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RESEARCH ARTICLE

Satellite cell activation and populations on single muscle-fiber cultures from adult zebrafish (*Danio rerio*)

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ABSTRACT

Satellite cells (SCs), stem cells in skeletal muscle, are mitotically quiescent in adult mammals until activated for growth or regeneration. In mouse muscle, SCs are activated by nitric oxide (NO), hepatocyte growth factor (HGF) and the mechanically induced NO–HGF signaling cascade. Here, the SC population on fibers from the adult, ectothermic zebrafish and SC responsiveness to activating stimuli were assessed using the model system of isolated fibers cultured at 27 and 21°C. SCs were identified by immunostaining for the HGF receptor, c-met, and activation was determined using bromodeoxyuridine uptake in culture or *in vivo*. In dose–response studies, SC activation was increased by treatment with the NO-donor drug isosorbide dinitrate (1 mmol l^{-1}) or HGF (10 ng ml^{-1}) to maximum activation at lower concentrations of both than in previous studies of mouse fibers. HGF-induced activation was blocked by anti-c-met antibody, and reduced by culture at 21°C. The effect of cyclical stretch (3 h at 4 cycles per minute) increased activation and was blocked by nitric oxide synthase inhibition and reduced by culture at 21°C. The number of c-met+ SCs per fiber increased rapidly (by 3 h) after stretching. The character of signaling in SC activation on zebrafish fibers, in particular temperature-dependent responses to HGF and stretch, gives new insights into the influence of ectothermy on regulation of muscle growth in teleosts and suggests the use of the single-fiber model system to explore the basis of fiber hyperplasia and the conservation of regulatory pathways between species.

KEY WORDS: Stem cells, Ectothermy, Proliferation, c-met, Hepatocyte growth factor

INTRODUCTION

Skeletal muscle function in fish is fundamentally important for survival using high- and low-speed swimming (Fauconneau et al., 1995), and the zebrafish [*Danio rerio* (Hamilton 1822)], an ectothermic teleost, is an important vertebrate model for studying development, physiology and pathology (Lieschke and Currie, 2007). In teleosts, muscle mass [over 90% white fibers (Wakeling and Johnston, 1999)] grows by increases in fiber number and size (Bird and Mabee, 2003; Buckingham and Vincent, 2009). The combination of hyperplasia and hypertrophy is termed indeterminate growth (Fauconneau and Paboeuf, 2001; Rowlerson and Veggetti, 2001; Johnston, 2006), and the growth potential is strongly influenced by temperature (Steinbacher et al., 2011). By comparison, in postnatal mammals, fibers hypertrophy and show accretion of post-mitotic myonuclei, but fibers do not increase in number (Petrella et al., 2008; O'Connor and Pavlath, 2007). Satellite cells (SCs) resident on fibers in adult mammals are quiescent (in the G_0 phase of cell division) until activated (Schultz et al., 1978; Mauro, 1961).

Once activated, SCs cycle and their daughter cells contribute to muscle growth and regeneration (Anderson, 2006). Two chemical mediators, nitric oxide (NO) and hepatocyte growth factor (HGF), and mechanical stretch (a culture model of exercise) activate SCs in mammals, as shown in cultures of dispersed SCs (Tatsumi et al., 2001; Tatsumi et al., 2002; Tatsumi et al., 2006; Tatsumi and Allen, 2008) and isolated fibers (Wozniak et al., 2003; Anderson and Wozniak, 2004; Wozniak and Anderson, 2007; Anderson et al., 2012; Anderson and Pilipowicz, 2002; Bischoff, 1975; Bischoff, 1986; Bischoff, 1990) and *in vivo* (Anderson, 2000; Anderson and Wozniak, 2004; Tatsumi and Allen, 2008). Calcium-dependent stretch-activated signals release NO from mechano-sensitive NOS-1μ, which induces HGF release from the extracellular matrix (Tatsumi et al., 2002; Yamada et al., 2006; Tatsumi et al., 2009; Hara et al., 2012). HGF binds to the c-met receptor on SCs and activation proceeds through p38 mitogen-activated protein kinase and phosphatidyl-inositol 3-kinase pathways (Shi and Garry, 2006). Activated SCs become myoblasts that upregulate muscle regulatory genes (Sabourin and Rudnicki, 2000; Kablar et al., 2003; Kuang et al., 2006) that, along with two muscle-specification genes, pax3 and pax7 (Seale et al., 2000; Kuang et al., 2006), regulate development and regeneration.

The SC population on fibers can be identified by staining for c-met protein as c-met transcripts are expressed by both quiescent and activated SCs (Cornelison and Wold, 1997); activity of the gene is essential in muscle development (Dietrich et al., 1999). The transcript is expressed in fast and slow muscle (Sonnenberg et al., 1993; Dietrich et al., 1999) and in activation in mouse muscle, c-met acts as an immediate-early gene (Wozniak and Anderson, 2007).

While the cascade of NO–HGF–c-met signaling is established for mammalian SCs, far less is known about SC activation in fish muscle. In the present study, the regulation of SC activation in growing adult fish muscle was investigated in fiber cultures from zebrafish. In this system, fiber interactions with resident SCs are preserved (Wozniak et al., 2005; Bischoff, 1990; Bekoff and Betz, 1977) and SC responses are independent of innervation, blood supply and other tissues. Experiments tested the hypotheses that SC activation on zebrafish muscle fibers is dependent on NO and HGF, that HGF responses are temperature-dependent and that stretch activation acts via NO release. Although zebrafish are a model organism in studying Duchenne and other muscular dystrophies (Lieschke and Currie, 2007; Berger et al., 2010; Guyon et al., 2007; Gupta et al., 2012; Guyon et al., 2003), this is the first physiological study on isolated zebrafish fibers to examine SC activation *in vitro*.

RESULTS

Zebrafish were similar in length (34.1±0.6 mm, mean ± s.e.m.) and mass (360±30 mg) in all experiments. Intact fibers observed for

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List of abbreviations

BrdU	bromodeoxyuridine
FBS	fetal bovine serum
HGF	hepatocyte growth factor
ISDN	isosorbide dinitrate
LN	N^{ω}-nitro-L-arginine methyl ester
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PM	proliferation medium
SC	satellite cell

counting exhibited regular sarcomeres and ranged in length and width. Single cells were not observed on the substrate and cultures contained very little, if any connective tissue or fiber or vessel fragments. SCs were identified by positive nuclear or chromosomal bromodeoxyuridine (BrdU) staining (dark gray to black in intensity) or by c-met+ cytoplasm (Fig. 1).

In vivo time-course studies characterized SC activation and the c-met+ SC population. SCs on adult zebrafish fibers were more activated than noted on adult mouse fibers. Immediately after plating, fibers displayed SCs labeled by prior incorporation of BrdU. The number of activated, BrdU+ SCs per fiber varied over time (P<0.0001, one-way ANOVA; Fig. 2A) and increased significantly (P<0.03) from 1.8±0.1 at 0 h to a peak of 2.4±0.1 at 5 h incubation. The level of activation decreased by 12 h and rose to a second peak at 20 h. The two peak values (5 and 20 h) were not different, and both were higher than the level at 12 h.

The mean number of c-met+ SCs per fiber (Fig. 2B) increased by approximately 40% (1.4-fold) over time in culture (P<0.0001) from 6.3±0.3 immediately after plating, to 8.9±0.3 after 24 h in culture at 27°C. This suggested that some SC proliferation occurred in the absence of treatment

In vitro experiments examined the SC responses to activating stimuli (NO, HGF and stretch), the effect of temperature on those responses, and the requirement for c-met signaling in activation. Dose–response graphs of the level of SC activation for isosorbide dinitrate (ISDN; 0–2.5 mmol l^{-1}; Fig. 3A) or HGF (0–25 ng ml^{-1}; Fig. 3B) show a significant effect of treatment (P<0.0001, one-way ANOVA) with 1 mmol l^{-1} ISDN (P<0.001 versus control) and 10 ng ml^{-1} HGF (P=0.003 versus control). The control level of SC activation was lower at 21°C than 27°C (P<0.0001; compare Fig. 4A and 4B), and there was a significant interaction between the effect of HGF treatment and temperature as the activation response was smaller at 21°C than at 27°C (P<0.001). The presence of anti-c-met antibody in fiber cultures inhibited HGF-induced activation at both 27 and 21°C, although treatment with anti-c-met antibody alone did not change the level of SC activation from the control cultures at either temperature.

The effect of mechanical stretch on SC activation and the NO dependence of stretch activation were also examined in five groups of cultures at 27 and 21°C: control, stretch, stretch plus ISDN, stretch plus N^{ω}-nitro-L-arginine methyl ester (LN), and stretch plus ISDN plus LN (Fig. 5). Stretch experiments on zebrafish fibers showed significant activation by mechanical stretching, similar to that reported for the mouse (Wozniak et al., 2003; Wozniak and Anderson, 2007). At both temperatures, stretch for 3 h increased activation (P<0.001); activation was increased by treatment with the NO-donor drug ISDN (P<0.05 versus stretch group) at both 27 and 21°C, although at 21°C this increase was negligible. NOS inhibition by LN treatment blocked stretch activation (P<0.01 from stretch group), and this was greater at the lower temperature as SC activation in stretch plus LN cultures was slightly greater at 27°C (not 21°C) than in the control group of fibers (P<0.05). Treatment with LN did not inhibit ISDN-induced SC activation (P<0.001 versus controls at both 27 and 21°C).

The c-met+ SC population increased rapidly in response to the 3 h stretch protocol (P<0.0001, ANOVA) compared with 0 h fibers fixed right after plating (P<0.001) and to fibers cultured for 3 h without stretch (P<0.001; Fig. 6). The number of c-met+ SCs per fiber increased further between 3 and 24 h of incubation for cultures that were stretched or not stretched in the first 3 h (P<0.0001 for both comparisons). The frequency distributions of c-met+ SCs per fiber show population shifts upward after 3 h stretch (P<0.01 versus baseline at 0 h; Fig. 6C).

DISCUSSION

Life-history, genotypic and phenotypic differences between mammals and teleosts suggest distinctive regulation of muscle SCs across species, because of the crucial nature of SCs in muscle development, growth and regeneration. Results of experiments on zebrafish muscle using the single-fiber model draw attention to important aspects of SC regulation related to NO– and HGF–c-met signaling. The prevalence of activated SCs *in vivo* was higher (1.5–2.5 BrdU+ SCs per fiber) than previously reported for SCs on mouse fibers (0.1–0.2 BrdU+ SCs per fiber) immediately after plating. In addition, the maximum activation response by stimulated SCs on zebrafish fibers *in vitro* occurred at higher concentrations of NO and lower concentrations of HGF than for mouse fibers in culture. Stretch activation, the essential role of c-met signaling in

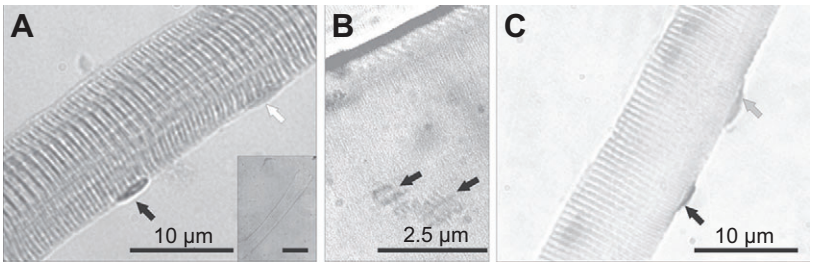

Fig. 1. Immunostaining of satellite cells (SCs) on zebrafish fibers. (A) Fiber shows a bromodeoxyuridine-positive (BrdU+) nucleus inside an SC (black arrow) and an unstained pale-gray nucleus in a second SC (white arrow), fixed 24 h after plating (inset is the negative control). (B) Part of a fiber showing two mitotic figures with BrdU+ chromosomes (arrows) at 48 h post-plating. This pilot experiment with an extended time course was used to confirm the location of BrdU incorporation into DNA. (C) Fiber fixed 24 h after plating and immunostained for c-met receptor shows one SC with dark-black c-met+ cytoplasm that also covers the nucleus (black arrow). A second SC has medium-gray (not black) staining of the cytoplasm (gray arrow), and was not counted as stained for c-met. Myonuclei (inside fibers) showed only background light gray or no staining for c-met or BrdU. Original magnification: A,C at ×400 (inset at ×100; scale bar, 20 µm); B at ×1000.

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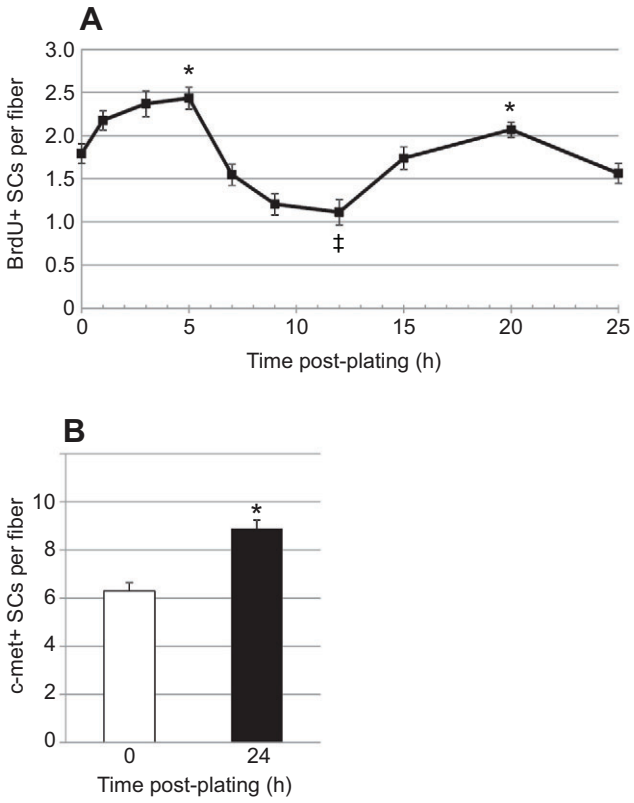

Fig. 2. *In vivo* time-course studies of BrdU+ and c-met+ SC populations on zebrafish fibers. (A) The number of BrdU+ SCs per fiber (the index of activation; means ± s.e.m.) as a function of incubation time (h) post-plating. Zebrafish were injected with BrdU 2.5 h prior to euthanasia, and fibers were plated 4.5–5.0 h after euthanasia. There were 45–131 fibers per group (typically in three to five replicate dishes). Peaks at 5 and 20 h of incubation were both significantly different from the 0 h time point (**P*<0.05) and the minimum at 13 h differed from activation at the 0, 5 and 20 h time points (‡*P*<0.001). (B) The population of SCs on fibers at 0 and 24 h of incubation post-plating shows a significant 1.5-fold increase in the population of c-met+ SCs per fiber (**P*<0.05), assessed by immunostaining for the c-met receptor for hepatocyte growth factor (HGF).

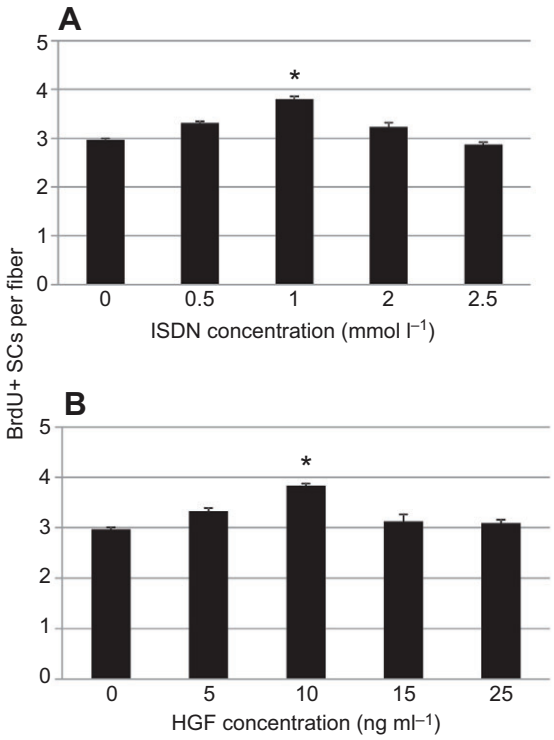

Fig. 3. SC activation by isosorbide dinitrate (ISDN) and HGF. Dose–response graphs of the level of SC activation (BrdU+ SCs per fiber; means ± s.e.m.) resulting from treatment with various concentrations of (A) ISDN (0–2.5 mmol l^{-1}) and (B) HGF (0–25 ng ml^{-1}). The dose-dependent response of SC activation to both ISDN and HGF was significant at 1 mmol l^{-1} ISDN (**P*<0.001) and 10 ng ml^{-1} HGF (**P*=0.003) compared with control cultures without treatment.

activation, and heterogeneity among SC on zebrafish fibers were similar to features in mouse-fiber cultures, with distinct effects of lower temperature to attenuate activation. These novel findings, discussed in more detail below, are the first step to closing a notable gap in the literature related to the impact of indeterminate growth and ectothermy on regulation of muscle growth in teleosts versus mammals (Johnston et al., 2011). They encourage further studies to identify the cellular and molecular mechanisms that distinguish regulation of muscle cell differentiation and growth between mammals (by fiber hypertrophy) and fish (indeterminate growth with both hyperplasia and hypertrophy of fibers).

Experiments designed to track activated SCs by their BrdU incorporation in adult zebrafish *in vivo* revealed that 9- to 10-fold more SCs were activated in the absence of stimuli at the time of plating than noted previously in fibers from wild-type control adult mice (Wozniak et al., 2003). The cell-cycle period was interpreted as 15 h (the time between two peaks in the number of BrdU+ SCs per fiber). The high numbers of activated SCs on fibers of similar dimensions from two species (e.g. length 0.28±0.01 mm) is consistent with growth being indeterminate in adult fish and ongoing increases in fiber number by hyperplasia. An asynchronous SC population likely explains the progressive increase in SC activation from plating to the first peak at 5 h, while the second peak of activated SCs is interpreted as progeny of those labeled in the first peak. Although activated SCs can migrate away from fibers in culture after HGF stimulation (because of activity of the motogenic c-met receptor), there were very few single cells observed on the plating surface and none were BrdU+. The second peak shown in Fig. 2A may also include the response of a slower-cycling population of SCs labeled during the brief availability of BrdU *in vivo*, suggesting SC heterogeneity in zebrafish, as reported for cultured mouse muscle cells (Collins et al., 2005; Deasy et al., 2003) and fibers (Wozniak et al., 2003; Wozniak and Anderson, 2007). BrdU labeling would also decline as the BrdU label was diluted in a second S-phase or dispersed as euchromatic nuclei after mitosis, because condensed chromosomes retained moderate staining for BrdU for at least two cell cycles (Fig. 1B). Thus the characterization of *in vivo* SC activation and size of the c-met+ SC population show heterogeneity similar to SCs on mouse muscle fibers, but identified that there are distinctly higher numbers of activated SCs on fish than mouse fibers, and that they are regulated to maximal activation at different NO and HGF concentrations.

The population of SCs on fibers was characterized using anti-c-met staining, based on experiments identifying c-met gene expression and protein in both quiescent and activated SCs (Cornelison and Wold, 1997) and subsequent applications of this marker protein in fiber-culture studies (Wozniak et al., 2003; Wozniak and Anderson, 2007). The number of c-met+ SC increased 1.5-fold after 24 h in culture, confirming that activated SCs went through a cell cycle during that time. A calculated per-fiber ratio, 1.8 BrdU+ SCs divided by 6.4

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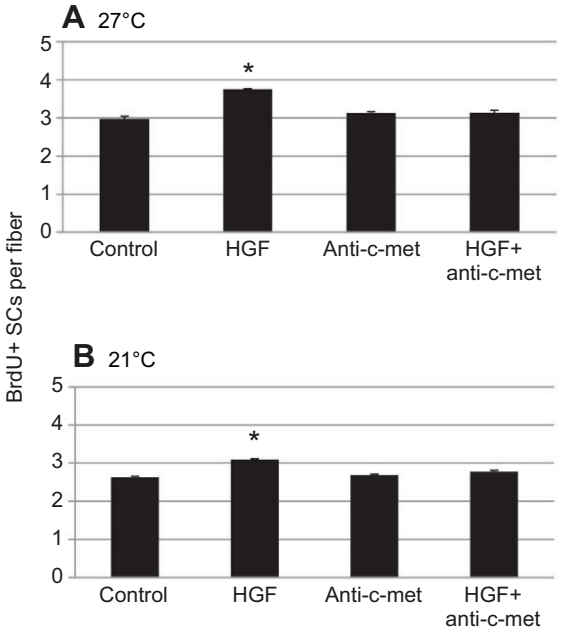

Fig. 4. Temperature study on HGF-induced SC activation. Bar graphs of the level of SC activation at (A) 27°C and (B) 21°C. Each panel illustrates activation (BrdU+ SCs per fiber; means ± s.e.m.) in control cultures and in cultures after treatment with HGF (10 ng ml^{-1}), anti-c-met antibody (1 µg ml^{-1}) or both HGF and anti-c-met antibody. The number of BrdU+ SCs per fiber was counted in 95–108 fibers per treatment group (in four replicate cultures per group). HGF induced significant activation (*P<0.0001, two-way ANOVA with repeated measures, at both 27 and 21°C) that was blocked by anti-c-met at both temperatures (no difference between the group of fibers treated with HGF plus anti-c-met and control fibers). Activation in fibers cultured at 21°C was lower than the comparable treatment groups at 27°C (P<0.0001).

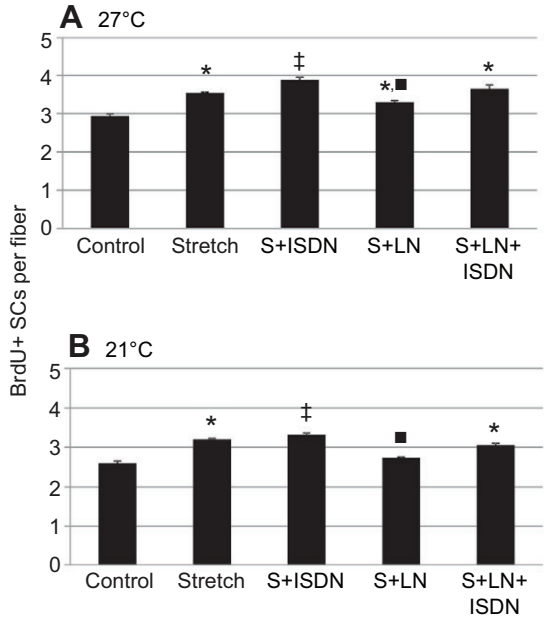

Fig. 5. Stretch-induced SC activation and effects of nitric oxide synthase (NOS) inhibition. Bar graphs showing the level of SC activation by stretch, with or without NOS inhibition and/or the NO-donor ISDN at (A) 27°C and (B) 21°C. Each panel shows activation (BrdU+ SCs per fiber, means ± s.e.m.) in control cultures and in cultures after treatment with either a stretch protocol (3 h stretch followed by 21 h of culture without stretch) or the stretch protocol (S) plus ISDN (1 mmol l^{-1}), NOS inhibition with N^{ω}-nitro-L-arginine methyl ester (LN; 0.2 µg ml^{-1}) or LN plus ISDN. Activation was increased by stretch (*P<0.001 versus controls) and was higher in fibers treated with stretch and ISDN (‡P<0.05 from stretch-only group), although at 21°C this statistical increase was negligible. LN reduced stretch activation (■P<0.01 from stretch group), and ISDN reversed the effects of NOS inhibition on stretch activation (for S+LN+ISDN group, * indicates significant difference from control fibers, P<0.05).

c-met+ SCs, indicates that approximately 30% of SCs were proliferative at the time of plating. The numerical difference between the two counts (i.e. roughly 4.6 SCs per fiber) represents three other populations: quiescent SCs in G_0 and activated SCs in the G_1 and G_2–M phases of the cell cycle. The rise in the number of c-met+ SCs per fiber over 24 h in culture is therefore explained as the sum of c-met+ SC doubling after initial activation *in vivo* augmented by a smaller number of new c-met+ SCs following activation in culture, presuming that c-met acts as an early-immediate gene in zebrafish muscle, as reported for the mouse (Wozniak and Anderson, 2007). The closest comparison from mouse-fiber cultures of the proliferative proportion of SCs indicates that approximately 15% of SCs were activated by 24 h after plating [mean of 0.2 BrdU+ and 1.38 c-met+ SCs per fiber reported previously (Wozniak et al., 2003)].

In fiber-culture experiments with BrdU uptake *in vitro*, dose–response patterns of SC activation showed bell-shaped curves with optimal activating concentrations at 1 mmol l^{-1} ISDN (an NO-donor drug) and 10 ng ml^{-1} HGF. Compared with previous studies on SC activation on mouse fibers (Wozniak and Anderson, 2007; Anderson and Pilipowicz, 2002), SCs on zebrafish fibers showed peak activation in response to lower ISDN and lower HGF levels (optimal at 25 ng ml^{-1} in mouse fibers). The zebrafish SCs also showed these 20–25% increases in activation, over and above the relatively high levels of activation in control dishes (3 BrdU+ SCs per fiber). While higher relative increases were noted in experiments with adult mouse fibers (comparing fibers cultures with and without treatment), untreated wild-type mouse fibers typically demonstrated 0.2–0.4 BrdU+ SCs per fiber in control conditions (Wozniak et al., 2003; Wozniak and Anderson, 2007). Even in the absence of dystrophin or NOS-1 from the cytoskeleton, which increased activation, there were only 0.8–1.0 BrdU+ SCs per fiber in fibers from dystrophic or NOS-1-deficient mice (Wozniak and Anderson, 2007). Notably, the dose–response pattern of an initial increase and then decline with increases in ISDN and HGF recalls the pattern in mouse fiber cultures (Wozniak and Anderson, 2007) and mice *in vivo* (Wang et al., 2009). It would be interesting to explore the possible impact of very high HGF concentrations (10–500 ng ml^{-1}) on SC proliferation on zebrafish fibers as a mechanistic basis for SC regulation in growing teleost muscle, as those levels of HGF prevent proliferation of mouse SCs in dispersed-cell cultures by increasing myostatin expression (Yamada et al., 2010). c-met receptor was demonstrated to be an essential mediator for activation in experiments using anti-c-met neutralizing antibody to block c-met receptor activity in response to HGF. Future experiments will examine the role of Delta-1-Notch signaling in SC proliferation on zebrafish fibers, as Delta-1 is upregulated after injury to mouse muscle and promotes SC proliferation (Conboy and Rando, 2002).

Studies on temperature and SC activation in zebrafish fibers were of interest as zebrafish is an ectothermic (cold-blooded) species. Biochemical processes during growth are temperature sensitive in cultures of mammalian cells (Watanabe and Okada, 1967). Thus, it is reasonable to consider that the unit under study in mammalian muscle-fiber cultures would be similarly affected by temperature. In addition, SC activation by extrinsic signals originating from fibers

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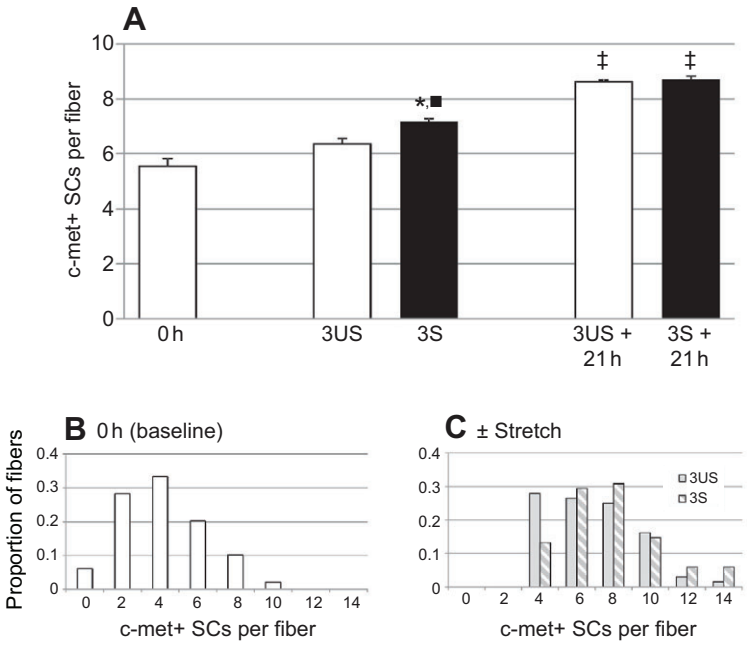

Fig. 6. Changes in SC population during stretch over 24 h. Graphs showing (A) the size of the c-met+ SC population in different treatment groups, and (B,C) the frequency distributions of c-met+ SCs per fiber for the same groups. (A) The number of c-met+ SCs per fiber (means ± s.e.m.) in fibers immediately after plating (0 h), cultured for 3 h without stretching (3US) or with a 3 h stretch protocol (3S), or cultured without or with the 3 h stretch protocol and incubated for an additional 21 h without stretching (3US + 21 h and 3S + 21 h, respectively). The number of c-met+ SCs per fiber increased between 0 h and 3S-treated fibers (**P*<0.001), and was higher in stretched than unstretched fibers at the 3 h time point (3S versus 3US, ▪*P*<0.001). The number of c-met+ SCs per fiber increased further by 24 h in culture, regardless of whether fibers were stretched for the first 3 h (‡*P*<0.0001 from all other groups). (B,C) The proportion of fibers (*y*-axis) plotted as a frequency distribution of the number of c-met+ SCs per fiber for groups represented in A. (B) Distribution at time 0 h, fixed immediately after plating and (C) distributions at 3 h incubation comparing groups with stretch (hatched gray bars) and without stretch (gray bars) (*P*<0.01, chi-squared analyses). The distribution of the c-met+ SC population was significantly higher in both groups in C compared with that at 0 h (shown in B).

and the environment (including mechanical signals) may introduce an additional feature of interest. Although numerous studies outline temperature-dependent effects on larval development that affect later muscle-growth capacity in teleosts (Steinbacher et al., 2011; Gutierrez de Paula et al., 2013; Schnurr et al., 2013; Garcia de la Serrana et al., 2012; Kullgren et al., 2013), it was only recently reported that the balance between metabolic performance and metabolic cost establishes physiological condition and critical swimming speed (Vergauwen et al., 2013; Vergauwen et al., 2010). Indeed, the two-way signaling between SCs and muscle fibers has not been investigated previously in teleosts, despite the important gap in our understanding of processes enabling their indeterminate growth (fiber hyperplasia and hypertrophy) in comparison to restriction to fiber hypertrophy (without hyperplasia) in postnatal mammals. Therefore, a distinct temperature-dependent regulation of SC activation was anticipated for fiber cultures isolated from ectothermic zebrafish compared with mammalian fibers in culture. Culturing at 21°C, a lower temperature than that used to rear the fish (27°C), decreased the number of activated, BrdU+ SCs per fiber in both control and HGF-treated cultures, although HGF increased SC activation at both temperatures. This change occurred in the absence of innervation or blood supply that, *in vivo*, would provide systemic regulation to reduce metabolic rate at lower temperatures. The notably acute reduction in SC activation at 21°C versus 27°C may be due to changes in enzyme activity before or after ligand binding to the c-met receptor. However, phosphorylation of matrix metalloproteinase 3, its activity to release HGF from the matrix surrounding fibers (Yamada et al., 2008; Tatsumi and Allen, 2008) and HGF synthesis by the fiber may be susceptible to temperature-dependent regulation. In addition, downstream from HGF-c-met binding, steps in the RAS pathway, subsequent activation of MAPK (Marshall, 1995; O'Brien et al., 2004) or the binding affinity of HGF binding to c-met itself may also be sensitive to lower temperature. Nonetheless, results of experiments to block HGF–c-met binding and study the effects of lower temperature indicated that HGF is a potent and essential signaling molecule in SC activation on zebrafish fibers. The reported effects of temperature during larval development on later muscle growth in other teleosts (Johnston and Temple, 2002; Brodeur et al., 2003; Cole et al., 2004; Johnston and Hall, 2004) would predict that acclimation to a lower temperature *in vivo*, before fibers are isolated for culture, would have even larger and/or more persistent impact on SC activation. Present results indicate that the single-fiber model system is ideal for investigating short- and long-term effects of environmental temperature changes on ontogenetic plasticity (Georga and Koumoundouros, 2010; Johnston et al., 2000; Johnston and Temple, 2002; Johnston, 2006; Johnston et al., 2011) and adaptive physiology (Johnston and Altringham, 1985; Rowlerson and Veggetti, 2001; Rowlerson et al., 1997), as muscle growth is fundamental to species function.

The NO dependence of activation by a mechanical stimulus was examined by experiments using stretch-mediated activation with or without effects of a nitric oxide synthase (NOS) inhibitor, LN. In 24 h fiber cultures, SC activation was increased over control levels by cyclical stretching for the first 3 h of incubation. Stretch activation was increased further by adding the NO-donor ISDN to cultures and reduced by NOS inhibition. The effect of exogenous NO (via ISDN) compensated for the LN inhibition of stretch activation mediated by NOS activity. By 24 h in culture, an increase in the number of c-met+ SCs per fiber suggested that SCs proliferated after activation. However, SC proliferation does not account for the higher number of c-met+ SCs per fiber in those fibers fixed immediately after 3 h of stretch compared with the group fixed after culturing for 3 h without stretch. Studies of c-met transcript expression with and without cycloheximide treatment, as conducted for mouse-fiber cultures (Wozniak and Anderson, 2007), will be required to confirm that the c-met receptor in zebrafish SCs acts as an early-immediate gene where rapid translation follows stimulus-mediated assembly of pre-transcribed mRNA. In mouse-fiber cultures, stretching for only 30 min was sufficient to increase the tissue level of c-met mRNA expression and the number of c-met-expressing SCs per fiber (Wozniak and Anderson, 2007). It is puzzling that the number of c-met+ SCs per fiber did not differ between 24 h cultures that were or were not stretched for the first 3 h in culture. It is possible that c-met+ SCs in the stretched group differentiated more quickly toward myocytes and downregulated the level of c-met protein. Such downregulation occurs in pax7-

expressing myogenic cells with the onset of myogenin expression (Seale et al., 2000) and the level of c-met protein rapidly decreases during differentiation of myocytes into myotubes in regenerating mouse muscle *in vivo* (Tatsumi et al., 1998). It would be interesting to examine the behavior of SCs marked by c-met expression and BrdU incorporation along with other proteins such as myogenin and syndecans 3 and 4 (adhesion factors on SC membranes used in myocyte fusion), as their expression overlaps with c-met (Cornelison et al., 2004; Rapraeger, 2000; Cornelison et al., 2001) and members of the Notch signaling pathway (Luo et al., 2005).

The present results showed important species differences in the size and activity of the SC pool, and the relative level of SC activation induced by HGF, NO and stretch, and distinctive temperature-dependent responses to HGF and mechanical activation of SCs on zebrafish fibers. Experiments on the time course of myogenic proliferation and differentiation on zebrafish fibers are now required to determine the expression profiles of staining for pax7, MyoD, myogenin, myostatin, HGF, metalloproteinases and cyclins that may be involved in the mechanism of the distinctive SC regulation in zebrafish fibers, for example, to establish heterogeneity among BrdU+ and unlabeled SCs. On the basis of current findings, focus on the contributions of lineage specification, activation, proliferation and differentiation on fibers to phenotypic changes in teleost development and aging would be of particular interest. The impact of cytoskeletal maturation in fibers on the regulation of SC function will also be important in examining the impact of ectothermy on fiber development and growth capacity. This is seen as particularly important in determining the potential role of SC behavior in defining the capacity for fiber growth by hyperplasia and hypertrophy in zebrafish versus its restriction to fiber hypertrophy in other taxa such as the mouse. In mouse muscle, such interplay affects development and disease progression, the scope of SC heterogeneity, and growth and regenerative capacity (Janke et al., 2013; Leiter and Anderson, 2010; Leiter et al., 2012). Fiber-type specific differences in the NO–HGF–c-met signaling pathway may also be revealed by future studies on fibers, as muscle growth and power are so highly dependent on fast-twitch fibers in fish. The capacity of SCs as stem cells, for proliferation and differentiation is reported to vary *in vitro* in dispersed cultures of SCs derived from fast and slow mammalian muscles (Lagord et al., 1998), so there may be differential regulation of SC 'fate' following activation signaling in fish versus mouse fiber cultures.

MATERIALS AND METHODS

Fish fiber cultures

Zebrafish were reared at 27°C under a natural photoperiod as approved by a University of Manitoba protocol, F12-034. Fish were anesthetized using tricaine methanesulfonate (MS-222; 40 mg 125 ml^{-1}). For *in vivo* labeling of activated SCs, fish were injected (intraperitoneal) with BrdU [0.04 ml of BrdU at 1 mg ml^{-1} in phosphate buffered saline (PBS)] (Berger et al., 2010) and returned to water for 2.5 h before euthanasia with an overdose of MS-222 (80 mg 125 ml^{-1}). Body length and mass were recorded. Skin was sprayed with 70% ethanol and blotted dry. Fibers were dissected according to the reported protocol (Anderson et al., 2012) with slight modifications. The skinned body was incubated for 2 h in 0.2% collagenase (Sigma-Aldrich, St Louis, MO, USA) mixed in proliferation medium (PM) containing L-15 medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS), 2% chick embryo extract, 1% antibiotic-antimycotic and 0.1% gentamycin. After digestion, each fish was transferred to a 60 mm glass Petri dish of fresh PM and cleaned of connective tissue and blood vessels. The fish body was triturated using the wide end of a glass Pasteur pipette (Fisher, Hampton, NH, USA) to free individual fibers; loose bones, scales, fat, etc. were removed under a dissecting microscope. Fibers isolated from two fish were pooled for use in each experiment to reduce the influence of individual variation among fish, cleaned further by gravity sedimentation (Anderson et al., 2012) and re-suspended in PM.

Fibers plated onto cleaned coverslips pre-coated with 50 µl collagen (Advanced BioMatrix, Inc., San Diego, CA, USA) were placed into 35 mm plastic Petri dishes (VWR International, Radnor, PA, USA) cooled on ice. Dishes were incubated at 27°C for 50 min to gel the collagen before fibers were covered carefully with 500 µl of basal medium [L-15 medium with 20% controlled replacement serum-2 (Sigma-Aldrich), 1% FBS, 1% antibiotic/antimycotic and 0.1% gentamycin] supplemented with BrdU (10 mg ml^{-1}, to label DNA synthesis) and various treatments (see below), and returned to the incubator. In a representative experiment, up to 35–40 dishes were prepared, each holding a coverslip with 20 or more plated fibers. Fibers from fish injected with BrdU *in vivo* were isolated as above, and cultured in medium without BrdU. There was a 4.5–5.0 h period from euthanasia until isolated fibers were plated. By comparison, this interval is 6.5–7.5 h in the mouse, because of the complexity of the multi-pennate flexor digitorum brevis muscle that is typically used in such studies, the dissection required to isolate the muscle, and the extent of cleaning required to remove tendons, other connective tissue and blood vessels during separation of fiber bundles. A similar number of mouse fibers can be isolated and pooled from six to eight flexor digitorum brevis muscles (three to four mice); the dissection even to expose the muscle is more complex and the fiber yield is lower. Further details of numerous differences in the fiber-isolation procedures between fish and more well-established mouse fiber cultures were developed to minimize the impact of isolation and plating, in order to study as 'native' a system as possible, as reported previously (Anderson et al., 2012).

Fibers were cultured at 27 or 21°C for 0–24 h, then coverslips were rinsed in PBS, fixed (20 min in 5% glacial acetic acid in 100% ethanol) and air dried. Fixed fibers were stored in dishes with 2 ml of 0.01 mol l^{-1} Tris-buffered saline (pH 8.4) containing 1% horse serum, and placed in humid chambers at 4°C until immunostaining.

For experiments using cyclical stretching, fibers were plated on six-well BioFlex-collagen type I culture plates (BF-3001C, FlexCell International Corporation, Hillsborough, NC, USA) that were recoated with collagen, and gelled for 50 min, as above. Cultures were stretched at four cycles per minute for 3 h on a FlexCell vacuum-tension system (FX-4000), as reported for mouse fibers (Wozniak and Anderson, 2007; Wozniak et al., 2003). A protocol of 20% lengthening along the radius of each well was employed to model movement in high-demand swimming. After 3 h of stretch (at 27 or 21°C), plates were removed from the FlexCell vacuum mat and incubated at the same temperature for another 21 h before fixation. Fibers plated in the same dishes and incubated without stretch served as controls.

Experiments

SC activation *in vivo* was examined using fibers isolated from fish injected with BrdU 2.5 h before euthanasia. Fibers were cultured at 27°C for 0–24 h; two to three dishes were removed from the incubator at different times post-plating and fixed before immunostaining for BrdU incorporation to assess the time course of activation. Time-dependent changes in the population of SCs on fibers were examined in fiber cultures fixed either immediately after plating or after 24 h in culture. SCs were identified by immunostaining for c-met.

Dose–response experiments were used to test whether zebrafish SCs responded to the two chemical stimuli that activate SCs in mouse muscle. Fibers were cultured in media containing BrdU (10 mg ml^{-1}) and either the NO-donor drug, ISDN (0–2.5 mmol l^{-1}; Sigma-Aldrich), or exogenous HGF (0–25 ng ml^{-1}; Sigma-Aldrich). The requirement for HGF–c-met binding during SC activation was explored using anti-c-met neutralizing antibody (1 µg ml^{-1}, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), as reported previously (Tatsumi et al., 1998). The temperature dependence of this signal was examined in parallel cultures at 27 and 21°C, and compared with untreated controls at the same temperature. HGF (10 ng ml^{-1}) was added to half the dishes in each group.

The stretch-activation response of SCs on fibers was examined using cultures at 27 or 21°C in medium containing BrdU and one of the following: (1) no treatment (controls), (2) stretch (3 h), or (3) stretch plus 1 mmol l^{-1} ISDN or the nonspecific NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (LN; Sigma-Aldrich, 0.2 µg ml^{-1}), or both LN and ISDN. After 3 h of

stretch, fibers were cultured for an additional 21 h (i.e. 24 h in total) at the same temperature before fixation and staining.

The effect of stretch on the population of SCs resident on fibers was explored using c-met immunostaining on fibers from one of five groups: fibers fixed immediately after plating (0 h incubation), 3 h without stretch, 3 h stretch, 3 h stretch then 21 h incubation without stretch, or 24 h incubation without stretch. Fibers were fixed and immunostained to detect c-met protein.

Immunostaining

Incorporation of BrdU into activated SC nuclei was detected by immunostaining (Mizunoya et al., 2011; Anderson and Pilipowicz, 2002) with anti-BrdU antibody (Roche Applied Science, Penzberg, Germany, 1:50). c-met+ SCs were detected using anti-c-met primary antibody (Santa Cruz, 1:50). Biotin-conjugated anti-mouse (for BrdU) and anti-rabbit (for c-met) IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, both at 1:200) and the streptavidin-horseradish peroxidase method using diaminobenzidine (Sigma-Aldrich) were used to visualize black staining in BrdU+ nuclei or c-met+ cytoplasm, as reported elsewhere (Anderson and Pilipowicz, 2002; Wozniak et al., 2003). Post-mitotic myonuclei inside fibers had only background staining much lighter than dark gray. The ability to make reproducible counts of darkly stained cells during fiber observations was developed using a count–recount approach while the observer developed standard criteria for positive staining, and count–recount variation in recorded experiments was <5%. Coverslips processed without primary antibody served as negative controls in each experiment.

Fibers on coded coverslips were viewed at 400× under an Olympus BH2 microscope (Olympus Canada Inc., Markham, ON, Canada). The number of SC nuclei stained dark gray to black was counted per fiber without knowledge of treatment group, for ~20 fibers per coverslip. By focusing up and down through each fiber, SCs could be identified as separate from the fiber. The total number of labeled SC nuclei was observed on the fibers that were encountered in systematic scanning, back and forth across the coverslip in lines separated by one 400× field. Some coverslips held fewer than 20 fibers because of fiber loss during stretching and the numerous rinse steps in fixation and staining procedures. Counts were recorded only for SCs on intact single fibers displaying regular sarcomeres. Fibers that overlapped were not counted, and dishes with debris or showing >5% broken and/or hypercontracted fibers were not counted. Final groups, therefore, ranged from 55 to 138 fibers per group, originally isolated in a population of fibers from two fish, depending on the number of dishes per group, the number of fibers isolated and plated, the number of fibers retained through processing, the number of overlapping fibers, and whether such fibers appeared in the systematic scan of individual coverslips. Note that each figure represents results from a separate experiment on two independent fish. Dose–response, temperature-dependence and stretch experiments were repeated two to three times on independent preparations of fibers, each isolated from two fish; individual figures represent single experiments.

Statistical analysis

Counts were compiled in Microsoft Excel spreadsheets, decoded into treatment groups and analyzed using appropriate statistical tests for the significance ($P<0.05$) of treatment effects. Statistics were performed on fiber number and not on the number of fish in a given experiment. Statistical design used one- or two-way ANOVA (including repeated measures, e.g. for temperature). Least significant difference tests were used *post hoc* (if warranted by significant F-values) for multi-group means comparisons in individual experiments, and t-tests were used to analyze data from experiments with two groups. Chi-squared statistics were used to analyze frequency distributions. All analyses were conducted after consultation with a statistician and as reported previously (Wozniak and Anderson, 2007). The number of BrdU+ or c-met+ SCs per fiber did not differ among cultures in repeat experiments or between coverslips and FlexCell dishes. Symbols in graphs indicate pair-wise differences.

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.Z. contributed to the design and execution of the experiments, interpretation of findings being published, and drafting and revising the article. J.E.A. contributed to the conception and design of the experiments, interpretation of the findings being published, and drafting and revising the article.

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