

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull

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ABSTRACT

Zebrafish have an unlimited capacity to regenerate bone after fin amputation. In this process, mature osteoblasts dedifferentiate to osteogenic precursor cells and thus represent an important source of newly forming bone. By contrast, differentiated osteoblasts do not appear to contribute to repair of bone injuries in mammals; rather, osteoblasts form anew from mesenchymal stem cells. This raises the question whether osteoblast dedifferentiation is specific to appendage regeneration, a special feature of the lepidotrichia bone of the fish fin, or a process found more generally in fish bone. Here, we show that dedifferentiation of mature osteoblasts is not restricted to fin regeneration after amputation, but also occurs during repair of zebrafish fin fractures and skull injuries. In both models, mature osteoblasts surrounding the injury downregulate the expression of differentiation markers, upregulate markers of the pre-osteoblast state and become proliferative. Making use of photoconvertible Kaede protein as well as Cre-driven genetic fate mapping, we show that osteoblasts migrate to the site of injury to replace damaged tissue. Our findings suggest a fundamental role for osteoblast dedifferentiation in reparative bone formation in fish and indicate that adult fish osteoblasts display elevated cellular plasticity compared with mammalian bone-forming cells.

KEY WORDS: Dedifferentiation, Osteoblast, Bone, Fracture, Injury, Zebrafish, Lepidotrichia, Skull, Osteocalcin, Kaede

INTRODUCTION

In contrast to mammals, adult fish and urodele amphibia (salamanders and newts) can efficiently regenerate appendages (fins and limbs) after amputation. Bone is a major element of appendages, and the ability to regenerate bony structures during fin and limb regeneration appears to be essentially unlimited (Hall, 2005; Han et al., 2005). Bone repair also occurs in adult mammals, but internal bone defects can only be healed below a critical size (Hall, 2005), and regeneration of bony elements lost due to amputation does not occur. Interestingly, even in salamander limbs the capacity to regenerate bone after amputation is higher than the ability to repair internal bone defects, which – similar to mammals – can only be repaired below a critical size (Satoh et al., 2010). Intriguingly, transplantation of blastemal cells, undifferentiated

progenitors that form after limb amputation, into a critical size bone defect can rescue repair of such bone injuries (Satoh et al., 2010). Thus, it is conceivable that different cellular mechanisms underlie bone defect repair and bone regeneration in the context of an amputated appendage.

Identification of the cellular source of regenerating appendages has been a long-standing issue in regeneration research. Although many adult tissues contain stem cells, whose activation and expansion could drive regeneration, adult differentiated cells can also represent sources of newly forming tissue (Tanaka and Reddien, 2011). Indeed, we and others have recently discovered that mature osteoblasts dedifferentiate to lineage-restricted osteoblast progenitors and thus provide an important source of newly forming bone during regeneration of the zebrafish caudal fin (Knopf et al., 2011; Sousa et al., 2011). Dedifferentiation, ‘a process in which a cell loses its specialized morphology, function and biochemistry to initiate cell division and reverts to a less differentiated state in order to redifferentiate again’ (Hall, 2005), is generally assumed to occur during salamander and teleost fish appendage regeneration (Monaghan and Maden, 2013). Recently, genetic lineage-tracing techniques have indeed shown that muscle regenerates from dedifferentiating muscle fibers in regenerating limbs of newts, but surprisingly not in axolotls, indicating that the cellular mechanisms of regeneration are diverse even within fairly closely related animal groups (Sandoval-Guzman et al., 2014).

Intriguingly, genetic lineage tracing has shown that mature osteoblasts do not contribute to bone repair in mammals (Park et al., 2012), and dedifferentiation of mature osteoblasts is not considered to occur in mammalian bone biology [with the possible exception of pediatric osteosarcomas (Pereira et al., 2009)]. Instead, during mammalian fracture healing, new osteoblasts appear to be supplied from mesenchymal stem cells, which, however, are restricted to only give rise to osteoblasts *in vivo* (Park et al., 2012). Thus, mature differentiated cells represent an important source of regenerating bone in adult fish, but not during fracture repair in mammals.

This raises the following possibilities: (1) osteoblast dedifferentiation is associated with appendage regeneration (which does not happen in mammals), but does not occur during bone repair (which is common to fish and mammals); (2) it only occurs in the bones of the fish fin (the lepidotrichia), which have been suggested to represent a distinct type of skeletal tissue not found in mammals based on their extracellular matrix composition (Mari-Beffa et al., 2007); or (3) teleost bone-forming cells are more plastic than mammalian osteoblasts, and their dedifferentiation is an important feature during fin regeneration and repair of internal bone defects in all teleost bones.

Here, by introducing injuries in bone tissue of the zebrafish fin and skull, we have addressed these questions and found that osteoblast dedifferentiation in teleosts does not only operate during appendage regeneration, but also during repair processes caused by other types of injury.

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RESULTS

Fin ray fractures – a new model of bone repair

To test whether osteoblast dedifferentiation is a process specific to appendage regeneration or whether mature osteoblasts are also involved in repair of more limited bone injuries, we induced fractures in the intramembranous bony fin rays of the zebrafish caudal fin. Fin rays contain segmented dermal bones (lepidotrichia) composed of two concave hemirays facing each other. These hemirays enclose fibroblasts, blood vessels, nerves and pigment cells and are covered by epidermis. A single layer of osteoblasts lines the bone matrix on the in- and outside of the hemirays (Knopf et al., 2011). By applying mechanical force, we induced distinct transverse cracks within a single segment of the bony rays, which typically affected only one of the two hemirays. These fractures differ from a recently developed crush injury model, in which several bone fragments were produced at the site of damage, in addition to the actual fracture crack (Sousa et al., 2012). We did not stabilize these fractures and assayed the repair process by repeated imaging over a period of several weeks. At 1 day post injury (dpi) and 2 dpi we observed a thickening of the tissue at the fracture site (Fig. 1A, arrow; supplementary material Fig. S1A, asterisk; see Table S1 for experimental numbers), which mostly represented epidermal tissue, as in *keratin4:AmCyan* transgenic fish, an epidermal reporter line (Wehner et al., 2014), *keratin4* promoter

activity could be detected in most of the cells of this swelling (Fig. 1B). In contrast to crush-injured fins (Sousa et al., 2012), the thickened epidermis was restricted to the fractured segment, illustrating different fracture severities. At 6–7 dpi, a thickening had formed that consisted of collagen fibers, as indicated by blue staining with Acid Fuchsin Orange G (AFOG) and Toluidine Blue (Fig. 1A,C; supplementary material Fig. S1B) (Constantine and Mowry, 1968). This ‘hard callus’ also stained positive for Alizarin Red and Calcein and thus represented calcifying bone matrix (Fig. 1D,E). Osteoblasts staining positive for the pan-osteoblast antigen *Zns5* (Johnson and Weston, 1995) lined the callus matrix, which, however, at 6 dpi did not contain Osteocalcin protein, indicating that the tissue had not yet transformed to mature bone tissue (Fig. 1F), in contrast to 28 dpi, when Osteocalcin protein was detected in the callus (Fig. 1G). At 4 weeks post injury, the fracture crack was not visible in whole-mount views of fractured fin rays any longer (Fig. 1A). However, longitudinal sections revealed that the old bone was still distinguishable from the surrounding new bone matrix and still contained a crack (Fig. 1H). Injured segments displayed a thickened appearance (Fig. 1A, arrowhead) even at 17 weeks and 1 year post injury (supplementary material Fig. S1C,D). We conclude that non-stabilized fractures to lepidotrichia bone are repaired by deposition of new bone matrix, but that the old bone of the fracture itself does not

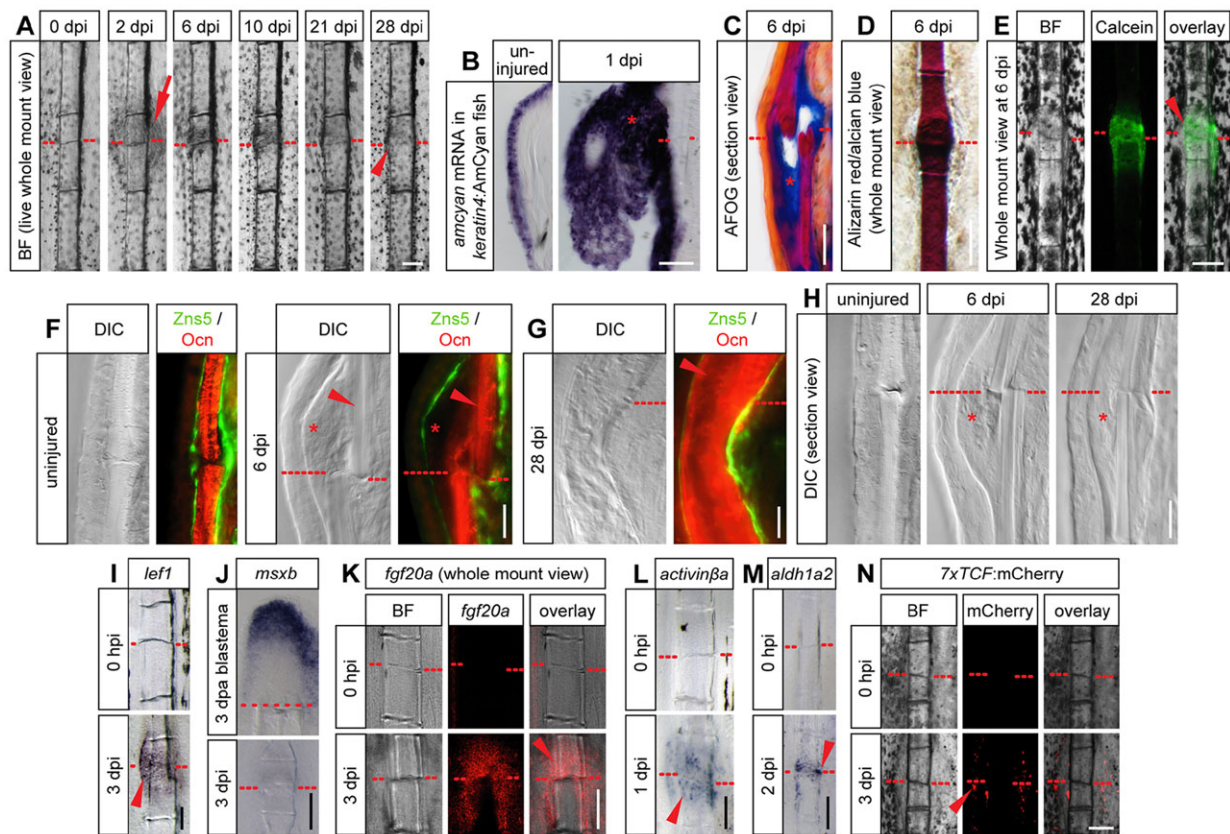


Fig. 1. Fracture model in zebrafish bony fin rays. (A) Whole-mount view of the same fractured fin ray at different time points post injury. Red dashed line, fracture; arrow, epidermal thickening; arrowhead, thickened bone. (B) Epidermal thickening at 1 dpi identified by *in situ* hybridization against *amcyan* mRNA in *keratin4:AmCyan* transgenic fish. (C) Longitudinal section view of an AFOG-stained fin hemiray at 6 dpi with collagen staining blue (asterisk). (D) Whole-mount view of an Alizarin Red/Alcian Blue stained fin ray at 6 dpi. The lack of blue staining indicates intramembranous ossification. (E) Whole-mount view of a fin ray at 6 dpi stained with Calcein indicates calcium incorporation into the mineralizing callus. (F,G) Immunofluorescence on longitudinal sections of individual hemirays stained for the pan-osteoblast marker *Zns5* and Osteocalcin (Ocn), labeling mature bone matrix. Note the absence of Osteocalcin in the callus tissue at 6 dpi (asterisk), but presence at 28 dpi (arrowhead). DIC, differential interference contrast. (H) Longitudinal sections of individual fractured hemirays. Asterisk, hard callus. (I–M) Expression of the indicated genes detected by chromogenic (I,J,L,M) or fluorescent (K) whole-mount *in situ* hybridizations on fractured rays or amputated fins (J). (N) mCherry fluorescence is induced in fractured *7xTCF:mCherry* Wnt reporter fish at 3 dpi. Scale bars: 100 μ m in A,D,E,I–N; 50 μ m in B,C; 20 μ m in F–H. BF, brightfield.

reunite. Furthermore, remodeling processes that would restore the original bone morphology appear to be slow, similar to those observed in human fracture repair, where remodeling continues up to several years after a fracture (Cameron et al., 2013).

We next asked whether lepidotrichia fractures induce a similar molecular response to fin amputation. During regeneration after amputation a multilayered wound epidermis forms, which surrounds the blastema, a population of proliferative, lineage-restricted progenitor cells characteristic to regenerating appendages (Lee et al., 2009; Monaghan and Maden, 2013). *lef1* marks a subregion of the wound epidermis (Poss et al., 2000a), and robust *lef1* expression was also induced in tissue surrounding fractures at 3 dpi (Fig. 1I). By contrast, while we could readily detect expression of the regeneration blastema marker *msxb* after amputation, expression at fractures was neither detected by whole mount *in situ* hybridization (Fig. 1J) nor qRT-PCR (data not shown), which differs from previously reported results using more severe crush injuries (Sousa et al., 2012). However, signaling pathways known to be required for fin regeneration appear to be activated in response to fractures as well. In particular, we detected expression of *fgf20a* (Fig. 1K), *activinβa* (*inhbaa* – ZFIN) (Fig. 1L), *aldh1a2* (*raldh2*, the rate-limiting enzyme in retinoic acid synthesis; Fig. 1M) and activity of Wnt/β-catenin signaling as reported by the *7xTCF-Xla.Siam:nlsMCherry^{la5}* (*7xTCF:mCherry*) transgenic line (Moro et al., 2012) (Fig. 1N). This indicates that repair of fin ray fractures requires a similar signaling environment to regeneration after amputation, despite the lack of *msxb* expression. Notably, FGF, Wnt, RA and Activin pathway components all have been shown to be involved in mammalian bone homeostasis or repair (Nagamine et al., 1998; Barnes et al., 1999; Schmid et al., 2009; Komatsu et al., 2010; Conaway et al., 2013), illustrating their relevance both in mammalian and zebrafish fracture repair.

Osteoblasts dedifferentiate in response to fin ray fractures

We next asked whether mature osteoblasts dedifferentiate in response to lepidotrichia fracture. Osteocalcin is specifically expressed in differentiated osteoblasts, and *osteocalcin:GFP* transgenic fish can be used to label mature osteoblasts *in vivo* (Gavaia et al., 2006; Knopf et al., 2011). In the adult caudal fin, GFP fluorescence was found in the center of every fin ray segment, but was absent from the segment boundaries (Fig. 2A). In response to fracture, reporter fluorescence was progressively lost in the injured segment starting at 1 dpi (arrow in Fig. 2A; see quantification in supplementary material Fig. S2A), as well as in the segments directly neighboring the fractured segment starting at 2 dpi (arrowheads in Fig. 2A, quantification in Fig. 2B; supplementary material Fig. S2A). Thus, downregulation of reporter expression is observed in segments not directly affected by the injury. By contrast, reporter activity was neither significantly reduced in other segments of the injured fin ray nor in uninjured fin rays of the same fin, indicating that the response is not systemic (Fig. 2B; supplementary material Fig. S2A,B). qRT-PCR on RNA isolated from injured plus adjacent segments showed that endogenous *osteocalcin* expression was also reduced in response to fracture (Fig. 2C).

We next asked whether *osteocalcin:GFP* levels dropped as a result of loss of osteoblasts. Cells that failed to express *osteocalcin:GFP* in the vicinity of the fracture continued to express *Zns5* (Fig. 2D), and their number did not significantly differ from that in uninjured fins (supplementary material Fig. S2C). Furthermore, although the number of apoptotic cells in the epidermis covering the fractured bone was significantly increased in the injured and directly adjacent segments, this was not the case in the fibroblast and osteoblast populations (Fig. 2E,F). Moreover, necrotic cells were

mainly detected in epidermal tissue but not in the osteoblast layer (supplementary material Fig. S2D-F). Together, these data indicate that mature osteoblasts do not undergo enhanced cell death but downregulate *osteocalcin* expression in a distance up to roughly 500 μm from a bone fracture.

Next, we tested whether fractures also resulted in upregulation of pre-osteoblast markers. Expression of a *runx2:GFP* reporter was present at very low levels in osteoblasts of the adult fin, except for a small population of cells at the growing tip of each fin ray (Fig. 2G). After fracture, *runx2b* expression could be detected in the bone surrounding the fracture, as shown by whole-mount *in situ* hybridization against *gfp* RNA in *runx2:GFP* transgenic fish (Fig. 2H), as well as by qRT-PCR against endogenous *runx2b* (Fig. 2I). We additionally found Tenascin C, which is expressed in mesenchymal condensations prefiguring osteoblasts during development, and represents an indicator of cell migration and tissue rearrangement (Chiquet-Ehrismann and Tucker, 2004), to be induced in fibroblasts and osteoblasts after fracture (Fig. 2J,K). Together, these results indicate that mature osteoblasts in the zebrafish fin ray adopt the gene expression characteristics of pre-osteoblasts following bone fracture.

In contrast to postproliferative, *osteocalcin*⁺ osteoblasts, pre-osteoblasts are cycling cells. We thus checked whether osteoblasts would start to proliferate in response to fracture. Although hardly any osteoblasts went through S-phase of the cell cycle in uninjured fins, the number of BrdU-incorporating osteoblasts rose to 20% in the injured segment, and enhanced proliferation was induced up to at least 500 μm from the fracture site, thus also in adjacent bony segments (Fig. 2L,M). It is noteworthy that incorporation of BrdU indicating S-phase reentry was already significantly elevated in dedifferentiating osteoblasts at 1 dpi, contrasting with the observation that induction of proliferation was delayed during fracture repair after crush injury (Sousa et al., 2012).

Osteoblasts in the stump of the amputated fin change their morphology in the process of dedifferentiation (Knopf et al., 2011). To test whether this is also the case after fracture we performed transmission electron microscopy (TEM) on fractured fin rays. At 1 dpi, bone-lining osteoblasts had not considerably altered their morphology in comparison to uninjured fin ray osteoblasts (Fig. 2N; data not shown). By contrast, at 6 dpi osteoblasts displayed a less elongated nucleus and overall a more rounded shape (Fig. 2N). The cells had also formed prominent extracellular matrix, which is suggestive of a secretory cell status (ECM in Fig. 2N). Notably, at 4 weeks post injury, osteoblasts still showed a rounded secretory morphology (supplementary material Fig. S2G), whereas later, at 8 weeks post injury, cells had again adopted their pre-injury morphology, characterized by a highly elongated nucleus (supplementary material Fig. S2G).

Altogether, these results indicate that osteoblasts respond to fin ray fracture by downregulation of late and induction of early osteogenic genes, by re-entry into the cell cycle and by loss of their specialized morphology. Thus, mature osteoblasts dedifferentiate in fractured fins as they do in response to amputation (Knopf et al., 2011).

Osteoblasts actively migrate to the site of injury

We have recently provided evidence based on lineage tracing that dedifferentiated osteoblasts move toward the amputation plane in response to fin amputation (Knopf et al., 2011). As expression of Tenascin C, which has anti-adhesive properties, was induced after fin ray fracture, we asked whether osteoblasts migrate toward a fracture as well. To test this we made use of a transgenic line expressing the photoconvertible protein Kaede under control of

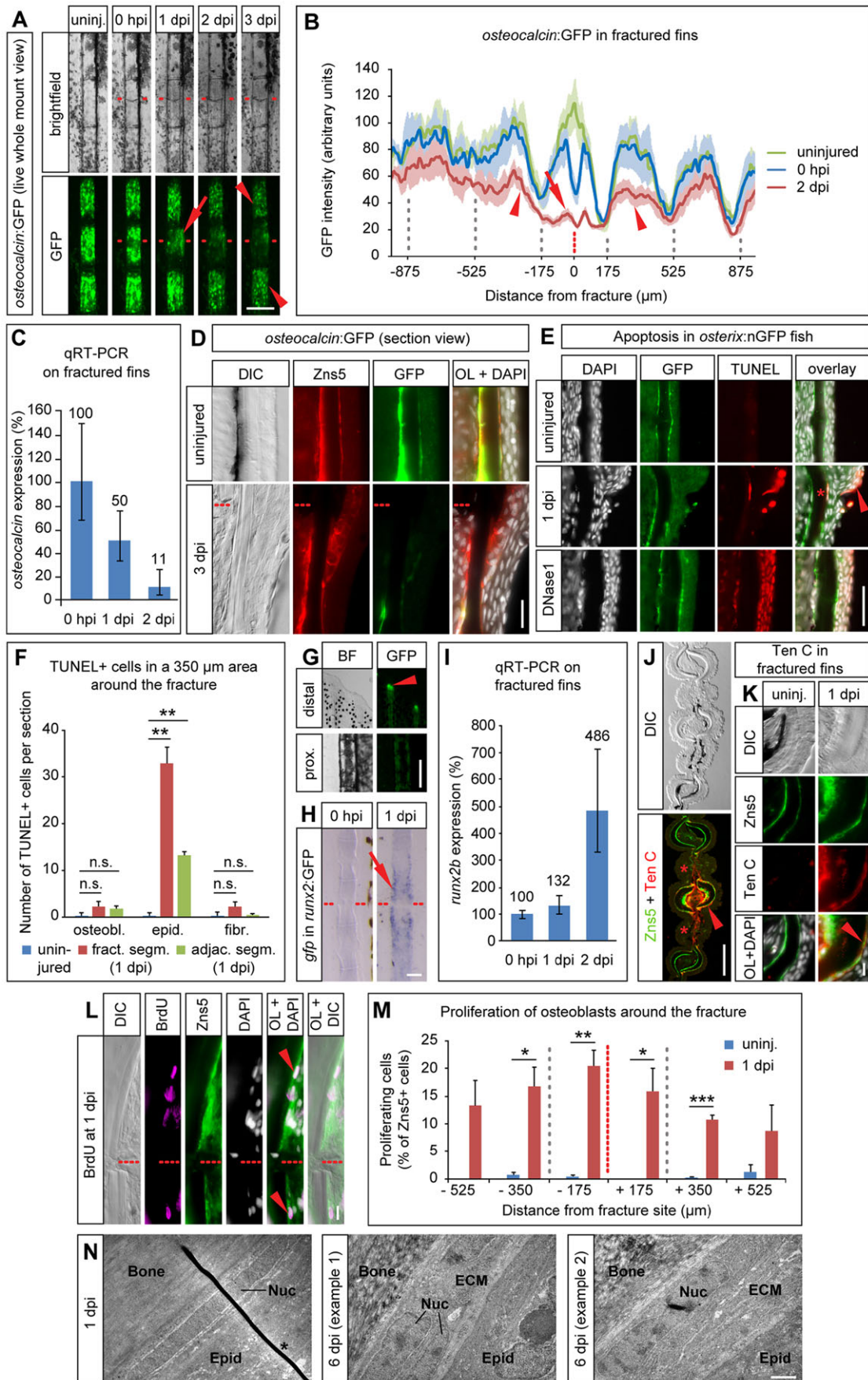


Fig. 2. See next page for legend.

Fig. 2. Osteoblasts in the fractured bony fin ray undergo dedifferentiation.

(A) Live whole-mount view of a fractured fin ray in the same *osteocalcin*:GFP transgenic fish imaged at different time points. GFP levels drop in the fractured (arrow) and adjacent (arrowheads) segments. (B) Quantification of *osteocalcin*:GFP expression showing average GFP intensity \pm s.e.m. Arrow, fractured segment; arrowheads, adjacent segments. GFP intensity is not reduced by introduction of the fracture, except at the direct fracture site (compare blue curve with green curve). Gray dashed lines, segment boundaries. (C) Endogenous *osteocalcin* levels determined by qRT-PCR on fractured plus adjacent segments and shown relative to the levels at 0 hpi. Error bars, s.d. (D) Longitudinal section view of *osteocalcin*:GFP uninjured and 3 dpi hemirays stained with antibodies against GFP and Zns5. In contrast to uninjured fins, at 3 dpi GFP can be hardly detected in Zns5+ osteoblasts. OL, overlay. (E) Apoptotic cells detected by TUNEL staining in longitudinal sections of *osterix*:nGFP fish are mainly found in the epidermal cell layer overlying the fracture (arrowhead) and only occasionally in osteoblasts (asterisk). DNase1-treated sections were used as positive control. (F) Quantification of experiment shown in E. Error bars, s.e.m. $**P \leq 0.01$ Student's *t*-test; n.s., not significantly different. (G) GFP expression in uninjured *runx2*:GFP transgenic fish is largely confined to the distalmost tips of the growing fin rays. prox., proximal. (H) Whole-mount *in situ* hybridization against *gfp* in *runx2*:GFP transgenic fish fin rays at 0 hpi and 1 dpi reveals induction of transgene expression at the fracture site (arrow). (I) Endogenous *runx2b* levels determined by qRT-PCR on fractured rays plus adjacent segments and normalized to the level at 0 hpi. Error bars, s.d. (J) Anti-Tenascin C and Zns5 antibody staining on cross-sections of three bony fin rays, the central ray of which had been fractured a day earlier. Tenascin C expression is present in osteoblasts and fibroblasts of the fractured fin ray (arrowhead) and extends into the fibroblast population of the interray (asterisks). (K) Magnified view of staining as in J. (L) Zns5+ osteoblasts in the proximity of the fracture incorporate BrdU (arrowheads). Longitudinal sections. (M) Quantification of experiment shown in L. BrdU+ Zns5+ cells were counted in bins of 175 μ m. Gray dashed lines, segment boundaries. Error bars, s.e.m. $*P \leq 0.05$. $**P \leq 0.01$. $***P \leq 0.001$, Student's *t*-test. (N) Osteoblasts change their ultrastructure upon injury, determined by TEM. ECM, extracellular matrix; Nuc, nucleus/nuclei. *, artefact from staining procedure. Scale bars: 200 μ m in A,G; 20 μ m in D; 50 μ m in E,H; 100 μ m in J; 10 μ m in K,L; 1 μ m in N.

regulatory regions of the *entpd5* gene, an osteoblast-specific factor required for skeletal mineralization (Huitema et al., 2012). In adult caudal fins, *entpd5*:Kaede was expressed in osteoblasts in the center of each fin ray segment (Fig. 3A). We have previously shown that osteoblasts located at the segment centers are *osteocalcin*+ (Knopf et al., 2011), suggesting that *entpd5* expression in the adult fin is specific to mature osteoblasts. We first tested whether this line can be used to confirm osteoblast migration in response to fin amputation. Indeed, when we photoconverted osteoblasts from green to red in a single fin ray segment and amputated the fin in the adjacent segment, converted cells moved toward and beyond the amputation plane within 2 days (Fig. 3A; supplementary material Fig. S3, arrowhead).

We then photoconverted a population of osteoblasts in one fin ray segment, introduced a fracture in the same fin ray and followed osteoblast behavior by repeated imaging during the following days. When a fracture was introduced in the segment adjacent to the one with photoconverted cells (red line and right fin ray in Fig. 3B; supplementary material Fig. S3), these osteoblasts moved toward the fractured segment and crossed the segment boundary by 1 dpi (arrowheads in Fig. 3B; supplementary material Fig. S3). During the following day, photoconverted cells were present closer to the fracture site, and some reached the injured site at 3 dpi (right fin ray in Fig. 3B; supplementary material Fig. S3). By contrast, photoconverted osteoblasts one segment further away from the fracture did not change their position (blue line and blue asterisk in Fig. 3B; supplementary material Fig. S3). These results indicate that dedifferentiating osteoblasts in a distance of roughly one segment (about 350 μ m) from the fracture start to move toward

the injury within 24 h of the damage, which is similar to what we have observed during bone regeneration after amputation (Knopf et al., 2011).

Movement of osteoblasts toward the fracture could be due to active migration or passive displacement caused by cell proliferation. To distinguish between these possibilities, we performed live time-lapse confocal microscopy of photoconverted osteoblasts in *entpd5*:Kaede fish. We found that osteoblasts displayed dynamic cell morphology and that individual osteoblasts changed their position relative to other cells (Fig. 3C; supplementary material Movies 1 and 2). By contrast, osteoblasts showed no signs of motility in uninjured fin rays (supplementary material Movie 3). This strongly suggests that osteoblasts actively migrate toward a fracture.

To further confirm that osteoblasts move toward the fracture, we lineage traced *osterix*+ (*sp7* – ZFIN) osteoblasts. We used *osterix*:CreERT2-p2a-mCherry \times *hsp70l:loxP* DsRed2 *loxP* nlsEGFP (in short *hs*:R to nG) double transgenic fish, in which 4-hydroxytamoxifen (4-HT)-induced Cre activity excises a *loxP*-flanked DsRed Stop cassette and thus allows for GFP expression in recombined cells and their progeny in response to heat shock (Halloran et al., 2000; Hans et al., 2009; Knopf et al., 2011). 4-HT treatment of adult fish before fracture resulted in stochastic labeling of osteoblasts with GFP after heat shock (Fig. 3D). Genetically labeled green cells accumulated in the area of the fracture callus within 2 dpi (Fig. 3D), indicating that *osterix*+ cells or their progeny move toward the injury site after fin ray fracture.

Redifferentiation of osteoblasts after fracture

We next characterized the temporal and spatial profiles of osteoblast redifferentiation during fracture healing and compared it to those observed in regenerating fins. Onset of osteogenesis as indicated by elevated GFP fluorescence in *runx2*:GFP transgenic fish occurred at the fracture site at the same time as in fin regenerates, namely at 2 days post amputation (dpa) and post injury (dpi), respectively (Fig. 3E; supplementary material Fig. S4A). Likewise, onset of osteoblast commitment, as indicated by *osterix*:nGFP expression, occurred at 3 days post lesion in both amputated and fractured fins (Fig. 3E; supplementary material Fig. S4B). The timing of late osteogenesis as indicated by reduced *runx2b* and increased *osteocalcin* expression (Hartmann, 2009), however, appeared to differ between regeneration after amputation and fracture repair. Although GFP reporter activity in *osteocalcin*:GFP transgenic fish was robustly induced at 6 dpa in regenerating fins, expression was only detected in about half of fractured rays at this time (arrow in Fig. 3E showing lack of expression versus arrowhead in supplementary material Fig. S4C showing presence of expression) and robust expression was detected in fractures only by 10 dpi. Concomitantly, while regenerating fins down-regulated *runx2*:GFP expression by 6 dpa, expression was maintained until 10 dpi in fractures (arrowhead in Fig. 3E; supplementary material Fig. S4A). We conclude that osteogenesis is initiated at the same speed in response to bone fractures and bone amputation, but that progression to mature osteoblasts is slower in fractures.

Osteoblast dedifferentiation occurs in injured skull bone

Having found that osteoblast dedifferentiation happens in response to fin ray fractures, we wondered whether it only occurs in lepidotrichia bones, which are specific to bony fishes and have no equivalent in mammals (Mari-Beffa et al., 2007). To test this, we assayed osteoblast behavior in response to injury of the dermal bone of the skull. We targeted the frontal and parietal bones of the zebrafish cranial vault, which are homologous to the corresponding

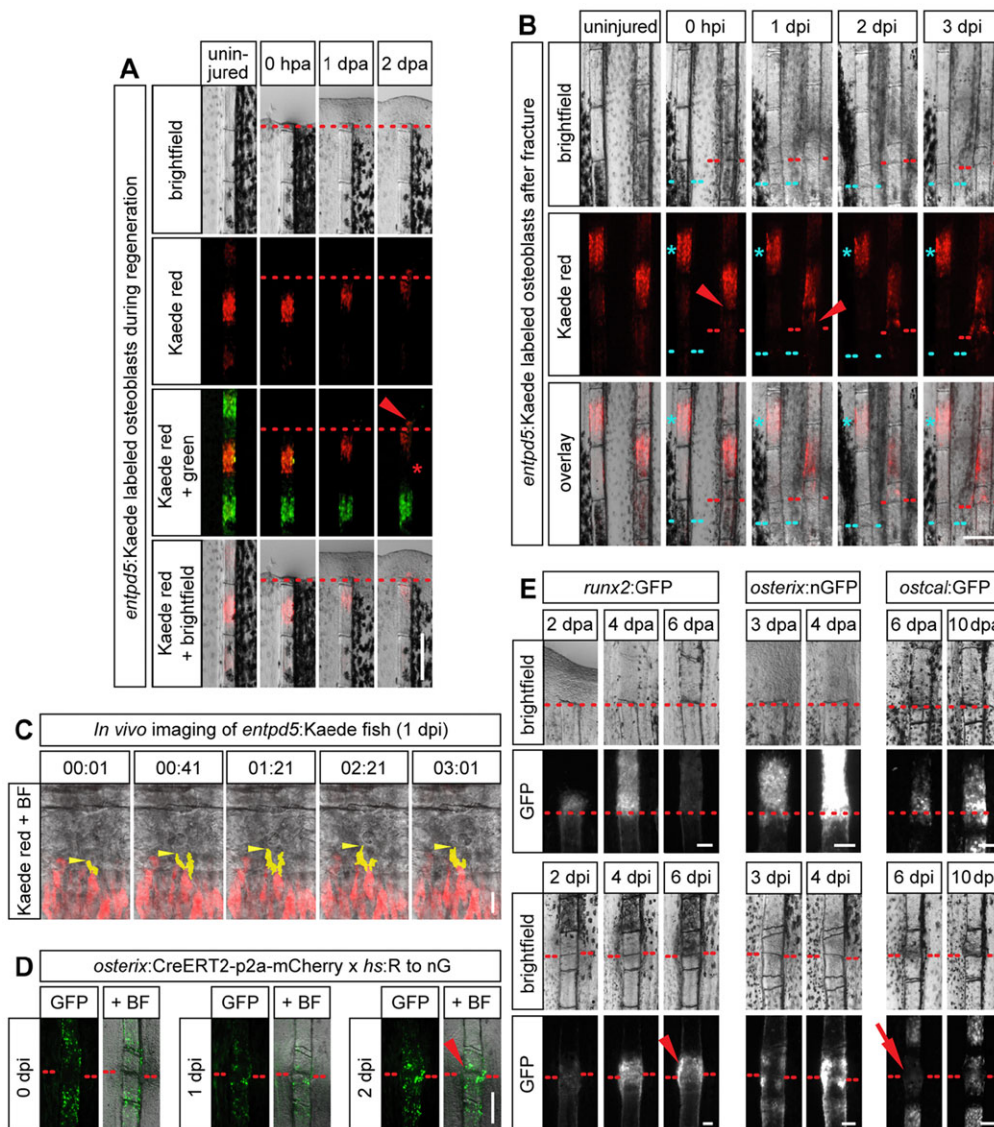


Fig. 3. Dedifferentiated osteoblasts migrate toward the injury site, where they redifferentiate. (A) *entpd5:Kaede* is expressed in mature osteoblasts in the center of the bony fin ray segments. In response to fin amputation, photoconverted osteoblasts (red) migrate to and beyond the amputation plane (arrowhead), as seen by repeated imaging of the same fish. At the same time the gap devoid of labeled osteoblasts at the segment boundary widens (asterisk). (B) Photoconverted osteoblasts in *entpd5:Kaede* transgenic fish in a segment adjacent to a fracture (right fin ray, arrowhead) or in the second segment from the fracture (left ray, asterisk). Osteoblasts migrate toward the fracture only from the segment adjacent to it. (C) Still images from a confocal time-lapse movie (supplementary material Movie 1) of migrating photoconverted Kaede+ osteoblasts in close proximity to the fracture. Protrusions of one cell are highlighted in yellow, its leading edge with an arrowhead. (D) *osterix+* cells or their progeny accumulate at the fracture at 2 dpi (arrowhead), as revealed by stochastic Cre-mediated genetic labeling of osteoblasts in *osterix:CreERT2-p2a-mCherry⁺hs:R* to nG fish. (E) Comparison of transgene expression in *runx2:GFP*, *osterix:nGFP* and *osteocalcin:GFP* fins during regeneration after amputation (upper panels) versus repair after fracture (lower panels). *ostcal*, *osteocalcin*. Scale bars: 200 μ m in A,B,D; 20 μ m in C; 100 μ m in E.

bones in the neurocranium of mammals. In analogy to established protocols for calvarial injuries in rodents (Spicer et al., 2012), we removed a circular piece of bone with a diameter of 0.5 mm by using a microdrill in the *os frontale* (Fig. 4A,B), although peripheral injury of the *os parietale* could sometimes not be avoided (Fig. 4A). These drill injuries healed quickly. By 3 dpi, small regions staining positive for both Alizarin Red and Calcein were detected in the injured area, suggesting new bone formation (Fig. 4B,C). By 7 dpi the lesions were almost completely filled with matrix, and by 14 dpi matrix formation as indicated by Calcein incorporation had largely returned to baseline levels (Fig. 4B,C).

We next injured the *os frontale* of *osteocalcin:GFP* transgenic fish to test whether mature osteoblasts downregulate *osteocalcin* expression. To reduce pigment obscuring the GFP signal, we introduced the *osteocalcin:GFP* line into the *casper* double mutant fish, which lack melanophores and iridophores (White et al., 2008). The GFP signal intensity in bone tissue close to the injured region was reduced at 3 dpi and more so at 7 dpi, as seen in whole-mount heads of live fish that were imaged repeatedly (Fig. 4D). GFP pixel intensity plots revealed that GFP intensity was significantly reduced up to a distance of at least 300 μ m from the edge of the drill injury (Fig. 4E). Transverse cryosections immunostained with anti-Zns5

and anti-GFP antibodies revealed that uninjured skull bones were lined by a single layer of osteoblasts on both the external and internal surface of the bone (Fig. 4F). All osteoblasts staining positive for the pan-osteoblast marker Zns5 also appeared to be positive for *osteocalcin:GFP*, indicating that the vast majority of osteoblasts lining the adult skull bones are fully differentiated and that no or very few undifferentiated progenitors exist in these osteoblast layers (Fig. 4F). Sections spanning the border zone between noninjured bone and the drill hole (bracket in Fig. 4F,G) at 7 dpi revealed a thin layer of bone matrix in the injury area that was lined by a single layer of Zns5+ osteoblasts on both the external and internal surface (arrow in Fig. 4F) as well as Zns5+ osteoblasts lining the bone adjacent to the injury (arrowhead in Fig. 4F). Osteoblasts covering the newly formed bone in the injury were largely devoid of *osteocalcin:GFP* expression, indicating that these had not yet differentiated by 7 dpi (Fig. 4F). Intriguingly, *osteocalcin:GFP* expression was also weak in the osteoblasts lining the bone adjacent to the injury, being reduced to about 50% of the level detected in noninjured skull bones, confirming osteoblast dedifferentiation observed in live fish (Fig. 4F; supplementary material Fig. S5). At 7 dpi, osteoblasts in the injury area strongly expressed *runx2:GFP* (Fig. 4G), and *osterix:nGFP* at the periphery

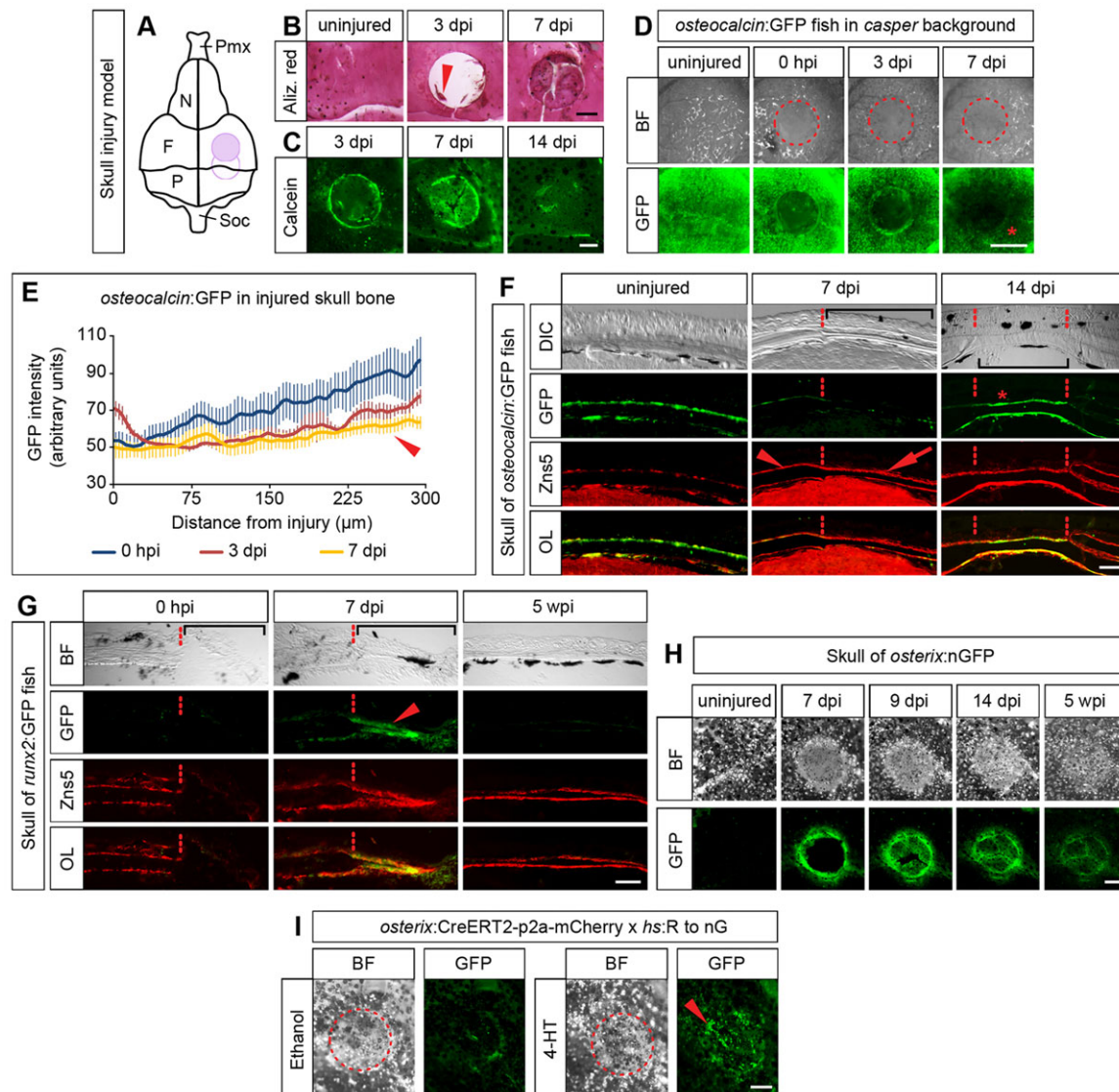


Fig. 4. Osteoblasts in dermal bone of the skull dedifferentiate following injury. (A) Scheme illustrating the bone injury (500 μ m hole) in the *os frontale* (purple filled circle) or at the boundary between the *os frontale* and *os parietale* (empty circle) of the dorsal skull. F, *os frontale*; N, *os nasale*; P, *os parietale*; Pmx, *os praemaxillare*; Soc, *os supraoccipitale*. (B) Whole-mount view of uninjured and injured skulls at different times post injury stained with Alizarin Red. Arrowhead, islands of bone matrix. (C) Calcein staining of whole-mount injured skulls at different times post injury. (D) Reduction of GFP signal intensity in *osteocalcin:GFP* transgenic fish in proximity to the drill injury (asterisk), revealed by repeated live imaging of the same fish. (E) Quantification of experiment shown in D. Error bars, s.e.m. (F,G) Immunofluorescence of transverse sections of the dorsal skull stained for GFP and Zns5. Bracket, injury site; dashed line, injury boundary; arrowhead, osteoblasts in uninjured area; arrow, osteoblasts lining newly formed matrix in injury area. (F) *osteocalcin:GFP* fish. (G) *runx2:GFP* fish. Arrowhead, GFP+ osteoblasts lining newly formed matrix in injury area. (H) Skull of an *osterix:nGFP* fish before and after injury. (I) *osterix+* osteoblasts contribute to repair of skull bone after injury. In *osterix:CreERT2-p2a-mCherry* \times *hs:R* to nG double transgenic fish genetically labeled osteoblasts (bright nuclear signals) cover the injury site at 7 dpi (arrowhead). Only background fluorescence is detected in vehicle (ethanol)-treated fish. Scale bars: 200 μ m in B,C,H,I; 500 μ m in D; 50 μ m in F,G.

of the injury (Fig. 4H). By 14 dpi, osteoblasts still expressed *osterix:nGFP* (Fig. 4H), but also had further matured as indicated by *osteocalcin:GFP* expression (Fig. 4F). Finally, by 5 weeks post injury, osteoblasts had ceased to express *runx2:GFP* (Fig. 4G) and displayed reduced *osterix:nGFP* expression (Fig. 4H).

We next tested whether surrounding *osterix+* osteoblasts contribute to bone healing in the injured skull. After irreversible genetic labeling of adult osteoblasts in *osterix:CreERT2-p2a-mCherry* \times *hs:R* to nG fish, we injured the zebrafish skull bone and checked for the presence of labeled osteoblasts in the injured area at 7 dpi. Whereas only background signal was observed in the vehicle-treated negative control, labeled osteoblasts characterized

by nuclear GFP were found covering the injury in fish treated with 4-HT (Fig. 4I). This shows that *osterix+* osteoblasts contribute to dermal bone repair in injured skull zebrafish skull bones.

Together, these data indicate that skull injuries heal via dedifferentiation of adjacently located mature osteoblasts, migration of dedifferentiated osteoblasts into the injury site and subsequent re-differentiation along the osteoblast lineage. Thus, osteoblast plasticity is not specific to lepidotrichia, but also occurs in dermal skull bone.

DISCUSSION

Our findings indicate that osteoblast dedifferentiation is neither restricted to the context of appendage regeneration nor to the special

bone type of lepidotrichia, but is likely to occur in response to internal injury of any dermal bone in zebrafish. Thus, mature fish osteoblasts display high plasticity and represent an important source of newly forming bone both after internal injury and appendage amputation. By contrast, differentiated osteoblasts in mammals are unlikely to be involved in bone repair. Although there is evidence for participation of postmitotic osteoblasts lining bone surfaces (bone lining cells) in mammalian bone formation after stimulation with either parathyroid hormone or FGF2 (Dobnig and Turner, 1995; Power et al., 2004; Kim et al., 2012), a recently developed microfracture model of the mouse calvaria and knee indicates that there is no or very low contribution of Osteocalcin⁺ cells to bone repair (Park et al., 2012). Rather, mesenchymal stem cells appear to be the precursors of newly forming osteoblasts during fracture repair in mammals (Park et al., 2012). Whether all osteoblasts in regenerating or healing fish bone are derived from mature cells via dedifferentiation remains to be shown; thus, a contribution of stem cells to fin regeneration or fracture repair cannot be ruled out. Of note, when osteoblasts are genetically depleted from zebrafish fins, bone can regenerate rather normally after amputation, implying that an alternative source of osteoblasts does exist; however, this has not been identified (Singh et al., 2012). Nevertheless, it is clear that in unperturbed conditions mature osteoblasts are an important source of healing bone in teleost fish, but not in mammals. It remains possible that in response to injury, mammalian mature osteoblasts do undergo dedifferentiation apparent by loss of *osteocalcin* and induction of *runx2* expression, yet they fail to migrate and do not contribute to injury repair. Adult mammalian skull-cap-derived osteoblasts reduce *osteocalcin* expression and re-enter the cell cycle when placed in culture (Lian and Stein, 1995), suggesting a dedifferentiation potential. Thus, it will be important to decipher the molecular mechanisms that allow for osteoblast dedifferentiation in zebrafish and to test whether the same mechanisms are able to induce osteoblast dedifferentiation in mammalian bone tissue, which could enhance bone healing. Considering both the speed of bone repair and the cost-efficiency of haltering zebrafish in the laboratory, we propose that skull injury models in zebrafish will, in addition to fin amputation and fracture, be very useful to investigate various aspects of vertebrate bone biology. Thus, the zebrafish injury models characterized here will aid in understanding the key mechanisms of bone repair and regeneration, one of which might be the plasticity of local differentiated bone cells.

MATERIALS AND METHODS

Fin amputations, fractures and skull injuries

Fin amputations were performed as previously described (Poss et al., 2000b) and fish were returned to 28.5°C water afterwards. To induce fin ray fractures, fish were anesthetized in 0.02% tricaine and placed laterally onto a 2% agarose-coated Petri dish. An injection needle (BD Becton Dickinson, 0.3×13 mm) was pushed onto the middle of a bony fin ray segment with slight pressure, until a transverse crack was introduced. A single fracture in the center of the fin ray segment was produced, which was usually limited to one of the two hemirays. Segments located three segments proximal to the bifurcation of the dorsal fin rays 3, 7 (sometimes 8, if fin ray 7 was heavily pigmented) and ventral fin ray 3 were chosen (usually three fractures per fish). Occasionally, we introduced a fourth and fifth fracture into the fins. For cryosections and qRT-PCR, up to eight (central) fin rays were fractured, whereas the outer ones were left untouched. Skull injuries were caused in anesthetized fish placed upright in a sponge using a microdrill (Fine Science Tools, 0.5 mm). All experiments with zebrafish were approved by the states of Saxony and Baden-Württemberg and performed in accordance to animal guidelines of TU Dresden and Ulm University.

Transgenic fish lines

The *osteocalcin*:GFP (*Ola.Osteocalcin.1:EGFP^{hu4008}*), *osterix*:nGFP (*Ola.Sp7:nls-GFP^{zfl32}*), *runx2*:GFP (*Hsa.RUNX2-Mmu.Fos:EGFP^{zfl259}*), *osterix*:CreERT2-p2a-mCherry (*Olsp7:CreERT2-p2a-mCherry^{uid8}*), *hs*:R to nG (*hsp70l:loxP DsRed2 loxP nlsEGFP^{uid9}*) and *keratin*:AmCyan [*keratin4:irtTAM2(3F)-p2a-AmCyan^{ulm5}*] fish lines have been described elsewhere (Knopf et al., 2011; Wehner et al., 2014). Creation of the *entpd5*:Kaede fish line was performed as described previously (Huitema et al., 2012; Bussmann and Schulte-Merker, 2011).

Quantification of GFP expression

Fin ray segments and skulls of live fish anesthetized with 0.02% tricaine were imaged repeatedly using identical settings. Pictures were acquired using a Leica MZ16 FA stereomicroscope equipped with a QIMAGING RETIGA-SRV camera. Intensity measurements along fin rays were made with the Plot profile tool in ImageJ Software version 1.45s along rectangular regions of interest whose width corresponded to that of individual fin rays. Intensity measurements with the Plot profile tool of injured heads in live fish were made along eight radial lines (anterior, anterior-left, left, posterior-left, posterior, posterior-right, right, anterior-right) starting from the edge of the injured bone. To quantify fluorescence on antibody-stained sections of injured skulls, intensity measurements were made along the stained tissue (curved lines, Plot profile tool). To normalize for differences in transgene expression levels between individual fish, all values were normalized against the average pixel intensity of uninjured sections.

Conversion of Kaede and *in vivo* imaging of *entpd5*:Kaede fish

To convert Kaede⁺ cells from green to red fluorescence, distinct fin ray segments of anesthetized *entpd5*:Kaede fish were illuminated with a UV laser (100% intensity, 400 Hz, frame averaging 14 times) connected to a Leica SP5 I confocal laser scanning microscope. After documentation of the converted cells with the stereomicroscope, fin ray fractures were introduced in adjacent segments or segments located two segments apart. Fin rays were imaged during the following days to reveal migration of converted cells. For *in vivo* imaging, fish were anesthetized and immobilized by covering the trunk and tail fin with 2% agarose. Fish were intubated with tubing (polyethylene tubing PE-160/10, OD=1.57 mm, ID=1.14 mm, Harvard Apparatus) which was connected to fresh fish system water and 0.02% tricaine, respectively. Depending on the intensity of gill movements fish were either supplied with pure system water or tricaine solution. Image acquisition was performed every 5 or 20 min, respectively, using a Leica SP5 confocal microscope equipped with a 20× dipping lens.

Osteoblast fate mapping

Olsp7:CreERT2-p2a-mCherry^{uid8}; *hsp70l:loxP DsRed2 loxP nlsEGFP^{uid9}* double transgenic fish were injected intraperitoneally either once (fin ray fractures) or repeatedly on three consecutive days (skull injuries) with 10 μl of 2.5 μM 4-hydroxytamoxifen (Sigma) or with the vehicle control ethanol. Seven days later fish were heat shocked at 37°C for 1 h and roughly 12 h later analyzed for recombination by the appearance of GFP⁺ cells. Fish were injured and photographed right after the insult (0 hpi) and at different time points post-injury. Fish were heat shocked roughly 12 h before each imaging to reveal GFP⁺ cells.

Proliferation studies with BrdU

Fish were incubated in 5 mM 5-bromo-2-deoxyuridine (Sigma) in fish system water for 8 h, after which the fins were fixed in 4% PFA.

Immunofluorescence

Fin and head cryosections were prepared as described (Knopf et al., 2011; Grandel et al., 2006). Immunofluorescence on fin cryosections was performed according to Knopf et al. (2011). A similar protocol was performed on skull cryosections (14 μm), except that the methanol treatment and blocking with calf plus horse serum were omitted. Antibodies used are listed by Knopf et al. (2011). In addition, rabbit anti-Osteocalcin (anti-ArBGP, gift of ML Cancela, Centre of Marine Sciences, Faro,

Portugal) was used at 1:250. Detection of BrdU was performed as described (Knopf et al., 2011).

In situ hybridizations

Chromogenic whole-mount *in situ* hybridization was performed according to Knopf et al. (2011). Fluorescent whole-mount *in situ* hybridization was performed with the TSA Plus kit (Perkin Elmer) according to the manufacturer's instructions.

In situ hybridization on cryosections was performed as follows: slides were dried for 1-3 h at room temperature (RT), treated with ice-cold MetOH for 15 min, rehydrated with TBST (15 mM NaCl, 5 mM Tris, 0.1% Tween 20, pH 7.5) twice for 10 min each, incubated with Proteinase K (5 µg/ml; Invitrogen) in TBST for 10 min, washed with TBST four times for 5 min each. Slides were then incubated with denatured probe (70°C for 10 min, then put on ice) against *amCyan* mRNA at 55°C o/n in a humidified chamber containing Solution A (150 mM NaCl, 15 mM sodium citrate, pH 6, 50% deionized formamide, 0.1% Tween 20). Slides were washed with Solution A three times (1×15 min, 2×30 min) at 55°C, followed by two washes for 30 min each with TBST at RT. Sections were blocked in 10% NCS (newborn calf serum, Sigma Aldrich) in TBST for at least 1 h at RT, incubated with anti-Dig-AP coupled antibody (Roche) at 1:2000 in 10% FCS in TBST o/n at 4°C. Slides were then washed with TBST four times for 30 min each, equilibrated with freshly prepared NTMT (100 mM Tris HCl pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween 20) and incubated in the dark with staining solution [400 µl/slide containing 1.4 µl NBT and 2.7 µl BCIP (both Roche) in NTMT]. Staining was stopped with four washes of PBT (10 min each) and slides were postfixed with 4% PFA in PBS for 15 min at RT or at 4°C o/n. Slides were washed with PBS and mounted with 70% glycerol.

Staining with AFOG, Alizarin Red/Alcian Blue and Calcein, and TEM

To stain sections with AFOG, slides were dried at RT for 1-2 h, incubated in Bouin's solution first for 2 h in 60°C, then for an additional 60 min at RT. Slides were washed in running water for 30 min, rinsed in 1% phosphomolybdic acid for 5 min and washed in dH₂O for 4-5 min, after which they were placed in AFOG staining solution (1 g Analine Blue, 2 g Orange G, 3 g Acid Fuchsin in 200 ml dH₂O, pH 1.09) for 4-5 min. Slides were rinsed in dH₂O for 2 min, dehydrated in 2×5 min 95% and 100% ethanol each, placed in Xylen for 2×2 min and embedded with Cytoseal.

Combined Alizarin Red/Alcian Blue staining was performed on methanol-treated (series of 70, 50 and 30% in PBS) fins. After rehydration with PBS for 2×20 min and water for 5 min, fins were stained with Alcian Blue (Sigma Aldrich) staining solution (Part A: 0.02% Alcian Blue in 50 mM MgCl₂) for 2 h at RT. Fins were then treated with an ethanol series (90, 50 and 30% in 50 mM MgCl₂) and washed for 2 h in water. Alizarin Red (Sigma Aldrich) staining solution (Part B: 0.5% Alizarin Red in 10% glycerol) was added to the fins and incubated for 1 h. Fins were cleared with a decreasing potassium hydroxide:glycerol series (3:1 o/n, 1:1 o/n, 1:3 o/n) and mounted with 80% glycerol for imaging. Fins were then embedded for cryosectioning (see above).

To detect calcium deposition in fin fractures and skull injuries, live fish were incubated for 20 min in 100 ml of 0.2% Calcein (Sigma Aldrich) in dH₂O (pH 7.5), rinsed in water once and washed in water for additional 20 min.

Toluidine Blue staining and TEM were performed according to Knopf et al. (2011).

Quantitative PCR (qRT-PCR)

For qRT-PCR eight central bony fin rays were fractured approximately three segments proximal to the bifurcation point (12 fish per sample). The fractured plus the two adjacent segments were harvested using a scalpel. RNA isolation, cDNA transcription and determination of relative mRNA expression was performed as described previously (Knopf et al., 2011). For quantitative PCR of *runx2b* the following intron spanning primers were used: 171 bp; ACACCCAGACCCTCACTCAG; GACAGCGGAGTGGTGGAG.

TUNEL assay

TUNEL staining on cryosections was performed with the ApopTag Red *In Situ* Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions. *osterix:nGFP (OISp7:nlsGFP^{zfl132})* fish were used, in which osteoblasts are labeled by nuclear GFP. GFP was visualized by immunofluorescent anti-GFP staining (see above) preceding the TUNEL assay.

Necrosis staining

Necrotic cells were detected as described with modifications (Roger et al., 1996). In brief, live fish with fractured fin rays were incubated for 20 min in 100 µg/ml Acridine Orange stock solution (Sigma Aldrich) and 2 µg/ml ethidium bromide (Roth). Fish were washed twice for 20 min in PBS, after which they were transferred to fishwater for another 20 min. Fins were fixed in 4% PFA for several hours at RT, washed several times with PBS and imaged with a Leica SP5 confocal microscope. Necrotic cells incorporate both Acridine Orange and ethidium bromide, and fluoresce brightly orange (Roger et al., 1996; Renvoize et al., 1998; Watanabe et al., 2007).

Statistics and experimental numbers

Information on sample sizes and further statistical information can be found for all experiments in supplementary material Tables S1 and S2.

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.G., F.K. and D.W. performed experiments and analyzed data. F.K. and G.W. designed experiments and wrote the paper. L.F.A.H. and S.S.-M. supplied unpublished fish lines.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105817/-/DC1>

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