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Localization, Distribution and Structure of Muscle Fibres Using Specific Antibody Markers in the zebrafish, *Danio rerio* (Hamilton, 1822)

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Abstract: The zebrafish *Danio rerio* is a popular model species for genetic and early development studies. It is relatively easy to maintain in laboratory, has a high fecundity rate and produces transparent embryos. Here, we characterise muscle development in early life stage zebrafish using paraffin sections of embryos and larvae treated with haematoxylin and eosin staining, and whole mount fluorescent immunohistochemistry. We found variations in the distribution of muscle mass throughout the body, with the greatest proportion of muscle mass found in the tail. Our data also showed for the first time the reaction of antibodies (protein expression) in muscle at early life stages of development. Whole mount fluorescent immunohistochemistry staining with three markers (PAX7, MF20 and F59) suggests that muscle development starts even earlier than previously suggested at the embryonic stage (1 dpf).

Keywords: PAX7, MF20, F59, myosin.

Introduction

The zebrafish, *Danio rerio*, has long been regarded as an ideal model species for both molecular and developmental biological studies due to its high fecundity and relatively short generation time; the total period from fertilization to hatching takes just 2-3 days (Kimmel *et al*., 1995) which is short compared with other fish species such as the mangrove killifish (*Kryptolebias marmoratus*) which takes 13 days to reach its hatching period (Mourabit *et al*., 2011). Moreover, it has orthologous genes with humans that share similar developmental paths (Barbazuk *et al*., 2000). In particular, zebrafish embryos are extremely well suited for studying muscle development as they are transparent, develop externally and a large proportion of the body is comprised of somatic muscle.

Muscle induction initiates during early developmental stages through a process called myogenesis. Several genes are involved in myogenesis including PAX7. PAX7 is a member of the PAX family that are involved in muscle development (Chihara *et al*., 2015). PAX7 is expressed during the early stages of development in the nucleus of progenitor cells and can bind with other factors such as PAX3 to form a heterodimer.

Many genes are implicated in muscle formation in the zebrafish, some of which are expressed inside the nucleus, e.g. the transcription factor PAX7. PAX7 is important for myoD and myf5 (early muscle transcription factor genes) expression (Weinberg *et al*., 1996; Row *et al.*, 2018). PAX7 also plays a major role in the regulation of other transcriptions factors that activate muscle genes at early stages of development; for instance, PAX7 regulates MyoD and MYF5 at 70%-75% epiboly (Bassel Duby & Olson, 2006; Ochi & Westerfield, 2007). MF20 and F59 are anti-myosin heavy chain antibodies. Myofibrils, the basic units of muscle cells, are made up of sarcomeres. Sarcomeres are comprised of two main protein filaments, actin and myosin, which are responsible for muscle contraction (Dew *et al*., 2014). As there are few studies in the literature on early muscle development in fish, we set out to further characterise muscle development during early life stages including the dynamic interaction of muscle protein expression.

In this study, we used three types of antibodies, PAX7 (to detect the progenitors of muscle) and MF20 and F59 (to detect myosin) and carried out histological cross sections of zebrafish embryos to illustrate the structure of muscle in early life stages of development. F59 binds to myosin heavy chain isoforms while MF20 specifically connect to light meromyosin and sarcomere myosin (Dew *et al*., 2014). So that, here in the field of muscle research, we concentrated on some specific proteins involved in muscle development at early stages of zebrafish development.

In zebrafish, muscles are generated from mesoderm during the blastula and gastrula stages. Specifically, muscle emanates from the paraxial mesoderm that later creates somites at the somitogenesis stage (Ochi & Westerfield, 2007; Sharma *et al.*, 2019). Somites posteriorly develop into dermis and muscles. There are two types of muscle, fast muscle and slow muscle (Rescan *et al*., 2001; Kocaefe *et al*., 2005). Both the dorsal lateral and the ventral germ ring switch to trunk muscle and tail muscle respectively (Kudoh *et al*., 2004). The somites then differentiate, into one of three regions, the myotome, the dermatome and sclerotome. The myotome and the dermatome are found together in a dorsal structure called the dermamyotome and later separate into dermatome which evolves to form the dermis, and myotome which participates largely to trunk skeletal muscle (Macintosh *et al*., 2006).

Materials & Methods

Fish Husbandry

During November 2019, wild type zebrafish embryos maintained under laboratory conditions prepared to mimic their natural habitat (28°C, pH7.3) reared in a 50×50×30 cm tank loaded with 60L water with circulation and aeration. Embryos were fed with dried egg yolk once a day. The fish occasionally spawned and deposited eggs on an artificial plastic grass in the early morning. For sampling purposes, all fish were sacrificed by terminal anesthesia with MS-222 (tricaine methanesulfonate) solution (0.004%). All work was carried out under the licence number BE1683.

Determination of muscle mass distribution using Paraffin wax and Harris haematoxylin / Eosin (HE) staining

Whole embryos (3dpf) and larvae (20dpf) (*n* =15 fish per life stage) were kept for 4-5 days

in 4% paraformaldehyde (PFA), dissolved in PBS, subsequently dehydrated and embedded in paraffin wax and sectioned transversely to 5µm thickness using a rotary microtome (Leica Biosystems RM2125 RTS). Slides were stained with Harris Haematoxylin/Eosin (Thermo Scientific), then embedded with DPX mounting medium (National Diagnostics). Images of histological sections were photographed using a LEICA DM1000 microscope.

Measurement of muscle mass

Muscle mass of body parts (anterior, trunk and tail (Fig.1)) were calculated based on the total physical mass using Image j (version 1.52 p). The relative mass of muscle ratio was calculated in histological sections using the following formula :

$$\text{Ratio of muscle mass} = \frac{\text{muscle area}}{\text{section area}} \times 100$$

Whole mount fluorescent immunohistochemistry (WMFI) was performed in order to assess the expression of proteins (PAX7, MF20, F59) associated with muscle development in early life stages

Whole mount fluorescent immunohistochemistry was carried out at 1dpf to identify muscle induction, specifically the expression of proteins associated with muscle development in early life stages. Embryos (*n* = 10 fish) were washed two times with PBS for10 minutes each and then incubated for one hour with blocking solution (5% BSA, 5%

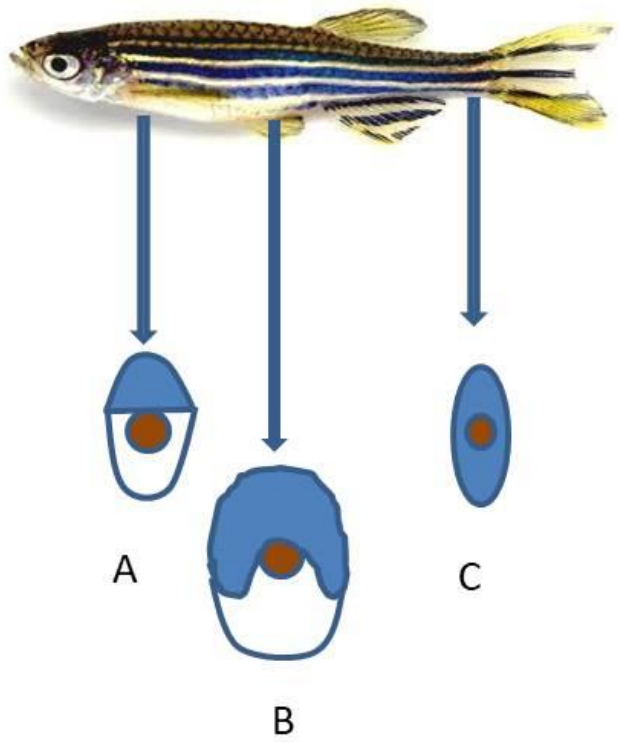

Fig. (1): Distribution of muscle mass in zebrafish embryos, shown through cross sections taken near the head (A) the trunk (B) and the tail (C). The proportion of muscle mass (in blue) can be seen to be greater in the tail compared to other parts of the body.

heat-inactivated Bovine Serum/HI-BS in PBS). Next, primary antibodies were added including F59 (Developmental Studies Hybridoma Bank/DSHB) at 1/20 dilution, MF20 (DSHB) at 1/20 dilution and PAX7 (DSHB) at 1/20 dilution and were left to incubate overnight. These were then washed with blocking solution three times for one hour each and incubated with anti-mouse IgG 488(1:1000) (Life Technology) and Hoechst (1:2000) (Life Technology) overnight. Embryos were then washed with blocking solution three times for one hour each and with 1x PBS including 0.1%TritonX-100 (PBSTx) two times for 15 minutes each. Images were taken using a confocal microscope (LSM 510, Laser module).

Results

Muscle development during early life stages

Cross sections of paraffin wax with Haematoxylin and Eosin stains from larvae at 3dpf and 20dpf revealed the structure of the hypaxial and epaxial. Skeletal muscle and spinal cord were shown to extend to the end of the tail at 3 dpf, similarly to what is seen at 20 dpf. Differences in muscle development showed that the myosepta was still not complete at 3 dpf (Fig. 2A), while myosepta and myomere were clearly developed at 20 dpf embryos (Fig. 2B). The muscle mass was distributed in different parts of the body (head, trunk and tail) showing the symmetrical division on both sides (Fig. 3).

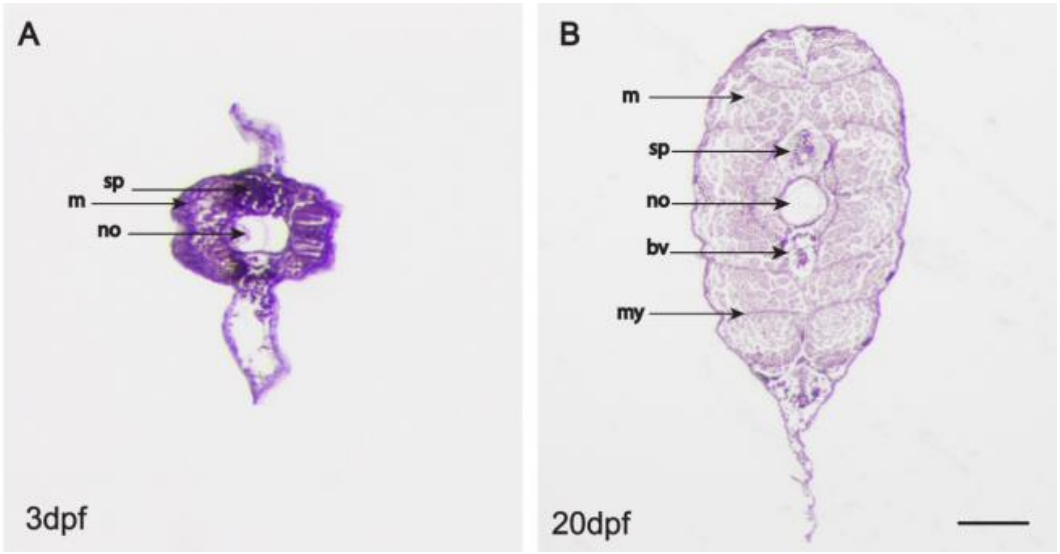

Fig. (2): Cross sections in the tail of the zebrafish, *D. rerio,* using paraffin wax with Haematoxylin - Eosin staining at 3dpf (A) and 20dpf(B) showing muscle structures. dpf, day post fertilization; bv, blood vessel; my, myocepta; m, myomere ; no, notochord; sp, spinal cord. The dorsal side is at the top of the image. Scale bar = 200µm.

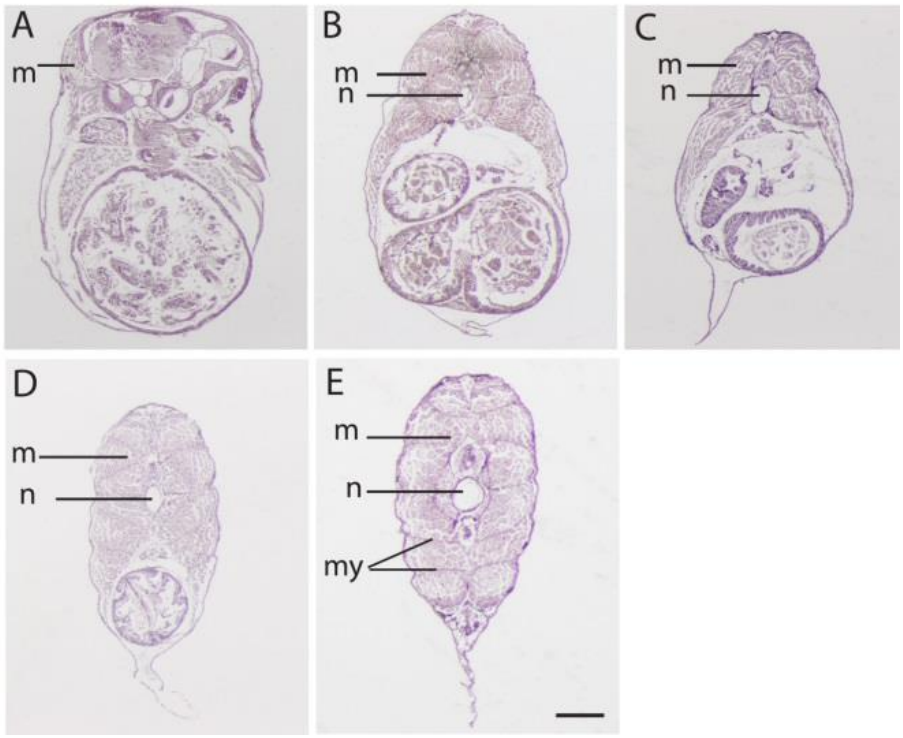

Fig. (3): Cross sections of the zebrafish, *D. rerio,* using paraffin wax with Haematoxylin - Eosin staining at 20dpf showing the distribution of muscle mass throughout the body. (A) anterior, (B and C) trunk, (D) posterior, (E) tail. my, myocepta (E) ; m, myomere (A-E); n, notochord (B-E). The dorsal side is at the top of the image. Scale bar= 200µm.

Localization of muscle mass

Cross sections of paraffin wax with Haematoxylin and Eosin stains showed the muscle mass distribution through body parts (Fig.3, A-E). The ratio of muscle mass to total body area was found to be higher in the tail (81%) and in the posterior part of the trunk (68%), whereas the ratio began to recede towards the head (36% in mid trunk, 33% in anterior trunk and 3% in the head (Fig. 4).

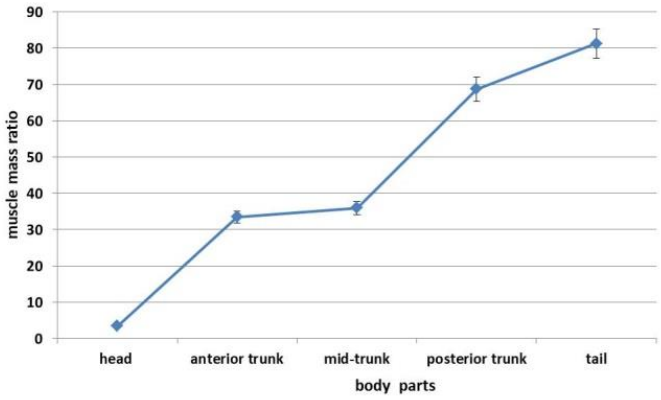

Fig. (4): Histogram of muscle mass ratio distribution in the head, anterior trunk, mid trunk, posterior trunk and tail of the zebrafish, *D. rerio,* The muscle mass ratio increases toward the posterior parts of body.

The expression of muscle development proteins in the tail of zebrafish during early life stages

Myosin localization was clearly distinguished along the length of the myofibrils (Fig. 5A). Likewise, fluorescent immunohistochemistry using MF20 (a myosin heavy chain antibody) stained the myofibril. However, this was much more localized in a small region of myofibrils, specifically in the myocepta (Fig. 5B). This revealed the domain of protein expression determining the regions of distribution of muscle forming proteins. The PAX7 antibody was expressed in the nucleus of progenitors at 1dpf (Fig. 5C) and represents its role in the regulation of myogenic differentiation of muscle cells.

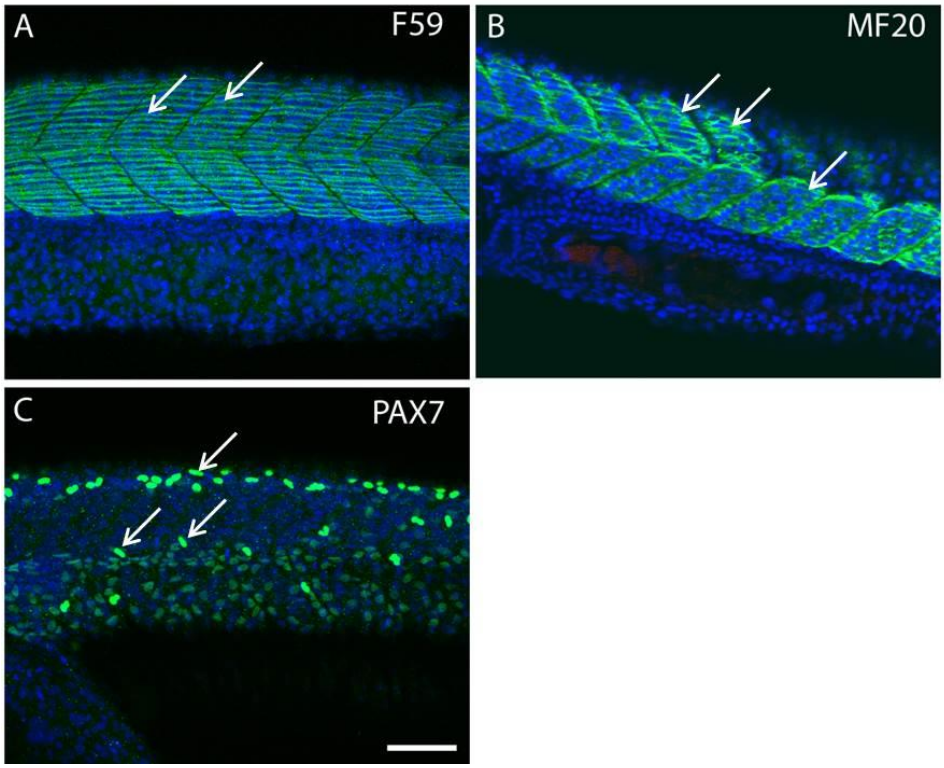

Fig. (5): Whole mount fluorescent immunohistochemistry in the zebrafish, *D. rerio,* at 1 dpf using F59, MF20 and PAX7 antibodies. F59 (A) and MF20 (B) show myosin signals while PAX7 (C) shows progenitors signals. Arrows refer to proteins expression. Scale bar = 100µm.

Discussion

Fish muscle distribute as a few long sheet that located on both sides of the body extending from head to tail, the long muscle consist of transverse sheet of connective tissue that divided into segments named myotomes, the shape of myotomes have order depend on the fish species (Dunajski, 1979). Whilst there have been many studies on muscle development using the zebrafish, *D. rerio*, as a genetic tool, the exact mechanisms of muscle development through muscle protein synthesis are still to be elucidated. In this study we assessed muscle mass distribution in the zebrafish embryo at 20 dpf stage where the muscle formation were completed, on the 1 dpf stage, it has been focusing on muscle protein expression using three types of muscle antibodies as markers, one to specifically stain

the nucleus of cell progenitors and the other for myosin heavy chain detection. At 1 dpf the musculature is mainly organized in the somites along the back of the fish (van Raamsdonk *et al.*, 1982).

The relative distribution of muscle mass in the trunk and tail occupies most of these physical parts. This is due to the requirement of these parts in fish motility during swimming and feeding. Our results indicate a higher ratio of muscle mass in the tail and posterior trunk. This is because this a region free of an abdominal cavity and plays a significant role in motility performance during swimming. The relative area of muscle which covers the whole dorsal and lateral line is closely related to the dorsal and paired fins. The skeletal muscles emanated from the mesoderm layer which is the main domain of muscle genes expression. In this study, the PAX7 antibody was shown to specifically target prospective heavy chain muscle cells. In addition, the timing of PAX7 expression was observed at 1dpf. This is contradictory to a previous study which first detected PAX7 expression in zebrafish at 27hpf (Hammond *et al.*, 2007). Different isoforms of heavy chain myosin are expressed in different domains depending on the function of muscle activity. This may evolve from a single gene or many genes in one family, and/or an alternative splicing event. Our results targeted antigen with a specific antibody to distinguish myosin heavy chain localization in zebrafish embryos at 1 dpf. The F59 antibody labelled fast twitch muscles in whole mount zebrafish embryos. Our investigation found fast twitch muscle formation in zebrafish embryos at 1 dpf, which is earlier than that cited by other researchers. For example, Doganli *et al.* (2016) described myosin heavy chain protein expression using F59 antibody between 1-5dpf. Liu *et al.* (2009) used F59 antibody to stain myosin heavy chain for detecting a number of muscle fibers at 26 hpf. F59 is a highly specific antigen for heavy chain myosin and is designed as a monoclonal antibody for skeletal muscles. Myosin plays a role with actin in eukaryotic cells in muscle cell movement and contractile ability. F59 reveals the sarcomere structure of skeletal muscles that orientate in a parallel pattern given the functional mission of muscle contraction. MF20 antibody is specifically used to detect sarcomere myosin (Dew *et al.*, 2014). Our results revealed clear MF20 antibody binding in myosin in general, with high expression in the myocepta.

Conclusions

Overall, we conclude that the ratio of muscle mass to total body area was found to be higher in the tail, both F59 and MF20 antibodies detected myosin protein expression in zebrafish at 1dpf, showing the proteins expression in myofibrils and myocepta units. In addition, PAX7 detected myogenic precursors at the same stage confirming the progressive activity of PAX7 in regulation of myogenic differentiation during this stage.

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Conflict of interest: The authors declare that they have no conflict of interest.

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توزيع وانتشار وتركيب الالياف العضلية باستخدام بعض الاجسام المضادة المتخصصة في سمكة الزيبرا *Danio rerio* (Hamilton, 1822)

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المستخلص: تعد سمكة الزيبرا نموذج شائع في الدراسات الوراثية والتطورية في المراحل الجنينة الاولية فمن الممكن تربيتها بسهولة نسبيا في المختبر وهي ذات معدل خصوبة عالي وتنتج اجنة يمكن ملاحظتها من خلال القشرة الخارجية الشفافة للبيضة. في الدراسة الحالية تم تشخيص تطور العضلات في المراحل الاولى لسمكة الزيبرا باستخدام مقاطع شمع البرافين النسجية للاجنة واليرقات باستخدام صبغتي الهيماتوكسلين والايوسين والصبغات المناعية المفلورة. درس توزيع وانتشار العضلات في منطقة الراس والجزء الامامي والوسطي والخلفي من الجذع ومنطقة الذنب، حيث اظهرت كتلة العضلات في منطقة الذنب والجزء الخلفي من الجذع اعلى نسبة مؤكدة ان استخدام هذه الاجزاء مهم في النشاط الحركي في سباحة السمكة. كذلك بينت الدراسة الحالية ولاول مرة باستخدام فعالية الاجسام المضادة في كشف نشوء بعض انواع البروتينات العضلية في المراحل الاولى من التطور. استخدمتPAX7 وF59 و MF20 كصبغات مناعية مفلورة لكشف بعض انواع البروتينات العضلية. اقترحت الدراسة بان هذه البروتينات العضلية تبدا بالنشوء والتطور خلال المراحل المبكرة بعد الاخصاب (24 ساعة بعد عملية الاخصاب).

الكلمات المفتاحية:. F59، MF20، PAX7، المايوسين.