Adult Zebrafish as a Model System for Cutaneous Wound-Healing Research

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Upon injury, the skin must quickly regenerate to regain its barrier function. In mammals, wound healing is rapid and scar free during embryogenesis, whereas in adults it involves multiple steps including blood clotting, inflammation, re-epithelialization, vascularization, and granulation tissue formation and maturation, resulting in a scar. We have established a rapid and robust method to introduce full-thickness wounds onto the flank of adult zebrafish, and show that apart from external fibrin clot formation, all steps of adult mammalian wound repair also exist in zebrafish. Wound re-epithelialization is extremely rapid and initiates with no apparent lag phase, subsequently followed by the immigration of inflammatory cells and the formation of granulation tissue, consisting of macrophages, fibroblasts, blood vessels, and collagen. The granulation tissue later regresses, resulting in minimal scar formation. Studies after chemical treatment or with transgenic fish further suggest that wound re-epithelialization occurs independently of inflammation and fibroblast growth factor signaling, whereas both are essential for fibroblast recruitment and granulation tissue formation. Together, these results demonstrate that major steps and principles of cutaneous wound healing are conserved among adult mammals and adult zebrafish, making zebrafish a valuable model for studying vertebrate skin repair.

INTRODUCTION

Full-thickness wounds to the skin must be promptly repaired to prevent blood loss and contamination of underlying tissues by foreign particles and pathogens. Cutaneous wound healing in adult mammals is a complex, multistep process involving overlapping stages of blood clot formation, inflammation, re-epithelialization, granulation tissue formation, neovascularization, and remodeling, usually leaving a scar behind (Martin, 1997; Singer and Clark, 1999; Shaw and Martin, 2009). In comparison, wounds in mammalian embryos heal by rapid re-epithelialization and in the absence of inflammation, granulation tissue formation, and scarring (Redd *et al.*, 2004).

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Abbreviations: dpw, days post wounding; FGF, fibroblast growth factor; hpw, hours post wounding; PBS, phosphate buffered saline

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Wound-healing studies in mammalian systems, although of high medical relevance, are costly, technically challenging, and time consuming. Given that major principles of wound repair are conserved, using "lower" organisms would aid in initial steps of the study. During recent years, the zebrafish (*Danio rerio*) has emerged as a model organism for various aspects of human development and disease (Lieschke and Currie, 2007; Li *et al.*, 2011). Wounds in the epidermis of zebrafish and mammalian embryos use similar principles to close (Redd *et al.*, 2004). However, cutaneous wound repair in adult zebrafish has not been studied as yet. During embryonic and larval stages, the zebrafish epidermis is bilayered, consisting of a flattened superficial layer known as the enveloping layer or periderm, which forms tight junctions and fulfils particular barrier functions (Kiener *et al.*, 2008), and a basal layer, which is attached to the underlying basement membrane (Sonawane *et al.*, 2005). A multilayered epidermis is only obtained during metamorphosis, commencing at ~20 and 25 days after fertilization. At the same time, fibroblasts invade the dermis, take over collagen production from basal keratinocytes, and form localized thickenings (dermal papilla) to initiate scale formation (Sire *et al.*, 1997; Le Guellec *et al.*, 2004; Sire and Akimenko, 2004).

Studies in mammalian systems have led to the identification of a number of cytokines and growth factors, which regulate the initial blood clotting response, re-epithelialization, inflammation, or granulation tissue formation (Werner and Grose, 2003; Barrientos *et al.*, 2008). Fibroblast growth factors (FGFs) comprise a family of structurally related secreted proteins that

signal through high-affinity transmembrane tyrosine kinase receptors (FGFr1-4) (Ornitz and Itoh, 2001). In mammals, the expression of FGF1, FGF2, FGF5, FGF7, and FGF10 was found in normal and wounded skin, and the expression of all these FGFs increased after skin injury (Werner and Grose, 2003; Barrientos *et al.*, 2008). Loss of FGF2 signaling by application of blocking antibodies (Broadley *et al.*, 1989) or in *Fgf2* mutant mice (Ortega *et al.*, 1998) results in decelerated wound closure and compromised granulation tissue formation, whereas topical application of FGF2 to wounds of diabetic mice increases granulation tissue formation and wound-healing capacity (Greenhalgh *et al.*, 1990).

In this paper, we describe the development of an assay for studying *in vivo* wound healing using zebrafish as a model system. By using a laser, full-thickness wounds can be quickly and reproducibly introduced on the flank of adult zebrafish. Wounds are re-epithelialized extremely rapidly and independently of blood clot formation and inflammation. Furthermore, granulation-like tissue is formed and later largely cleared, resulting in minimal scar formation. Chemical treatment and transgenic studies reveal essential roles of wound inflammation and FGF signaling for granulation tissue formation, demonstrating genetic and mechanistic conservation of vital wound-healing processes between fish and mammals.

RESULTS

The organization of unwounded skin in the trunk of adult zebrafish

Histological and immunofluorescent analyses with a variety of markers (Supplementary Figure S1 online) demonstrate that the trunk skin of adult zebrafish is composed of overlapping scales, each of which is wrapped by a thin layer of dermal fibroblasts and a multilayered epidermis. Epidermis and dermis are separated by a basement membrane, and dermis and underlying muscle are separated by a layer of subcutaneous adipocytes.

Full-thickness skin wounds are re-epithelialized within hours

We have established a rapid and reproducible technique for introducing wounds of ~2 mm in diameter onto the flank of adult zebrafish, using a clinical dermatology laser (Figure 1a). A vital dye penetration assay, where methylene blue is absorbed by damaged tissue but not undamaged or regenerated epidermis, reveals rapid reestablishment of the barrier by 12 hours post wounding (hpw) (Figure 1b–e). Sections reveal that introduced wounds have initially lost all epidermal and dermal cells, including the scales, and the subcutaneous adipocytes, whereas underlying muscle tissue is undamaged (Figure 1f and i). At 7 hpw, a thin neoepidermis covers most of the wounded surface (Figure 1g and j) and by 24 hpw the wound is completely re-epithelialized, with a neoepidermis of multiple cell layers (Figure 1h and k).

Wounds exhibit a strong inflammatory response, granulation tissue formation, and neovascularization

To analyze the inflammatory response of adult zebrafish, we made use of transgenic lines expressing GFP under the control of the *mpx* promoter to label neutrophils (*Tg(mpx:GFP)*) (Renshaw *et al.*, 2006), or the *lyz* (formerly called *lysC*) promoter, labeling leukocyte lineages that include neutrophils and macrophages (*Tg(lyz:DsRED2/GFP)*) (Hall *et al.*, 2007; Feng *et al.*, 2010). At 24 hpw, wound inflammation is massive and readily visible at lowest magnification (Supplementary Figure S2 Figure). Live imaging was performed to study the kinetics of inflammation. Indirect colabeling of the neoepidermis using the methylene blue penetration assay at 4 hpw reveals that neutrophils remain behind the leading edge of the re-epithelializing epidermis (Figure 2a–d). Consistently, in *Tg(mpx:GFP)/Tg(lyz:DsRED2)* double transgenic fish, inflammatory cells are only present in marginal regions but absent from the center of the wound at 4 hpw (Figure 2e). More inflammatory cells are present at 8 hpw, when the wound is largely re-epithelialized (Figure 2f and i). During the following days, numbers of inflammatory cells slowly drop, leaving mainly macrophages in the wound at 4 days post wounding (dpw) (Figure 2g–i).

During mammalian wound healing, inflammation, and re-epithelialization coincide with granulation tissue formation, characterized by the invasion of fibroblasts, macrophages, and blood vessels into the wound space underneath the neoepidermis (Singer and Clark, 1999). Histologically, a granulation-like tissue in zebrafish wounds is first visible at 2 dpw (Figure 2j and k), reaches maximal size at 4 dpw (Figure 2j and l), and starts to regress again at 6 dpw (Figure 2j, m and n). *col1a2 in situ* hybridization reveals that the granulation tissue largely consists of fibroblasts, which are hardly present at 24 hpw (Figure 2o and p) but in large numbers at 4 dpw (Figure 2q and r). Immunofluorescent analysis at 4 dpw further reveals the presence of collagen type I fibers (Figure 2s) and macrophages in the granulation tissue (Figure 2h and u), whereas neutrophils are largely confined to the neoepidermis (Figure 2t). Furthermore, analysis of *Tg(fli1a:EGFP)* fish shows weak wound neovascularization at 4 dpw (Figure 2v–x), which becomes more prominent at 6 and 8 dpw (Figure 2v, y and z).

Adult wounded zebrafish exhibit minimal scar formation

Analysis at later stages after wounding demonstrates the progressive regression of the granulation tissue so that at 10 dpw very few cells can be seen beneath the neoepidermis. New scales are forming, and the collagen distribution resembles that of an unwounded region (Figure 3a–c). Analysis of *Tg(mpx:GFP)* (Figure 3d and e) and *Tg(fli1a:EGFP)* (Figure 3f and g) transgenic fish at 10 dpw reveals a reduction in the number of leukocytes and blood vessels back to levels as in unwounded skin. At 28 dpw, the healed wound (Figure 3h) is largely indistinguishable from the unwounded skin (Figure 3i), with a normal epidermis and dermal compartment, and with completely recovered subcutaneous adipocytes and scales (Figure 3m–o). Even the striped pigmentation is largely recovered (Figure 3j–l). The only remaining indication of the wound is a small region of collagen deposition in the muscle layer, as indicated by acidic fuchsin orange G staining (arrowed in Figure 3h), although this was not apparent in all cases.
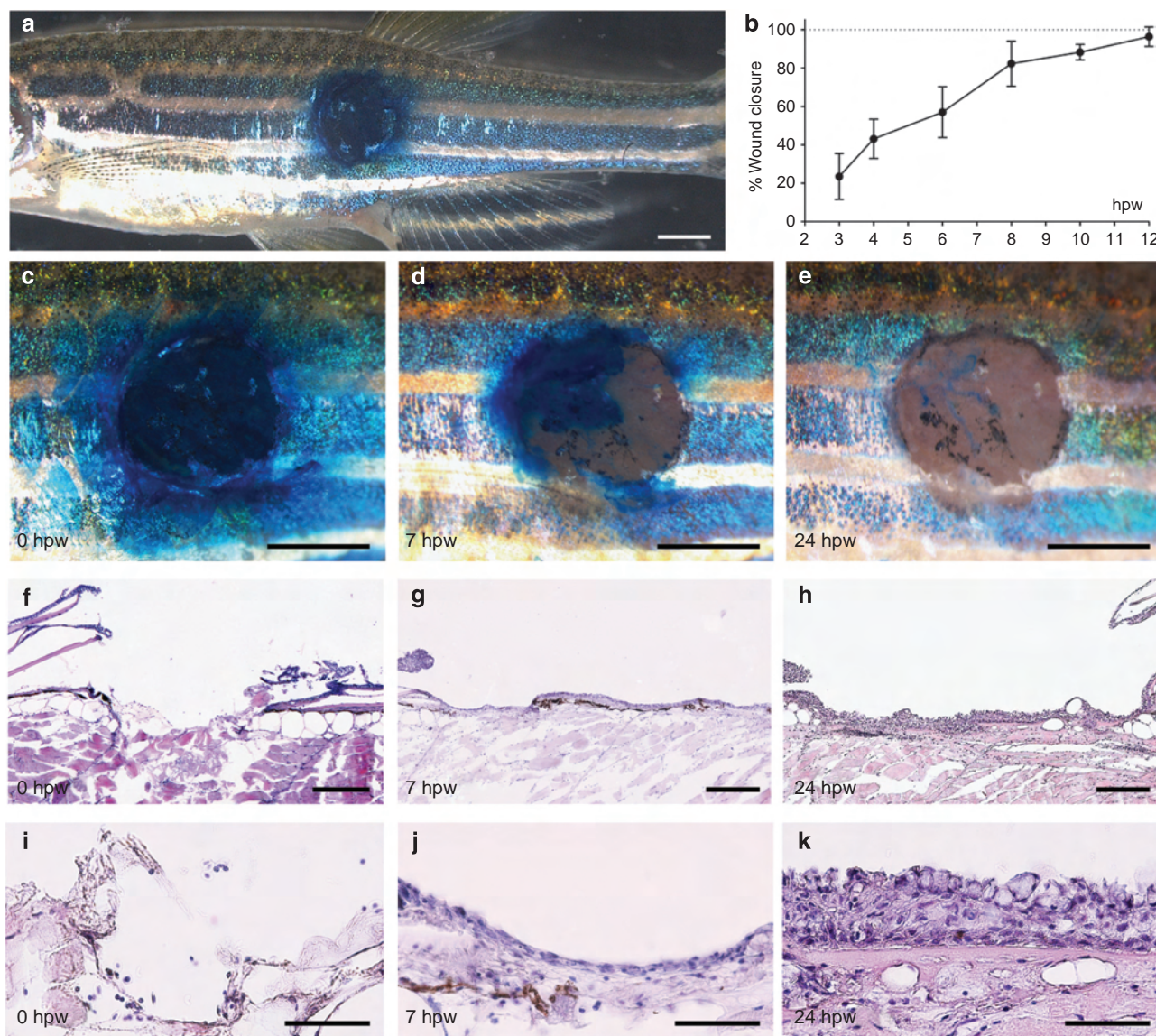
Figure 1. Wounds of adult zebrafish undergo rapid re-epithelialization. (**a**) Overview of the left flank of an adult zebrafish with an ∼2 mm circular wound stained with methylene blue. (**b**) Graphical illustration of time course of wound closure (see Material and Methods); mean values of closure and SD were determined for at least six individuals per time point using Excel software. (**c–e**) Magnified superficial views of the wound. At 0 hours post wounding (hpw) (**a**, **c**), methylene blue penetrates the entire wound, whereas the epidermal barrier has been partly recovered by 7 hpw (**d**) and fully recovered by 24 hpw (**e**). (**f–k**) Hematoxylin and eosin (H&E) staining of longitudinal sections through the wound reveals the removal of epidermis, dermis, and scales by the applied wounding protocol (**f**, **i**; $n=4$). At 7 hpw, a thin neoepidermis is observed on the surface of the wound (**g**, **j**; $n=6$). At 24 hpw, the recovered epidermis appears restratified (**h**, **k**; $n=10$). Bars: **a**, **c–e** = 1 mm; **f–h** = 200 μm; **i–k** = 50 μm.

## Wound depth affects the extent of tissue regeneration

In order to determine whether the extent of regeneration in the adult fish was due to the shallowness of the wounds we introduced, we also analyzed deeper wounds, reaching into the muscle tissue (Supplementary Figure S3 online). According to the vital dye assay and histological analysis at 8 and 24 hpw, deeper wounds re-epithelialize at normal rates, yielding a fully restratified wound epidermis (Supplementary Figure S3a–i online). Furthermore, dermis and scales are fully regenerated at 28 dpw (Supplementary Figure S3m–o online). In contrast, the regeneration of the lost muscle tissue beneath the deeper wounds is incomplete, with

damaged regions often filled with additional subcutaneous adipocytes (Supplementary Figure S3m–o online). Furthermore, pigmentation regenerates in more irregular patterns (Supplementary Figure S3j–l online). This indicates that also in deeper wounds, the skin and its appendages readily regenerate with minimal scarring.

Blood clotting and inflammation have no role in re-epithelialization of adult zebrafish wounds

To gain insights into functional interdependences of the different steps of wound healing and into possible evolutionary conservation of molecular control mechanisms, we carried
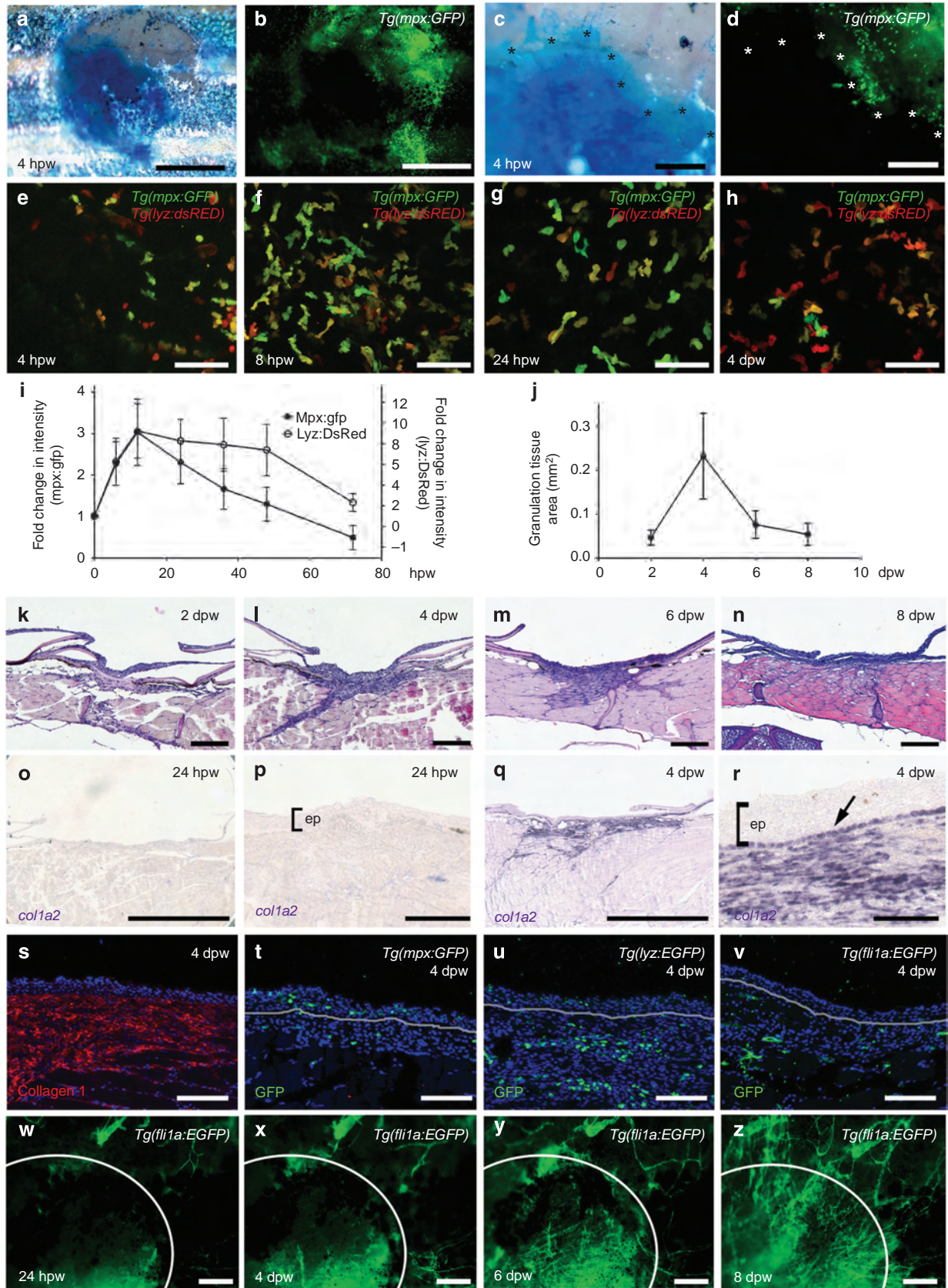
out a series of chemical treatments and transgenic analyses. In adult mammals, the formation of an external fibrin clot is a critical initial wound-healing response, serving as a temporary seal for the damaged tissue, a source of signals that stimulate later processes of wound healing, and a substrate for invading cells. We treated adult fish with 150 μM sodium warfarin, an inhibitor of the blood clotting process, which has previously been used in zebrafish (Jagadeeswaran and Sheehan, 1999;
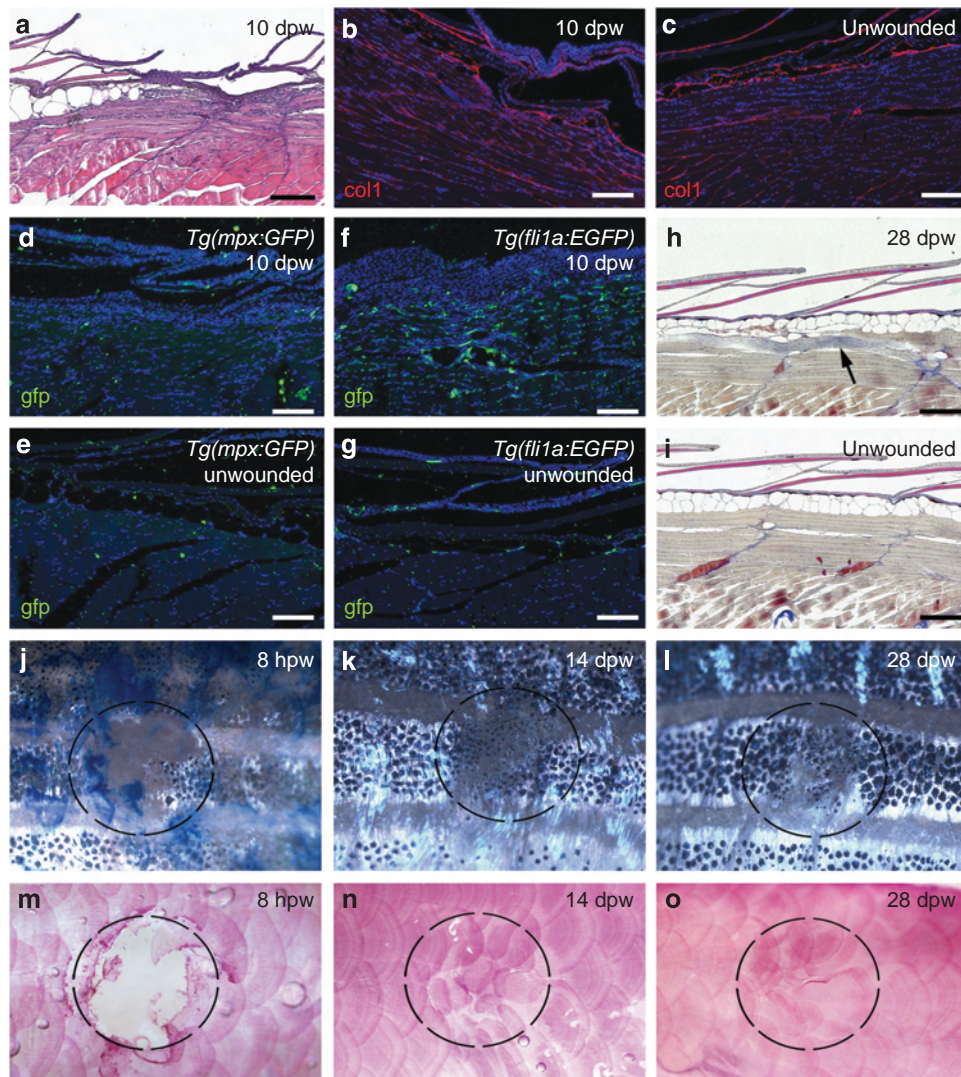
Figure 3. Adult zebrafish exhibit minimal scar formation. (**a**) Hematoxylin and eosin (H&E) staining at 10 days post wounding (dpw) reveals minimal remaining granulation tissue and newly forming scales. (**b, c**) At 10 dpw, minimal collagen1 deposition is observed beneath the regenerating scales (**b**), similar to an unwounded region (**c**) (*n* = 6/6). (**d–g**) *Tg(mpx:GFP)* (**d, e**; *n* = 4/4) and *Tg(fli1a:EGFP)* (**f, g**; *n* = 4/4) transgenic fish at 10 dpw display normal number of leukocytes (**d**) and reduced numbers of blood vessels (**f**) at the regenerating wound site when compared with an unwounded region (**e, g**). (**h, i**) Acidic fuchsin orange G (AFOG) staining at 28 dpw indicates the complete recovery of epidermis, dermis, scales, and adipocytes with rarely occurring collagen deposits within the muscle layer beneath (**h**; *n* = 4/4), compared with unwounded fish (**i**). (**j–o**) Superficial views of wounded fish demonstrate the almost complete recovery of stripe pattern (**j–l**) and scales (**m–o**; alizarin red) by 28 dpw (*n* = 6/6). Dashed circles mark the position of the wound. Bar: **a, h, i** = 500 μm; **b–g** = 200 μm. col1, collagen1.

◀

Figure 2. Adult zebrafish exhibit a strong inflammatory response and granulation tissue formation. (**a–d**) Brightfield (**a, c**) and fluorescent (**b, d**) images of a *Tg(mpx:GFP)* fish at 4 hpw; GFP-positive neutrophils are present in re-epithelialized (methylene blue excluding) regions of the wound (edges marked by asterisks in **c** and **d**; *n* = 6/6). (**e–h**) Live images of the wound center of *Tg(mpx:GFP)i114/Tg(lyz:DsRED2)117* double transgenic fish reveal inflammatory cells, with a progressive relative increase of macrophages (**h**). (**i, j**) Graphical illustrations of time course of wound inflammation (**i**) and granulation tissue formation (**j**); mean values and SD of relative fluorescent intensities (**i**) or granulation tissue areas (**j**) were determined for at least six individuals per time point using Excel software. (**k–n**) Hematoxylin and eosin (H&E) staining reveals formation of granulation tissue beneath the wound from 2–6 days post wounding (dpw), which then regresses (8 dpw, n). (**o–r**) *col1a2* expression beneath the wound is sparse at 24 hours post wounding (hpw) (**o, p**; *n* = 4/4) but very prominent at 4 dpw (**q, r**; *n* = 6/6). In addition to dermal fibroblasts, *col1a2* is expressed by basal keratinocytes of the neoepidermis (indicated by arrow in *r*). (**s–v**) Immunofluorescence analysis at 4 dpw reveals collagen 1 deposition (**s**; *n* = 4/4), leukocytes (**t, u**; *n* = 4/4), and blood vessels (**v**; *n* = 4/4) within the granulation tissue. *mpx*-positive neutrophils are also present in the neoepidermis (**t**; *n* = 4/4). (**w–z**) Superficial views of a *Tg(fli1a:EGFP)* fish shows progressive wound vascularization from 24 hpw to 8 dpw (*n* = 4/4). Scale bars: **a, b, o, q** = 1 mm; **c–d, w–z** = 250 μm; **e–h** = 50 μm; **k–n** = 500 μm; **p, r, s–v** = 100 μm. ep, epidermis; GFP, green fluorescent protein.

Hanumanthaiah *et al.*, 2001) and which causes localized internal bleeding of treated fish (Figure 4m–p). However, warfarin treatment has no effect on the re-epithelialization of introduced wounds, which close at the same rate and yield a multilayered neoepidermis as in controls (Figure 4a–l). In addition, we failed to detect an external fibrin clot during our histological examinations (Figure 2), indicating that re-epithelialization of cutaneous zebrafish wounds occurs independently of blood clotting. Similarly, abrogation of inflammation by treatment of *Tg(mpx:GFP)* and *Tg(mpx:GFP)/Tg(lyz:DsRED2)* fish with hydrocortisone has no effect on the rate of epidermal barrier recovery (Figure 5), suggesting that re-epithelialization is uncoupled from wound inflammation.

Impairment of inflammation and transgenic inhibition of FGF signaling results in a compromised granulation tissue formation
In mammals, formation of granulation tissue has been shown to depend both on wound inflammation (Leibovich and Ross,

1975; Eming *et al.*, 2009) and FGF2 function (Broadley *et al.*, 1989; Ortega *et al.*, 1998). Indeed, hydrocortisone-induced impairment of inflammation in wounds of the zebrafish results in reduced granulation tissue (Figure 6a–d). To investigate the role of FGF signaling during granulation tissue formation, we made use of *Tg(hsp70l:dnfgfr1-EGFP)* transgenic zebrafish for temporally controlled ubiquitous expression of a C-terminally truncated mutant form of Fgf receptor 1 in which the cytoplasmic tyrosine kinase domain is replaced by GFP. Upon dimerization with endogenous receptors, this truncated receptor is predicted to block all FGF receptor subtypes in a dominant-negative manner, as shown for Fgfr1-, Fgfr2-, and Fgfr4-dependent processes in larval and adult zebrafish (Lee *et al.*, 2005; Lepilina *et al.*, 2006). Heat-shock treatment of *Tg(hsp70l:dnfgfr1-EGFP)* fish results in a normal neoepidermis, but almost complete absence of granulation tissue at 4 dpw (Figure 6f, I and j), whereas granulation tissue of normal size is present in heat-shocked nontransgenic control fish (Figure 6e,
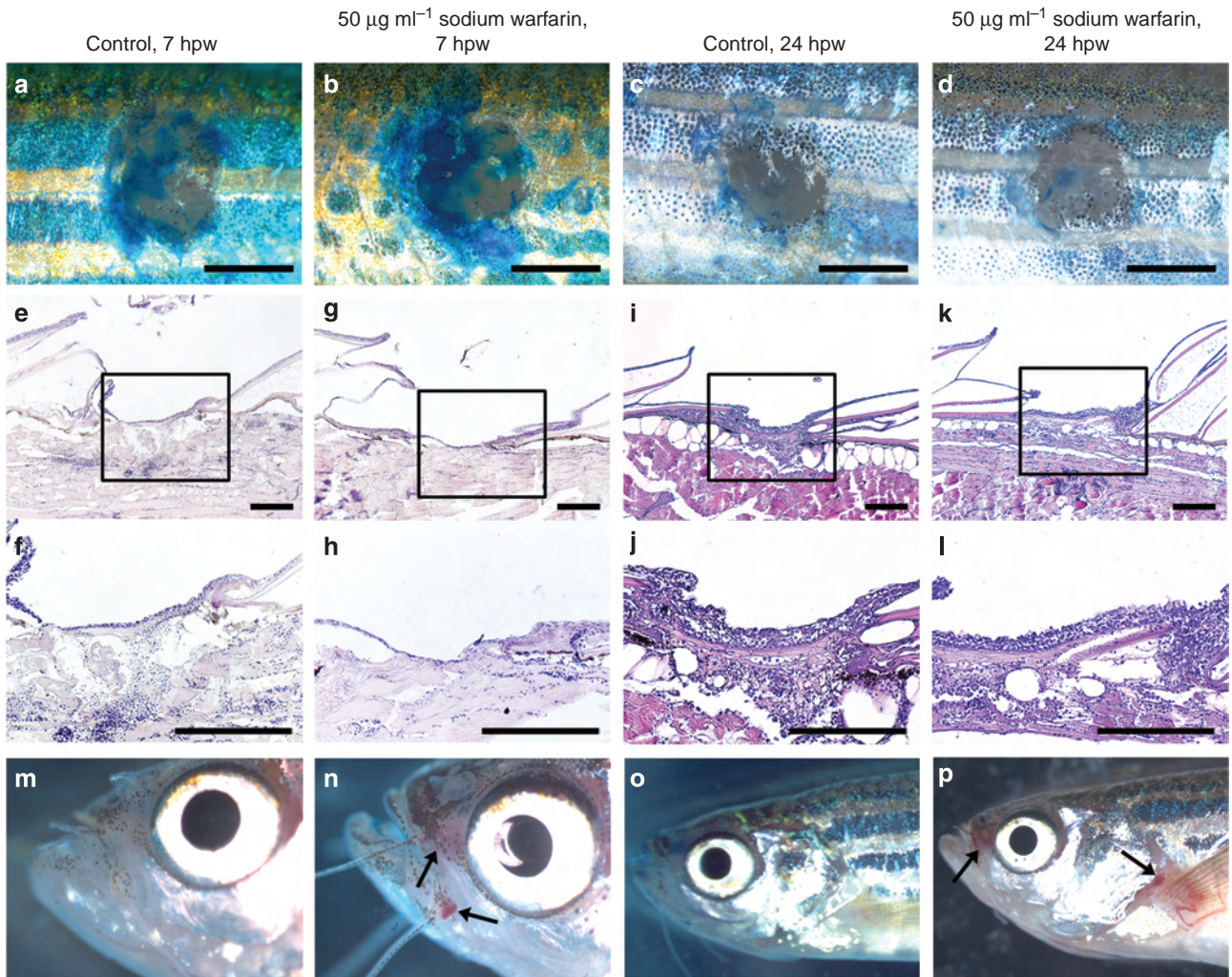
Figure 4. Blood clot formation has no role in the re-epithelialization process. Methylene blue penetration assay (**a–d**) and hematoxylin and eosin (H&E)-stained longitudinal sections (**e–l**) of wounds from control fish (**a, c, e, f, i, j**) and fish treated with 150 μM sodium warfarin (**b, d, g, h, k, l**), demonstrating no differences in the degree of barrier recovery or wound re-epithelialization at 7 hours post wounding (hpw) (**a, b, e–h**) or 24 hpw (**c, d, i–l**) (*n* = 18/18), even though warfarin-treated fish show localized internal bleeding, particularly around the mouth, gills, and pec fins at 7 and 24 hpw (**m–p**). Bar: **a–d** = 1 mm; **e–l** = 100 μm.
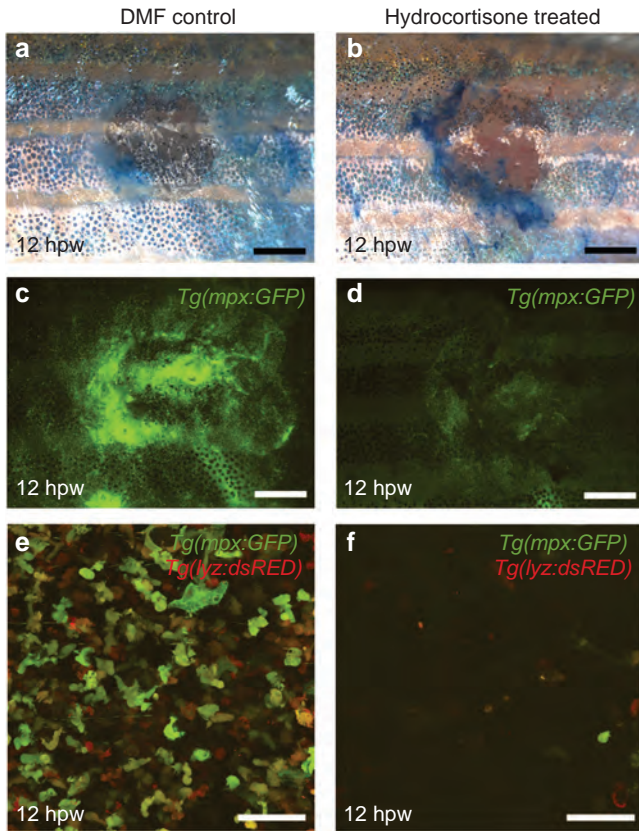
Figure 5. Reducing the inflammatory response does not affect re-epithelialization. (**a–f**) *Tg(mpx:GFP)* (**a–d**) and *Tg(mpx:GFP)/Tg(lyz:DsRED2)* fish (**e**, **f**) treated with 275 μM hydrocortisone show no delay in re-epithelialization (**a**, **b**; *n* = 19/19), although the number of inflammatory cells at the wound is clearly reduced at 12 hours post wounding (hpw) when compared with control fish (**c–f**). Bar: **a–d** = 1 mm; **e–f** = 50 μm.

g and h). *col1a2 in situ* hybridization analysis further indicates the absence of wound fibroblasts in heat-shocked *Tg(hsp70l: dnfgfr1-EGFP)* fish (Figure 6k and l). However, blockage of FGF signal transduction does not affect wound macrophage numbers at 1 dpw (Figure 6m–p) or wound vascularization (Figure 6q–t).

DISCUSSION
The different steps of wound repair in adult mammals and fish
Mammalian wound healing involves several phases and processes that overlap in time: blood clotting, inflammation, re-epithelialization, granulation tissue formation, neovascularization, and tissue remodeling/scar resolution (Singer and Clark, 1999; Shaw and Martin, 2009). Our data indicate that, apart from blood clot formation, all processes also take place during wound repair in adult zebrafish. However, the time course of individual process initiation appears slightly different. In mammals, blood clotting occurs first, providing chemotactic factors that attract inflammatory leukocytes and extracellular matrix proteins that serve as a migration substrate for immigrating cells. Neutrophils cleanse the wounded area and are eventually phagocytosed by macrophages, which enter the wound slightly later (Kim *et al.*, 2008).

Macrophages further secrete various growth factors to attract keratinocytes, fibroblasts, and blood vessels into the wound, promoting re-epithelialization, granulation tissue formation, and vascularization (Singer and Clark, 1999; Shaw and Martin, 2009).

Although there is some debate over the lineage specificity of the *lyz* promoter in zebrafish, it has been suggested that *mpx*-expressing cells are neutrophils and *lyz*-expressing cells are macrophages (Feng *et al.*, 2010). Therefore, our results suggest that, as in mammals, macrophages remain in healing wounds longer than neutrophils and that inflammation precedes granulation tissue formation and vascularization (Figure 2). However, re-epithelialization starts even before inflammation and in the absence of blood clotting (warfarin treatment; Figure 4), inflammation (hydrocortisone treatment; Figure 5), or granulation tissue (Figure 6). Thus, re-epithelialization seems to be largely independent of ECM proteins supplied by the blood clot or the granulation tissue. This might be because of a predominant impact of tissue-autonomous extension movements within the re-epithelializing epidermis (RR and MH, unpublished data), a process that is likely to also occur in wounds of mammalian embryos (Caddy *et al.*, 2010). In addition, re-epithelialization is independent of wound debridement by neutrophils and chemotactic signals from macrophages. Interestingly, also in mouse, wounds close with almost normal rates in the absence of neutrophils and macrophages (Dovi *et al.*, 2003; Martin *et al.*, 2003), suggesting that, similar to our findings in zebrafish, re-epithelialization does not require an inflammatory response.

Conserved roles of inflammation and FGF signaling for granulation tissue formation in mammals and fish
In contrast, inflammation seems necessary for fibroblast recruitment, granulation tissue formation, and wound vascularization both in mammals and fish (Figure 6). Thus, hydrocortisone treatment in zebrafish compromises granulation tissue formation similar to the effects caused by similar treatments in mammals (Leibovich and Ross, 1975) and pointing to the existence of fibroblast-stimulating signals from inflammatory cells both in fish and mammals. However, as the effects of hydrocortisone treatment may not be restricted to inflammation, more sophisticated transgenic ablation experiments, as performed in the mouse (Lucas *et al.*, 2010), will be necessary to provide ultimate proof for the requirement of inflammatory cells and to identify the relevant stimulatory signals. Our data obtained upon global transgenic inhibition of FGF signal reception point to a thus far not further specified FGF as essential for granulation tissue formation, again in agreement with results obtained by the loss of FGF2 function in mouse (Broadley *et al.*, 1989; Ortega *et al.*, 1998). In contrast, FGF signaling is dispensable for wound re-epithelialization, inflammation, and vascularization, pointing to a direct effect of FGF signaling on fibroblasts. Indeed, cultured mammalian wound fibroblasts can directly respond to FGF2 (Sasaki, 1992; Rahimi *et al.*, 2005), whereas fibroblasts of the zebrafish granulation tissue show strong expression of the FGF receptor *fgfr1a* (CK and MH, unpublished results). FGFs are well-known stimulators of
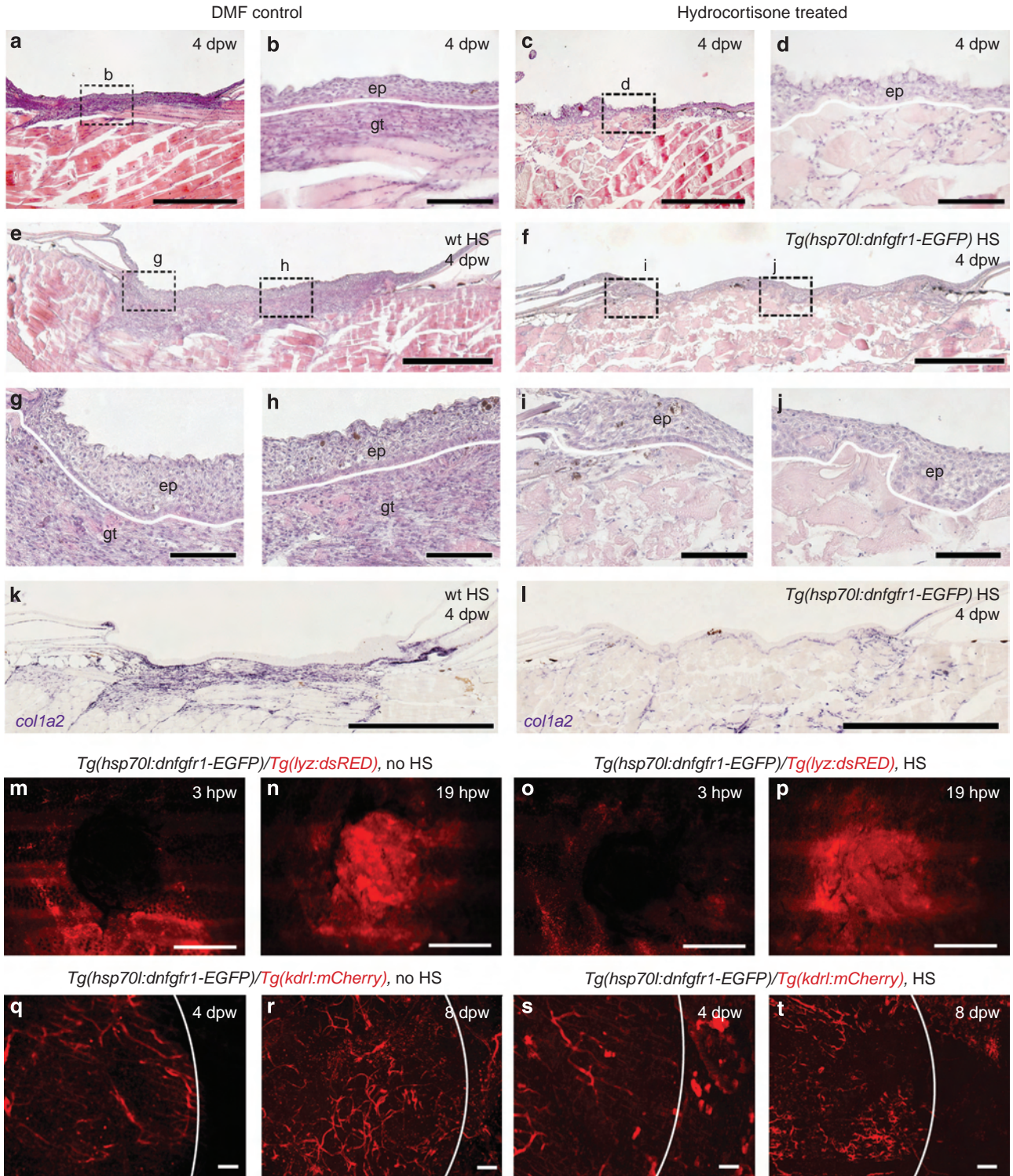
Figure 6. Inflammation and fibroblast growth factor (FGF) signaling are required for granulation tissue formation. (**a–d**) Hydrocortisone-treated fish at 4 days post wounding (dpw), lacking granulation tissue beneath the wound (**c, d**; $n = 9/9$) in comparison with DMF-treated controls (**a, b**). (**e–j**) Hematoxylin and eosin (H&E) staining demonstrates granulation tissue beneath the wound epidermis in heat-shocked wild-type fish (**e, g, h**; $n = 6/6$) but not in the heat-shocked *Tg(hsp70l:dnfgfr1-EGFP)* fish (**f, i, j**; $n = 6/6$) at 4 dpw. (**k, l**) *In situ* hybridization analysis reveals normal *col1a2* expression in a heat-shocked wild-type fish (**k**; $n = 4/4$), but strongly reduced expression in a heat-shocked *Tg(hsp70l:dnfgfr1-EGFP)* fish at 4 dpw (**l**; $n = 4/4$). (**m–t**) Analysis of *Tg(hsp70l:dnfgfr1-EGFP),Tg(lyz:dsRED)* (**m–p**) or *Tg(hsp70l:dnfgfr1-EGFP), Tg(kdrl:HSRAS-mCherry)* (**q–t**) double transgenic fish reveals normal inflammatory responses (**m–p**) and normal vascularization (**q–t**) in non–heat-shocked (**m, n**; $n = 8/8$; **q, r**; $n = 8/8$) and heat-shocked *Tg(hsp70l:dnfgfr1-EGFP)* fish (**o, p**; $n = 8/8$; **s, t**; $n = 8/8$). Bar: **a, c, e, f** = 500 μm; **b, d, g–j, q–t** = 100 μm; **k–p** = 1 mm. ep, epidermis; gt, granulation tissue; HS, heat-shocked; wt, wild-type.

angiogenesis (Lieu *et al.*, 2011). The fact that FGF signaling is dispensable for the neovascularization of zebrafish wounds might be because of other related signals, such as vascular endothelial growth factors, which are crucially involved in the vascularization of mouse wounds (Barrientos *et al.*, 2008).

Together, this points to an evolutionary conservation of crucial molecular mechanisms underlying granulation tissue formation in different vertebrate classes.

Adult zebrafish exhibit scar-free skin regeneration

A striking difference between mammalian and fish wound repair is that wound-tissue remodeling in zebrafish is completed with minimal scarring (Figure 3 and Supplementary Figure S3 online). The granulation tissue resolves completely, inflammation terminates, and blood vessels regress within 10 dpw. Even in deeper wounds with damage of underlying muscle, the skin regenerates almost perfectly, whereas the damaged muscle does not (Supplementary Figure S3 online).

We can only speculate about the mechanisms of the perfect skin remodeling and possible differences between fish and mammals. Macrophageless PU.1 null neonatal mice repair wounds without an apparent fibrotic response (Martin *et al.*, 2003). Furthermore, the capability of scar-free wound healing during mouse embryogenesis is lost at the same stage (E15) when first inflammatory responses can be observed. On the basis of these observations, a causative relationship between inflammation and scarring has been proposed (Martin and Parkhurst, 2004). Our finding of scar-free wound healing in adult zebrafish, despite a massive inflammatory response, speaks against this notion. The speed of re-epithelialization, the lack of cornification in outer epidermal layers (Supplementary Figure S1 online), which only evolved in higher, land-based vertebrates, and the exposure to a liquid environment could be crucial factors in this regeneration capability. In mammals, similar conditions apply to mucosal injuries and to fetal cutaneous wounds, which, strikingly, also close rapidly and with minimal scar formation (Szpaderska *et al.*, 2003; Rolfe and Grobbelaar, 2012).

The adult zebrafish as a valuable *in vivo* model of cutaneous wound repair

In our opinion, the described combination of shared and class-specific features of wound healing in fish and mammals makes the adult zebrafish a valuable model for comparative studies, which should also increase our understanding of mammalian wound repair and the cellular, molecular, and genetic bases of human wound-healing pathologies. Compared with mammals, in which the different processes of wound healing largely overlap in time, they occur in a more subsequent manner in zebrafish, which should allow us to better dissect direct from indirect effects obtained after chemical or genetic interference. This is exemplified above for the roles of inflammation and FGF signal reception during granulation tissue formation. The established zebrafish wounding protocol is robust and fast and can be used for large-scale forward genetic screening. In addition, the adult zebrafish seems highly suitable for treatments with chemical inhibitors, which

are simply added to the water, allowing to screen libraries for drugs interfering with wound healing or alleviating particular wound-healing pathologies.

MATERIALS AND METHODS
Zebrafish and wounding

Adult zebrafish were wounded at an age of 6–12 months. The transgenic lines used, Tg(krt4:egfp)gz7, Tg(mpx:GFP)i114, Tg(lyz:;EGFP) nz117, Tg(lyz:dsRED2)nz50, Tg(fli1a:EGFP)y1, Tg(kdrl:HSRAS:mCherry) s896, and Tg(hsp70l:dnfgfr1-EGFP)pd1, have been described previously (Gong *et al.*, 2002; Lawson and Weinstein, 2002; Lee *et al.*, 2005; Renshaw *et al.*, 2006; Hall *et al.*, 2007; Chi *et al.*, 2008).

For wounding, adult fish were anesthetized in 0.13% Tricaine (w/v) and laid on Whatman paper soaked in system water. A full-thickness wound was introduced onto the left flank directly anterior to anal and dorsal fins (Figure 1a). An Erbium:YAG MCL29 Dermablate dermatology laser (Asclepion, Jena, Germany) was set to a frequency of 5 Hz and two pulses with a strength of 500 mJ for smaller (20–30 mm) or 600 mJ for larger (30–40 mm) specimens were applied, resulting in a pulse strength of 7.1 or $8.5 \, \mathrm{J \, cm^{-2}}$, respectively. For heat shock–induced transgene activation, *Tg(hsp70l:dnfgfr1-EGFP)* fish were transferred from 28 °C to prewarmed water at 40 °C for 1 hour, returned to water at 28 °C, and wounded 4 hours later. Heat-shock treatments were repeated every 24 hours.

All zebrafish experiments were approved by the national animal care committee (LANUV Nordrhein-Westfalen; 8.87-50.10.31.08.130; 84-02.04.2012.A253) and the University of Cologne.

Tissue-labeling procedures

For methylene blue penetration, fish were anesthetized in 0.13% Tricaine and placed on Whatman paper soaked with water. A drop of an aqueous 0.1% (w/v) methylene blue solution was applied to the wound for 1 minute, followed by extensive washing. For quantification, open (blue) and total wound areas were measured using ImageJ software (NIH, Bethesda, MD).

For histological and immunofluorescence analyses adult zebrafish were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) overnight at 4 °C then washed with 1x PBS. For whole-mount immunofluorescence analysis, fish were washed for several hours in dH_20 and blocked in PBS + 10% fetal calf serum. Antibody incubations were carried out in PBS + 10% fetal calf serum and washes in PBS + 0.5% Triton-X. Mineralized bone was stained with alizarin red as described (Walker and Kimmel, 2007).

For sectioning, samples were fixed in 4% paraformaldehyde, decalcified in 0.5 M EDTA (pH 7.4) at room temperature for 5 days, dehydrated in a graded series of alcohols, cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany), and embedded in paraffin. Sections of 8 μm thickness were stained with hematoxylin and eosin, acidic fuchsin orange G, or periodic acid-Schiff reaction, using standard protocols. For quantification of granulation tissue sizes, consecutive sections of wounds were stained with hematoxylin and eosin. Sections comprising the center of the wound were selected and granulation tissue areas measured using ImageJ.

Immunofluorescence analysis on paraffin-embedded tissue was performed using standard protocols. Primary antibodies used were: anti-p63 (1:100, sc-8431, Santa Cruz, Dallas, TX), anti-gfp (1:100, A10262, Invitrogen, Carlsbad, CA), and anti-col1 (1:200, ab23730, Abcam, Cambridge, UK).

In situ hybridization on paraffin-embedded sections was performed as described previously (Hyde *et al.*, 2007). *col1a2* complete cDNA was excised from EST clone IMAGp998C1714602Q and cloned into pCMV-SPORT6.1. Probe was synthesized with *Sma*I and T7 RNA polymerase.

Drug treatments

Adult zebrafish were treated with sodium warfarin (150 μM; A4571—Sigma) or hydrocortisone (275 μM; H4001—Sigma Aldrich, St Louis, MO) in fish system water. In each case, fish were treated with the drug for at least 12 hours before wounding. The drug-containing water was exchanged every 24 hours when necessary.

Imaging

Transgenic fish were anesthetized in 0.13% Tricaine (w/v) and photographed using a Zeiss Apotome, Zeiss Confocal (LSM710 META, Carl Zeiss, Jena, Germany), or Leica M165 FC microscope (Leica, Solms, Germany). For quantification of inflammation, entire wounds of double transgenic *Tg(mpx:GFP)i114/Tg(lyz:dsRED2)nz50* fish were repetitively imaged at indicated time points using identical settings. Mean fluorescent intensity values of the wounded area were measured using ImageJ and expressed as fold change in intensity compared with that directly after wounding.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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