Development 137, 871-879 (2010) doi:10.1242/dev.043885

IGF signaling between blastema and wound epidermis is required for fin regeneration

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SUMMARY

In mammals, the loss of a limb is irreversible. By contrast, urodele amphibians and teleost fish are capable of nearly perfect regeneration of lost appendages. This ability depends on direct interaction between the wound epithelium and mesenchymal progenitor cells of the blastema. It has been known for decades that contact between the wound epithelium and the underlying blastema is essential for successful regeneration. However, the underlying mechanisms are poorly understood. Here, we show that upon amputation the blastema induces expression of the ligand Igf2b, which then activates IGF signaling specifically in cells of the adjacent apical epithelium. Inhibition of IGF signaling by either morpholino antisense technology, or by specific chemical inhibitors of Igf1 receptor function NVP-AEW541 and NVP-ADW742, impairs fin regeneration. At the cellular level, this block in regeneration is reflected by a lack of the distinctive basal epithelium, increased apoptosis in the wound epidermis and reduced proliferation of blastema cells. Furthermore, induction of the blastemal and wound epidermal markers cannot be supported in the absence of IGF signaling. These data provide evidence that Igf2b expressed in the blastema promotes the properties of the adjacent wound epidermis, which subsequently are necessary for blastema function. Thus, IGF signaling upregulated upon fin amputation represents a signal from the blastema to the wound epithelium, a crucial step in appendage regeneration.

KEY WORDS: IGF, Zebrafish, Regeneration, Fin, Blastema, Wound epidermis

INTRODUCTION

Studies in urodele amphibians and teleost fish identified a conserved sequence of events promoting limb and fin regeneration, which do not take place in a nonregenerative mammalian limb (Brockes and Kumar, 2005; Iovine, 2007; Nakatani et al., 2007; Stoick-Cooper et al., 2007a; Yokoyama, 2008). Following amputation, the injured stump is repaired by rapid migration of epidermal cells over the amputation surface (Campbell and Crews, 2008; Poss et al., 2003). Subsequently, a mass of mesenchymal proliferating progenitor cells, called a blastema, accumulates at the plane of amputation. The blastemal cells act like stem cells: they provide descendant cells that build the regenerate while retaining their own undifferentiated, proliferating identity in the niche underneath the wound/apical epidermis (Gurley and Sánchez Alvarado, 2008). The interruption of the contact between the wound epidermis and the blastema prevents regeneration (Brockes and Kumar, 2008; Campbell and Crews, 2008; Carlson, 2007). Hence, the mechanisms mediating communication between the two tissues are of central interest in the field of regenerative biology.

The zebrafish is a well-established model organism to study mechanisms of regeneration in vertebrates (Akimenko et al., 2003; Iovine, 2007; Nakatani et al., 2007; Poss et al., 2003). Particularly, the caudal fin represents an exceptionally valuable model of rapid and perfect recreation of a complex organ. The caudal fin is easily accessible and convenient for surgery. It consists of 17-18 parallel bony rays spanned by soft interrays. After amputation of the fin, each ray independently forms an outgrowth to regenerate on its own, even after ablation of the other adjacent rays. Thus, each individual fin provides multiple experimental replicates. Furthermore, the fin can be re-amputated several times, and the regeneration process will be repeated each time.

The process of fin regeneration has been described to occur in three phases: wound healing, blastema formation and regenerative outgrowth. Following amputation within the first 12 hours, the injured stump is repaired by rapid migration of epidermal cells over the amputation surface. The mesenchymal tissues located in the vicinity of the resection site undergo disorganization and display enhanced cell proliferation. This process is followed by the formation of the blastema at 24-36 hours post-amputation (hpa). The blastema constitutes a mesenchymal apical growth zone that is maintained underneath the wound/distal epidermis for the duration of the regenerative outgrowth phase, which takes approximately 2 weeks. With elongation of the outgrowth, the cells of the proximal blastema exit this domain and begin differentiation to re-establish the missing structures in the proximodistal direction.

To understand the genetic basis of fin regeneration, several approaches have been used: mutagenesis screens (Gurley and Sánchez Alvarado, 2008; Johnson and Weston, 1995) candidate-gene strategies (Akimenko et al., 2003; Stoick-Cooper et al., 2007a), suppression subtractive hybridization (Padhi et al., 2004) and microarray analysis (Schebesta et al., 2006; Yin et al., 2008). Progress in the last decade led to the identification of several key molecular regulators of blastema formation. Among of them there is a suite of signaling molecules (Stoick-Cooper et al., 2007a). The administration of retinoic acid causes teratogenic effects and impairs fin regeneration (White et al., 1994). The ligand Fgf20a is required for wound epidermis formation and for mesenchymal proliferation (Whitehead et al., 2005). Shh and BMP signaling pathways play a role in the proliferation and/or differentiation of scleroblasts that produce dermal bones (Laforest et al., 1998; Quint et al., 2002). The Activin-βA/TGFβ pathway is required for normal wound repair and

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Accepted 15 January 2010

blastema proliferation (Jaźwińska et al., 2007). Both canonical and noncanonical Wnts influence blastemal proliferation and patterning of the outgrowth (Stoick-Cooper et al., 2007b). The chemokine Sdf1a (Cxcl12a – Zebrafish Information Network) controls epithelial cell proliferation in regenerating fins (Dufourcq and Vriz, 2006). This long list of the signaling molecules supports the hypothesis that molecular mechanisms of organ regeneration rely on secreted factors mediating cell-cell communication.

The current study identifies another signaling pathway that is required for fin regeneration: namely, Insulin-like growth factor (IGF) signaling. We demonstrate that the ligand *insulin-like growth factor 2b* (*igf2b*) is transcriptionally induced in the blastema, whereas signal-responsive cells are in the wound epidermis. Inhibition of Igf1 receptors blocked initiation and progression of fin regeneration. Closer examination of the cellular causes of the phenotype revealed a lack of ordered basal epithelium, enhanced apoptosis in the wound epidermis, and decreased proliferation of the blastemal cells. The induction of the wound epidermal and blastema markers, such as *msxB*, *fgf20a*, *lef1* and *wnt5b* cannot be supported in absence of IGF signaling. Furthermore, we established the interray wound-healing assay to show the requirement for IGF signaling for closure of superficial wounds. These findings reveal the requirement of IGF signaling for the interaction between the wound epidermis and underlying mesenchyme during fin regeneration.

MATERIALS AND METHODS

Animal procedures

The following zebrafish strains were used in this study: wild-type AB strain (Oregon) and a *devoid of blastema* (*dob*) mutant (Whitehead et al., 2005). Fish 6-24 months of age were anaesthetized in 0.1% tricaine (Sigma-Aldrich), and caudal-fin amputations were performed with razor blades. Animals were allowed to regenerate for various times at 28.5°C. NVP-ADW742 and NVP-AEW541 compounds (Novartis Pharma AG) were dissolved in DMSO at 10 mM stock concentration and were added in fish water for a final concentration of 5 μM. The control fish were in water with 0.05% DMSO. For proliferation analysis, the fish were incubated for 6 hours in fish water containing 50 μg/ml of BrdU (Sigma-Aldrich). Activin/TGFβ inhibitor SB431542 (Tocris) was used as previously described (Jaźwińska et al., 2007).

Morpholino knockdown

Vivo-porter coupled morpholino oligonucleotides (MOs) (Gene Tools) were used at 1.5 mM for injection. The MO sequences were as follows (5′ to 3′): igf2b ATGATGTTTTAGTTGGTCCTCCATG, igf1ra TCGCTGTTCC-AGATCTCATTCCTAA, igf1rb TGTTTGCTAGACCTCATTCCTGTAC, control mispaired TCcCTcTTCCAcATCTCATTgCTtA.

MOs were injected in the dorsal half of the fin regenerate at 48 hpa. The other uninjected half was considered as the internal control in order to monitor the normal growth. Immediately after injection the fins were photographed using a Leica MZ16 stereomicroscope and Leica camera (DFC480). The same fins were again photographed at 24 and 48 hours after morpholino delivery.

The software ImageJ 1.42h was used for the measurement of the regenerate area. In order to calculate the percentage area of the outgrowth between the injected and non-injected part, the values were inserted in the following formula: $(Exp_{3\ days\ or\ 4\ days} - Exp_{2\ days})/(Cont_{3\ days\ or\ 4\ days} - Cont_{2\ days}) \times 100$, where Exp is the area of the outgrowth of the MO-treated regenerate and Cont is the area of the corresponding outgrowth of the uninjected control half.

In situ hybridization

To generate antisense probes, portions of the coding sequences of genes were cloned by the PCR amplification of zebrafish cDNA. The reverse primers were synthesized with an addition of the promoter for T3 polymerase. The following forward (F) and reverse (R) primers were used (5′ to 3′): igf2b (NM_001001815), F CCATACCTGCTCGAGAACAGAC and R, AGGATGGGAGGCAGCTTG (573 bp); msxB (NM_131260), F GAGAATGGGACATGGTCAGG and R GCGGTTCCTCAGGAT-AATAAC (721 bp); fgf20a (NM_001037103), F GGGTCTCAT-TTCGTCCTCAC and R AAGCTCAGGAACTCGCTCTG (540 bp); wnt5b (NM_130937), F GCCAGCTCTATCAGGACCAC and R AGACCCAGTGGTTTCATTGC (704 bp); lef1 (NM_131426), F CAGTCACGACGCAGCTAGAC and R CTCTGGCCTGTACCTGAAGC (879 bp); igf1ra (NM_152968), F ATCTCGGCCACATCTTTGTC and R TGATGACAGCTACGATCACG (859 bp); igf1rb (NM_152969), F GTTTCAGCCACGGGTTTG and R GCCTGTCATTGTTTCGGTTC (880 bp).

Digoxigenin-labeled RNA antisense probes were synthesized from PCR products with the Dig labeling system (Roche). In situ hybridization of whole fins was performed as described (Poss et al., 2000b) with 15 minute proteinase K (20 μg/ml, Sigma-Aldrich) treatment. In situ hybridization on fin cryosections was according to (Smith et al., 2008).

Hematoxylin and Eosin staining

Fins were fixed in 4% paraformaldehyde in PBS, dehydrated and embedded in paraffin blocks. Sections were cut at the thickness of 8 μm, rehydrated and stained with Mayer's Haemalum for 12 minutes. The nuclear staining was differentiated for 5 seconds in 0.37% HCl prepared in 70% ethanol, and the slides were washed in tap water for 10 minutes. The staining of proteins was obtained by incubation for 10 minutes in 0.1% Eosin Y solution in water with a drop of acidic acid, followed by a rapid wash in water. The sections were dehydrated in a water/ethanol series, cleared in xylol, and mounted in Entelan medium (Merck).

Immunohistochemistry

The following primary antibodies were used: rabbit anti-phospho- Igf1r (Tyr 1161) at 1:50 (Santa Cruz); rat anti-BrdU at 1:200 (Abcam); rabbit anti-Tenascin C at 1:500 (USBiological); rabbit anti-active-Caspase-3 at 1:10000 (Abcam). The following secondary antibodies were used at a concentration of 1:500: goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 488 (Molecular Probes) and goat anti-rabbit Cy3-conjugated (Jackson ImmunoResearch).

Fins were fixed in 4% paraformaldehyde in PBS, cryosectioned and immunostained as previously described (Poss et al., 2000b). The images were taken with confocal microscopy (Leica TCS SP5). For quantification of BrdU positive nuclei, we calculated the proportion of immunostained cells per total number of DAPI-stained nuclei. For active-Caspase-3, the inhibitor-induced change was calculated as a fold-difference measured as fluorescence staining. The measurements were performed using the software ImageJ 1.42h.

Quantitative real-time PCR analysis

cDNA synthesis and RT-PCR reactions were performed as previously described (Jaźwińska et al., 2007). The values and expression ratios were normalized to β-actin. Following primers were used (5′ to 3′): β-*actin* (NM_131031), F TTGCTCCTTCCACCATGAAG, R CTTGCTTGCT-GATCCACATC (123 bp); *igf2b* (NM_001001815), F GCAGGTCAT-TCCAGTGATGC, R TCTGAGCAGCCTTTCTTTGC (103 bp); *igf1ra* (NM_152968), F AACGGGTCTTGGACTGAACC, R GGCCACGT-AGAAGGCATTATC (78 bp); *igf1rb* (NM_152969), F CAGAGCC-TGAGAAGCACGAG, R AAACAACACTCCTCCACAATCC (105 bp).

Western blot

Fin regenerates were collected in 10 mM Tris-HCl, 2 mM EDTA, pH 8.0. Tissues were disrupted by ultrasonication for 20 seconds, the suspension centrifuged (13,000 ***g***, 4°C, 30 minutes) and the supernatant was recovered. Protein concentrations were determined by the Bradford method. Protein samples were separated by SDS-PAGE (12.5%) and transferred onto nitrocellulose membrane using semi-dry blotting apparatus (Bio-Rad). Membranes were stained with Ponceau S to check for even loading of the gels and then further processed as described before (Chen et al., 2006) using total-Akt antibody (1:1000, Cell Signaling Technology) and phospho-Akt antibody (1:1000, Ser 473, Cell Signaling Technology).

RESULTS

IGF signaling pathway is activated during fin regeneration

Fin regeneration requires a relatively rapid increase of tissue mass followed by differentiation. It is likely that this short-term growth event depends on growth-promoting factors. Because of the paradigm of IGF signaling as a developmental regulator of organ growth and homeostasis (Edgar, 2006), we decided to evaluate the role of the IGF pathway during fin regeneration in zebrafish. Transcriptional induction of *insulin-like growth factor 2b* (*igf2b*) in regenerating fins was identified in our Affymetrix microarray analysis (Jaźwińska et al., 2007). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) demonstrated a twofold increase (2.21±0.16, n=3, P<0.05) of *igf2b* transcript already during the wound-healing phase at 8 hpa, compared with cut fins collected immediately after amputation at 0 hpa (Fig. 1F). Further upregulation of fourfold (4.43±0.45, n=3, P<0.01) was identified during blastema formation at 24 hpa, and it reached a fivefold increase (5.21±0.59, n=3, P<0.01) during the outgrowth phase at 72 hpa (Fig. 1F). To visualize the localization of *igf2b* transcript in fins, we performed in situ hybridization on whole fins and on fin cryosections. The expression of the gene was detected in the blastema at different stages of regeneration: at 24, 48 and 72 hpa (Fig. 1A-E). No *igf2b* expression was detected in fins collected immediately after amputation (at 0 hpa) or in uncut fins (data not shown). The qRT-PCR and in situ hybridization studies demonstrate that *igf2b* is transcriptionally induced during fin regeneration.

IGFs are evolutionarily conserved secreted proteins that signal through a type 1 tyrosine kinase receptor, Igf1r (Chitnis et al., 2008). Ligand binding to the Igf1r leads to the autophosphorylation of three tyrosine residues in the catalytic domain of the receptor, which is required for the activation of the downstream signaling cascades. To visualize the activation of the IGF pathway, we used a specific antibody against phosphorylated Igf1r (p-Igf1r). We found that this antibody labeled the basal layer of the epidermis of uncut fin, except the distalmost fin margin (Fig. 2A-C). In regenerating fins during blastema formation and the outgrowth phase, p-Igf1r was also detected in the multilayered wound epidermis (Fig. 2D,E). We concluded that IGF signaling is activated in the wound epidermis of the regenerating fins.

To determine whether the induction of Igf1r signaling is dependent on known regeneration factors, such as Fgf20a and Activin-βA, we visualized p-Igf1r in *fgf20a* (*devoid of blastema*, *dob*) (Whitehead et al., 2005) mutant fins and in fins treated with the inhibitor of the TGFβ/Activin pathway (10 μM SB431542) (Jaźwińska et al., 2007). We did not observe a difference in p-Igf1r immunostaining when the non-regenerating fins were compared with the control fins at 24 hpa (data not shown). This suggests that Igf1r signaling in the wound epidermis is independent of FGF and TGFβ/Activin-βA signaling.

Inhibition of Igf1r impairs fin regeneration

In contrast to the presence of a single *igf1r* gene in mammals, two duplicated genes, named *igf1ra* and *igf1rb*, are present in zebrafish (Maures et al., 2002). In the zebrafish embryo, the duplicated receptors have overlapping functions, although they are not strictly redundant (Maures et al., 2002). We examined the temporal expression profiles of both receptors in the regenerating fins by qRT-PCR assay. Both transcripts were detected at 0, 24 and 72 hpa, with relatively unchanged levels at all stages examined (see Fig. S1A in the supplementary material). However, the *igf1rb* mRNA concentrations were approximately twofold higher than the concentrations of *igf1ra* mRNA. We next analyzed the spatial expression profiles of Igf1 receptors by in situ hybridization assay. Positive signal for mRNA of both receptors was detected in a ubiquitous fashion in fins at 3 days post-amputation (dpa) (see Fig. S1B-D in the supplementary material). These analyses demonstrated that the two Igf1 receptors are expressed in uncut and regenerating fins.

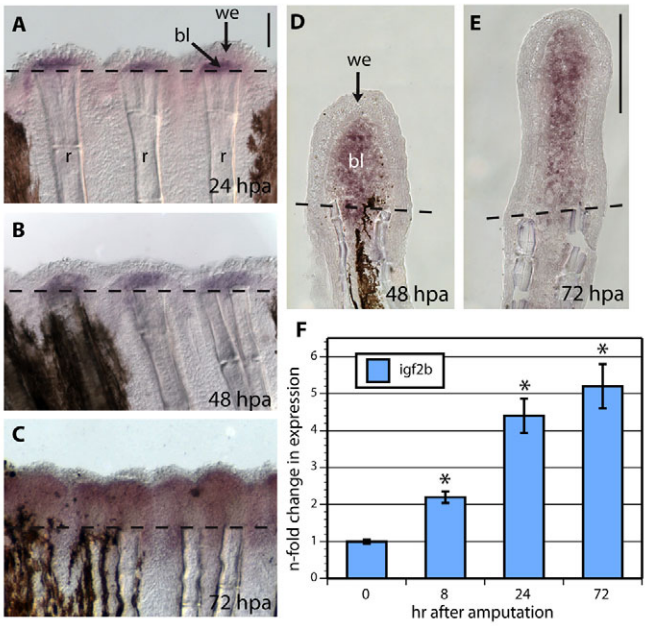

Fig. 1. *igf2b* is expressed in the blastema of regenerating fins. (**A-C**) Whole-mount in situ hybridization with *igf2b* mRNA antisense probe (purple) reveals gene expression at the distal region underneath the wound epidermis at 24 (A), 48 (B) and 72 (C) hpa. Dashed lines indicate the amputation plane. The regenerated tissue is located above the dashed lines. (**D**,**E**) In situ hybridization performed on cryosections at 48 and 72 hpa demonstrates *igf2b* expression in the blastema. (**F**) Quantitative RT-PCR determination of *igf2b* mRNA in regenerating fins at 8, 24 and 72 hpa relative to control fins at 0 hpa, which as calibrator samples were normalized to 1.00. Error bars represent s.e.m. *P<0.001; n=3 samples, each was prepared from 10-15 fins. bl, blastema; r, ray with skeletal elements; we, wound epidermis. Scale bars: 100 μm in A,E.

In order to examine the role of IGF signaling during fin regeneration, we used specific MO antisense oligomers. Recently, the MO technology has been optimized for in vivo delivery, by covalently linked delivery moiety, which enables cell penetration (Morcos et al., 2008). In this study, we used in vivo MOs to block translation of the transcripts encoding the ligand *igf2b* and both receptors *igf1ra* and *igf1rb* by injection into the dorsal half of fin regenerates at 48 hpa. The remaining ventral half of the regenerate served as control tissue. The effects of MOs were assessed at 24 and 48 hours post-injection (hpi) by comparison of the outgrowth size of the injected and non-injected fin halves. Fin tissue injected with control MO displayed nearly normal outgrowth compared with the non-injected regenerate (Fig. 3A) (at 24 hpi 91.6±3.3% of control; at 48 hpi 94.0±4.0% of control, n=10, P>0.05). Injection of *igf2b* MO or a combination of *igf1ra* and *igf1rb* MOs resulted in 42 and 48% reduction of the regenerate at 24 hpi, respectively (Fig. 3B-F) (*igf2b* MOs at 24 hpi 41.5±7.1% of control; *igf1rab* MOs at 24 hpi 48.4±4.1% of control, n=10, P<0.01). We concluded that MO-mediated knockdowns of *igf2b* and *igf1rab* impair fin regeneration.

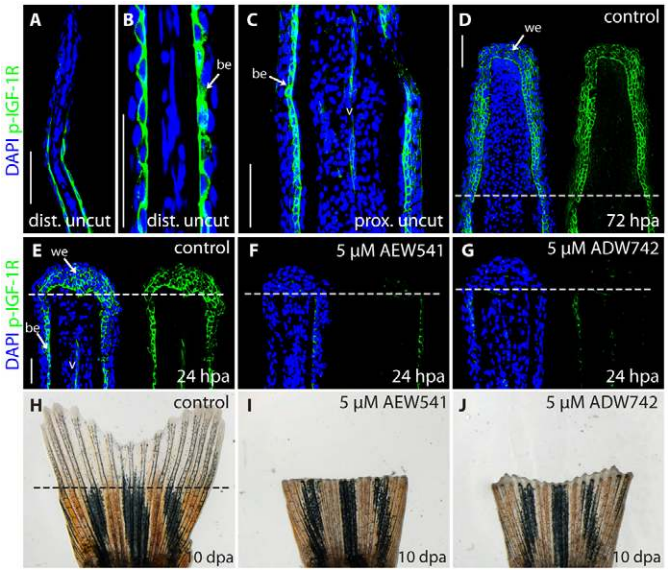

Fig. 2. NVP-AEW541 and NVP-ADW742 block Igf1r signaling in zebrafish fins, resulting in inhibition of fin regeneration. (**A-G**) Longitudinal fin sections stained with phospho-Igf1r antibody (green) and DAPI (blue) to visualize nuclei. (A,B) A distal part of an uncut fin shown at different magnifications. A single cell layer of the basal epidermis labeled with phospho-Igf1r antibody. The distal tip of the fin is devoid of phospho-Igf1r staining. (C) A proximal part of an uncut fin. Phospho-Igf1r antibody marks the basal epidermis and blood vessel (v). (D) A regenerative outgrowth at 72 hpa. The wound epidermis contains phospho-Igf1r-positive cells. The dashed line indicates the amputation plane. (E) A control fin at 24 hpa with phospho-Igf1r-positive cells in the wound epidermis, the basal epidermal layer of the stump, and in the endothelial cells of the blood vessels. Treatment with 5 μM NVP-AEW541 (F) or 5 μM NVP-ADW742 (G) severely reduces phospho-Igf1r staining in the fins at 24 hpa. (**H-J**) Whole caudal fins at 10 dpa display blocked regeneration after treatment with 5 μM NVP-AEW541 (I) or 5 μM NVP-ADW742 (J), compared with control (H). be, basal epidermis; v, blood vessel; we, wound epidermis. Scale bars: 50 μm in A,C,D,E.

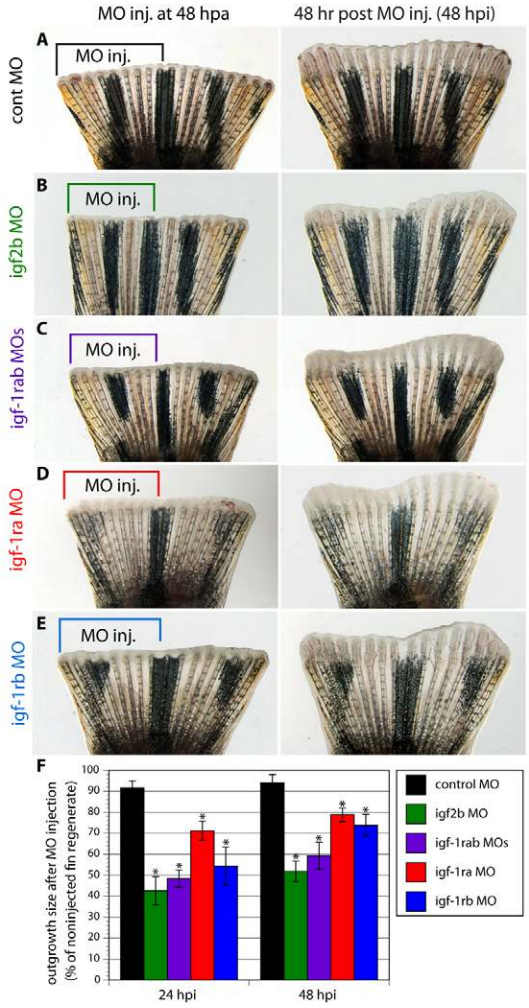

Fig. 3. Morpholino-mediated *igf2b* and *igf1rab* knockdowns impair fin regeneration. (**A-E**) Fins microinjected into their dorsal half with control (A), *igf2b* (B), *igf1ra* + *igf1rb* (C), *igf1ra* (D) and *igf1rb* (E) MOs. The remaining non-injected half of the fin served as an internal control to assess the normal growth rate. The left panels show fins shortly after injection and the right panels show the same fins at 48 hpi. The control MO does not affect the size of the outgrowth relative to the uninjected side (A). The specific MOs impair regeneration, leading to an asymmetric shape of the regenerate (B-E). (**F**) Bar chart comparing the surface of regenerates that regrew within 24 and 48 hours after MO injection in relation to the uninjected fin regenerate. The injected:uninjected regenerate surface is significantly reduced by delivery of specific MOs. *n*=10 fins per group; **P*<0.01.

To further investigate the role of IGF signaling during fin regeneration, we implemented a pharmacological approach using two specific inhibitors of the Igf1r. NVP-ADW742 and NVP-AEW541 have been shown in mammalian systems to specifically block Igf1r function by interfering with its ATP-binding site (Garcia-Echeverria et al., 2004; Mitsiades et al., 2004). A high conservation of the entire kinase domain between the mammalian and both zebrafish Igf1ra and Igf1rb amino acid sequences (92% identity) suggests that the inhibitors might also be functional in zebrafish. To test the effectiveness of both inhibitors in the fin, we examined phosphorylation status of Akt, which is a major downstream effector of Igf1r in mammals and in zebrafish (Chitnis et al., 2008; Pozios et al., 2001). Western blot analysis indicated a marked reduction in the levels of phosphorylated Akt after treatment of regenerating fins with 5 μM of NVP-AEW541 or 5 μM NVP-ADW742 for 24 hours, compared with DMSO-treated controls (see Fig. S2 in the supplementary material). The total levels of Akt protein were similar in all tested samples. To further examine the specificity of the inhibitors in the fins, we visualized phosphorylation of Igf1r after the exposure to the inhibitors for 12 and 24 hours. We found that treatment with 5 μM of either of the chemicals for 24 hours diminished p-Igf1r, as shown by immunofluorescence of fin sections (Fig. 2E-G). A reduction of p-Igf1r signal was also detected after administration of the inhibitors for 12 hours (data not shown). These results demonstrate that NVP-AEW541 and NVP-ADW742 block the IGF signaling pathway in adult zebrafish fin.

To test the requirement of IGF signaling during fin regeneration, we amputated the fins and treated the fish with 5 μM NVP-AEW541 and 5 μM NVP-ADW742 for 10 days, the time that is normally sufficient to restore most of the lost part of the appendage (Akimenko et al., 2003; Poss et al., 2003). Among ten fish that were treated with NVP-AEW541, eight fins displayed a complete regenerative block and two fins developed a small (200-500 μm long) undifferentiated irregular outgrowth (Fig. 2I). Similar results

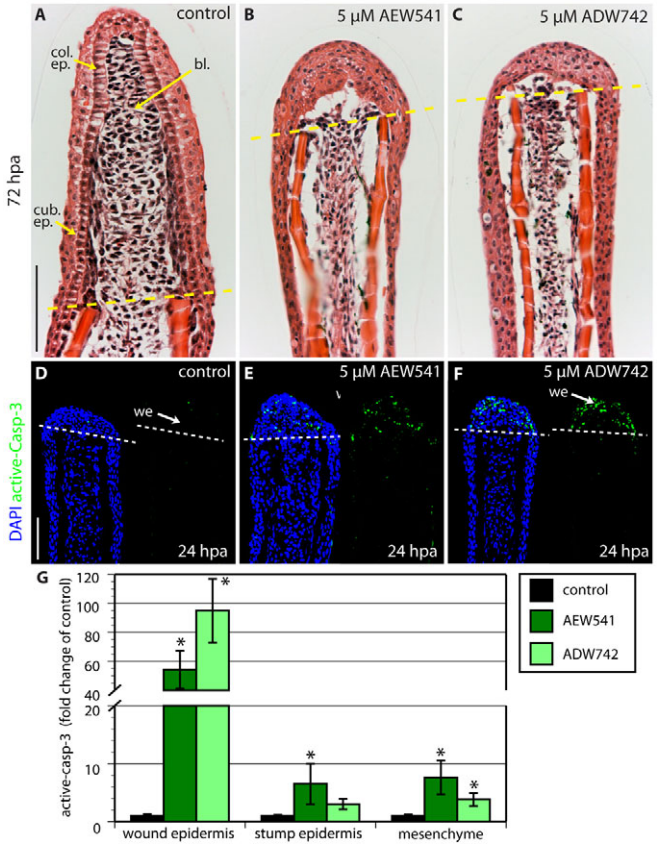

Fig. 4. Igf1r signaling is required for the establishment of the wound epidermis. (**A-C**) Longitudinal sections of fins at 72 hpa stained with Hematoxylin and Eosin. The dashed line marks the amputation plane. In control fin regenerates, the basal epithelium consists of ordered linear cuboidal epithelial cells in the proximal part and columnar epithelial cells at the apical end. The blastema is also indicated by an arrow (A). Fins treated with either NVP-AEW541 (B) or NVP-ADW742 (C) lack a distinctive basal layer of the wound epidermis and the blastema. (**D-F**) Longitudinal sections of fins at 24 hpa stained with active-Caspase-3 antibody (green) and a nuclear marker DAPI (blue). Control fins display only individual active-Caspase-3-positive cells (D). Exposure to either NVP-AEW541 (E) or NVP-ADW742 (F) markedly enhances cell apoptosis specifically in the wound epidermis. (**G**) Quantification of active-Caspase-3 in control and inhibitor-treated fins at 24 hpa. n=6; *P<0.01. bl., blastema; col. ep., columnar epithelial cells; cub. ep., cuboidal epithelial cells; we, wound epidermis. Scale bars: 50 μm.

were obtained for NVP-ADW742-treated fish: of ten fish, seven fins completely failed to regenerate and three fins carried a small irregular outgrowth (Fig. 2J). These results demonstrate that IGF signaling is required for zebrafish fin regeneration.

Blocking IGF signaling affects the wound epidermis

To determine the cellular nature of regenerative failure, we examined histology of control and inhibitor-treated regenerates by Hematoxylin-Eosin staining at the stage of blastema formation at 30 hpa and during the regenerative outgrowth phase at 72 hpa. All control and experimental fins revealed normal re-epithelialization of the wound: several layers of epithelial cells covered the resection site and formed a thickened apical cap at 30 hpa (see Fig. S3A-C in the supplementary material). However, the architecture of the basal layer of the wound epidermis appeared abnormal. Specifically, the basal epithelium in control regenerates consists of ordered cuboidal or columnar cell layer, which is required for normal regeneration. The inhibitor-treated fins lacked such a distinctive basal epithelium at 30 and 72 hpa (Fig. 4A-C and see Fig. S3A-C in the supplementary material). To investigate the effect of Igf1r inhibitors on molecular markers of the wound epidermis, we visualized *wnt5b* and *lef1* mRNA (Poss et al., 2000a; Stoick-Cooper et al., 2007b). In situ hybridization of fins at 30 and 72 hpa revealed an absence of both marker genes after treatment with Igf1r inhibitors (data not shown). Thus, IGF signaling is required for the establishment of the wound epidermis, which is essential for fin regeneration.

To determine the requirement of IGF signaling for the properties of the normally established wound epidermis, we performed a drug-shift experiment. Fins were first allowed to develop a small outgrowth and to establish a wound epidermis for 30 hpa in normal conditions, and then they were exposed to Igf1r inhibitors for additional 24 hours. In control regenerating fins at the outgrowth phase, *wnt5b* is expressed in the basal layer of the apical epithelium and the distal tip of the blastema, whereas *lef1* transcript localizes to the lateral/proximal domain of the basal wound epithelium and to the distal blastema (Poss et al., 2000a; Stoick-Cooper et al., 2007b). Both genes were downregulated after 24 hour treatment with the Igf1r inhibitors starting at 30 hpa (Fig. 5G-J and see Fig. S5 in the supplementary material) (n=4). Despite the molecular defects after the drug-shift, the architecture of the wound epidermis remained unaffected in these fins, as visualized by histological analysis (see Fig. S3D-F in the supplementary material). We concluded that IGF signaling is required to maintain the intrinsic molecular properties of the basal wound epithelium.

IGFs are potent survival factors in normal and malignant cells (Kurmasheva and Houghton, 2006). To test the effect of NVP-AEW541 or NVP-ADW742 on cell survival in the regenerating fins, we assessed the activation of Caspase 3, which is one of the crucial executioners of cell death. The control fins at 24 hpa contained only few scattered active-Caspase-3-positive cells (Fig. 4D) (n=6). The fins that were exposed to the inhibitors of Igf1r displayed approximately 55- to 95-fold enhanced cell apoptosis specifically in the wound epidermis (Fig. 4D-G) (n=6), whereas the stump epidermis and mesenchyme displayed a relatively lower increase in apoptosis, which was up to sevenfold of the control (Fig. 4D-G). Interestingly, we did not detect a massive cell apoptosis in the drug-shift experiment, where the fins were treated with the inhibitors for 24 hours starting at 30 hpa (see Fig. S3G-I in the supplementary material). We conclude that IGF signaling acts as a survival factor for a specific subset of cells during the formation of the wound epidermis.

Immunofluorescence revealed phosphorylated Igf1r in the basal layer of mature uninjured epidermis (Fig. 2B,C). This finding suggests a role for IGF signaling in fin homeostasis. To test this hypothesis, uncut fins were exposed to NVP-AEW541 or NVP-ADW742 for 3 days, and fin morphology and cell death were analyzed. The histological analysis did not reveal abnormal appearance of the fin, but the activation of Caspase 3 was enhanced up to threefold in the epidermis compared with control fins (see Fig. S4 in the supplementary material; n=6). This finding indicates that IGF signaling plays a role as a survival factor in uninjured epidermal tissue of the fin. However, the effect of IGF signaling on mature epidermis is much smaller when compared with the massive apoptosis in the wound epidermis at 24 hpa (Fig. 4D-G).

To further examine the function of IGF signaling in regulation of epithelial wound healing, we established an interray wound-healing assay. The interrays connect adjacent rays of the fin (Akimenko et

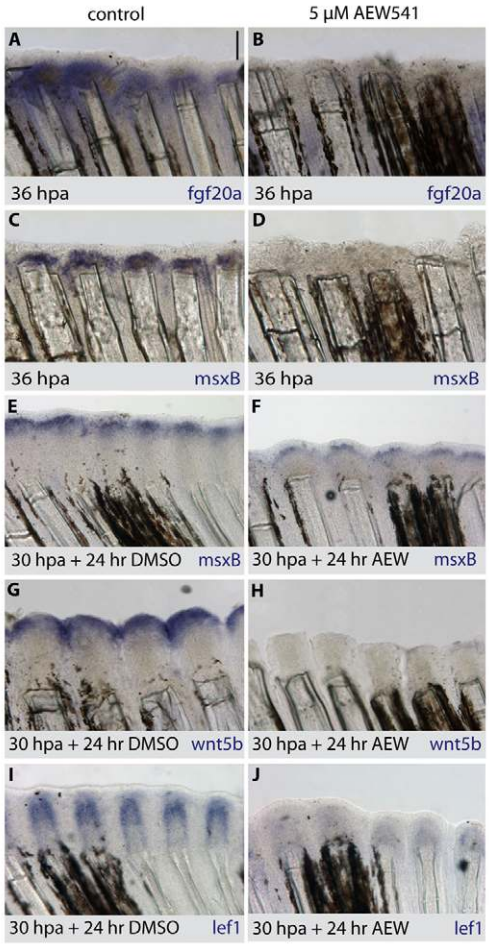

Fig. 5. IGF signaling is required for the expression of blastemal and wound-epidermal marker genes. Whole-mount in situ hybridization of fins with antisense probes (purple). (**A-D**) In control fins at 36 hpa, *fgf20a* and *msxB* are strongly detected in the blastema of the outgrowth (A,C). In fins treated with NVP-AEW541, the expression of the blastemal marker genes is almost undetectable (B,D). (**E-J**). Drug-shift experiment (30 hours at normal conditions and 24 hours with treatment). Control fins express *msxB* in the blastema (E), *wnt5b* in the apical wound epidermis and distal tip of the blastema (G), and *lef1* in the proximal wound epithelium and distal blastema (I). Exposure to NVP-AEW541 (F,H,J) strongly diminished expression of these genes. Scale bar: 100 μm.

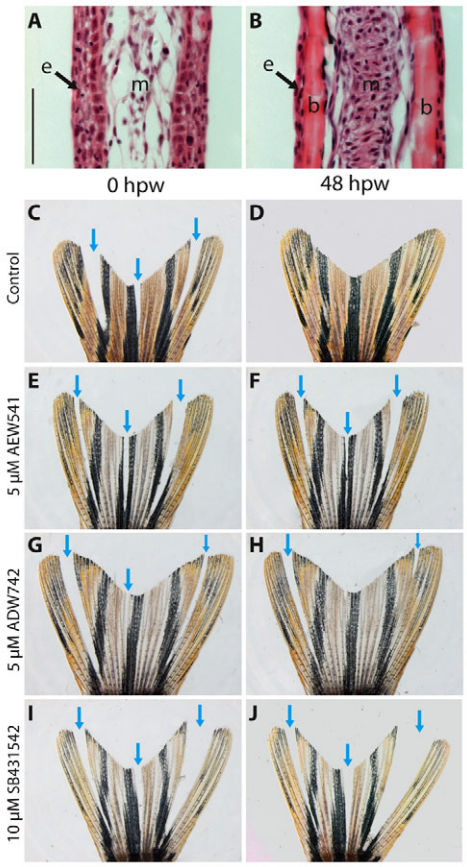

Fig. 6. Interray wound-healing assay demonstrating the role of IGF and Activin/TGFβ signaling in wound closure. (**A**,**B**) Hematoxylin and Eosin staining of longitudinal fin sections. (A) Interrays lack skeletal elements, have multilayered epidermis, and very loose mesenchyme. (B) Rays contain bones covered by thin epidermis. Mesenchyme appears as a compact aggregation of interconnected fibroblasts. (**C-J**) Bright-field images of injured fins. Arrows indicate incisions between the bony rays. These interray notches were closed after 48 hours in control fins (C,D). Exposure to either NVP-AEW541 (E,F) or NVP-ADW742 (G,H) markedly impaired wound healing. Treatment with the inhibitor of Activin/TGFβ signaling, SB431542, completely blocked wound closure (I,J). b, bones; e, epidermis; m, mesenchyme; hpw, hours post-wounding. Scale bar: 50 μm.

al., 2003). They consist of multilayered epidermis and loose mesenchymal tissue containing few cells in comparison with the compact mesenchyme of the rays (Fig. 6A,B). We performed three incisions of the interrays per fin, which were fully healed within 24 to 48 hours (Fig. 6C,D) (n=9). Our previous studies demonstrated the role of TGFβ/Activin signaling in the repair of the deformed interrays (Jaźwińska et al., 2007). Here, we tested the function of TGFβ/Activin signaling in healing of the cut interrays. Inhibition of this signaling pathway with a specific inhibitor, SB431542, did not affect re-epithelialization of the wound margin but completely blocked the wound closure (Fig. 6I,J) (n=9). Next, we used the interray wound-healing assay to examine the effect of IGF signaling. We found that the treatment with NVP-AEW541 and NVP-ADW742 partially impaired wound closure (Fig. 6E-H) (NVP-AEW541: of nine wounds, four partially healed and five did not heal; NVP-ADW742: of nine wounds, four were well healed, three were partially healed, and two did not heal). This result demonstrates that IGF signaling is required for normal healing of wounded epidermis.

Igf1r signaling is essential for blastema formation

Mesenchymal cell proliferation and tissue remodeling are key mechanisms of blastema formation during epimorphic regeneration (Akimenko et al., 2003; Poss et al., 2003). To test cell proliferation at the onset of blastema formation, DNA-replicating cells were labeled with BrdU for 6 hours before fin collection at 24 hpa. Mesenchymal cell proliferation was similar in control and experimental fins (Fig. 7). To visualize the disorganized mesenchyme, we used antibody against Tenascin C, which is an extracellular glycoprotein demarcating the distal mesenchymal

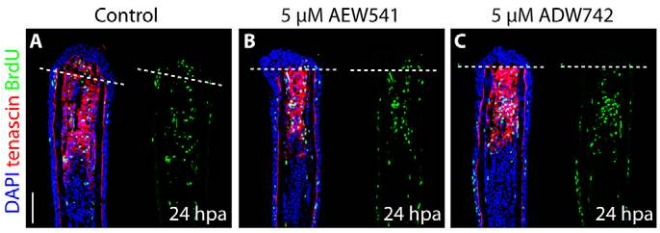

Fig. 7. Inhibition of IGF signaling does not affect cell proliferation and Tenascin C expression at the onset of blastema formation. (**A-C**) Fin regenerates at 24 hpa triply stained with BrdU antibody (green), Tenascin C antibody (red) and DAPI (blue). The dashed line demarcates the amputation plane. At this stage of regeneration, no difference in cell proliferation or Tenascin C expression is detected between the control (A) and Igf1r inhibitor-treated fins (B,C). Scale bar: 50 μm.

region of the regenerating vertebrate appendages (Jaźwińska et al., 2007; Onda et al., 1991). We did not observe any difference in the size and the intensity of the Tenascin C domain between control and experimental fins at 24 hpa (Fig. 7). Thus, IGF signaling does not seem to influence the extent of mesenchymal proliferation and remodeling at the onset of blastema formation.

The first defect in mesenchymal proliferation after Igf1r inhibitor-treatment was detected at 36 hpa (Fig. 8A-C). The proliferation of epidermal cells was similar in control and experimental fins. At this time point, all the control fins developed a conical blastema protruding beyond the amputation plane (Fig. 8A). None of the inhibitor-treated fins displayed an outgrowth (Fig. 8B,C). These data demonstrate that IGF signaling has a mitogenic role during blastema formation.

To determine the requirement of IGF signaling for the function of the normally established blastema, we performed a drug-shift experiment. Fins were first allowed to develop the blastema for 30 hpa in normal conditions, and then they were exposed to the Igf1r inhibitors for the next 24 hours. For the last 6 hours of the experiment, the fish were kept in the presence of BrdU to label dividing cells. We assessed BrdU-incorporation in nuclei located up to about 350 μm to the distal tip of the fin sections. We observed that the loss of IGF signaling led to approximately 80% reduction in proliferation of mesenchymal cells (Fig. 8D-G) (control 31.4±3.0%; NVP-AEW541-treated fins 7.5±1.3%; NVP-ADW742-treated fins 5.9±2.1%; n=6; P<0.01). In contrast to mesenchyme, the overlying epidermis displayed slightly enhanced proliferation in comparison to control (Fig. 8D-G) (control 15.4±0.9%; NVP-AEW541-treated fins 18.8±1.4%; NVP-ADW742-treated fins 17.1±2.0%). Interestingly, cell proliferation was not altered by exposure to the inhibitors for 6 or 12 hours starting at 30 or 48 hpa (data not shown). This indicates that IGF signaling does not have an immediate and/or direct effect on the mitogenic property of mesenchymal cells. The expression of Tenascin C in these fins remained similar to control, indicating normal level of tissue remodeling (Fig. 8D-F).

To determine the effect of Igf1r inhibitors on molecular specification of the blastema, we performed in situ hybridization. At 36 hpa, *msxB* and *fgf20a* mark blastema cells (Akimenko et al., 1995; Whitehead et al., 2005). No expression of these genes was apparent in fins that were treated with NVP-AEW541 or NVP-ADW742 for 24 hours before fin collection (Fig. 5A-D and data not shown) (n=6). Moreover, the expression of *msxB* mRNA was significantly reduced in the drug-shift experiment, in which the fins

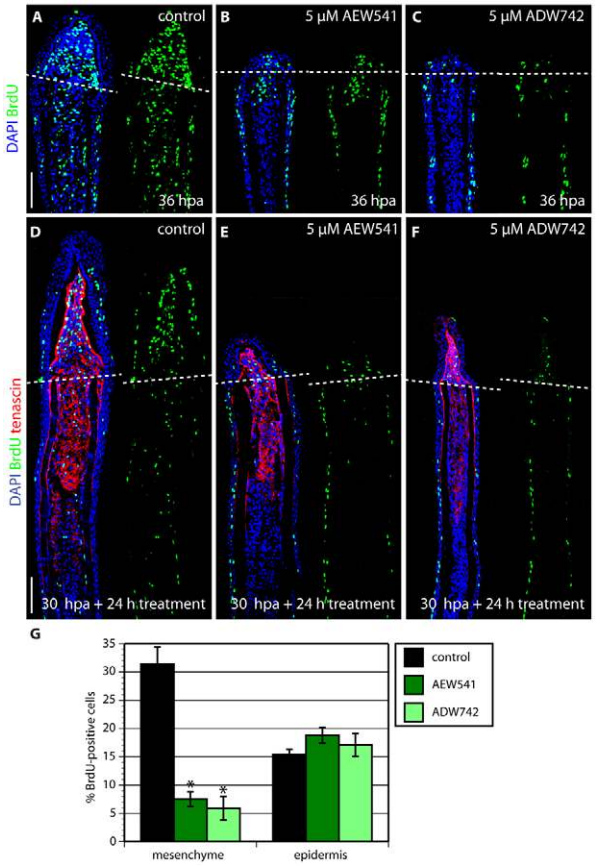

Fig. 8. IGF signaling is required for proliferation of blastema cells. (**A-C**) Longitudinal sections of fins at 36 hpa stained with BrdU antibody (green) and DAPI (blue). Fins treated with either NVP-AEW541 (B) or NVP-ADW742 (C) display decreased mesenchymal proliferation and no blastemal outgrowth, compared with control (A). (**D-F**) Longitudinal sections of fins after a drug shift: 30 hours at normal conditions followed by 24 hour treatment with 0.05% DMSO (control) (D), 5 μM NVP-AEW541 (E) and 5 μM NVP-ADW742 (F), triply stained with BrdU antibody (green), Tenascin C antibody (red) and DAPI (blue). The dashed line demarcates the amputation plane. In control fins, the blastema displays massive proliferation (D). The smaller size of the outgrowth and reduction of blastemal proliferation demonstrates a block of normally initiated regeneration after the exposure to the drug. (**G**) The percentage of BrdU-positive cells relative to total number of cells in regenerating fins after DMSO (control) or inhibitor treatment for 24 hours starting at 30 hpa. The counted nuclei were located up to ~350 μm from the distal tip of the fin sections. Error bars represent the s.e.m. n=6; *P<0.01. Scale bars: 50 μm.

were treated with the inhibitor for 24 hours starting at 30 hpa (Fig. 5E,F) (n=5). These data indicate that IGF signaling regulates expression of blastemal genes required for regeneration.

Our data shown in Fig. 5E-J and Fig. 8G demonstrate that inhibition of Igf1r for 24 hours, starting at 30 hpa, is sufficient to affect the expression of the molecular markers and proliferation of the blastema cells. To further examine the role of IGF signaling during the outgrowth phase, we analyzed the morphology of the regenerates in this drug-shift experiment. Starting at 30 hpa, the regenerating fins were treated with NVP-AEW541 or NVP-ADW742 for 5 days, and the fins were photographed. The inhibition of IGF signaling resulted in approximately 82% reduction of the regenerate area in comparison with control (see Fig. S6 in the

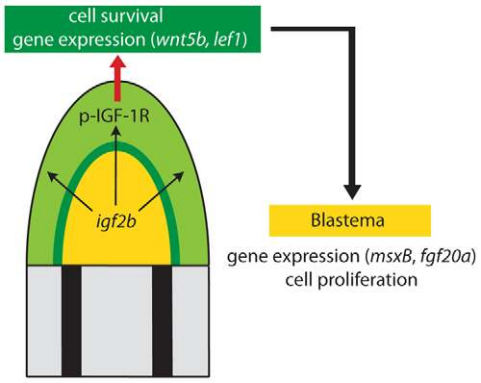

Fig. 9. IGF signaling mediates interactions between the wound epidermis and the blastema. Schematic representation of a regenerating fin with the blastema in yellow and the wound epidermis in green. The basal layer of the wound epidermis is shown in dark green. The black bars represent skeletal structures, and the gray fields are parts of the stump. *igf2b* is expressed in the blastema. The ligand triggers Igf1r signaling in the wound epidermis, promoting cell survival and supporting molecular specification of the basal layer of the wound epidermis. The properties of the wound epidermis acquired upon Igf1r signaling are required for normal blastema functions, including cell proliferation and expression of blastema marker genes.

supplementary material) (NVP-AEW541 18.9±5.8% of control regenerate; NVP-ADW742 17.5±4.0% of control regenerate; n=3; P<0.001). The impaired regenerates of the inhibitor treated fins displayed abnormal differentiation of the tissue, as seen by poorly developed skeletal tissue and weaker pigmentation (see Fig. S6G-I in the supplementary material). We concluded that IGF signaling is required for the progression of the fin regeneration.

DISCUSSION

The direct interaction between the specialized wound epidermis and the blastema is an absolute requirement for vertebrate limb regeneration (Campbell and Crews, 2008). The present study identifies a fundamental role of IGF signaling in mediating reciprocal communication along the epidermis-blastema axis. Our data support a model, in which information between adjacent domains is transmitted through secreted growth factors (Fig. 9). *igf2b* is transcriptionally upregulated in the mesenchyme underlying the wound epidermis. Molecular mechanisms that induce this expression are not known. Secreted Igf2b ligand activates paracrine signaling in the wound epithelium, as detected by phosphorylated Igf1r kinase receptor. This activation is required for the formation of the distinctive basal epithelial layer and for the expression of wound epithelial molecular markers *wnt5b* and *lef1*. Although we did not observe enhanced activation of Igf1r receptor in the blastema, the inhibition of its signaling impaired cell proliferation in the blastema and expression of blastemal markers *msxB* and *Fgf20a*. These observations indicate that the effects of IGF on the blastema are cell non-autonomous. Accordingly, the activation of Igf1r signaling in the wound epidermis indirectly influences underlying blastemal cells. We propose that IGF signaling is required to support intrinsic properties of the wound epidermis, which are needed to stimulate the regenerative response of the blastemal cells.

It has been known for decades that the interruption of the contact between the wound epidermis and the blastema blocks appendage regeneration in amphibians (Mescher, 1976; Tassava and Garling, 1979; Thornton, 1957). However, it is still little understood how the wound epidermis is involved in stimulating or allowing limb regeneration. One of the best-known characteristics of a wound epidermis is a production of diffusible growth factors that can pass to the underlying mesenchyme. Studies in amphibian limb and in zebrafish fin regeneration have shown that members of the Wnt family are expressed in the apical epithelium of regenerating organs (Kawakami et al., 2006; Stoick-Cooper et al., 2007b; Yokoyama et al., 2007). Elegant experiments involving transgenic animals have demonstrated that Wnt signaling regulates cell proliferation of the blastema. Our findings are consistent with recently emerging evidence suggesting that the properties of the wound epidermis are modulated by signals coming from the underlying mesenchymal cells. This view has been proposed based on a phenotype caused by a mutation of the *fgf-20a* gene in zebrafish, *dob*, which is characterized by a defective wound epidermis and a lack of the blastema (Whitehead et al., 2005). *fgf-20a* is expressed in mesenchyme underneath the apical epithelium. Recently it has been shown that FGF signaling modulates the Sdf1/Cxcr4 system in regenerating fins (Bouzaffour et al., 2009). The chemokine *sdf1* is expressed in the blastema, but the signal-receiving cells are located in the epidermis (Dufourcq and Vriz, 2006). Thus, divergent signaling systems influence interactions between the blastema and the wound epidermis.

The landmark structures of epimorphic regeneration, the specialized wound epithelium and the blastema, failed to form in amputated fins treated with Igf1r inhibitors. Interestingly, the preparation phase, which precedes the formation of these structures, was unaffected by the inhibition of IGF signaling. The early regenerative responses, such as wound re-epithelialization, enhanced BrdU incorporation and Tenascin C expression were unchanged in fins that were treated with Igf1r inhibitor during the first 24 hpa. This data indicate that the initial cell cycle re-entry and mesenchymal remodeling can take place even in the absence of the specialized wound epithelium. Proliferation of mesenchymal cells preceding blastema formation has been shown to depend on the FGF or Activin/TGFβ systems (Jaźwińska et al., 2007; Whitehead et al., 2005). IGF signaling does not influence the impact of these early signals on mesenchymal cell proliferation before blastema formation.

It has long been recognized that animals with a capacity for appendage regeneration also display an extraordinary wound healing potential (Brockes and Kumar, 2008). Here, we established an interray wound-healing assay to test the requirement of Activin/TGFβ and IGF for healing of superficial wounds in the zebrafish fin. First we showed that interray incisions along the proximodistal axis of the fin are rapidly and perfectly closed. However, wound closure was completely blocked by the loss of Activin/TGFβ signaling, and it was partially impaired by the Igf1r inhibitors. This brings a consideration of the molecular similarities between epimorphic regeneration and healing of skin wounds that are not associated with a regenerative outgrowth. Urodele amphibians can also heal their skin wounds without the appearance of scars (Roy and Levesque, 2006). In the axolotl limb, superficial wounds with experimentally augmented innervation lead to the formation of a blastema-like structure that derives from de-differentiated dermal fibroblasts (Endo et al., 2004; Satoh et al., 2007). It will be interesting to characterize the contribution of dermal fibroblasts in wound healing and blastema formation in the zebrafish fin.

Studies in mammalian model systems highlight the importance of IGF signaling in normal skin homeostasis and in promoting wound healing (Edmondson et al., 2003; Semenova et al., 2008; Werner and Grose, 2003). IGF action enhances cell proliferation, survival and migration of keratinocytes. Deficiency in Igf1 is associated with

decreased epidermal thickness. However, overexpression of IGF ligands or their receptor has been recognized as a cause of tumorogenesis (Chao and D'Amore, 2008; Pollak et al., 2004). Thus, IGF ligands can act either as pro-regeneration or pro-cancer effectors. It remains to be shown how the IGF system is fine-tuned in zebrafish for the purpose of regeneration.

Acknowledgements
We are grateful to Franscesco Hofmann, Novartis Pharma AG for providing NVP-AEW541 and NVP-ADW742 compounds. We thank C. Weber for fish care; M. Kaczorowski for excellent technical assistance; W. Blum for help with western blot; M. Celio, M. Affolter and B. Müller for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation, grant number: 310000_120611.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.043885/-/DC1

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