GATA-6 mediates human bladder smooth muscle differentiation: involvement of a novel enhancer element in regulating α-smooth muscle actin gene expression

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Kanematsu A, Ramachandran A, Adam RM. GATA-6 mediates human bladder smooth muscle differentiation: involvement of a novel enhancer element in regulating α-smooth muscle actin gene expression. Am J Physiol Cell Physiol 293: C1093–C1102, 2007. First published July 11, 2007; doi:10.1152/ajpcell.00225.2007.—Hollow organs exposed to pathological stimuli undergo phenotypic modulation characterized by altered expression of smooth muscle contractile proteins and loss of normal function. The molecular mechanisms that regulate smooth muscle differentiation, especially in organs other than the vasculature, are poorly understood. In this study, we describe a role for the GATA-6 transcription factor in regulation of human bladder smooth muscle differentiation. Knockdown of endogenous GATA-6 in primary human bladder smooth muscle cells (pBSMC) led to decreased mRNA levels of the differentiation markers α-smooth muscle actin (α-SMA), calponin, and smooth muscle myosin heavy chain. Similar effects were obtained following downregulation of GATA-6 by forskolin-induced elevation of intracellular cAMP levels. Forskolin treatment of pBSMC abolished recruitment of GATA-6 to the α-SMA promoter in vivo and reduced activity of human α-SMA promoter-directed gene expression by >60%. This inhibitory effect was rescued by enforced expression of wild-type GATA-6 but not by a zinc-finger-deleted mutant, GATA-6-ΔZF, which lacks DNA-binding ability. In silico analysis of a region of the human α-SMA promoter, described previously as a transcriptional enhancer, identified a putative GATA-binding site at position −919/−913. Point mutation of this site in SMA-Luc abrogated GATA-6-induced activation of promoter activity. Together, these results provide the first evidence for a functional role for GATA-6 in regulation of bladder smooth muscle differentiation. In addition, these findings demonstrate that GATA-6 regulates human α-SMA expression via a novel regulatory cis element in the α-SMA promoter-enhancer.

The smooth muscle cell (SMC) phenotype is regulated, in part, by changes in the transcriptional program (4, 38). The GATA transcription factors are a highly conserved family of zinc finger proteins that mediate tissue-specific gene expression. They can be broadly classified into transcriptional regulators of the hematopoietic lineage (GATA-1, -2, -3) or of the mesodermal and/or endodermal lineages (GATA-4, -5, -6) (reviewed in Ref. 31). Of the latter group, all three GATAs have been implicated in regulation of cardiac muscle phenotype, both during development and in response to pathological stimuli (29, 31, 32, 52). Both GATA-5 and GATA-6 are expressed in SMC (33, 34). Although the functional significance of GATA-5 expression in smooth muscle has not been addressed, GATA-6 has been implicated in regulation of SMC contractile protein expression in vascular SMC, suggesting that the protein is an important contributor toward maintenance of the differentiated state (10, 43, 49, 50, 53). However, inhibition of GATA-6 by expression of a dominant negative engulfed GATA-6 fusion protein or conditional inactivation of GATA-6 expression in SMC by tissue-specific targeting of Cre recombinase led to little or no change in expression of genes encoding contractile proteins in vascular SMC, arguing against a role for GATA-6 in smooth muscle differentiation (25, 26). Thus the specific mechanisms whereby GATA-6 might participate in regulation of the differentiated smooth muscle phenotype and the extent to which GATA-6 activity is tissue or organ specific remain poorly understood.

In this study, we demonstrate that GATA-6 mediates smooth muscle differentiation in primary culture SMC derived from the human urinary tract. We identify GATA-6 as a direct transcriptional regulator of α-SMA expression in this cell type.

EXPERIMENTAL PROCEDURES

Cell culture. After receiving institutional review board approval and parental consent, we obtained human bladder tissue from patients undergoing ureteral reimplantation for vesicoureteral reflux but with no bladder abnormality otherwise. Tissue was processed for isolation of cells or immunohistochemical analysis as described previously (5). Briefly, primary culture human bladder smooth muscle cells (pBSMC) were isolated by explant from fragments of bladder muscle tissue and propagated as described previously (5). Cells were maintained in low-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air-5% CO2.

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CO₂. All experiments were performed on cells between passages 2 and 5. Based on immunocytochemical staining, the majority of cells stained positively for α-SMA and calponin (data not shown). Rat pBSMC were isolated from neonatal rat bladders by enzymatic dispersion as described previously (39) and maintained in medium with 0.5 µg/ml puromycin for ~2 wk until no more uninfected control cells survived. Puromycin-resistant cells were passaged for assays. Three independent sets of stable cell lines were generated for the assays described.

Promoter-reporter assays. The human disabled-2 (Dab-2) promoter-reporter construct (pGL3-Dab2) was a kind gift from Dr. Jer-Tsong Hsieh, University of Texas Southwestern Medical Center (56). The human α-SMA promoter-reporter (pCI-α-SMA-Luc) and appropriate negative and positive control constructs (pCI and pCI-Luc) were a generous gift from Dr. Michael Keogh, Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School (24). Site-directed mutagenesis of pCI-α-SMA-Luc was performed by overlap PCR as described previously (39). Promoter constructs were transfected along with GATA-6 expression vectors or pEGFP-N1 (Clontech) as control into HEK-293 cells or rat pBSMC as follows. HEK-293 cells were transfected in DMEM-10% FBS. pBSMC were transfected under serum-free conditions for 1 h as described previously (24). Cells were treated with forskolin in M199 + 10% FBS 24 h later. Lysates were harvested 48 h posttransfection, and luciferase activity was measured using the luciferase assay reagent (Promega, Madison, WI).

Chromatin immunoprecipitation. Formaldehyde-cross-linked chromatin was obtained from pBSMC and sheared as described previously (14) before immunoprecipitation. Briefly, sheared chromatin was precleared with preimmune mouse serum and protein G beads (22) and then immunoprecipitated with 5 µg of mouse anti-GATA-6 antibody (R&D Systems) or mouse IgG1 (Sigma). The enrichment of GATA-binding sequences in eluted DNA was quantified by real-time PCR and expressed as a percentage of input DNA (14, 20). Primers used for PCR are listed in Table 1.

Electromobility shift analysis. Electromobility shift analyses (EMASs) for GATA-6 were carried out with 3–5 µg of nuclear extract and 0.5 ng of 32P-labeled oligonucleotide probe in a 20-µl reaction volume containing 25 mM Tris-HCl, pH 8, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM EGTA, and 1% Triton X-100 supplemented with protease and phosphatase inhibitors. Nuclear extracts were obtained according to the method of Dignam as described previously (39). Immunoblot analysis was performed as described previously (45).

Construction of GATA-6 expression vectors. Sequences corresponding to the short and long isoforms of human GATA-6 (6) were amplified from pBSMC cDNA using the Expand long-template PCR reaction system supplemented with 5% dimethylsulfoxide and ligated into the BglII site of pCMV-HA vector (Clontech Laboratories, Mountain View, CA). A mutant form of GATA-6 lacking the entire NH₂-terminal zinc finger domain and part of the COOH-terminal zinc finger domain (ΔZF-GATA-6) was a kind gift from Dr. Kenneth Walsh, Boston University School of Medicine (40). The ΔZF-GATA-6 coding region was subcloned into pCMV-HA for expression analyses.

Generation of BSMC with stable knockdown of GATA-6. To suppress expression of endogenous GATA-6 in human pBSMC, double-stranded 21-base length oligonucleotides targeting the 3′-untranslated region of the human GATA-6 transcript were designed and subcloned into the lentiviral vector pLKO.1-puro (46), a generous gift from Dr. William C. Hahn, Dana-Farber Cancer Institute. The sequence of the GATA-6-targeting oligonucleotide was 5′-GGCGTACGAGAAGGCTAT CCTT-3′. A control vector containing sequence targeting green fluorescent protein (GFP: 5′-GGTCAGCTCCGGGAGGAGCC-3′) was also generated, as described elsewhere (46). An additional control vector comprising a scrambled sequence (42) was purchased from Addgene (Cambridge, MA). Several candidate sequences targeting GATA-6 were screened by transient analyses in 293T cells. Based on the efficacy of reduction in expression of endogenous GATA-6 in 293T cells, sequences were selected for subsequent packaging into viral particles. To generate viral supernatants encoding GATA-6-specific or control short hairpin RNA (shRNA), 293T cells were cotransfected with the desired lentiviral construct and the packaging and envelope plasmids pCMV.R8.91 and pCMV-BS-VSV-G. Viral supernatants were retrieved 72 h posttransfection, passed through a 0.45-µm filter, and immediately used for infection. Human pBSMC were infected in the viral supernatant for 4 h and then maintained in medium with 0.5 µg/ml puromycin for ~2 wk until no more uninfected control cells survived. Puromycin-resistant cells were passaged for assays. Three independent sets of stable cell lines were generated for the assays described.
A JC TCT-3'); GATA mut, containing mutations (underlined) in the consensus GATA sites (5'-CAC TTC TTA ACA GAA AGT CTT AAC TCT-3'); G1 wt, corresponding to the GATA-6 binding motif present in the α-SMA enhancer (5'-GGG ATT ATG GAG ATT AGA ATT CGA GAC-3'); and G1 mut, containing a point mutation (underlined) in the α-SMA GATA-6 motif (5'-GGG ATT ATG GAG GTT AGA ATT CGA GAC-3').

Statistical analysis. Where appropriate, comparisons between experimental groups were performed using Student’s t-test. P < 0.05 was considered significant.

RESULTS

GATA-6 mRNA was shown previously to be expressed in the urogenital ridge and bladder of the developing mouse (33). However, its expression in tissues of the human urinary tract has not been described. Initially, we performed qualitative RT-PCR to determine which members of the mesodermal cohort of GATA factors (GATA-4, -5, -6) were expressed in pBSMC. As shown in Fig. 1A, pBSMC express GATA-5 and GATA-6 mRNA, whereas GATA-4 mRNA levels were negligible, consistent with the reported expression pattern of GATA factors in the developing urinary tract (33, 34). Immunoblot analysis indicated that pBSMC express two molecular forms of GATA-6 (Fig. 1B), consistent with previous descriptions of two GATA-6 isoforms arising from the use of alternative promoters and/or initiation codons (6, 7). Based on the migration pattern relative to ectopically expressed GATA-6, endogenous GATA-6 expressed in human pBSMC predominantly comprises the short isoform. Despite detection of GATA-5

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F/R, forward or reverse primer; Dab-2, disabled-2; α-SMA, α-smooth muscle actin; SM-MHC, smooth muscle-myosin heavy chain; wt, wild type; mut, mutant.
mRNA in pBSMC, we were unable to detect GATA-5 protein in these cells (data not shown). Immunofluorescence imaging demonstrated that GATA-6 localized primarily to nuclei in cultured cells and tissues (Fig. 1C).

Recent reports asserted that a dominant inhibitor of GATA-6 activity, or genetic ablation of the protein, had minimal effect on expression of α-SMA, SM-MHC, and calponin in vascular SMC (25, 26). To assess whether this was the case in the SMC of the bladder, we employed RNA interference to knock down GATA-6 protein levels and evaluated GATA-6 target gene expression using real-time RT-PCR. Suppression of GATA-6 expression led to a reduction in α-SMA, calponin, and SM-MHC mRNA levels (Fig. 2A), suggesting that GATA-6 plays an important role as a transcriptional regulator of contractile protein expression. Parallel changes in protein expression for α-SMA and calponin (not shown) were also observed (Fig. 2B). GATA-6 knockdown also reduced levels of the known GATA-6 target Dab2 (35). In contrast, cyclin D1 mRNA levels were increased in SMC lacking GATA-6 expression, suggesting that effects on gene expression from GATA-6 knockdown were more extensive and were not restricted to genes specific to the muscle lineage.

As an independent test of the effect of GATA-6 downregulation on the SMC gene expression pattern, we exploited the observation that elevation of intracellular cAMP levels can decrease GATA-6 protein levels (21, 30, 36). Initially, we determined the effect of increasing cAMP on GATA-6 expression in SMC. Elevation of intracellular cAMP levels by exposure of cells to the direct adenylate cyclase activator forskolin (Fig. 3A, ii) or to the cAMP analog 8-BrcAMP (Fig. 3A, iii) led to a time- and dose-dependent reduction in GATA-6 protein levels. A similar effect was observed with DBcAMP (not shown). GATA-6 knockdown reduced levels of the known GATA-6 target Dab2 (35). In contrast, cyclin D1 mRNA levels were increased in SMC lacking GATA-6 expression, suggesting that effects on gene expression from GATA-6 knockdown were more extensive and were not restricted to genes specific to the muscle lineage.

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shown). The forskolin-induced loss of GATA-6 expression did not result from apoptotic effects on cells, since flow cytometry analysis revealed no change in the extent of the sub-G1 peak following forskolin treatment (data not shown).

Alterations in intracellular cAMP levels are known to lie downstream of both adrenergic and cholinergic receptor activation (2, 15, 51). To determine whether activation of these receptors could affect GATA-6 levels in pBSMC, we first performed real-time RT-PCR to assess relative receptor expression. As shown in Fig. 3B, the predominant species observed in pBSMC in culture and in human detrusor tissue (data not shown) were the β2-adrenergic and M2 muscarinic acetylcholine receptors. Treatment of pBSMC with isoproterenol, a β2-adrenergic agonist and inducer of cAMP synthesis, downregulated GATA-6 levels (Fig. 3B, ii). This reduction could be reversed in the presence of carbachol (CCh), a muscarinic agonist (iii). In each case, GATA-6 and GAPDH expression were assessed as described in A. C: pBSMC were treated with 50 μM Fsk for 24 h. Total RNA from control and Fsk-treated cells was reverse-transcribed, and cDNAs were amplified by semiquantitative real-time PCR using gene-specific primers as described in text. Data represent the degree of change in gene expression in treated vs. untreated cells following normalization to GAPDH (i). Lysates of control and Fsk-treated cells were resolved by SDS-PAGE and blotted with antibodies to GATA-6, α-SMA, and GAPDH (ii). Dab2, human disabled-2; SM-MHC, smooth muscle myosin heavy chain.

Fig. 3. Elevation of intracellular cAMP levels downregulates GATA-6 expression in human BSMC. A: schematic summarizing the effect of cAMP manipulation on GATA-6 protein levels (i). Confluent and quiescent pBSMC were treated with the indicated doses of forskolin (Fsk; iii) for 8 or 24 h or 8′-bromoadenosine-3′,5′-cyclic monophosphate (8-BrcAMP; iii) for 24 h. Whole cell lysates were resolved by SDS-PAGE and blotted with antibodies to GATA-6 or GAPDH. B: relative expression of β-adrenergic and muscarinic (M2 and M3) acetylcholine receptors in pBSMC was determined by real-time RT-PCR (i). Data are presented as ratios to the housekeeping gene, GAPDH. pBSMC were exposed to increasing doses of isoproterenol (Iso), a β-adrenergic agonist (ii). Untreated cells (C) or Fsk (100 μM)-treated cells were included as negative and positive controls, respectively. pBSMC were exposed to 10−6 M Iso for 8 h in the absence or presence of 10−4 M carbachol (CCh), a muscarinic agonist (iii). In each case, GATA-6 and GAPDH expression were assessed as described in A. C: pBSMC were treated with 50 μM Fsk for 24 h. Total RNA from control and Fsk-treated cells was reverse-transcribed, and cDNAs were amplified by semiquantitative real-time PCR using gene-specific primers as described in text. Data represent the degree of change in gene expression in treated vs. untreated cells following normalization to GAPDH (i). Lysates of control and Fsk-treated cells were resolved by SDS-PAGE and blotted with antibodies to GATA-6, α-SMA, and GAPDH (ii). Dab2, human disabled-2; SM-MHC, smooth muscle myosin heavy chain.
following GATA-6 knockdown by RNA interference (RNAi) (see Fig. 2A). Downregulation of α-SMA protein was also evident in forskolin-treated SMC. Together, these observations suggest that GATA-6-mediated regulation of contractile protein expression in bladder SMC is sensitive to intracellular cAMP levels.

To determine the effect of cAMP on transcriptional regulation of α-SMA by GATA-6, we employed a human α-SMA promoter-luciferase reporter construct. The fragment comprises 1 kb upstream of the transcription start site and incorporates the proximal core promoter and a distal enhancer element (24). Initially, we evaluated GATA-6-mediated stimulation of the α-SMA promoter in the highly transfectable HEK-293 cell line and observed greater than fourfold induction of promoter activity relative to control (Fig. 4A). Based on our observations that elevation of intracellular cAMP decreased GATA-6 levels (Fig. 3), we tested whether forskolin caused a concomitant reduction in α-SMA promoter activation in pBSMC. As shown in Fig. 4B, forskolin treatment led to a >60% decrease in reporter activity, whereas a constitutively active luciferase construct under control of the cytomegalovirus (CMV) promoter was unaffected by forskolin treatment. Notably, the forskolin-induced decrease in α-SMA promoter activity could be rescued almost completely by ectopic expression of wild-type GATA-6, but not by a zinc finger-deleted mutant, GATA-6ΔZF, that lacks DNA-binding ability (Fig. 4C, i), suggesting that direct binding of GATA-6 to the promoter is required for its regulatory activity. The inability of GATA-6ΔZF to rescue α-SMA promoter activity was not a result of variation in protein levels, since the deletion mutant was expressed at levels comparable to those of the wild-type protein (Fig. 4C, ii). Together, these findings suggest that GATA-6 directly regulates the α-SMA promoter in a cAMP-sensitive manner.

Our observation that α-SMA mRNA levels decreased following downregulation of GATA-6 protein levels, as well as the ability of GATA-6 to activate α-SMA reporter activity, suggested that GATA-6 was recruited to the α-SMA promoter in vivo. The α-SMA gene is known to be regulated at the transcriptional level through the binding of serum-response factor (SRF) and one or more coregulatory factors to Careg boxes located within the core promoter region (10, 37, 53, 55). In addition, Keogh et al. (24) identified a region within the human α-SMA promoter located at −999/−890 relative to the transcriptional start site that behaved as an enhancer in SMC. To determine whether this region might be targeted by GATA-6, we analyzed the −999/−890 fragment in silico using MatInspector (9) and identified a putative GATA-binding site at position −919/−913 (AGATTA). To determine whether GATA-6 was recruited to the human α-SMA promoter in vivo, we performed chromatin immunoprecipitation (ChIP) and amplified the eluted DNA with primers flanking either the core or enhancer GATA sites. GATA-6 bound to both the α-SMA core promoter and enhancer regions (Fig. 5A, top). In addition, elevation of SMC cAMP levels by forskolin treatment abolished GATA-6 binding to both sites (Fig. 5A, bottom) and was accompanied by a reduction in α-SMA transcription as measured by real-time RT-PCR (Fig. 5B).

The identification of a putative GATA binding site (AGATTA) in the enhancer region of the α-SMA promoter suggested a role for this motif in GATA-6 recruitment. To address this question, we performed EMSA using nuclear extracts of pBSMC and oligonucleotides incorporating the GATA site. As shown in Fig. 6A, i, we observed robust complex formation with labeled probe corresponding to the GATA binding site at −919/−913. The complex was competed out with the corresponding unlabeled α-SMA GATA or consensus GATA oligonucleotides, but not with mutant versions of these sequences (Fig. 6A, i). In addition, we observed immunodepletion of the complex in the presence of GATA-6 antibody but not control IgG (Fig. 6A, ii), consistent with the presence of GATA-6 in the complex.

Having confirmed GATA-6 binding to the GATA site-containing enhancer region by ChIP and EMSA analyses, we next investigated the functional consequence of mutating this site on α-SMA promoter activity. We engineered a point mutation in the enhancer GATA motif (AGATTA→AGGTTA) in the SMA-Luc construct and compared basal and GATA-6-activated transcription from the wild-type and mutant α-SMA promoters. As shown in Fig. 6B, point mutation of the GATA site did not affect basal transcription but abrogated GATA-6-induced promoter activation. Together, these findings implicate GATA-6 as a regulator of α-SMA expression that acts through a specific site in the enhancer region.
DISCUSSION

In this study, we have demonstrated that GATA-6 mediates differentiation of primary human bladder SMC and regulates \( \alpha \)-SMA expression via a novel \( \textit{cis} \) element in the \( \alpha \)-SMA promoter-enhancer. The evidence supporting these conclusions is as follows: 1) downregulation of GATA-6 protein levels in primary bladder SMC by RNA interference led to a reduction in mRNAs encoding multiple smooth muscle differentiation marker proteins; 2) reduction of GATA-6 levels by elevation of intracellular cAMP levels suppressed \( \alpha \)-SMA promoter activation, mRNA expression, and protein production; 3) GATA-6 was recruited to the human \( \alpha \)-SMA promoter in vivo; 4) cAMP-mediated inhibition of \( \alpha \)-SMA promoter activity could be rescued by enforced expression of wild-type GATA-6 but not by a DNA-binding incompetent GATA-6 mutant; and 5) point mutation of the GATA-binding motif in the \( \alpha \)-SMA enhancer abolished GATA-6 binding as shown by EMSA and abrogated GATA-6-induced promoter activation. Although GATA-6 mRNA has been reported in bladder SMC in the developing mouse (33, 34), the functional role of this factor in the bladder has not been explored, and its role as a mediator of smooth muscle differentiation in general has been in dispute (25, 26). Our demonstration that loss of endogenous GATA-6 expression by genetic or pharmacological means resulted in downregulation of \( \alpha \)-SMA and other contractile proteins strongly implicates GATA-6 in maintenance of the differentiated, contractile phenotype in bladder smooth muscle.

The specific molecular mechanisms that govern smooth muscle differentiation in hollow organs other than the vasculature are almost completely unknown. Recent findings from Li et al. (28) provided evidence for SRF as a key regulator of bladder smooth muscle differentiation during murine development. In that study, SRF expression was noted in the developing bladder mesenchyme and smooth muscle before the appearance of \( \alpha \)-SMA. These observations are consistent with findings from vascular smooth muscle, where SRF regulates the expression of several smooth muscle-specific genes via...
CArG boxes located within their core promoter regions (55). Since SRF is expressed ubiquitously, specificity of smooth muscle marker expression is achieved through the interaction of SRF with one or more binding partners, including myocardin (55), GATA-6 (37), and the cysteine-rich LIM-only protein CRP1 and CRP2 (10). Consistent with these findings, we observed recruitment of GATA-6 to the core promoter region of the human α-SMA promoter in pBSMC as shown by ChIP. Interestingly, elevation of intracellular cAMP has been reported to inhibit SRF activity (12, 19) in addition to reducing GATA-6 protein levels (21, 30). Thus it is possible that the observed inhibition of α-SMA promoter activity in pBSMC treated with forskolin may result from negative regulation of both GATA-6 and SRF. In support of this possibility, deletion analysis of the α-SMA promoter indicated that although loss of the distal GATA binding site prevented rescue of forskolin-induced repression, its absence did not preclude inhibition of α-SMA promoter activity by forskolin (data not shown). This latter effect is likely mediated, at least in part, through inhibition of SRF (12, 19). Despite the potential involvement of SRF, however, the demonstration that ectopic expression of GATA-6 could recover α-SMA promoter activity almost completely strongly implicates GATA-6 as a dominant regulator of α-SMA expression in bladder smooth muscle.

In addition to its interaction with the core promoter, we also observed binding of GATA-6 in a region ~1 kb upstream of the α-SMA transcriptional start site (TSS). This region has been implicated previously in α-SMA transcriptional regulation, with the demonstration that the region from −999/−890 functioned as a transcriptional enhancer in SMC (24). However, the mechanism underlying its function is unknown. With the exception of the current report, the promoter constructs used in other studies of human α-SMA gene regulation terminated proximal to −999/−890 (3, 11, 41) and so were studied out of context and, as this study shows, in the absence of an endogenous regulatory feature that elicits activity from a regulator of smooth muscle differentiation. We have shown that point mutation of a putative GATA binding motif within this region prevented GATA-6-mediated activation of the promoter, demonstrating that GATA-6 binding to this site is required for α-SMA expression in SMC. Thus our study is the first to identify a specific transcription factor that is recruited to the α-SMA enhancer and that regulates its function. In addition to its obvious relevance to regulation of smooth muscle phenotype, this observation may also have implications for cells undergoing epithelial-mesenchymal transition (EMT). The EMT is implicated in several physiological and pathological conditions, including development, fibrosis, and tumor cell metastasis (44, 47) and is characterized by increased α-SMA expression. It is tempting to speculate that factors recruited to the enhancer region of the α-SMA promoter may elicit tissue-specific and context-specific regulation of α-SMA expression.

Another important implication of our findings is that stimuli that alter intracellular cAMP levels, such as effectors of the autonomic nervous system, are likely to be important regulators of GATA-6-mediated expression of α-SMA and possibly other contractile proteins in hollow organs. Intracellular cAMP levels in SMC are controlled, at least in part, by the balance between neurotransmitters such as β-adrenergic agonists that activate adenylate cyclase and muscarinic cholineric agonists that inhibit the enzyme (2). Based on our observations, under conditions where cholinergic stimuli predominate and cAMP levels are attenuated, expression of GATA-6 and its target genes/proteins is elevated. Conversely, β-adrenergic stimulation of SMC, which upregulates cAMP, decreases GATA-6 and its targets. These observations are consistent with a model where GATA-6 acts as a switch to regulate α-SMA expression downstream of autonomic inputs. Consistent with this hypothesis, conditions characterized by excessive contraction of smooth muscle, such as certain types of urinary tract dysfunction, can be alleviated by administration of agents that restore the appropriate balance between β-adrenergic and muscarinic cholineric signaling in bladder SMC and normalize smooth muscle contractility (17, 18, 23).

The complexity underlying specification of SMC phenotype is only beginning to be appreciated. Although SMC are classified broadly into those of vascular or visceral origin, even SMC from the same tissue type display striking differences in gene expression profiles. For example, expression profiling of SMC isolated from different locations within the vasculature revealed numerous differentially expressed genes (13). One such gene, PRISM, was found to regulate SMC fate by transcriptional repression of differentiation-inducing genes, including GATA-6, and activation of proliferative genes (13). GATA-6 itself is not expressed in SMC from all smooth muscle-containing tissues, showing robust expression in SMC of the vasculature and bladder wall but no expression in gut or uterine smooth muscle (33). Although its functional significance remains to be determined, this restricted expression pattern is consistent with a tissue-specific role for GATA-6 and may also reflect differential expression of factors that regulate the expression of GATA-6 itself.

In summary, we have demonstrated that GATA-6 is an important regulator of human bladder smooth muscle differentiation. We have also identified a new mode of regulation of α-SMA expression in primary human bladder SMC, via GATA-6 binding to a novel cis-acting element in the human α-SMA promoter-enhancer. Together, these observations provide a point of departure for future studies aimed at understanding the GATA-6-dependent signaling web in the bladder.

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