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Research paper

Cloning of zebrafish *Mustn1* orthologs and their expression during early development

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ABSTRACT

Mustn1 is a small nuclear protein that is involved in the development and regeneration of the musculoskeletal system. Previous work established a role for *Mustn1* in myogenic and chondrogenic differentiation. In addition, recent evidence suggests a potential role for *Mustn1* in cilia function in zebrafish. A detailed study of *Mustn1* expression has yet to be conducted in zebrafish. As such, we report herein the cloning of the zebrafish *Mustn1* orthologs, *mustn1a* and *mustn1b*, and their expression during zebrafish embryonic and larval development. Results indicate a 44% nucleotide identity between the two paralogs. Phylogenetic analysis further confirmed that the Mustn1a and 1b predicted proteins were highly related to other vertebrate members of the Mustn1 protein family. Whole mount *in situ* hybridization revealed expression of both *mustn1a* and *1b* at the 7-somite stage through 72 hpf in structures such as Kupffer's vesicle, segmental mesoderm, head structures, and otic vesicle. Additionally, in 5 day old larva, *mustn1a* and *1b* expression is detected in the neurocranium, otic capsule, and the gut. Although both were expressed in the neurocranium, *mustn1a* was localized in the hypophyseal fenestra whereas *mustn1b* was found near the posterior basicapsular commissure. *mustn1b* also displayed expression in the ceratohyal and ceratobranchial elements of the pharyngeal skeleton. These expression patterns were verified temporally by q-PCR analysis. Taken together, we conclude that *Mustn1* expression is conserved in vertebrates and that the variations in expression of the two zebrafish paralogs suggest different modes of molecular regulation.

1. Introduction

Mustn1 (originally termed as Mustang) was previously identified and cloned from mRNA isolated from a rat fracture callus and encodes for a 9.6 kDa nuclear protein. Its up-regulated temporal expression during fracture repair was localized to multiple cell types within the callus, including periosteal osteoprogenitors, osteoblasts and proliferating chondrocytes (Lombardo et al., 2004). Further analysis during skeletal development in rat revealed expression predominantly in the musculoskeletal system; mesenchymal condensations of limbs, vertebral perichondrium, mesenchymal cells of the intervertebral discs (Lombardo et al., 2004), in mouse somites and skeletal muscles (Liu et al., 2010) and mouse limb buds, branchial arches and tail bud (Gersch and Hadjiargyrou, 2009).

Functional perturbation analyses using RNA interference indicated that *Mustn1* plays a critical role in both skeletal myogenesis and craniofacial chondrogenesis. More specifically, targeted down-regulation of *Mustn1* in mouse C2C12 myoblasts abolished myofusion and myotube formation correlating with dramatic mRNA and protein decreases of both myofusion and myogenic markers, including the myogenic regulating factors MyoD and Myogenin (Liu et al., 2010). Similarly, down-regulation of *Mustn1* in rat pre-chondrocytic cells *in vitro* suppresses chondrogenic differentiation accompanied with decreases in both, proteoglycan production and in *Sox9*, *ColII* and *Col X* mRNA (Gersch and Hadjiargyrou, 2009). Lastly, treatment of Xenopus embryos with a *Mustn1* antisense morpholino in craniofacial and dorsal axial tissues resulted in small or absent eyes, a shortened body axis and tail kinks. In addition, cranial *Sox9* mRNA expression was also reduced and led to the loss of differentiated cartilaginous head structures such as the ceratohyal and pharyngeal arches (Gersch et al., 2012).

The zebrafish model has become a powerful genetic tool for understanding developmental processes as well as human disease, with over 70% of human genes having a zebrafish orthologue (Howe et al., 2013). Furthermore, studies utilizing the zebrafish model in craniofacial patterning (Mork and Crump, 2015) and muscle development (Ochi

Abbreviations: aa, amino acid(s); cDNA, DNA complementary to RNA; kDa, kilodalton(s).

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and Westerfield, 2007; Jackson and Ingham, 2013) have provided insights into human biology. In addition to previous studies implicating *Mustn1* function in craniofacial chondrogenesis and muscle development in frog and mouse, recent evidence suggests a potential role for *Mustn1* in cilia function in zebrafish (Choksi et al., 2014). Another zebrafish study utilized the *mustn1b* promoter in an investigation into whether SPARC/Osteonectin expression is regulated by DNA methylation of its promoter (Torres-Núñez et al., 2015). Previously, we also reported on both, the analysis of the murine *Mustn1* promoter (Liu and Hadjiargyrou 2006) as well as its use to drive GFP expression in a transgenic mouse (Krause et al., 2013). Despite these studies, a detailed analysis of *Mustn1* expression has yet to be undertaken in zebrafish. Herein we report on the cloning of the zebrafish *Mustn1* orthologs, *mustn1a* and *1b*, as well as the characterization of their expression patterns during early embryonic and larval development.

2. Materials and methods

2.1. Zebrafish

This study was carried out in accordance to protocols approved by the NYIT College of Osteopathic Medicine Institutional Animal Care and Use Committee (NYITCOM IACUC). Wild-type AB/TU animals were used for all studies. Embryos were cultured in 0.0045% phenylthiourea (PTU) after 24 h post-fertilization (hpf) to inhibit pigmentation and were staged according to Kimmel et al. (1995). Addition of PTU after 24 hpf does not interfere with craniofacial development (Bohnsack et al., 2011).

2.2. Identification and cloning of mustn1a and mustn1b

To identify zebrafish *Mustn1* homologs, BLAST searches of the zebrafish genome (GRCz10) were conducted using mouse (NP_852055) and human (NP_995325) *Mustn1*. The database searches identified putative *mustn1a* (NM_001326444; located on chromosome 6) and *mustn1b* (NM_001197053; located on chromosome 11) sequences. For isolating full-length clones, cDNA from total RNA extracted from 24 hpf embryos was used as template for PCR using the primer sequences: *mustn1a* forward, acaacgtacacaacagtcagtgtac, reverse, agaatggtcctacagtgttacttcc; *mustn1b* forward, tcaactcagccaaaatgtcacagc, reverse, atcacacttggtgatttcctcagg. Sequence alignments were performed using Clustal Omega (McWilliam et al., 2013) and processed in Jalview (Waterhouse AM1 et al., 2009). Needleman-Wunsch two sequence global alignment was used to determine percent nucleotide identity between *mustn1a* and *mustn1b*.

2.3. In situ hybridization

Whole mount *in situ* hybridization was performed as previously described (Thisse et al., 1993). Sequenced *mustn1a* and *mustn1b* cDNA was cloned into a GATEWAY (Life Technologies) compatible pBluescriptII KS + vector for antisense probe production. Antisense RNA was produced from linearized vector using T3 RNA polymerase (New England Biolabs).

2.4. RNA extraction

Total RNA was extracted from dechorionated embryos using Trizol (Life Technologies). Approximately, 50 embryos per time point were placed in 1 ml Trizol reagent and vortexed for 30 s. After 5 min at room temperature, 200 μl of phenol/choroform/isoamyl alcohol (25:24:1, *v*/v) was added and briefly mixed by vortexing. Samples were centrifuged at 4 °C for 15 min at 12,000 × *g*. The aqueous phase was isolated and 500 μl of cold isopropanol was added and the mixture was incubated at room temperature for 10 min followed by precipitation and centrifugation at 4 °C, 12,000 × *g*. The pellet was washed in 75% cold ethanol and centrifuged for 5 min 4 °C at 12,000 x g. The final pellet was dried, resuspended in RNase free water and incubated at 55 °C for 10 min followed by storage at −80 °C. RNA concentration and purity was determined by using the Nanodrop and agarose gel electrophoresis, respectively.

2.5. Quantitative PCR (q-PCR)

RNA from 7-somite, 15-somite, 24 hpf, 48 hpf, 72 hpf, 120 hpf embryos was used to generate cDNA using the Transcriptor Universal cDNA Master Kit (Roche) following manufacturer's directions. Quantitative real time polymerase chain reaction (q-PCR) was carried out using SYBR Green I Master kit (Roche) and following manufacturer's directions. All reactions were carried out in a in a LightCycler 480 (Roche) following a standard q-PCR protocol used previously in our laboratory (Gersch et al., 2005; Gersch and Hadjiargyrou, 2009; Komatsu and Hadjiargyrou, 2004; Komatsu et al., 2007; Liu and Hadjiargyrou, 2006, Zhong et al., 2006; Liu et al., 2010). Primers used for q-PCR: *mustn1a* forward, atgtctcaactgggcgaaca; reverse, acttcctcttggtgggtttg; *mustn1b* forward, tgtcacagccggaggttaag; reverse, tcatttcccaaacacacttc; *ef1a* forward, ctggaggccagctcaaacat; reverse, atcaagaagagtagtaccgctagcattac. All PCR reactions were performed with an annealing temperature of 55 °C. The sizes of the expected amplicons were verified by gel electrophoresis. All data were normalized to a housekeeping gene (*ef1a*, NM_200009; Tang et al., 2007). Each PCR reaction was performed six times as technical replicates. Changes were considered statistically significant when $p < 0.05$ as determined by Mann–Whitney analysis using SPSS (Version 22, SAS Institute, Cary, NC).

3. Results

3.1. Cloning and characterization of zebrafish mustn1 genes

BLAST searches of the zebrafish genome (zebrafish assembly 10) using mouse and human *Mustn1* amino acid sequence revealed two potential orthologs, *mustn1a* (NM_001326444; chromosome 6) and *mustn1b* (NM_001197053; chromosome 11). Using the zebrafish database sequence for *mustn1a* and *mustn1b* as a template, full-length clones were obtained from total RNA isolated from embryos 24 h post-fertilization (hpf) as described in materials and methods. Sequencing of full-length clones confirmed the identity of *mustn1a* and *mustn1b*. Analysis of the *Mustn1* orthologs determined *mustn1a* shared 44% nucleotide identity with *mustn1b* (Fig. 1A). Both paralogs were 71% identical at the amino acid level (Fig. 1B). Predicted amino acid sequences from *mustn1a* and *mustn1b* genes showed significant homology with other vertebrate Mustn1 proteins (Fig. 1B). For example, using BLAST, zebrafish Mustn1a showed an amino acid identity of 63% to human, 61% to xenopus and chimp, 60% to dog, chicken and cow, 56% to mouse, and 54% to rat. Similarly, zebrafish Mustn1b showed an amino acid identity of 69% to dog, 68% to chimp and cow, 64% to chicken, and 63% to human, mouse and rat. Phylogenetic analysis further confirmed the zebrafish Mustn1a and Mustn1b predicted proteins were highly related to other members of the Mustn1 protein family (Fig. 1C). Both zebrafish Mustn1 proteins contain the conserved nuclear localization sequence (NLS) found in other vertebrate homologs (Fig. 1B) but the NLS in *mustn1a* appeared to be further diverged.

3.2. mustn1a and mustn1b mRNA distribution in early zebrafish development

To determine the expression profiles of *mustn1a* and *mustn1b* during early development, we conducted whole mount *in situ* hybridization studies at different embryonic and larval stages. *mustn1a* mRNA expression was detected in different domains from the 7-somite stage through 72 hpf (Fig. 2). At the 7-somite stage, *mustn1a* expression was detected throughout the embryo (Fig. 2A) with expression detected in Kupffer's