

# Dynamic somite cell rearrangements lead to distinct waves of myotome growth

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The myogenic precursors responsible for muscle growth in amniotes develop from the dermomyotome, an epithelium at the external surface of the somite. In teleosts, the myogenic precursors responsible for growth have not been identified. We have used single cell lineage labeling in zebrafish to show that anterior border cells of epithelial somites are myogenic precursors responsible for zebrafish myotome growth. These cells move to the external surface of the embryonic myotome and express the transcription factor Pax7. Some remain on the external surface and some incorporate into the fast myotome, apparently by moving between differentiated slow fibres. The posterior cells of the somite, by contrast, elongate into medial muscle fibres. The surprising movement of the anterior somite cells to the external somite surface transforms a segmentally repeated arrangement of myogenic precursors into a medio-lateral arrangement similar to that seen in amniotes.

**KEY WORDS:** Myotome, Anterior border cells, Cell fate, Paraxial mesoderm, Somite, Dermomyotome, Zebrafish, Fast muscle, Pax7, Myogenesis, Teleost

## INTRODUCTION

Vertebrate axial skeletal muscle first develops in a compartment of the somite called the myotome. In amniotes the myotome grows from a supply of myogenic precursors in the dermomyotome, an epithelium external to the myotome (Scaal and Christ, 2004). The transcription factors Pax3 and Pax7 are expressed in mitotically active muscle progenitors in the dermomyotome (Relaix et al., 2004). As these cells differentiate into muscle they express myogenic regulatory factors (MRFs) such as MyoD, Myf5 and myogenin (Relaix, 2006).

Recently, we and others proposed that cells expressing Pax3 and Pax7 on the surface of the teleost myotome are myogenic precursors (Devoto et al., 2006; Groves et al., 2005; Steinbacher et al., 2006). Here, we combine lineage tracing and gene expression analysis to test whether these Pax3- and Pax7-expressing cells are myogenic precursors and to identify their origin in zebrafish. We show that cells along the anterior border of epithelial somites move to the external surface of the developing myotome and express Pax7. A subset of these later differentiates into muscle fibres of the lateral myotome. We discuss the similarities and differences between the cellular basis for myotome growth in amniotes and in zebrafish.

## MATERIALS AND METHODS

### Embryos, immunohistochemistry, birthdating and morphometrics

Zebrafish (*danio rerio*) were raised according to standard procedures. MF20 and Pax7 antibodies were obtained from Developmental Studies Hybridoma Bank (DSHB) (Bader et al., 1982; Kawakami et al., 1997). Prox1 antibodies were from Chemicon, whereas MEF2, Myf5 [which recognises MyoD and not Myf5 in zebrafish (Hammond et al., 2007)] and myogenin were from Santa Cruz Biotechnology. Antibody labeling and BrdU birthdating was performed as previously described (Barresi et al., 2001; Barresi et al., 2000; Devoto et al., 2006; Feng et al., 2006). For phalloidin labeling, dehydration was omitted and Triton was substituted for Tween. Labeled embryos were

visualised and photographed using a Zeiss LSM confocal microscope. Morphometric analysis of fast fibre cross-sectional area was done as previously described (Johnston et al., 2003); slow fibres were omitted from this analysis.

### Time-lapse and cell labeling

Fluorescent images of six-somite embryos carrying the histone2A.F/Z:GFP transgene (Pauls et al., 2001) were captured every 2 minutes for 2 hours. For lineage tracing, cells were injected in 12-somite embryos as previously described (Devoto et al., 1996). Embryos were viewed and photographed using a Zeiss Axioplan compound microscope and a Zeiss LSM 510 confocal microscope.

## RESULTS AND DISCUSSION

### Cell rearrangements in the epithelial somite

The first indication of myogenic specification in zebrafish is in the segmental plate, when adaxial cells along the notochord express MRF genes (Fig. 1A) (Weinberg et al., 1996). Somites in zebrafish form as epithelial balls surrounding a core of mesenchymal cells (Fig. 1D,E). After somites form, only posterior epithelial cells express MyoD (Fig. 1A) (Coutelle et al., 2001). This suggests that posterior cells have committed to myogenesis. By contrast, Pax7 mRNA is first detectable in anterior cells approximately 2 hours after segmentation (Feng et al., 2006; Hammond et al., 2007; Seo et al., 1998). Three hours after segmentation, Pax7 and myogenin immunoreactivity is strongest in non-overlapping subsets of somite cells, with Pax7-positive cells restricted to lateral and anterior domains of the somite and myogenin-positive cells restricted to medial and posterior domains (Fig. 1B). Some myogenin-positive nuclei appear to be in elongating cells, which are in the medial posterior region of the somite just lateral to elongated adaxial cells that span the antero-posterior length of the somite (Fig. 1B). At the end of the segmentation period (24 hours), Pax7-expressing cells are restricted to the external somite surface (Fig. 1C) (Devoto et al., 2006; Feng et al., 2006; Groves et al., 2005; Hammond et al., 2007).

The change in Pax7 distribution suggests that cells move from the anterior to the external surface of the somite. In the midtrunk (somites 9 to 14), the anterior of the second to last formed somite (ss II) (Ordahl, 1993) consists of a readily identifiable row of 7 to 9 cells along the medio-lateral axis, from the anterior-most adaxial cell to the lateral border of the somite (Fig. 1E). We call these anterior

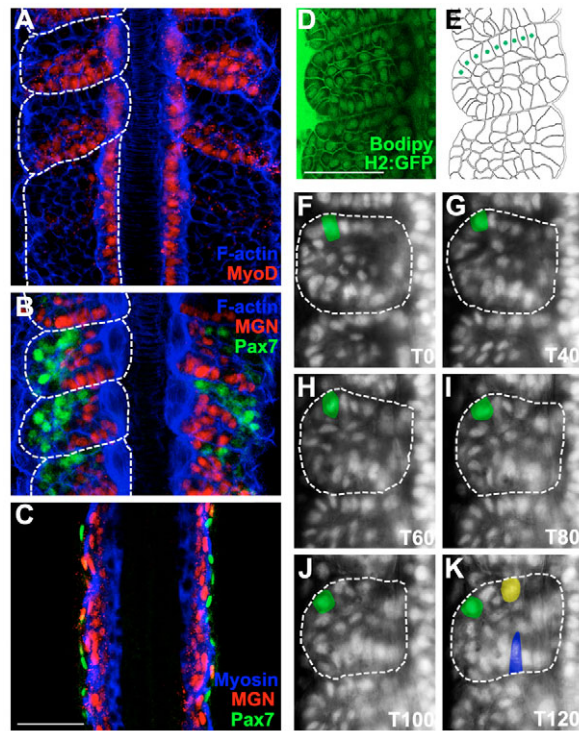
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**Fig. 1. Distinct anterior and posterior domains in the epithelial somite.** (A) In a 12S (15 hours) embryo, MyoD (red) is expressed in nuclei in the posterior of newly formed somites and in adaxial cells of the anterior segmental plate. (B) In the anterior of a 14S (16 hours) embryo, myogenin labeling (red) is in the posterior and medial somite, whereas Pax7 (green) is restricted to anterior and lateral. (C) In a 24-hour embryo, Pax7-labeled nuclei (green) are on the external myotome surface, whereas myogenin labeled nuclei (red) are more medial. (D) Bodipy ceramide-stained Histone H2:GFP 12S embryo. (E) Cells from the embryo in D are outlined, anterior border cells (ABCs) are marked by green dots. (F–K) Selected time-lapse images of green, GFP-labeled nuclei. T, time in minutes from the commencement of analysis. An individual ABC is pseudo-coloured green and tracked through individual frames. (K) An additional ABC is pseudo-coloured yellow in the same medio-lateral position as an elongating posterior cell in blue. (A–K) Dorsal views, anterior to the top, the midline is in the center in A–C, at the right in D–K. Scale bars: 50  $\mu$ m in C for A–C, and 50  $\mu$ m in D for D–K.

border cells (ABCs). We used embryos expressing nuclear localised green fluorescent protein (GFP) (Pauls et al., 2001) to follow groups of ABCs during the time when the distribution of Pax7-positive cells changes. In all embryos, ABCs moved laterally during the first few hours after segmentation, and those that reached the lateral surface moved posteriorly (Fig. 1F–K). During this time, medial and posterior somite cells elongated into muscle fibres (Fig. 1K). We conclude that during the first few hours after becoming incorporated into a somite, the posterior cells begin to elongate into muscle fibres, whereas ABCs instead remain as undifferentiated cells that shift from the anterior to the lateral somite surface.

### ABCs develop into Pax7-positive dermomyotome cells

We used lineage tracing to follow the movement of ABCs for longer periods of time, and to test whether they express Pax7, and later differentiate into muscle fibres. Four to eight hours after

becoming incorporated into a somite, most ABCs gave rise to non-elongated cells on the external surface of the somite ( $n=64/70$ ; Fig. 2B,G), whereas a few gave rise to muscle fibres ( $n=7/70$ ; Table 1). The position and morphology of these ABC progeny closely resembles what Waterman named ‘external cells’ (Waterman, 1969).

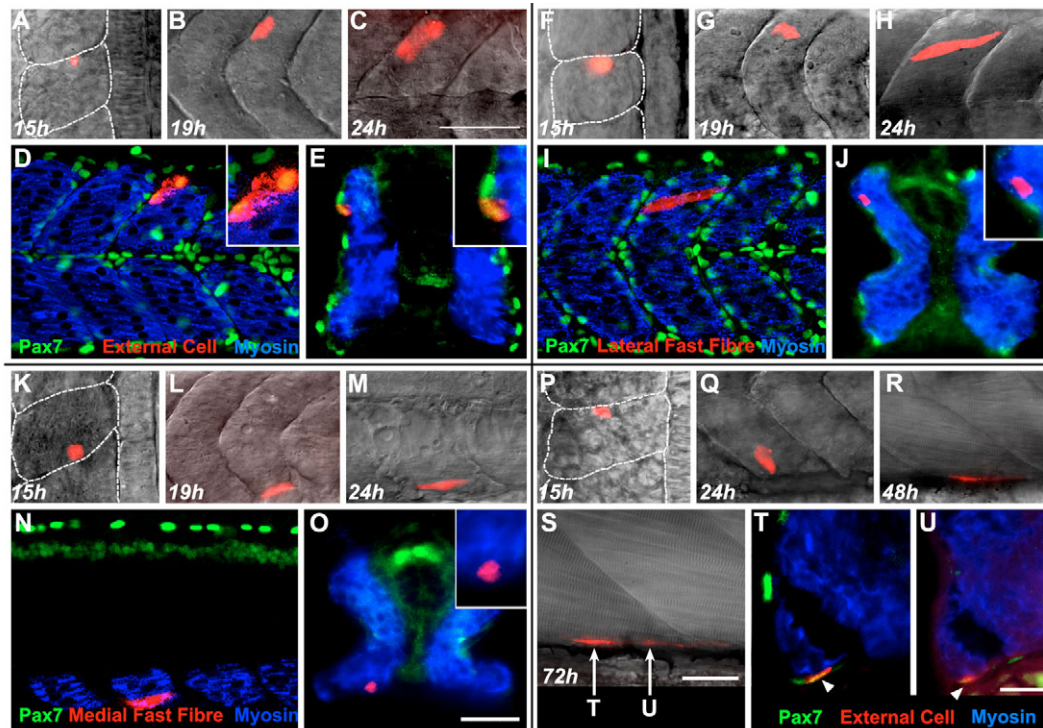
Ten to 15 hours after becoming incorporated into a somite (the 24–30-hour stage), more than half ( $n=39/70$ ) of ABCs had given rise to elongated cells resembling muscle fibres (Fig. 2G,H). All of those tested were myosin positive ( $n=10/10$ ; Fig. 2J). Muscle fibres derived from ABCs were always fast fibres on the medial surface of the superficial slow muscle cells (Fig. 2J, Fig. 3A). Because virtually all ABCs became external cells (Fig. 2B,G, Table 1), and over half later differentiated into muscle fibres (Fig. 2H, Table 1), we conclude that external cells are derived from ABCs and become lateral fast muscle fibres. The interface between slow and fast fibres has been described as a germinal layer (Rowlerson and Veggetti, 2001). Our data indicate that the germinal layer derives from ABCs that move laterally to become external cells, and then back medially to become muscle fibres.

In contrast to ABCs, posterior cells differentiated very early into elongated muscle fibres. Five hours after injection posterior cells had not moved laterally, and instead had elongated medially within the myotome (Fig. 2L). Differences in timing of elongation were not related to initial medio-lateral position of the injected cells – ABCs and posterior cells at similar distances from the midline elongated at quite different times (data not shown). Unlike ABCs, all injected posterior cells had elongated, and/or fused with other cells to span the anterior-posterior length of the somite before 24 hours ( $n=6/6$ ). Elongated cells derived from posterior cells were always deep within the myotome (Fig. 2M,O, Fig. 3A), and expressed MyHC ( $n=6/6$ ; Fig. 2N,O). Thus, posterior cells and ABCs give rise to separate waves of myogenesis and generate muscle fibres in distinct myotome locations.

We next wished to determine whether ABCs develop into Pax7-expressing cells. Seven to 12 hours after injection, half of injected ABCs developed into Pax7-positive cells on the external surface ( $n=14/27$ ; Fig. 2D,E). One injected ABC gave rise to both a Pax7-positive cell and a muscle fibre. Like Waterman’s external cells (Waterman, 1969), Pax7-positive injected cells are extremely flattened and occupy the external surface of the myotome in close apposition to superficial muscle fibres (Fig. 2E). Most of the Pax7-negative cells derived from ABCs were elongated muscle fibres ( $n=9/12$ ) that may have already downregulated Pax7 expression. A few Pax7-negative cells had a morphology similar to external cells ( $n=3/12$ ). We suspect that these non-elongated, Pax7-negative cells would have later expressed Pax7, but it is possible that a minority of ABCs develop into cells on the external surface of the myotome that never express Pax7. We observed no strict correlation between the medio-lateral position of an ABC and the likelihood of it developing into a Pax7-positive precursor (data not shown).

The zebrafish myotome expands during larval stages as new muscle fibres are added (Rowlerson and Veggetti, 2001). Pax7-positive cells remain on the surface of the myotome into the larval period and beyond (Fig. 2R,S and data not shown). To determine whether ABCs also contribute to these late Pax7-positive potential myogenic precursors, we allowed some embryos with rhodamine-injected ABCs to develop into the early larval period. Some injected ABCs remained as undifferentiated cells on the external surface of the myotome as long as we observed. After approximately 48 hours, these external cells assume a more protracted morphology, and are





**Fig. 2. Distinct fates of ABCs and posterior cells.** (A-E) ABCs give rise to external cells. (A) The injected ABC (red). (B) Four hours later, this ABC had given rise to a cell on the external somite surface. (C) Ten hours after injection, 2 cells remain on the surface of the somite. (D) Both cells (red) are Pax7-positive [colocalisation of rhodamine and Pax7 (green) in the nucleus appears yellow] on the surface of the myotome (blue). (E) Transverse section of one labeled cell shown in D. (F-J) ABCs give rise to muscle. (F) The injected ABC (red). (G) Four hours later, this cell is on the external somite surface. (H) Ten hours after injection, this ABC formed an elongated fibre. (I) The elongated muscle fibre (red) is obliquely oriented, like fast fibres, Pax7-negative (green), and is superficial within the myotome (blue). (J) Transverse section of the embryo in I, the labeled cell is a lateral fast fibre. (K-O) Posterior cells elongate early. (K) The injected posterior cell. (L) Four hours later, the posterior cell had elongated, deep within the somite. (M) Ten hours after injection, the posterior cell had formed an elongated muscle fibre. (N) The elongated fibre (red) is Pax7-negative (green), and is deep within the myotome (blue). (O) Transverse section of the embryo in N, the labeled cell is a very medial fast fibre. (P-U) External cells persist into the larval stage. (P) The injected ABC (red). (Q) Ten hours after injection, this cell is on the external surface of the myotome. (R) Thirty-four hours after injection, this cell had generated multiple cells. (S) Fifty-seven hours after injection, all injected cells remain on the external surface, arrows show the position of sections shown in T,U. (T,U) The injected cell gave rise to two Pax7-positive (yellow) cells on the surface of the myotome (blue). A,F,K and P are dorsal views, anterior to the top, the notochord is at the right of the image. B-D,G-I,L-N and Q-S are lateral views, with anterior to the left and dorsal to the top. E,J,O,T,U are transverse sections with dorsal to the top. Scale bars: 50  $\mu\text{m}$  in C for A-C,F-H,K-M,P-R; 50  $\mu\text{m}$  in O for D,E,I,J,N,O; 25  $\mu\text{m}$  in S; 25  $\mu\text{m}$  in U for T,U.

found primarily at the dorsal and ventral extremes of myotome, as well as at the horizontal myoseptum. They continue to express Pax7 (Fig. 2T,U).

### ABCs enter the myotome between existing fibres

In amniotes, early myogenic precursors downregulate Pax7 expression as they enter the myotome from the dorso-medial, ventro-lateral, rostral and caudal lips of the early dermomyotome (Ben-Yair et al., 2003; Relaix et al., 2005). Later myogenic precursors from the centre of the dermomyotome enter the myotome directly, retaining Pax7 expression for a prolonged time. These cells are responsible for fetal muscle growth and the generation of muscle satellite cells. We charted the position of Pax7-positive nuclei within the myotome to provide an indication of where external cells enter the myotome in zebrafish. We found that Pax7-positive nuclei within the myotome were excluded from rostral and caudal myotome ends, and were instead found scattered along the central region of the myotome (Fig. 3B,C). This suggests myogenic precursors enter the myotome directly, by moving between superficial slow muscle fibres along their length, but not around their ends. These Pax7-positive cells within the myotome are usually positive for MRFs (data not

shown) (Devoto et al., 2006). We suspect that these cells differentiate soon after entering the myotome, and are not equivalent to late myogenic precursors derived from the centre of the dermomyotome in amniotes.

### Medial to lateral expansion of the myotome

The above gene expression and fate map data indicate that early-differentiating posterior cells give rise to medial fast fibres, whereas later-differentiating ABCs give rise to lateral fast fibres. Consistent with these observations, medial fast fibres became post-mitotic prior to lateral fast fibres. Early BrdU exposure led to BrdU-positive muscle fibre nuclei throughout the entire medio-lateral extent of the myotome (Fig. 3D). By contrast, late BrdU exposure led to BrdU-positive nuclei only along the lateral edge of the fast myotome, just medial to slow muscle fibres (Fig. 3E).

To confirm these results we used fibre cross-sectional area measurements to map the distribution of new fast fibres [very few slow fibres are added (Barresi et al., 2001)]. At 48-hour, 72-hour and 96-hour stages, smaller diameter [presumptive new (Veggetti et al., 1993)] fast fibres were in the lateral myotome, whereas larger diameter fast fibres were in the medial myotome (Fig. 3F-H).

**Table 1. Fates of injected anterior border cells**

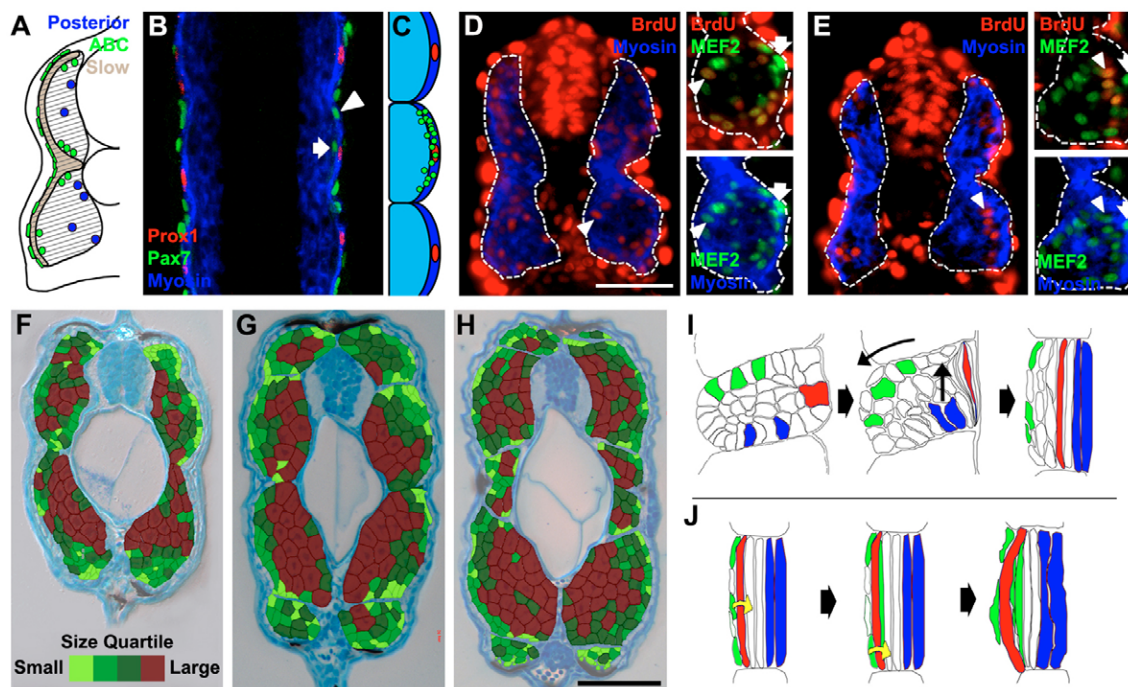
	Time after segmentation		
	4-8 hours	10-15 hours	34-200 hours
External cells	62 (89%)	31 (43%)	6 (18%)
External cells and muscle fibres	2 (3%)	7 (10%)	3 (9%)
Muscle fibres	5 (7%)	32 (46%)	23 (72%)
Unknown	1 (1%)	0 (0%)	0 (0%)
Totals	70 (100%)	70 (100%)	32 (100%)

### CONCLUSIONS AND PERSPECTIVE

In sum, zebrafish myogenesis proceeds in distinct waves. First, posterior cells of the epithelial somite and adaxial cells generate an embryonic myotome, while ABCs form a layer of undifferentiated myogenic precursors external to this myotome (Fig. 3I). Second, some of these external cells generate new fast fibres lateral to the earlier formed, deep, fast fibres, probably by moving between and not around pre-existing slow fibres (Fig. 3J).

Zebrafish external cells are very similar to early amniote dermomyotome cells. Both are specified in the epithelial somite, express Pax3 and Pax7 and give rise to both muscle fibres and

more Pax7-expressing external cells. In addition to their shared position, gene expression and fate, external cells and early dermomyotome cells are proliferative, and Hedgehog promotes their differentiation into muscle (Buckingham, 2006; Feng et al., 2006; Hammond et al., 2007; Johnson et al., 1994). We do not yet know if later zebrafish external cells also share features with later amniote dermomyotome cells, which give rise to dermis, endothelial cells, cells that migrate into limbs, and cells that enter the myotome as mitotically active, Pax7-positive myogenic precursors (Yusuf and Brand-Saber, 2006). The zebrafish dermis is acellular until 4 weeks of life (Le Guellec



**Fig. 3. Distinct waves of myogenesis and a model for myotome development.** (A) A summary of the fates of injected ABCs and posterior cells at 24 hours. Some injected ABCs (green) have formed differentiated fast muscle fibres, all are between superficial slow fibres and the earlier formed, fast fibres. All posterior cells (blue) have differentiated into medial fast fibres. (B) Dorsal view of a 24-hour embryo labeled for Pax7 (green), Prox1 (red) and myosin (blue). Slow fibre nuclei (red, Prox1) are at the surface of the myotome (blue, myosin). Most Pax7-positive nuclei (green) are outside of the myotome, but some are within the slow (arrowhead) or fast (arrow) domains of the myotome. (C) Schematic showing the location of Pax7-positive nuclei that are within the myotome. The Pax7-positive nuclei within the myotome (MyHC-positive domain) from 30 different somites (somites 9-11 in a 24-hour embryo) are shown. (D) Transverse section through a mid-trunk somite (S9-12) of a 24-hour embryo treated with BrdU from the 5S stage (11 hours 40 minutes) to 24 hours. BrdU-positive nuclei (red, arrowheads) are found throughout the myotome (MyHC, blue), except for the superficial slow nuclei (arrows) (Barresi et al., 2001). Insets show muscle nuclei labeled with MEF2 (green). (E) Transverse section through a mid-trunk somite (S9-12) of a 24-hour embryo treated with BrdU from the 20S stage (19 hours) to 24 hours. BrdU-positive nuclei in the myotome are found solely within the lateral fast myotome (arrowheads). (F-H) Fibres with a smaller cross-sectional area are found laterally at 48 hours (F), 72 hours (G) and 96 hours (H); superficial slow fibres were excluded from this analysis. Relative cross-sectional area is shown according to size quartiles: light-green fibres, smallest 25%; dark-red fibres, largest 25%. (I, J) Models of somite cell movements and fates, viewed from dorsal (the midline is to the right). (I) Posterior cells (blue) become the medial fast fibres and ABCs (green) move laterally, becoming external cells expressing Pax7. (J) External cells then move medially into the myotome to become lateral fast fibres. During this time, adaxial cells (red) are displaced laterally and become superficial slow muscle fibres. Scale bars: 50  $\mu$ m in D, H.

et al., 2004); it remains unknown whether external cells in zebrafish contribute to dermis or other cell types later in larval life.

Unlike the amniote dermomyotome, zebrafish external cells derive from the anterior border of the epithelial somite. Interestingly, in *Xenopus*, anterior epithelial somite cells also move to the lateral surface, during the process of somite rotation (Afonin et al., 2006). Whether these cells become external cells (Grimaldi et al., 2004) is unknown. However, in other amphibians (Keller, 2000), as well as in chick (Selleck and Stern, 1991; Stern and Canning, 1990) and in mouse (Eloy-Trinquet and Nicolas, 2002), it is clear that the dermomyotome (external cells) do not derive from the anterior border of the epithelial somite. Our results demonstrate that although the cellular movements underlying the initial development of external cells in teleosts and the dermomyotome in amniotes may have diverged, the presence of an external myogenic precursor population is conserved among vertebrate lineages.

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