EXPLORING THE ROLE OF ACTINOTRICHIA DURING FIN REGENERATION

by

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Zebrafish are capable of robustly regenerating injured limbs and organs. After amputation of zebrafish fins, a blastema is formed which is subsequently populated by Wnt-producing "niche" cells beginning at 3 days post amputation (dpa). This coincides with the timeline of actinotrichia production, as actinotrichia collagens begin to be produced between 2 and 3 dpa. Actinotrichia are unique spoke-like collagen structures that stabilize the fin tip while bony rays are still forming. It is thought that actinotrichia treadmill proximally and are degraded as they come into proximity of newly mineralizing bone. Actinotrichia remain present in the distal tips of unamputated fins, and it is thought that they act in support of fin tissue in areas without mature bone. In larval zebrafish, fin mesenchymal cells produce and orient actinotrichia through cellular protrusions. We sought to determine whether, in the regenerating adult fin, Wnt-producing "niche" cells similarly wrap actinotrichia in actin-rich protrusions, and to determine pathways that directly regulate these interactions. We explored two lines of inquiry; (1) How do actinotrichia and niche cells interact? And (2) What factors promote actinotrichia formation? Through time course imaging of a regenerating fin, we concluded that actinotrichia components appear at 2dpa, which is earlier than previously recorded. To explore actinotrichia-niche cell interactions, we stained and imaged fin sections. We observed niche cells colocalizing with actinotrichia, as well as actinotrichia appearing to wrap around dach-positive niche cells. In our

exploration of the factors promoting actinotrichia formation, we used Wnt RNA-seq data and drug treatments to conclude that the Wnt and TGF-β pathways are not directly upstream of actinotrichia production. Our findings provide contributions toward a better understanding of regeneration, which has exciting implications for improving human healing.

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Introduction

Zebrafish robustly regenerate lost or damaged organs, including fins, heart, and scales. Within 4 weeks of amputation, the zebrafish caudal fin regenerates back to its original size and shape, regardless of the extent of the tissue removed (Morgan, 1900). Humans lack the ability to regenerate, so the regeneration-competent zebrafish model is used to study the mechanisms behind regeneration with the ultimate goal of supplementing new therapeutic approaches to human healing. Upon amputation, the zebrafish fin first forms a regenerative 'blastema,' composed of osteoblasts and activated mesenchymal cells that control the extent of outgrowth (Wehner *et al*., 2014). As the fin continues to regenerate, unique collagenous structures termed actinotrichia extend outwards parallel to growth while the bony rays are still forming (König *et al.*, 2018; Mari-Beffa *et al*., 1989). Knockdown of actinotrichia components actinodin 1 and 2 (And1 and And2) results in the loss of actinotrichia and impaired fin folds. The pectoral fin truncation present in these knockdown embryos mirrors the polydactyly observed in early tetrapod fossils, suggesting that the loss of both actinoids, and the resulting loss of actinotrichia, contributed to the evolutionary fin-to-limb transition (Zhang *et al*., 2010).

Actinotrichia are collagen-rich fibrils that provide scaffolding that osteoblast cells then use to deposit the bone matrix (Wood, 1982). Actinotrichia components include both collagenous and non-collagenous components, such as Collagen I, Collagen II, Actinodin-1, and Actinodin-2 (Zhang *et al*., 2010). Mutations with a loss of the collagens that make up actinotrichia, specifically col2a1b and col1a1a, result in a significant reduction of fin fold area and delayed development. Additionally, the mutant col1a1 or *chihuahua* displayed a phenotypic malformation of the skeleton. In the adult fins, the overall fin was shorter and the lepidotrichia were thicker with only one bifurcation per ray. Additionally, this mutant possessed significantly

smaller actinotrichia in both size and numbers, implying that actinotrichia are necessary to support the tip of the fin during regeneration (Durán *et al*., 2011).

In larval zebrafish, fin mesenchymal cells are found beneath the basal epidermis, where they produce and orient actinotrichia through cellular protrusions (Kuroda *et al*., 2020). Larval mesenchymal cells appear to interact with actinotrichia in cell culture (Kuroda *et al*., 2020). Additionally, electron micrographs of regenerating carp tail fins show fibroblast-like protrusions from connective tissue cells wrapping around actinotrichia (Böckelmann & Bechara, 2009). However, there is no current data on whether these same actinotrichia-niche cell interactions occur in regenerating adult zebrafish.

Previous work in the lab has shown that mesenchymal cells in adult zebrafish fins display similar protrusions to those observed in larval zebrafish and in other regeneration-competent species of fish (unpublished data). We termed these cells as "niche cells," identifying them as a subset of activated mesenchymal cells that produce Wnt. Wnt signaling is essential for continued fin regeneration (Kawakami *et al*., 2006; Stewart *et al*., 2019; Wehner *et al*., 2014). We performed modeling using differential equations to show that niche cell-derived Wnt controls the extent of fin regeneration (Stewart *et al*., 2019). Longfin mutant zebrafish sustain their niche cell population, prolonging the fin regeneration period (Stewart et al., 2019). A separate genetic model of fin overgrowth, the "another longfin" mutant, possesses abnormally long actinotrichia in both regenerating and un-amputated caudal fins (König *et al*., 2018).

Together, these findings indicate that actinotrichia interact with niche cells in order to play an important role in fin growth and regeneration. However, how niche cells and actinotrichia interact, and the specific purpose of this interaction remains unclear. I focused on the two questions of how actinotrichia and niche cells interact, and what promotes the production

of actinotrichia. I hypothesized that Wnt signaling promotes niche cells to produce and align actinotrichia through cellular protrusions. I believe that the mesenchymal cell protrusions aligning actinotrichia in larval fins, along with the presence of cellular protrusions in adult niche cells, indicate that cellular protrusions are also aligning actinotrichia in adult fins during regeneration. Additionally, the presence of growth defects in mutant zebrafish with a loss of actinotrichia components suggests that actinotrichia promote proper regenerative outgrowth.

Developing a better understanding of the ways in which actinotrichia interact with niche cells to assist in regeneration will aid new developments in human healing. The fin-to-limb transition correlates with both a loss of actinotrichia, and a loss of regenerative abilities. A better understanding of this relationship could lead to new healing therapies for bone growth and repair.

Results

How do actinotrichia and niche cells interact?

Our first goal was to characterize actinotrichia observationally. Cell interactions with actinotrichia had not been previously studied in adult zebrafish, but previous research in zebrafish larvae and adult carp showed interactions between actinotrichia and mesenchymal niche cells (Böckelmann & Bechara, 2009; Kuroda *et al*., 2020). This suggested that there would be observable interactions in adult zebrafish as well. We began by testing an antibody against collagen II, a component of actinotrichia, to observe actinotrichia on both whole mount and sectioned slides. The col2a1 antibody had been previously used to mark actinotrichia on frozen slides, and we hypothesized that it would also work with paraffin tissue sections (Durán *et al*., 2011). We conducted a series of experiments testing various antigen retrieval methods, including trypsin, EDTA, and Immunosaver antigen retrieval solutions. We found that the col2a1 antibody was only able to successfully stain paraffin sections when using trypsin antigen retrieval.

We next wanted to observe actinotrichia using col2a1 antibody over regenerative time. Previous research indicated that actinotrichia formation occurs by 3dpa (Durán *et al*., 2011). Therefore, we hypothesized that the col2a1 stain time course would show actinotrichia appearing between our 2dpa and 4dpa timepoints. We stained regenerating whole fins at 2, 4, 7, 10, and 14dpa for Collagen II (using col2a1 antibody), actin (using phalloidin), Dachshund transcription factor (Dach, using DACH1 antibody), and nuclei (using Hoechst 33342) (Figure 1A-E). Dach transcription factor marks regenerating fin "niche" cells (Stewart *et al*., 2019), the groups of cells which we hypothesized interact with actinotrichia. We concluded that the actinotrichia stained by col2a1 were not present at 2dpa, but appeared by 4dpa and lengthened over regenerative time, which aligned with our hypothesis (Figure 1A-E). We also observed smaller immature collagen

fibrils distally. The larger and more mature collagen fibers appeared to generally align parallel to the direction of regeneration, while the immature fibrils were much more varied in their directionality (Figure 1F).

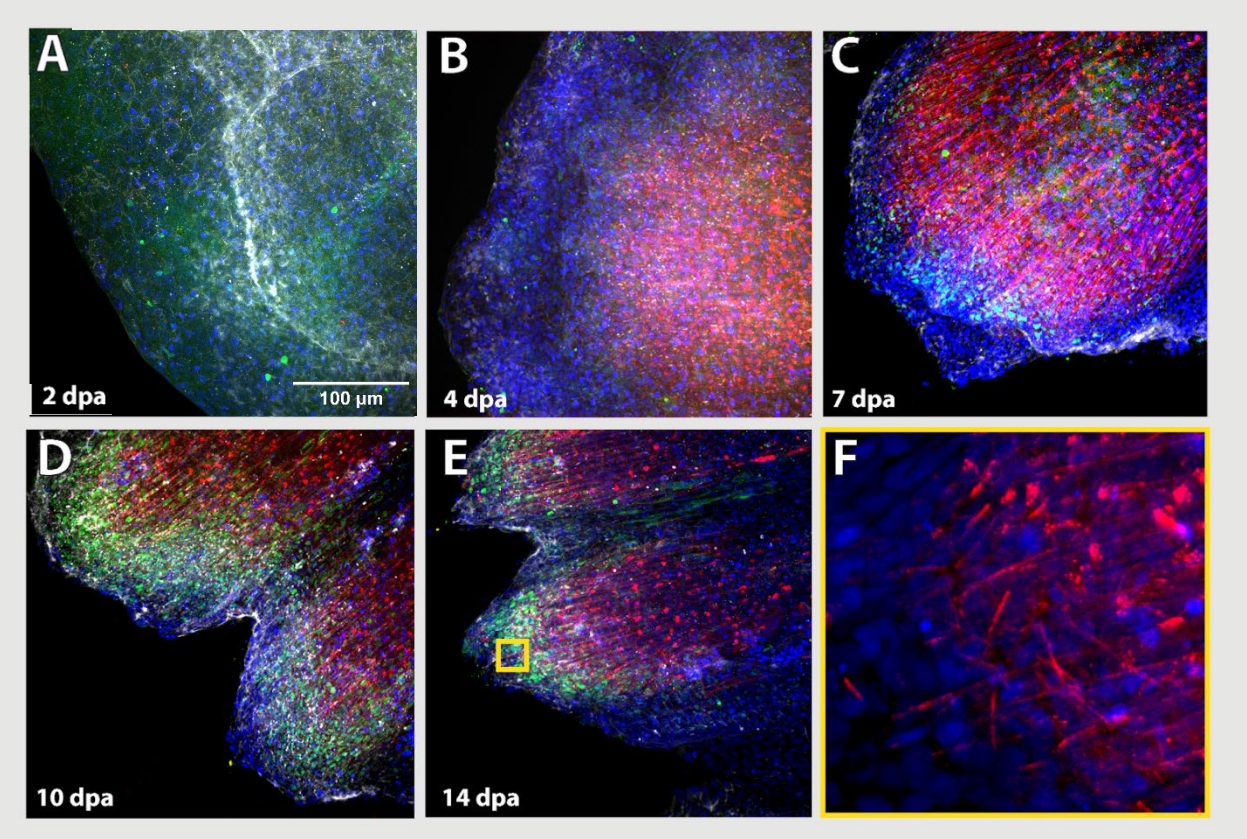

Figure 1. Immunofluorescence of whole regenerating fins between 2 and 14dpa.

Actinotrichia appear by 4dpa and lengthen over regenerative time. Colors represent phalloidin (white), Dach (green), nuclei (blue), and col2a1 (red). No actinotrichia are present at 2dpa (A). Collagen II, an actinotrichia component, appears by 4dpa (B). Actinotrichia lengthen and continue to be present through 14dpa (C-E). Small actinotrichia fibrils were observed distally (F).

We were interested in examining these smaller fibers more closely and predicted that a fluorescent reporter line would be a more specific marker of actinotrichia and better highlight smaller, immature fibrils. Previous imaging done with the *Tg*[5xand1(2k):And1-GFP] line

(Nakagawa *et al*., 2022), hereafter referred to as And1-GFP, appears to mark actinotrichia more brightly and clearly than our col2a1 whole mount immunofluorescence staining, potentially due to differing distribution of collagen II and And1 proteins within actinotrichia. We acquired the And1-GFP line and performed whole mount imaging of And1-GFP/+ fish. We found that this fluorescent line showed actinotrichia brighter and more completely than the col2a1 stain (Compare Figure 1 to Figure 2). Using this new line, we wanted to examine early regeneration again, this time focusing more closely on the first four days of regeneration, when actinotrichia first appeared. We hypothesized that actinotrichia would appear at 3dpa, and we would be able to see the previously-unstudied small, immature fibrils that would develop into mature actinotrichia over time. We amputated caudal fins of 6 And1-GFP/+;*sost:nls-Eos*/+ fish and imaged fin regeneration daily for 4 days. We found that immature actinotrichia appeared at 2dpa, which was earlier than had previously been reported (Figure 2B). We also concluded that, as expected, immature actinotrichia appeared first and later developed into longer, thicker, mature actinotrichia. (Figure 2B-D)

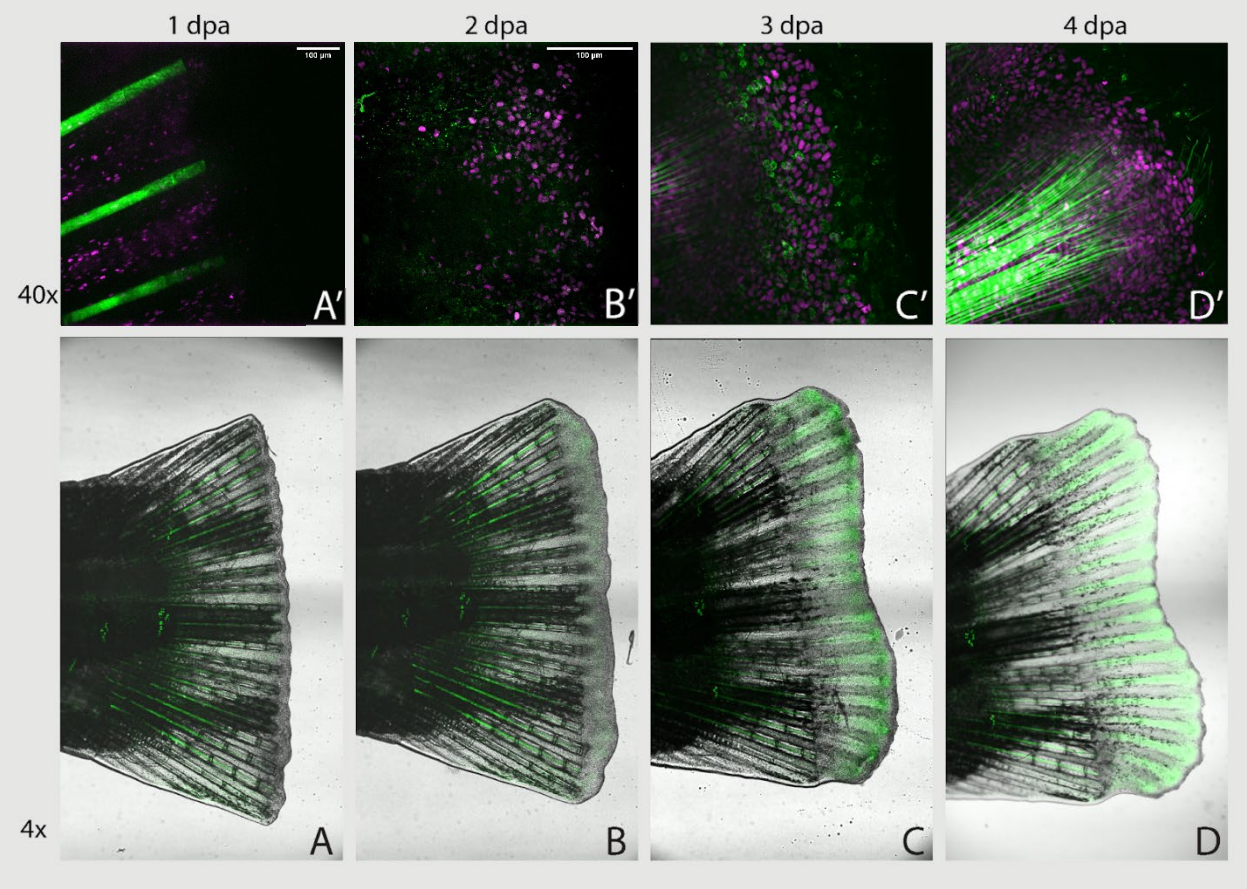

Figure 2. Whole regenerating fins of And1-GFP/+;*sost:nls-Eos*/+ fish between 1 and 4dpa.

Actinotrichia appear at 2 dpa and lengthen over regenerative time. Colors represent And1-GFP (green) and Sost-Eos (magenta). No actinotrichia are present at 1dpa (A'). Actinodin-1, an actinotrichia component, appears by 2dpa (B'). Actinotrichia lengthen and persist through 4dpa (B'-D').

After solidifying our methods for observing actinotrichia in the fin, we wanted to move further into exploring the relationship between actinotrichia and niche cells. In zebrafish larvae, mesenchymal cells appear to use actin-rich cellular protrusions to orient actinotrichia parallel to the fin rays as the fin extends distally (Kuroda *et al*., 2020). Additionally, in adult carp, fibroblast membrane protrusions wrap around actinotrichia in the regenerating tail fin (Böckelmann & Bechara, 2009). Based on this evidence, we hypothesized that niche cells also interact with

actinotrichia in adult zebrafish through cellular protrusions. We performed whole mount immunofluorescence for collagen II, Dach, actin, and nuclei on 5dpa caudal fins. We used confocal imaging with Airyscan detector to vastly improve resolution and observe niche cell-actinotrichia interactions up close. We found that distal niche cells are closely associated with actinotrichia (Figure 3A-A'). Because actin, a component of the cell cytoskeleton, and col2a1, an actinotrichia component, colocalize so closely, we concluded that niche cells do wrap actinotrichia in cellular protrusions (Figure 3B-B''). Distal niche cells appear to interact more with small or immature actinotrichia fibrils, whereas proximal niche cells may interact more with mature actinotrichia.

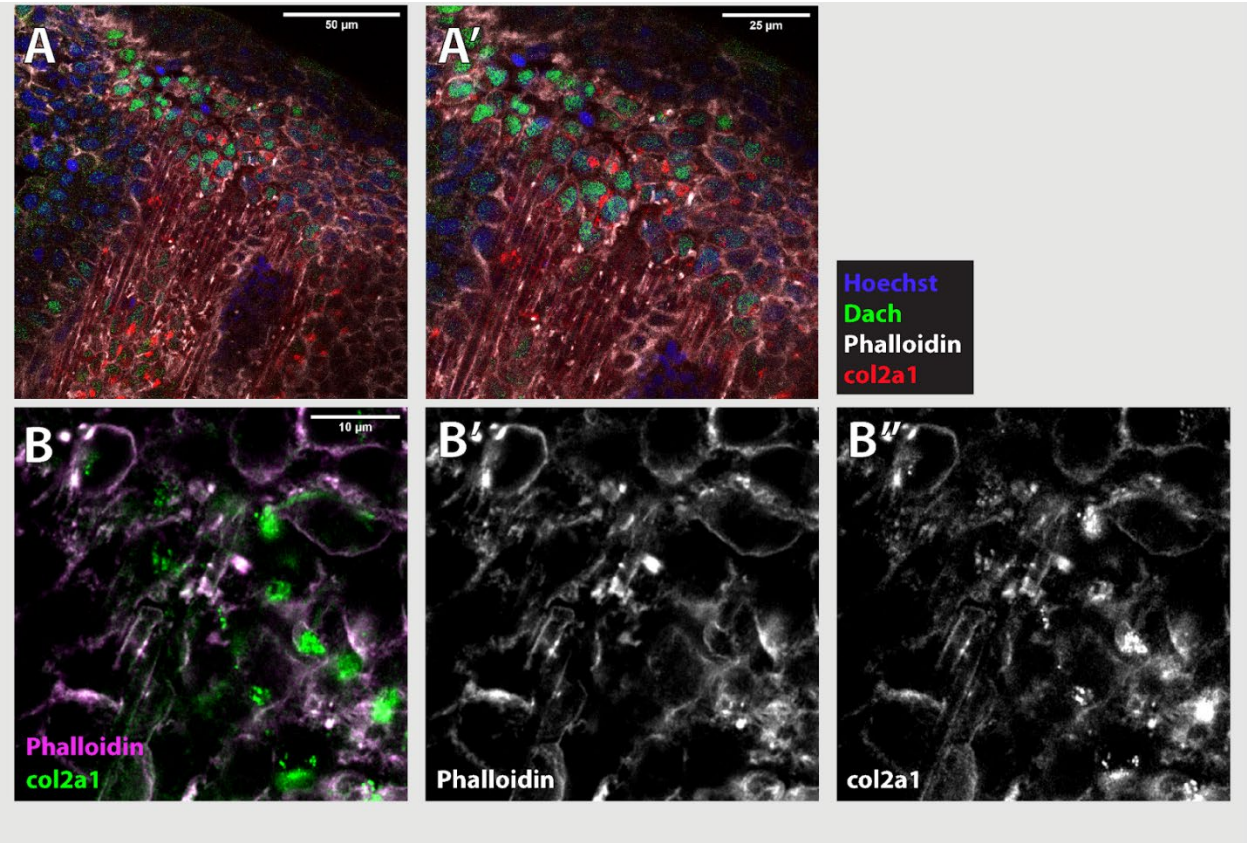

Figure 3. Mesenchymal cells wrap actinotrichia with actin-rich protrusions

Phalloidin and col2a1 stains overlap closely in the 5dpa regenerating fin. Niche cells (green, marked by Dach) at the distal fin tip associate closely with col2a1-marked actinotrichia (A,A'). Further zooms show a great degree of colocalization of actin and collagen II (B-B'').

Based on these promising results, we wanted to observe the interactions between actinotrichia and niche cells in the And1-GFP/+ fish. We stained longitudinal sections of 5dpa caudal fins with Dach, Phalloidin, and Hoechst (Figure 4A). Imaging these sections, we found niche cells stained by Dach that were surrounded by the actinotrichia component And1 (Figure 4B-B''). From this observation, we concluded that our earlier observation was correct, and there are clear interactions between actinotrichia and niche cells in regenerating fins, which supports

our hypothesis that niche cells and actinotrichia interact in regenerating adult zebrafish fins similarly to how they do in larval fins.

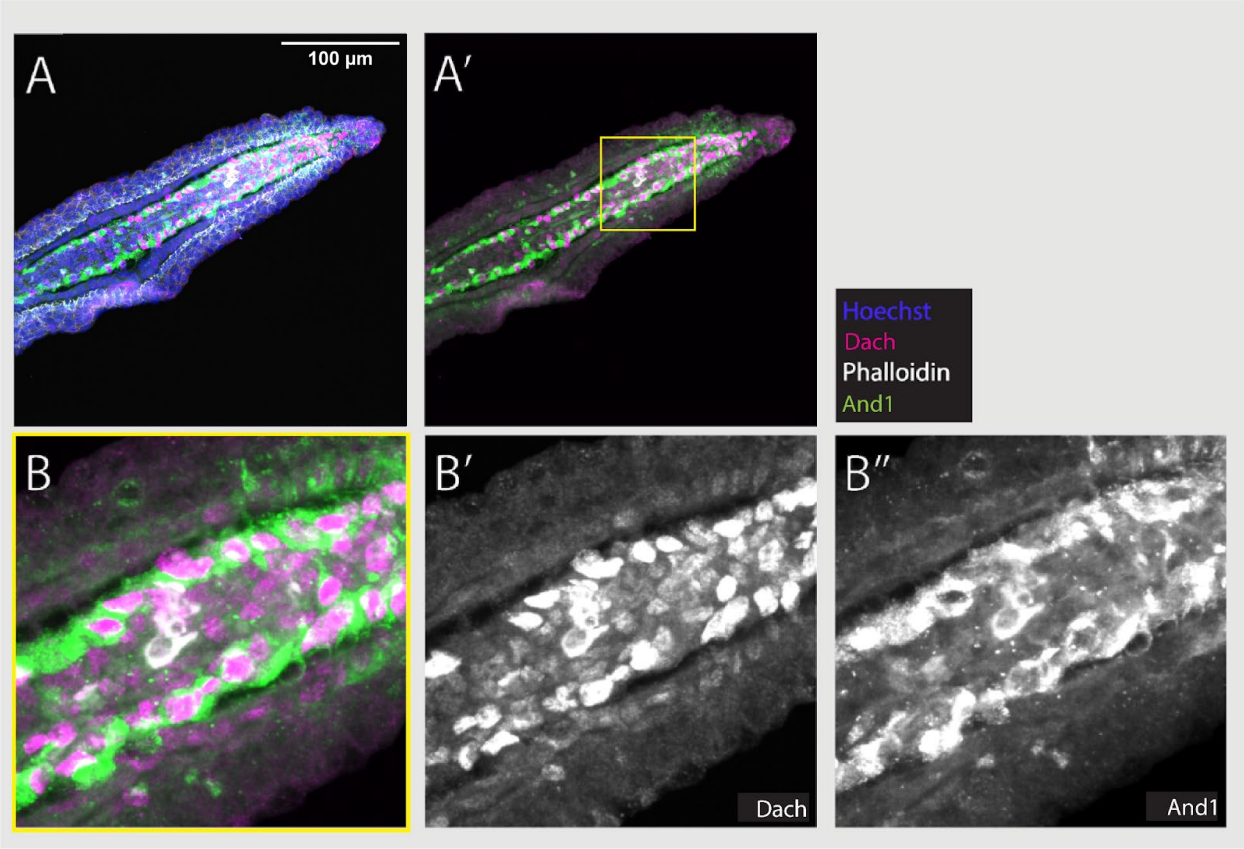

Figure 4. Longitudinal section immunostaining of And1-GFP/+ fish reveal clear interactions between actinotrichia and mesenchymal niche cells

Longitudinal sections are stained with Hoechst (blue), Dach (magenta), and Phalloidin (gray) (A). Dach stains (magenta) appear to be wrapped by actinotrichia component And1 (green) (A'). Further zooms show the extent to which actinotrichia are surrounding the niche cells (B-B'').

We also wanted to investigate through which proteins niche cells interact with actinotrichia. We hypothesized that niche cells could interact with actinotrichia through focal adhesions. In order to test this, we stained slides with phosphorylated Focal Adhesion Kinase (pFAK) and col2a1 antibodies. We observed focal adhesions present in the basal epidermis but did not see any focal adhesions in the mesenchymal cells that were close to the collagen

microfibrils stained by col2a1 (Figure 5). Therefore, we concluded that actinotrichia-niche cells interactions do not occur through focal adhesions.

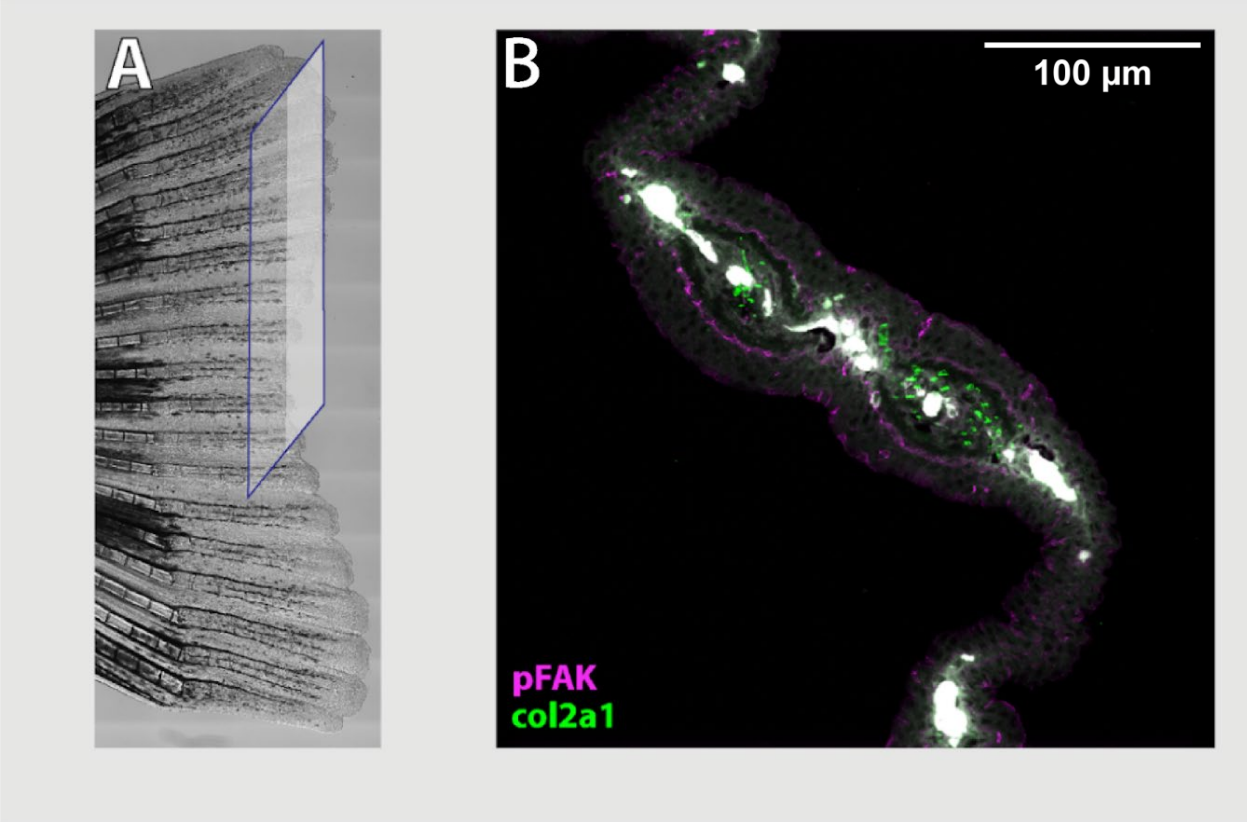

Figure 5. Mesenchymal cells associate with actinotrichia, but not through focal adhesions

Distal transverse section of a regenerating caudal fin. Schematic of transverse section orientation (A). Fin rays stained with col2a1, an actinotrichia component, and phospho Focal Adhesion Kinase (pFAK) (B). Focal adhesions were observed in the basal epidermis but not in mesenchymal calls nearby to collagen microfibrils. Bright white blobs in panel B represent autofluorescence of blood cells and should be ignored.

What factors promote actinotrichia formation?

We hypothesized that actinotrichia production could occur downstream of Wnt signaling, as Wnt is essential for continuation of fin regeneration (citation). I searched the PubMed database for the term “actinotrichia” and examined resulting papers to identify genes which have

been shown to be actinotrichia components or control actinotrichia production. This resulted in a list of 22 actinotrichia-related genes (Table 1). I analyzed existing RNA sequencing data on Wnt-C59 (a Wnt inhibitor) vs DMSO (control) treated regenerating fins and found that Wnt inhibition does not meaningfully change expression of actinotrichia-related genes (Figure 6A-B). Of the 22 genes from the literature review, only three showed significant changes in expression. These significant genes were *and4*, *bmp2b*, and *shh* (Figure 6C). From this data, we concluded that Wnt does not directly control actinotrichia production.

Gene	Description	Citation
and1	actinotrichia components	König *et al*., 2018
and2	actinotrichia components	König *et al*., 2018
and3	actinotrichia components	König *et al*., 2018
and4	actinotrichia components	König *et al*., 2018
col2a1b	actinotrichia components	Durán *et al*., 2011
col9a1c	actinotrichia components	Nakagawa *et al*., 2023
col1a1a	actinotrichia components	Durán *et al*., 2011
lh1	enzyme	Durán *et al*., 2011
fgfr1	fgf signaling pathway	König *et al*., 2018
fgf20a	fgf signaling pathway	König *et al*., 2018
wnt5b	BMP signaling pathway	Thorimbert *et al*., 2015
id1	BMP signaling pathway	Thorimbert *et al*., 2015
id3	BMP signaling pathway	Thorimbert *et al*., 2015
shh	involved in actinotrichia formation	Quint *et al*., 2002
epi3	specific part of and1, early mesenchymal-specific repressor	Phan *et al*., 2019
mmp13a	knockdown reduces Collagen II and Actinodin 1 expression	Li *et al*., 2020
mmp2	activated on mesenchymal cell protrusions, remodels the ECM associated with maturation of actinotrichia	Wyatt & Crawford, 2021
serpinh1b	codes for Hsp47, Hsp47 knockdown affects the organization and localization of actinotrichia	Bhadra & Iovine, 2015
fndc3a	Fin regeneration in mutants is hampered by interference with actinotrichia formation	Liedtke *et al*., 2019
hmcn2	remodel the ECM of the fin fold intraepidermal space at the level of actinotrichia	Feitosa *et al*., 2012
fbln1	remodel the ECM of the fin fold intraepidermal space at the level of actinotrichia	Feitosa *et al*., 2012
bmp2b	Disruption causes a loss of actinotrichia	Quint *et al*., 2002

Table 1. Genes related to actinotrichia

The table contains a list of genes collected through a literature review of papers found through keyword “actinotrichia” on PubMed. 58 papers were reviewed and 22 genes related to actinotrichia were identified.

Figure 6. RNA sequencing of Wnt-inhibited fins suggests that actinotrichia production is not directly downstream of Wnt signaling.

We used existing RNA sequencing data collected from proximal (pink) and distal (yellow) regenerates of 4dpa Wnt-C59 and DMSO-treated fins (A). Combining data for all actinotrichia related genes showed no significant change in expression ($p = 0.991$ for proximal, $p = 0.881$ for distal, 2-tailed T-test) (B). Only 3 individual actinotrichia-related genes change in expression under Wnt inhibition (C). We conclude that Wnt signaling is not immediately upstream of transcription of most actinotrichia components.

Inhibition of Transforming growth factor-β (TGF-β) signaling through the small molecule SB431542 has previously been shown to inhibit And1 expression (König *et al*., 2018). Thus, we decided to test whether TGF-β signaling is directly upstream of actinotrichia production using the And1-GFP line. We included Wnt inhibition through Wnt-C59 treatment as a negative control, where we know that fins will regenerate shorter, but not due to a direct effect on actinotrichia production (Figure 6). FK506 treatment was included as a positive control. As treatment with FK506, a calcineurin inhibitor, leads to regeneration of longer fins (Kujawski *et*

al., 2014), we expected to observe longer or more actinotrichia. We performed drug treatments on regenerating And1-GFP/+;*sost:nls-Eos*/+ zebrafish between 7 and 9dpa, treating for 4 hours per day. The treatment groups were 500 nM Wnt-C59, 20 μm SB431542, 500 nM FK506, and DMSO as a control. The *sost:nls-Eos* line, which expresses nuclear-localized Eos fluorescent protein in mesenchymal cells, was included to allow us to visualize whether niche cell interaction with actinotrichia changed as a result of these treatments. At 10dpa, we imaged regenerating caudal fins and subsequently analyzed the images using ImageJ. We quantified the size of the regenerated area and found that the fish treated with FK506 regenerated longer fins than DMSO control on average, while Wnt-C59 and SB431542 treatment did not significantly change regenerated area (Figure 7A). As expected, we observed that the FK506 regenerated area was larger than the DMSO control, as FK506 drug treatment causes the regeneration of long fins. We also observed an increase in Eos+ nuclei and a lack of joint formation in the distal-most regenerated tissue (not shown), which indicated that the FK506 treatment was successful in disrupting calcineurin signaling. Wnt-C59 and SB432542 treated fish did not regenerate shorter fins as expected, which could be due to the short treatment duration. We quantified the number of actinotrichia on one hemiray of ray 3 and found that FK506-treated fish had less mature actinotrichia than the other cohorts ($p = 0.0918$), which was also unexpected (Figure 7B). Finally, we noted qualitatively that the distal, disorganized, and presumably immature actinotrichia microfibrils present in the DMSO control were consistently absent from the Wnt-C59 and SB431542 drug treated fish. In contrast, distal actinotrichia microfibrils qualitatively appear to be increased under FK506 treatment (Figure 7C).

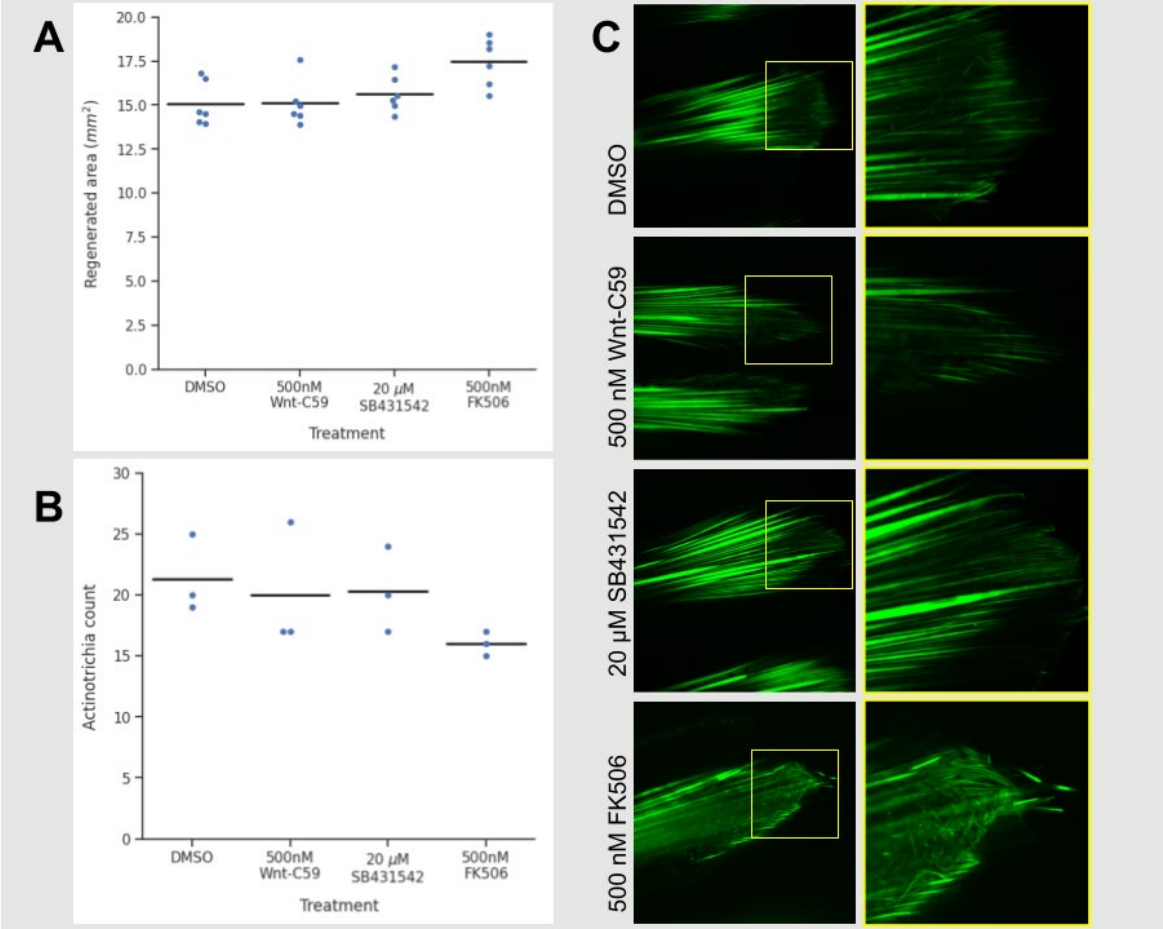

Figure 7. Drug treatments of regenerating fins suggest that the presence of new actinotrichia fibrils is more important for outgrowth than the quantity of mature actinotrichia.

We performed drug treatments between 7 and 10dpa and imaged at 10dpa. Using ImageJ we calculated regenerated area (mm^2) and counted the total actinotrichia on ray 3. Data points from individual fish were graphed with blue points, with the mean of each treatment represented by the black horizontal line. FK506 drug treated fish on average had longer fins than the DMSO control, while Wnt-C59 and SB432542 treatments showed no change (A). The actinotrichia count of the FK506 fish was much lower than the actinotrichia count of the fish treated with the DMSO control, while Wnt-C59 and SB43152 showed no change in comparison to control (B). Fish treated with DMSO and FK506 had actinotrichia microfibrils that appeared immature and disorganized, while the fish treated with Wnt-C59 and SB432542 lacked these fibrils and only had mature actinotrichia (C).

Methods

Caudal fin amputations

Zebrafish were anesthetized using tricane and placed in a petri dish on the microscope. Fins were flattened into a relaxed position with a paintbrush. A razor blade was used to cut straight across the fin longitudinally. The cut was made at the ends of the second procurrent rays. When collecting regenerated fins, the second cut was made proximal to the first cut in order to collect all of the regenerated tissue. After amputating, the fish were revived in system water and placed back into their original tanks.

Preparing paraffin-embedded caudal fin sections

Amputated fins were collected by re-amputation proximal to the original amputation plane and fixed overnight in glass vials with 4% PFA-PBS. Tissue was then rinsed in PBS and decalcified for 4 days in 500 mM EDTA pH 8. Fins were dehydrated in an ethanol series and moved through xylenes and into paraffin wax. Fins were then embedded in paraffin blocks, sectioned at 7μm, and arranged on Matsunami MAS SUMAS1190 slides.

Paraffin section immunofluorescence

Slides were washed in xylenes and 100% ethanol and then hydrated through an ethanol series, ending in distilled water. The slides were then moved to antigen retrieval solution in a plastic Coplin jar and heated for 5 minutes in a pressure cooker. For EDTA-Tween 20 antigen retrieval, the antigen retrieval solution contained 1 mM EDTA pH 8 and 0.1% tween 20. After antigen retrieval and cooling, the slides were blocked for one hour in blocking solution (10% milk in 1x PBS/0.1% Tween 20). Primary antibodies in blocking buffer were added to the slides with a coverslip and slides were incubated at 4°C overnight. The next day, the coverslip was

carefully removed, and the slides were placed back in the Coplin jar for 2 quick 1x PBS/0.1% Tween-20 (PBST) washes. Secondary antibodies in blocking buffer were added to each slide with a coverslip and incubated at room temperature for 45-60 minutes. The coverslips were then removed, and the slides washed in PBST. Slides were stained with 1:3000 Hoechst 33342 in PBST, and finally washed twice in 1x PBS. The stained sections were then mounted using 1 drop of slowfade diamond and a coverslip, which is secured in place with a layer of clear cosmetic lacquer along the edges of the coverslip. Sections were imaged using a Nikon spinning disk microscope and processed in ImageJ. Minimum and maximum intensity values were kept constant within experiments.

Whole mount fin immunofluorescence

Amputated fins were harvested into ice-cold 4% PFA-PBS and fixed for 30 mins. Fins were rinsed in 1xPBS, then washed for 45 minutes, changing the solution every 15 minutes. Fins were then blocked in blocking buffer for 3 hours (1xPBS + 1% TritonX-100 + 3% BSA + 10% DMSO). Primary antibody solution was then added in blocking buffer at 4°C overnight. The next day a series of three 1xPBS + 1% TritonX-100 washes were performed for 2 hours each, before the secondary antibody solution was added in blocking buffer overnight. The next day, the 1xPBS + 1% TritonX-100 2-hour wash series was repeated, before the fins were stained with 1:3000 Hoechst 33342 in PBS + 1% TritonX-100. Finally, the Hoechst stain was removed through three PBS + 1% TritonX-100 washed, and fins were subsequently mounted on a glass slide. To mount, the fins were spread out and slowfade diamond was added with a coverslip, which was secured in place with a layer of clear cosmetic lacquer along the edges of the coverslip. Immunostained fin tissue was then imaged either using a Nikon spinning disk microscope, or, for Airyscan imaging, a Zeiss 770 confocal microscope with AiryScan detector.

Identifying actinotrichia-related genes and analysis of Wnt-inhibition RNA sequencing data

A literature review was conducted on PubMed using the keyword "actinotrichia". All search results were carefully reviewed, and all genes related to actinotrichia mentioned were recorded, along with a short description. Existing Wnt-inhibition RNA sequencing data was then reviewed, and the data for all listed genes from the literature review was compiled. Data included comparisons of gene expression between Wnt-inhibited and non-Wnt-inhibited fish in both distal and proximal areas. Data was then analyzed in Google Sheets. A two-tailed, two-sample paired T-test was performed on both the proximal and distal data from each gene to determine significance, as well as the averages of all actinotrichia related genes.

Drug treatments

24 adult *Tg*[5xand1(2k:And1-GFP]/+;*sost:nls-Eos*/+ zebrafish caudal fins were amputated and allowed to regenerate in standard housing for 7 days. Fish were then separated into 4 groups of 6 and underwent drug treatments for 4 hours before being moved to flow-through racks in separate tanks for each treatment group. The treatment groups were 500 nM Wnt-C59, 20 μm SB431542, 500 nM FK506, and DMSO as a control. 4-hour drug treatments were repeated at 8 and 9 dpa. At 10 dpa, regenerated caudal fins were imaged using a Nikon spinning disk microscope. For each fish, a 4x stitched image of brightfield and 488 nm fluorescence was obtained. For at least 3 fish in each group, Eos protein was irreversibly converted to far-red fluorescence using 405 laser excitation on the 40x laser objective. Then, 40x z stacks of the distal tip of ray 3, with And1-GFP in the 488 channel and nls-Eos in the 561 channel, were acquired. After imaging, fish were revived in system water and placed back on the flow-through rack for subsequent experiments. Images were processed using ImageJ software.

To quantify regenerated area, the polygon selection tool was used to trace the outline of the fin from the amputation line to the distalmost tissue, and the area within the polygon was recorded. The multi-point tool was used to count the number of mature actinotrichia. Representative slices of distal actinotrichia microfibrils were chosen by finding the slice with the most visible actinotrichia. P values were calculated by a two tailed T test comparing each drug to the DMSO control values.

Discussion

Regeneration in zebrafish is a captivating subject of study because of the implications to improve human healing. Indications that actinotrichia fibrils are necessary for proper outgrowth during fin regeneration in zebrafish suggests that actinotrichia play an important role in regeneration, and that they could perhaps be harnessed to support human bone growth and healing. Therefore, understanding how actinotrichia support fin outgrowth is extremely valuable. Actinotrichia appear at 2.5 dpa in regenerating adult zebrafish fins (Durán *et al*., 2011). Presumably, they support fin outgrowth by providing a scaffolding for the blastema before the development of mature bone. In our exploration of actinotrichia, we first looked into actinotrichia dynamics of the regenerating caudal fin over time. We concluded that actinotrichia microfibrils first appeared at 2 dpa, which is earlier than previously reported (Figure 2). We next looked into the interactions between actinotrichia and niche cells with the hypothesis that niche cells align actinotrichia through cellular protrusions in adult zebrafish during regeneration, much like how mesenchymal cells appear to orient actinotrichia in larval zebrafish cell cultures (Kuroda *et al*., 2020). We found colocalization of niche cell actin filaments with collagen II in whole mount fin tissue (Figure 3) and actinotrichia surrounding niche cells in And1-GFP/+ fin sections (Figure 4), and thus concluded that niche cells interact with actinotrichia in regenerating adult zebrafish. We also found that these interactions do not occur through focal adhesions (Figure 5). These results support our theory that the interactions between actinotrichia and niche cells occur similarly in regenerating adult zebrafish to zebrafish larvae, and thus the actinotrichia may be guided and oriented by niche cells to promote proper fin growth.

Our second line of inquiry regarded the question: What factors promote actinotrichia production? We initially investigated the Wnt signaling pathway, because we have previously

shown that Wnt signaling is essential to maintain niche cells (Stewart *et al*., 2019). After performing a literature review to develop a list of genes related to actinotrichia, we used existing RNA sequencing data to compare the expression of these genes between short term Wnt-C59 treated and DMSO treated regenerating fins. We concluded that Wnt inhibition does not directly affect actinotrichia production (Figure 6).

We next investigated whether TGF-β signaling could be more directly involved with actinotrichia production, as inhibition of TGF-β through the small molecule SB431542 inhibits expression of And1, an actinotrichia component (König *et al*., 2018). We performed drug treatments using SB431542, Wnt-C59 as a negative control, FK506 as a positive control, and DMSO. We quantified these results and found that neither SB431542 nor Wnt-C59 had significant impacts on the overall regenerated area or the number of actinotrichia per ray. FK506, however, showed larger regenerated areas and had less mature actinotrichia (Figure 7A-B). We believe that the Wnt-C59 and SB431542 treatments did not result in shorter fins because the duration of the drug treatments was not long enough to show significant changes in the length of outgrowth. Looking closely at the distal tip of the third ray, we observed that distal, quiescent niche cells in particular appear to be closely associated with smaller And1-GFP-marked fibrils, which we assume are immature actinotrichia fibrils. Supporting this, these fibrils are not aligned in the proximal-distal orientation as mature fibrils are, but rather appear to be oriented in all directions. We noted that the fins treated with SB431542 and Wnt-C59 consistently lost their immature actinotrichia fibrils, which were present in the FK506 treated fins and the DMSO control. Much of the mature actinotrichia present in these fins were likely produced before the drug treatments began, and the changes in immature fibrils are more likely to be due to the impact of the drugs. Based on the RNA seq data and the drug treatments, we conclude that both

Wnt and TGF-β signaling pathways may indirectly affect production of actinotrichia. The drug treatments also showed a decreased actinotrichia count in the longer fins resulting from FK506 treatments, which was an unexpected result as actinotrichia were previously observed to be longer in the "another longfin" mutant (König *et al.*, 2018). It may be that mature actinotrichia count does not correlate with outgrowth, and it is rather the immature fibrils that are most important in supporting the regenerating fin. It is also possible that insufficient production of actinotrichia components before FK506 treatment led to the opposite effect on mature actinotrichia compared to long-finned mutants due to the short treatment time.

The results of these experiments have led to further questions regarding the role of niche cells in actinotrichia formation. Imaging the And1-GFP line during normal regeneration and during drug treatments identified potential actinotrichia microfibrils associated with distal niche cells which were oriented in all directions. This suggests that actinotrichia microfibrils could be aligned into mature actinotrichia fibrils by niche cells, as occurs in larval fin fold development (Kuroda *et al.*, 2020). Performing live imaging over 12 hours of regeneration in and1-GFP/+;sost:nls-Eos fish is an essential future experiment, as it would allow us to determine whether these smaller fibrils eventually become mature actinotrichia or have another role, and, if so, whether fusion into mature actinotrichia may be mediated by niche cells. It is also essential to identify which niche cell membrane components interact with actinotrichia. Another future experiment would be to try integrin inhibitor drug treatments, which would allow us to explore which proteins and receptors niche cells use in their interactions with actinotrichia, and what the effect of losing this interaction would be. Finally, we assume that actinotrichia have an essential role in fin regeneration, but this has not been adequately shown. Injection of And1 and And2 morpholinos into the blastema to knockdown gene function would allow us to observe how the

loss of actinotrichia components affects fin regeneration. These experiments would allow us to better understand the function of actinotrichia in regenerating adult zebrafish fins. Understanding how interactions between actinotrichia and niche cells affect regenerating adult zebrafish fins will aid new developments in bone healing therapies. A better understanding of this relationship will help to determine whether interaction of growth-promoting cells with actinotrichia or collagen fibrils could be repurposed to improve regenerative medicine therapies.

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