression of hormone receptor genes in target tissue cells. Oxytocin, the neuropeptide responsible for triggering uterine contractions and lactation [60], is no exception. Oxytocin receptor is present in many organs, suggesting roles in a variety of biological processes [61,62]. In the present study, we have found that oxytocin receptor is expressed specifically in uterus SMCs but none of the other SMCs examined in this study (Figure 4E). This uterus-specific expression can help to explain the unique sensitivity of the uterus to oxytocin during parturition and the clinical usefulness of oxytocin agonists or antagonists, respectively, to induce or prevent labor.

Uterus SMCs also have especially high expression levels of many genes that encode the contractile machinery of SMCs, including tropomyosin 1 and 2, calponin, caldesmon 1, SM-α-actin, MYH9 myosin, heavy and light chain polypeptide, and phospholamban (Figure 4E). These genes are widely considered as cell-type lineage markers for all SMCs; indeed, expression of SM-α-actin was used in this study as a criterion to insure that all cells in our cultured samples were SMCs. The unusually high level of expression of these proteins in uterine SMCs, however, suggests not only that there are quantitative differences in the force-generating apparatus and the contractile capacities among different SMCs but also that these differences may reflect the distinct mechanical requirements of each SM cell type. The uterus, for example, must generate enough force and pressure within a short time frame to deliver the fetus through the birth canal during labor. The SMCs of the adjacent cervix, which relaxes and expands during parturition, express the genes encoding the contractile apparatus at levels significantly lower than that in the uterine SMCs (Figure 4E), despite the overall similarity in the gene expression patterns of uterine and cervical SMCs (Figures 1A and 2B).

Gene Expression Programs Induced by Serum Stimulation of Vascular SMCs Are Linked to Prognosis in Some Human Cancers

SMCs can undergo remarkable phenotypic modulations in response to environmental stimuli, and the association between the occurrence of these transitions and the onset of various human diseases is well established. An example of this phenomenon is the phenotypic change from the quiescent "contractile" to the activated "synthetic" state of vascular SMCs associated with serum exposure. Vascular SMCs are usually shielded from the circulating blood by a layer of overlying endothelial cells. When the integrity of the endothelial cell barrier is compromised, vascular SMCs come into direct contact with all of the components of blood, including serum constituents generated by activation of the coagulation cascade. Although serum is a complex and not fully defined mixture, exposure to serum, the soluble fraction of coagulated blood, represents a physiologically relevant stimulus associated with various forms of vascular injury. Scenarios that can lead to the exposure of vascular SMCs to serum include inflammation; injury; the development of faulty, leaky blood vessels in tumors [63]; and acute endothelial damage from balloon angioplasty [64].

We investigated the response to serum as a simple, controlled, ex vivo model of the temporal response of vascular SMCs to vascular injury induced by serum exposure. Coronary artery SMCs were first placed under replicative quiescence in DMEM media with 0.1% serum for 48 h, then exposed to fully supplemented media, DMEM with 10% serum [65], and the ensuing temporal program of gene expression was followed by analyzing samples at 1, 3, 6, 12, and 24 h with DNA microarrays. We derived the SMC serum responses by performing zero-transformation against three time zero samples. A comparison of the SMC serum response to a previously defined fibroblast serum response [66] (Figure S1) revealed that there was a high degree of similarity between the serum responses of these two cell types and that several gene clusters share similar induction kinetics (clusters 1, 3, and 5 in Figure S1). There were also some distinct features associated with each cell type. For example, several genes induced by serum only in SMCs were implicated in atherosclerosis. A cluster of genes involved in cholesterol biogenesis (cluster 7) was noted to be repressed by serum exposure in fibroblasts but not SMCs [66].

To systemically define a gene signature reflecting the serum response of vascular SMCs, we used SAM to identify 534 unique genes (653 gene elements), with an FDR of 1%, that distinguish three serum-starved vascular SMC samples from five serum-exposed vascular SMC samples (Figure 5A). All the selected genes were induced upon serum exposure. The SMC serum-response signature shares relatively few features with previously defined gene expression signatures of cellular responses to physiological stimuli: 39 genes in common with the fibroblast serum-response signature [66], 19 genes in common with the proliferation signature [67], and 22 genes in common with the epithelial hypoxia response signature [36].

To investigate the possible contribution of the vascular SMC serum-response program in the progression and phenotypes of human cancers, we defined a quantitative
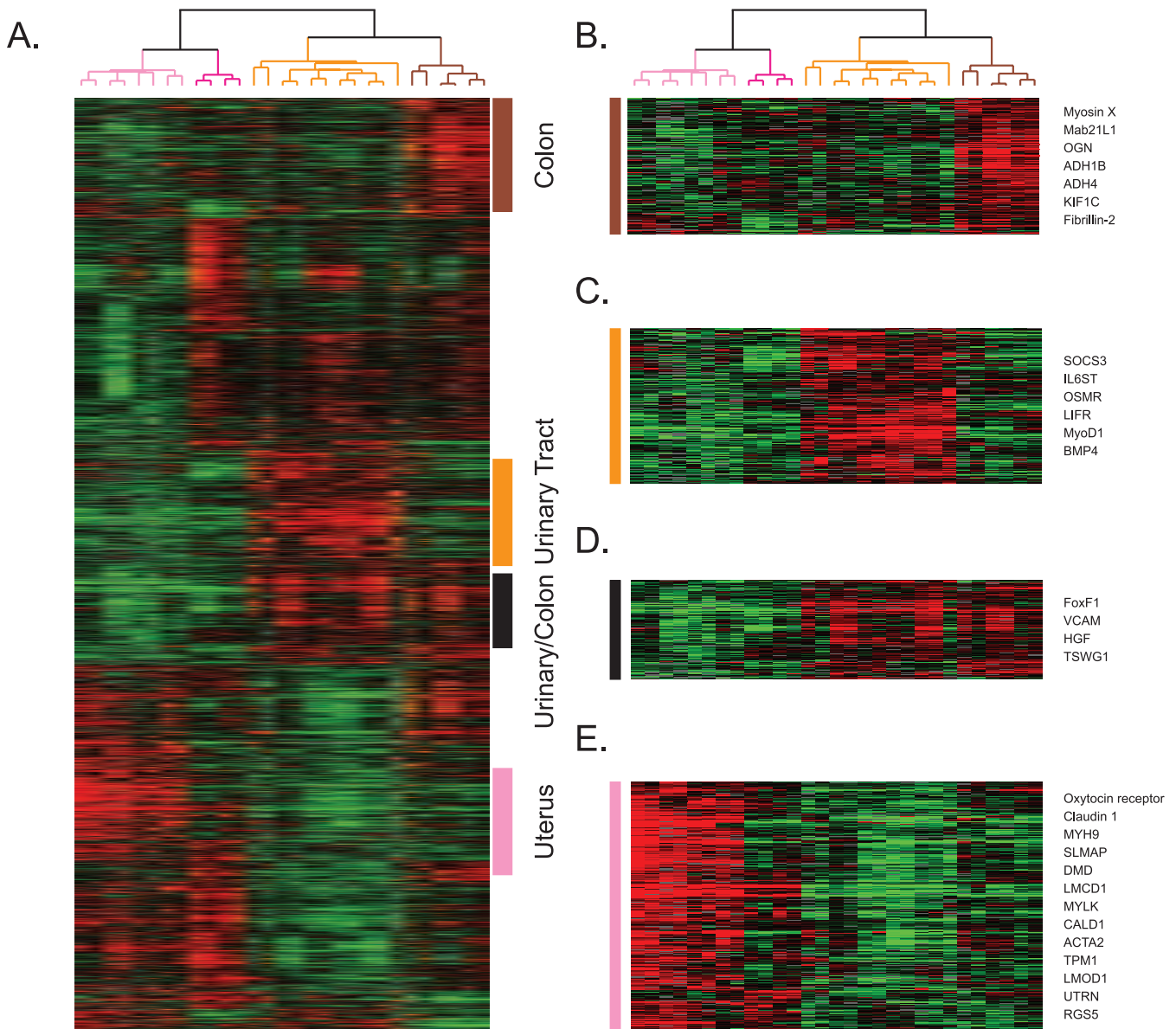
Figure 4. Colon-, Urinary Tract-, and Uterus-Specific Gene Expression programs

(A–D) Colon-, urinary tract-, cervix- and uterus-specific gene expression identified by a multi-class SAM analysis are shown in (A) and names of select genes are expanded in (B–E). Named genes are colon specific (B, brown bar), urinary tract specific (C, orange bar), urinary/colon specific (D, black bar) and uterus specific (E, pink bar). Complete data can be found on the supplementary Web site and SMD.

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"SMC serum-response score" for each sample by simply averaging the relative gene expression levels (logarithmic scale) of the 534 genes in the vascular SMC serum-response gene signature. Calculating the average expression level allowed a quantitative and unbiased determination of the activity of the SMC serum-response program, which was likely to be more stable than the expression of any particular genes in the expression program. This approach also provided a metric that could be applied to tumor samples based on their gene expression patterns to gauge the degree to which a program similar to the vascular SMC serum response was active in each tumor. We first evaluated the vascular SMC serum-response signature in a Stanford breast cancer study representing expression profiles of 85 samples containing normal breast tissues, fibroadenomas, and 78 locally advanced

breast cancers with associated extensive clinical and molecular data [68]. Among the vascular SMC serum-response genes, 68 genes were well measured in 80% of the breast samples and used to determine their SMC serum scores (Figure 5B). When we split all ductal adenocarcinoma samples based on their SMC score, 41 samples had an SMC score lower than zero while 44 samples had an SMC score higher than zero (Figure 5B). To investigate the significance of this separation, we compared samples with respect to overall and relapse-free survival. The patients with high SMC scores had significantly lower overall survival ($p = 0.0063$) and relapse-free survival ($p = 0.0007$) based on a Cox-regression model (Figure 5C).

To assess the consistency and prognostic significance of the SMC serum-response signature in an independent set of
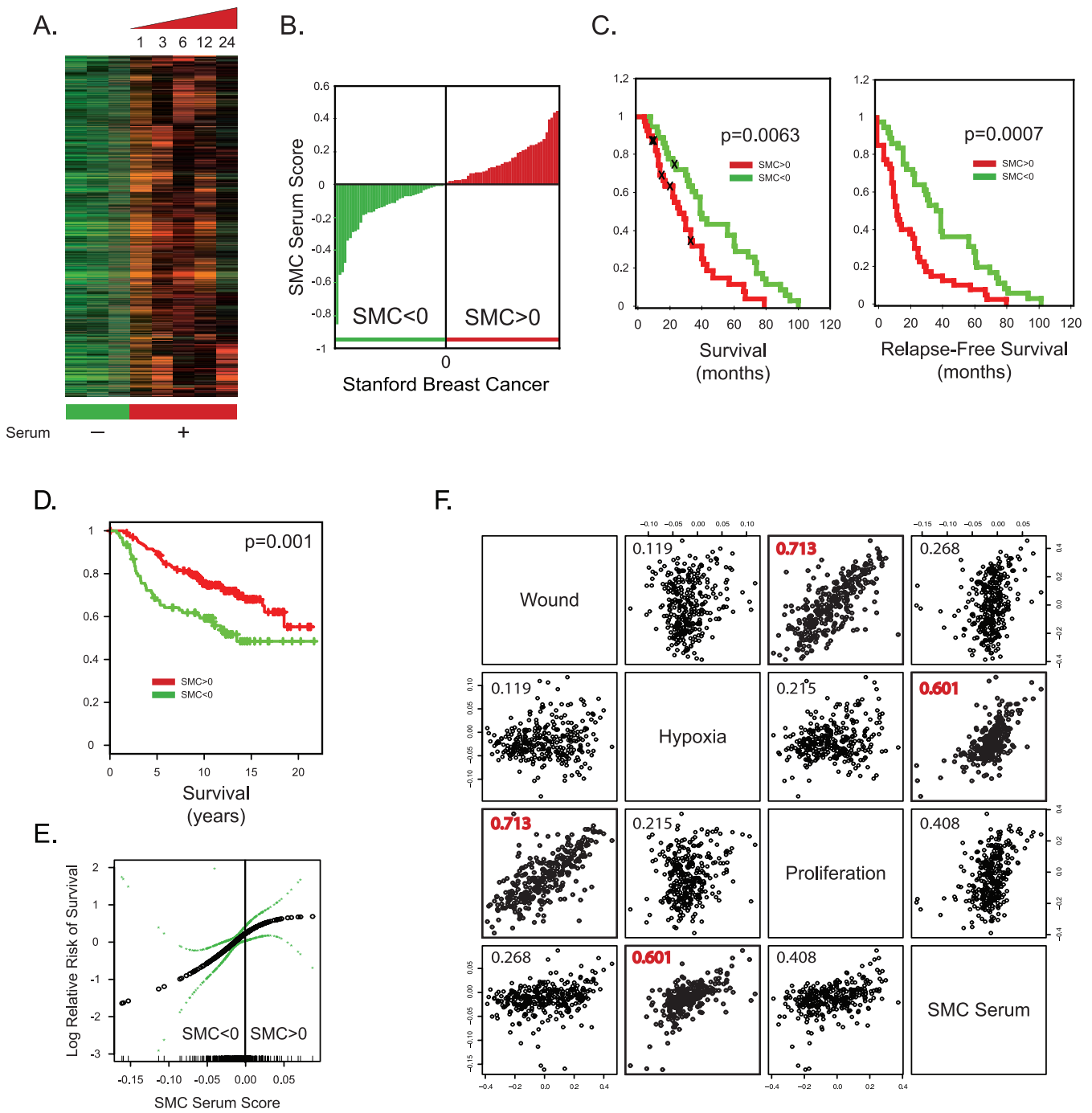
Figure 5. Analysis of Vascular SMC Serum Response and Its Prognostic Significance in Human Cancers

(A, B) Expression of the 653 genes in the SMC serum-response signature in coronary SMCs before (green) and after (red) serum exposure at indicated time points (A). The SMC serum-response scores of the Stanford breast tumor collection (B) were calculated based on the average expression values of the serum-response signature genes. The tumors were separated based on whether SMC serum-response scores were positive (red, right) or negative (green, left).

(C, D) With the threshold value of the SMC serum-response signature set at zero for classification of patients into high (red) and low (green) SMC serum-response groups, the Kaplan-Meier analysis shows significant differences in survival and time to recurrence between the two groups of samples in the Stanford (C) and NKI (D) breast cancer datasets.

(E) Correlation of the quantitative and continuous SMC serum-response score (x-axis) of each tumor sample (black circle) in the NKI breast cancer dataset versus the (log) relative risk (y-axis) of mortality.

(F) Scatter plots showing the relationship between the wound-response [66], hypoxia-response [36], proliferation [67], and SMC serum-response signatures. Each point in the scatter plots represents a single one of the 295 tumors analyzed in the NKI dataset. The overall correlation between each pair of expression signatures across this set of 295 samples is indicated in each panel.

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breast cancer samples, we analyzed a published data set from the Netherlands Cancer Institute (NKI), which consisted of 295 early-stage breast cancer samples (stage I and II). Of the 653 genes that comprised the vascular SMC serum-response gene cluster in our microarray dataset, 640 were also represented in the microarrays used in the NKI study. The expression of these 640 genes in the 295 breast cancer samples allowed the separation of all the tumors into two distinct groups based on the SMC serum-response score (Figure 5D). Tumors in the high SMC serum-response group were associated with poorer overall survival ($p = 0.001$) (Figure 5C) than those in the low SMC serum-response group, confirming the prognostic value of the serum-response gene expression program of vascular SMCs. While thresholding the score at zero produced a striking discrimination of tumor phenotypes, this cut-off point was somewhat arbitrary. To investigate whether an alternative model based on the SMC serum-response signature might improve predictive power, we fit a multivariate Cox model including the SMC serum score in a quantitative form. These curves estimated the differential contribution of the SMC serum score to the (log) relative risk in a continuous fashion (Figure 5E). The results showed a strong positive correlation between the clinical risks with the SMC scores over a wide range of SMC serum scores in which most of the data occurred. Similarly, this SMC serum-response signature was associated with significantly shorter survival in a separate study of ovarian cancers, separating 72 patients with advanced ovarian cancer into groups with markedly different clinical outcomes (M. E. Schaner, personal communication).

Previous studies have shown that gene expression signatures related to cellular proliferation [67], a fibroblast serum response (wound) [66] and a hypoxia response [36] identified breast cancer patients with significantly poorer survival. Although there is rather little overlap in the genes comprising the gene expression signatures of these three distinct biological processes, their efficacy in providing clinical insights in the same set of tumor samples raises the possibility that they may be related. To investigate their potential relationship, we calculated the scores of 295 NKI breast tumors based on the four different gene signatures (Figure 5F). Then we calculated the pair-wise correlation of signature scores to determine their potential relationship. As reported previously, the hypoxia score is only weakly correlated with the fibroblast wound-response (corr = 0.11) or proliferation scores (corr = 0.215) [36]. In contrast, the SMC Serum score was highly correlated with the hypoxia response score (corr = 0.601). This result suggests that the breast cancers with an activated hypoxia response also tend to have an activated program related to the vascular SMC serum response. The vascular SMC serum response might reflect vascular injury; perhaps the hypoxia response and SMC serum response are both elevated in a subset of breast tumors with vascular abnormalities and poor tumor oxygenation. A similar correlation between the hypoxia and SMC serum-response signatures was observed among the breast cancer samples characterized at Stanford (Figure S2). Interestingly, although the fibroblast wound-response signature was also elicited by ex vivo serum exposure, there was only a weak correlation between the fibroblast wound-response score and SMC serum-response score in these cancers (corr = 0.268).

To test whether the vascular SMC serum response contrib-utes anything new and useful to clinical decision making or complements previously established prognostic factors, we evaluated it in a multivariate Cox model that included other established prognostic and clinical factors in the NKI breast cancer dataset. Although it had significant prognostic value by itself, the vascular SMC serum-response signature contributed little additional predictive power when chemotherapy, ER status, tumor size, grade, angioinvasion, and age were included in the predictive model. Therefore, the prognostic information represented by the vascular SMC serum-response signature was already contained in, and perhaps functionally linked to, the classical prognostic factors (Table S1).

Discussion

It has been claimed that there are approximately 200–400 cell types in the human body [69]. The aims of this study were to begin to examine how many distinct cell types are truly encompassed by the moniker "smooth muscle cell" by systematically comparing gene expression patterns in smooth muscle cells from diverse anatomical sites, and to search for links between these characteristic gene expression patterns and the differentiation, functional specialization, and contribution to pathology of SMCs. The results show that SMCs native to different anatomic sites have distinct, reproducible gene expression patterns that persist through many generations of ex vivo culture in standard media, suggesting that these SMCs comprise distinct differentiated cell types. Similar observations have been made about fibroblasts and endothelial cells in our previous studies [21,25] and for many other superficially similar cell types, notably lymphocytes, whose underlying diversity has emerged through molecular characterization and has been shown to have implications for our fundamental understanding of disease processes [70]. Thus, despite their apparent similarity in morphology and function, many of the cell types that compose the stroma and the vascular systems of various anatomical locations and structures in the human body have diverse, distinct differentiation programs and molecular specializations. Our results on the diversity of fibroblasts [25], endothelial cells [21], smooth muscle cells (in this study), and blood cells [70] highlight the unexpected large number of cell types in the human body and emphasize the need for a better understanding of the fine specialization of these different cell types. The molecular and physiologic differences among superficially homogenous cells may help explain the variations in the physiological behavior of cell types and provide insights into the genetic networks that lead to regional differentiation and to the final local specialization of the architecture and function of human tissues. This depth of understanding will almost certainly have wide-reaching implications for human biology.

Several biological themes emerged in our analysis of SMC gene expression. First, although important common mechanisms for regulating the expression of SMC lineage markers are shared by all SMCs [3], unique differentiation programs characteristic of SMCs at distinct anatomic sites were reflected in the pervasive differences in gene expression patterns. Several growth factors and transcription factors are able to activate SMC differentiation programs [3]; the relative importance and contribution of each growth or transcription

factor may vary by SMC subtype. For example, the expression of vascular SMC-specific genes appears to be partially controlled by high TGF-β activity. It is known that TGF-β can trigger SMC differentiation by activating the expression of many SMC lineage genes [71] through the TGF-β control elements present in their promoters [72]. During blood vessel formation, TGF-β is the crucial molecular signal through which endothelial cells recruit splanchnic mesoderm cells and then induce them to differentiate into SMCs [73,74]. The relatively high level of expression of both TGF-β and its receptor may account for the characteristic biosynthetic phenotype of vascular SMCs, as many genes involved in the biosynthesis, trafficking, and modification of ECM proteins are downstream target genes of TGF-β 27]. The preferential expression of ligands, receptors, and other signaling components of the TGF-β signaling pathway by vascular SMCs [75–77] suggests that this feature of the vascular SMC expression program might be maintained via an autocrine mechanism. In contrast, the low level of TGF-β activity in visceral SMCs, reflected in the low level of expression of TGF-β signaling pathway–related genes, suggests that their differentiation programs may be less reliant on TGF-β. Several visceral SMC-specific transcription factors (such as MRF2 (also known as ARID5B) and PBX1) possess the ability to trigger SMC differentiation [6,9] and are likely to contribute to the differentiation programs of these SMCs instead. This divergence of SMC subtype differentiation programs and molecular mechanisms has been found to account for differential SM–major histocompatibility complex and CRP-1 (also known as CEBPE) activation in various SMC subtypes [78,79].

Second, the anatomic site–specific differentiation of SMCs may help explain the particular susceptibility of specific tissues and organs to specific pathogenic processes. For example, vascular SMCs, but not visceral SMCs, express large sets of genes implicated in fibrogenic matrix deposition, tissue remodeling, hypoxia response, and inflammatory responses. These biological processes are implicated in pathogenesis of human diseases affecting SMCs, such as atherosclerosis, hypertension, and asthma. For example, the unique vulnerability of arterial SMCs to atherosclerosis may be related in part to the high levels at which they express TGF-β and the inflammatory response genes (Figure 3B), and the relatively low expression level of a group of genes involved in glutathione biosynthesis in vascular SMCs might contribute to their relative vulnerability to damage from oxidative stress [80].

Most patients with pulmonary artery hypertension have elevated EDN1 levels, and pharmacological blockage of EDN1 activity leads to clinical improvement and better prognosis [81]. EDN1 receptor A mediates a contractile response to EDN1 in SMCs; the high intrinsic expression of its transcript in pulmonary artery SMCs suggests that pulmonary arteries may be especially sensitive to the vasoconstrictive activity of circulating EDN1 [82]. The differential expression of fibrillin 1 and fibrillin 2 in SMCs may be related to the anatomic specificity of diseases that result from defects of these proteins. Fibrillin 1 is expressed exclusively in vascular SMCs while fibrillin 2 is found also in colon SMCs (Figure 2B). Mutations in fibrillin 1 lead to Marfan's syndrome, in which major defects affect the vascular structures, in agreement with vascular SMC expression of fibrillin-1 (Figure 2B). Mutations in fibrillin 2, on the other hand, lead to congenital

contractural arachnodactyly. In addition to vascular defects, congenital contractural arachnodactyly patients suffer from abnormalities in the digestive tract, such as duodenal atresia, esophageal atresia, and intestinal malrotation [83]. Thus, links between differential gene expression and disease phenotypes may provide a basis for the localization of defects in syndromes, paralleling similar findings in fibroblasts [25].

Third, specialized gene expression programs reflecting anatomically specific differentiation may underlie the distinct mechanical properties of the SMCs in each tissue. For example, uterine SMCs express genes encoding contractile proteins and sarcomere units at especially high levels, but cervical SMCs, while otherwise very similar, do not. The uterus-specific expression pattern may be related to the uterus' unique need to generate contractile force during parturition and points to a uterus-specific mechanism for fine tuning expression of genes broadly expressed by cells of SMC lineage. Although SRF/myocardin levels are similar in all SMCs, variations in the expression levels of other regulators may account for these fine-tuned differences. For example, the oxytocin receptor, expressed primarily in uterus SMCs, may play such a role, given its ability to trigger muscle differentiation programs [84].

Fourth, variations in gene expression patterns suggest regulatory mechanisms underlying position-specific differentiation. For example, the patterns of expression of a small number of *HOX* genes can recapitulate the same anatomic clustering of SMCs that was originally achieved with all of the genes in our study, suggesting that positional information encoded in the pattern of expression of the *HOX* genes may play an important role in determining the distinct molecular phenotypes of SMCs at different sites. The expression of the BMP4 and LIF/OSM receptor in urinary tract SMCs may be a vestige of their developmental origins and suggests the possibility that these cells may retain some of the developmental plasticity of their progenitors. Corollary to all of our findings on differential gene expression in SMCs is the idea that topographically regulated genes, which can be studied ex vivo in cultured cells, may provide an excellent starting point for dissecting the molecular pathways involved in the development of individual tissues and organs.

Finally, we have found that a gene in vitro expression program elicited in vascular SMCs by short-term exposure to serum, an in vitro model of vascular injury [3], is predictive of elevated risk of progression in a variety of human carcinomas. Expression of the vascular SMC serum-response program in human carcinomas may reflect aberrant properties of the tumor vasculature, which can often have biochemical, structural, and compositional abnormalities that result in defective and leaky endothelial cells. Consistent with this possibility, tumors with a gene expression pattern resembling the SMC serum response tend to also exhibit a strong hypoxia response [36], another characteristic of tumors with defective tumor vasculature [19,85]. Such vascular dysfunction may impede drug delivery and create tumor microenvironments that favor metastasis. The possibility that the SMC serum-response signature could identify patients with defective tumor blood vessels, who might benefit from the emerging cancer therapeutics that target tumor vasculature (e.g., the VEGF-specific antibody, bevacizumab), deserves further investigation [19,86,87].

The functional and regulatory specializations revealed by

the global gene expression patterns of SMCs from different tissues are of particular clinical importance because SMCs are among the cells most frequently targeted by drugs—e.g., for the reduction of airway resistance, the regulation of blood pressure, or for the induction or inhibition of peristalsis, urination, or labor. Most current treatments affect SMCs indirectly through the autonomic nervous system, and these treatments sometimes have undesirable off-target side effects. Perhaps more specific interventions, with fewer side effects, might be achieved by directly targeting the intended smooth muscle groups. A notable example of such a specifically targeted treatment is provided by the use of oxytocin to induce uterine contraction, by targeting the oxytocin receptor, whose expression in smooth muscle is largely limited to the uterus. Although this study provides only an initial survey of the molecular diversity and heterogeneity of SMCs, it is clear that the genes encoding many potentially "drugable" targets—ion channels, adhesion molecules, and G-protein signaling receptors—are selectively expressed in different SMCs. This fact makes them particularly interesting as potential targets for cell type–specific therapeutics. A more comprehensive study of global expression patterns in anatomically distinct SMCs is likely to uncover additional potential targets for selective SMC-directed therapeutics.

Supporting Information

Figure S1. Gene Expression Program of Serum Responses of Fibroblasts and Smooth Muscle Cells

(A, B) Hierarchical clustering of a total of 1,866 elements that display a greater than 3-fold change in mRNA expression in more than one sample when fibroblasts and SMCs are exposed to serum. The triangles represent the time course of the indicated cell types. Identified clusters of genes with unique expression patterns are marked by vertical bars and expanded in (B) with the names of representative genes shown on the right.

Found at doi:10.1371/journal.pgen.0030164.sg001 (1.3 MB PDF).

Figure S2. A Scatter Plot Showing the Relationship between the Hypoxia-Response [36] and the SMC Serum-Response Signatures in the Stanford Breast Cancer Dataset [68]

Found at doi:10.1371/journal.pgen.0030164.sg002 (66 KB PDF).

Table S1. Multivariate Analysis of Classical Prognostic Factors and SMC Serum-Response Signature

Found at doi:10.1371/journal.pgen.0030164.st001 (16 KB XLS).

Table S2. Gender and Age Distribution of SMC Samples

Found at doi:10.1371/journal.pgen.0030164.st002 (18 KB XLS).

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