Sulf1 modulates BMP signaling and is required for somite morphogenesis and development of the horizontal myoseptum

Jason R Meyers [a],*, Jessica Planamento [a], Pierson Ebrom [a], Neil Krulewitz [a], Emma Wade [b], Mary E. Pownall [b]

[a] Biology Department, Colgate University, Hamilton, NY, United States
[b] Biology Department, University of York, York, United Kingdom

ABSTRACT

Heparan sulfate proteoglycans (HSPGs) are glycosylated extracellular or membrane-associated proteins. Their unbranched heparan sulfate (HS) disaccharide chains interact with many growth factors and receptors, modifying their activity or diffusion. The pattern of HS sulfation can be altered by the enzymes Sulf1 and Sulf2, secreted extracellular 6-O endosulfatases, which remove specific sulfate groups from HS. Modification by Sulf enzymes changes the binding affinity of HS for protein such as ligands and receptors, affecting growth factor gradients and activities. The precise expression of these sulfatases are thought to be necessary for normal development. We have examined the role of the *sulf1* gene in trunk development of zebrafish embryos. *sulf1* is expressed in the developing trunk musculature and as well as in midline structures such as the notochord, floorplate and hypochord. Knockdown of *sulf1* with antisense morpholinos results in poor differentiation of the somitic trunk muscle, loss of the horizontal myoseptum, lack of pigmentation along the mediolateral stripe, and improper migration of the lateral line primordium. *sulf1* knockdown results in a decrease in the number of Pax7-expressing dermomyotome cells, particularly along the midline where the horizontal myoseptum develops. It also leads to decreased *sdf1/cxcl12* expression along the mediolateral trunk musculature. Both the Pax7 and *cxcl12* expression can be restored by inhibition pharmacological inhibition of BMP signaling, which also restores formation of the myoseptum, fast muscle development, and pigmentation patterning. Lateral line migration and neuromast deposition depend on *sdf1/cxcl12* and FGF signaling respectively, both of which are disrupted in *sulf1* morphants. Pharmacological activation of FGF signaling can rescue the spacing of neuromast deposition in these fish. Together this data indicate that *sulf1* plays a crucial role in modulating both BMP and FGF signaling along the developing myoseptum to coordinate the morphogenesis of trunk musculature, associated pigment cells, and lateral line neuromasts.

© 2013 Elsevier Inc. All rights reserved.

Introduction

Somites are a defining feature of vertebrate embryos. They are transient structures, apparent as blocks of epithelial cells that progressively segment from the posterior mesoderm and lie in a paraxial position adjacent to the neural tube and notochord. Somite cells give rise to all the skeletal muscle in the body, as well as the ribs, vertebrae, and the dermis of the skin (Christ et al., 1998). In addition, the somites provide cues for migrating axons and in this way also give a segmental pattern to the peripheral nervous system (Keynes and Stern, 1984). The several different cell types known to derive from the initially naive epithelial somite are specified by local cell signaling (Emerson, 1993).

In vertebrates, skeletal muscle cells will differentiate as one of two physiologically distinct fiber types: fast or slow. In zebrafish, the decision for a cell to generate fast or slow muscle is made early in development while the somites are still forming (Devoto et al., 1996). Fate-mapping studies showed that the adaxial cells, positioned adjacent to the notochord, give rise to slow muscle cell types. As somites form, most adaxial cells migrate laterally to become superficial slow fibers (SSF), while a few adaxial cells remain adjacent to the notochord and become muscle pioneer cells (MPCs). MPCs are characterized by the expression of *engrailed* (Hatta et al., 1991; Ekker et al., 1992). Medial fast fiber (MFF) precursors develop in close proximity to the MPCs and also express *engrailed*, but at a lower level (Hatta et al., 1991). When MPCs differentiate they remain mononucleate and stretch from the notochord to the lateral somite surface, in this way foreshadowing the horizontal myoseptum, a structure which divides each somite into dorsal and ventral sections and gives somites

their characteristic chevron-shape. Mutants without a horizontal myoseptum develop somites with a characteristic U-shape (Van Eeden et al., 1996). Some of these mutants also lack a notochord, such as *no-tail* (Halpern et al., 1993) and *floating-head* (Talbot et al., 1995), while in other U-mutants the notochord is present, such as *sonic-you* and *you-too* (Van Eeden et al., 1996).

The horizontal myoseptum provides guidance for the migration of the posterior lateral line (PLL) primordium, which migrates posteriorly from the otic vesicle, along the horizontal myoseptum, depositing neuromasts at regular intervals (Whitfield, 2005). In addition, the horizontal myoseptum coordinates the migration of a mediolateral stripe of melanocytes (Svetic et al., 2007). MPCs and the horizontal myoseptum therefore play essential organizational roles for somite structure which subsequently imparts guidance cues to pattern other developing systems.

Hedgehog signaling from the notochord induces adjacent adaxial cells to a slow muscle fate(Blagden et al., 1997; Hirsinger et al., 2004). In response to high levels of Sonic hedgehog (Shh), some cells will activate high levels of *engrailed* expression and become MPCs, while neighboring cells receive less Shh signal, express lower levels of *engrailed*, and become MFFs (Wolff et al., 2003). The ability of hedgehog signaling to specify different cell types in a concentration-dependent manner is supported by experiments in other developmental models and suggests it behaves as a morphogen (Torroja et al., 2005; Dessaud et al., 2008). Superimposed on the activating Shh signal, BMP signaling from dorsal and ventral parts of the myotome has been shown to restrict *engrailed* expression to the central region where MPCs form (Dolez et al., 2011). The antagonistic effects of Shh and BMP signaling have been further elucidated at the level of the enhancer element that drives *engrailed* expression in the MPCs (Maurya et al., 2011). In addition, heparan sulfate proteoglycans (HSPGs) have been found to be essential for the ability of BMP to pattern the myotome (Dolez et al., 2011).

HSPGs are essential for many cell signaling pathways important for development, including hedgehog and BMP signaling (Lin, 2004). HSPGs consist of a protein core to which glycosaminoglycan (GAG) chains are attached. These unbranched chains of disaccharide repeats can be modified, or not, by sulfation; this results in a high degree of structural heterogeneity and allows HSPGs to bind many different proteins (Turnbull et al., 2001). The secreted sulfatase enzymes Sulf1 and Sulf2 can act at the cell surface to remodel HSPG structure by specifically removing a sulfate group from the 6-O position of glucosamine in heparan sulfate (HS) chains. This modification changes the affinity of HS for ligands and receptors and impacts cell signaling (Lai et al., 2004; Ai et al., 2006; Freeman et al., 2008).

sulf1 and two *sulf2* genes have been described in zebrafish and have been shown to be expressed in the central nervous system and the somites during early development (Gorsi et al., 2010), though their function in control of developmental signaling has not be examined. Here we use antisense morpholino oligonucleotides to knock-down *sulf1* and find abnormal somite development, with a loss of the horizontal myoseptum. These embryos also display disrupted migration of the PLL primordium and a failure of pigment cells to migrate. Pharmacological inhibition of BMP signaling rescues the formation of the horizontal myoseptum, and restores the normal migration of the PLL primordium and pigment cells. We conclude that *sulf1* acts an essential modulator of BMP signaling and is required for normal morphogenesis of the zebrafish somite.

Methods

Fish lines and general care

Wild-type fish were of the TL strain, and were obtained from the Zebrafish International Resource Center (ZIRC; Eugene, OR).

In *Tg*(*cldnb1:lyn-gfp*) fish, GFP is expressed in the cells of the lateral line, including the migrating primordium (Haas and Gilmour, 2006), and was a kind gift of Darren Gilmour. In *Tg*(*BMPRE:eGFP*) fish, GFP is expressed under control of repeated SMAD-mediated BMP response elements (Collery and Link, 2011), and were a kind gift of Brian Link. Fish were maintained on a 14:10 light:dark cycle at 28.5 °C unless otherwise stated. All care and use of fish was approved by the Animal Care and Use Committee at Colgate University.

Morpholino injections

Anti-sense morpholino oligonucleotides were designed against the start site and the splice boundary between intron 2 and exon 3 of the zebrafish *sulf1* gene, using the following sequences:

sulf1 start AMO: CATCATGGGACTGCGAACGCGAATC
sulf1 splice AMO: ATCCTGACACACAAGACAGACAACA

Morpholinos were reconstituted to 1 mM in water, and were injected using a picoinjector (Harvard Apparatus, Holliston MA) to deliver 1 pmol of the *sulf1* start AMO or 0.25 pmol of the *sulf1* splice AMO to zebrafish embryos at 1–2 cell stage. Fish were then returned to incubation in E3 medium (Westerfield, 2007). To confirm that the splice morpholino altered levels of correctly spliced mRNA, 15 uninjected and 15 *sulf1* splice AMO injected fish were collected at 24 hpf and frozen in liquid nitrogen. RNA was extracted using PureLink RNA isolation kit (Life Technologies, Grand Island, NY), and cDNA generated using DyNAmo cDNA Synthesis kit (Thermo Scientific, Lafayette, CO). cDNA was amplified via PCR using primers spanning two different exons in *sulf1* or the housekeeping gene HPRT1.

Pharmacological manipulation

To inhibit BMP signaling, zebrafish embryos were incubated in 5 μM dorsomorphin (EMD Millipore, Billerica MA, 10 mM stock in DMSO) or 2 μM LDN193189 (Selleck Chemical, Houston TX, 10 mM stock in DMSO) diluted in E2 medium (15 mM NaCl, 0.5 mM KCl, 1 mM MgSO$_4$, 0.15 mM KH$_2$PO$_4$, 0.05 mM Na$_2$HPO$_4$, 1 mM CaCl$_2$, 0.7 mM NaHCO$_3$) beginning at 12 hpf (Boergermann et al., 2010). Control embryos were treated with 0.1% DMSO. To enhance FGF signaling, we incubated zebrafish embryos 2.5 μM BCI (EMD Millipore), a Dusp1/6 inhibitor (Molina et al., 2009) beginning at 30 hpf.

Time-lapse microscopy

To directly observe migration of the lateral line primordium, conducted time-lapse microscopy as in Haas and Gilmour (2006). Briefly, we anesthetized 24 hpf Tg(cldnb:lyngfp) zebrafish embryos, which express membrane tagged GFP in all lateral line cells, with 0.003% tricaine (Sigma-Aldrich, St. Louis MO), and embedded them in 0.75% low-melt agarose in E3 media on coverglass, then imaged on a Zeiss 710 confocal laser scanning microscope. Images were acquired every 15 min for approximately 18 h.

Immunocytochemistry and in situ hybridization

Fish were fixed with 4% paraformaldehyde, and then processed either for whole-mount labeling or cryosectioning. For whole-mount labeling, fish were treated with acetone at −20 °C for 10 min, rehydrated in PBS with 0.1% Tween-20 (PBST), blocked in 10% normal goat serum, incubated in primary antibodies over-night, washed with PBST, then incubated in secondary antibodies