Krüppel-like Factor 4 (KLF4/GKLF) Is a Target of Bone Morphogenetic Proteins and Transforming Growth Factor β1 in the Regulation of Vascular Smooth Muscle Cell Phenotype*  

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Vascular smooth muscle cell (VSMC) differentiation and phenotypic modulation is characterized by changes in mRNA expression for smooth muscle (SM) marker contractile proteins such as α-SM actin and SM22α. Transforming growth factor β1 (TGF-β1) is a potent VSMC differentiation factor; however, it is not known if other TGF-β-superfamily members, in particular the bone morphogenetic proteins (BMPs), modulate VSMC phenotype. Here we demonstrate that a large subset of TGF-β-superfamily members and their type I receptors are differentially co-expressed as VSMC phenotype changes during fetal/neonatal development and that BMP2, -4, and -6 reciprocally regulate SM-marker mRNA and protein expression in vitro. BMP2 and BMP6 decrease expression of the SM markers α-SM actin, SM22α, and calponin in rat VSMCs, whereas BMP4 increases their expression. The effects of BMP-2, -4, and -6 on SM marker gene transcription are mediated through a consensus TGF-β-controlling element, the TCE, which is common to regulatory regions of SM-marker genes. Moreover, co-treatment experiments revealed that BMP-2, -4, and -6 each inhibit TGF-β1-modulated increases in SM22α reporter gene activity. Regardless of whether they positively or negatively regulate SM marker expression, TGF-β1 and BMP-2, -4, and -6 all induced binding of the Krüppel-like transcription factor, GKL4/KLF4, to the TGF-β control element. Induction of KLF4 was confirmed by immunocytochemistry and Western blotting, which revealed that a lower molecular weight KLF4 protein is induced after treatment with TGF-β-superfamily members. Taken together, our results demonstrate that multiple members of the TGF-β superfamily act in concert to modulate VSMC phenotype.

Vascular smooth muscle cells (VSMCs)1 in the adult arterial media exhibit a differentiated phenotype characterized by expression of smooth muscle (SM) contractile genes such as SM22α, SM-myosin heavy chain (SM-MHC), α-SM actin, and calponin (1, 2, 4). In contrast, intimal VSMCs, commonly associated with diseased vascular tissue in adults, express low levels of these genes and resemble less differentiated fetal or neonatal VSMCs (1–4). Although genes characteristic of the differentiated VSMC phenotype have been identified, the morphogenetic factors that regulate VSMC phenotype remain largely unelucidated (5). Identification of these regulators may shed light on the mechanisms of VSMC phenotype modulation in the etiology of vascular diseases.

Transforming growth factor β1 (TGF-β1) exerts multiple effects on VSMCs in vitro, including a concentration-dependent effect on proliferation and stimulation of extracellular matrix protein synthesis (1, 6–8). Additionally, TGF-β1 potently up-regulates expression of SM-contractile marker genes in cultured VSMCs, pluripotent C3H10T1/2, and neural crest stem cells (9–11). Exploration of the molecular mechanism whereby TGF-β1 up-regulates α-SM actin gene expression in vitro led to the identification of a TGF-β control element (TCE), which is present in multiple SM-marker gene promoters and shares sequence similarity with the Sp1 recognition site (9). The 10-bp TCE element is required for α-SM actin promoter activity in vitro and SM22α promoter activity in vivo (9, 12). The effects of TGF-β1 on VSMC phenotype have been widely investigated; however, few studies have focused on the effects of other TGF-β-superfamily members, in particular the bone morphogenetic proteins (BMPs) in the regulation of VSMC phenotype. BMPs were initially isolated based on their ability to induce ectopic bone and cartilage formation in vivo in muscle tissue or subcutaneous sites of rodents (13). However, BMP expression studies as well as the analysis of BMP mouse knockout models suggest that BMPs exert a broad range of biological activities from cell proliferation, differentiation, and apoptosis to roles during development and that functional differences exist between different BMPs (14–16). BMP signaling is mediated via heterochimeric complex formation with type I and type II serine/threonine kinase receptors, which transduce their signals via SMAD proteins (17–19). Evidence suggesting a role for BMPs in VSMC biology is growing. Arterial expression of BMP2 at day 9.5 post-coitus and BMP4 and -6 at day 10.5 post-coitus in mice (20, 21) correspond with the temporal expression of the SM differentiation marker genes, h1 calponin, SM22α, and SM-MHC (22–24). Furthermore, BMP6 expression has been documented in both human intimal and medial VSMCs (25). Functionally, BMP7 has been implicated in maintaining VSMC phenotype in occluded rat renal arteries, whereas BMP2 instructs 10% of neural crest stem cells to differentiate into SMCs (11, 26). Expression of the type I TGF-β-superfamily receptors ALK2, -3, -5, and -6 has been shown in  

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1 The abbreviations used are: VSMCs, vascular smooth muscle (SM) cells; SMC, smooth muscle cells; TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; EMSAs, electrophoretic mobility shift assays; ALK, activin-like kinase; RT, reverse transcriptase; TCE, TGF-β controlling element; SM-MHC, smooth muscle myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid.
cultured adult rat VSMCs (27). ALK3 and ALK6 serve as BMP receptors, ALK5 functions as a TGF-β receptor, and ALK2 can be bound by activin and BMP7 (28, 29). Importantly, mutations in ALK1 result in human type II hereditary hemorrhagic telangiectasia, a condition characterized by multiple vascular defects thought to result from a deficiency in VSMC differentiation and recruitment, whereas mutations in the BMP type II receptor cause pulmonary hypertension (30, 31). Downstream from the receptors, BMP signaling molecules, SMADs, have been shown to be essential for vascular development. SMAD5 null mice lack an organized vasculature and present with large blood vessels containing a paucity of VSMCs, whereas SMAD6 knockout mice exhibit aortic ossification and increased blood pressure (32–34). In total these studies strongly suggest a role for the BMPs in the regulation of VSMC phenotype and/or vascular development.

Because they differentiate/mature, VSMCs progress through a phenotypic continuum that can be divided into fetal, neonatal, and adult stages. During fetal development, cells commit to the VSMC lineage at days 9–10 post-coitus, initiate expression of SM-marker genes, and proliferate rapidly (5, 22, 23, 24). Neonatal VSMCs exhibit a reduced proliferative index, increased SM-marker expression, and increased matrix synthesis, whereas adult VSMCs maintain basal expression levels of the SM-markers (5). Understanding how VSMC phenotypic modulation is regulated during development is particularly important, because intimal VSMCs associated with diseased vascular tissue express a phenotype that resembles that of normal medial VSMCs during fetal and neonatal development (5).

In this study we demonstrate that multiple TGF-β-superfamily members and their receptors are dynamically co-expressed during VSMC differentiation and can reciprocally regulate SM-differentiation marker expression via a TCE-dependent mechanism. In addition, the activity of TGF-β1 can be modulated by other members of the TGF-β superfamily. Mechanistically, TGF-β-superfamily members, whether positive or negative regulators of SM-marker gene expression, induce binding of the transcription factor KLF4/GKLF to the TCE element, demonstrating the importance of multiple TGF-β-superfamily members in VSMC phenotypic regulation.

EXPERIMENTAL PROCEDURES

Morphogens—Materials were obtained from common commercial suppliers unless otherwise stated. Human recombinant BMP-2, -4, and -6 were kindly provided by the Genetics Institute (Cambridge, MA). BMP-2, -4, or -6 (50 ng/ml), or vehicle (phosphate-buffered saline, 0.1% bovine serum albumin) were cloned into the TA-vector (Invitrogen) and sequenced to confirm identity. Levels of expression were determined on a Packard Instant Imager, which determines real time cpm of designated areas. All experiments analyzed in this manner were normalized to GAPDH.

Northern Blotting—RNA (10 μg) was electrophoresed on agarose gels (1.5%) containing 2.2 M formaldehyde and ethidium bromide for 20 h at 42 °C for 5 min. The lysates were then passed through a 25-gauge needle 3 times. Total RNA was quantified using a Bio-Rad protein assay system according to the manufacturer’s instructions. Equal amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) using a Transblot-SD semi-dry transfer cell. Membranes were incubated with antibodies to α-SM actin (3RD2), SM22α (3RF10), and 18 S probes were labeled using an Amersham Biosciences oligo-labeling kit (4). Hybridization was carried out at 65 °C followed by washing in 0.1× SSC, 0.1% SDS at 65 °C, and quantification was performed on a Packard Instant Imager and normalized to 18 S.

Western Blotting—VSMCs were solubilized in 100 μl of 0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.0004% (w/v) bromphenol blue and incubated at 100 °C for 5 min. The lysates were then passed through a 25-gauge needle 5 times. Total protein in each lane was quantified using a Bio-Rad protein assay system according to the manufacturer’s instructions. Equal amounts of protein were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) using a Transblot-SD semi-dry transfer cell. Membranes were incubated with antibodies to α-SM actin (Sigma clone 1A4, 1:1000 dilution), calponin (Sigma clone hCP, 1:1000 dilution) or GKLF (Santa Cruz, T16x, 1:1000 dilution).

Electrophoretic Mobility Shift Assay (EMSA) DNA binding experiments were carried out essentially as described above, except that the final protein extracts (prepared as described in McCaffery and Jackson (35)) were resolved on gels.

Transient Transfections—The pCB80 (p80) rat SM22α and pCB55 (p55) promoter fragments (36) were cloned into FGL-3 luciferase reporter vector (Promega). The TECE element was mutated using the Stratagene QuikChange site-directed mutagenesis kit. Constructs were confirmed by sequencing and DNA for transfections was prepared using EndoFree DNA preparation kits (Qiagen).

Rat VSMCs were seeded at a density of 6.6 × 10³ cells/60-mm plate and 20 h later transfected with 4 μg of DNA mixed with 20 μl (3 mg/ml) Superfect Reagent (Qiagen) for 2 h. The media was then changed, and the cells were treated with TGF-β1 (2.5 ng/ml), BMP-2, -4, and -6 (50 ng/ml), or vehicle (phosphate-buffered saline, 0.1% bovine serum albumin) for 48 h. Forty-eight hours post-transfection, cells were harvested in lysis buffer (Roche Molecular Biochemicals). Relative luciferase activity was determined on a luminometer and normalized to total protein levels. Four independent experiments were carried out. Experiments differed only in terms of treatment, as we have observed that viral promoters interfere with SM differentiation-marker promoter responsiveness due to interactions with CARG boxes present in these promoters (37). Data were analyzed using Student’s t test, and differences were considered significant for p < 0.05.

Electrophoretic Mobility Shift Assay (EMSA)—Growth arrested VSMCs were treated with TGF-β1 (2.5 ng/ml) or vehicle for 4 h, and nuclear extracts were prepared as in McCaffery and Jackson (35). A double-stranded DNA probe (50 ng) was labeled with α-32P(CTP (50 μCi) using Klenow, and unincorporated nucleotides were removed from the probe using a nucleotide removal kit (Qiagen).
Sequences of the oligonucleotides are given in Table I. A 20-μl binding reaction contained 10 μg of nuclear extract, 1× binding buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 50% v/v glycerol), 30,000 cpm labeled probe, 100 ng of poly(dA•dT)poly(dA•dT), and 50 ng of cold competitors where indicated. Binding reactions were incubated for 20 min at room temperature and resolved on 5% polyacrylamide gels. Supershift assays were performed as above except that a GKL, BTEB2, SP1, or AML1 antibody (Santa Cruz, GKL, T16x, BTEB2 (A-16x), AML1 (C-19x)) (2 μg/reaction) was added for 1 h before addition of the labeled probe.

**Immunocytochemistry**—Adult rat VSMCs seeded at a density of 10,000 cells/well on 4-well chamber slides were allowed to attach and were then serum-starved for 72 h before TGF-β1 or BMP2 treatment. Immunocytochemistry was carried out according to manufacturer’s instructions using a GKLF-specific or BTEB2 antibody and a fluorescently-tagged anti-goat secondary antibody (Sigma).

**RESULTS**

**Differential Expression of TGF-β and BMPs in Rat Neonatal Development**—Previously we demonstrated by Northern blotting that a number of SM-marker genes including α-SM actin, calponin, SM-MHC, and SM22α, were up-regulated during neonatal development (4). To confirm this expression pattern using RT-PCR, primers specific to calponin, SM-MHC, and SM22α were used in PCR reactions with cDNA derived from purified aortic VSMCs. This analysis confirmed up-regulation of SM markers between day 1 and week 2 of neonatal development (Fig. 1). Subsequently, the same RT-PCR system was used to examine the developmental expression profiles of TGF-β1–3, and the BMPs were identified above. All three TGF-β isoforms displayed similar expression profiles and were up-regulated during early neonatal development (neonatal day 1 to week 2) with high expression maintained until week 8 (Fig. 2). However, in the adult (week 12), expression of TGF-β2 and -3, and to a lesser extent TGF-β1, declined (Fig. 2).

Before examining BMP expression profiles during development, we determined which BMPs are expressed by adult rat VSMCs by using degenerate oligonucleotides (Table I) designed to conserve BMP sequences to amplify cDNA generated from these cells. This revealed that BMP2–6, activin βA, and GDF5 (growth differentiation factor) (data not shown) are co-expressed by adult rat VSMCs. RT-PCR profiling established that BMP2–7 and activin βA exhibit reproducible dy-
Dynamic expression patterns during VSMC differentiation (Fig. 3). BMP5 and -7 expression was high during fetal development and decreased during neonatal development. In contrast, expression of BMP2, -3, -4, and -6 and activin A was low during fetal development, up-regulated during neonatal development, and maintained at high levels until week 8. In the adult (week 12) expression of BMP4 and -6 declined, whereas expression of BMP2 and -3 and activin A remained elevated (Fig. 3). Although GDF-5 was identified in the degenerate PCR screen, it was not possible to amplify a GDF5 PCR product from these samples.

Co-expression of Type I TGF-β BMP Receptors and Their Ligands in Rat Neonatal Development—Because TGF-β isoforms and BMPs display distinct expression profiles during VSMC differentiation, we examined whether the expression of TGF-β isoforms and BMP type I receptors was also differentially regulated. This revealed that ALK2, -3, -5, and -6 and their ligands are co-expressed during VSMC differentiation (Fig. 4). The expression pattern of the BMP receptor ALK6 was similar to that of BMP2, -3, -4, and -6, with the greatest expression during neonatal development. ALK3, also a BMP receptor, was expressed during the neonatal phase of development, but its expression peaked specifically at week 2 (Fig. 4). In contrast ALK5, a TGF-β receptor, and ALK2 an activin/BMP7 receptor were expressed constitutively during development.

TGF-βs and BMPs Differentially Regulate α-SM Actin and SM22α mRNA Expression—Co-expression of TGF-β1–3, BMP2–7, and their receptors during VSMC development suggested a putative role for these factors in the regulation of VSMC phenotype. TGF-β1 and activin A have been shown to up-regulate α-SM actin gene expression (9, 38), but the effects of TGF-β2, TGF-β3, and the BMPs on VSMC differentiation marker gene expression are unknown. We chose to evaluate the effects of BMP2, -4, and -6 because these BMPs were made available to us and are expressed in normal adult vasculature.

Confluent adult rat VSMCs were growth-arrested and treated with TGF-β (2.5 ng/ml) or vehicle for 48 h. All three TGF-β isoforms increased SM22α and α-SM actin mRNA expression relative to vehicle-treated VSMCs (Fig. 5A). In contrast, BMP2 and -6 (50 ng/ml) down-regulated α-SM actin and SM22α expression (Fig. 5B), whereas BMP4 up-regulated expression levels of these genes relative to vehicle-treated cells (Fig. 5B).
TGF-β1 and TGF-β3 up-regulated SM actin protein expression (Fig. 6A). In contrast, BMP2 and -6 down-regulated and BMP4 up-regulated expression of SM actin and another SM differentiation marker, calponin (Fig. 6B). The level of expression of SM-MHC protein was also analyzed but was too low to detect (not shown).

To determine whether the observed changes in protein levels require new protein synthesis, VSMCs were treated with TGF-β superfamily members for 48 h in the presence of cyclohexamide (10 μg/ml). Cyclohexamide treatment blocked the effects of the TGF-β-superfamily members on SM actin protein expression, indicating that changes in protein levels are dependent on new protein synthesis (Fig. 6, A and C).

The TCE Element Is Required for BMP Responsiveness of the SM22α Promoter—Transient transfections were carried out using SM22α promoter constructs p80 and p55 linked to a luciferase reporter (Fig. 7A) (36). The p80 construct spanned bases −303 to +65 and has been shown to be 7-fold more active in VSMCs than the longer p55 construct (−1515 to +65) and two shorter (−193 to +65 and −117 to +65) constructs (36). The longer p55 construct was less active than p80 presumably due to upstream silencer sequences (36).

Transactivation assays with both the p55 luc and p80 luc promoter constructs revealed that BMP-2, -4, and -6 all repress SM22α promoter activity in VSMCs (Fig. 7, B and C). Down-regulation of SM22α promoter activity by BMP2 and -6 correlates with the effects of these ligands on SM-marker expression at the mRNA and protein levels (Figs. 5 and 6). Down-regulation of SM22α promoter activity by BMP4 contrasts with the effects of BMP4 on SM22α mRNA and protein levels (Figs. 5 and 6), suggesting that other promoter elements upstream of those included in the longer p55 construct are required for BMP4 enhancement of SM22α promoter activity. This contrasted with TGF-β1, which enhanced SM22α promoter activity of both the p55 and p80 constructs.
To determine whether the TCE element is required for BMP responsiveness of the SM22α promoter, the TCE element was mutated. Mutation of the TCE element abolished BMP-induced repression (Fig. 7C), indicating that an intact TCE element is required for BMP-regulated SM22α promoter repression.

To address whether the BMPs can modulate TGF-β1-induced SM22α promoter activity, the wild type SM22α p80 promoter construct was transfected in VSMCs followed by treatment with TGF-β1 in combination with BMP2, -4, or -6. These studies demonstrated that BMP-2, -4, and -6 can negatively regulate TGF-β1-induced increases in SM22α promoter activity (Fig. 7D).

**TGF-β1 and BMPs Enhance SMC Nuclear Protein Binding to a Cis-regulatory TCE—EMSA**s performed with nuclear proteins prepared from VSMCs treated with TGF-β1 or BMP2, -4, or -6 all formed three specific shifted complexes, c1-c3, with the TCE probe (Fig. 8A). Although c1-c3 were also apparent in assays with proteins from vehicle-treated cells, a greater degree of c1-c3 formation was observed in experiments utilizing nuclear protein extracts derived from cells treated with TGF-β1 superfamily members (compare lane 2 to lanes 5 and 8). Formation of these complexes was competed out by the addition of excess cold wild type TCE competitor. Excess of a mutated TCE cold competitor did not affect formation of the three complexes (Fig. 8A).

**KLF4 Is the Predominant Protein in TCE Complexes c1 and c2**—The TCE has been shown to bind Sp1 and the Krüppel-like transcription factors BTEB2 (KLF5) and GKLK (KLF4) (12). To determine whether these proteins are constituents of the VSMC TCE binding complexes, supershift assays were performed with antibodies specific for Sp1, BTEB2, and GKLK. An AML1 antibody was used as a negative control (Fig. 8B). Of the three specific complexes formed, the two upper complexes, c1 and c2, were competed out by the GKLK-specific antibody in the control TGF-β1- and BMP2-treated nuclear extracts (lanes 4, 8, and 12). No supershift or competition was observed with the Sp1 (not shown), BTEB2, or AML1 antibodies, thus identifying GKLK and an unknown lower migrating protein complex (c3) as the predominant TGF-β1- and BMP-induced protein that binds to the TCE.

**Members of the TGF-β Superfamily Induce the Expression of a Shorter Isoform of GKLK/KLF4**—Western blotting revealed that after treatment of VSMCs with TGF-β1, BMP2, and activin A, a lower molecular mass GKLK isoform was induced (Fig. 9A). A single 52-kDa GKLK protein was observed with the control extract, and a doublet containing an additional smaller GKLK protein was observed in extracts from cells treated with TGF-β1 superfamily members. It is possible that the lower molecular mass GKLK protein is a component of the c2 complex induced by TGF-β1 or BMP2 shown in Fig. 8; however, further characterization of the c2 complex is required before definitive conclusions can be drawn. Further analysis excluded phosphorylation and acetylation as the cause of the size differences between the GKLK protein bands (not shown).

Immunocytochemical assays further demonstrated the induction of GKLK/KLF4 by TGF-β1 and BMP2 above the levels in vehicle-treated cells (Fig. 9B). In contrast, BTEB2 expression was not observed in treated or control adult rat VSMCs (not shown).

**DISCUSSION**

In this study we demonstrated that multiple TGF-β-superfamily members can modulate expression of VSMC phenotypic
markers via a common cis-acting TCE element and that BMPs are able to modulate the effect of TGF-β1 on the transactivation of a SM-specific gene, SM22α. Modulation of SM-marker expression, whether positive or negative, was associated with the induction of a smaller GKLF/KLF4 protein that binds to the TCE element, a sequence common to a number of SM-specific gene promoters.

In vivo TGF-β-superfamily members, which are important modulators of VSMC phenotype, are differentially co-expressed during fetal/neonatal to adult development. This suggests that the ratio of different family members during development is important for VSMC phenotypic change and stability. It follows that a shift in the expression ratios of the different family members may act as a catalyst in the induction of vascular disease.

Recently, transgenic mice bearing an SM22α-lacZ promoter construct with a mutated TCE element revealed that the TCE element is essential for the in vivo expression of the SM-marker SM22α (12). In our system BMP-2, -4, and -6 were shown to transcriptionally repress SM22α promoter activity. The effects of BMP2 and -6 were consistent with results seen at the mRNA level; however, BMP4 repression of SM22α promoter activity was in contrast to its induction of SM22α mRNA. The difference between the transcriptional reporter assays and the mRNA levels is probably due to the presence of unidentified enhancer elements not present in the promoter regions covered by the p80 and p55 reporter constructs.

Utilization of a p80 reporter construct containing a mutated TCE element established that an intact TCE element is required for BMP-modulated SM22α promoter repression. The discovery of TCE-dependent BMP-modulated SM-marker expression suggested that the BMPs might compete with TGF-β1. This was validated in vitro as co-treatment of VSMCs transfected with the p80 reporter, with TGF-β1, and BMP2, -4, or -6 showed that each of these BMPs decreased TGF-β1-induced SM22α promoter activity, emphasizing the importance of the expression levels of multiple TGF-β-superfamily members in the regulation of VSMC phenotype.

Both positive and negative regulators of SM22α promoter activity induced TCE binding of VSMC nuclear proteins. Based on work by Adam et al. (12), we speculated that TGF-β-superfamily members may induce binding of different Krüppel-like transcription factors to the TCE. Adam et al. (12) identify GKLF as a TCE-binding protein using yeast one-hybrid analysis and showed that GKLF expression is down-regulated by TGF-β1. Co-transfection assays in C3H10T1/2 cells revealed that GKLF-repressed TGF-β1 induced α-SM actin and SM22α.
Regulation of VSMC Phenotype by TGF-β-Superfamily Ligands

Fig. 8. Analysis of TCE binding proteins. A, a competitive EMSA showing the effects of TGF-β1 and BMP2 treatment on protein complex formation on the TCE of the SM22α promoter. Nuclear extracts were obtained from adult rat VSMCs treated with vehicle (V), TGF-β1 (T1), or BMP2 (B2). Competitors were added to the gel shift reactions as follows. Lanes 2, 5, and 8, no competitor DNA; lanes 3, 6, and 9, 50 ng of wild type double-stranded competitor DNA; lanes 4 and 7, 10–50 ng of mutant double-stranded competitor DNA. Shifted complexes c1-c3 are marked. B, supershift analysis to determine proteins binding in complexes c1-c3. Nuclear extracts from above were used in supershift reactions containing the following additional antibodies. –, no antibody control; B, BTEB2; G, GKLF; A, AML1. Shifted complexes are labeled c1-c3. Lanes 2, 6, and 10, no antibody; lanes 3, 7, and 11, BTEB2 antibody; lanes 4, 8, and 12, GKLF antibody; lanes 5, 9, and 13, AML1 antibody.

Fig. 9. GKLF induction by TGF-β-superfamily members. A, Western blot showing the induction of GKLF after treatment of rat VSMCs with members of the TGF-β superfamily. Shown are vehicle (lane 1), TGF-β1 (lane 2), BMP2 (lane 3), BMP4 (lane 4), BMP6 (lane 5), BMP7 (lane 6), and activin A (lane 7)-treated nuclear extracts respectively. B, immunocytochemistry showing the effect of TGF-β and BMP2 on endogenous levels of GKLF/KLF4 expression. Panels depict control, TGF-β1, and BMP2-treated adult rat VSMCs.

cross-reacted with other KLF family members because 10 of the 12 amino acids of the peptide used to generate it are identical to GKLF (12). BTEB2 was originally identified as a marker of dedifferentiated VSMCs because it activates transcription of SMemb/nonmuscle myosin heavy chain-B and has been shown to have a role in vascular remodeling after injury (39). It is highly expressed during vascular development but is down-regulated in adult vessels; therefore, its absence in cultured adult VSMCs in serum-free conditions is not unexpected.

In this study we identified a shorter GKLF variant that was induced after TGF-β and BMP2 treatment. It is likely that the different GKLF variants are the result of post-translational modification of GKLF; however, this difference was not due to phosphorylation or acetylation. GKLF has previously been shown to contain both activation and repression domains (40). Because both positive and negative regulators of SM markers induce the smaller variant, it is likely that additional transacting factors are involved in SM-promoter regulation. These additional factors may bind directly to the GKLF complex or to other cis elements within SM-promoters.

In addition to defining a downstream target of TGF-β and BMP-modulated transcriptional regulation in VSMCs, this is the first report to describe the expression patterns of TGF-β-superfamily type I receptors and their ligands during neonatal VSMC differentiation. The dynamic in vivo expression profiles of these family members and their receptors further supports their role as major effectors of VSMC phenotype. TGF-β isoforms displayed similar expression patterns during neonatal development; however, expression of TGF-β2 and TGF-β3 declined in the adult. Isoform-specific differences in expression have previously been described in rat medial VSMCs 48 h after balloon injury, where TGF-β1 and -3 mRNA expression was elevated, whereas TGF-β2 mRNA expression declined (41). Although TGF-β isoforms have similar expression patterns and effects on SM-marker expression, it is possible that they differentially regulate other processes such as fibrogenesis or cell proliferation (42).

BMP5 and -7 were maximally expressed during fetal development, whereas BMP2, -3, -4, and -6 were up-regulated during neonatal development. Because little is known about the effect of the BMPs on VSMCs, it is difficult to speculate from these expression profiles as to the role of each factor. During neonatal development, a number of significant changes occur in VSMCs, including increased extracellular matrix, SM-contractile protein expression, and decreased proliferation (5). Re-
cently, the effects of BMP2 and BMP7 have been examined in injured arteries (20, 43). BMP7 maintained VSMC phenotype in occluded rat renal arteries, and BMP2 was shown to inhibit VSMC proliferation after balloon injury to rat carotid arteries (26, 43). We showed that BMP2 expression increased during the neonatal phase of development, a time when VSMC proliferation occurs (26, 43). We showed that BMP2 expression increased during this phase, yet in vitro BMP2 down-regulated SM-marker expression. Thus, BMP2 may regulate other aspects of VSMC phenotype. Further studies into this phenomenon could provide insight into BMP modulation of VSMC phenotype. Further supporting the notion that a balance of positive and negative regulators determines VSMC phenotype (5).

In summary, this study has provided mechanistic insight into BMP modulation of VSMC phenotype. Further studies into the roles played by specific BMPs in regulating VSMC phenotype are now required, given the correlation of VSMC phenotypic modulation with vascular disease.

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