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Fgf signalling controls diverse aspects of fin regeneration

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ABSTRACT

Studies have shown that fibroblast growth factor (Fgf) signalling is necessary for appendage regeneration, but its exact function and the ligands involved during regeneration have not yet been elucidated. Here, we performed comprehensive expression analyses and identified *fgf20a* and *fgf3*/*10a* as major Fgf ligands in the wound epidermis and blastema, respectively. To reveal the target cells and processes of Fgf signalling, we performed a transplantation experiment of mesenchymal cells that express the dominant-negative Fgf receptor 1 (*dnfgfr1*) under control of the heat-shock promoter. This mosaic knockdown analysis suggested that Fgf signalling is directly required for fin ray mesenchyme to form the blastema at the early pre-blastema stage and to activate the regenerative cell proliferation at a later post-blastema stage. These results raised the possibility that the early epidermal Fgf20a and the later blastemal Fgf3/10a could be responsible for these respective processes. We demonstrated by gain-of-function analyses that Fgf20a induces the expression of distal blastema marker *junbl*, and that Fgf3 promotes blastema cell proliferation. Our study highlights that Fgfs in the wound epidermis and blastema have distinct functions to regulate fin regeneration cooperatively.

KEY WORDS: Zebrafish, Fin regeneration, Fgf, Blastema, Wound epidermis

INTRODUCTION

The regenerative abilities of tissues in higher vertebrates, including mammals, are limited, but several vertebrate species, such as urodele amphibians and teleost fish, retain remarkable capacity for tissue regeneration, which is known as epimorphic regeneration. Numerous morphological and histological studies have shown that epimorphic regeneration of appendages such as limbs, tails and fins proceeds through several distinct steps: wound closure and healing, formation of a thick epidermis termed 'wound epidermis', formation of proliferating mesenchymal cells termed 'blastema', and tissue outgrowth (Akimenko et al., 2003; Poss et al., 2003; Kawakami, 2010). In the case of zebrafish fin regeneration, recruited blastemal cells are detected at 18 h post-amputation (hpa; Poss et al., 2000), but apparent blastema cell proliferation and outgrowth is observed after 2 days post-amputation (dpa) and thereafter.

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Whereas the blastema plays a key role in regeneration by supplying new cells within the regenerated tissue, significance of the wound epidermis has been less clear. A number of studies have suggested that regeneration is impaired or delayed when normal formation of the wound epidermis is disturbed by insertion of the amputated limb into the body cavity, by skin grafting or by surgical removal of the wound epidermis (Goss, 1956; Thornton, 1957; Mescher, 1976; Tassava and Garling, 1979; Campbell and Crews, 2008). Thus, it is conceivable that the wound epidermis plays an essential role during regeneration, but the exact function of the wound epidermis remains unknown.

During the last decade, studies have been focused on identifying the molecular basis of regeneration. In particular, studies on zebrafish have pointed to the involvement of a variety of signalling molecules such as insulin-like growth factor (Chablais and Jazwinska, 2010), Notch (Grotek et al., 2013; Munch et al., 2013), bone morphogenetic protein (Smith et al., 2006), retinoic acid (Blum and Begemann, 2012), Wnt (Stoick-Cooper et al., 2007; Wehner et al., 2014), activin βA (Jazwinska et al., 2007), Sonic hedgehog (Quint et al., 2002), stromal cell-derived factor 1 (Bouzaffour et al., 2009), laminin (Chen et al., 2015), Kit ligand (Tryon and Johnson, 2014), adenosine (Rampon et al., 2014) and hydrogen peroxide (Gauron et al., 2013). Notably, several studies have suggested that Fgf signalling is necessary for fin regeneration (Poss et al., 2000; Lee et al., 2005, 2009; Wills et al., 2008). Furthermore, a genetic screen by Whitehead et al. (2005) showed that the temperature-sensitive mutant of *fgf20a* does not promote formation of the blastema in zebrafish at a non-permissive temperature, indicating that Fgf20a is at least an essential Fgf ligand during fin regeneration. In spite of the crucial role of Fgf signalling in regeneration, the exact mechanism of Fgf action, including the responsible Fgf ligands and their functions, is poorly understood.

In this study, we performed comprehensive expression analyses of Fgf ligands and showed that *fgf20a* is expressed in the wound epidermis from an early stage of blastema formation when the expression of *msxb* (*msx1b*) and *msxc* (*msx3*) genes and the blastema cell proliferation are not activated, whereas *fgf3* and *fgf10a* are differentially activated in the blastema at the following stage concurrently with blastema cell proliferation. Using a cell-transplantation experiment of mesenchymal cells with inducible expression of *fgfr1*, we showed that Fgf signalling is directly required for the fin ray mesenchyme to form the blastema at the early pre-blastema stage and to activate the regenerative cell proliferation at a later post-blastema stage. Furthermore, we explored the specific functions of Fgf ligands by gain-of-function analyses and demonstrated that Fgf20a does indeed induce the distal blastema marker *junbl* (*junbb*) (Yoshinari et al., 2009; Ishida et al., 2010), and that Fgf3 promotes cell proliferation in the blastema explants. Our study reveals the diverse roles of Fgf signals during fin regeneration in zebrafish and how Fgfs in the wound epidermis and blastema cooperatively regulate regeneration.

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RESULTS

The Fgf ligands that are expressed during fin regeneration

To identify the Fgf ligands that are involved in fin regeneration, we conducted comprehensive expression analysis by means of reverse-transcription polymerase chain reaction (RT-PCR; Fig. S1A). A low level of expression was detected for some Fgf genes in uncut fins, which may represent a basal expression for normal tissue growth and/or maintenance. However, genes of several Fgf ligands – *fgf3*, *fgf7*, *fgf10a*, *fgf10b*, *fgf12a*, *fgf12b*, *fgf13a*, *fgf20a*, *fgf20b* and *fgf24* – were apparently upregulated in response to tissue amputation. Notably, only expression of *fgf20a* and *fgf20b* was detectable after 1 dpa (Fig. S1A; Fig. 1A), a stage when the blastema is forming, but cell proliferation in the blastema has not yet begun (Fig. S2).

We further determined the tissue localisation of expression of Fgf genes by tissue-specific RT-PCR analysis using the RNAs isolated from the blastema and wound epidermis (Fig. 1B; Fig. S1B). The specificity of RNA samples was confirmed by the expression of epidermal and blastemal markers, *p63* (*tp63*) and *msxc*, respectively (Akimenko et al., 1995; Fig. S3). Although cross-contamination with cells between the above samples is inevitable, this approach enabled us to analyse quickly the epidermal or blastemal expression of the Fgf genes in question. Among the regeneration-associated Fgfs, a group of ligands – *fgf3*, *fgf7*, *fgf10a*, *fgf10b* and *fgf12b* – was principally induced in the blastema, whereas another group, including *fgf12a*, *fgf13a*, *fgf20a*, *fgf20b* and *fgf24*, was principally induced in the epidermis.

The RT-PCR analyses indicated that *fgf20a* and *fgf20b* were the earliest Fgf genes activated in the wound epidermis. Other studies have suggested that *fgf20a* expression is localised to the blastema and/or wound epidermis (Whitehead et al., 2005; Lee et al., 2009). The cause of this ambiguity is thought to be the low resolution of *in situ* hybridisation (ISH) signals. To confirm the tissue localisation of *fgf20a* expression, we utilised a transgenic (Tg) zebrafish line with an enhancer trap (Nagayoshi et al., 2008), in which an enhanced green fluorescent protein (EGFP) cassette was inserted in the vicinity of the *fgf20a* gene (Fig. S4A). In the Tg line, EGFP expression was detected in response to amputation in cells around the amputation site as early as 12 hpa, a stage when the blastema formation is still not evident. EGFP expression became localised to the basal layer of the wound epidermis after 2 dpa and eventually was restricted to cells at the posterior margin of the fin after regeneration (Fig. 1C). Although a few EGFP-positive cells were also seen within fin rays at 12 hpa, it was not clear whether or not this reflects the endogenous *fgf20a* expression. Such cells were not observed at later stages (Fig. 1C). The principal EGFP expression was colocalised with the epidermal marker p63 (Fig. 1D), and such tissue localisation is in agreement with the results of the RT-PCR analysis (Fig. 1B). We also repeated the ISH analysis and confirmed that the endogenous *fgf20a* expression coincided with EGFP expression (Fig. S4B). Furthermore, EGFP expression in rhombomere neurons at the prim-15 stage (Fig. S4C) is also consistent with the previously reported pattern of *fgf20a* expression (Terriente et al., 2012). Taken together, these data supported the notion that *fgf20a* expression during fin regeneration is recapitulated by the enhancer trap zebrafish line, and that Fgf20a is an Fgf ligand that is locally expressed in the basal layer of the wound epidermis.

The blastema is composed of several distinct cell types, such as non-proliferating or slowly proliferating distal blastema cells, highly proliferative proximal blastema cells, osteogenic cells including the osteoblast, etc. (Fig. S5). To reveal the cellular localisation of expression of the blastema Fgf genes, we performed an ISH analysis

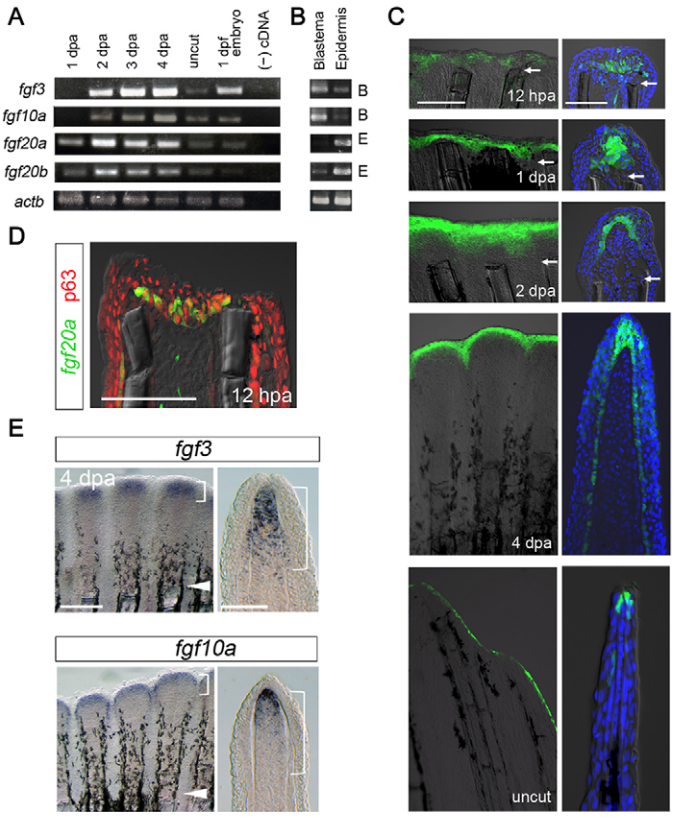

Fig. 1. The expression of Fgf ligands during zebrafish fin regeneration. (A) RT-PCR analysis of the expression of *fgf3*, *fgf10a*, *fgf20a* and *fgf20b* at 1, 2, 3 or 4 dpa and in uncut fins. The presented data is a part of comprehensive analysis shown in Fig. S1. The number of PCR cycles was 30. *actb1*, *β-actin1* primers. (B) RT-PCR analysis of tissue-specific expression of Fgf genes in the blastema and wound epidermis at 2 dpa. The number of PCR cycles was 35. Fgf genes that are mainly expressed in the blastema or wound epidermis are indicated as B (blastema) or E (epidermis) to the right of the panel. (C) Localisation of *fgf20a* expression in the wound epidermis, as visualised by the enhancer trap line, *HGn21A*. Enhanced green fluorescent protein (EGFP)-positive cells gave rise to the basal epidermal cells at 2 dpa. In the 'no-regeneration' state (uncut fin), EGFP was detected in the distal tip of the epidermis. Tissue sections (right panels) were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI). Arrows indicate the site of amputation. Scale bars: 200 µm for whole-mount analysis (left panels); 50 µm for sections (right panels). (D) Initiation of *fgf20a* expression in the epidermal cells. A cryostat section of *HGn21A* regenerate at 12 hpa stained with the antibody against p63, a marker of epidermal cells. Scale bar: 50 µm. (E) Whole-mount *in situ* hybridisation (ISH) analysis of *fgf3* and *fgf10a* expression at 4 dpa (left panels) and their respective tissue sections (right panels). Expression of these Fgf genes was strongest in the distal region of the blastema, though the region of *fgf3* expression was broader than that of *fgf10a*. Arrowheads mark amputation sites; brackets indicate approximate areas of the blastema. Scale bars: 200 µm (left); 50 µm (right).

of *fgf3*, *fgf7*, *fgf10a*, *fgf10b* and *fgf12b*. Many of these did not yield a reliable ISH signal, but only *fgf3* and *fgf10a* were clearly detectable in the blastema (Fig. 1E; Fig. S6). The expression of *fgf3* and *fgf10a* was the strongest in the distal blastema.

Early Fgf signalling acting on the mesenchyme induces blastema formation

Because of the loss-of-blastema phenotype of the *fgf20a* mutant (Whitehead et al., 2005) and localisation of *fgf20a* expression to the wound epidermis, it may be hypothesised that the wound epidermis regulates blastema formation via an early Fgf20a signal. To verify the role of early Fgf signalling, we re-evaluated the effects of

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inhibition of Fgf signalling in a Tg zebrafish that overexpresses dominant-negative Fgf receptor 1 (*dnfgfr1*) under control of the heat shock protein 70 (*hsp70l*) promoter (Lee et al., 2005). When Fgf signalling was blocked starting at 0 dpa, the fins actually showed a loss-of-blastema phenotype at 2 dpa, as in the *fgf20a* mutant (Fig. S7A-D). Before the onset of regenerative cell proliferation (Fig. S2), a basal level of cell proliferation at 1 dpa detected by 5-ethynyl-2′-deoxyuridine (EdU) incorporation was not affected by the inhibition of Fgf signalling (Fig. S7E,F). We also examined apoptosis in the Fgf signalling-inhibited fins, but no significant increase in apoptosis was observed (Fig. S7G,H). These observations support the notion that the early Fgf signalling before 2 dpa, which is mainly mediated by Fgf20a, is required for blastema formation. More intriguingly, *fgf20a* expression in the wound epidermis was reduced by inhibition of Fgf signalling (Fig. S7D), suggesting that Fgf signalling also participates in maintenance of *fgf20a* expression in the wound epidermis.

Given that early Fgf signalling is required for blastema formation, we next investigated whether it acts on the fin ray mesenchymal cells to induce blastema formation. To this end, we performed a cell-transplantation experiment, in which donor blastema cells carrying the transgenes *hsp70l:dnfgfr1* and *Olactb:loxP-dsR2-loxP-EGFP* (Yoshinari et al., 2012), which drives the DsRed2 expression in ubiquitous cell types including the osteoblasts (Fig. S8), were transplanted into the host blastema (Fig. 2A). After the transplantation and regeneration, progenies of the transplanted cells mostly contributed to the regenerated mesenchymal cells (Fig. 2B), but only a few cells (11 out of 226) to the Zns5-positive osteogenic cells including osteoblasts (Fig. 2C). This situation enabled us to inhibit Fgf signalling in the genetically labelled mesenchymal cells at a desired time point and to track their behaviour.

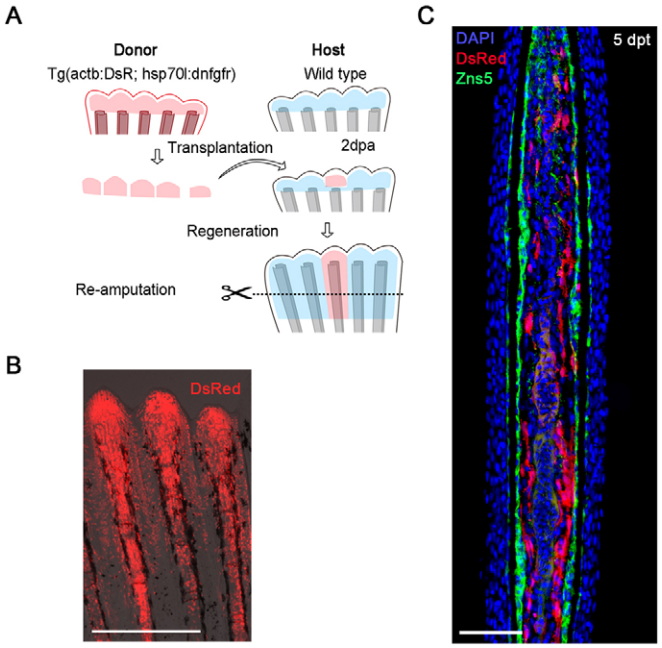

Fig. 2. Generation of a mosaic fin by a blastema transplant. (A) The procedure of the blastema transplantation and mosaic analysis. Donor blastema cells were taken from the double-transgenic zebrafish of Tg(*hsp70l:dnfgfr1*) and Tg(*Olactb:loxP-dsR2-loxP-EGFP*); the latter transgene drives ubiquitous expression of *DsRed2* (Yoshinari et al., 2012). The regenerated fins are a chimera of host cells and transplanted donor cells. (B) A whole-mount fluorescent view of the mosaic fin at 5 days post-transplantation (dpt). The fin contains progenies of the transplanted cells expressing the *dsRed2*. Scale bar: 500 µm. (C) A longitudinal tissue section of the mosaic fin at 5 dpt stained with an antibody against Zns5, a marker of osteogenic cells (green), and DAPI (blue). The transplanted cells (red) mostly gave rise to mesenchymal cells (11 Zns5-positive cells out of 226 DsRed2-positive cells, counted from 12 sections, from independent transplantations in four different fish). Scale bar: 50 µm.

The mosaic fins were allowed to regenerate until 7 dpa when the progenies of the transplanted cells were stably integrated and were re-amputated to initiate regeneration in the presence or absence of heat shock. With normal Fgf signalling (no heat shock), the labelled mesenchymal cells distally migrated to initiate the blastema formation (Fig. 3A, −HS; Fig. S9). By contrast, Fgf non-responsive cells were rarely observed within the blastema, which consisted of wild-type cells (Fig. 3A-C, +HS). Besides the reduced contribution of Fgf non-responsive cells to the blastema, overall regeneration, including the expression of a blastema marker, *msxc*, and the number and distribution of the Zns5-positive osteogenic cells were not changed (Fig. 3D-G). These observations suggest that the early Fgf signalling before 2 dpa is directly required for the fin ray mesenchymal cells to migrate and form blastema.

Action of later Fgf signalling is required for blastema cell proliferation

The later activation of several Fgf genes including *fgf3* and *fgf10a* in the blastema (Fig. S1; Fig. S6) raised a possibility that these Fgfs may perform additional functions during regeneration. We evaluated the overall function of later Fgf signalling after blastema formation by a stage-specific inhibition of Fgf signalling using Tg(*hsp70l:dnfgfr1*). When inhibition of Fgf signalling started at 3 dpa, a stage after the blastema formation, regeneration was immediately suppressed accompanying the decrease of blastema and the genes that mark the distal blastema (Fig. S10A-D). No significant increase in apoptosis was observed by the later suppression of Fgf signalling (Fig. S10E,F). However, cell proliferation in the blastema was severely decreased by the inhibition of Fgf signalling (Fig. S10G,H), indicating that the later Fgf signalling after 2 dpa is required for cell proliferation in the blastema. This function of later Fgf signalling is in contrast to that of early Fgf signalling, which did not affect basal cell proliferation in the epidermis and mesenchyme (Fig. S7E,F). In addition, the later blockage of Fgf signalling downregulated the expression of epidermal *fgf20a* as in Fig. S7D as well as the blastemal *fgf3* and *fgf10a*, suggesting that Fgf signalling is also required for maintenance of the expression of epidermal and blastemal Fgf ligands.

Given the later role of Fgf signalling in blastema cell proliferation, we further confirmed whether a direct Fgf signal to the fin ray mesenchyme is required for it. To do this, we again performed the transplantation experiment of *hsp70l:dnfgfr1* cells. However, the inhibition of Fgf signalling was started at 2 dpa when the expression of *msxc* and blastema Fgfs and the blastema cell proliferation have initiated. Without heat shock (Fgf signalling is normal), progenies of the transplanted cells contributed to formation of the entire region of regenerated tissue including the blastema (Fig. 4A, −HS). By contrast, after heat shock the number of *dnfgfr1*-expressing cells rapidly diminished in the blastema (Fig. 4A, +HS). EdU labelling confirmed that most of the Fgf non-responsive cells (Fig. 4B, green staining) were outside of the proliferation zone (Fig. 4B, brackets) and thus less proliferative (Fig. 4C). Except for the reduced contribution of Fgf non-responsive cells to the newly formed regenerate, overall cell proliferation within regenerates was not changed in the mosaic fins

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with inhibition of Fgf signalling (Fig. 4D). When the fin ray harboured many *dnfgfr1*-expressing cells, the length of the regenerate was slightly reduced in the operated fin rays (Fig. 4A, −HS). Altogether, these observations confirmed that the reception of Fgf signal by the fin ray mesenchyme is necessary for maintenance of its regenerative cell proliferation.

Fgf20a overexpression induces distal blastema markers

The early onset of *fgf20a* expression, its epidermal localisation during regeneration (Fig. 1; Fig. S1), and the inability to form the blastema in *fgf20a* mutant (Whitehead et al., 2005) strongly suggest that epidermal Fgf20a could be responsible for the blastema induction and maintenance. To prove the specific function of Fgf20a, we conducted

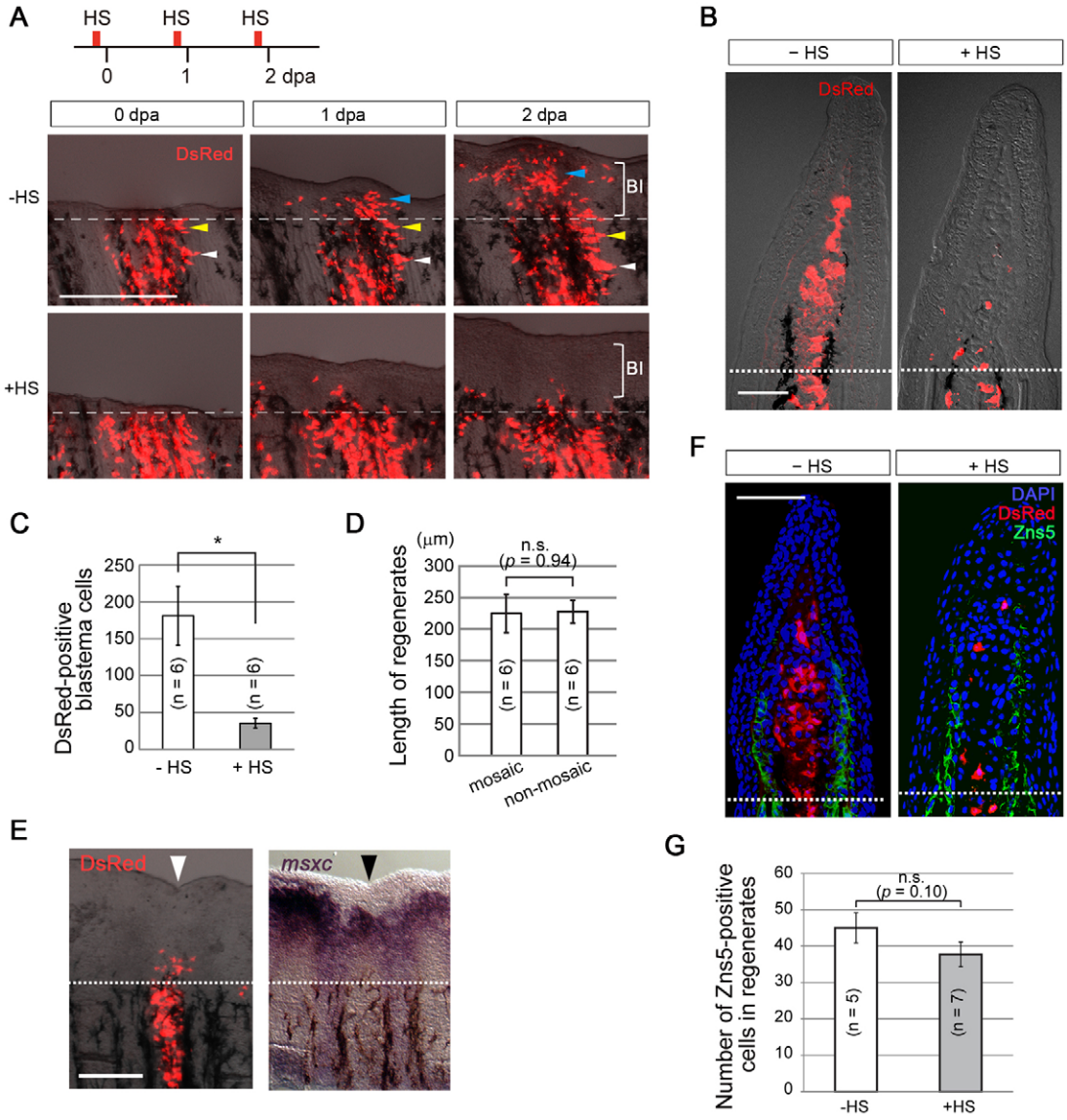

Fig. 3. Action of early Fgf signalling on mesenchymal cells induces blastema formation. (A) Tracking of the behaviour of labelled mesenchymal cells from 0 to 2 dpa. With intact Fgf signalling, numerous DsRed2-positive mesenchymal cells migrated in the distal direction and contributed to the blastema (−HS; 7/7 successful transplants), but few Fgf non-responsive cells participated in blastema formation (+HS; 8/8 successful transplants). Bl, blastema. Dashed lines, site of amputation. The coloured arrowheads respectively indicate the corresponding cell populations between different regeneration stages. (B) Tissue sections of representative samples at 2 dpa. In the absence of heat shock (−HS), progenies of the transplanted cells contributed to the blastema. By contrast, after heat shock the Fgf non-responsive cells stayed near the amputation plane (+HS). Dotted lines, site of amputation. (C) Quantification of the DsRed2-positive cells in the blastema in the presence (+HS) or absence (−HS) of heat shock. *n*, the number of fin rays that successfully contained many DsRed2-positive mesenchymal cells and were used for quantification (five fish in total, respectively). **P*<0.02. (D) Measurement of the length of regenerates of mosaic or non-mosaic fin rays. The heat shock was applied daily, and the lengths of the regenerates were measured at 2 dpa. No significant differences were observed in spite of the impaired contribution of Fgf non-responsive cells in the mosaic fin rays. *n*, the number of fin rays that successfully contained many DsRed2-positive mesenchymal cells and were used for quantification (five fish in total, respectively). (E) Expression of *msxc* in the mosaic fin. *msxc* expression was nearly normal at 2 dpa in the mosaic regenerate in spite of the reduced contribution of the Fgf non-responsive cells to the blastema (*n*=5/5 successful transplants). Black and white arrowheads indicate the mosaic fin ray. Dotted lines, site of amputation. (F) Distribution of osteoblasts detected by the anti-Zns5 antibody in the mosaic regenerate at 2 dpa. The distribution of osteoblasts was not affected in spite of the impaired contribution of Fgf non-responsive cells in the mosaic fin rays. Dotted line, site of amputation. (G) Quantification of the Zns5-positive cells shown in F. *n*=the number of sections used for the quantification, which were derived from four independent transplantations. In C,D,G, error bars denote mean± s.e.m.; Student's *t*-test was performed to assess statistical significance. n.s., not significant. Scale bars: 200 μm (A,E); 50 μm (B,F).

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gain-of-function experiments using a Tg zebrafish that overexpresses *fgf20a* under control of the *hsp70l* promoter. The Tg ubiquitously expressed *fgf20a* in both epidermal and blastemal compartments after heat shock (Fig. S11A). However, no drastic differences in length of the regenerating fin were observed between the Tg and wild-type fish that were given daily heat shocks (data not shown).

To analyse the role of Fgf20a in blastema induction, we performed an ISH analysis of *msxc* and *junbl*, genes that are expressed in the distal blastema. In comparison with the distally restricted expression of *msxc* in wild-type fins, the *fgf20a*-overexpressing fins showed a broader *msxc* expression (Fig. 5A,B). Significantly, the expression of *junbl*, an earlier blastema marker (Ishida et al., 2010), was strongly and ectopically upregulated in and around the blastema (Fig. 5C,D), suggesting that *fgf20a* overexpression causes mesenchymal cells to acquire the distal blastema identity. This situation is complementary to the

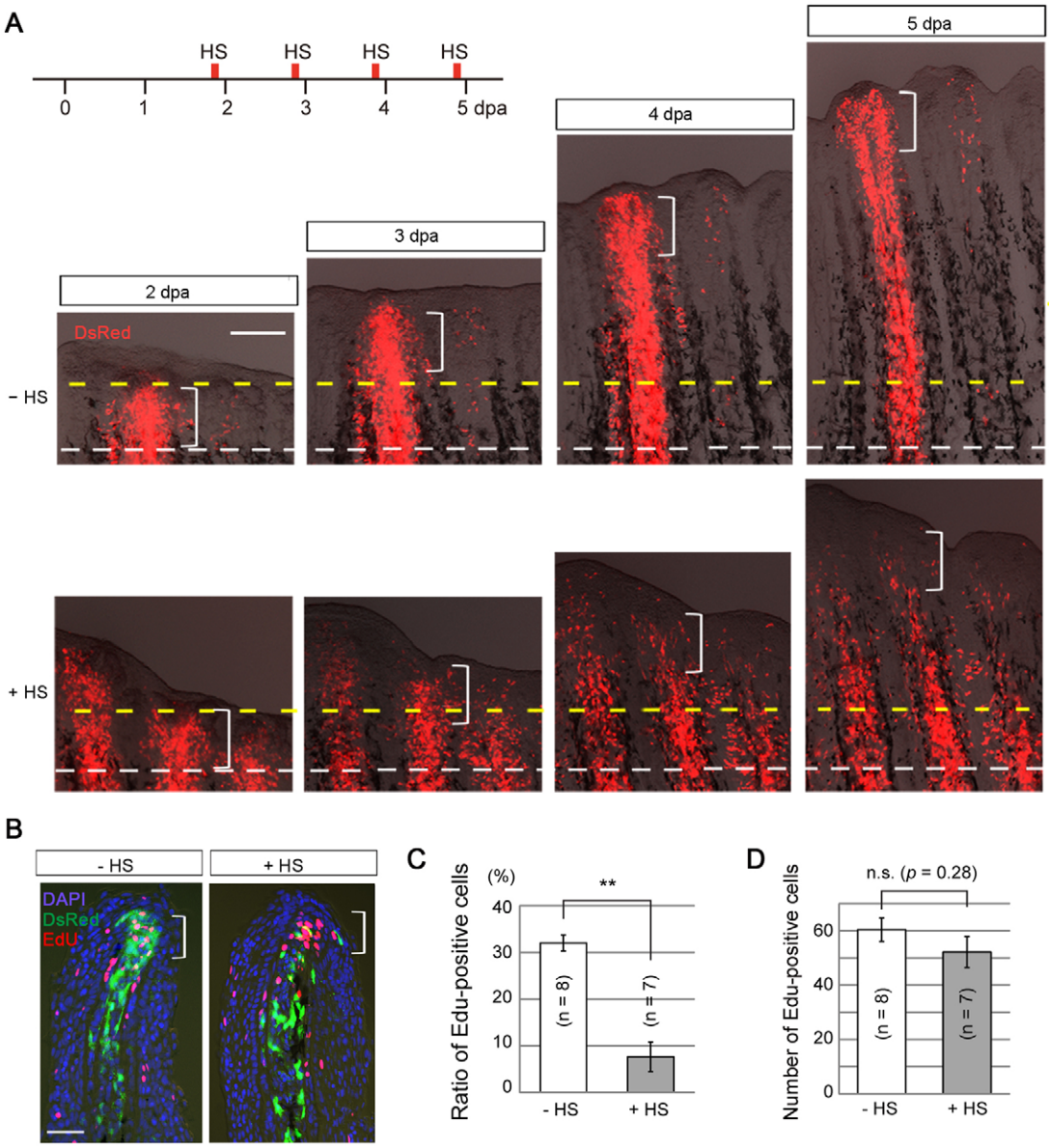

Fig. 4. Action of later Fgf signalling on mesenchymal cells is required for cell proliferation. (A) Tracking of the behaviour of labelled cells from 2 to 5 dpa. Normally (−HS, i.e. normal Fgf signalling), the DsRed2-labelled mesenchymal cells increased in number and contributed to the entire region of a regenerate after 3 dpa (*n*=9/9 successful transplants), whereas Fgf non-responsive mesenchymal cells rapidly retracted from the blastema in the newly regenerated region (+HS; *n*=8/8 successful transplants). White dashed lines, the site of amputation; yellow dashed lines, the distal end of the blastema at 2 dpa and its corresponding sites at subsequent stages; brackets, the approximate area of the blastema (200 µm from the distal tip). Scale bar: 200 µm. (B) Detection of proliferating cells by EdU labelling at 4 dpa. Labelling lasted for 6 h before sampling. With intact Fgf signalling (−HS), many DsRed2-positive (green) cells were located in the proliferation zone (brackets) and were also positive for EdU. With inhibition of Fgf signalling (+HS), most of DsRed2-positive cells were found outside the proliferation zone and were not showing EdU staining. The tissue sections were counter-stained with DAPI. Scale bar: 20 µm. (C) Measurement of the proportion of EdU-positive cells among progenies of the transplanted cells. Approximately 30% of the cells were EdU-positive in the absence of heat shock, but this ratio decreased to 5% by the inhibition of Fgf signalling. *n*, the number of fin rays that successfully contained many DsRed2-positive mesenchymal cells and were used for quantification (five fish in total, respectively). Error bars represent mean±s.e.m. Student's *t*-test was performed to assess statistical significance. ***P*<0.001. (D) Determination of the total number of EdU-positive cells in the regenerates in C. Overall cell proliferation was not different between the samples with (+HS) and without (−HS) heat shock. Error bars represent mean±s.e.m. Student's *t*-test was performed to evaluate statistical significance. n.s., not significant.

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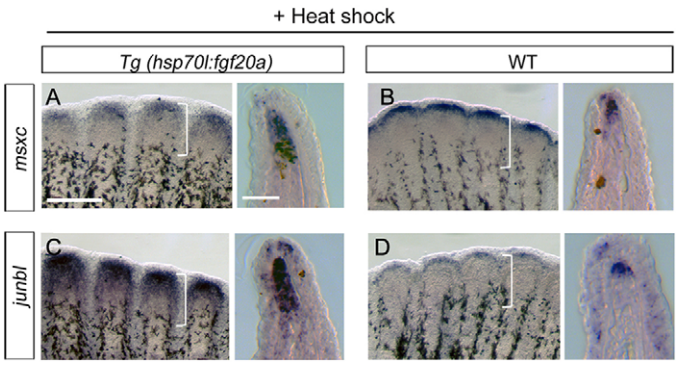

Fig. 5. Fgf20a directs formation of the distal blastema. (A,B) ISH analysis of the distal blastema marker *msxc* in *fgf20a*-overexpressing Tg and wild-type (WT) fins at 4 dpa. The *msxc* expression in the *fgf20a*-overexpressing Tg fins was broader than that in WT fins (*n*=10/10). (C,D) ISH analysis of *junbl*, an earlier blastema marker. *junbl* expression showed striking upregulation in the Tg fins (*n*=10/10). The colour reaction was terminated at the same time in the Tg and WT fins. Brackets, the approximate area of the blastema (200 µm from the distal tip). Scale bars: 200 µm (left); 50 µm (right).

no-blastema phenotype of the *fgf20a* mutant (Whitehead et al., 2005) and to the early blockage of Fgf signalling (Fig. 3A; Fig. S7).

Fgf3 overexpression stimulates cell proliferation in the blastema

Considering the expression of *fgf3* and *fgf10a* in the blastema and the role of later Fgf signalling for blastema cell proliferation, it is thought that the blastema Fgfs including Fgf3 and Fgf10a could be responsible for cell proliferation. To test this possibility, we took advantage of a zebrafish Tg(*hsp70l*:*fgf3*) and overexpressed *fgf3* during regeneration (Fig. S11B). As in the case of *fgf20a* overexpression, when *fgf3* was overexpressed, we did not observe a drastic difference in length of the regenerating fin in the Tg overexpressing *fgf3* (data not shown).

To further examine the effect of *fgf3* overexpression on cell proliferation, we assayed cell proliferation by EdU incorporation, but no apparent increase of EdU-positive cells in the amputated fish fins was detected (data not shown). It was thought that cell proliferation in the regenerates may be saturated even in the absence of *fgf3* overexpression. Therefore, we developed a blastema cultivation assay, in which the blastemas were separated from the wound epidermis and cultured in a collagen gel (Fig. 6A). In the cultured blastema without the epidermis, cell proliferation rapidly decreased within a few hours accompanying a downregulation of the distal blastema marker *junbl* (Fig. S12).

When the blastema explants, which were cultured for 6 h at 28°C, were then incubated at 37.5°C to induce *fgf3* expression, an apparent increase of the EdU incorporation was detected (Fig. 6B,C). The *fgf3* overexpression did not affect cell death in the explants (Fig. S13), therefore the increase of EdU-positive cells was not due to the survival of apoptotic cells by Fgf3. This result demonstrated that Fgf3 does indeed have a mitogenic effect on the blastema mesenchymal cells.

We also tested the effect of *fgf20a* overexpression on cell proliferation. Significantly, *fgf20a* overexpression also induced cell proliferation in the blastema explants (Fig. 6B,C). Because *fgf20a* overexpression induced the distal blastema marker *junbl* and *fgf3* (Fig. 5; Fig. 6D), we suggest that *fgf20a* overexpression activates cell proliferation via the induction of *fgf3*. Importantly, despite the similar phenotype of *fgf3* and *fgf20a* overexpression in cell proliferation, *fgf3* overexpression did not induce expression of the blastema markers *junbl* or *msxc* (Fig. 6E). This indicates that Fgf3, unlike Fgf20a, does not have a blastema-inducing function.

DISCUSSION

Despite the increasing number of signals that are shown to be involved in regeneration, the mechanisms by which such signals regulate appendage regeneration are largely unknown. In this study, we aimed to determine the exact mechanism of Fgf signalling during zebrafish fin regeneration and revealed that Fgf20a in the wound epidermis and Fgf3 in the blastema regulate distinct processes of regeneration and cooperatively ensure appendage regeneration (Fig. 7).

The role of the wound epidermis in the induction of blastema formation via the Fgf20a signal

The blastema and the wound epidermis are the principal components of epimorphic regeneration in urodeles and teleost fish. The importance of the wound epidermis has been suggested in many ways (Goss, 1956; Thornton, 1957; Mescher, 1976; Tassava and Garling, 1979), but its exact role has long been unclear. In this study, we showed that *fgf20a* expression is localised to the wound epidermis from the early stage of regeneration before the onset of blastema cell recruitment. We further showed that Fgf signalling at this stage is necessary for induction of the blastema, and that Fgf20a induces the distal blastema gene *junbl*. Taken together, these data strongly suggested that epidermal Fgf20a is an Fgf ligand that acts on the fin ray mesenchyme to promote formation of the blastema.

Fgf20 is a recently identified subfamily of Fgfs that is expressed in the brain (Ohmachi et al., 2000). Recently, it has been demonstrated that *Fgf20* knockout mice do not show dermal condensation, which is required for hair follicle formation, and thus the defect results in the loss of guard hair (Huh et al., 2013). Furthermore, it has been reported that the Scaleless chicken mutant, which lacks feathers and limb scales, harbours a nonsense mutation in the *Fgf20* gene (Wells et al., 2012). Moreover, the temperature-sensitive *fgf20a* zebrafish mutant shows gradual fin degeneration (Wills et al., 2008) and malformation of bony tissues such as the skull and scales (Cooper et al., 2013). These phenotypes across species are consistent with the notion that Fgf20 plays a conserved role as an essential epidermal signal during appendage development and regeneration in vertebrates.

In addition to the wound epidermis and blastema, recent studies have highlighted the role of nerves in regeneration. Meda et al. (2016) have used a denervation strategy and demonstrated that sensory neurons are responsible for controlling H_2O_2 levels. Furthermore, Simões et al. (2014) have shown that the expression of genes, including *fgf20a*, was altered upon denervation. These data suggest that redox regulation by nerves may play an important role for tissue regeneration through regulation of regeneration-associated genes such as *fgf20a*.

Functions of Fgf3 and Fgf10a during regeneration

In contrast to the epidermal *fgf20a* expression, expression of *fgf3* and *fgf10a* was detected in the distal blastema. A number of studies have suggested that Fgfs, including Fgf3 and Fgf10a, have mitogenic activity (Konishi et al., 2006; Shin et al., 2006; Wang et al., 2007; Itoh and Ornitz, 2008); however, their functions during fin regeneration have not yet been explored. In this study, we performed a gain-of-function analysis of *fgf3* in the cultured blastema explants and demonstrated that Fgf3 promotes cell proliferation. Intriguingly, Fgf3 overexpression did not show an

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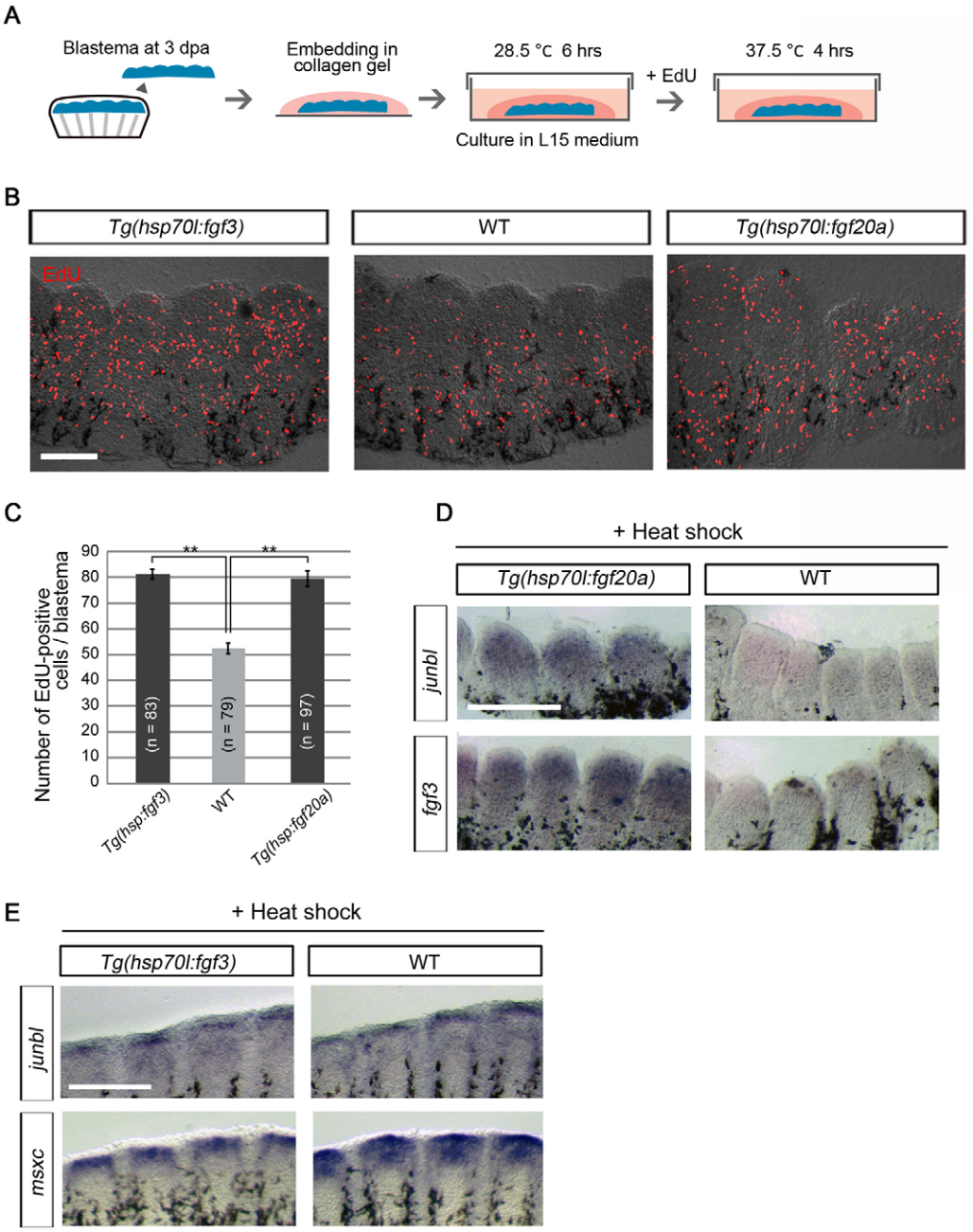

Fig. 6. Expression of *fgf3* promotes proliferation of blastema cells. (A) The cell proliferation assay procedure using a blastema explant. (B) EdU incorporation in the blastema explants from Tg overexpressing *fgf3* or *fgf20a*, and wild-type (WT) zebrafish. EdU was applied for 6 h before sampling. A significant increase in the number of EdU-positive cells in the *fgf3*- and *fgf20a*-overexpressing explants was observed. (C) Quantification of EdU incorporation in the explants. Error bars represent mean±s.e.m. Student's *t*-test was performed to assess statistical significance. $^{**}P<0.01$. *n*, the number of blastema, which were taken from six WT fish, six Tg(*hsp70l:fgf3*) or eight Tg (*hsp70l:fgf20a*). (D) Whole-mount ISH analysis of *junbl* and *fgf3* in *fgf20a*-expressing and WT explants after 20 h at 28.5°C plus 4 h at 37.5°C. A significant increase of *junbl* (*n*=5/5 explants) or *fgf3* (*n*=5/5 explants) was observed. It is thought that the induction of *fgf3* expression by Fgf20a activated cell proliferation. (E) Whole-mount ISH analysis of *msxc* and *junbl* in *fgf3*-expressing and WT fins at 4 dpa. Unlike the *fgf20a*-overexpressing fins (Fig. 5), the *fgf3*-overexpressing fins did not show an increase in expression of *msxc* (*n*=6/6) or *junbl* (*n*=6/6). Scale bars: 100 µm (B); 200 µm (D,E).

increase of cell proliferation in uninjured fins (Fig. S14). It seems that only the blastema cells have a competence to Fgf signalling probably due to the presence of Fgf receptor expression (Poss et al., 2000). Although we did not assess the mitogenic activity of Fgf10a, it is reasonable to assume that this protein could possess a function overlapping with that of Fgf3 (Konishi et al., 2006; Shin et al., 2006).

It has been reported that a mutation in the mouse *Fgf10* gene causes a complete loss of fore- and hindlimbs, and that a mutation in zebrafish *fgf10a* displays a loss of pectoral fin (Min et al., 1998; Sekine et al., 1999; Norton et al., 2005). In the case of regeneration, application of exogenous Fgf10 protein to non-regenerative *Xenopus* limb stumps successfully stimulates regeneration of well-patterned limb structures (Yokoyama et al., 2001). These studies suggest that Fgf10 plays an essential role in the development and regeneration of vertebrate appendages.

Studies in chicks and mice have also shown that Fgf10 expression is activated by epidermal Fgfs in the developing limb, and that it reciprocally signals to the overlying ectoderm to maintain expression of epidermal Fgfs (Ohuchi et al., 1997; Xu et al., 1998). Intriguingly, we also observed that blockage of Fgf signalling results in downregulation of epidermal *fgf20a* and blastemal *fgf10a* and *fgf3* (Fig. S7D; Fig. S10D), suggesting that a similar positive-feedback mechanism involving Fgf10a may operate during fin regeneration.

In addition to Fgf20a, Fgf3 and Fgf10a, other Fgfs may have additional roles in tissue homeostasis. In salamanders, it has been shown that the juxtaposition of anterior and posterior limb tissue plus innervation is necessary and sufficient to induce complete limb regeneration (Nacu and Tanaka, 2011). Nacu et al. (2016) have recently shown that FGF8 and sonic hedgehog provide complementary cross-inductive signals that are required for limb

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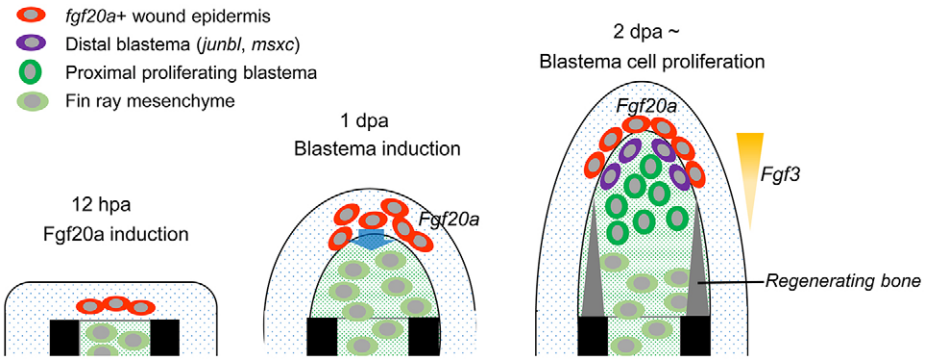

Fig. 7. Diagram of the proposed roles of Fgfs during fin regeneration. The early Fgf signalling mainly mediated by Fgf20 from the wound epidermis induces blastema formation by acting on the underlying mesenchymal cells. At a later stage, after blastema formation, Fgf3, which is upregulated in the blastema, functions as a mitogen and promotes cell proliferation.

outgrowth. Though we did not detect *fgf8* as one of the regeneration-induced Fgf ligands, *fgf8a* expressed in the uninjured epidermis (Fig. S1) could provide a positional cue together with other signals.

Mesenchymal cells in fin rays are the target of Fgfs

In this study, we used a novel approach for dissecting the target cells and analysing the functions of Fgf signalling during regeneration. According to a procedure described previously (Yoshinari et al., 2012), we transplanted *hsp70l*:*dnfgfr1* blastema cells that ubiquitously express the *β-actin1:dsRed2* transgene into a wild-type blastema region. Most of the progeny cells of the transplanted blastema gave rise to fin ray mesenchymal cells (but not osteogenic cells) after regeneration (Fig. 2C). Therefore, by re-amputating fins, we were able to specifically inhibit the action of Fgf signals to the fin ray mesenchymal cells at a desired time point and examine the effect by live cell imaging. Using this mosaic knockdown approach, we successfully demonstrated a direct role of Fgf signals on the mesenchymal cells and observed the effect on their behaviour under live conditions.

Cell transplantation has generally been performed during the early embryonic stage, but targeting the transplanted cells to specific tissues has been difficult. Blastema transplantation is expected to be an efficient method for manipulating a specific signal in labelled mesenchymal cells. Using a similar approach with other signalling molecules in future studies, we will be able to further dissect the cellular behaviour and responses to these signals and elucidate how the signals in question regulate appendage regeneration.

Conclusions

In this study, we revealed that Fgf20a is a signal from the wound epidermis for initiating distal blastema formation. We also showed that blastemal Fgf3 serves as a signal that promotes regenerative cell proliferation of blastema cells. Though it has been known that Fgf signalling is necessary for regeneration, our study firstly elucidated the responsible Fgf ligands and the mechanisms by which fin regeneration is regulated. Given the importance of epidermal Fgf20a for initiation of blastema formation and regeneration, questions remain regarding how *fgf20a* expression is regulated during regeneration. Identification of upstream regulators of *fgf20a* and characterisation of the cross-talk with other signalling pathways will further advance our understanding of the regeneration process.

MATERIALS AND METHODS

Fish husbandry and fin amputation

Zebrafish of a wild-type line, which has been maintained in our facility for more than 10 years by inbred breeding, were kept in a recirculating system in a 14 h day/10 h night cycle at 28.5°C in accordance with the Animal Research Guideline of Tokyo Institute of Technology. The Tg(*hsp70l*:*dnfgfr1-EGFP*)^{pd1} line was obtained from the Zebrafish International Resource Center (ZIRC). The Tg(*hsp70l*:*fgf3*)^{x27} line was a generous gift from Dr Bruce B. Riley. Experiments with adult fin regeneration were performed on 3- to 12-month-old zebrafish. The fish were anaesthetised with tricaine (3-aminobenzoic acid ethyl ester; Sigma) and their caudal fins were cut with a scalpel. For reproducibility, fins were amputated in a straight line in the middle of the central fin ray. The regenerating fins were collected at appropriate time points for further analysis. Heat-shock induction of *dnfgfr1*, *fgf3* or *fgf20a* was performed at 38°C for 1 h in a small water bath. Heat-shock treatment of fish was performed at 6 h before amputation and every 24 h subsequently. For quantitative analysis of cell proliferation, the third to fifth fin rays from the dorsal or ventral side were used for making tissue sections and scoring.

RT-PCR analysis

Total RNA samples were isolated from regenerating fin tissues (three to six fins) using TRIzol (Gibco BRL). For isolation of the wound epidermis and blastema, the regenerating fins at 2-3 dpa were incubated with 25 mg/ml pancreatin (WAKO) in Hank's solution and mechanically split into the epidermal and mesenchymal parts with fine needles and/or forceps. cDNAs were synthesised using the Thermoscript RT-PCR Kit (Invitrogen) with random hexamer as primers. The synthesised cDNAs were diluted to 100 µl with 0.1× TE buffer (1 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at −30°C. PCR was performed with rTaq polymerase and primers listed in Table S1. Forward and reverse primers were designed on separate exons so that only PCR products from cDNAs were amplified. We performed the PCR more than four times using 30, 33, 35 and 37 cycles and obtained similar results.

Whole-mount ISH analysis

Whole-mount ISH was performed according to a previously described protocol (Thisse and Thisse, 2008). After the colour reaction, the samples were mounted with 80% glycerol. For RNA probe synthesis, the Fgf coding sequences were amplified by PCR using KOD polymerase (Toyobo) and cloned into the Zero Blunt TOPO vector (Clontech). Primers for cloning of the Fgf genes are listed in Table S2.

Detection of proliferating cells

Cell proliferation was detected by labelling with Edu using the Click-iT EdU Imaging Kit (Life Technologies). Briefly, zebrafish were incubated in a solution containing 50 µM EdU in fish water (0.3% artificial sea salt, 0.0001% Methylene Blue) for 12 or 6 h at 28.5°C. EdU was detected according to the manufacturer's instructions. The samples were mounted with 80% glycerol containing 25 mg/ml triethylenediamine (DABCO, Nacalai Tesque) as an anti-fading reagent. The labelled cells were counted using the acquired confocal *z*-stack images.

Transplantation of blastema cells

The blastema transplantation was performed as described previously (Yoshinari et al., 2012). The zebrafish lines Tg(*Olactb*:*loxP-dsR2-loxP-EGFP*) (Yoshinari et al., 2012) and Tg(*hsp70l*:*dnfgfr1*) (Lee et al., 2005) were crossed to generate the double-Tg line. The caudal fin of the double-Tg line was amputated and allowed to regenerate until 2 dpa. The blastema and wound epidermis were manually separated with 30-gauge needles in Hank's

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solution and cut into small pieces. The donor blastema was transplanted into the host blastema region by means of a transplantation glass needle. The operated fins were allowed to regenerate for 5 days and re-amputated for analysis of the behaviour of DsRed2-labelled mesenchymal cells.

Immunostaining and histological analysis

Tissues were fixed with 4% PFA (overnight, 4°C) and then briefly washed twice with PBS containing 0.1% Triton X-100 (PBTx), dehydrated using methanol, and stored at −30°C. Immunostaining was performed on tissue sections according to a standard protocol (Ishida et al., 2010). Polyclonal anti-DsRed antibody (632393, Invitrogen) and anti-p63 antibody (ab97865, Abcam) were used at 1:1000. Anti-Zns5 antibody (Developmental Studies Hybridoma Bank) was used at 1:200. The sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 µg/ml in PBTx; Invitrogen) and mounted with 80% glycerol containing DABCO. Images were taken using a confocal microscope (Fluoview FV1000, Olympus).

Generation of the Tg(*hsp70l:mCherry-2a-fgf20a*) line

The Tg zebrafish line that expresses *fgf20a* under control of the *hsp70l* promoter was generated by injecting a plasmid, pTol2 (*hsp70l:mCherry-2a-fgf20a*), into fertilised eggs. The construct was made by replacing the *creERt2* sequence of the *pT2(hsp70l:mCherry-2a-creERt2)* (Yoshinari et al., 2012) with the *fgf20a* coding sequence. The Tg line, which is simply referred to as Tg(*hsp70l:fgf20a*) in this paper, was identified by the expression of mCherry after heat shock and was used for analyses after two generations of out-crosses with wild-type fish.

In vitro cell proliferation assay of the blastema explant

Regenerating tissues were collected from the Tg(*hsp70l:fgf3*), Tg(*hsp70l:fgf20a*) and wild-type fish at 3 dpa. After removal of the wound epidermis, the blastema was embedded into a collagen gel (Cellmatrix type I-A, Nitta Gelatin). The embedded explants were placed in L15 medium containing 100 U/ml penicillin G (Nacalai Tesque) and 100 µg/ml streptomycin (Nacalai Tesque) and incubated at 28.5°C for 6 h. For EdU labelling, the tissues were cultured in the presence of 10 µM EdU at 37.5°C for 4 h. The EdU-labelled cells were counted using the acquired confocal *z*-stack images.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.S., Y.Y. and N.H. conducted the experiments; E.S. and A.Kawakami. analysed the data; G.A. and K.K. generated the *fgf20a* enhancer trap zebrafish line; E.S. and A. Kawakami designed the experiments and wrote the paper; and A. Kudo supervised the research group. All the co-authors read and approved the final version of the manuscript.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.140699.supplemental

References

Akimenko, M. A., Johnson, S. L., Westerfield, M. and Ekker, M. (1995). Differential induction of four msx homeobox genes during fin development and regeneration in zebrafish. *Development* **121**, 347-357.

Akimenko, M. A., Marí-Beffa, M., Becerra, J. and Géraudie, J. (2003). Old questions, new tools, and some answers to the mystery of fin regeneration. *Dev. Dyn.* **226**, 190-201.

Blum, N. and Begemann, G. (2012). Retinoic acid signaling controls the formation, proliferation and survival of the blastema during adult zebrafish fin regeneration. *Development* **139**, 107-116.

Bouzaffour, M., Dufourcq, P., Lecaudey, V., Haas, P. and Vriz, S. (2009). Fgf and Sdf-1 pathways interact during zebrafish fin regeneration. *PLoS ONE* **4**, e5824.

Campbell, L. J. and Crews, C. M. (2008). Wound epidermis formation and function in urodele amphibian limb regeneration. *Cell. Mol. Life Sci.* **65**, 73-79.

Chablais, F. and Jazwinska, A. (2010). IGF signaling between blastema and wound epidermis is required for fin regeneration. *Development* **137**, 871-879.

Chen, C.-H., Merriman, A. F., Savage, J., Willer, J., Wahlig, T., Katsanis, N., Yin, V. P. and Poss, K. D. (2015). Transient laminin beta 1a induction defines the wound epidermis during zebrafish fin regeneration. *PLoS Genet.* **11**, e1005437.

Cooper, W. J., Wirgau, R. M., Sweet, E. M. and Albertson, R. C. (2013). Deficiency of zebrafish fgf20a results in aberrant skull remodeling that mimics both human cranial disease and evolutionarily important fish skull morphologies. *Evol. Dev.* **15**, 426-441.

Gauron, C., Rampon, C., Bouzaffour, M., Ipendey, E., Teillon, J., Volovitch, M. and Vriz, S. (2013). Sustained production of ROS triggers compensatory proliferation and is required for regeneration to proceed. *Sci. Rep.* **3**, 2084.

Goss, R. J. (1956). Regenerative inhibition following limb amputation and immediate insertion into the body cavity. *Anat. Rec.* **126**, 15-27.

Grotek, B., Wehner, D. and Weidinger, G. (2013). Notch signaling coordinates cellular proliferation with differentiation during zebrafish fin regeneration. *Development* **140**, 1412-1423.

Huh, S.-H., Narhi, K., Lindfors, P. H., Haara, O., Yang, L., Ornitz, D. M. and Mikkola, M. L. (2013). Fgf20 governs formation of primary and secondary dermal condensations in developing hair follicles. *Genes Dev.* **27**, 450-458.

Ishida, T., Nakajima, T., Kudo, A. and Kawakami, A. (2010). Phosphorylation of Junb family proteins by the Jun N-terminal kinase supports tissue regeneration in zebrafish. *Dev. Biol.* **340**, 468-479.

Itoh, N. and Ornitz, D. M. (2008). Functional evolutionary history of the mouse *Fgf* gene family. *Dev. Dyn.* **237**, 18-27.

Jaźwińska, A., Badakov, R. and Keating, M. T. (2007). Activin-betaA signaling is required for zebrafish fin regeneration. *Curr. Biol.* **17**, 1390-1395.

Kawakami, A. (2010). Stem cell system in tissue regeneration in fish. *Dev. Growth Differ.* **52**, 77-87.

Konishi, M., Asaki, T., Koike, N., Miwa, H., Miyake, A. and Itoh, N. (2006). Role of Fgf10 in cell proliferation in white adipose tissue. *Mol. Cell. Endocrinol.* **249**, 71-77.

Lee, Y., Grill, S., Sanchez, A., Murphy-Ryan, M. and Poss, K. D. (2005). Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration. *Development* **132**, 5173-5183.

Lee, Y., Hami, D., De Val, S., Kagermeier-Schenk, B., Wills, A. A., Black, B. L., Weidinger, G. and Poss, K. D. (2009). Maintenance of blastemal proliferation by functionally diverse epidermis in regenerating zebrafish fins. *Dev. Biol.* **331**, 270-280.

Meda, F., Gauron, C., Rampon, C., Teillon, J., Volovitch, M. and Vriz, S. (2016). Nerves control redox levels in mature tissues through Schwann cells and hedgehog signaling. *Antioxid. Redox Signal.* **24**, 299-311.

Mescher, A. L. (1976). Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J. Exp. Zool.* **195**, 117-128.

Min, H. S., Danilenko, D. M., Scully, S. A., Bolon, B., Ring, B. D., Tarpley, J. E., DeRose, M. and Simonet, W. S. (1998). Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes Dev.* **12**, 3156-3161.

Munch, J., Gonzalez-Rajal, A. and de la Pompa, J. L. (2013). Notch regulates blastema proliferation and prevents differentiation during adult zebrafish fin regeneration. *Development* **140**, 1402-1411.

Nacu, E. and Tanaka, E. M. (2011). Limb regeneration: a new development? *Annu. Rev. Cell Dev. Biol.* **27**, 409-440.

Nacu, E., Gromberg, E., Oliveira, C. R., Drechsel, D. and Tanaka, E. M. (2016). FGF8 and SHH substitute for anterior–posterior tissue interactions to induce limb regeneration. *Nature* **533**, 407-410.

Nagayoshi, S., Hayashi, E., Abe, G., Osato, N., Asakawa, K., Urasaki, A., Horikawa, K., Ikeo, K., Takeda, H. and Kawakami, K. (2008). Insertional mutagenesis by the Tol2 transposon-mediated enhancer trap approach generated mutations in two developmental genes: tcf7 and synembryn-like. *Development* **135**, 159-169.

Norton, W. H. J., Ledin, J., Grandel, H. and Neumann, C. J. (2005). HSPG synthesis by zebrafish Ext2 and Extl3 is required for Fgf10 signalling during limb development. *Development* **132**, 4963-4973.

Ohmachi, S., Watanabe, Y., Mikami, T., Kusu, N., Ibi, T., Akaike, A. and Itoh, N. (2000). FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. *Biochem. Biophys. Res. Commun.* **277**, 355-360.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M. et al. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235-2244.

Poss, K. D., Shen, J., Nechiporuk, A., McMahon, G., Thisse, B., Thisse, C. and Keating, M. T. (2000). Roles for Fgf signaling during zebrafish fin regeneration. *Dev. Biol.* **222**, 347-358.

Poss, K. D., Keating, M. T. and Nechiporuk, A. (2003). Tales of regeneration in zebrafish. *Dev. Dyn.* **226**, 202-210.
Quint, E., Smith, A., Avaron, F., Laforest, L., Miles, J., Gaffield, W. and Akimenko, M.-A. (2002). Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of sonic hedgehog and bmp2b or exposure to cyclopamine. *Proc. Natl. Acad. Sci. USA* **99**, 8713-8718.
Rampon, C., Gauron, C., Meda, F., Volovitch, M. and Vriz, S. (2014). Adenosine enhances progenitor cell recruitment and nerve growth via its A2B receptor during adult fin regeneration. *Purinergic Signal.* **10**, 595-602.
Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al. (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21**, 138-141.
Shin, M., Noji, S., Neubüser, A. and Yasugi, S. (2006). FGF10 is required for cell proliferation and gland formation in the stomach epithelium of the chicken embryo. *Dev. Biol.* **294**, 11-23.
Simões, M. G., Bensimon-Brito, A., Fonseca, M., Farinho, A., Valério, F., Sousa, S., Afonso, N., Kumar, A. and Jacinto, A. (2014). Denervation impairs regeneration of amputated zebrafish fins. *BMC Dev. Biol.* **14**, 49.
Smith, A., Avaron, F., Guay, D., Padhi, B. K. and Akimenko, M. A. (2006). Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblast differentiation and function. *Dev. Biol.* **299**, 438-454.
Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N. and Moon, R. T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* **134**, 479-489.
Tassava, R. A. and Garling, D. J. (1979). Regenerative responses in larval axolotl limbs with skin grafts over the amputation surface. *J. Exp. Zool.* **208**, 97-110.
Terriente, J., Gerety, S. S., Watanabe-Asaka, T., Gonzalez-Quevedo, R. and Wilkinson, D. G. (2012). Signalling from hindbrain boundaries regulates neuronal clustering that patterns neurogenesis. *Development* **139**, 2978-2987.
Thisse, C. and Thisse, B. (2008). High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59-69.
Thornton, C. S. (1957). The effect of apical cap removal on limb regeneration in Amblystoma larvae. *J. Exp. Zool.* **134**, 357-381.
Tryon, R. C. and Johnson, S. L. (2014). Clonal analysis of kit ligand a functional expression reveals lineage-specific competence to promote melanocyte rescue in the mutant regenerating caudal fin. *PLoS ONE* **9**, e102317.
Wang, X. P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V., Maas, R. L., Chuong, C.-M., Schimmang, T. and Thesleff, I. (2007). An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS Biol.* **5**, 1324-1333.
Wehner, D., Cizelsky, W., Vasudevaro, M. D., Özhan, G., Haase, C., Kagermeier-Schenk, B., Röder, A., Dorsky, R. I., Moro, E., Argenton, F. et al. (2014). Wnt/beta-catenin signaling defines organizing centers that orchestrate growth and differentiation of the regenerating zebrafish caudal fin. *Cell Rep.* **6**, 467-481.
Wells, K. L., Hadad, Y., Ben-Avraham, D., Hillel, J., Cahaner, A. and Headon, D. J. (2012). Genome-wide SNP scan of pooled DNA reveals nonsense mutation in FGF20 in the scaleless line of featherless chickens. *BMC Genomics* **13**, 257.
Whitehead, G. G., Makino, S., Lien, C.-L. and Keating, M. T. (2005). fgf20 is essential for initiating zebrafish fin regeneration. *Science* **310**, 1957-1960.
Wills, A. A., Kidd, A. R., III, Lepilina, A. and Poss, K. D. (2008). Fgfs control homeostatic regeneration in adult zebrafish fins. *Development* **135**, 3063-3070.
Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R. I., Ornitz, D. M., Leder, P. and Deng, C. (1998). Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* **125**, 753-765.
Yokoyama, H., Ide, H. and Tamura, K. (2001). FGF-10 stimulates limb regeneration ability in Xenopus laevis. *Dev. Biol.* **233**, 72-79.
Yoshinari, N., Ishida, T., Kudo, A. and Kawakami, A. (2009). Gene expression and functional analysis of zebrafish larval fin fold regeneration. *Dev. Biol.* **325**, 71-81.
Yoshinari, N., Ando, K., Kudo, A., Kinoshita, M. and Kawakami, A. (2012). Colored medaka and zebrafish: transgenics with ubiquitous and strong transgene expression driven by the medaka beta-actin promoter. *Dev. Growth Differ.* **54**, 818-828.