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Fgf regulates dedifferentiation during skeletal muscle regeneration in adult zebrafish

Alfonso Saera-Vila, Phillip E. Kish, Alon Kahana *

Department of Ophthalmology and Visual Sciences, Kellogg Eye Center, University of Michigan, Ann Arbor, MI, USA

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ABSTRACT

Fibroblast growth factors (Fgfs) regulate critical biological processes such as embryonic development, tissue homeostasis, wound healing, and tissue regeneration. In zebrafish, Fgf signaling plays an important role in the regeneration of the spinal cord, liver, heart, fin, and photoreceptors, although its exact mechanism of action is not fully understood. Utilizing an adult zebrafish extraocular muscle (EOM) regeneration model, we demonstrate that blocking Fgf receptor function using either a chemical inhibitor (SU5402) or a dominant-negative transgenic construct (dnFGFR1a:EGFP) impairs muscle regeneration. Adult zebrafish EOMs regenerate through a myocyte dedifferentiation process, which involves a muscle-to-mesenchyme transition and cell cycle reentry by differentiated myocytes. Blocking Fgf signaling reduced cell proliferation and active caspase 3 levels in the regenerating muscle with no detectable levels of apoptosis, supporting the hypothesis that Fgf signaling is involved in the early steps of dedifferentiation. Fgf signaling in regenerating myocytes involves the MAPK/ERK pathway: inhibition of MEK activity with U0126 mimicked the phenotype of the Fgf receptor inhibition on both muscle regeneration and cell proliferation, and activated ERK (p-ERK) was detected in injured muscles by immunofluorescence and western blot. Interestingly, following injury, ERK2 expression is specifically induced and activated by phosphorylation, suggesting a key role in muscle regeneration. We conclude that the critical early steps of myocyte dedifferentiation in EOM regeneration are dependent on Fgf signaling.

1. Introduction

De novo regeneration of injured or degenerated tissues and organs carries great promise for curing a multitude of debilitating disorders, potentially replacing the need for organ transplantation and creating new opportunities for treating many chronic disorders. Unfortunately, mammals in general, and humans in particular, have a rather limited capacity to regenerate. The reasons for that are not well understood, since most mammalian tissues contain resident stem cells that at least in vitro can give rise to progenitor cells with regenerative capabilities. Muscle injury and degeneration is a particularly important cause of morbidity and mortality, and the ability to regenerate muscle, whether skeletal or cardiac, would completely alter the therapeutic landscape. Despite the presence of skeletal muscle stem cells (i.e. satellite cells) that can repair and maintain muscles (at least until they exhaust in degenerative diseases, such as Duchenne Muscular Dystrophy [1,2]), mammalian skeletal muscles cannot regenerate and replace muscle tissue following extirpation.

Fish and amphibians have a much greater capacity to regenerate, which appears to rely less on resident stem cells and much more on cell reprogramming and dedifferentiation [3]. Indeed, adult zebrafish are able to regenerate both skeletal [4] and cardiac muscle [5,6], as well as retina [7], spinal cord [8], liver [9] and fin [10,11]. Our lab recently characterized a robust regenerative pathway in adult zebrafish extraocular muscles (EOMs) – a form of skeletal muscle– which is driven by myocyte dedifferentiation with no significant contribution of satellite cells [4]. Thus, we can now utilize a novel model for studying the mechanistic underpinnings of myocyte reprogramming via dedifferentiation.

Fibroblast growth factors (Fgfs) are a family of polypeptide growth factors composed, in vertebrates, by over twenty members [12]. Fgfs exert their biological pleiotropic functions by activating the Fgf receptor (Fgfr) tyrosine kinase, except for Fgf11–Fgf14 which function intracellularly via Fgfr-independent mechanisms [13]. Fgf signaling controls embryonic development and morphogenesis, maintains tissue homeostasis, regulates organ function, and promotes wound healing [14]. The roles of Fgf in skeletal muscle development, repair, and regeneration have been extensively studied. Thus, Fgfs regulate muscle development through autocrine and paracrine mechanisms in vitro [15] and

Abbreviations: EdU, 5-ethynyl-2′-deoxyuridine; EOM, extraocular muscle; hpi, hours post injury; dpi, days post injury; Fgf, fibroblast growth factor; Fgfr, fibroblast growth factor receptor; LR, lateral rectus; Tc, cell cycle length; Ts, length of S-phase; MAPK, mitogen-activated protein kinase; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase kinase.

* Corresponding author at: 243 KEC, 1000 Wall St., Ann Arbor, MI 48105, USA.
E-mail address: akahana@med.umich.edu (A. Kahana).

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several steps of myogenesis in limb development [16]. Specifically in zebrafish embryos, Fgf signaling plays a role in morphogenesis and cell fate determination of myotomes [17], and is required for fast muscle differentiation [18]. Furthermore, it has been shown that Fgfs promote satellite cell proliferation [19–21] and, therefore, plays important roles in the muscle response to injury [22,23].

Among their many functions, Fgfs also play a key role in tissue repair and regeneration in zebrafish [9,10,24–26], amphibians [27,28] and even mammals [29,30]. Since Fgfs play a universal role in regulating tissue regeneration and zebrafish EOMs regenerate by myocyte reprogramming and dedifferentiation, we directly tested whether Fgfs are important in EOM regeneration, and whether their role is primarily early, during cell reprogramming and proliferation, or late during migration and redifferentiation. Here we report that blocking Fgfr resulted in severely delayed regeneration, and that Fgf signaling is most important during the early events of myocyte dedifferentiation. We further demonstrate that Fgf exerts its effects through the MAPK/ERK signaling pathway, and that ERK2 is a candidate for a critical role in the process.

2. Materials and methods

2.1. Zebrafish (Danio rerio) rearing and surgeries

All animal work was performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the University of Michigan Committee on the Use and Care of Animals, protocol 06034. Sexually mature adult (4–18 month old) zebrafish were spawned in our fish facility and raised per standard protocol at 28 °C with a 14-h light/10-h dark alternating cycle.

Adult zebrafish were anesthetized (0.05% Tricaine Methanosulfate) and about 50% of the LR muscle was surgically excised, i.e. myectomy [4]. The remaining muscle following surgery (48.42% ± 4.9%, average ± SD) is represented in the figures as a grey area. The length of the regenerating muscle was quantified by craniectomy as described previously [4]. Regeneration is represented as the relative size of the injured LR muscle normalized to the length of the uninjured LR muscle (representing 100%). All experiments were performed using 5 fish per experimental group and/or time point, unless stated otherwise in the text and/or figure legend.

To label muscles with a marker other than EGFP, transgenic α-actin-mcherry and double transgenic α-actin-mcherry/hsp70-dnFGFR1a:EGFP fish [31] were generated by co-injecting 100 pg acta1-mCherry plasmid (kindly provided by Dr. Thomas Hall) with 50 pg transposase mRNA into 1-cell stage wild type or hsp70-dnFGFR1a:EGFP embryos. For each fish line, mCherry positive fish were crossed to one another to generate F1 embryos, which were raised and used for experiments.

2.2. Drug treatments

SU5402 (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO as a 17 mM stock and added to fish water at a final concentration of 17 μM as described [10], tanks were kept in the dark. U0126 (Fisher Scientific, Pittsburgh, PA) was dissolved in DMSO as a 25 mM stock and added to fish water at a final concentration of 25 μM [32]. Up to 5 fish were treated in 1 l of water, tanks were maintained at 28.5 °C and drug solutions were replaced every 24 h. Drug treatments were performed immediately after surgery and no significant mortality was noted.

2.3. Adult heat induction experiments

Heat-shock treatments were performed on adult zebrafish directly in the housing racks using our customized system, as described [33]. Fish were exposed to warm water (38.0–38.3 °C) for 1 h daily to drive the ectopic expression of dnFGFR1a:EGFP [31]. The transgenic fish line hsp70-GFP fish [34] was used as a technical control. All experiments were performed using 5 fish per experimental group and/or time point, unless stated otherwise in the text and/or figure legend.

2.4. Specimen processing

Zebrafish heads were excised and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Decalcification was performed using either Morse's solution or 10% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS, pH 7.2–7.4). Fixed and decalcified tissues were cryopreserved with 20% sucrose in PBS, embedded in OCT (Fisher Scientific), frozen and evaluated microscopically using coronal frozen sections (12 μm) as described previously [4].

2.5. Staining and biochemical analysis

Immunostaining was performed as described [4]. Briefly, slides were washed in PBS for 5 min and placed in blocking solution (5% goat serum in PBS + 0.2% Tween, PBST) for 30 min. Slides were incubated in a humid chamber overnight at 4 °C in primary antibody (rabbit anti-phospho-p44/42 MAPK [ERK 1/2], #4370 from Cell Signaling Technology, Danvers, MA) diluted to 1:200 in PBST + 1% goat serum. Slides were again washed 4 times for 5 min in PBST and then incubated in the dark with Alexafluor 647-conjugated goat anti-rabbit secondary antibody (Invitrogen) diluted 1:1000 in PBST + 1% goat serum. Nuclei were stained with DAPI and coverslipped with ProLong Gold Antifade Reagent. Negative control experiments with no primary or secondary antibodies were performed. Sections from 5 different fish per experiment were stained.

Western blots were performed following standard protocols. Injured or uninjured LR muscles from 10 to 15 fish were pooled and homogenized in lysis buffer containing protease (cOmplete, Roche Diagnostics Corporation, Indianapolis, IN) and phosphatase (PhosSTOP, Roche Diagnostics Corporation) inhibitors. The transgenic α actin-EGFP fish were used to visualize the muscles. Samples were sonicated and centrifuged at 10,000*g* for 10 min at 4 °C. Supernatant was collected and protein concentration determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and bovine serum albumin (BSA) as standard. Equal amounts of protein (20 μg) were loaded on 7.5% (ERK) or 12.5% (caspase 3) SDS polyacrylamide gels covered with a 3.9% stacking polyacrylamide gel and separated at 130 V for 1 h using Mini-Protean III (Bio-Rad, Hercules, CA). Proteins were then electroblotted into PVDF membranes (Bio-Rad) by wet transfer (Mini Trans-Blot® Cell, Bior-rad) at 100 W for 1 h. Membranes were blocked for 1 h at room temperature with 5% BSA in TBST and incubated overnight at 4 °C with primary antibody diluted in blocking solution. Anti-γ-tubulin antibody (1:10,000, T5326) was obtained from Sigma-Aldrich, active caspase 3 antibody (1:500, ab13847) from abcam (Cambridge, MA), anti-p44/42 MAPK (ERK 1/2) (1:1000, #9107) and anti-phospho-p44/42 MAPK (ERK 1/2) (1:2000, #4370) were purchased from Cell Signaling Technology. Membranes were washed in TBST and incubated with IgG-horseradish peroxidase conjugate secondary antibody (1:10,000, anti-mouse #7076 and anti-rabbit #7074 from Cell Signaling Technology) at room temperature for 1 h. Detection of signal was done using WesternBright ECL HRP substrate (advansta, Menlo Park, CA) and an Azure c500 Western Blot Imaging System (azure biosystems, Dublin, CA). Densitometric quantification of the bands was done with ImageJ software (http://rsbweb.nih.gov/ij/). The intensity of the protein of interest is normalized to the intensity of the tubulin band and represented in arbitrary units.

2.6. EdU incorporation assays

Cellular proliferation was assessed by intra-peritoneal (IP) injections of 5-ethynyl-2′-deoxyuridine (EdU) and standard detection methods [35]. Fish were anesthetized and injected with EdU (20 μl, 10 mM EdU in PBS) at 26 h post injury (hpi) and sacrificed 4 h later (30 hpi). For