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Myomaker mediates fusion of fast myocytes in zebrafish embryos

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ABSTRACT

Myomaker (also called Tmem8c), a new membrane activator of myocyte fusion was recently discovered in mice. Using whole mount *in situ* hybridization on zebrafish embryos at different stages of embryonic development, we show that myomaker is transiently expressed in fast myocytes forming the bulk of zebrafish myotome. Zebrafish embryos injected with morpholino targeted against myomaker were alive after yolk resorption and appeared morphologically normal, but they were unable to swim, even under effect of a tactile stimulation. Confocal observations showed a marked phenotype characterized by the persistence of mononucleated muscle cells in the fast myotome at developmental stages where these cells normally fuse to form multinucleated myotubes. This indicates that myomaker is essential for myocyte fusion in zebrafish. Thus, there is an evolutionary conservation of myomaker expression and function among Teleostomi.

1. Introduction

Myocyte fusion is a fundamental process for myofibre formation during development, post-natal muscle growth and muscle regeneration. During myogenesis, mononucleated myoblasts exit the cell cycle, express muscle-specific transcription factors and start differentiation. Once differentiated, mononucleated myogenic cells, called myocytes, are able to fuse with one another to generate syncytial myofibres. Critical steps in myocyte fusion include recognition, adhesion, and membrane fusion. However, the identification and mechanisms of action of essential cell surface proteins regulating these different steps are far from being completely known.

A lot of knowledge in myocyte fusion arises from studies in the fruit fly *Drosophila melanogaster*. In this model organism, each muscle is composed by a single myofibre that originates from fusion of several fusion competent myocytes (FCM) with one founder cell (FC). The founder cell expresses specific membrane receptors such as Kin of IrreC (Kirre) and Roughest (Rst) [10,14] whereas FCMs express counter-receptors Sticks and stones (Sns) and Hibris (Hbs) [1,2]. The mutually exclusive expression of these cell surface receptors in two distinct subtypes of myocytes is the molecular basis of fusion regulation in drosophila.

In zebrafish and mice, nephrin has been identified as the homologous protein of drosophila Sns [11]. Functional studies have shown that nephrin loss of function blocks myocyte fusion in zebrafish embryos and in cultured mouse muscle cells [11]. A protein homologous to drosophila kirre has also been identified in zebrafish [12]. This protein, termed Kirrel3l is crucial for myocyte fusion in zebrafish embryos as shown by knockdown of the protein using antisense morpholino [12]. Nevertheless, the role of Kirrel in mammalian myocyte fusion is not confirmed yet and remains under debate [4]. A receptor ligand pair (Jam-b/Jam-c) restricted to deuterostomes, has also been shown to be essential for syncytial muscle fiber formation in zebrafish as demonstrated by the phenotype of mutant embryos lacking either protein [6]. Whether Jam-b/Jam-c controls cell fusion in mammalians is currently unknown. Taken together, these data suggest that common and distinct molecular mechanisms are involved in myocyte fusion across species.

Recently, a muscle-specific transmembrane protein, called myomaker, has been shown to be necessary for myocyte fusion in mice. Myomaker is transiently expressed during myogenesis and its invalidation through homologous recombination prevents multi-nucleated muscle fibers formation [5]. Myomaker protein sequence is highly conserved across vertebrate organisms [5]. In this study, we described myomaker gene expression in zebrafish and showed that myomaker acted as an essential activator of myocyte fusion in fish.

2. Materials and methods

2.1. Zebrafish husbandry

Wild-type zebrafish (*Danio rerio*) were raised in our facilities (INRA LPGP, Rennes) and maintained under oxygen saturation in

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a recirculating water system at 27 ± 1 °C, pH 7.5. Zebrafish were exposed to a photoperiod of 16 h light/8 h dark. All investigations were conducted according to the guiding principles for the use and care of laboratory animals and in compliance with French and European regulations on animal welfare (DDSV approval #35-31).

2.2. Isolation of zebrafish myomaker cDNA

Total RNA were extracted from adult zebrafish trunk and reverse transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystem). This cDNA preparation was used as a template to amplify by PCR a fragment encompassing 923 nucleotides of zebrafish myomaker cDNA. Sense primer (GCC ATT TCT CTG AGT CGC AA) and antisense primer (CTG TCC CTG TGA ATA AGT TGT) were designed from zebrafish myomaker sequence (NM_001002088.1). PCR were performed using Go Taq Hot Start Green kit (Promega, M5122). Amplified product cDNA was inserted in vector using pGEM-T Easy Vector kit (Promega) and sequenced to confirm its identity.

2.3. Whole mount in situ hybridization

Embryos were removed from their chorion by a 3 min incubation in a solution containing 1/100 (wt/vol) Pronase (Sigma, P6911) pre-warmed to 28 °C. Afterwards, they were fixed in 4% paraformaldehyde overnight and stored in methanol at −20 °C until use. Digoxigenin- and fluorescein-labeled antisense RNA probes were synthesized from PCR-amplified templates using appropriate RNA polymerases. Whole mount *in situ* hybridization were performed using standard protocol [15] with an INSITU PRO VS automate (INTAVIS AG). Depending on the embryonic stage, different incubation times (0–30 min) were chosen for proteinase K treatment. Double whole mount *in situ* hybridizations were performed as previously reported [7]. Briefly, digoxigenin- and fluorescein-labeled riboprobes were mixed together in hybridization buffer. After reaction with anti-digoxigenin alkaline phosphatase Fab fragments, the first color staining was performed with NBT-BCIP (Boehringer Mannheim). Alkaline phosphatase was inactivated by incubation with 0.1 M glycine–HCl (pH 2.2) twice for 10 min. After extensive washing with PBS, the embryos were incubated with anti-fluorescein alkaline phosphatase Fab fragments. The second color staining was performed with Fast Red substrate (Boehringer Mannheim). For histological examinations of sections, samples were embedded in 2.5% gelatine and 2% agar in distilled water. Blocks were sectioned at 35 μm on a Leica vibratome. Section images were obtained using a Nikon ECLIPSE 90i microscope.

2.4. Morpholino treatment

Freshly fertilized eggs were injected with morpholinos at one to two-cell stage. Morpholinos (Gene Tools) were dissolved in sterile water at a concentration of 2 mM. Two anti-Tmem8c morpholinos (MO1 and MO2), one anti-p53 morpholino and one morpholino random control were used in our experiments. Anti-Tmem8c MO1 was designed to bind 3 bases 5′ of the predicted start codon in the Tmem8c transcript, whereas the MO2 target sequence locates 26 nucleotides upstream of the MO1 binding site. Morpholinos sequences were as follows: MO1: TCT TGG CGA TAA ACG CTC CCA TTG C; MO2: ACT CAG AGA AAT GGC CTC GGG ATT T.

Typically, 400 eggs were injected with the myomaker morpholino and the control morpholino for each experiment. Embryos were injected with approximately 4 nl of MO1 (200 μM) or MO2 (250 μM) diluted in sterile water with 0.1% phenol red. About the same number of eggs was injected with a random control morpholino at the concentration of 250 μM. To ensure that phenotype specificity is due to knock down of myomaker and not to non-specific induction of apoptosis, embryos were co-injected with p53 morpholino at 1.5-fold the concentration of MO anti-myomaker.

The injected eggs were cultured at 28 °C, and embryos were fixed in 4% paraformaldehyde overnight at 24 h of development. Zebrafish embryos were permeabilized in 0.3% Triton in PBS solution for 3 h. Cell nuclei were stained with 4,6-diamidino-2-phenindole (DAPI) at the concentration of 0.01 mg/ml for 30 min. To delimit the myofibre contour, phalloïdin staining coupled with fluorescent dye were used at the concentration of 50 μg/ml for 30 min.

2.5. Image acquisition and processing

Confocal microscopy images were collected using a Leica TCS SP8 MP microscope and LAS AF software. Whole mount *in situ* images were obtained using a macroscope NIKON AZ 100 coupled with NIKON Digital Sight DSRi1 camera and using NIS-Elements D 3.2 software. Entire images were adjusted for contrast, brightness, dynamic range, and resampled to a standardized resolution (300 dpi) using ImageJ software.

3. Results and discussion

3.1. Myomaker is transiently and exclusively expressed in fast somitic myogenic cells during embryogenesis

A sequence homologous to mouse myomaker has been previously reported [5]. Blast analysis using mouse myomaker sequence (NP_079652.1) against the zebrafish genome indicated that a single gene orthologous to mouse myomaker (73% identity) was present in zebrafish genome despite the additional genome duplication that occurred at the origin of the teleosts lineage. Myomaker (Tmem8c) (NP_001002088.1) displayed only 33% and 31% identity with Tmem8a (NP_001139040.1) and Tmem8b (XP_005165500.1) respectively. From zebrafish myomaker coding nucleotide sequence (NM_001002088.1), we generated a specific riboprobe for *in situ* hybridization and designed morpholinos for functional studies. *In situ* hybridization experiments showed that myomaker transcript was not detected at the early stages of somitogenesis (15 hpf) (data not showed), but became evident in 20 hpf embryos (Fig. 1A and E). At this latter stage, myomaker expression was observed within most of the somites with the exception of the newly formed posterior somites. Myomaker transcript was not detected in paraxial mesoderm from which somites are formed (Fig. 1). As somitogenesis proceeded along an anteroposterior axis, myomaker labeling appeared progressively in more caudal somites (Fig. 1). The initial detection of myomaker transcript in somites of 20 hpf embryos indicated that the inception of myomaker transcription follows that of kirrel and Jam-b/Jam-c, two other transmembrane proteins involved in muscle cell fusion [6,12]. Thus, it is likely that myomaker acts in later steps of the fusion process than kirrel and Jam-b/Jam-c. Consistently, [5] have reported that myomaker is involved in the membrane fusion process in mouse whereas kirrel and Jam-b/Jam-c have a role in recognition and adhesion between muscle cells in zebrafish.

Two major distinct populations of muscle progenitors involved in the primary myogenesis can be distinguished in the developing somite: the adaxial cells next to the notochord and the lateral cells of the posterior somitic compartment [13]. The adaxial cells migrate laterally during somite maturation to form the superficial slow muscle fibers, whereas the lateral cells of the posterior compartment elongate and differentiate into medial fast fibers. Contrasting to fast muscle precursors that fuse with each other to

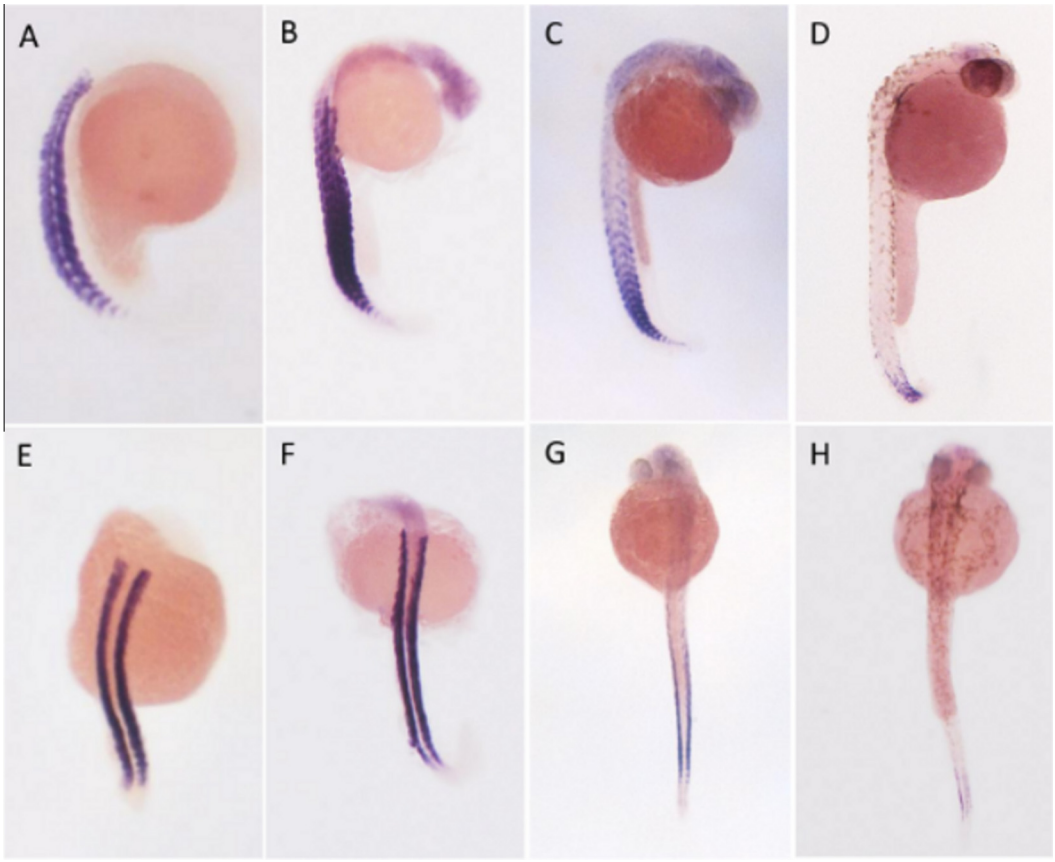

Fig. 1. Myomaker is transiently expressed in somites during embryogenesis. Lateral views (A–D) and dorsal views (E–H) are presented. Myomaker was expressed in rostral somites of 20 hpf embryos (approximately 20–22 somites, A and E). In 24 hpf embryos (somitogenesis is completed, B and F), the labeling has progressed up to the most caudal somites. In 30 hpf embryos (C and G), the labeling is excluded from rostral somites. Myomaker labeling is no longer present in somites at 35 hpf (D and H).

form multinucleated myofibres, slow muscle precursors differentiate into mononucleated myofibres during embryonic myogenesis [9]. Given the difference in their ability to fuse, we further examined the early expression of myomaker in fast and slow muscle cells. For this purpose we performed double *in situ* hybridization using an antisense myomaker riboprobe and an antisense slow myosin heavy chain myosin 1 (smhc1) [3] specific to slow muscle cells. Thereby, we observed that adaxial slow muscle cells expressing smhc1 did not transcribe myomaker (Fig. 2A). In contrast, myomaker expression was clearly evident in lateral fast muscle cells (Fig. 2A). Absence of myomaker expression in slow muscle cells was also observed after slow cells have completed their migration towards the surface of the myotome (Fig. 2B). This indicated that only fast muscle cells that fuse to form multinucleated myofibres expressed myomaker during embryonic myogenesis. After 24 hpf, the somitic expression of myomaker declined in a rostro-caudal progression to become completely undetectable after 35 hpf. Therefore myomaker gene is transitory expressed during embryonic development (Fig. 1) as reported in mice [5]. Given that muscle growth in fish involves several phase of myogenesis [8], we cannot however rule out the possibility that myomaker is reactivated in post embryonic stages. In this regard, it is interesting to note that myomaker is reactivated in adult mice after muscle injury suggesting that myomaker function is not restricted to developmental stages [5].

In conclusion, the transient expression of myomaker in zebrafish early somites is reminiscent of that reported in mice [5] suggesting a conservation of expression pattern of myomaker ortholog among Teleostomi.

3.2. Myomaker is essential for fusion of fast myocytes in zebrafish

Thus, the expression of myomaker is compatible with a role in the fusion of fast muscle cells. Using antisense morpholino directed against myomaker, we investigated the role of myomaker in zebrafish developing somite.

At early stage (before completion of somitogenesis), injected embryos did not present notable phenotype. In particular, the segmentation of the paraxial mesoderm was normal. Moreover, embryos injected with myomaker morpholinos hatched later than wild-type embryos (approximately 58 h versus 48 hpf). Four days post hatching, wild-type embryos were able to swim whereas morphants stayed at the bottom of the dish exhibiting only sporadic movements even under tactile stimulation, a phenotype suggesting a muscle dysfunction (see movie in Supplemental data). These observations are in agreement with those reported in myomaker null mice embryos that were alive until full-term of embryonic development but exhibited skeletal muscle deficiency [5].

To better characterize the causes of the reduced locomotor activity, confocal analysis were carried out on embryos at 24 hpf. At this stage, wild type zebrafish somites displayed syncytial fast fibers containing up to four nuclei (Fig. 3A). Confocal analysis showed that embryos injected with either of the two morpholinos failed to form multinucleated fast fibers; instead, fast muscle cells remained distinct and mononucleated (Fig. 3B). Co-injection of myomaker-morpholino together with p53-morpholino led to the same phenotype to that one obtained with the injection of myomaker-morpholino alone indicating that myomaker-morpholino did not induce the p53-dependant apoptotic pathway. Also

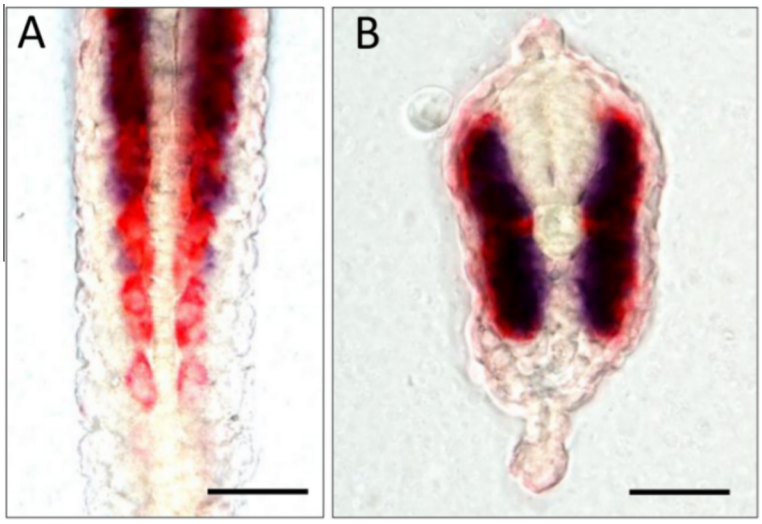

Fig. 2. Myomaker is exclusively expressed in fast somitic myogenic cells. Dual color *in situ* hybridization for slow myosin heavy chain 1 (red) and myomaker (dark blue). (A and B) stage 24 hpf zebrafish embryos. (A) Frontal section of posterior trunk: adaxial slow muscle cells do not express myomaker which is transcribed laterally in fast muscle cells. (B) Transverse section of anterior trunk: the slow muscle cells that have completed their lateral migration are persistently not labeled whereas muscle cells of the fast myotome express robustly myomaker transcript. Scale bars represent 50 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

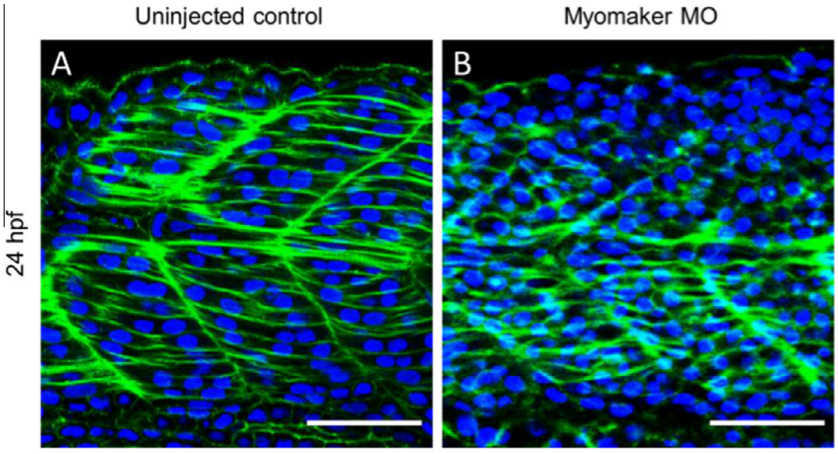

Fig. 3. Myomaker is essential for fast myocyte fusion. Confocal microscopy images of fast-twitch muscle in uninjected (A) and myomaker MO1 morpholino injected (B) embryos at 24 hpf. Sagittal view: loss of myomaker prevents muscle cell fusion and formation of multinucleated fast myofibres. Embryos are stained with DAPI (blue) and phalloidin-Alexa488 (green) to stain nuclei and F-actin respectively. Scale bars represent 50 μM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

zebrafish embryos injected with random morpholino led to a wild-type phenotype. These observations showing that knock-down of myomaker prevents fast myocyte fusion in zebrafish are in agreement with the muscular phenotype of mice with null homozygous mutations of the myomaker gene [5]. This phenotype is also similar to those induced by knock-down or mutations of fusogenic genes such as Jam-b, Jam-c and kirrel3l both involved in myogenic cell fusion in zebrafish [6,12].

Altogether, our results show that myomaker is transitory expressed in the myotome and is essential for fast myocyte fusion in zebrafish. Thus, there is a high degree of evolutionary conservation of myomaker expression and function among Teleostomi. The precise mechanisms by which myomaker controls myogenic cell fusion in vertebrates remain to be elucidated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.093.

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