Comparative Expression Profiling Reveals an Essential Role for Raldh2 in Epimorphic Regeneration*  

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Zebrafish have the remarkable ability to regenerate body parts including the heart and fins by a process referred to as epimorphic regeneration. Recent studies have illustrated that similar to adult zebrafish, early life stage larvae also possess the ability to regenerate the caudal fin. A comparative microarray analysis was used to determine the degree of conservation in gene expression among the regenerating adult caudal fin, adult heart, and larval fin. Results indicate that these tissues respond to amputation/injury with strikingly similar genomic responses. Comparative analysis revealed raldh2, a rate-limiting enzyme for the synthesis of retinoic acid, as one of the most highly induced genes across the three regeneration platforms. In situ localization and functional studies indicate that raldh2 expression is critical for the formation of wound epithelium and blastema. Patterning during regenerative outgrowth was considered to be the primary function of retinoic acid signaling; however, our results suggest that it is also required for early stages of tissue regeneration. Expression of raldh2 is regulated by Wnt and fibroblast growth factor/ERK signaling.

Injury, disease, and aging all result in a loss of tissue and reduced quality of life. Numerous human conditions could be significantly improved if therapies that encourage tissue regeneration were available. Most adult tissues and organs, especially in humans and other mammals, have lost their regenerative potential. As a result, injury to a tissue or organ usually results in permanent damage from scarring to disability. The field of regenerative medicine is aimed at developing strategies to restore individual cell types, complex tissues, or structures that are lost or damaged. Currently, one of the main approaches in the field of regenerative medicine is to guide the process of differentiation of stem cells into specific cell types and then into complex structures (1). Alternatively, another strategy is to determine how certain organisms have retained the ability to regenerate their tissues, organs, and appendages (2, 3). By understanding the molecular pathways that differentially function in these "lower" animals, we will be in a stronger position to uncover why mammals fail to react to injury with a regenerative response.

Lower vertebrate model systems such as urodele amphibians and teleost fish have the remarkable ability to regenerate organs such as the heart, spinal cord, retina, and limbs/fins (2, 3). In recent years, zebrafish has been established as a research model for the identification of molecular signaling pathways that govern the process of regeneration. Adult zebrafish caudal fin regeneration occurs by epimorphic regeneration, which involves reprogramming and differentiation of blastema cells to different cell types to restore the tissue to its original form (2, 4–6). A genetic zebrafish mutant study revealed that Fgf20a is absolutely required for the initiation and formation of blastema, whereas recent reports suggest that Wnt/β-catenin signaling seems to act upstream of FGF signaling (7, 8). Even though major progress has been made in the identification of some of the essential pathways for regeneration such as FGF,3 Wnt, and Activin-βA (actβA) signaling, most would agree that we are still at the early stages of gene discovery (5, 7–10).

Similar to adult zebrafish, early life stage larvae have the ability to regenerate amputated caudal fins through the formation of the wound epithelium and blastema (7, 11–16). Also, similar to the adult zebrafish, chemical inhibition of FGFR1 by SU5402, aryl hydrocarbon receptor activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin, and glucocorticoid receptor activation by beclomethasone-abrogated larval fin regeneration (13–15, 17), suggesting that there are similarities at the cellular and molecular levels between adult and larval regeneration. Because many of the experimental advantages of zebrafish lie at the earliest life stages, the study of larval regeneration during this experimentally tractable life stage is enticing.

A comprehensive microarray analysis of adult zebrafish fin and adult heart regeneration identified some conserved genomic responses to amputation in these distinct regeneration models (18, 19). This suggests that the pathways essential for the initiation of regeneration may be conserved. To identify

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S8 and Figs. S1–S2.

2 Both authors contributed equally to this work.

3 The abbreviations used are: FGF, fibroblast growth factor; RA, retinoic acid; ERK, extracellular signal-regulated kinase; dpa, days post amputation; dpf, days post fertilization; qRT, quantitative reverse transcription; hpa, hours post amputation; raldh2, retinaldehyde dehydrogenase 2; Citral, 3,7-di-methyl-2,6-octadienal; DEAB, 4-diethylaminobenzaldehyde; ANOVA, analysis of variance; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
whether there are corresponding similarities in the regenerative
gene expression response in the early life stage model, we
conducted broad-based microarray analysis of larval and adult
fin regeneration and compared the gene expression changes.
Comparative analysis between larval and the adult fin regenera-
tion systems indicated a high degree of similarity between the
two gene expression profiles. When the larval gene list was
compared with the published zebrafish heart regeneration gene
list, similar patterns of gene expression changes were revealed.
Because the tissue architecture of larval fin, adult fin, and heart
are very different, the significant commonality in the gene
expression changes must be reflective of conserved molecular
signaling. To demonstrate the power of the larval fin regenera-
tion model, we analyzed the role of a candidate gene and per-
formed functional studies.

EXPERIMENTAL PROCEDURES

Zebrafish Lines and Care—For the larval fin regeneration
studies, fertilized eggs were obtained from AB strain zebrafish
(University of Oregon, Eugene, OR). For the adult in situ
hybridization study, 2-month-old AB strain zebrafish were
used. The fin amputations were performed as previously
described (15, 16, 20, 21). The Tg(hsp70l:tcf3-GFP) line was
obtained from ZIRC.

Chemicals—The retinoic acid (RA) synthesis inhibitors 4-di-
ethylaminobenzaldehyde (DEAB) and 3,7-dimethyl-2,6-octa-
dienal (Citral) were purchased from Sigma. The amputated lar-
vae were exposed to DEAB and Citral at final concentrations
of 250 and 25 μM, respectively, and the solutions were changed
daily until 3 days post amputation (dpa). The ERK1/2 inhibitor
U0126 was purchased from EMD Biosciences (San Diego, CA).
U0126 was continuously exposed at a final concentration of
3 dpa. The larval fin tissue was used to generate biotinylated comple-
mentary RNA (cRNA) using the Two-Cycle Target Labeling kit
(Affymetrix, Santa Clara, CA). Briefly, RNA samples were
reverse transcribed using a T7-(dT)24 primer and Superscript II
reverse transcriptase (Invitrogen) and double-stranded cDNA
was synthesized. This was then used as a template for in vitro
transcription for another round of double-stranded cDNA syn-
thesis. For the adult fin regeneration study, 2.5 μg of total RNA
was used to generate biotinylated cRNA for each treatment
group using the One-Cycle Target Labeling kit (Affymetrix,
Santa Clara, CA). From the double-stranded cDNA, biotin-
ylated cRNA was synthesized using T7 RNA polymerase and
a biotin-conjugated pseudouridine containing nucleotide
mixture provided in the IVT Labeling Kit (Affymetrix, Santa
Clara, CA). For both larval and adult fin regeneration exper-
iments, 10 μg of purified and fragmented cRNA from each
experimental sample was hybridized to zebrafish genome
arrays (Zebrafish430_2) according to the Affymetrix GeneChip
Expression Analysis Technical Manual (701021 Rev. 5). Arrays
were scanned with an Affymetrix scanner 3000. For data anal-
ysis, the Affymetrix cel files were imported into GeneSpring 7.1
software (Agilent Technologies, Palo Alto, CA). The files were
GC-RMA processed to discount for background signal and
each transcript was normalized to the median signal to allow
comparison between arrays on a relative scale for each gene.
The differential effect of time on regeneration was performed
by comparing the nonregenerating fin tissue (0 dpa) to other
time points by one-way ANOVA assuming equal variance
employing Benjamini and Hochberg multiple testing correc-
tions (p < 0.05). Only genes that were at least 1.7-fold dif-
ferentially expressed from the 0 dpa gene levels were consid-
ered for analysis. The annotation of genes was performed by
considering the sequence similarity to known mammalian
proteins that was determined by conducting a BLAST search of
each Affymetrix probe set against the Sanger data base.
Additionally, other databases such as GenBankTM (BLAST)
and the Zebrafish Affy Chip Annotation Project at Children’s
Hospital Boston were utilized. The fold-difference values from microarray data of the published heart regener-
ation study were on a base 2-logarithm scale and those values
were transformed to normal numbers for comparison with
our studies. Experiments were MIAME certified, and the raw
data are listed at the National Center for Biotechnology
Information (NCBI) Gene Expression Omnibus (GEO) (series
record GSE10188).

Morpholinos—The fluorescein-tagged raldh2 morpholino
(Gene Tools, Philomath, OR) was used to transiently knock
down raldh2 expression. The sequence of raldh2 morpholino is
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5′-GGTTACCTACTGAGGCCATCGGC-3′. Morpholinos were diluted to 3 mM in 1× Danieau’s solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6) as described (22). A standard control morpholino (Gene Tools, Philomath, OR) (5′-CTTACCTAGTTACAATT-TATA-3′) was used. Approximately 2 nl of 0.3 mM morpholino solution was microinjected into the embryos at the 1–2 cell stage. The fin tissue of control and raldh2 morphants were amputated at 2 dpf and exposed and allowed to grow for 3 days at 28 °C.

Quantitative RT-PCR—Total RNA was isolated in triplicate from the regenerating fins at 0, 1, 2, and 3 dpa (n = 150/group). From the larval fin RNA, cDNA was prepared from 100 ng of total RNA per group using Superscript II (Invitrogen) and oligo(dT) primers in a 20-μl volume. Quantitative RT-PCR (qRT-PCR) was conducted using gene-specific primers (supplemental Table S8) with the Opticon 2 real-time PCR detection system (MJ Research, Waltham, MA). Briefly, 1 μl of cDNA was used for each PCR in the presence of SYBR Green, using the DyNAmo SYBR Green qPCR kit according to the manufacturer’s instructions (Finnzymes, Espoo, Finland). All samples were normalized to their β-actin abundance. Quantitative differences between biological samples were determined by normalizing all samples to a common reference sample. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were conducted to ensure formation of specific products. Significant differences of mRNA abundance were assessed by one-way ANOVA on log10 transformed data using Tukey method (p < 0.05) (SigmaStat, Chicago, IL).

Whole Mount in Situ Hybridization—In situ hybridization was performed on the regenerating fin at the respective time points as described previously (16). The mvp, msxe, and dlx5a probes were obtained from Atsushi Kawakami (16). The raldh2 probe was prepared by cloning the cDNA by RT-PCR from the RNA isolated from the adult whole zebrafish. The wnt10a probe was a gift from Gilbert Weidinger (Biotechnological Center, Technical University of Dresden, Dresden, Germany). The embryos were reared in phenylthiourea (Sigma) at a final concentration of 100 μM at 24 h post fertilization to inhibit formation of pigmentation.

Cell Proliferation Assay—The cell proliferation assay was conducted as previously described (14) on regenerating fin tissue after pulse labeling with BrdUrd (Roche Applied Science) for 6 h starting from 24 or 48 h post amputation (hpa). BrdUrd assay was performed on vehicle- or DEAB-exposed amputated larvae at the respective time points. After 6 h of incubation with BrdUrd at 28 °C, the larvae were fixed in 4% paraformaldehyde overnight. The fixed larvae were dehydrated with methanol and then stored in methanol at −20 °C. Briefly, immunohistochemistry was conducted on the stored larvae by rehydrating with a graded methanol/PBST (phosphate-buffered saline (PBS) and 0.1% Tween 20) series. The larvae were then treated with proteinase K in PBST for 20 min at room temperature and then rinsed several times with PBST. The larvae were refixed in 4% paraformaldehyde for 30 min and then washed several times in water, followed by quick rinses in 2N HCl and incubation in 2N HCl at room temperature for 1 h. After several washes, the larvae were then blocked with 1% normal goat serum in PBST for 1 h at room temperature and then incubated with anti-BrdUrd antibody (1:100; G3G4; Developmental Studies Hybridoma Bank, Iowa City, IA) overnight at 4 °C. After 4 or more 30-min washes with PBST, the larvae were incubated with a secondary antibody (1:1000; Alexa 546-conjugated goat anti-mouse; Molecular Probes, Eugene, OR) for 4 h at room temperature. The larvae were then washed 4 times for 30 min in PBST and visualized by epifluorescence microscopy. The BrdUrd-labeled fluorescent cells were quantified with the acquired images using ImagePro Plus software program (Media Cybernetics, Inc., Silver Spring, MD).

RESULTS

Structural Morphogenesis of Larval to Adult Fin—Although both larvae and adult zebrafish regenerate their caudal fins following amputation, it is clear that there are structural differences in the regenerating tissues between the two life stages. Bright field imaging revealed that the lepidotrichia (fin rays) are not yet present in larvae at 5 dpf. Instead, the larval fin primordia contained an abundance of actinotrichia (composed of collagenous fibrils) that populate the tissue. It is also noteworthy that the larval fin at this stage is not vascularized as revealed by in vivo imaging of the Fli1-GFP transgenic line. This led us to ask when the larval fin takes on adult fin morphology. To answer this question, fin developmental progression was systematically assessed to identify the structural morphogenesis until the fin developed an adult-like phenotype (supplemental Fig. S1). Although vasculature in the trunk was functional with strong blood flow in 3- and 7-day-old zebrafish, vascularization of the caudal fin was not apparent until after 10 dpf. At ~10 dpf, the posterior end of the notochord begins to bend dorsally, and soon after, clusters of actinotrichia gather, like corn stalks tied with twine, to form ray-like structures ventral to the notochord (supplemental Fig. S1). Concomitant with the formation of rays, the vasculature forms along these rigid tracks. By 19 dpf, 18 rays had developed, became vascularized, and innervated (neuronal immunohistochemistry data not shown). By ~3 weeks, the caudal fin appears similar to the adult morphologically, with fully formed vasculature including intersegmental vascular loops. These studies illustrate significant structural differences between the adult and 2-day-old larval fin structures, yet at both life stages, the animals are equally able to regenerate their fin tissues following amputation.

Comparative Microarray Analysis Revealed Common Signaling Pathways during Zebrafish Regeneration—Because regeneration is an orchestrated process of molecular events, we designed a broad-based microarray study to identify the gene expression changes that occur specifically in the isolated regenerates over time in larval fin tissues. The differential gene expression profiles for 1, 2, and 3 dpa were created by filtering for genes that were at least 1.7-fold differentially abundant relevant to the non-regenerating fin (0 dpa). One-way ANOVA was conducted for statistical significance and a total of 1851 transcripts were altered in at least one regeneration time point from 0 dpa (Fig. 1A). From the 1851 genes, a shorter gene list was created and annotated by filtering for genes that were at least 2.5-fold differentially abundant at any regenerating time.
These transcripts were grouped into functional categories such as wound healing and immune response, signal transduction, extracellular matrix, and cell adhesion (Fig. 1B). Our results were consistent with the previous studies conducted using RNA isolated from adult zebrafish caudal fins and the adult hearts (18, 19). This prompted us to perform a comparative genomic analysis across three different regeneration platforms. We first compared the amputation-initiated gene expression changes between the larval and adult fin regenerates. As was done with the larval expression data, an adult fin regeneration expression list was created by filtering transcripts that were at least 1.7-fold differentially expressed at 1, 3, or 5 dpa when compared with the non-regenerating 0 dpa fin. Statistical significance was analyzed by one-way ANOVA and a total of 3762 transcripts were changed at 1, 3, or 5 dpa from 0 dpa. The larval and adult fin lists were cross-compared and 658 transcripts (~36% of the larval list) were identified as common (Fig. 1C). We further narrowed the common list by filtering for the transcripts that were at least 1.7-fold differentially abundant at 1 dpa in the larval gene list and this reduced the number of genes to 341. To acquire more meaningful data, we then analyzed the pattern of gene expression changes by assessing the similarity in gene regulation between the larval and adult gene lists. Of the 341 transcripts that were common in both lists, 109 and 107 genes were similarly induced and repressed, respectively, which comprised about 64% resemblance in the pattern of gene regulation between the two regenerating tissue platforms (supplemental Table S2). Similar to the previous adult regeneration studies, many genes involved in wound healing, signal transduction, transcriptional regulation, and extracellular matrix components were regulated in both fin regeneration models (supplemental Table S3).

The common gene expression profile identified during these two distinct fin regeneration models directed us to compare the larval fin regeneration genomic response to the response in the regenerating adult heart. We utilized the published data from the study performed on zebrafish regenerating heart (18), in which a total of 662 genes were differentially expressed in the regenerating zebrafish heart in at least one of the three time points, 3, 7, or 14 dpa (18). The larval fin regeneration expression list was compared with the adult heart regeneration list and we identified 189 common gene expression changes (Fig. 1D). Of these genes, 116 were similarly induced and 18 were similarly repressed, which constitutes about 89 and 31% similarity in the gene regulation, respectively, between larval fin and adult heart regeneration.

FIGURE 1. Comparative genomic analysis during zebrafish regeneration. A, heat map illustrating the changes in gene expression during the progression of larval fin regeneration. Non-amputated fin tissue at 2 dpf (0 dpa) was used as the control to compare with the regenerating fin at 1, 2, and 3 dpa. B, the genes that were at least 2-fold differentially expressed were grouped based on the known function of the proteins. Comparative gene expression profiling was performed between larval fin, adult fin, and adult heart regeneration systems in zebrafish. The Venn diagram comparing the genes that were modulated during regeneration between C, larval and adult fin regeneration; D, larval fin and adult heart regeneration; and E, larval fin, adult fin, and adult heart regeneration models.
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FIGURE 2. In situ localization of raldh2 in the larval and adult regenerating fin tissue. A, the mRNA localization of raldh2 was performed in the regenerating fin tissue at various time points in the larval fin tissue. The expression was detected in the larval regenerate as early as 4 hpa and continuously until 3 dpa. The raldh2 is clearly expressed beneath the wound epithelium at 4 and 8 hpa and in the blastema region at 12 and 24 hpa. Similar to the microarray and qRT-PCR data, raldh2 is highly expressed at 2 dpa when compared with the other time points. B, the expression of raldh2 is present in the distal blastema region in the adult regenerating fin at 3 dpa and the transcript is completely absent in the uncut adult fin.

(supplemental Table S4). This suggests the existence of conserved biomolecules that are generally required for tissue regeneration (supplemental Table S5).

To further mine regeneration expression data, we performed a comparative analysis across three regeneration platforms, the two fin regeneration models and the zebrafish heart regeneration system. The goal was to identify the gene expression changes that are similarly modulated after amputation (Fig. 1D). A total of 91 genes were common and 54% of these genes were similarly regulated across all three platforms (supplemental Table S6). A number of genes that were induced or repressed in either adult fin or heart regeneration were validated by qRT-PCR with the larval fin RNA (supplemental Fig. S2). Wound healing transcripts such as galectin 9, cathepsin S, C, and B were similarly regulated indicating that the immediate response to amputation is conserved across the three regeneration platforms. The induction of raldh2 was validated by qRT-PCR with the larval fin RNA (supplemental Fig. S2). In situ localization of raldh2 in regenerating larval fins revealed expression as early as 4 hpa continuing through 72 hpa (3 days post amputation) (Fig. 2A). raldh2 was expressed during blastema formation (4, 12, and 24 hpa) suggesting a possible role in the development of the blastema. Although not quantitative, raldh2 signal intensity at 48 and 72 hpa was notably high, consistent with the microarray and qRT-PCR data (supplemental Table S1 and supplemental Fig. S2), suggesting the importance of this rate-limiting enzyme to the post-blastema phase of regeneration. In the adult regenerating fin tissue, raldh2 was localized in the distal blastemal region just beneath the wound epithelium at 3 dpa (Fig. 2B). This is consistent with the expression of retinoic acid receptor γ in adult regenerating fin tissue beneath the wound epithelium (26), depicting the overlapping expression of RA signaling forms. Two members of the Maf protein family such as krml2 and krml2.2, which are involved in the control of cellular differentiation were also similarly regulated across three different platforms (23, 24). The extracellular matrix components timp2 and mmp14 were highly induced indicating the importance for a proper foundation for the proliferating cells to migrate and adhere in a regulated fashion. We also identified many genes such as fgf20a, msxe, msxc, and wnt5b that have been previously reported in different zebrafish fin regeneration studies in this larval fin regeneration microarray analysis (supplemental Table S7). Most importantly, raldh2 (retinaldehyde dehydrogenase 2) was one of the genes that was highly induced across three regeneration models. The profound induction of this gene in the epicardium after amputation of zebrafish heart has been recently reported (18, 25). This is significant as the caudal fin and heart are morphologically completely different, yet at the level of gene expression, common genomic responses to amputation were observed, again suggesting that there are likely conserved “regenerative mechanisms.”

raldh2 is highly expressed during caudal fin regeneration—From the comparative genomic analysis, raldh2, a rate-limiting enzyme for RA synthesis was one of the highly induced genes during regeneration across the three regeneration platforms.
members in the regenerating fin tissue. In support of our data, previous mRNA localization studies have revealed that \textit{raldh2} is very highly expressed in epicardium surrounding the ventricle, atrium, and outflow tract as early as 1 dpa after partial ventricular amputation in zebrafish heart (25). Together, the qRT-PCR data and the mRNA localization studies confirm the enhanced expression of \textit{raldh2} during caudal fin regeneration in zebrafish.

**FIGURE 3.** Inhibition of RA signaling impairs wound epithelium and blastema formation and blocks fin regeneration. A–C, the caudal fin of 2-day-old larvae were amputated and exposed to vehicle, DEAB or Citral, and the regeneration potential was assessed at 3 dpa. DEAB and Citral exposure completely blocked the regenerative progression. D and E, control and \textit{raldh2} morphants were amputated and allowed to grow for 3 days at 28 °C. \textit{raldh2} repression impaired regeneration. The expression of wound epithelium marker \textit{dlx5a} and blastema marker \textit{msxe} were assessed in the regenerating fin at 1 dpa by \textit{in situ} hybridization in the vehicle, DEAB- and Citral-exposed larvae. G, H, L, and M, DEAB- and Citral-exposed larvae failed to express \textit{dlx5a} or \textit{msxe} when compared with the vehicle-exposed larvae (F and K). The expression of \textit{dlx5a} and \textit{msxe} in the regenerating fin at 1 dpa were highly reduced in the \textit{raldh2} morphants (N and O) in comparison with the standard control morphants (I and J). P and Q, regenerative growth was quantified by measuring the distance from the plane of amputation to the tip of regenerating tissue (n = 6 representative images). Both DEAB- and Citral-exposed larvae and the \textit{raldh2} morphants had significantly less regenerative growth (p < 0.001). The respective values represent the mean ± S.E. and the asterisk refers to the statistically significant difference (one-way ANOVA). R, co-exposure of RA with DEAB rescued the inhibition of regeneration, and there was statistical difference in regeneration between DEAB alone and RA + DEAB (#, p < 0.001). All these experiments were conducted multiple times and the images are representative of more than 50 animals. DMSO, dimethyl sulfoxide.
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**FIGURE 4. Inhibition of RA signaling impacts cell proliferation during larval fin regeneration.** A, the amputated larvae exposed to vehicle or DEAB and Citral were incubated with BrdUrd at 24–30 and 48–54 hpa. The bracket represents the area analyzed for quantifying the proliferating cells. B, cell proliferation was quantified between vehicle or DEAB- (n = 7) and Citral (n = 9)-exposed larvae. The respective values represent the mean ± S.E. (one-way ANOVA and Tukey method). The BrdUrd-labeled cells were significantly reduced in DEAB- and Citral-exposed larvae at 24–30 and 48–54 hpa when compared with the vehicle (p < 0.001). C, the control and raldh2 morphants were amputated and BrdUrd assay was performed as described above. D, quantification of the cell proliferation between control and raldh2 morphants. There was significant reduction in the number of proliferating cells in the raldh2 morphants at both 24–30 and 48–54 hpa when compared with the control morphants (p < 0.001). All the parameters were measured using the Image Pro-Plus software (Media Cybernetics, Silver Spring, MD).

Raldh2 Is Required for Larval Fin Regeneration—As raldh2 is highly induced in three regeneration systems, we hypothesized that if Raldh2 is critical for regeneration, inhibition of RA synthesis by specific inhibitors should block regeneration. The larvae at 2 dpf were amputated and exposed continuously to a specific Raldh2 inhibitor, DEAB (250 μM) and a RA synthesis inhibitor, Citral (25 μM). The larvae exposed to DEAB and Citral were not able to accomplish regeneration, and the measurement studies clearly indicate the inhibitory effect on regeneration (Fig. 3, A–C and P). To further demonstrate the specific requirement of raldh2 during regeneration, we utilized the available raldh2/neckless mutant, but the larvae were severely deformed making regeneration assessments impossible. As an alternative, we performed morpholino antisense repression of raldh2 and analyzed the regeneration potential of the raldh2 morphants. Our previous larval fin regeneration studies demonstrated that morpholinos can be effectively delivered at the one-cell stage and efficacy lasts for several days (14, 15). Because complete knockdown of raldh2 is detrimental to normal embryonic development and leads to early mortality (27), we titrated the amount of raldh2 morpholino to only partially repress Raldh2 expression, and optimized the concentration of the raldh2 morpholino to a level that did not affect normal fin development (data not shown). The control morphants completely regenerated their fin tissue after amputation at 3 dpa, whereas the raldh2 morphants failed to regenerate (Fig. 3, D, E, and Q). As the primary function of Raldh2 is the synthesis of RA, we next tested whether the inhibitory effect on regeneration by DEAB could be reversed using exogenous RA. All-trans-RA (0.01 μM) was co-exposed with DEAB immediately after caudal fin amputation for 24 h. The use of exogenous RA rescued the inhibition of regeneration by DEAB (n = 32/46) (Fig. 3R). Similarly, we successfully rescued the impaired regeneration of raldh2 morphants by ~50%, using exogenous RA (data not shown). Of note, RA (at 0.01 μM) itself did not affect larval fin regeneration (data not shown). These results clearly suggest that raldh2 expression is required for fin regeneration.

Inhibition of raldh2 Impairs Wound Epithelium, Blastema, and Cell Proliferation during Larval Fin Regeneration—To understand the phase(s) of regeneration affected by the inhibition of RA signaling, we performed *in situ* analysis with dlx5a and msxe, markers that define the wound epithelium and the blastema, respectively. The expression of dlx5a and msxe was lost in the DEAB- and Citral-exposed larvae at 1 dpa (Fig. 2, F–H and K–M). To confirm the result that Raldh2 expression is essential for proper formation of wound epithelium and blastema, we further performed mRNA localization studies with the same markers in raldh2 morphants. Very similar to the RA synthesis inhibitors, the expression of both dlx5a and msxe were significantly reduced in the regenerating fin tissue of the raldh2 morphants when con-
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24–30 and 48–54 hpa when compared with the vehicle-exposed larvae (Fig. 4, A and B). We also performed BrdUrd incorporation assay on raldh2 morphants to identify whether cellular proliferation is similarly affected using the anti-sense approach. Similar to the DEAB-exposed larvae, raldh2 morphants had a significant reduction in the number of BrdUrd-labeled cells at 24–30 and 48–54 hpa when compared with the standard control morphants (Fig. 4, C and D). It is noteworthy that the inhibitory effects on cell proliferation are similar between DEAB-exposed larvae and raldh2 morphants with a reduction of BrdUrd-labeled cells at the posterior and ventral side of the notochord (14, 16). Altogether, these results suggest that the expression of raldh2 is required for cell proliferation at different regenerative stages.

Wnt Signaling Regulates Raldh2 Expression during Fin Regeneration—Because Raldh2 is functionally important for fin regeneration, it is important to begin to identify the factors that control raldh2 expression. The functional importance of Wnt signaling during zebrafish adult fin regeneration was recently reported (7, 10). First, to analyze whether Wnt signaling has any functional role during larval fin regeneration, we used a heat shock-inducible dominant negative transgenic zebrafish line (Tg(hsp70: ΔTCF-GFP) that simultaneously expresses GFP and inhibits Wnt/β-catenin signaling. Two-day-old larvae were unable to regenerate fin tissue at 3 dpa (Fig. 5, A and F). The expression of raldh2, dlx5a, and msxe was affected in hsp70:ΔTCF-GFP transgenic larvae (Fig. 5, B and C), suggesting similar requirement for Wnt signaling during both larval and adult fin regeneration. To directly test whether Wnt signaling regulates the expression of raldh2, in situ hybridization was performed on hsp70:ΔTCF-GFP transgenic larvae. Raldh2 was completely absent in hsp70:ΔTCF-GFP transgenic larvae at 1 dpa, indicating the regulatory role for Wnt signaling (Fig. 5D). To confirm that Wnt signaling was inhibited after heat shock in the hsp70: ΔTCF-GFP transgenic larvae, we quantitatively measured the expression of known Wnt target genes. qRT-PCR revealed that

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FIGURE 6. Expression of raldh2 is dependent on FGF and ERK1/2 signaling during fin regeneration. A, the expression of raldh2 was not present in the SU5402-exposed larval regenerating fin tissue at 1 dpa. B, vehicle-exposed larvae were completely regenerated by 3 dpa, whereas, U0126-exposed larvae failed to regenerate. C and D, the wound epithelium marker dlx5a and blastema marker msxe are not expressed in the U0126-exposed larvae. E, Raldh2 is not expressed in the regenerating fin of U0126-exposed larvae. F, the expression of mvp, an ERK1/2 target gene, was completely lost in U0126 larvae. G, analysis of fgf20a in the regenerating fin tissue by qRT-PCR. H, by measuring the length of new fin tissue, it is clear that regeneration is significantly inhibited in U0126-exposed larvae (p < 0.001). DMSO, dimethyl sulfoxide.

axin2, dkk1, and cyclin D1 were significantly down-regulated in hsp70:ΔTCF-GFP transgenic larvae (Fig. 5E). Because Wnt signaling is considered to be upstream of all known molecular signaling pathways identified during fin regeneration, we proposed that wnt10a expression should not be affected by Raldh2 inhibition. In situ analysis revealed comparable wnt10a expression between vehicle, DEAB- and Citral-exposed larvae, indicating that wnt10a is unaffected by the inhibition of RA signaling (Fig. 5G).

FGF and ERK1/2 Signaling Is Required for the Expression of Raldh2—One of the most well studied pathways in zebrafish regeneration is FGF signaling (5, 8, 15, 16). Similar expression of fgf20a was identified in the larval regenerating fin, underscoring the commonality at the molecular level between the adult and larval fin regeneration systems (supplemental Tables S1 and S3 and Fig. 6G). To identify whether FGF signaling controls raldh2 expression, in situ hybridization was conducted on SU5402 (a chemical inhibitor of FGFR1) (5) exposed larvae at 1 dpa. The raldh2 expression was completely absent in the SU5402-exposed animals, suggesting that FGF signaling regulates its expression (Fig. 6A). Furthermore, because FGF signaling is mediated through the phosphorylation of ERK1/2, we used an ERK1/2 inhibitor (U0126) to determine whether activation of ERK1/2 is required for the expression of raldh2. As the role of ERK1/2 signaling on fin regeneration has not been previously reported, 2 dpf larvae were amputated and exposed to vehicle or U0126 (100 μM) continuously for 3 days. The U0126-exposed larvae failed to regenerate the amputated fin tissue and results in a complete loss of dlx5a and msxe expression in the regenerates at 1 dpa (Fig. 6, B–D and H). Similar to SU5402, inhibition of ERK1/2 activation completely abolished the expression of raldh2 (Fig. 6F). The inhibition of ERK1/2 signaling by U0126 was validated by analyzing the expression of mvp, an ERK target gene (Fig. 6F) (28). These results suggest that the FGF signaling pathway, possibly through the phosphorylation of ERK1/2 is required for the expression of raldh2 during larval fin regeneration in zebrafish.

RA Signaling Is Sufficient to Rescue the Inhibitory Effect of FGFR1 and ERK1/2 Inhibitor on Larval Fin Regeneration—Our raldh2 expression analysis following Wnt and FGF signaling inhibition suggests that RA signaling is downstream to the Wnt and FGF pathways. It has been reported that Wnt is upstream to FGF signaling (7). To directly determine whether RA signaling is downstream to FGF signaling, we asked whether the inhibitory effect of SU5402 could be overcome by exogenous RA. Amputated larvae at 2 dpf were co-exposed with SU5402 and exogenous RA continuously for 3 days. Exogenous administration of RA rescued SU5402-mediated impairment of regeneration suggesting that RA signaling is downstream to FGF pathway (Fig. 7, A and C). Because ERK1/2 signaling is downstream to FGF signaling, we analyzed whether RA could rescue the inhibitory effect of ERK1/2 inhibitor. U0126-exposed larvae did not elicit inhibition of regeneration in the presence of exogenous RA, suggesting that RA signaling is downstream
to ERK1/2 signaling (Fig. 7, B and D). This is a significant finding as it demonstrates a necessary role for RA signaling for larval fin regeneration.

**DISCUSSION**

Because the early life stages of zebrafish are amenable to molecular and genetic techniques, the development of the larval fin regeneration model provides a unique platform to rapidly identify the genes required for regeneration (11, 13–16). Comparative gene expression analysis revealed significant common gene expression changes in larval fin, adult caudal fin, and heart regenerating tissues, suggesting common molecular pathways choreographing the regeneration process.

The physiological progression of fin regeneration in larvae and adults is similar, as both initiate with the formation of a wound epithelium, blastema formation, and the distal to proximal propagation of cell proliferation (16, 29). Furthermore, there is also growing evidence to suggest that heart regeneration in zebrafish has a high degree of commonality with fin regeneration with respect to the order of events that occur after a surgical wound. Both tissues regenerate through the blastema formation followed by proliferation of cells to complete outgrowth (2, 18, 25, 30). Gene expression of *msxB* and *msxC* encoding homeo-domain containing transcription factors are re-induced in regenerating zebrafish hearts as early as 3 dpa, and also in regenerating fin blastema (2, 18, 25, 30). Additionally, the expression pattern of *notch1b* and *deltaC*, members of the Notch signaling pathway, are induced very early after heart amputation and in the regenerating fin blastema (30). None of the four genes described above were detected in the non-amputated fin or heart tissue, indicating that the re-induction of these genes was specific to the regenerating tissue. Together, the current literature supports the existence of conserved molecular mechanisms across the three different regenerative platforms.

Recent studies illustrate that a proper balance of Wnt/β-catenin signaling is also critical for the formation and proliferation of blastema cells (7, 10). This is consistent with our result observed in the larval model: when canonical Wnt signaling is blocked, the formation of wound epithelium and blastema are blocked (Fig. 5, A–C). Moreover, Wif1, a feedback regulator of the Wnt signaling pathway was one of the repressed transcripts in both adult and larval fin regeneration models suggesting that the Wnt signaling pathway is well regulated during regeneration. Moreover, a significant number of Wnt target genes were identified in both the larval and adult fin regeneration gene expression list (supplemental Table S3).

FGF signaling is one of the well studied signaling pathways during zebrafish regeneration. The necessity of FGF signaling during adult zebrafish fin and heart regeneration was demonstrated with the use of the FGFR1 inhibitor (SU5402) and the transgenic line (*hsp70:dn-fgfr1*) that expresses the dominant
negative FGFR1 protein upon heat shock (5, 25, 31). Predictably, the larval fin regeneration system also requires FGF signaling because SU5402 also blocked the early life stage regeneration (15, 16). Moreover, fgf20a, that was identified as an initiator of blastema formation in adult regenerating fin (8), is also highly induced in the larval fin tissue (Table S1). Even though fgf20a was detected by microarray analysis and qRT-PCR, the expression was too low to detect by in situ hybridization in the larval fin tissue. During zebrafish heart regeneration, among the several FGF ligands tested, only fgf17b was strongly expressed in the cardiomyocytes at the apical edge of the regenerating heart tissue (25). The expression of different FGF ligands in these tissues is not unexpected considering the diversity of the regenerating tissues. But the data strongly indicates that epimorphic tissue regeneration requires functional FGF signaling in the early stages of the regenerative process.

Raldh2, a rate-limiting enzyme for RA synthesis was one of the most highly induced genes in all three regeneration models. A profound induction of raldh2 in the epicardium after zebrafish heart amputation has been reported (18, 25). This is significant as the caudal fin and heart are morphologically different, yet at the level of gene expression, common genomic responses to amputation were observed. RALDH2 enzyme activity is also highly induced in NG-2 cells after spinal cord injury in rats (32). raldh2 is also expressed in the skin and perichondrium and in perivascular cells in cartilage during deer antler regeneration (33). Whole body regeneration from a miniscule blood vessel fragment has been illustrated in the colonial urochordate Botrylloides leachi, and the homologue of the RA receptor and raldh-related gene were exclusively expressed in blood cells in the regeneration niches, suggesting the ancestral conservation of RA signaling during regeneration and body restoration events (34).

The functional role of RA signaling during amphibian and zebrafish regeneration has been studied for decades and RA is even referred to as a regeneration-inducing molecule (26, 35–40). RA is mainly characterized as a signaling molecule that is required for the vertebrate pattern formation both in developing and regenerating tissues. Amphibian regeneration studies revealed that exposure of regenerating axolotl and urodele limbs to RA results in the modification of positional memory in the proximodistal axis and caused patterning defects such as duplication of the stump (41, 42). Similarly, exposure of zebrafish with RA during fin regeneration resulted in remarkable morphological effects suggesting that exogenous RA can re-specify patterns in the regenerating fin tissue (26). Most of the regeneration studies with RA signaling are related with the patterning of the structures during regeneration. Our chemical inhibition studies suggest that RA signaling is indeed required for the complete formation of wound epithelium and blastema. Moreover, we also illustrated the sufficient role of RA signaling during larval fin regeneration. The complete understanding of the RA signaling requirement for wound epithelium and blastema formation requires further studies.

Because the expression of raldh2 was continuously present from 4 hpa to 3 dpa, we presume that the requirement of RA signaling is continuous from the initiation of regeneration through pattern formation and regenerative outgrowth (Fig. 2). However, the increased expression of raldh2 at 2 dpa raises the possibility for a distinct flux of RA signaling (Fig. 2 and supplemental Fig. S2), and suggests a dual phase of RA signaling during regeneration. In support of our proposal, vertebrate limb developmental studies in mice have illustrated the existence of an early phase of RA signaling to initiate forelimb development, followed by a late phase of RA signaling required to develop the apical ectodermal ridge fully along the distal ectoderm to complete the limb outgrowth (43). Moreover, studies with the raldh2/neckless zebrafish mutant revealed that RA signaling is required for the induction of the pectoral fin field and also to establish a prepattern of anteroposterior fates in the condensing fin mesenchyme (44). Therefore, in addition to the well-established functional role of RA signaling during the regenerative outgrowth, it is also essential in the early stages of regeneration, suggesting the existence of two phases of RA signaling during regeneration.

Finally, we illustrated that the expression of raldh2 is regulated by Wnt and FGF/ERK signaling and that RA signaling is downstream of FGF/ERK signaling during zebrafish fin regeneration (Fig. 7). Even though multiple signaling pathways are active during regeneration, the functional interactions required to accomplish epimorphic regeneration are still not completely understood. Collectively, these studies reveal that the regenerative response choreographing epimorphic tissue regeneration is conserved, which offers opportunities to use multiple models to unravel the regenerative program.

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