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Expression and Promoter Analysis of a Highly Restricted Integrin Alpha Gene in Vascular Smooth Muscle

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Abstract

Full genome annotation requires gene expression analysis and elucidation of promoter activity. Here, we analyzed the expression and promoter of a highly restricted integrin gene, *Itga8*. RNase protection and quantitative RT-PCR showed *Itga8* to be expressed most abundantly in vascular smooth muscle cells (SMC). Transcription start site mapping of *Itga8* revealed the immediate 5' promoter region to be poorly conserved with orthologous sequences in the human genome. Further comparative sequence analysis showed a number of conserved non-coding sequence modules around the *Itga8* gene. The immediate promoter region and an upstream conserved sequence module were each found to contain a CArG box, which is a binding site for serum response factor (SRF). Luciferase reporter assays revealed activity of several *Itga8* promoter constructs with no apparent restricted activity to SMC types. Further, neither SRF nor its coactivator, Myocardin (MYOCD), was able to induce several distinct *Itga8* promoter constructs. Transgenic mouse studies failed to reveal *Itga8* promoter activity indicating distal regulatory elements likely control this gene's in vivo expression profile. Interestingly, although the promoter was unresponsive to SRF/MYOCD, the endogenous *Itga8* gene showed increases in expression upon ectopic MYOCD expression even though knockdown of SRF both in vitro and in vivo failed to demonstrate a corresponding change in *Itga8*. Collectively, these data demonstrate that *Itga8* expression is CArGSRF independent, but MYOCD dependent through an as yet unknown sequence module that is distal from the promoter region.

1. Introduction

Understanding how cell type identity is established requires the identification of genes whose expression is restricted to a given cell lineage as well as the characterization of promoter and enhancer sequences that confer cell-restricted expression. For example, smooth muscle cell (SMC) lineages are defined by the expression of dozens of cell-restricted genes that encode for proteins involved with contraction and the cytoskeleton, transcription, and cell signaling (Miano 2003; Owens et al 2004). This program of differentiation is thought to be usurped in various disease settings wherein SMC revert to a synthetic and highly proliferative state (Hershenson et al 2008; Owens et al 2004). Thus, there is increasing interest in understanding the transcriptional regulatory circuits involved with the

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maintenance of SMC differentiation and how these circuits are perturbed in disease states. Several SMC differentiation genes are directly regulated by the widely expressed transcription factor, serum response factor (SRF). SRF binds the decamer sequence CC(A/T)$_6$GG which is found in the immediate vicinity of over half of the SMC-restricted genes defined to date and a growing number of genes involved in cytoskeletal processes (Miano 2003; Olson and Nordheim 2010; Owens et al 2004). SRF interacts with scores of coregulators but the SMC- and cardiac muscle-restricted coactivator, myocardin (MYOCD) (Wang et al 2001), is of particular interest given its necessity (Huang et al 2008; Li et al 2003) and sufficiency (Long et al 2008) for biochemical and functional attributes of vascular SMC.

Alpha 8 integrin (ITGA8) belongs to a class of transmembrane proteins that form heterodimers with beta subunits (Hynes 2002). ITGA8 binds to the beta 1 integrin subunit (ITGB1), and together they serve as cell-surface receptors for tenascin C, fibronectin, vitronectin, osteopontin, and nephronectin (Brandenberger et al 2001; Schnapp et al 1995b). Functions for ITGA8 include cell-cell communication, cell-matrix interaction, and cell migration. Genetic inactivation of *Itga8* in mice results in defective kidney morphogenesis during development (Haas et al 2003; Müller et al 1997). We previously identified *Itga8* as a retinoid-responsive gene in a suppressive subtractive screen of primary rat aortic SMC (Chen et al 2001). In this report, we analyzed the expression of the murine and human *ITGA8* genes and show a preferential expression for aortic SMC tissue in both species. Comparative genomics facilitated the elucidation of chromosomal synteny and homologous sequences across the 200-kb locus. Further wet lab assays and bioinformatics tools allowed us to define the *Itga8* promoter, which appears to exhibit poor specificity in vitro and no measureable activity in transgenic mice. Luciferase reporter assays showed the promoter to be unresponsive to SRF and MYOCD despite the presence of a conserved SRF-binding CArG element in an upstream module. Unexpectedly, while loss in *Srf* had no effect on *Itga8* expression in vitro or in vivo, ectopic MYOCD elevated *Itga8* transcripts suggesting a CArG-independent sequence module that is responsive to MYOCD transactivation. Collectively, our data demonstrate a vascular SMC-restricted integrin gene that is SRF-independent, yet is activated by the SRF coactivator MYOCD.

2. Materials and methods

2.1. mRNA expression analyses of Itga8

We first used an RNase protection assay of mouse tissues harvested from FVB/NJ mice. Individual tissues were snap frozen in liquid nitrogen, homogenized with a Polytron PT10/35 (Brinkman), and total RNA isolated with TRIzol (Invitrogen). A riboprobe was generated with the following primers corresponding to the murine *Itga8* mRNA sequence (lower case letters here and below represent a clamp followed by a restriction enzyme site): forward primer, 5'-gatacgaattcATCTAGCGGGCTCAACAGGC-3', complementary to nucleotides +1 to +20 relative to the mapped transcription start site; reverse primer, 5'-gatacaagcttTTCCCGAACGCTGTTCACCCG-3', complementary to nucleotides +281 to +301. *Pfu* DNA polymerase was used to perform PCR amplification with the following conditions: 95°C for 3 min; 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; and 72°C for 7 min. PCR products were analyzed in 1% agarose gel, purified, cut with *EcoRI* and *HindIII* and cloned into pBSKII+. The vector was then linearized with *EcoRI* to serve as a template for in vitro transcription (Ambion) using alpha ^{32}PUTP (NEN Life Sciences). RNase protection was performed on 10 ug total RNA from mouse tissues using HybSpeed RPA kit (Ambion, Austin, TX) and radiolabeled riboprobes to the aforementioned *Itga8*, the SMC-restricted *Tagln1* (Li et al 1996), and *18 S* as an internal control for equal RNA loading. Products were resolved on 5% denaturing polyacrylamide gel and detected using X-OMAT film (Kodak). Quantitative RT-PCR was done as described

(Long et al 2009) on independent mouse tissues using the following primers to *Itga8* mRNA: forward primer, 5'-TGACACCACCAACAACAGG-3'; reverse primer, 5'-AGTTCTCCAGTGATACAAAGGG-3'. Northern blotting of store bought human tissue blots (Clontech) was done using a discriminating probe to human *ITGA8* mRNA.

2.2. Bioinformatics analysis

Genomic sequences corresponding to human, rat, and mouse *ITGA8* were downloaded from the UCSC Genome Browser (Kent et al 2002) and analyzed for conservation using VISTA alignment as described (Mayor et al 2000). Sequence modules adjacent to the mapped transcription start site were further interrogated using ClustalW. The Genetics Computer Group's FINDPATTERNS algorithm was used to determine whether any one of more than 1,200 permutations of the CArG box was present and conserved between human and mouse *ITGA8*.

2.3. Transcription start site mapping of Itga8

The transcription start site (TSS) of *Itga8* was mapped using First Choice RLM-RACE kit (Ambion Inc.) per the company's protocol. Total RNA was harvested from FVB/NJ mouse aorta using TRIzol (Invitrogen) and 10 ug was reverse transcribed to generate a cDNA template. Three antisense primers were synthesized (IDT, Skokie, IL) corresponding to the 5' untranslated region and coding sequence, with two rounds of nested PCR. The first PCR was carried out using the gene-specific outer primer GSP1; 5'-TTCTACGATATCCGGCTGGC-3' and the kit supplied outer adapter primer (AP1). The following PCR conditions were used: 94°C for 3 min; 35 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min; and 72°C for 7 min. The second PCR was carried out using gene-specific inner primer GSP2; 5'-GACATAAGCTTTTGAACGCCAGACACGCA-3' and kit supplied inner adapter primer 2 (AP2). The following PCR conditions were used: 94°C for 3 min; 35 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1.5 min; and 72°C for 7 min. PCR reactions were run ± 5% DMSO (to facilitate amplification of GC rich region), and analyzed on 1% agarose gel. PCR products were gel purified (Qiagen) and directly sequenced. An independent RNase protection assay was done using a probe similar to that used in Figure 1A only there was overlapping 5' sequence that would not be expected to hybridize to endogenous *Itga8* transcripts. Reactions were resolved on 6% denaturing polyacrylamide gel along with sequence reactions performed using 2 g of the original riboprobe template plasmid (Sequenase 2.0, Amersham) and 2 picomoles primer of sequence 5'-TTCCCGAACGCTGTTCAC-3'.

2.4. Cloning of Itga8 promoter

A mouse BAC (RP23-255H18) containing the 5' promoter and most of the *Itga8* locus was selected based upon sequence alignment, thus eliminating the need for a colony screen. This BAC was used to create all promoter constructs. A common reverse primer 5'-gatacaagcttTTCCCGAACGCTGTTCACCCG-3' (beginning 9 nucleotides upstream of the ATG methionine) was used in conjunction with several forward primers to generate a series of truncated murine *Itga8* promoter constructs (Fig. 4A). The position of the 5' end of forward primers (of length 21 nucleotides plus the gatacctcgag clamp/restriction site) is indicated with triangles (Fig. 4A). A 4,234 nucleotide promoter (not shown) construct was generated by ligating a *KpnI*/*StuI* digested fragment (from BAC clone) into an existing *Itga8* promoter construct for further study (below).

2.5. Transient transfections and luciferase assay

Pac1 SMC, Hek293, and Cos7 cells were grown to 70% confluency in 24-well culture dishes with 10% FBS in DMEM and refed with complete medium two hours prior to

calcium-phosphate mediated transfection (Graham and Van der Eb 1973). Briefly, quadruplicate wells were transfected with 0.5ug empty luciferase reporter (basic, Promega) or different *Itga8* promoter constructs in the presence of 50 ng renilla reporter as a control for transfection efficiency and cell number. We also co-transfected SRFVP16 or MYOCD_v3 (Imamura et al 2010) to assess the effects of each transactivator on *Itga8* promoter activity. The Mod1 sequence module was cloned upstream of the -578 *Itga8* promoter construct to ascertain whether there was any enhancer activity associated with this module.

After 24 hours, cells were rinsed twice with cold DPBS and lysates prepared using Dual Luciferase passive lysis buffer (Promega). 20 ul cell lysates were assayed for luciferase and renilla activity using a tube luminometer and relative normalized light units (RLUs) were plotted using Graphpad Prism 5.0 software.

2.6. SRF knockdown and MYOCD overexpression studies

We used varying MOI of adenovirus carrying MYOCD (Long et al 2008), dominant negative SRF (Miano et al 2000), a short hairpin RNA to SRF (Streb and Miano 2005), or MRTFA (kind gift from Dr. Paul Herring) in Pac1 SMC, primary mouse aortic SMC or human coronary artery SMC (HCASM) to assess effects on *ITGA8* mRNA expression by qRT-PCR or conventional (gel) RT-PCR. Controls included equivalent amounts of adenovirus with either lacZ or a short hairpin RNA to EGFP. In brief, cells were grown to 70% confluency in designated medium and then transduced with replicative defective adenovirus in 2% FBS plus DMEM for one hour at 37°C. Cultures were refed with complete medium overnight and then total RNA was isolated for RT-PCR as above. For in vivo *Srf* knockdown studies, mice carrying floxed *Srf* alleles (Miano et al 2004) and the *Myh11-Cre^{ERT2}* mouse (Wirth et al 2008) were treated five consecutive days with either sunflower oil or 33 μg/g tamoxifen (injected i.p.). Two weeks later, aortic tissue RNA was isolated for qRT-PCR analysis of *Itga8*, *Cnn1*, or *Srf* mRNA. PCR primers for expression analysis are available upon request.

3. Results

3.1. Itga8 mRNA Expression Profiling

Previous work suggested a restricted pattern of ITGA8 protein expression in vascular and visceral SMC lineages (Schnapp et al 1995a). However, there has been a limited analysis of the mRNA expression of *Itga8*. Accordingly, we utilized three independent assays to carefully assess *Itga8* mRNA expression across multiple tissue types. First, an RNase protection assay of mouse tissues revealed a striking enrichment for *Itga8* transcripts in aortic SMC tissue with much lower expression in all other tissues analyzed (Fig. 1A). The detection of two transcripts with the 5' riboprobe likely reflects alternative usage of a distinct TSS. The tissue profile of *Itga8* mRNA expression was different from the SRF-dependent SMC marker, *Tagln1*, which exhibited abundant expression in both vascular and visceral SMC tissues, particularly bladder and uterus (Fig. 1A). In a second independent assay, quantitative RT-PCR demonstrated similar enrichment for *Itga8* in aortic tissue with nearly 100-fold greater expression than all other tissues analyzed (Fig. 1B). Moreover, independent qRT-PCR expression studies showed that the relative expression of *Itga8* mRNA in intestine was nearly 10-fold lower than that observed in aortic tissue whereas the opposite was true for the CArG-dependent SMC-restricted genes, *Cnn1* and *Myh11* (Fig. 1C). Finally, Northern blotting of multiple human tissues demonstrated a prominent ~7.5-kb *ITGA8* mRNA transcript in aorta with virtually undetectable levels of expression across all other tissues (Fig. 1C). Together, these extensive expression data are consistent with a previous study done in rat tissues (Chen et al 2001) and extend the latter data to show selective

enrichment of *Itga8* transcripts in vascular versus visceral SMC types of human and mouse origin.

3.2. Itga8 Comparative Genomics and Transcription Start Site Mapping

A previous report documented the genomic structure and sequence variations within the human *ITGA8* locus (Ekwa-Ekoka et al 2004). Here, we examined the conservation of *Itga8* genomic sequence between human, mouse and rat. VISTA plotting revealed limited *Itga8* sequence homology between mouse and human across more than 200-kb; there was expected homology within the 30 exons that comprise the *Itga8* gene (Fig. 2A). The intronic regions are characterized by stretches of low homology, interspersed by relatively short regions (one to several hundred base pairs) of greater than 70% interspecies homology (Fig. 2A). Graphically, these peaks appear identical to the peaks that represent coding exonic sequence, though closer inspection of the raw sequence, and comparison of those to expressed sequence data (EST's), reveals that these peaks are not likely part of an alternately spliced form of *Itga8*. Both mouse (chromosome 2qA1) and human (chromosome 10p13) *ITGA8* genes are flanked at the centromeric end by orthologous loci annotated by NCBI. Notable differences are that the 5' intergenic distance is 35kb in the human, while ~15kb for the mouse. There is no synteny with respect to flanking genes in the telomeric direction (Fig. 2B).

We used 5' RACE and RNase protection assays to identify the major TSS for the mouse and human *ITGA8* genes. In both cases, there is a major TSS proximal to the initiating methionine, and a minor start site farther upstream. The major start site was observed to "slide" somewhat, though in the majority of RACE clones, the TSS mapped to 247 and 200 nucleotides upstream of the mouse and human initiating methionine, respectively (Fig. 3). Surprisingly, the immediate 5' promoter region showed little sequence homology between human and mouse *Itga8* (Fig. 3, top). In fact, out of 30 SMC-restricted genes, only the *Eln* (66% homology over 116 nucleotides) and *Myh11* (70% homology over 118 nucleotides) promoters had lower human-mouse homologies than *Itga8* (71% homology over 159 nucleotides). However, ClustalW alignment of two flanking modules (Mod1 and Mod2) revealed one (Mod1) to contain a conserved SRF-binding CArG box (Fig. 3) suggesting that *Itga8* may be under control of the SRF/MYOCD transcriptional switch as occurs with a growing number of SMC-restricted genes (Miano 2003).

3.3. Itga8 Promoter Sequence and Activity

Sequence analysis of the mouse 5' promoter region revealed several SMC-related transcription factor binding sites such as E boxes and a CArG-like box of little sequence homology to other orthologous sequences (Fig. 4A). There is no canonical TATA box or CCAAT box present, nor is there strong adherence to a consensus initiator sequence surrounding the TSS (Fig. 4A). To assess murine *Itga8* promoter activity in vitro, we transfected truncated promoter constructs into either a SMC line (Pac1) or several non-SMC types. The data revealed prominent promoter activity of each construct with little specificity for the Pac1 SMC line (Fig. 4B). Further deletion of the *Itga8* promoter revealed near complete loss in transcriptional activity between nucleotides −142 and −18 of the TSS (data not shown). To ascertain whether CArG-containing Mod1 (Fig. 3) is responsive to the SRF/MYOCD switch, we co-transfected Pac1 SMC with a Mod1-luciferase reporter ± SRFVP16 or Myocardin. No induction of the Mod1 reporter was seen with either SRFVP16 or Myocardin (Fig. 4C). On the other hand, two known SRF-dependent promoters, SM22 (Li et al 1997) and Calponin (Miano et al 2000), were induced by both transactivators (Fig. 4C). We next determined whether SRF/MYOCD could transactivate the −4,234 *Itga8* promoter, which harbors a proximal CArG box (Fig. 4A) and the distal conserved CArG box in Mod1 (Fig. 3). While both SRF and MYOCD potently transactivated the *Lmod1* promoter (Nanda

and Miano 2012), neither one induced the −4,234 *Itga8* promoter or shorter promoter constructs (Fig. 5). Finally, because in vitro promoter data do not always reflect in vivo activity, we introduced a −1,218 *Itga8* lacZ reporter gene into the pronucleus to assess lacZ staining in embryonic mice. Although we obtained several transgenic founders (n=12), 10/12 exhibited no detectable lacZ staining and two showed a mosaic pattern of staining with no resemblance to a SMC activity profile (data not shown). These results suggest that while the *Itga8* promoter is demonstrably active in vitro, distal elements are missing to coordinate SMC-restricted promoter activity in vivo.

3.4. Effects of SRF/MYOCD on Endogenous Itga8 mRNA Expression

The above *Itga8* promoter data suggest that despite the presence of CArG elements, the SRF/MYOCD switch is inactive over the *Itga8* locus. To formally prove this point, we evaluated *Itga8* mRNA expression under a variety of conditions where SRF/MYOCD could be modulated. As expected, known SRF target genes such as *Cnn1* are reduced upon knockdown of SRF with either a short hairpin RNA to SRF (Fig. 6A) or through Cre-mediated inactivation in mice (Fig. 6B). We also saw reductions in *Cnn1* upon transduction of cells with an adenovirus carrying a dominant negative *Srf* transgene (data not shown). However, irrespective of the approach to reduce wildtype SRF levels/activity, there was no corresponding decrease in endogenous *Itga8* mRNA (Fig. 6A–6B). These data are consistent with the inability of SRF to transactivate the *Itga8* promoter (Fig. 4C, 5B). Unexpectedly, ectopic MYOCD expression resulted in a consistent increase in *Itga8* mRNA levels across rat, mouse, and human SMC (Fig. 6C and data not shown). Interestingly, the closely related MRTFA coactivator (Wang et al 2002) had no measureable effect on endogenous ITGA8 mRNA (Fig. 6D). However, MYOCD dose-dependently increased levels of *ITGA8* mRNA in human SMC (Fig. 6D). Thus, while *Itga8* appears to be independent of SRF, the SRF coactivator MYOCD has a potentiating effect on *Itga8* mRNA levels indicating a novel mode of action.

4. Discussion

A major goal of the genomics revolution is to delineate the expression profile of every transcribed sequence and elucidate regulatory elements governing each transcript's expression profile. Such information will be of enormous value as we sort through the increasing number of sequence variants (mostly in the non-protein coding genome) linked to disease. In this report, we have demonstrated that *Itga8* mRNA is expressed abundantly in aortic SMC tissue of mouse or human origin. Comparative genomics reveals a moderately sized *Itga8* locus with low homology outside the protein coding region, including the 5' promoter. We note the presence of at least two CArG boxes in the upstream *Itga8* promoter region which exhibit neither in vitro nor in vivo SMC-restricted activity. Despite the presence of CArG boxes, the *Itga8* promoter constructs analyzed were completely refractory to SRF/MYOCD-dependent transactivation. However, ectopic MYOCD expression consistently elevated endogenous *Itga8* mRNA levels in several species of SMC. Collectively, these results demonstrate the presence of an aortic SMC enriched gene that is SRF-independent and MYOCD-dependent.

We previously cloned *Itga8* in an expression screen for genes induced by the anti-proliferative agent, all-trans retinoic acid (atRA) (Chen et al 2001). Since atRA exerts its biological effects on SMC by promoting their quiescent state (Miano and Berk 2000), we hypothesized that *Itga8* may be part of the normal SMC differentiation program. Indeed, studies in human and mouse tissues (this report) and rat tissues (Chen et al 2001) show clearly that *Itga8* mRNA expression is most abundant in adult aortic SMC that exhibit a contractile phenotype. The mRNA expression data reported here are somewhat incongruent with a previous study showing an apparent abundance of ITGA8 protein in intestinal SMC,

though there was reported weak expression in uterine SMC (Schnapp et al 1995a). We have been unable to demonstrate reliably consistent ITGA8 protein expression with commercial antisera; nevertheless, it is possible that the limited mRNA expression of *Itga8* mRNA in visceral SMC tissues such as bladder and uterus may coincide with stable expression of the protein. Further work will be needed with multiple antibodies to ITGA8 to definitively conclude expression of this integrin subunit at the protein level.

The SMC differentiated phenotype is characterized by a molecular signature of gene expression that includes various cyto-contractile genes, transcription factors, and signaling molecules (Miano 2003; Owens et al 2004; Spin et al 2004). Most of these markers are expressed similarly in both visceral (hollow organ) and vascular SMC. However, there are some notable exceptions. Telokin and smoothelin A are expressed more abundantly in visceral versus vascular SMC (Herring et al 2001; van Eys et al 2007). Conversely, smoothelin B is primarily expressed in vascular SMC (van Eys et al 2007). It appears as though *Itga8* is also restricted to vascular SMC as the level of mRNA expression in visceral SMC-containing tissues such as bladder, intestine, and uterus is very low. The functional significance of such differential expression of genes between visceral and vascular SMC lineages is unclear but may relate to the unique contractile properties of vascular versus visceral SMC.

MYOCD is part of a molecular switch for the induction of SMC contractile genes (Chen et al 2002). The majority of such genes contain one or more CArG box to which SRF/MYOCD binds (Miano 2003; Owens et al 2004). In this study, we used comparative genomics analysis of the *Itga8* gene to reveal a proximal CArG box in the immediate 5' promoter region as well as a more distal conserved CArG box. However, neither SRF nor MYOCD could activate the *Itga8* promoter in cultured cells suggesting that the CArG elements are non-functional. This is not surprising since there are thousands of conserved CArG boxes across the genome, some of which are inactive (Benson et al 2011). Consistent with this notion, we show here that *Srf* loss-of-function in cultured cells and in inducible knockout mice results in little change in *Itga8* mRNA. It is noteworthy that the only other vascular SMC-restricted gene (smoothelin B) is also independent of SRF for promoter activity (Rensen et al 2006). Interestingly, although *Itga8* expression and promoter activity is independent of SRF, we found that MYOCD overexpression increased endogenous *Itga8* in mouse, rat, and human vascular SMC. At present, we cannot discern between a direct effect of MYOCD over a novel regulatory element or an indirect effect through a conventional SRF-CArG-dependent pathway. Nevertheless, the data do suggest that MYOCD works independently of CArG-SRF over the *Itga8* locus. There are previous reports of MYOCD working to stimulate gene expression in an SRF-independent manner through distinct cis regulatory elements (Qiu et al 2005; Wang et al 2011). In this context, it will be of interest to define all MYOCD-dependent genes that are SRF-independent. Such information will likely reveal novel MYOCD interacting partners and their regulatory elements. Finally, studies examining Cre-mediated excision of *Myocd* in adult aortic tissue did not reveal a corresponding decrease in *Itga8* mRNA (data not shown). This finding would imply that baseline *Itga8* mRNA levels are independent of normal MYOCD expression. Alternatively, there may be a compensatory effect from a related MYOCD family member, though data reported here would suggest MRTFA not to be a candidate compensatory molecule.

The *Itga8* promoter, while demonstrably active in vitro, exhibits no specificity for SMC in cultured cells. Further, we were unable to demonstrate any discernible activity of the *Itga8* promoter in transgenic mice. This would suggest that the 1,218 nucleotide promoter construct used in vivo was insufficient for activity and that elements distal to these sequences are necessary for in vivo *Itga8* expression. In this context, there is considerable data showing the functionality of distal regulatory elements in the control of gene expression

(Giraldo and Montoliu 2001; Heaney and Bronson 2006; Long and Miano 2007). Thus, future analysis of the in vivo regulatory control of *Itga8* should employ the use of bacterial artificial chromosomes that will capture a larger genomic landscape and hence provide a better opportunity to demonstrate in vivo activity of this vascular SMC-restricted gene.

In summary, we have defined in multiple species a vascular SMC-restricted gene, *Itga8*, which is part of the molecular signature for this cell type's differentiated phenotype. We show through comparative genomics the conservation of an upstream CArG box that does not appear to confer SRF-dependent expression in vitro or in vivo. However, the SRF cofactor MYOCD is shown to induce the endogenous *Itga8* gene. An important future direction will be to identify the MYOCD-responsive regulatory site and its DNA-binding transacting factor.

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Highlights for Kitchen et al, “Expression and Promoter Analysis of a Highly Restricted Integrin Alpha Gene in Vascular Smooth Muscle”

- *Itga8* mRNA is highly specific for aortic SMC tissue in human and rodent
- *Itga8* promoter is active in vitro, but inactive in transgenic mice
- *Itga8* promoter contains a CArG box but is unresponsive to SRF/MYOCD
- Inactivation of SRF results in little change in endogenous *Itga8* expression
- Ectopic MYOCD induces endogenous *Itga8* expression

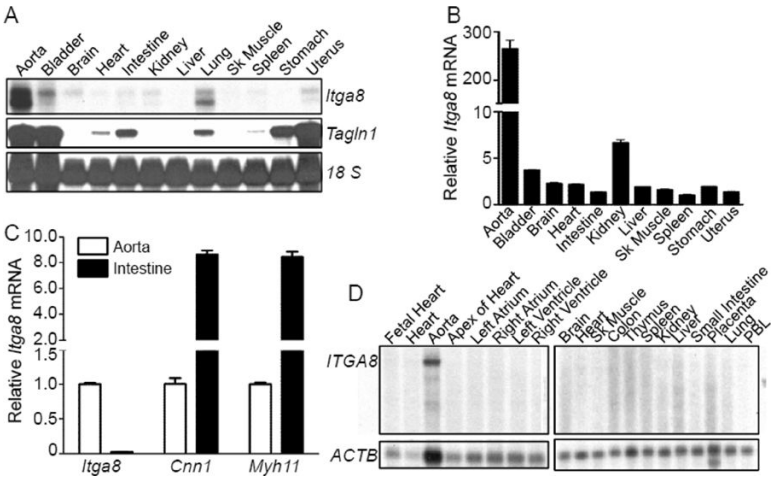

Figure 1. Tissue distribution of *Itga8* mRNA

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(A) RNase protection of *Itga8* mRNA in indicated adult mouse tissues. Note the over-abundance of *Itga8* mRNA in aorta versus other tissues as compared to *Tagln1* (aka SM22). (B) qRT-PCR of *Itga8* mRNA in independent mouse tissues (B) as well as aorta versus intestine (C). Note the differential expression of *Itga8* mRNA between aorta and intestine versus that of *Cnn1* and *Myh11*. (D) Northern blotting of comprehensive human tissues demonstrating similar enrichment of *ITGA8* mRNA levels in aortic muscle tissue versus all other tissues. All expression data are representative of multiple experiments performed by independent investigators.

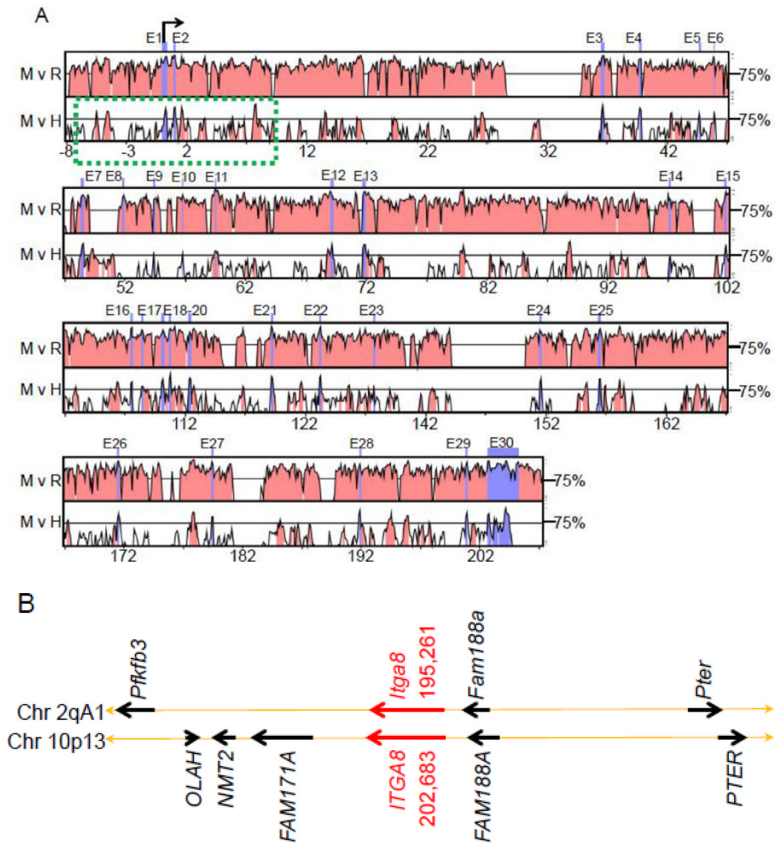

Figure 2. Comparative sequence and chromosomal analysis of *ITGA8*

Kitchen et al,

(A) VISTA plot of *ITGA8* and flanking sequences between mouse (M) versus rat (R) (top panels) and mouse versus human (H) (bottom panels). The green dashed boxed area is shown below in Figure 3. (B) Chromosome position of orthologous genes between human (Chr 10) and mouse (Chr 2). There is chromosomal synteny between species with *ITGA8* in the centromeric direction (pointing right) but not in the telomeric direction (pointing left). Schematic generated from the UCSC Genome Browser (http://genome.ucsc.edu/). Numbers below *ITGA8* represent the size of each transcribed gene.

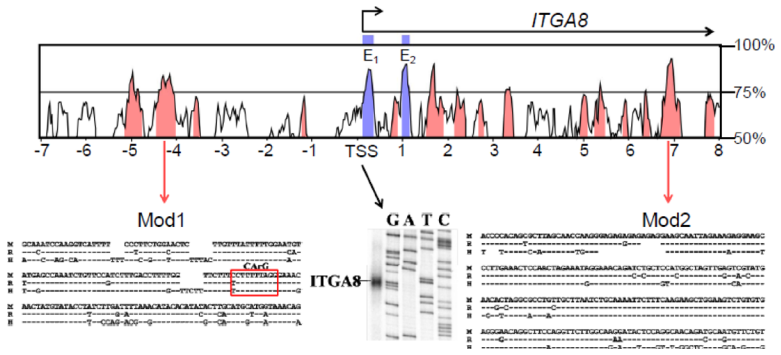

Figure 3. *ITGA8* transcription start site mapping and conservation of genomic sequence

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A VISTA plot (corresponding to the green dashed boxed region in Fig. 2A) of human versus mouse 5' *ITGA8* is shown with the first two exons (in blue). The x-axis represents the human coordinates of *ITGA8* and the y-axis indicates the percent homology between human and orthologous mouse sequences. The pink peaks represent intronic and upstream non-coding sequences with at least 75% of homology over 100 bp of sequence as in Figure 2A above. The bent arrow represents the mapped transcription start site (TSS, shown below in an RNase protection assay). Two conserved non-coding sequence modules (Mod1 and Mod2) are indicated by the arrows with corresponding sequence homology between mouse, rat, and human. A conserved CArG-like element is boxed (in red) in Mod1 (see below).

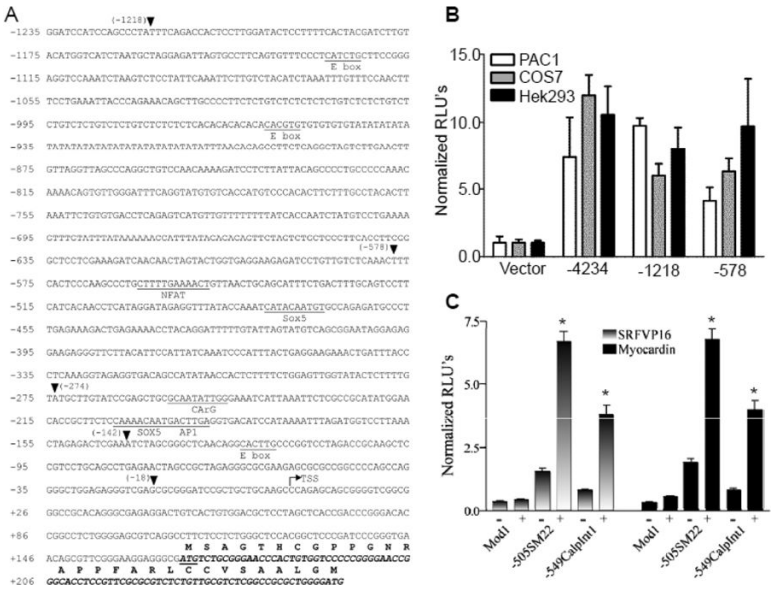

Figure 4. *Itga8* promoter sequence and activity

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(A) Nucleotide sequence of the mouse *Itga8* promoter and 5' coding sequence (bold italics and underlined). The 5' end of several promoter construct used in luciferase assays is indicated by a triangle and the relative position to the TSS is indicated in parenthesis. Putative binding sites predicted by TRANSFAC and GCG software are indicated as is the mapped TSS. (B) Deletion constructs of the mouse *Itga8* promoter corresponding to positions labeled in panel A were tested in the indicated cell lines and the luciferase activity normalized to a renilla reporter gene is shown. (C) Co-transfection experiments with either SRFVP16 or Myocardin (MYOCD_v3, (Imamura et al 2010)) and either Mod1 of *Itga8* fused to the −578 promoter or the SMC-restricted promoters SM22 or Calponin (Calp). All luciferase experiments were repeated multiple times in independent experiments and were statistically analyzed by one-way ANOVA with Tukey's posthoc test. Asterisks indicate $p < 0.05$ compared to corresponding empty vector control.

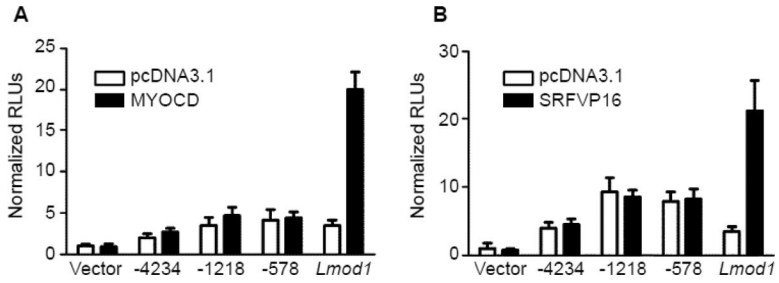

Figure 5. SRF and Myocardin effects on *Itga8* promoter activity

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Pac1 SMC were transfected with pGL3 Basic (vector) or the same luciferase backbone containing either −4,234, −1,218, or −578 nucleotides of the 5' promoter of *Itga8* in the absence or presence of MYOCD_v3 (A) or SRFVP16 (B). A Leiomodin 1 (*Lmod1*) promoter construct known to be SRF and MYOCD dependent (Nanda and Miano 2012) was included as a positive control. Data are representative of two independent studies done in quadruplicate.

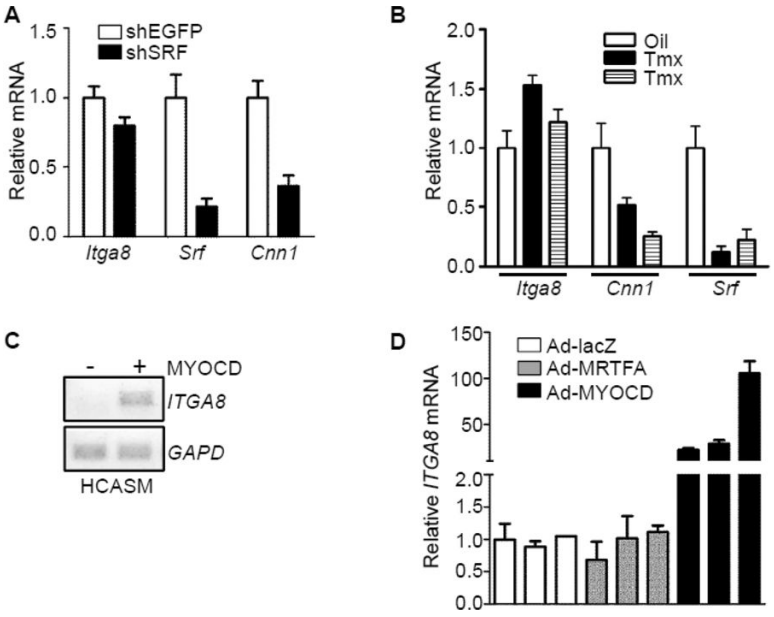

Figure 6. SRF and Myocardin effects on endogenous *Itga8* expression

Kitchen et al,

(A) Pac1 SMC were transduced with either shSRF or shEGFP adenovirus and endogenous *Itga8* mRNA analyzed by qRT-PCR. The expression of Itga8 was normalized to a house-keeping gene (*Ppia*) that showed no changes in expression across treatments. (B) Mouse aortic tissue in which *Srf* was deleted through Tamoxifen (Tmx) induced Cre-mediated excision was analyzed 3 weeks post Tmx or oil treatment by qRT-PCR. Two independent aortic samples treated with Tmx are shown as is an oil control. Note obvious decrease in *Srf* as well as known SRF target gene, *Cnn1*, but no similar decrease in *Itga8*. (C) Conventional (gel) RT-PCR of *ITGA8* mRNA in human coronary artery SMC (HCASM) transduced with 30 MOI of either Ad-lacZ (−) or Ad-MYOCD (+). This study is representative of at least three independent experiments on different isolates of HCASM. (D) Similar study as in panel C only HCASM were transduced with increasing titers (10, 30, or 100 MOI) of indicated viral constructs. This experiment was repeated once in HCASM and in several rat and mouse SMC lines (not shown).