Cellular dynamics of regeneration reveals role of two distinct Pax7 stem cell populations in larval zebrafish muscle repair

Tapan G. Pipalia1,4, Jana Koth1,2,4, Shukolpa D. Roy1, Christina L. Hammond1,4, Koichi Kawakami3 and Simon M. Hughes1,4,

ABSTRACT
Heterogeneity of stem cells or their niches is likely to influence tissue regeneration. Here we reveal stem/precursor cell diversity during wound repair in larval zebrafish somitic body muscle using time-lapse 3D confocal microscopy on reporter lines. Skeletal muscle with incision wounds rapidly regenerates both slow and fast muscle fibre types. A swift immune response is followed by an increase in cells at the wound site, many of which express the muscle stem cell marker Pax7. Pax7+ cells proliferate and then undergo terminal differentiation involving Myogenin accumulation and subsequent loss of Pax7 followed by elongation and fusion to repair fast muscle fibres. Analysis of pax7a and pax7b transgenic reporter fish reveals that cells expressing each of the duplicated pax7 genes are distinctly localised in uninjured larvae. Cells marked by pax7a only or by both pax7a and pax7b enter the wound rapidly and contribute to muscle wound repair, but each behaves differently. Low numbers of pax7a-only cells form nascent fibres. Time-lapse microscopy revealed that the more numerous pax7b-marked cells frequently fuse to pre-existing fibres, contributing more strongly than pax7a-only cells to repair of damaged fibres. pax7a-marked cells are more often present in rows of aligned cells that are observed to fuse into a single fibre, but more rarely contribute to nascent regenerated fibres. Ablation of a substantial portion of nitroreductase-expressing pax7b cells with mitomycin d prior to wounding triggered rapid pax7a-only cell accumulation, but this neither inhibited nor augmented pax7a-only cell-derived myogenesis and thus altered the cellular repair dynamics during wound healing. Moreover, pax7a-only cells that did not regenerate pax7b cells, suggesting a lineage distinction. We propose a modified founder cell and fusion-competent cell model in which pax7a-only cells initiate fibre formation and pax7b cells contribute to fibre growth. This newly discovered cellular complexity in muscle wound repair raises the possibility that distinct populations of myogenic stem cells contribute differentially to repair in other vertebrates.

INTRODUCTION
Efficient wound repair is key to vertebrate survival and thus under strong evolutionary selection. In skeletal muscle, wounds, surgery, degenerative diseases, or even the high forces generated during running downhill, trigger damage that is repaired from satellite cells, resident muscle stem cells that lie beneath the basal lamina of healthy muscle fibres (Mauro, 1961). During repair, satellite cells activate to form proliferative myoblasts. Some lineage descendants of these satellite cell-derived myoblasts regenerate fibres by cell cycle exit, terminal differentiation and fusion, but other satellite cell progeny self-renew, returning to quiescence. Molecular mechanisms involved in satellite cell-dependent muscle fibre repair are increasingly understood, mainly through studies in rodents and in tissue culture cells (reviewed in Ciciliot and Schiaffino, 2010; Yin et al., 2013). For example, recent studies have highlighted the importance of the transcription factor Pax7 as a marker of satellite cells and a key regulator of the repair process itself (Gunther et al., 2013; Seale et al., 2000; von Maltzahn et al., 2013). It remains unclear, however, whether all satellite cells are equal or whether distinct classes of muscle precursor cell (MPC) contribute to distinct aspects of muscle regeneration.

The difficulty of imaging the muscle repair process in the live animal has hampered efforts to analyse muscle stem cell contributions to repair. With this in mind, a number of groups have turned to the zebrafish, in which optical clarity permits lineage tracing and monitoring of individual identified cells in vivo over long periods. Like other teleosts, zebrafish efficiently repair muscle wounds (Knappe et al., 2015; Li et al., 2013; Ott et al., 2012; Rodrigues et al., 2012; Rowlerson et al., 1997; Seger et al., 2011) and accumulation of Pax7-expressing cells in wounds has been described (Knappe et al., 2015; Seger et al., 2011). Zebrafish models of several muscle-degenerative diseases have been developed (Bassett et al., 2003; Gupta et al., 2011, 2012; Ruparelia et al., 2012; Szal et al., 2012; Wallace et al., 2011) and their regeneration analysed (Seger et al., 2011). Moreover, satellite cells marked by Pax7 have been reported in a variety of teleost species, including zebrafish (Hollway et al., 2007; Zhang and Anderson, 2014; reviewed in Siegel et al., 2013).

Developmentally, satellite cells originate from the dermomyotome of the somite, a transient embryonic structure that is also marked by expression of Pax7, and its close parologue Pax3 (Gros et al., 2005; Kaspar-Duchossoy et al., 2005; Relaix et al., 2005). The teleost equivalent of dermomyotome, an external cell layer of Pax3- and Pax7-expressing cells on the lateral somite surface, exists in zebrafish and contributes to muscle surface (Devoto et al., 2006; Groves et al., 2005; Hammond et al., 2007; Hollway et al., 2007; Stellabotte et al., 2007; Waterman, 1969). Dermomyotomal cells reside on the somite.
RESULTS

Time course of muscle repair in larval zebrafish

Zebrafish larvae expressing GFP in specific muscle fibre types were wounded by unilateral needle insertion into the epaxial somite. Tg(9.7kb smyhc1:gfp)i104, in which the slow myosin heavy chain 1 enhancer drives GFP labelling of ~20 mononucleate superficial slow muscle fibres in each somite (Elworthy et al., 2008), and Tg(-2.2myl2:2:gfp)i135, which labels underlying multinucleate fast fibres (von Hofsten et al., 2008), were used to analyse fibre loss and repair in individual fish over time (Fig. 1A-C). Upon lesion, GFP fluorescence rapidly diminished in the disrupted fibres. At 1 day post-wounding (1 dpw), significant loss of labelled fibres was observed in one to three somites in each transgenic line. Contralateral and adjacent somites seemed unaffected (Fig. 1A,B and data not shown). By 2 dpw, small smyhc1-GFP and mylz2-GFP fibres were observed spanning the wound region. Reappearance of GFP in both slow fibre monolayer and underlying fast muscle was significant by 3 dpw (Fig. 1A-C). Although fibres generally re-integrated correctly into the original somite structure, some misplaced slow fibres were observed deep in the wound site (Fig. 1D-D∗). Kaede photoconversion-based cell tracking revealed that the vast majority of labelled cells at the injury site were lost and replaced by weakly fluorescent cells between 2-4 dpw (Fig. 1E), thereby showing that Kaede tracing was not suitable to determine the source of regenerated muscle fibres (Fig. 1E). Analysis of wounded larvae stained with phalloidin and Hoechst 33342 confirmed the loss of structural components of muscle (Fig. S1A). Nuclei within the lesion were rapidly lost and then reaccumulated at the wound site from 2 dpw onwards (Fig. S1B; Fig. 1F). Thus, damage to somitic muscle fibres is rapidly repaired, consistent with previous reports (Rodrigues et al., 2012; Seger et al., 2011). These findings show that the cell biology of muscle wound repair is open to time-lapse analysis in zebrafish embryos.

To understand muscle cell behaviour during regeneration in more detail, 3.5 days post-fertilisation (dpf) larvae labelled in nuclei with histone-GFP and plasma membrane with mCherry were analysed by time-lapse confocal microscopy for 8.5 days (n=5) (Fig. 2; Movie 1). Again, we observed a clear disruption of muscle structure immediately after wounding and fluorescence decreased (Fig. 2). By 24 hours post-wounding (hpw), the shape of nuclei within the wound was more heterogeneous than in equivalent unwounded regions. Cells with small round GFP+ nuclei, morphologically unlike those in unwounded fibres, arose in the wound region. Between 24-72 hpw, some of these cells had intense membrane mCherry signal, moved rapidly, and were probably leukocytes (Fig. 2; Movie 1 and see below). Less motile rounded GFP+ nuclei persisted beyond 72 hpw, becoming more numerous over subsequent days (Movie 1), with some showing mitotic profiles. Membrane mCherry signal diminished in the wound after 48 hpw, paralleling degradation of fibre components (Fig. 1E). From 3-8 dpw, some nuclei within the wound had higher GFP signal than their neighbours in adjacent unwounded imaged somites, potentially reflecting synthesis of new histone-GFP during cell proliferation (Fig. 2). By 120 hpw (5 dpw), rows of aligned nuclei were observed in the wound (Fig. 2). Despite some persistent disorganisation of the wounded somite, aligned elongated nuclei characteristic of mature fibres accumulated after 5 dpw. Like Kaede, membrane mCherry remained low in the regenerating wound, presumably because regenerating fibres could not re-synthesise the markers due to a lack of residual injected RNA. Taken together, the time course of the events during larval zebrafish muscle regeneration parallels that of adult mammal muscle regeneration.

Rapid epidermal closure and leukocyte infiltration to muscle wounds

Avoidance of bacterial infection is a key element of the response to injury. We observed that epidermal lesions closed rapidly, within 1 h in a purse-string fashion in the case of single somite-width needle lesions (Fig. S2A-C). Moreover, as in the case of simple epidermal wounds or muscle degeneration (Richardson et al., 2013; Walters et al., 2009), leukocytes (marked by hz and mpx transgenes and therefore probable neutrophils) infiltrated the wound within 2 hpw (Fig. S2D,E). Brightly mCherry-fluorescent cells, putative phagocytes, entered the wound within 20 min (Fig. S2F). These appear to be invading leukocytes that transiently occupied the wounded somite, constituting a small fraction of the ~160 total nuclei in an epaxial somite at 48 hpw, and then leave during the 36-60 hpw period (Fig. 1E; Fig. S2E,F). Thus, most nuclei in regenerating somites are not leukocytes.

Nuclear loss and recovery during muscle regeneration

Despite the invasion of leukocytes, total nuclear number transiently decreased in wounded epaxial somites shortly after injury and remained reduced at 24 hpw, presumably resulting from the degradation of damaged tissue (Fig. 1F; Fig. S1). Thereafter, average nuclear number recovered, reaching 125% of control or adjacent uninjured somites (Fig. 1F). In somites with smaller wounds, nuclear number did not increase, whereas larger wounds generally led to a significant excess of nuclei compared with adjacent unwounded somites (Fig. 1F). We conclude that proliferation and/or migration of cells into the myotome contribute to the regeneration of somitic muscle.

Wounding triggers proliferation and differentiation of Pax7 cells within the somite

In addition to muscle fibres, at the time of wounding, somites contained mononucleate cells, many of which are marked by the muscle stem/precursor cell marker Pax7 (Hammond et al., 2007; Hollway et al., 2007; Minchin et al., 2013; Stellabotte et al., 2007; Windner et al., 2012). These Pax7+ cells are originally distributed
on the lateral myotome surface and concentrate at the dorsal and ventral edges of the somite and the horizontal and vertical myosepta (HZM and VMZ; Windner et al., 2012). Subsequently, small numbers of Pax7+ cells arise in the deep central myotome (Minchin et al., 2013) (Fig. 3A). Upon making a large wound, the number of Pax7+ cells was rapidly reduced and then recovered by 1 dpw (Fig. 3A,B,D; Fig. S3A). At 1-3 dpw, an increased proportion of Pax7+ cells were in S-phase, as assayed by EdU pulse labelling (Fig. 3B,C,F). To demonstrate that proliferative cells contribute to new muscle fibres in wounds, larvae marked with membrane-targeted GFP were continuously exposed to EdU from 3 hpw to 3 dpw. At regenerating wounds, large numbers of nuclei were observed, most of which were EdU+ and new fibres at the wound contained multiple EdU+ nuclei (Fig. S4). Fibres in adjacent unwounded somites contained few EdU-labelled and many unlabelled nuclei (Fig. S4), indicative of a low rate of MPC fusion to muscle fibres during growth. Most nuclei in regenerating muscle wounds had undergone S-phase after wounding. Thus,
proliferation of Pax7+ cells contributes to recovery in somite cell numbers.

The location of Pax7+ cells changed after wounding. Whereas in control or adjacent unwounded somites, Pax7+ cells remained predominantly at somite borders and HMZ, numerous Pax7+ cells arose in the central myotome after wounding (Fig. 3B,C,E; Fig. S3A), consistent with the observations of Seger et al. (2011). Furthermore, in wounded somites, Pax7+ cells at the VMZ recovered more slowly than those in the central myotome, even though Pax7+ cells at the VMZ rapidly entered S-phase after wounding (Fig. 3E; Fig. S3D,E). Analysis of cells at the VMZ of wounded somites, paralleling the loss of all cell types examined (data not shown). However, at both 1 and 3 dpw, more Myog+ cells were present in the central region of damaged somites than in undamaged adjacent somites (Fig. 3G,1). Moreover, the fraction of Pax7+ cells co-expressing Myog was increased compared with adjacent unwounded somites, as was the number of cells expressing Myog alone (Fig. 3G, Fig. S3B,C). Similar co-expression of Pax7 and Myog has been reported previously (Day et al., 2009; Devoto et al., 2006). These findings suggest that, despite the increased proliferation of the Pax7+ cell population, Pax7+ cells in wounds were more frequently undergoing terminal differentiation than those in unwounded regions.

**Pax7-expressing cells contribute to muscle regeneration**

To verify that Pax7+ cells contribute to muscle regeneration, we employed fish labelled with a pax7a:GFP BAC transgene (Mahalwar et al., 2014; S. Alsheimer, PhD thesis p. 249, Universität Tübingen, 2012). Prior to wounding, and in control and adjacent unwounded somites, the reporter labelled cells on the somite borders, as well as xanthophores and cells in the dorsal neural tube. To examine the response of MPCs specifically, pax7a:GFP was bred onto a pfeffer mutant background that substantially reduces xanthophore number (compare Fig. 4A with Fig. 5A) (Odenthal et al., 1996). In large wounds, most pax7a:GFP signal was lost at the wound site, consistent with ablation of many MPCs (Fig. S6A). pax7a:GFP cells re-accumulate at 1 dpw, divide and migrate, gradually invading the wound and contributing to fibres near the wound edge by 2 dpw (Fig. S6). Correlating with the extent of wound and time course of pax7a:GFP cell invasion, repair rate varied. However, by 6 dpw muscle seemed regenerated and some pax7:GFP cells remained undifferentiated after recovery (Figs S6, S7). Thus, pax7a-expressing MPCs participate in muscle wound repair.

**Pax7a- and Pax7b-expressing cells behave differently**

We observed a mismatch between transgenic pax7a:GFP labelling and Pax7 immunoreactivity. In large wounds, Pax7 protein was detected in more cells within the central wound by 1 dpw, than expressed pax7a:GFP (Fig. 3B,D; Fig. 4B; Fig. S6A, Fig. S7). Zebrafish have duplicated Pax7 paralogues, pax7a and pax7b, which are both expressed in somitic cells (Minchin and Hughes, 2008). We therefore examined a pax7b splicing trap reporter line (gsAlzGFFD164A;5xUAS:EGFP; Fig. S8) for the response of cells expressing pax7b to muscle wounding. At 3 dpf in this line (hereafter referred to as pax7b:GFP), strong GFP was observed in cells in or near the myosepta, both VMZ and HMZ. GFP was also detected in numerous superficial fast muscle fibres (Fig. 4A) (Minchin and Hughes, 2008). This markedly contrasted with pax7a:GFP, which rarely marked fibres in unwounded conditions (Fig. 4A). Conversely, pax7a:GFP strongly marked xanthophores, but pax7b:GFP was weaker in these cells. Instead, pax7b:GFP was observed in numerous dermomyotome cells overlying the slow fibres (Fig. 4A). Whereas pax7a:GFP cells predominated at the dorsal edge of the myotome, pax7b:GFP cells were concentrated at the HZM (Fig. 4A,B). Thus, pax7a:GFP and pax7b:GFP were differentially regulated, prompting the question of their expression in wounds.

Time-lapse 3D confocal microscopy of wounds revealed differences between pax7a:GFP and pax7b:GFP MPCs. Upon wounding, both pax7a:GFP+ and pax7b:GFP+ cells were reduced in
numbers, but already by 1 dpw re-accumulated in the wound region (Fig. 4B). Like pax7a:GFP+ cells, pax7b:GFP+ cells were observed to proliferate (Fig. 4C). Short time-lapse analyses showed that both pax7a:GFP+ and pax7b:GFP+ cells often migrate into the myotome from somite borders (data not shown). Fibre labelling in wounds was consistently more pronounced in pax7b:GFP than in pax7a:GFP fish. Whereas about half of new pax7b:GFP+ fibres were brighter than surrounding marked mononucleate cells, this was not the case for pax7a:GFP+ fibres (Fig. 4B,D; Fig. S6C, Fig. S7). In wounds, pax7b:GFP+ cells were observed to fuse to large, presumably pre-existing, fibres, as well as to contribute to thin nascent fibres (Fig. 4E). Fusion to pre-existing fibres was very rarely observed for pax7a:GFP+ cells (data not shown). Most strikingly, pax7a:GFP rarely marked large regenerating fibres within wounds, and then only weakly, suggesting that the GFP in nascent fibres is not stable enough to persist in larger maturing fibres (Fig. 4D). In contrast, pax7b:GFP readily marks maturing fibres in wounds, both superficial and deep, and these were brighter than with pax7a:GFP, suggesting that pax7b:GFP-expressing cells contribute to the growth of regenerated or damaged fibres (Fig. 4D,E). To summarise, cells marked by either pax7a or pax7b each contribute to larval muscle wound repair.

To understand the different behaviour of pax7a- and pax7b-marked cells better, pax7a:GFP;gSAIzGFFD164A;5xUAS:RFP (pax7a:GFP;pax7b:RFP) larvae were examined. To reduce the complexity of wound repair dynamics, smaller focal wounds were made by fine needle insertion into a local region of a single somite. Such wounds had a more uniform repair time course and facilitated imaging and were therefore used in all subsequent experiments. MPCs contained either pax7a:GFP, pax7b:RFP or both (Fig. 5A,D). Most pax7a:GFP-only cells were located at the dorsal myotome edge or VMZ, whereas most pax7b:RFP-only cells were located at the HZM (Fig. S9A). Upon wounding, both pax7a:GFP and pax7b:RFP cells accumulated in the wound at 1 dpw, with most cells expressing both markers. Cells expressing only pax7a:GFP were also observed within wounds (Fig. 5B; Fig. S9B). GFP in pax7a:GFP-only cells was in general brighter than in dual-labelled cells. Counts revealed that pax7a:GFP-only and pax7a:GFP:pax7b:RFP dual-labelled MPCs accumulated in wounded somites at 1 dpw (Fig. 5D). pax7b:RFP-only cells did not increase in numbers and were rare within wounds. Thus, the presence or absence of pax7b:RFP distinguished two MPC populations within wounds.

In contrast to 1 dpw, by 2 dpw, each gene marked regenerated fibres within the wound region (Fig. 5B). Almost all newly formed fibres in pax7a:GFP;pax7b:RFP larvae at 2 dpw had detectable RFP and GFP, although their GFP was generally weak (Fig. 5B; Fig. S9B, see also Movies 2, 3). Moreover, clusters of large fibres

---

**Fig. 3. Rapid recovery of Pax7-expressing cells in wounded somites through proliferation and relocation enhances differentiation in central myotome.** Wild-type zebrafish larvae wounded at 3 dpf in epaxial somites 16-18 (yellow brackets) were analysed at the indicated times post-wounding by confocal immunodetection of Pax7 with EdU (A-F) or Myogenin (G,I) or in situ mRNA hybridisation for myf5 (H), shown in lateral view, anterior to left, dorsal to top. Blue boxes are magnified. (A-C) Diminished numbers of Pax7+ cells after wounding (A) are rapidly replaced (B) and show increased proliferation (B,C). (D-G) Pax7+ cells were counted in 2-4 wounded and 2-4 adjacent unwounded somite regions per larva and averaged to yield a value for each animal. VMZ, vertical myoseptum. Mean±s.e.m values from four larvae (D,E,G) or the number indicated (F). Statistical analysis is shown in Fig. S3A. (H) myf5 mRNA adjacent to a hypaxial wound (outlined by dots). Note the lack of myf5 mRNA in unwounded somites at this stage. (I) Pax7+ Myog+ nuclei (white arrowheads) generally have lower Myog signal than Pax7+ Myog+ cells (yellow arrowheads). Scale bars: 50 µm.
acquired labelling in the wound region, but not elsewhere, and tended to look red. Such newly marked fibres augmented the pre-existing red fibres. (Fig. 5B; Movie 2). These data show that pax7a:GFP-only cells behave differently from pax7b:RFP cells (most of which also express pax7a:GFP), with the latter contributing more strongly to fibres in wounds.

To compare the contribution of each cell population to fibre repair, the response of mononucleate MPCs in identified wounded fish was examined at 1 dpw and again at 2 dpw. At 1 dpw, pax7b:GFP cells were numerous and frequently observed in rows of up to seven cells within the wound region aligned with fibres, but subsequently disappeared, being replaced by marked fibres. The orientation of such rows subtly changed with depth within the somite, matching the orientation of fast fibres in unwounded somites (Fig. 5C and data not shown). In contrast, although the bright pax7a:GFP-only MPCs were motile, no groups had more than four aligned MPCs (Fig. 5C,E). Moreover, such MPCs within wounds at 1 dpw did not form large numbers of pax7a:GFP-only fibres at 2 dpw (Fig. 5B,C). Thus, dual-labelled cells aligned and seemed to fuse more often than pax7a:GFP-only cells.

To understand whether disappearance of dual-labelled cells reflected loss of markers, cell death, migration or fusion, we performed continuous time-lapse analysis between 1 and 2 dpw. Dual-labelled MPCs were highly dynamic and frequently fused to pre-existing fibres (Fig. 6; Movie 2). Upon fusion, GFP and some RFP immediately filled the host fibre, as predicted from the rapid cytoplasmic GFP diffusion in fibres (Bajanca et al., 2015). However, the fortuitous localisation of a portion of the RFP in punctate structures within some MPCs allowed us to track the location of the fusing MPC and its integration over an hour into the fibre outline (Fig. 6A,B). pax7b-reporter+ cell appearance in the wound region followed by fusion. Disappearance of small bright GFP+ cells amongst the superficial red fibres (arrows) and muscle fibres (arrows) over the ensuing days. Asterisk indicates persistence of a deep fast fibre marked by pax7b-reporter prior to wounding. Unwounded somites also accumulate small numbers of marked mononucleate cells (magenta arrowheads). (C) Time series confocal slices showing pax7b-reporter+ cell division (arrowheads) prior to wounding. (D) Magnified confocal slices showing wounding (yellow brackets) and repair. Note the stronger fibre labelling (arrows) with pax7b-reporter than with pax7a:GFP, relative to mononucleate cells (arrowsheads). (E) Time series confocal slices showing superficial (s/f, left panels) and deep (right panels) pax7b-reporter+ cell appearance in the wound region followed by fusion. Disappearance of small bright GFP+ cells amongst the superficial fibres (red arrowheads) correlated with appearance of small bright GFP+ cells in deep regions (blue arrowheads, centre). Loss of some small deep cells then correlated with appearance of weakly GFP-labelled fibres (blue arrows; rightmost panel). Asterisks indicate a deep fast fibre marked by pax7b-reporter prior to wounding. Scale bars: 50 µm.
dual-labelled MPCs are rapidly dividing, differentiating and fusing to fibres, consistent with the abundant labelling of fibres by \textit{pax7b}:\textit{RFP} (and weak \textit{pax7a}:\textit{GFP}), whereas \textit{pax7a}-only cells behave differently, aligning less in wounds and initiating fibre formation.

\textbf{Ablation of \textit{pax7b}-expressing cells reveals \textit{pax7a}:\textit{GFP} cell behaviour}

To examine the behaviour of \textit{pax7a}:\textit{GFP}-only cells in the absence of dual-labelled cells, we ablated \textit{pax7b}-expressing cells using the nitroreductase/metronidazole (NTR/MTZ) system (Curado et al., 2007). Treatment of \textit{pax7a}:\textit{GFP};\textit{gSAIzGFFD164A};\textit{UAS-E1b:gal4};\textit{UAS:NTR-}\textit{mCherry} larvae with MTZ overnight eliminated most \textit{mCherry}-labelled MPCs and led to numerous phagocytes containing red debris in the ventral regions (Fig. S10). MTZ had no effect on larvae in the absence of \textit{NTR-mCherry} cells (blue arrowheads). Dual-labelled somite cells (magenta arrowheads) concentrate on VNz. Note the lack of \textit{Pax7} cells in the deep myotome at this stage. The \textit{pax7b}-reporter labelled cells strongly in somites, and also weakly in dorsal neural tube (NT).

\textbf{NTR-\textit{mCherry} cells, but substantial numbers of \textit{pax7a}:\textit{GFP}-only cells remained at 3 dpf. Thus, MTZ efficiently and selectively eliminates most \textit{pax7b}-expressing cells.}

When untreated larvae were wounded, numerous \textit{NTR-mCherry}-labelled fibres arose within the wound at 2 dpw (Fig. 7A,B). In contrast, when MTZ-treated larvae were wounded, few \textit{pax7b}:\textit{NTR-mCherry}-labelled fibres arose within the wound, consistent with the ablation of most \textit{pax7b}:\textit{NTR-mCherry} MPCs (Fig. 7A,B; Table S1). Nevertheless, remaining \textit{pax7a}:\textit{GFP}-only cells accumulated in the wound (Fig. 7A). Strikingly, however, no recovery of \textit{NTR-mCherry}-labelled cells was observed until at least 3 dpw, demonstrating that \textit{pax7a}:\textit{GFP}-only cells did not give rise to \textit{pax7b}-expressing cells (Fig. 7A,B). Within wounds, \textit{pax7a}:\textit{GFP}-only cells formed thin nascent fibres expressing GFP, both in MTZ-treated and untreated larvae (Fig. 7A,B; Table S1). At 1 and 2 dpw, \textit{pax7a}:\textit{GFP} cells were more numerous in wounded somites than in their presence, suggesting rapid proliferation of remaining \textit{pax7a}:\textit{GFP} cells (Fig. 7B; Table S1 and data not shown). Nevertheless, the extra \textit{pax7a}:\textit{GFP}-only MPCs did not give rise to additional \textit{GFP}-only fibres compared with non-ablated injured controls, at least prior to 3 dpw, the latest time point examined (Fig. 7B; Table S1). In contrast, MTZ greatly reduced formation of new dual-labelled fibres, consistent with the reduction in \textit{pax7b}-expressing MPCs (Fig. 7B; Table S1). Surprisingly, despite the absence of \textit{pax7b}:\textit{NTR-mCherry}-labelled fibres and lack of compensating increase in \textit{pax7a}:\textit{GFP} fibres, the gross morphology of wounds in MTZ-treated fish did not appear worse than that of untreated wounded larvae at 2 dpw (data not shown). These results show that \textit{pax7a}:\textit{GFP}-only cells do not convert to \textit{pax7b}-expressing cells within wounds and do not substitute for the depletion of the latter cells by enhanced differentiation.
Fusogenicity of pax7b-expressing cells

As already described (Fig. 5C,E, Fig. 6), pax7b:GFP MPCs align more often in rows than pax7a:GFP-only cells, suggesting different potential fusogenic behaviour. Several additional differences between pax7a:GFP-only and the pax7b:GFP MPCs support this view. Firstly, whereas both populations of precursor cells had increased in number to a similar extent at 1 dpw, by 2 dpw pax7b:GFP cell numbers had returned to the unwounded value, but bright pax7a:GFP cell numbers remained elevated (Fig. 7C), indicating a greater tendency to differentiate and fuse in pax7b:GFP MPCs. Secondly, the decrease in pax7a:GFP MPC numbers between 1 and 2 dpw was, within counting error, similar to the number of new pax7a:GFP fibres formed; there were no ‘missing’ MPCs. In contrast, the decrease in pax7b:GFP MPC numbers was greater than the increase in the number of pax7b:GFP fibres at 2 dpw; the difference we termed ‘missing’ MPCs (Fig. 7C). As neither apoptosis nor gradual loss of label of pax7b:GFP cells were observed (e.g. Movie 2), the apparently ‘missing’ fraction of pax7b:GFP MPCs might have fused with one another to form multinucleate fibres. Lastly, when a GFP+ MPC fused to a pre-existing fibre, the GFP signal immediately became much weaker. This argues that large fibres with bright fluorescence derive from fusion of multiple marked MPCs; such bright fibres are rare in pax7a:GFP but more common pax7b:GFP fish (Fig. 4B). Taken together, these data suggest that pax7a:GFP cells contribute less to fibre repair in wounds than do pax7b:GFP cells.

Fig. 6. Fusion of pax7a- and pax7b-reporter cells to existing myotubes during wound repair. Extended orthogonal projection views of an epaxial somite wound in a pax7a:GFP; pax7b:gal4;UAS:RFP 4 dpf larva showing individual pax7a:GFP:pax7b:RFP dual-labelled (yellow) MPCs fusing to existing unlabelled (A) or RFP+ (B) fibres from Movies 2 and 3. The whole image was non-linearly enhanced and brightness corrected to compensate for bleaching and facilitate tracking of individual cells, as described in Materials and Methods. (A) At 25.5 hpw, prior to fusion, an MPC had uniform cytoplasmic GFP and diffuse cytoplasmic and vesicular RFP (arrow). 10 min later, cytoplasmic GFP and RFP have now filled the whole cytoplasm of a large adjacent previously unlabelled fibre (brackets), whereas the vesicular RFP remains localised (arrowhead) and integrates into the fibre in the succeeding 50 min (see Movie 2, blue MPC). Transverse II shows the same view as Transverse I, but with the fusing fibre marked (dots). (B) At 39.5 hpw, two MPCs (magenta and yellow arrows), fuse simultaneously to the same large adjacent myotube (arrowheads; Movie 2, magenta and yellow MPCs). (C) At 32.3 hpw, a dual-labelled MPC (arrowhead; Movie 3, white MPC) that originated from the anterior somite border, divided and then migrated along a recently formed GFP+ nascent myofibre (arrow). 10 min later the MPC has fused into the nascent fibre, as shown by RFP loss from the MPC and increase in the fibre. The fused cell remains distinct at 10 min, but merges into the nascent fibre by 20 min. Process shown in merge and single colour lateral (dorsal up, anterior left) and transverse (dorsal up, medial left) views. Blue lines indicate range of extended orthogonal projection views.
DISCUSSION
Our findings on muscle wound repair lead to three major conclusions. First, that the process and timing of muscle repair in larval zebrafish has great similarities to that in adult mammalian muscle. Second, that duplicated \textit{pax7a} and \textit{pax7b} genes in zebrafish provide molecular markers of MPC cell lineage heterogeneity. Third, that each population of MPCs had specific behaviours in wound repair that suggest a modified founder cell/fusion competent myoblast model operates in vertebrates.

Visualisation and conservation of muscle wound repair in vertebrates
Our characterisation of the time course of muscle regeneration in zebrafish larvae reveals remarkable similarities with that in adult fish and mammals. Extending previous studies of larval zebrafish muscle repair (Seger et al., 2011; Knappe et al., 2015), we show that epidermal wounds close within hours and leukocytes marked by transgene reporters of both neutrophil (\textit{mpx}) and macrophage (\textit{lyz}) genes infiltrate the muscle abundantly but transiently for around two days, a time course comparable with that observed in other model organisms and human (Ciciliot and Schiaffino, 2010). Pax7-marked MPCs are triggered to enter the wound, divide and undergo terminal differentiation involving Myogenin upregulation, and regenerate muscle fibres. In small wounds, new muscle fibres form from 1 dpw and show significant repair by 2 dpw, which is somewhat faster than reported in mammalian models (Ciciliot and Schiaffino, 2010). However, in larger wounds, comparable with those analysed in mammalian systems, we observe slower repair, with fibres regenerated progressively from the wound edge and taking around a week, a time course comparable with that in the large wounds generally studied in other species (Ciciliot and Schiaffino, 2010). As satellite cell-based muscle repair is a synapomorphy of...
vertebrates (Chen et al., 2006; Hollway et al., 2007; Zhang and Anderson, 2014), our findings validate use of zebrafish to study mechanisms of muscle regeneration.

Hitherto, direct visualisation of MPC fusion during muscle repair has not been reported. Our imaging of MPC fusion directly to both pre-existing and nascent fibres in wounded regions shows that the cell biology of fusion and regeneration in a 3D mesenchymal tissue in vivo is accessible in the zebrafish. Fusion has been imaged in detail in Drosophila muscle development (Kim et al., 2015; Richardson et al., 2008); from our initial analysis, the vertebrate process appears similar. We find that MPCs fuse laterally to fibres at any point along their length, and the process is rapid, occurring in a few minutes and as little as 3 h after the final MPC mitosis. Both slow and fast fibres regenerate, but our analysis focused on the multinucleate fast fibres; how MPCs regenerate slow fibres remains to be determined.

**Pax7 genes as molecular markers of MPC cell lineage heterogeneity**

Our studies reveal several zebrafish MPC populations, based on differential expression of the pax7a and pax7b genes in distinct somitic locations, lack of interconversion between MPC sub-populations and different cell behaviours in response to wounding. Endogenous pax7a and pax7b genes are differentially expressed in embryonic MPCs, with pax7b expressed in early dermomyotome precursors in the anterior somite border and pax7a expressed later (Hammond et al., 2007; Minchin and Hughes, 2008). Genetic marking confirmed the differential expression and revealed that MPCs expressing pax7a:GFP-only, pax7b:reporter-only or both are found in larvae. In the absence of antibodies specific to each Pax7 protein it is unclear whether the reporters reflect endogenous protein accumulation. Nevertheless, the markers can be used to track the fate of each MPC type.

Within wounds, pax7a:GFP-only cells participate in repair, forming nascent fibres, but their GFP rapidly diminishes in regenerated fibres, apparently by dilution as newly formed fibres enlarge. In contrast, pax7b-reporters (we used several) persist in regenerated fibres. pax7b-only cells are rare in wounds; most pax7b: RFP MPCs also contain detectable pax7a:GFP. These dual-labelled cells frequently fuse to fibres. As Pax7 immunoreactivity was not observed in muscle fibres, this persistence of pax7b:GFP/RFP is best explained by perdurance of GFP/RFP, perhaps from more abundant or ongoing MPC fusion. In summary, within wounds, pax7b expression distinguishes pax7a-only and dual-labelled MPC populations.

Each MPC population is stable within wounds. Interconversion was not observed in time-lapse studies. Moreover, even when pax7b-expressing cells are ablated, pax7a-only MPCs do not regenerate dual-labelled MPCs. The two populations express pax7a:GFP differently. In general, pax7a:GFP-only MPCs tend to have more GFP than dual-labelled MPCs. Consequently, without sensitive equipment the pax7a:GFP-only cells are preferentially detected in the GFP channel and appear to form mostly small nascent fibres. Although all MPCs in wounds express some level of pax7a:GFP, pax7b:GFP marks more, and larger, regenerated fibres. This suggests that pax7b:GFP perdures better than pax7a: GFP and/or that more pax7b:GFP cells fuse. Taken together, the data argue that the two populations of MPCs represent distinct cell lineages that respond differently to wounding.

In aggregate, the two MPC populations explain the results observed with Pax7 protein. Confirming previous studies (Knappe et al., 2015; Seger et al., 2011), we show that Pax7+ cells are more abundant than bright pax7a:GFP-MPCs, accumulate and proliferate in muscle wounds and express markers of myogenic progression, such as Myf5 and Myogenin. Our quantitative analysis showed that around half of all somitic Pax7+ cells in wounded somites co-express Myogenin, a marker of terminal differentiation. As ∼40% Pax7+ were in S-phase at 1 dpw, it seems that most non-differentiating Pax7+ cells must be proliferating rapidly, explaining the increase in Pax7+ cells and recovery of cell number in wounds. Congruently, cells marked by either pax7a or pax7b reporters often divided in wounds. Even in large wounds, when repair is nearing completion, both MPC populations recover at the somite borders, as expected of muscle stem cells.

When pax7b:NTR-mCherry-marked MPCs are ablated they do not recover, nor do pax7a:GFP-marked MPCs contribute more GFP to repaired fibres in wounds. Strikingly, however, recovery of overall muscle morphology in these small wounds was not grossly defective. Whether a regeneration defect in pax7b-reporter MPC-ablated muscle causes a persistent change in myotome cellularity will require further quantitative analyses.

The range of MPC migration is a key factor affecting muscle growth, regeneration and the effectiveness of gene and cell therapies (Bentzinger et al., 2014; Hughes and Blau, 1990; Negroni et al., 2015; Partridge et al., 1989). Our data show that at the time of wounding (~3 dpf), there are few Pax7+ cells deep within the body of the myotome, but within a few hours after injury pax7a- and pax7b-reporter-labelled cells migrate towards and deep into the wounded region. Moreover, whereas Pax7+ cell numbers in the central region of the somite regained or exceeded control levels within 1 dpw, there was a striking delay in the recovery of Pax7+ cells on the VMZ, consistent with migration of a proportion of the cells into the body of the regenerating somite from the borders. Although new fibre formation during larval growth occurs in specific somitic locations (Barresi et al., 2001; Johnston et al., 2009), we observed efficient regeneration irrespective of wound location within the epaxial somite, demonstrating that resident MPCs can rapidly reach most somite regions. The wounded zebrafish somite might provide a suitable in vivo screening system for factors regulating MPC migration.

In unwounded somites, pax7a-only cells accumulate at VMZ and the dorsal and ventral myotome edges. After wound regeneration, we observed particularly numerous pax7a:GFP cells at the posterior border of regenerated somites, a location suggested to contain abundant myogenic precursors in various fish species (Marschallinger et al., 2009). Conversely, pax7b-reporter cells are less abundant in these locations but are more numerous at the HZM and in cells scattered over the lateral myotome surface, the location of the zebrafish dermomyotome (Devoto et al., 2006). In amniotes, cells derived from the central and border dermomyotome regions behave differently, but both express Pax7 (Ben-Yair and Kalcheim, 2005; Buckingham and Relaix, 2007; Gros et al., 2005; Schienda et al., 2006). The expression of pax7a:GFP alone in certain neural tube cells, also suggests sub-functionalisation of each gene during teleost evolution. To conclude, we hypothesise that subpopulations of Pax7+ MPCs corresponding to those we have revealed in zebrafish also exist in amniotes (Fig. 7D), and might have retained evolutionarily conserved functional roles in both muscle growth and wound repair.

**A modified founder cell/fusion competent cell model in vertebrates**

What roles do the two populations of MPCs play? In Drosophila, genetically defined individual founder cells initiate each fibre,
which then grows by fusion with numerous fusion-competent myoblasts (FCMs) (Atreya and Fernandes, 2008; Dutta et al., 2004; Rushton et al., 1995). Our data provide evidence for a modified founder cell/FCM system in zebrafish muscle regeneration. Founder cells have not been described in vertebrates. However, clones of vertebrate embryonic myoblasts only fuse to form small nascent fibres with few nuclei (Miller and Stockdale, 1986). Later-arising clones generate large multinucleate myotubes (Cossum et al., 1988; Miller and Stockdale, 1986). Moreover, the initial fusion events that form mammalian myotubes and the subsequent growth of myotubes by fusion are developmentally regulated (Horsley et al., 2001, 2003). We find that MPC expressing pax7a:GFP-only migrate early to wounds and differentiate to mark thin, presumably nascent, myotubes from 1 dpw onwards, even when pax7b-expressing cells are ablated. Such cells can have one or a few nuclei. In contrast, pax7b-expressing MPCs also migrate to wounds early, but rapidly contribute to both small and large fibres by 1 dpw, frequently fuse to pre-existing fibres and also align in rows reminiscent of fusing myoblasts. Most convincingly, our time-lapse Movie 3 captures the entire process of individual pax7a-only MPCs initiating a fibre and then a pax7b-expressing MPC fusing into the nascent fibre. Moreover, pax7b reporters persist in marking larger fibres at later stages of repair. The greater reduction in pax7b:GFP cell numbers between 1 and 2 dpw strongly suggests that many of them fuse with one another. All these data argue that, during regeneration, the less-abundant pax7b-only MPCs initiate nascent fibre formation, while more numerous pax7b MPCs contribute to the growth of nascent fibres and to the repair of damaged pre-existing fibres (Fig. 7D). Our vertebrate ‘modified founder cell hypothesis’ asserts that (1) a unique lineage of founder cells initiate formation of a fibre with or without fusion to one another, (2) the nascent fibre then grows by addition of myoblasts from a second distinct lineage of MPCs. In zebrafish larval regeneration, pax7a-only cells, and pax7b-expressing cells (most of which also express pax7a) behave like founder cells and FCMs, respectively.

In amniones, MPC heterogeneity has long been thought to underpin generation of distinct kinds of muscle cells during development (Cossum et al., 1983; Miller et al., 1985; Rutz and Hau schka, 1982; White et al., 1975). An advantage of the duplication of the pax7 gene in the zebrafish is that it reveals the distinct behaviour of pax7a:GFP-only and pax7b:GFP cells, suggesting they might represent distinct cell lineages with particular roles in myogenesis. Complex somitic myogenesis with distinct waves of fibre formation and a dermomyotome-like stem cell compartment arose prior to the divergence of teleost and amniote ancestors (Devoto et al., 2006). We hypothesise, therefore, that several Pax7+ lineages might have existed in the common ancestor and could persist in extant amniotes. However, as amniotes only have a single (unduplicated) Pax7 gene, distinction of two MPC sub-populations comparable to those marked with pax7a:GFP-only and pax7b:GFP cells, suggesting they might represent distinct cell lineages with particular roles in myogenesis.

**MATERIALS AND METHODS**

**Zebrafish lines, maintenance and manipulation**

Zebrafish (Danio rerio) embryos were kept and staged by standard methods (Kimmel et al., 1995; Westerfield, 2000). Tg(2.2mylz2:gapdh)155 (von Hofsten et al., 2008), Tg(9.7kb smyb:greenfluoroscein)156 (Ewerto hy et al., 2008), Tg(5xUAS:GFP) (Mahalwar et al., 2014), pax7a:GFP;5xUAS:GFP;5xUAS:EGFP or 5xUAS:RFP14 (Asakawa et al., 2008), Tg(UAS:Elb-Ntr-mCherry)264 (Davison et al., 2007), Tg(2.6a:HA2AVA-GFP)145 (Pauls et al., 2001), Tg(Ola:Actb:Ha:HRAS:EGFP)119 (Cooper et al., 2005) were maintained on King’s wild-type or AB background. Tg(6:GFP)117 (Hall et al., 2007) and Tg(mp5:GFP)111 (Renshaw et al., 2006) were on roy/mifia. Care and use of experimental animals complied with all relevant institutional and national animal welfare laws, guidelines and policies.

To label Pax7+ cells, we performed a transposon-mediated gene trap screen and identified the pax7b trap line in which the Gal4FF is integrated in the fourth intron of the pax7b gene: pax7b;5xG43C12134;5xUAS:EGFP (Asakawa et al., 2008). Kaede injections were as described (Minchin et al., 2013).

For wounding, dechorionated larvae were embedded on their side in 1% low melting point agarose (LMA) in embryo medium (EM) containing 160 mg/l MS222 anaesthetic (Westerfield, 2000) and damaged neural tube were avoided, as accidental damage of these tissues can trigger death. Controls were mounted but uninjured larvae from the same lay. After wounding, each fish was released from LMA, kept separately and wounded at 3.5 dpf. Larvae were treated overnight with 2.5-10 µM metronidazole at 2.5 dpf and wounded at 3.5 dpf.

**Immunodetection**

Larvae were fixed with 2% paraformaldehyde in PBS for 30 min to overnight depending on the stage. Immunodetection for slow myosin heavy chain (MyHC) (1:5, F59; Devoto et al., 1996), general MyHC (1:10, A4;1025; Blagden et al., 1997), Pax7 (1:5; DSIB; Kawakami et al., 1997), Myogenin (1:50, sc-576, Santa Cruz) and GFP (1:500, TP-401, Torrey Pines or 1:500, G1546, Sigma) was performed in PBS with 0.5-1% Triton X-100 (PBT) for between overnight and 5 days at 4°C on a rotary shaker, depending on larval age and antigen (Hinits and Hughes, 2007) followed by Alexa Fluor 488 or 555 secondary antibodies (1:1000: A21121 and A21428, respectively; Invitrogen). at least overnight (±Hoechst 33342) at 4°C. After Edu detection in Fig. S4, samples were blocked in 5% BSA, 3% normal goat serum, PBT for 20 min, incubated using Alexa Fluor 488-conjugated anti-GFP (1:500, A-21311, Molecular Probes) and Hoechst in block buffer (shaking at 4°C for 3-6 h), followed by 6×15 min washes in PBT. Phalloidin-Alexa Fluor 555 or phalloidin-Alexa Fluor 633 (1:1000, A34055 or A22284, Thermo Fisher) were used to stain F-actin. Larvae were mounted on glass slides under bridged coverslips in Citifluor AF1 or Vectashield (H-1000, Vector Laboratories). In situ mRNA hybridisation was as described (Groves et al., 2005).

**Imaging and data analysis**

Time-lapse fluorescence images were acquired on either a Zeiss LSM Exciter M1 or LSM780 with Zeiss 20X/1.0 objective using Zen software. Larvae were mounted in 1% low melting point agarose (LMA) in EM containing 160 mg/l MS222 anaesthetic (Westerfield, 2000)
and antibiotics (Sigma, P0781 used at 1:100) in a 60 mm Petri dish, flooded with EM (upright LSM) or 20 mm glass-bottom dish (inverted LSM). Where scan intervals allowed, larvae were removed from LMA and MS222 between time points and returned to a 28.5°C incubator. Image analysis and processing was done with Volocity 6.3 (PerkinElmer), Imaris 8.2 (Bitplane), Photoshop CSS (Adobe) and Fiji/ImageJ (NIH). In movies, to compensate for bleaching and facilitate cell tracking of individual cells, the whole image stacks were brightened and non-linearly enhanced by altering gamma in Imaris, so as to produce comparable brightness within the images at each time point. No quantitation was done on non-linearly manipulated data. For some live imaging experiments, embryos were injected at the 1-cell stage with ~150 pg RNA encoding plasma membrane-targeted mCherry (Shaner et al., 2004) yielding ubiquitously red cell membranes. Nuclear counts were means for all wounded or a similar number of adjacent unwounded somites from each animal. Half the cells on both VMZs and HZM were attributed to an epaxial somite. GFP+ cell numbers were analysed in epaxial somite before and after focal needle wounds in pax7a:GFP; pax7b:GFP or pax7a:GFP; pax7b:red reporter larvae from confocal image stacks repeatedly collected at 1.5-24 h intervals. Mononucleate cells were defined as a volume of uniform signal with little or no contact with some live imaging experiments, embryos were injected at the 1-cell stage with ∼150 pg RNA encoding plasma membrane-targeted mCherry (Shaner et al., 2004) yielding ubiquitously red cell membranes. Nuclear counts were means for all wounded or a similar number of adjacent unwounded somites from each animal. Half the cells on both VMZs and HZM were attributed to an epaxial somite. GFP+ cell numbers were analysed in epaxial somite before and after focal needle wounds in pax7a:GFP; pax7b:GFP or pax7a:GFP; pax7b:red reporter larvae from confocal image stacks repeatedly collected at 1.5-24 h intervals. Mononucleate cells were defined as a volume of uniform signal with little or no contact with some live imaging experiments, embryos were injected at the 1-cell stage with ∼150 pg RNA encoding plasma membrane-targeted mCherry (Shaner et al., 2004) yielding ubiquitously red cell membranes. Nuclear counts were means for all wounded or a similar number of adjacent unwounded somites from each animal. Half the cells on both VMZs and HZM were attributed to an epaxial somite. GFP+ cell numbers were analysed in epaxial somite before and after focal needle wounds in pax7a:GFP; pax7b:GFP or pax7a:GFP; pax7b:red reporter larvae from confocal image stacks repeatedly collected at 1.5-24 h intervals. Mononucleate cells were defined as a volume of uniform signal with little or no contact with some live imaging experiments, embryos were injected at the 1-cell stage with ∼150 pg RNA encoding plasma membrane-targeted mCherry (Shaner et al., 2004) yielding ubiquitously red cell membranes. Nuclear counts were means for all wounded or a similar number of adjacent unwounded somites from each animal. Half the cells on both VMZs and HZM were attributed to an epaxial somite. GFP+ cell numbers were analysed in epaxial somite before and after focal needle wounds in pax7a:GFP; pax7b:GFP or pax7a:GFP; pax7b:red reporter larvae from confocal image stacks repeatedly collected at 1.5-24 h intervals. Mononucleate cells were defined as a volume of uniform signal with little or no contact with some live imaging experiments, embryos were injected at the 1-cell stage with ∼150 pg RNA encoding plasma membrane-targeted mCherry (Shaner et al., 2004) yielding ubiquitously red cell membranes. Nuclear counts were means for all wounded or a similar number of adjacent unwounded somites from each animal. Half the cells on both VMZs and HZM were attributed to an epaxial somite. GFP+ cell numbers were analysed in epaxial somite before and after focal needle wounds in pax7a:GFP; pax7b:GFP or pax7a:GFP; pax7b:red reporter larvae from confocal image stacks repeatedly collected at 1.5-24 h intervals. Mononucleate cells were defined as a volume of uniform signal with little or no contact with...


