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Characterization of zebrafish intestinal smooth muscle development using a novel *sm22a-b* promoter

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Abstract

Smooth muscle cells provide structural support for many tissues and control essential physiological processes, such as blood pressure and gastrointestinal motility. Relatively little is known about early stages of intestinal smooth muscle development and its relationship to the development of the enteric nervous system, which regulates intestinal motility. Here, we report an evolutionarily conserved 523 base pair regulatory element within the promoter of the zebrafish *sm22a-b* (*transgelin1*) gene that directs transgene expression in smooth muscle cells of the intestine and other tissues. Comparative genomic analysis identified a conserved motif within this element consisting of two Serum Response Factor binding sites that is also present in the promoters of many mammalian smooth muscle genes. We established a stable line expressing GFP in smooth muscle cell and used this line to describe lineage relationships among cells within different intestinal smooth muscle layers and their co-development with the enteric nervous system (ENS).

Keywords

zebrafish; smooth muscle; sm22a; transgelin; promoter; enteric nervous system ENS; transgenic; intestine; evolutionary conserved element (ECR); serum response element (SRE); colourless (cls)

Introduction

Transgelin/Sm22 α (hereafter Sm22 α) is an actin binding protein involved in actin filament and stress fiber formation (Assinder et al. 2009). Mammalian Sm22 α is one of the earliest markers for committed smooth muscle progenitor cells and is expressed in differentiated smooth muscle cells in all tissues (Solway et al. 1995).

The zebrafish has two *sm22a* gene paralogues; *sm22a-a* (also referred to as *transgelin2*, AY398421) and *sm22a-b* (also referred to as *transgelin1*, AY934529) that arose as a result of an ancient whole genome duplication that occurred in ray finned fish (Amores et al.

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1998). During evolution, gene paralogues often acquire specialized functions or are expressed in a restricted subset of cell types (Prince, Pickett 2002). Consistent with this, the zebrafish *sm22α* paralogues have partially overlapping expression patterns; *sm22α-a* seems to be expressed early in what have been considered to be undifferentiated smooth muscle precursor cells (Yang et al. 2003) while *sm22α-b* is first expressed when these cells start to differentiate (Georgijevic et al. 2007). The two *sm22α* paralogues are co-expressed in vascular smooth muscle and possibly intestinal smooth muscle during embryonic and larval stages.

sm22α-b is one of the earliest expressed markers of differentiated zebrafish intestinal and vascular smooth muscle cells (Georgijevic et al. 2007). Similar to mouse *Sm22α* (Li et al. 1996), weak expression of zebrafish *sm22α-b* can also be detected in embryonic trunk muscle at 60hpf (hours post fertilization) but not at later developmental stages (4dpf; days post fertilization) (Georgijevic et al. 2007). While both proteins have nearly the same level of similarity to the mammalian proteins, the expression pattern of *sm22α-b* suggests that it is the orthologue of mammalian *Sm22α*.

Previous studies have identified promoter regions within the human, mouse and rat *Sm22α* genes (Camoretti-Mercado et al. 1998; Xu et al. 2003; Osbourn et al. 1995; Solway et al. 1995). To identify comparable regulatory elements in the zebrafish *sm22α-b* promoter capable of driving smooth muscle expression *in vivo*, we performed comparative genomic sequence analyses of the *sm22α-b* upstream region of 4 species of fish. This analysis identified several highly conserved elements that were subsequently tested *in vivo* for enhancer activity. We generate a stable transgenic line expressing GFP in smooth muscle cells and use this line to study the temporal intestinal smooth muscle development *in vivo*. We show that the enteric nerves mature before the smooth muscle cells but that smooth muscle develops independently from the ENS.

Results

To find an autonomous promoter fragment that can drive transgene expression in intestinal smooth muscle of developing zebrafish we used data from the zebrafish genome sequencing project to isolate 2 genomic fragments of the *sm22α-b* promoter by PCR: one was comprised of 3 kb (kilo base pairs) DNA 5' of the first exon, the other was an overlapping 5 kb fragment that extended in the 3' direction up to the second exon, which contains the translational ATG codon (Fig. 1 A). Both fragments were cloned into a Tol2-GFP vector (Kawakami, Shima 1999; Kawakami et al. 2004) and injected together with mRNA encoding the Tol2 transposase into 1-cell stage zebrafish embryos. Fluorescence microscopy of the injected embryos showed that either fragments could drive GFP expression in smooth muscle cells suggesting that all essential control elements are located before the first exon. With both constructs, weak GFP expression was first detectable at 72 hours post fertilization (hpf). Expression was considerably stronger at 4 days post fertilization (dpf) and 5 dpf.

Regulatory enhancer or suppressor elements are often conserved between species and are separated by divergent DNA with no known function. To identify such regulatory elements we compared sequence upstream of *sm22α-b* exon 2 translation initiation codon with 10 kb

of DNA fragments derived from the *sm22α-b* orthologues of the green spotted pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*) and three-spined stickleback (*Gasterosteus aculeatus*) using the multiple sequence alignment tool Mulan (Ovcharenko et al. 2005). This identified 6 evolutionary conserved regions (ECRs) that were present in at least 3 of the 4 species (see additional online information). In zebrafish, ECR 1–4 are located several thousand base pairs upstream of exon 1, ECR5 is adjacent to the 5' border of exon 1, ECR 6 is adjacent to the 5' border of exon 2 (Fig. 1A). To test for enhancer activities, DNA fragments containing each ECR were cloned into GFP expression constructs containing a basal promoter derived from the carp actin gene in a Tol2 vector (Kawakami, Shima 1999; Kawakami et al. 2004) using the multisite gateway system (Kwan et al. 2007). Only embryos injected with the ECR5 construct had smooth muscle GFP expression replicating the 3 kb and 5 kb expression constructs. Embryos injected with ECR6 occasionally showed weak GFP expression in the heart area, while no GFP expression was detected in embryos injected with the constructs containing ECR 1–4.

We next tried to identify possible transcription factor binding sites within ECR5. The low complexity of these small DNA motifs makes it difficult to predict specific sites in the promoter of a single species; comparative genomic sequence analyses between different species, however, can predict *cis*-regulatory elements, based on the assumption that only specific binding sites are preserved between species (phylogenetic footprinting (Müller et al. 2002)). A clustalW alignment of ECR5 of the four fish species shows 6 conserved domains in ECR5 (Fig. 1 B); domains 1, 2, 3 and 6 were conserved between all species, whereas domain 4 and 5 were not well conserved in zebrafish. However, many small sequence motifs within domains 4 and 5 of the other fish species could be found immediately upstream of the zebrafish ECR5 fragment (data not shown), this sequence was included in the ECR5 expression construct. We used the multiTF function of the Mulan sequence alignment program to identify transcription factor bindings sites, which are conserved within ECR5 of the 4 fish species. Using the default parameter (“optimized for function” (Ovcharenko et al. 2005)) Mulan predicted only two serum response factor (SRF) binding sites (CArG boxes) in domain 3 and 6 of ECR5 (TRANSFAC data-base). Using less stringent parameters (matrix similarity 0.85, high specificity matrices only) MultiTF predicted an additional ETS binding site in domain 6 (Fig. 1B) and several STAT binding sites in domain 1 (not shown). These findings are noteworthy because paired SRF and adjacent ETS binding sites are present in the promoters of many vertebrate smooth muscle genes (Pipes et al. 2006; Wang, Olson 2004).

Smooth muscle GFP expression with the ECR5 expression construct was significantly stronger than with the larger promoter constructs tested. Larvae that had been injected with this construct were therefore used to establish a stable transgenic line (Tg(*sm22α-b*: GFP)). Analysis of F1 progeny from several F0 injected embryos showed GFP expression patterns that recapitulated the previously reported expression pattern of the endogenous *sm22α-b* mRNA (Georgijevic et al. 2007) (Fig. 2 A–E). Within larvae of each of these lines, smooth muscle cells expressing GFP covered the whole intestine at 72 hpf and persisted throughout larval development (Fig. 2 A, B). At 4 dpf GFP was also detected in the dorsal aorta, weakly in the proximal cardinal vein (Fig. 2 D) and in the ventral aorta and the bulbous arteriosus of

the heart (Fig. 2 E). GFP expression was detected in trunk skeletal muscle at 65 hpf and in contrast to *sm22α-b* mRNA (Georgijevic et al. 2007) weak GFP expression was still present at 4 dpf (Fig. 2 A, B, D), most likely because of perdurance of stable GFP protein. GFP expression was also detected outside of the previously reported *sm22α-b* expression domain (Georgijevic et al. 2007). This included the rostral notochord in all lines examined, the cardiac atrium and ventricle (starting at 24 hpf) and the eye (Fig. 2 C, E). Expression in the ventricle and atrium could arise from the absence of an inhibitory regulatory element in our constructs since mRNA is not detected here at 4 dpf (Santoro et al. 2009). This may also apply to *sm22a-b* expression in the notochord and eye but it is also conceivable that expression was overlooked in previous RNA in-situ expression studies.

Development of zebrafish intestinal smooth muscle has so far only be described using methods that could not resolve three dimensional cellular morphology (Georgijevic et al. 2007; Wallace et al. 2005; Olden et al. 2008). Confocal analysis of GFP expression in developing smooth muscle cells of Tg(*sm22α-b*: GFP) larvae allowed us to visualize smooth muscle organization in vivo. Two regions of intestinal smooth muscle were examined: in the mid-intestine close to the posterior border of the anterior intestinal bulb, and in the posterior intestine towards the anus. 3D reconstruction of confocal images revealed that at later stages of development intestinal smooth muscle is comprised of two cell layers: an inner circular layer and an outer longitudinal layer (Fig. 3 C–F). First GFP positive cells are detectable in the anterior around 67 hpf (not shown) and in the posterior at 72 hpf (Fig 3 B). At this stage the majority of the cells are organized in a single layer, with only few present in a second outer layer. Presumably the inner cells are precursors of the circular layer cells while the outer cells are longitudinal layer cell precursors. At this stage the cells in the inner layer have a rounded shape (Fig 3 A, B) that by 4 dpf has progressed to a dorso/ventral spindle shape typical of mature circular smooth muscle (Fig. 3C–D). By 4 dpf the inner circular and outer longitudinal layers are clearly distinguishable (Fig. 3 C, D). Based on the intensity of GFP fluorescence and cellular morphology, the proximal longitudinal layer cells examined in the mid-intestine appeared to develop before the posterior longitudinal layer cells. The morphology and organization of the circular and longitudinal layers continues to develop through 6 dpf. At this stage the cells appear fully differentiated in both domains examined (Fig. 3 E, F). The cells of the circular layer project as single strands surrounding the intestinal epithelium, while the cells of the longitudinal layer form straight long cables above the circular layer.

Transposase mediated transgenesis using the Tol2 system promotes stable integration of transgenes into the genome. Expression constructs are flanked by fragments of transposable elements and injected together with mRNA that encodes the transposase. Although embryos are microinjected at the 1-cell stage, transgene expression is always found in a mosaic fashion, thus indicating delayed genomic integration of the transgene (Kawakami, Shima 1999; Kawakami et al. 2004). We found that we can further decrease the number of transgenic cells by injecting sub-optimal doses of DNA and the mRNA encoding the transposase. Using this approach, we frequently found larvae that expressed GFP in patches comprised of only 2–3 cells at 72 hpf (Fig. 4 A, B), the stage when GFP is first detectable in germline transgenic larvae (Fig. 3 A, B). One explanation of this finding is that zebrafish

embryos have a large population of smooth muscle progenitor cells that have undergone proliferative expansion prior to the stage when transgenes integrate into the genome using the Tol2 methodology. We took advantage of this variation of the Tol2 methodology to study the ontogeny of intestinal smooth muscle cell subtypes. We followed 10 patches of cells (in a total of 8 larvae) from 72hpf-5dpf using fluorescence microscopy and analyzed the development of smooth muscle cells within the circular and longitudinal layers. Consistent with our analysis of germline transgenic larvae (Fig. 3), the first GFP positive cells are detected at around 72 hpf develop into circular layer smooth muscle cells (Fig. 4 A–F), the first longitudinal layer cells are detected at 80 hpf, in the mid-intestine (Fig. 4 C) and in the posterior intestine at 4 dpf (Fig. 4 F). Interestingly, the circular and longitudinal layer GFP positive cells were always found in the same location in all patches examined; that is, we did not detect longitudinal layer cells that were not associated with circular layer cells at any of the stages we examined. This suggests that circular and longitudinal layer cells arise from a common progenitor and do not develop independently.

The localization of GFP in smooth muscle cells also allowed us to study the development of smooth muscle interactions with the enteric nervous system *in vivo*. We crossed the *Tg(sm22α-b:GFP)* line with a line expressing DsRed under the control of the *Xenopus* neural-specific beta tubulin (NBT) promoter (*Tg(sm22α-b:GFP, NBT:DsRed)*), which drives expression in most neurons (Peri, Nüsslein-Volhard 2008). We examined smooth muscle innervations at 72 hpf and 5 dpf (FIG. 5, see additional online data for 3D images). Surprisingly, these studies showed well developed enteric neurons that had established a network of axonal projections at the stage when morphological differentiation of the GFP positive smooth muscle cells had not occurred (72 hpf; Fig 5 A, B). At this stage the neuronal cell bodies and axons were located on the surface of the smooth muscle layers. At 5 dpf most axons and cell bodies were located in the longitudinal layer on top of the circular layer (Fig. 5 C, D).

The finding that the enteric nervous system matures before the longitudinal cell layer has formed opens the possibility that smooth muscle development relies on nervous input or nerves form a scaffold for migrating longitudinal layer cells. *Colourless (cls)* larvae have a mutation in the *sox 10* gene resulting in deficient migration and maturation of neural crest cells (Dutton et al. 2001). As a result, the neural crest derived enteric nervous system is nearly completely missing in *cls* mutants (Kelsh, Eisen 2000) (Fig. 5 E, F). We crossed the *Tg(sm22α-b:GFP, NBT:DsRed)* line to *cls* to examine the smooth muscle morphology. At 5 dpf no enteric neurons could be detected in *cls* mutants, but neither the circular or longitudinal smooth muscle layer showed obvious morphological defects (Fig. 5 E, F).

Discussion

Here we report the identification of an enhancer element from the zebrafish *sm22α-b (transgelin1)* gene that drives reporter gene expression in intestinal and vascular smooth muscle. When coupled with a basal promoter, such as from the carp beta-actin gene (Villefranc et al. 2007), this regulatory element drives transgene expression in a pattern that is nearly identical to the expression pattern of the endogenous *sm22α* gene (Georgijevic et al. 2007). Our study suggests that *sm22α-b* promoter fragment and the *Tg(sm22α-b:GFP)*

larvae will be useful tools to study smooth muscle development in the zebrafish system. Indeed, the resolution and morphology of the in-vivo imaging facilitated by fluorescent reporters and confocal microscopy shown here is superior to antibody staining of fixed tissue specimens which until now only identified sub-cellular structures and could not outline the entire muscle cells. Similarly, histological analyses of specimens processed for mRNA in-situ hybridization have even lower resolution.

As an example of the utility of the reporter line we generated, we were able to infer the lineage relationship of smooth muscle cells within the circular and longitudinal layers. Our findings in mosaic F0 transgenic larvae suggest the presence of a common smooth muscle progenitor cell that gives rise first to circular smooth muscle layer cells, followed by longitudinal layer cells. Although smooth muscle cells are considered to arise from lateral plate mesoderm (LPM) (Reichenbach et al. 2008), it was not known whether an initial wave of migrating LPM cells form circular smooth muscle layer, followed by a second wave of migrating LPM that forms the longitudinal layer, or whether both layers arise from common precursor cell. Our finding argues for the existence of a common progenitor cell.

The ECR5 regulatory element contained two SRF binding CArG boxes and one ETS binding site that are conserved in the *sm22α* promoter between the 4 fish species examined. These binding sites are also present in the promoters of most mammalian smooth muscle genes, including the human, mouse and rat *sm22α* promoters (Camoretti-Mercado et al. 1998; Xu et al. 2003; Osbourn et al. 1995; Solway et al. 1995). Two promoter bound SRF proteins can form a ternary complex with Myocardin, a strong transcriptional activator in smooth muscle (Pipes et al. 2006; Owens 2007; Wang, Olson 2004). The presence of a nearby ETS site is suggested to be involved in the inhibition of this complex by replacing myocardin with activated ETS domain proteins (Wang, Olson 2004). The Myocardin/SRF complex has been extensively studied in mammals since it is involved in the phenotypic switching from a differentiated to a proliferative state during smooth muscle injury, such as occurs in hypertension and arteriosclerosis (Pipes et al. 2006; Wang, Olson 2004; Hendrix et al. 2005). The definition of a small functional *sm22α* promoter in zebrafish will facilitate phylogenetic footprinting to the mammalian promoters and might lead to the discovery of new conserved binding sites and factors which are involved in smooth muscle differentiation and proliferation.

The development of the enteric nervous system has been studied extensively in zebrafish (Shepherd et al. 2004; Pietsch et al. 2006; Olsson et al. 2008; and others). Previously, it has been shown that the onset of expression of neuronal markers coincides with the appearance smooth muscle markers in the intestine (Wallace et al. 2005; Reichenbach et al. 2008; Olden et al. 2008). Our results here show that differentiated enteric neurons with well defined axonal projections are present before smooth muscle cells have adopted their mature spindle-like shape and before the longitudinal muscle layer has formed, indicating that enteric nervous system development precedes smooth muscle development. Some neurotransmitters are expressed before the onset of smooth muscle contraction (Olsson et al. 2008), suggesting further that the enteric neuromuscular junction matures before smooth muscle is functional. Motility assays show that in the unfed state, ENS deficient *cls* mutants develop only uncoordinated contractions (Kuhlman, Eisen 2007). Together, these findings

suggest that the ENS could be involved in smooth muscle differentiation. We show here, that *cls* mutant do not have obvious smooth muscle defects at 5dpf arguing against a role of the ENS in smooth muscle differentiation. Consistent with this we recently show that in the fed state, *cls* mutants have functional peristalsis albeit reduced compared with wild type larvae (Davuluri et al. 2010).

Material and Methods

Identification of sm22 α -b full length mRNA, and cloning of promoter fragments

For the large genomic fragments we designed primers for the promoters adjacent to the first exon of AY934529 or proximal to the 2nd exon (see below for sequences). A recently published sequence (BC122180) extends the 5' UTR of the gene, suggesting that the first primer is within the UTR of exon 1.

To identify ECRs we isolated 10 kb of upstream sequence from the green spotted pufferfish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*) and three-spined stickleback (*Gasterosteus aculeatus*) using the Sanger ensemble database (<http://www.ensembl.org/Multi/blastview>). sm22 α -b was distinguished from sm22 α -a by a higher degree of homology in these alignments. ECRs were identified by the multiple sequence alignment tool Mulan (<http://mulan.dcode.org/> (Ovcharenko et al. 2005)).

To generate the expression constructs, 3 kb and 5 kb promoter fragments were cloned into pT2XIG (Kawakami et al. 2004) replacing the actin promoter and intron. The ECRs were cloned into a 5' gateway vector containing FseI and AscI sites. The ECR/basal actin-GFP/Tol2 vector was put together with a multisite gateway system as described (Kwan et al. 2007; Villefranc et al. 2007).

Generation of mosaic and germline transgenic fish

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the animal welfare committee University of Pennsylvania School of Medicine. To generate F0 transgenic larvae, 20 ng/ul of the expression constructs and 20 ng/ul Tol2 Transposase mRNA were co-injected into newly fertilized zebrafish embryos as described (Kawakami et al. 2004; Kwan et al. 2007; Villefranc et al. 2007). For mosaic analysis we injected 10–15ng/ul DNA and mRNA.

Fluorescence and confocal microscopy

Fish were raised at 28°C in embryo media E3 and eggs were collected as described (Westerfield 1995). At least five larvae were analyzed per stage.

Confocal microscopy—To optimally visualize intestinal smooth muscle cells in 3 dpf larvae, it was necessary to remove the yolk sac with fine forceps while the larvae were in DMEM/ 100 mg/l Tricaine (MS222, tricaine methanesulfonate (Rombough 2007)). To reduce the time between yolk removal and image acquisition the larvae were mounted in DMEM and held in place by fine glass fibers; images were acquired immediately after yolk

removal. Intestinal morphology was comparable in larvae with and without yolk. To mount de-yolked larvae, one end of 2 parallel glass fibers were glued to a 60 mm glass bottom Petri dish with nail polish. Larvae were secured underneath the two parallel glass fiber. Movement of the larva was prevented by a third fiber perpendicular and below the parallel fibers.

To image intestinal smooth muscle cells at 4 dpf and later stages, larvae were anesthetized with 100 mg/l Tricaine in E3 and mounted in a matec glass bottom dish by covering with 0.8% low melting agarose (Sigma) heated to 40°C in E3/Tricaine. To inhibit intestinal smooth muscle contraction at these stages, the concentration of Tricaine was increased to about 1000 mg/l immediately prior to image acquisition. To avoid effects of preparation and mounting on the development of the larvae individual larvae were examined at each stage.

Images were acquired with an inverted compound microscope (Olympus IX71) connected to a Yokogawa CSU10 spinning disc confocal unit and Photometrics HQ2 camera. Metamorph software was used to capture images and ImageJ software for processing. 3D images were processed using Volocity 5.0 software. Deconvolution was applied to the images in Fig. 5 using Volocity and a calculated PSF. The Tg(*NBT:DsRed*) line also expresses in nerve cells and axons of the skin. These nerves are distinguishable from the ENS since they are located in a separate layer above the intestine. For visualization purposes this staining was removed before the 3D reconstructions using ImageJ.

Compound Microscopy—For lineage tracing experiments and whole mount imaging larvae were mounted on methyl cellulose in E3 containing 100 mg/l Tricaine (Rombough 2007) and visualized using an upright microscope (Olympus BX61). For lineage tracing larvae were rinsed in fresh E3 without Tricaine and allowed to develop until the subsequent time-point. 10 patches of cells in a total of 8 larvae were analyzed from 72hpf to 5dpf.

Primer and sequences

-2000 5': AGACGACAGAATAGAGAGGGCGGTGT

Exon1 3': CAGCAGCTTTATGTTTCAGCACGG

Exon2 3': CACCTGTGATATTGCTCATACATATAT

ECR5 5': GTAAACAAGACGAGCTGAACGCCATC

ECR5 3': ATAAAACACTCGGGGCCGAGAGAGAA

ECR5 sequence (GeneBank acc # HQ009523):

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GTAAACAAGACGAGCTGAACGCCATCTACTGTCCGATCATTAAATCTGCAGA
GGACTGTCCTCATTTCACTGACTGCATGTGTGTGTGTTTCAGCTCAGTGTCTTC
TGCAGAAGAGGGAATGTAGAAACGTGAGCTCTGTTCAACATCAGATGAGAG
AAACTGGAGTTACTGAACGTACAAATAAAGCCATGAAAACCTAGATGAGTGG
AGAAAATAAACTCCATCACTCAGGAAAACCTAAACGGACGAGAGCAGAAC
AGCTGGAAAACACTAATGAACAACAAGCAAACTATAGTTGGTGTGTGTGTGT
GTGTGTGTGTGTGTGTGTTCTCAGCACTGATGCTGTATGATCTGGTATTTGG
AGAATTCCTCTCTGTGAGCAGACCGCAGCTCTTGGCTCTCCTCCTATCCTCA
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TCACAGCTCTTCCCTAATAAGGCTGCGCTTCAGCAAAGGCCGGGCTTCAGG
 AAAACTGCGTCTCCTTTTAAGGTGCCAGTTTCCTTTTCTCTCTCGGCCCCGAG
 TGTTTTAT

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Amores A, Force A, Yan YL, Joly L, Amemiya C, Fritz A, Ho RK, Langeland J, Prince V, Wang YL, Westerfield M, Ekker M, Postlethwait JH. Zebrafish hox clusters and vertebrate genome evolution. *Science*. 1998; 282:1711–1714. [PubMed: 9831563]
- Assinder SJ, Stanton JA, Prasad PD. Transgelin: an actin-binding protein and tumour suppressor. *Int J Biochem Cell Biol*. 2009; 41:482–486. [PubMed: 18378184]
- Camoretti-Mercado B, Forsythe SM, LeBeau MM, Espinosa R, Vieira JE, Halayko AJ, Willadsen S, Kurtz B, Ober C, Evans GA, Thweatt R, Shapiro S, Niu Q, Qin Y, Padrid PA, Solway J. Expression and cytogenetic localization of the human SM22 gene (TAGLN). *Genomics*. 1998; 49:452–457. [PubMed: 9615232]
- Davuluri G, Seiler C, Abrams J, Alexander SJ, Pack M. Differential Effects of Thin and Thick Filament Disruption on Zebrafish Smooth Muscle Regulatory Proteins. *Neurogastroenterology and Motility*. 2010
- Dutton KA, Pauliny A, Lopes SS, Elworthy S, Carney TJ, Rauch J, Geisler R, Haffter P, Kelsh RN. Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development*. 2001; 128:4113–4125. [PubMed: 11684650]
- Georgijevic S, Subramanian Y, Rollins EL, Starovic-Subota O, Tang AC, Childs SJ. Spatiotemporal expression of smooth muscle markers in developing zebrafish gut. *Dev Dyn*. 2007; 236:1623–1632. [PubMed: 17474123]
- Hendrix JA, Wamhoff BR, McDonald OG, Sinha S, Yoshida T, Owens GK. 5' CArG degeneracy in smooth muscle alpha-actin is required for injury-induced gene suppression in vivo. *J Clin Invest*. 2005; 115:418–427. [PubMed: 15690088]
- Holmberg A, Olsson C, Holmgren S. The effects of endogenous and exogenous nitric oxide on gut motility in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol*. 2006; 209:2472–2479. [PubMed: 16788030]
- Holmberg A, Schwerte T, Pelster B, Holmgren S. Ontogeny of the gut motility control system in zebrafish *Danio rerio* embryos and larvae. *J Exp Biol*. 2004; 207:4085–4094. [PubMed: 15498954]
- Kawakami K, Shima A. Identification of the Tol2 transposase of the medaka fish *Oryzias latipes* that catalyzes excision of a nonautonomous Tol2 element in zebrafish *Danio rerio*. *Gene*. 1999; 240:239–244. [PubMed: 10564832]
- Kawakami K, Takeda H, Kawakami N, Kobayashi M, Matsuda N, Mishina M. A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev Cell*. 2004; 7:133–144. [PubMed: 15239961]
- Kelsh RN, Eisen JS. The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. *Development*. 2000; 127:515–525. [PubMed: 10631172]
- Kuhlman J, Eisen JS. Genetic screen for mutations affecting development and function of the enteric nervous system. *Dev Dyn*. 2007; 236:118–127. [PubMed: 17131406]

- Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien CB. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn*. 2007; 236:3088–3099. [PubMed: 17937395]
- Li L, Miano JM, Cserjesi P, Olson EN. SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ Res*. 1996; 78:188–195. [PubMed: 8575061]
- Müller F, Blader P, Strähle U. Search for enhancers: teleost models in comparative genomic and transgenic analysis of cis regulatory elements. *Bioessays*. 2002; 24:564–572. [PubMed: 12111739]
- Olden T, Akhtar T, Beckman SA, Wallace KN. Differentiation of the zebrafish enteric nervous system and intestinal smooth muscle. *Genesis*. 2008; 46:484–498. [PubMed: 18781646]
- Olsson C, Holmberg A, Holmgren S. Development of enteric and vagal innervation of the zebrafish (*Danio rerio*) gut. *J Comp Neurol*. 2008; 508:756–770. [PubMed: 18393294]
- Osborn JK, Weissberg PL, Shanahan CM. A regulatory element downstream of the rat SM22 alpha gene transcription start point enhances reporter gene expression in vascular smooth muscle cells. *Gene*. 1995; 154:249–253. [PubMed: 7890172]
- Ovcharenko I, Loots GG, Giardine BM, Hou M, Ma J, Hardison RC, Stubbs L, Miller W. Mulan: multiple-sequence local alignment and visualization for studying function and evolution. *Genome Res*. 2005; 15:184–194. [PubMed: 15590941]
- Owens GK. Molecular control of vascular smooth muscle cell differentiation and phenotypic plasticity. *Novartis Found Symp*. 2007; 283:174–191. discussion 191-3, 238-41. [PubMed: 18300422]
- Peri F, Nüsslein-Volhard C. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. *Cell*. 2008; 133:916–927. [PubMed: 18510934]
- Pietsch J, Delalande JM, Jakaitis B, Stensby JD, Dohle S, Talbot WS, Raible DW, Shepherd IT. *lessen* encodes a zebrafish trap100 required for enteric nervous system development. *Development*. 2006; 133:395–406. [PubMed: 16396911]
- Pipes GC, Creemers EE, Olson EN. The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. *Genes Dev*. 2006; 20:1545–1556. [PubMed: 16778073]
- Prince VE, Pickett FB. Splitting pairs: the diverging fates of duplicated genes. *Nat Rev Genet*. 2002; 3:827–837. [PubMed: 12415313]
- Reichenbach B, Delalande JM, Kolmogorova E, Prier A, Nguyen T, Smith CM, Holzschuh J, Shepherd IT. Endoderm-derived Sonic hedgehog and mesoderm *Hand2* expression are required for enteric nervous system development in zebrafish. *Dev Biol*. 2008; 318:52–64. [PubMed: 18436202]
- Rombough PJ. Ontogenetic changes in the toxicity and efficacy of the anaesthetic MS222 (tricaine methanesulfonate) in zebrafish (*Danio rerio*) larvae. *Comp Biochem Physiol A Mol Integr Physiol*. 2007; 148:463–469. [PubMed: 17643329]
- Santoro MM, Pesce G, Stainier DY. Characterization of vascular mural cells during zebrafish development. *Mech Dev*. 2009; 126:638–649. [PubMed: 19539756]
- Shepherd IT, Pietsch J, Elworthy S, Kelsh RN, Raible DW. Roles for GFRalpha1 receptors in zebrafish enteric nervous system development. *Development*. 2004; 131:241–249. [PubMed: 14660438]
- Solway J, Seltzer J, Samaha FF, Kim S, Alger LE, Niu Q, Morrissey EE, Ip HS, Parmacek MS. Structure and expression of a smooth muscle cell-specific gene, SM22 alpha. *J Biol Chem*. 1995; 270:13460–13469. [PubMed: 7768949]
- Villefranc JA, Amigo J, Lawson ND. Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev Dyn*. 2007; 236:3077–3087. [PubMed: 17948311]
- Wallace KN, Akhtar S, Smith EM, Lorent K, Pack M. Intestinal growth and differentiation in zebrafish. *Mech Dev*. 2005; 122:157–173. [PubMed: 15652704]
- Wang DZ, Olson EN. Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr Opin Genet Dev*. 2004; 14:558–566. [PubMed: 15380248]
- Westerfield, M. *The Zebrafish Book: A Guide for the Laboratory use of Zebrafish (Danio rerio)*. University of Oregon Press; 1995.

- Xu R, Ho YS, Ritchie RP, Li L. Human SM22 alpha BAC encompasses regulatory sequences for expression in vascular and visceral smooth muscles at fetal and adult stages. *Am J Physiol Heart Circ Physiol*. 2003; 284:H1398–H1407. [PubMed: 12521938]
- Yang XY, Yao JH, Cheng L, Wei DW, Xue JL, Lu DR. Molecular cloning and expression of a smooth muscle-specific gene SM22alpha in zebrafish. *Biochem Biophys Res Commun*. 2003; 312:741–746. [PubMed: 14680827]

Figure 1.
sm22α-b promoter constructs used in this study. A: Promoter constructs (red) and evolutionary conserved regions (ECR, orange) tested for enhancer activity. B: ClustalW alignment of ECR5 from *Tetraodon nigroviridis* (Tn), *Gasterosteus aculeatus* (Ga), *Oryzias latipes* (Ol), and *Danio rerio* (Dr). 6 (1–6) segments with high conservation are present in all 4 (1, 2, 3, 6) or 3 of the 4 species (4, 5). Segment 3 contains a SRF binding CArG box; segment 6 contains a CArG box and an ETS-protein binding site.

Figure 2.

ECR5 of the *sm22a-b* promoter can drive GFP expression in smooth muscle cells. At 72 hpf (A) expression is detectable in the intestine (arrowheads) heart (*), the rostral notochord ("N") and trunk muscle cells. Starting at 4 dpf (B–E) expression is present in the intestine (arrowheads B) in the notochord ("N", C) the dorsal aorta ("DA" D) and the cardinal vein ("CV" D). In the heart (E) GFP is expressed in the bulbus arteriosus ("BA"), the atrium ("A") and the ventricle ("V") and the ventral aorta ("VA"). (A, B, D lateral view, C dorsal, E ventral; scalebar in E corresponds to 250 μm in A–C 100 μm in D and 50 μm in E)

Figure 3.

Smooth muscle differentiation. GFP expression in smooth muscle of the middle (A, C, E) and posterior (B, D, F) intestine. A–F: large panel: maximum intensity projections of confocal scans through half of the intestine (lateral view); lower panels: top 15 μm to visualize longitudinal layer; right panels: z-section at the position indicated in the lower panel. The longitudinal cell layer is located on top of the circular but is less bright and thus appears below in the large panels. At 72 hpf (A, B) circular layer cells are present but appear undifferentiated, with a round morphology and projections to adjacent cells. Few

undifferentiated longitudinal layer cells are present (A, B lower panel). At 4dpf (C, D) and 6dpf (E, F) cells become increasingly directional and differentiate to long strands. Both outer longitudinal and inner circular layer are distinguishable. (scalebar in F: 20 μ m, all panels same scale)

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Figure 4.

Clonal analysis of smooth muscle development. Mosaic expression of the ECR5 -GFP transgene (*Tg(sm22α-b: GFP)*) in developing smooth muscle cells in a domain in the middle of the intestine (A, C, E, G) and the posterior intestine (B, D, F, H). Frequently patches of 2–3 cells can be found at 72 hpf (A, B). At 80 hpf the cells elongate in circular directions and first longitudinal layer cells become visible in the middle domain (arrowheads C). More longitudinal layer cells develop between 80 hpf and 96 hpf, they are always located close to circular layer cells. (scalebar in H: 25 μ m, all panels same scale)

Figure 5.

Development of enteric nervous system (ENS) and smooth muscle. 3D reconstruction of smooth muscle cells expressing GFP (green) and ENS cells expressing DsRed (red) (lateral view; insets: z-sections at position indicated by broken line). A, C, E middle, B, D, F posterior intestine at 72 hpf (A, B) and 5 dpf (C–F). Enteric neurons with well-defined axonal projections are detected at 72 hpf while the smooth muscle is still differentiating (A, B) and the cells have a round shape in the posterior (B). At 72 hpf ENS cells are positioned above the smooth muscle cells (A, B). At 5 dpf most ENS cells are located above the circular within the longitudinal smooth muscle layer (C, D) axons can often be seen in parallel to longitudinal layer cells. Smooth muscle differentiation appears normal in colourless (*cls*) mutant larvae without ENS (E, F) (scalebar in F: 20 μ m, all panels same scale)