Positive and Negative Acting Krüppel-like Transcription Factors Bind a Transforming Growth Factor Beta Control Element Required for Expression of the Smooth Muscle Cell Differentiation Marker SM22α In Vivo

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Running Title: TGFβ Control Element Dependent SM22α Gene Regulation

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SUMMARY

Transforming growth factor beta (TGFβ) is implicated in the regulation of smooth muscle cell (SMC) differentiation. We previously identified a novel TGFβ control element (TCE) in the promoters of SMC differentiation marker genes including α SM actin and SM22α. In this study, the importance of the TCE in regulation of SM22α gene expression in vivo was investigated by mutating it within the context of a mouse SM22α promoter-LacZ transgenic construct. Mutation of the TCE completely abolished SM22α promoter activity in arterial SMC as well as in developing heart and skeletal muscle. To identify the transcription factor(s) binding to the TCE we performed yeast one-hybrid cloning analysis and identified gut-enriched Krüppel-like factor (GKLF). However, co-transfection studies in cultured cells showed that GKLF repressed the TGFβ-dependent increases in SM22α and αSMA promoter activity. Furthermore, GKLF was not highly expressed in differentiated SMC in vivo and TGFβ down-regulated GKLF expression in de-differentiated cultured SMCs. In contrast, over-expression of a related factor, BTEB2, transactivated SM22α promoter activity. Thus our findings suggest a reciprocal role for related Krüppel-like transcription factors in the regulation of SMC differentiation through a TCE dependent mechanism.
INTRODUCTION

Smooth muscle cells (SMC), unlike their cardiac and skeletal muscle counterparts, do not terminally differentiate and can exhibit a range of different phenotypes that may underlie their contribution to the pathogenesis of vascular disorders [1]. In a healthy blood vessel the majority of the SMCs are located within the media where they express a contractile (or differentiated) phenotype and function to regulate vascular tone. However, during the development of vascular diseases such as restenosis, SMCs can down-regulate SM contractile proteins and revert to a non-contractile (or partially de-differentiated) phenotype reminiscent of that seen during early fetal development [2]. As such, there is considerable interest in identifying the various extracellular ligands that regulate the ability of SMCs to modulate their phenotype, and their molecular mechanism of action.

Transforming growth factor beta (TGFβ) is thought to regulate a number of the cellular events underlying the development of vascular lesions, including SMC differentiation [3]. TGFβ is not only released by platelets [4] and macrophages [5] at the sites of vascular injury, its mRNA and protein is also elevated in neointimal SMCs of injured rat carotid arteries and in human restenotic tissue [6, 7]. Although its role in complex vascular disorders is poorly understood, much more is known about the role of TGFβ during vascular development. For example, 50% of mice deficient in both alleles of TGFβ1 die in utero between 9.5 and 10.5 dpc from abnormalities in yolk sac vessel development caused, in part, by a failure of mesenchymal precursor cells to differentiate into vascular SMCs [8]. Similarly, disruption of SMAD5 and SMAD6, intracellular mediators of signaling by TGFβ superfamily ligands, has resulted in defects in vascular development [9, 10]. Consistent with these in vivo studies, TGFβ has been shown to stimulate
expression of \(\alpha\) SM actin, SM-myosin heavy chain (SM-MHC), and SM22\(\alpha\) in SMCs that have undergone partial de-differentiation (or modulation) in culture [11]. Moreover, TGF\(\beta\) has also been shown to induce expression of these SMC differentiation marker genes in a variety of non-SM precursor cell types in culture, including multi-potent embryonic 10T1/2 cells and neural crest cells [12, 13, 14, 15]. Although these observations indicate an important role for TGF\(\beta\) in promoting the differentiation of mesenchymal cells towards a contractile smooth muscle fate, and in regulating changes in VSMC phenotype, the molecular mechanisms responsible for this effect are unknown.

To understand the molecular control of SMC differentiation, our laboratory and others have studied expression of SM differentiation marker genes such as \(\alpha\) SM actin [16, 17], SM-MHC [18], and SM22\(\alpha\) [19, 20, 21]. SM22\(\alpha\) is a calponin-related protein that interacts with other contraction associated proteins, such as tropomyosin and F-actin [22]. SM22\(\alpha\) was shown to be expressed in all muscle lineages during early embryogenesis but becomes restricted to all SMC types in late fetal development, and it is highly expressed in these cells, in adult animals [20, 23]. The first 445 base pairs of the mouse SM22\(\alpha\) promoter has been shown to be sufficient to drive expression of a linked reporter gene in all three muscle lineages in transgenic mice [19, 21]. However, unlike the endogenous SM22\(\alpha\) gene that is expressed in virtually all SMC subtypes [20], this region of the promoter was only active in a subset of arterial SMCs throughout development [19, 21].

Previously, we investigated the molecular mechanisms whereby TGF\(\beta\) drives expression of SMC differentiation marker genes. Studies identified a novel positive acting TGF\(\beta\) control element (TCE) that was required for TGF\(\beta\)-induced activation of the \(\alpha\) SM actin promoter [11].
Interestingly, the promoters of multiple other SMC differentiation marker genes including SM22α and SM-MHC were also found to contain similar TCE regions [11]. We also demonstrated that the TCE was essential for TGFβ-induced α SM actin expression in non-SMCs [24], consistent with the observations that TGFβ can induce SM differentiation marker genes in a variety of mesenchymal-derived cells.

A major goal of our previous studies [11] was to identify the trans-acting factor(s) that bound to the TCE. Although the TCE resembles a cis element that binds members of the Sp1 family, we demonstrated that neither Sp1 nor Sp3 was present in the TGFβ-dependent shift complex formed with cultured SMC nuclear extracts [11]. However, high molar fold excesses of a cold consensus Sp1 binding oligonucleotide did compete with the TCE for factor binding, suggesting that Krüppel-like zinc-finger transcription factors other than Sp1 or Sp3 might bind to the TCE [11]. This observation is of interest since previous studies have demonstrated the importance of zinc-finger proteins in tissue differentiation [25].

In summary, there is clear evidence of the involvement of TGFβ in the regulation of SMC differentiation in vivo, and that TGFβ can stimulate expression of the SMC differentiation marker gene α SM actin in a variety of mesenchymal cells in culture through a TCE dependent mechanism [11, 24]. However, as yet there is a lack of evidence demonstrating a role for the TCE in the regulation of SMC differentiation marker gene expression in vivo, and the trans-acting factors that regulate TCE activity have not been identified. Thus, the present studies have addressed the following questions: 1) Does a TCE regulate SM22α promoter activity in vivo? 2) What trans-acting factor(s) bind to the TCE? Results demonstrated that the TCE was required for SM22α promoter activity in transgenic mice. Moreover, yeast one-hybrid cloning identified
gut-enriched Krüppel-like factor (GKLF or KLF4) as a TCE binding protein that can negatively regulate TGFβ-dependent SMC differentiation marker gene promoter activity in cultured cells. In contrast, a GKLF related factor, BTEB2, enhanced rather than repressed TGFβ dependent promoter activity. These results suggest that related Krüppel-like factors reciprocally regulate expression of multiple SMC differentiation marker genes through binding to the TCE.

EXPERIMENTAL PROCEDURES

Preparation of SMC Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)

SMCs from rat thoracic aorta were isolated and cultured as described previously [26]. Cells were plated at a density of $3 \times 10^3$/cm$^2$, grown to confluency in serum containing medium, then growth arrested for 4 days in serum-free medium [27] prior to stimulation with 2.5 ng/ml TGFβ (TGFβ1, R&D Systems) diluted with vehicle (4 mM HCl, 1 mg/ml bovine serum albumin) [11]. Nuclear extracts were prepared from SMC cultures stimulated with TGFβ or vehicle control for 4 hr according to the method of Dignam et al. [28] with the addition of protease inhibitors [16].

Oligonucleotides used for EMSAs were synthesized and HPLC purified commercially (Operon Technologies, Inc.). The following double-stranded oligonucleotides (only sense strand shown) were used either as a probe or cold competitor: SM22TCE, 5'-ggagtgagtgggccggccg-3'; SM22mut, 5'-ggagtatttttgcggccg-3'; αSMATCE, 5'-gaagctgtttgggagtcc-3'; αSMAmut, 5'-gaagctatttttgtgggatc-3'; CArGB, 5'-gaggtccctatatgtgtgtg-3'; core CACCC site derived from the sequence of the β-globin promoter,
5-agctagccacacctgaagct-3. EMSA probes were generated by end labeling 20 μM single-stranded oligonucleotides with 150 μCi [γ-32P]ATP (6000 Ci/mm) (DuPont NEN) using T4 polynucleotide kinase. Labeled single-stranded oligonucleotides were annealed and purified from unincorporated nucleotides using NucTrap Push columns (Stratagene). EMSAs were performed with 20 μl of binding reaction containing ~30pg (20,000cpm) labeled probe, 5 μg of nuclear extract (or 100 ng recombinant GKLF/BSA where indicated), and 0.25 μg of poly(dA-dT) in a 1 × binding buffer (12 mM HEPES (pH 7.9), 100 mM KCl, 5 mM MgCl₂, 4 mM tris-HCl (pH 7.5), 0.6 mM EDTA, 0.6 mM dithiothreitol, and 10% glycerol). Samples were incubated for 20 minutes at room temperature then subjected to electrophoresis on a 5% polyacrylamide gel (30:1 acrylamide/bis-acrylamide (Bio-Rad)) at 170 V in 0.5 × TBE (45 mM Tris borate and 1 mM EDTA).

Antibody super-shift reactions were set up as described above, however, 2 μl antibody was incubated with the sample at room temperature for 15 minutes after addition of the labeled probe. The monoclonal anti-BTEB2 antibody was a generous gift from Yamasa Corporation Diagnostics Division (Araoicho, Choshi Chiba, Japan) and was raised against a carboxy terminal peptide antigen described previously [29]. The polyclonal anti-GKLF antibody was purchased commercially (M-19X, Santa Cruz). The recombinant mouse GKLF protein was a generous gift from Dr. Vincent W. Yang (Johns Hopkins University School of Medicine, Baltimore, MD), and was produced as described previously [30].

**Generation and Analysis of Transgenic Mice**
The region of the mouse SM22α promoter from -445 to +65, previously shown to direct $\text{LacZ}$ expression in the arterial vasculature of transgenic mice [19, 21], was generated by PCR and sequenced [31] in its entirety to verify accuracy. This promoter region was coupled to a $\text{LacZ}$ reporter gene within the context of the pUC19 vector [32] (Fig. 2). Site directed mutagenesis of the TCE within this pUC19SM22-$\text{LacZ}$ construct (Fig. 2) was performed using the Ex-site mutagenesis kit (Stratagene Corp). The integrity and accuracy of the mutated construct was determined by sequencing [31].

Both the wild-type SM22-$\text{LacZ}$ and mutated SM22TCEmut-$\text{LacZ}$ transgenes were prepared for injection after purification of each plasmid DNA construct by alkaline lysis [33] followed by banding on two successive ethidium bromide-cesium chloride gradients. Removal of the pUC19 vector DNA was achieved by SacII/EcoRI digestion followed by agarose gel purification and multiple ethanol precipitations of each transgene. Transgenic mice were generated by conventional methods [34, 19] within the Transgenic Core Facility at The University of Virginia, Charlottesville. Transgenic mice were used to establish stable breeding founder lines with the presence of the transgene being assayed by polymerase chain reaction using genomic DNA purified from adult mouse tail clips [35]. Transgene expression and histological analyses were performed as described previously [19, 36].

**Yeast One-Hybrid Analysis**

Yeast one-hybrid analysis was carried out according to the MATCHMAKER one-hybrid protocol (CLONTECH, Inc., Palo Alto, CA). Briefly, three tandem copies of the 10 bp TCE ($5'$-gagtggccgg-3') were inserted upstream of the pHISi and pHISi-1 reporter plasmids
by designing two anti-parallel \(3 \times\) TCE oligonucleotides with an \(Eco\)RI and \(Xba\)I overhang (Operon Technologies, Inc.). Both oligonucleotides were annealed and sub-cloned into the \(Eco\)RI/\(Xba\)I digested reporter plasmids which were sequenced [31] to confirm the presence and integrity of the TCE target elements. Each TCE-reporter construct was linearized and integrated into the genome of competent YM4271 to generate yeast TCE-reporter stains whose background \(HIS3\) expression was ablated using 15 mM 3-aminotriazole. TCE-binding protein genes were identified by screening competent TCE-reporter strains with a mouse 17 dpc whole embryo cDNA/AD fusion library (MATCHMAKER pACT2 vector, CLONTECH). Library plasmid clones were isolated from yeast capable of growing in the absence of leucine and histidine (Zymoprep, Zymo Research), then transformed into competent DH5\(\alpha\) bacteria. Each cDNA insert was sequenced [31] and compared to known sequences in the GenBank database.

**Transient Transfections, Reporter Gene Assays, and DNA Constructs**

10T1/2 cells (CCL 226: American Type culture Collection, Manassas, VA) were grown and maintained in Eagle’s Basal medium (BME) with 10% FCS according to the suppliers instructions. Prior to transfection, 10T1/2 cells were plated at \(1 \times 10^4\) cells/well in 6-well plates and incubated in BME/10% FCS for 24 hr. Cells were transiently transfected (in duplicate) with approximately 2 \(\mu\)g of reporter plasmid and effector expression plasmid (normalized for equal copy numbers) using the Superfect transfection reagent (Qiagen). After a 2.5 hr incubation the medium was replaced with fresh BME containing 2% FCS, and TGF\(\beta\) (2.5 ng/ml) or vehicle were added. Cell extracts were prepared 48 hr later by a detergent lysis method (Promega), and...
β-galactosidase reporter enzyme activity measured using the β-galactosidase enzyme assay system (Promega). β-galactosidase activities were normalized for total protein content (BCA protein assay reagent, Pierce). Each transfection experiment was repeated three times, and the relative β-galactosidase activity data was expressed as the mean ± standard deviations. The GKL and BTEB2 expression plasmids each contained the entire coding region of rat GKL and BTEB2 respectively. These were cloned into the pcDNA3 expression vector (Invitrogen), and were a gift from Dr. Ryozo Nagai (Gunma University School of Medicine, Gunma, Japan). A Full-length mouse LKLF cDNA was a gift from the laboratory of Dr. Jerry Lingrel (Department of Molecular Genetics, University of Cincinnati, OH) and was cloned into pcDNA3 vector. Empty pcDNA3 vector was used as a control in co-transfection experiments. The α SM actin promoter-\(\text{LacZ}\) construct (p2600Int-LacZ) has been shown to direct \(\text{LacZ}\) expression in transgenic mice, and has been described previously [37].

RNA Isolation and Northern Blot Analysis

Northern blots were performed as previously described [11] using 2 µg/lane of poly A+ RNA from rat and mouse tissues (CLONTECH). RNA was prepared from vehicle or TGFβ stimulated cultured SMCs harvested using a 1× trypsin/EDTA solution (3 minutes at 37°C). Cells were washed twice in cold 1× PBS, and collected by centrifugation at 900 rpm. Total RNA was isolated by lysis in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM MgCl₂, and 0.5% (vol/vol) Nonidet P-40. Isolation of RNA from mouse small intestine +/- epithelium required homogenization of the tissue on ice in a lysis buffer containing 0.6 M Tris (pH 7.4), 0.3 M NaCl,
3 mM MgCl₂, and 0.5% Nonidet P-40. Nuclei and cell debris were pelleted at 10,000 g for 5 minutes, and the supernatant made 1.5% with SDS before being extracted twice with citrate buffered phenol (pH 4.5), and centrifuged at 10,000 g for 2 minutes. RNA was precipitated over-night and re-suspended in an appropriate volume of RNAase-free water. 50 µg of total RNA/lane was Northern blotted as described previously [11].

Membranes were hybridized at 65°C with a radiolabelled GKLF probe, then stringently washed in 0.1× SSC/0.1% SDS at 65°C. The 1.3 kb mouse GKLF probe (~4 ng) was labelled with [α-³²P]dCTP (DuPont NEN) using randomer hexamer priming and Klenow extension (Prime-It, Stratagene). Autoradiography was carried out at -80°C using X-Omat LS film (Eastman Kodak Co.). Standardization of RNA loading and transfer was achieved by re-probing Northern blots with a probe for 18 S rRNA.

**RT-PCR Analysis**

RNA was isolated from mouse small intestine +/- the epithelial layer as indicated above. cDNA was generated from 10 µg of total RNA by random priming with ProStar First Strand RT-PCR kit (Stratagene). PCR amplification of a 495 bp N-terminal coding region of mouse GKLF (position + 28 to +523) [38] was achieved using 22mer sense (5'-GAGAAGACACTGCGTCCAGCAG-3; 28-49) and anti-sense (5'-CAGATTCTCGGCTGTAGAGGAG-3; 502-523) oligonucleotide primers in a total reaction volume of 50 µl. The composition of the PCR mix was as follows; 2 µl cDNA sample, 1× PCR buffer, 1.5 mM MgCl₂, 200 µM each dNTP, 0.5 µM of each primer, and 2.5 units of Taq.
polymerase. Reactions were overlaid with mineral oil and subjected to 30 PCR cycles of 94°C (1 minute), 55°C (1 minute), 72°C (1 minute). Amplified cDNA products were resolved on 1% agarose gels then transferred onto nylon membrane by Southern blotting. Blots were hybridized at 65°C for 6 hr with a radiolabelled mouse GKLF probe (prepared as above), then stringently washed in 0.1× SSC/0.1% SDS at 65°C and exposed to film for 1-2 hours. Standardisation of GKLF PCR amplification was achieved by co-amplification of a GAPDH control.

**Western Blot Analysis**

50 µg of nuclear extracts from vehicle control, or TGFβ (2.5 ng/ml) stimulated SMCs were loaded on a 12% SDS-PAGE Mini-Protean gel (Bio-Rad) and subjected to electrophoresis at 200V. Proteins were transferred from the gel onto a polyvinylidene difluoride (PVD) membrane at 70 V for 2 hr. Blocking of the membrane and probing with appropriate antibodies were carried out according to a protocol provided by Santa Cruz. Affinity-purified goat polyclonal GKLF antibodies (M-19) (Santa Cruz) were used as primary antibodies at a 1:1000 dilution (1 µg/ml). Membranes were also immunoblotted with control goat serum to indicate any non-specific reactivity. Alkaline phosphatase conjugated rabbit anti-goat antibodies (Pierce) were used as secondary antibodies at a 1:20,000 dilution. Immunoreactive proteins were detected with Immuno-Star substrate (Bio-Rad).

**RESULTS**
The SM22α TCE Formed a TGFβ-Dependent EMSA Complex Identical to that Formed with the α SM Actin TCE

Figure 1a compares the 10 bp consensus TCE and flanking regions of rat and mouse α SM actin and SM22α promoters, and illustrates the strong degree of homology of the TCE, particularly in the first seven base pairs, within the promoters of these genes. Also shown is the close proximity of each TCE with respect to a CArG element contained in each promoter (Fig. 1a) that has been shown to be required for expression in vivo [32, 37]. We have previously shown that the α SM actin TCE forms a TGFβ dependent shift complex [11, 24]. Electrophoretic mobility shift assays (EMSA) using nuclear extracts from vehicle and TGFβ stimulated aortic SMCs demonstrated that the SM22α TCE formed a TGFβ-dependent shift complex identical in mobility to that of the α SM actin TCE (Fig. 1b, lanes 1-4). Introduction of four G to T mutations into the SM22α TCE completely abolished the formation of this complex in a manner identical to that previously shown for the α SM actin TCE (Fig 1b, lanes 5 and 6) that we previously demonstrated was required for promoter activity in cultured SMC [11].

The TCE was Required for SM22α Promoter Activity In Vivo

To investigate the importance of the TCE in the regulation of SM22α promoter activity in vivo, we mutated the TCE within the context of the mouse -445 SM22α promoter [19, 21], and generated transgenic mice with the wild-type and mutated promoter coupled to the LacZ reporter gene (Fig. 2). The wild-type (SM22α-LacZ) and TCE mutant (SM22TCEmut-LacZ) transgenic constructs tested are illustrated in Figure 2. The TCE mutations examined were shown to prevent TGFβ dependent factor binding to the α SM actin TCE [11], and SM22α TCE
Four independently derived stable transgenic mouse lines containing the SM22TCEmut-LacZ transgene were analyzed, and LacZ expression compared to transgenic mice harboring the wild-type SM22-LacZ transgene. Analysis of 11.5 and 13.5 dpc embryos, and adult aorta, demonstrated that the SM22α TCE mutations completely abolished the ability of the SM22α promoter to direct LacZ transcription in arterial SMCs (Fig. 3). Furthermore, the absence of transient LacZ expression in the somites (11.5 dpc), and heart during embryonic development indicated that the TCE was also required for SM22α promoter activity in developing skeletal and cardiac muscle lineages (Fig. 3). Identical results were obtained in four independent founder lines examined. We have found that nearly 100% of wild type -445 SM22α-LacZ transgenic mice show high-level expression in skeletal muscle, cardiac muscle, and arterial SMC during development. As such, the virtual absence of expression in four independent SM22TCEmut-LacZ founders provides compelling evidence that the TCE mutations and not an insertional locus effect were responsible for loss of promoter activity.

Identification of a TCE Binding Factor By Yeast One-Hybrid Cloning

To identify TCE interacting factor(s) we performed yeast one-hybrid cloning by screening a mouse 17.5 dpc whole embryo cDNA/GAL4 activation domain (AD) fusion library. The yeast indicator strain used contained the pHisi reporter plasmid with three head-to-tail repeats of the 10 bp TCE cloned upstream of the HIS3 selection marker. Twenty-eight strongly positive yeast colonies were obtained after screening the cDNA/GAL4AD library with this reporter strain. cDNAs from each yeast colony were sequenced. Most interesting of the clones identified was a 1265 bp cDNA which, based on sequence analysis, was shown to be the zinc
finger transcription factor gut-enriched Krüppel-like factor (GKLF or KLF4) (accession number U20344) [38]. The region of mouse GKLF represented by the one-hybrid clone is illustrated in Figure 4a. In addition to our identification of GKLF by one-hybrid cloning, two other criteria made it a strong candidate as a potential TCE binding factor. First, our previous studies suggested that TCE binding factor(s) were related to the Krüppel-like factor Sp1 [11]. Second, GKLF is known to bind a CACCC motif [39, 40], and this sequence is present within the TCE although in the reverse orientation (i.e. GTGGG) (Fig. 1a).

Recombinant GKLF Bound the Wild-Type but Not Mutated SM22α or β SM Actin TCEs

Although one-hybrid screening indicated that GKLF could activate a TCE containing promoter within yeast, this result does not provide direct evidence that GKLF can directly bind the TCE. Thus, we tested the ability of recombinant GKLF (recGKLF) to bind the TCE in EMSA (Fig. 4b). These assays employed a bacterially expressed, purified recGKLF protein [30, 41]. A 21 bp region of the β-globin promoter containing a CACCC-like motif previously shown to bind GKLF [39, 40] was used as a positive control for recGKLF binding. A 20 bp promoter region containing a CArG element (CArG B) was used as a negative control. Results demonstrated that recGKLF bound both the SM22α and β SM actin TCE (lanes 3 and 5). Binding was specific in that it was abolished by competition with cold probe (data not shown), or by mutation of either TCE (lanes 4 and 6). GKLF binding was also seen with the CACCC containing β-globin probe (lane 1), but not with the CArG B probe (lane 2). Additionally, the ability of an anti-GKLF antibody to supershift the GKLF-TCE complex is shown (lanes 9 and 10).
GKLF was previously identified as being highly expressed in gut tissues where it is thought to play a role in the regulation of growth arrest and differentiation [38]. However, our studies are the first to implicate its potential involvement in control of expression of SMC differentiation marker genes. As a first step to investigate its possible role in SMC differentiation, we examined GKLF expression in adult rat tissues by Northern blot analysis (Fig. 5a). Consistent with the findings of Shields et al. [38], GKLF was highly expressed in the lung and small intestine. In addition, we demonstrated that GKLF was expressed highly in testis, moderately in heart and spleen, and at low levels in skeletal muscle (Fig. 5a). To determine what proportion of GKLF expression in the small intestine originated from differentiated SMC, we generated primers to an N-terminal region of GKLF and used semi-quantitative RT-PCR analysis to compare GKLF expression in RNA samples isolated from whole mouse small intestine, and small intestine in which the epithelial layer had been removed (Fig. 5b). Results demonstrated that GKLF was expressed at very low levels in the SMC layer of the small intestine indicating that the majority of GKLF expression in the small intestinal sample (Fig. 5a) was derived from the epithelial layer, consistent with previous in situ hybridization studies [42, 38]. In addition, we performed our own in situ hybridization studies, and compared SM22α and GKLF expression during mouse embryonic development and in adult SM containing tissues. Similar to the findings of Garrett-Sinha et al. [42], results demonstrated that GKLF expression was highest in epidermal and epithelial cells, with expression in the skin first detectable at E10.5 (data not shown). However, GKLF was not expressed in any tissue that expressed the SM differentiation marker gene SM22α (data not shown). Taken together, results indicate that
GKLF is expressed at very low levels in differentiated SMC in vivo.

**GKLF Negatively Regulated the TGFβ-Dependent Increase in SM22α and α SM Actin Promoter Activity**

Results in Figure 4b demonstrate that GKLF can bind specifically to both the SM22α and α SM actin TCE. However, our observation that its expression is virtually absent from differentiated SMC tissues in vivo raises questions regarding the potential function of GKLF in regulating SM22α gene expression. Indeed, one possible hypothesis is that GKLF is a TCE dependent repressor of SMC differentiation marker gene expression. To examine this hypothesis, vehicle or TGFβ stimulated multi-potent embryonic 10T1/2 cells were transiently co-transfected with SM22α, or α SM actin promoter-LacZ reporter constructs, and a mammalian expression vector containing a GKLF cDNA (pcDNA3/GKLF). Treatment of precursor 10T1/2 cells with TGFβ has previously been shown to induce the expression of SM differentiation markers, including α SM actin and SM22α, and promote their differentiation towards a SMC-like lineage [12]. Thus, 10T1/2 cells represent an excellent system for studying the effect of GKLF on the TGFβ-induced increases in SM differentiation marker gene promoter activity. Results demonstrated that GKLF over-expression completely abolished the TGFβ-dependent increase in (-445) SM22αLacZ promoter activity (Fig. 6a). Similarly, GKLF also inhibited TGFβ stimulated increases in expression of an α SM actin promoter reporter construct designated p2600IntLacZ that we previously showed was sufficient to drive expression in transgenic mice in a manner similar to the endogenous gene [37] (Fig. 6b).
**TGFβ Decreased Expression of GKLF in Cultured SMC**

Transfection data demonstrating that GKLF suppressed TGFβ induced activation of SM22α and α SM actin suggested that TGFβ may decrease GKLF expression in cultured SMCs that have undergone partial de-differentiation. To test this, cultured SMCs were stimulated with TGFβ or vehicle for 24 hr and GKLF mRNA expression measured by Northern blot analysis (Fig. 7a). In addition, RT-PCR analysis was used to examine the effect of TGFβ on GKLF expression 3 and 8 hr after stimulation (Fig. 7b). In contrast to its low level of expression in differentiated SMCs in vivo, results demonstrated that GKLF was highly expressed in phenotypically modulated cultured SMC. However, GKLF expression was markedly reduced 3, 8 and 24 hr following TGFβ treatment. Furthermore, Western blot analysis of nuclear extracts isolated from vehicle or TGFβ stimulated SMCs using an antibody to the C-terminal DNA binding region of GKLF showed that TGFβ treatment reduced the level of 52 kDa GKLF protein (Fig. 7C). Interestingly, TGFβ treatment was associated with the appearance of a lower band that cross-reacted with this antibody, suggesting that TGFβ treatment may be associated with increased expression of a related factor.

**Evidence for the Presence of a GKLF-Related Factor in the TGFβ-Dependent TCE Shift Complex Formed with SMC Nuclear Extract**

Results of our preceding studies suggest that GKLF acts to repress rather than activate TCE activity. Indeed, it is possible that the level of GKLF expression as determined at the mRNA (Figs. 5 and 7) or protein level (Fig. 7c) may not correlate with its TCE binding activity. Thus, we also performed EMSA super-shift analysis with an antibody to GKLF (Fig. 9b). The
ability of this antibody to supershift a GKLF-TCE complex is demonstrated in Figure 4b (lane 10). Results showed that the GKLF antibody was unable to super-shift the TGFβ-dependent SMC complex formed with the TCE. Taken together, results provide convincing evidence that: 1) GKLF can bind specifically to the TCE, 2) over-expression of GKLF negatively regulates expression of multiple SMC differentiation marker genes, 3) TGFβ decreased GKLF expression at the mRNA and protein level, and there was an absence of detectable GKLF binding activity in the TCE shift complex formed with TGFβ treated SMC nuclear extracts. However, results of Western analysis in Figure 7c showing the appearance of anti-GKLF antibody cross-reactive bands in TGFβ simulated cells suggest that the TGFβ dependent TCE factor binding may be GKLF-related.

To further test this possibility, we determined whether a cold β-globin oligonucleotide that contains a CACCC motif known to bind GKLF-related proteins [43, 44, 45, 46] could compete for formation of the TCE complex. Consistent with this possibility, results showed that addition of increasing amounts of the cold β-globin probe resulted in a progressive decrease in formation of the TGFβ-dependent shift complex using both the α SM actin and SM22α TCE probes (Fig. 8).

The GKLF Related Factor BTEB2 Transactivates SM22α Promoter Activity

Recent studies have implicated a possible role for the GKLF-related basic transcriptional element binding protein (BTEB) factors in the transactivation of gene expression during SMC phenotypic modulation [29, 47]. To test whether BTEB2 might contribute to TGFβ dependent regulation of SM22α in vitro, multi-potent embryonic 10T1/2 cells were transiently co-
transfected with an SM22α promoter-\textit{LacZ} reporter construct, and a BTEB2 containing mammalian expression vector (pcDNA3/BTEB2) (Fig. 9a). In contrast to the repression effects of GKLF, BTEB2 over-expression enhanced the TGFβ-dependent increase in (-445) SM22α\textit{LacZ} promoter activity (Fig. 9a). Over-expression of another GKLF-related factor, LKLF, had no significant effect on SM22α promoter activity (data not shown). Moreover, the TGFβ dependent TCE EMSA shift complex was almost completely supershifted by an anti-BTEB2 antibody (Fig. 9b). It should be noted that the antibody used was raised against a peptide immunogen derived from the carboxy-terminal DNA binding domain of BTEB2 [29]. Since, this region is highly conserved amongst the KLF subfamily of Krüpple-like factors we cannot rule out the possibility that this antibody can cross-react with other KLF proteins in EMSA reactions. However, as reported by Watanabe et al [29], we found that this antibody was specific for BTEB2 in Western analysis (data not shown).

\textbf{DISCUSSION}

The major goals of the present study were: 1) to determine whether the TCE regulates SM differentiation marker gene expression \textit{in vivo}; and 2) to identify putative \textit{trans}-acting factors that regulate its activity. We have shown that the TCE is required for (-445) SM22α promoter activity in transgenic mice and, using yeast one-hybrid cloning, identified the Krüppel-like transcription factor GKLF as a TCE binding protein. However, GKLF over-expression was found to negatively regulate the TGFβ dependent increase in SM22α promoter activity in cultured cells. GKLF was not expressed in differentiated gut SMC \textit{in vivo} but was
highly expressed in partially de-differentiated (or phenotypically modulated) cultured SMC. Moreover, addition of TGFβ was shown to down-regulate endogenous GKLF expression in cultured SMC. Results also suggested that the TGFβ-dependent factor binding to the TCE in cultured SMC was a GKLF-related Krüppel-like factor. Indeed, we have demonstrated that the GKLF-related transcription factor, BTEB2, can trans-activate SM22α promoter activity.

Our studies are the first to show a critical role for the TCE in controlling the expression of a SM differentiation marker gene in vivo. Previous cell culture studies illustrating a role for the TCE in regulating SM differentiation marker gene expression were performed within the context of the rat α SM actin promoter [11, 24]. However, the TCE within the SM22α promoter is very similar to the α SM actin TCE with regards its sequence (100% identity in the 1st 7 bp of the consensus TCE (see Fig 1a)), its ability to form a TGFβ-dependent shift complex (Fig. 1b), and its proximity to a nearby CArG element in each promoter (Fig. 1a). Mutation of the TCE completely abolished 445 SM22α promoter activity in vivo, an effect identical to that observed when a nearby CArG element (CArG near) (Fig. 1a) was mutated [32]. Indeed, we previously demonstrated that the α SM actin CArG element (CArG A) was required in combination with the TCE to coordinately regulate TGFβ-induced α SM actin expression in cultured cells [11]. In addition to its effect on arterial SMCs, the TCE mutation also abolished SM22α promoter activity in skeletal and cardiac cells during early embryonic development (Fig. 3). This is in agreement with our previous findings showing that the TCE can regulate α SM actin gene expression in non-SMC types [24].

GKLF (also known as epithelial zinc finger (EZF) or Krüppel-like factor 4 (KLF4)) is a Krüppel-like transcription factor containing three C2H2 zinc fingers. GKLF is thought to play
an important role in regulating cell growth and differentiation [38]. Our studies provide the first evidence implicating GKLF in the regulation of SMC differentiation marker gene expression. GKLF is known to bind to a CACCC-like motif present in the β-globin, keratin 4, and Epstein-Barr virus promoters [39, 40]. Interestingly, this motif is found in a 7 bp region of the TCE (GAGTGGG) that is completely conserved in both the rat and mouse αSM actin and SM22α TCE (Fig. 1a). Moreover, we have demonstrated that recombinant GKLF can bind to the TCE, but not if this GAGTGGG region is mutated to TATTGTT (Fig. 4b). Thus, the conserved GAGTGGG region that contains a known GKLF binding motif appears to be an important determinant of the TCE binding site.

Previous studies have demonstrated that GKLF expression is highest in skin [42], epithelial cells of the gut [38], lung, and vascular endothelial cells [39]. Indeed, although our studies have shown that GKLF is expressed in phenotypically modulated cultured SMC, GKLF expression was not detectable in differentiated smooth muscle containing tissues in vivo. This inverse relationship between GKLF levels and SM differentiation is in accordance with our co-transfection studies suggesting a role for GKLF as a TCE-dependent repressor of SMC differentiation marker gene expression (Fig. 6). Indeed, we have also shown that addition of TGFβ to cultured SMC rapidly down-regulates endogenous GKLF expression (Fig. 7). These studies suggest that the ability of TGFβ to promote the differentiation of SMC may be due to a down-regulation of GKLF repressor activity. Moreover, although not directly tested, the inference is that GKLF may play an important role in suppressing SMC differentiation (e.g. following vascular injury) in cases where TGFβ is absent or inactive. Of interest, GKLF has also been shown to both transactivate [40] and repress [39, 41] the activity of a variety of gene
promoters including; human keratin 4 [40], thymidine kinase [39] and CYP1A1 (cytochrome P-450IA1) [41]. It has been suggested that the presence of both a repression and activation domain may allow GKLF to switch between acting as a positive or negative transcription factor, and may be controlled by the binding of another factor(s) [48]. As such, we initially speculated that GKLF could both repress and activate SMC differentiation genes in a TCE dependent manner. However, a number of our findings indicated that GKLF was only repressing through the TCE: 1) GKLF repressed both SM22α and α SM actin promoter activities in co-transfection studies, 2) GKLF was expressed at very low levels in differentiated SMCs in vivo, 3) TGFβ treatment resulted in decreased GKLF expression in cultured SMCs, 4) there was an absence of detectable GKLF binding activity in the TCE shift complex formed with TGFβ treated SMC nuclear extracts.

GKLF is a member of an expanding sub-family of Krüppel-like factors that includes EKLF [44], BKLF [45], LKLF [49] and the basic transcriptional element binding protein (BTEB) factors, all of which share extensive homology in their carboxy terminal DNA binding domains and the ability to bind to a CACCC motif [46, 45, 43, 29]. Indeed, several of our observations indicate that removal of GKLF repressor function alone is not sufficient for TGFβ dependent increases in expression of SM22α or α SM actin. First, TGFβ treatment was associated with a large increase in binding to a TCE shift complex. Second, although TGFβ down-regulated GKLF expression, we observed the appearance of lower molecular weight proteins that cross-reacted with GKLF antibodies in SMC nuclear extracts stimulated with TGFβ. Since the antibody used was raised against the C-terminus of GKLF it would be expected to react with the conserved DNA binding regions of other related factors. In support of this
observation, a cold β-globin oligonucleotide containing a CACCC motif known to bind GKLF-related factors was able to compete with TCE factor binding. As such, these data suggest that the ability of TGFβ to stimulate expression of SM22α and α SM actin involves the removal of GKLF repressor activity from the TCE, allowing binding of another closely-related, positive-acting factor.

In preliminary studies aimed at identifying the trans-activating KLF protein binding to the TCE we chose two potential candidates, LKLF and a BTEB factor, BTEB2. BTEB2 was particularly interesting since previous studies have demonstrated that it is abundantly expressed in SM containing tissues where it can trans-activate expression of the embryonic form of the smooth muscle myosin heavy chain B gene [29]. Co-transfection results demonstrated that LKLF had no significant effect on SM22α promoter activity, however, BTEB2 was found to trans-activate the SM22α promoter. Moreover, an antibody raised against the conserved carboxy-terminal DNA binding domain of BTEB2 was shown to supershift the TGFβ dependent SMC shift complex formed with the TCE. Although there is a possibility that this antibody may cross-react with other KLF proteins, these studies at least suggest that BTEB2 may be the trans-activating factor binding to the TCE in vitro. Indeed, further studies will be required to demonstrate; 1) that it is BTEB2 and not a related positive-acting KLF protein binding to the TCE, 2) what effect BTEB2 has on the transcription of SMC differentiation marker genes such as SM22α in vivo.

Our studies demonstrating the reciprocal effects of GKLF and BTEB2 on SM22α promoter activity suggest the possibility that TCE dependent regulation of SMC differentiation may be controlled by two closely related competing transcription factors. In support of this, a
recent study by Zhang et al. [41] demonstrated that GKLF suppresses the CYP1A1 gene promoter by competing with the binding of Sp1, a potent activator of CYP1A1. Reciprocal control of TCE dependent SMC differentiation may provide a molecular mechanism that could account for the phenotypic plasticity of SMC that is known to play a key role in vascular injury responses [1]. Alternatively, given that GKLF is expressed in many non-SM cell types as well as phenotypically modulated cultured SMC, our studies may have highlighted a general mechanism whereby GKLF acts to repress transcription of SM genes in non-SM cell types. Since we have as yet no direct evidence that GKLF controls vascular SMC phenotypic modulation in vivo, further studies will determine whether de-differentiated intimal vascular SMCs express higher levels of GKLF than their differentiated, medial counterparts, and whether knockout or over-expression of GKLF alters the differentiated state of SMC in vivo.

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*TGFβ Control Element Dependent SM22α Gene Regulation*
FIGURE LEGENDS

Figure 1. A, Comparison of the TCE containing regions of the mouse and rat SM22α and α SM actin promoters (boxed). B, EMSA demonstrating that the SM22α TCE forms a TGFβ-dependent shift complex similar to that of the α SM actin TCE. EMSA was performed using nuclear extracts from aortic SMCs +/- TGFβ (2.5 ng/ml) stimulation. 5 µg of nuclear extracts were incubated with a 20 bp radiolabelled α SM actin (lanes 1 and 2), SM22α (lanes 3 and 4) or mutated (mut) SM22α (lanes 5 and 6) TCE probe. Competition reactions using both cold SM22α or α SM actin TCE oligonucleotide duplexes indicated that the TGFβ-induced complex (arrowed) was specific for each TCE (data not shown).

Figure 2. Diagram illustrating the SM22α-LacZ reporter gene constructs used to generate transgenic mice. A pUC19SM22-LacZ construct containing the region of the mouse SM22α promoter from -445 to +65 was used as a template for site directed mutagenesis of the TCE region. The four G toT mutations introduced in the TCE mutant construct are shown (Δ mutant).

Figure 3. Results of analysis of LacZ expression in transgenic mice harboring the wild-type and TCE mutant SM22α-LacZ transgenes. Embryos were harvested at 11.5 dpc and 13.5 dpc and fixed in 2% formaldehyde/0.2% paraformaldehyde; adult aorta were isolated from perfusion fixed 6-week-old transgenic mice. Embryos/aorta were stained for β-galactosidase activity using 5-bromo-chloro-3-indolyl-β-D-galactopyranoside substrate, then cleared in benzyl benzoate:benzyl alcohol (2:1). Similar results were obtained in four independent founder lines.
**Figure 4.** Yeast one hybrid cloning identified a TCE interacting protein that showed 100% homology to the mouse GKLF. **A**, Diagram illustrating the region of mouse GKLF (mGKLF) (GenBank accession number U20344) [38] represented by the 1265 bp one-hybrid cDNA clone. **B**, EMSA analysis showing binding of recombinant GKLF with the wild type but not the mutated SM22α and α SM actin TCEs. EMSA was performed by incubating 100 ng recombinant GKLF (recGKLF) with radiolabelled oligonucleotide duplexes representing the region of the β-globin promoter containing the GKLF-binding CACCC motif (positive control, lane 1), CArG B (negative control, lane 2), wild-type and mutant (SM22 mut) SM22α TCE (lanes 3 and 4), and, wild-type and mutant (αSMA mut) α SM actin TCE (lanes 5 and 6). Bovine serum albumin (BSA) control (100 ng) was also independently incubated with wild-type SM22α and α SM actin TCE (lanes 7 and 8). The ability of anti-GKLF antibody to supershift the GKLF SM22α TCE complex is also demonstrated (lanes 9 and 10). The major GKLF shift complex is indicated with an arrow.

**Figure 5.** **A**, Northern blot analysis showing the expression pattern of the GKLF one-hybrid clone in 2 µg poly A+ RNA isolated from various adult rat tissues (lanes 1-8) and adult mouse small intestine (lane 9). **B**, Semi-quantitative RT-PCR analysis of GKLF in adult mouse small intestine + (whole)/- epithelium. cDNA was generated from total RNA isolated from both whole intestine and the smooth muscle layer after the epithelial layer had been removed. Note the low expression of GKLF in the smooth muscle layer of the small intestine. Amplification of a GAPDH control gene is also shown to indicate that the levels of cDNA were equivalent.

**Figure 6.** Transient co-transfection analysis of multi-potent embryonic 10T1/2 cells
demonstrating the repression effect of GKLF on TGFβ-induced SM22α (A) and α SM actin (B) promoter activity. The SM22α-LacZ (A) and p2600Int-LacZ (B) reporter plasmids (1 µg) were co-transfected with empty pcDNA3 vector (Control) or pcDNA3-GKLF expression plasmid (GKLF) (1 µg) into vehicle or TGFβ (2.5 ng/ml) stimulated 10T1/2 cells. Relative promoter activities were obtained 48 hours post transfection by normalizing β-galactosidase activity against total protein content. Data are expressed as means (columns) ± standard deviations (error bars).

**Figure 7.** Northern, RT-PCR and Western blot analysis demonstrating the effect of TGFβ on GKLF mRNA and protein expression in aortic SMCs. **A**, Northern blots containing 50 µg of total RNA isolated from quiescent cultures of SMC stimulated for 24 hr +(T) or -(V) TGFβ (2.5 ng/ml) were hybridized with a radiolabelled GKLF probe (upper panel). Blots were re-hybridised with an 18 S rRNA probe to verify similar RNA loadings and transfer for each lane (lower panel). **B**, cDNA was generated from RNA isolated from cultured SMC stimulated for 3 and 8 hrs +(T) or -(V) TGFβ. Semi-quantitative RT-PCR analysis was carried out using primers designed to amplify a 495 bp N-terminal region of mouse GKLF. Amplification of a GAPDH control gene is also shown to indicate that the levels of cDNA were equivalent. **C**, Western blot analysis for GKLF in nuclear extracts from growth-arrested aortic SMC cultures stimulated +(T) or -(V) TGFβ (2.5 ng/ml) for 4 hours.

**Figure 8.** EMSA showing competition with the TCE complex using a β-globin oligonucleotide duplex containing the CACCC motif known to bind a number of Krüppel-like transcription
factors including GKLF. Competition reactions were performed by adding 50 (lanes 3 and 8), 100 (lanes 4 and 9), and 250-fold (lanes 5 and 10) molar excess (relative to the radiolabelled DNA) of cold β-globin oligonucleotide duplexes to nuclear extracts from TGFβ-stimulated aortic SMCs before the addition of either radiolabeled SM22α (lanes 1-5) or α SM actin (lanes 6-10) TCE. An arrow indicates disappearance of the TGFβ-dependent shift complex in each case.

**Figure 9.** A. Co-transfection analysis demonstrating the trans-activating effect of BTEB2 on TGFβ-induced SM22α promoter activity in 10T1/2 cells. SM22α-LacZ reporter plasmid (1 µg) was independently co-transfected with empty pcDNA3 vector (Control), pcDNA3-GKLF (GKLF) and pcDNA-BTEB2 (BTEB2) expression plasmids (1 µg) into vehicle or TGFβ (2.5 ng/ml) stimulated 10T1/2 cells. Relative promoter activities are indicated. Data are expressed as means (columns) ± standard deviations (error bars). B. EMSA analysis demonstrating that a BTEB2 antibody can supershift the TGFβ dependent shift complex formed with the TCE. EMSA was performed by incubating a 20 bp radiolabelled SM22α TCE probe with 5 µg nuclear extracts from aortic SMCs - (lane 1)/+ (lanes 2-4) TGFβ (2.5 ng/ml) stimulation. Super-shift analysis was performed by adding 2 µl of anti-GKLF (lane 3) or BTEB2 (lane 4) antibodies to the EMSA reactions. The super-shifted BTEB2 complex is indicated (ss).
Fig. 1
Fig. 2
Fig. 4
Fig. 5
Fig. 6

A

Relative activity

Vehicle
TGFβ

Control
GKLF

SM22αLacZ

B

Relative activity

Vehicle
TGFβ

Control
GKLF

p2600IntLacZ
Fig. 7
Fig. 8
Positive and Negative Acting Krüppel-like Transcription Factors Bind a Transforming Growth Factor Beta Control Element Required for Expression of the Smooth Muscle Cell Differentiation Marker SM22α In Vivo

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