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REVIEW

Lessons on skeletal cell plasticity from studying jawbone regeneration in zebrafish

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Three major mesenchymal cell types have important roles in determining the shapes of vertebrate animals: bone-producing osteoblasts, cartilage-producing chondrocytes, and fat-producing adipocytes. Although often considered discrete cell types, accumulating evidence is revealing mesenchymal cells of intermediate identities and interconversion of cell types. Such plasticity is particularly evident during adult skeletal repair. In this Review, we highlight recent work in zebrafish showing a role for hybrid cartilage–bone cells in large-scale regeneration of the adult jawbone, as well as their origins in the periosteum. An emerging theme is that the unique mechanical and signaling environment of the adult wound causes skeletal cell differentiation to diverge from the discrete lineages seen during development, which may aid in rapid and extensive regeneration of bone.

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Introduction

Three structural cell types are characterized by their production of matrix of varying degrees of stiffness: soft (fat, adipocytes), hard (cartilage, chondrocytes) and hardest (bone, osteoblasts). These cells arise from mesenchyme derived from either mesoderm or a specialized population of ectoderm called the cranial neural crest. *In vitro*, mesenchymal cells isolated from diverse parts of the animal can generate all three lineages under the right inductive conditions. However, the *in vivo* correlates of these 'mesenchymal stem cells' are now being appreciated as a complex group of cell populations with varying degrees of potency.[1,2] For example, recently identified 'skeletal stem cells' in mouse generate chondrocytes and osteoblasts but not adipocytes,[3,4] yet other mesenchymal progenitors marked by *Col2a1*−, *Sox9*−, or *Aggrecan*-based Cre lines in mice generate all three lineages.[5]

The traditional view is that naïve multi-potent or bi-potent mesenchymal progenitors are induced to follow discrete lineages (**Figure 1**). In the case of osteoblasts, this includes upregulation of the Runx2 and then Sp7 transcription factors, followed by production of matrix genes such as Col1a1 and Spp1 that act as a substrate for mineralization, as well as endocrine factors such as Bglap.[6] For chondrocytes, Sox family members (Sox5/6 and Sox9/10) induce a distinct cohort of matrix proteins, such as Col2a1 and Aggrecan. In the growth plate, chondrocytes later express other proteins (for example, Col10a1 and Mmp13) associated with hypertrophy.[7] Adipocytes prominently express PPAR-γ and the important lipid-associated protein Perilipin.[8,9] However, accumulating evidence suggests that such molecular distinctions can be blurred. In mice, chick and zebrafish, common precursors for osteoblasts and chondrocytes, called 'osteochondroprogenitors', express both Runx2 and Sox9 factors.[10–12] In the chick calvaria, osteoblasts express *Sox9* and *Col2a1* mRNA weakly, although the proteins for both genes are not detected.[13] Similarly, osteoblasts for intramembranous bones in zebrafish express high levels of *col10a1* and low levels of *col2a1a*.[11,14] Reciprocally, developmental chondrocytes in zebrafish display weak expression of *col1a2* and an *sp7* transgene.[15] Altogether, these findings suggest that osteochondroprogenitors concurrently express chondrocyte and osteoblast programs (albeit at weak levels), with further differentiation into a specific lineage resulting in repression of the non-adopted lineage(s). Consistent with this view, lineage tracing in mice with conditional *Sox9*−, *Col2a1*− or *Aggrecan*-based Cre transgenes results in labeling of not only chondrocytes but also osteoblasts, bone marrow stromal cells, and in some cases adipocytes.[5]

Even after differentiation along discrete lineages, mesenchymal cell types are often observed to exhibit gene expression in common with other derivatives. During cartilage differentiation, for example, mesenchymal progenitors first form condensations and then transition through proliferative and hypertrophic chondrocyte states. However, hypertrophic

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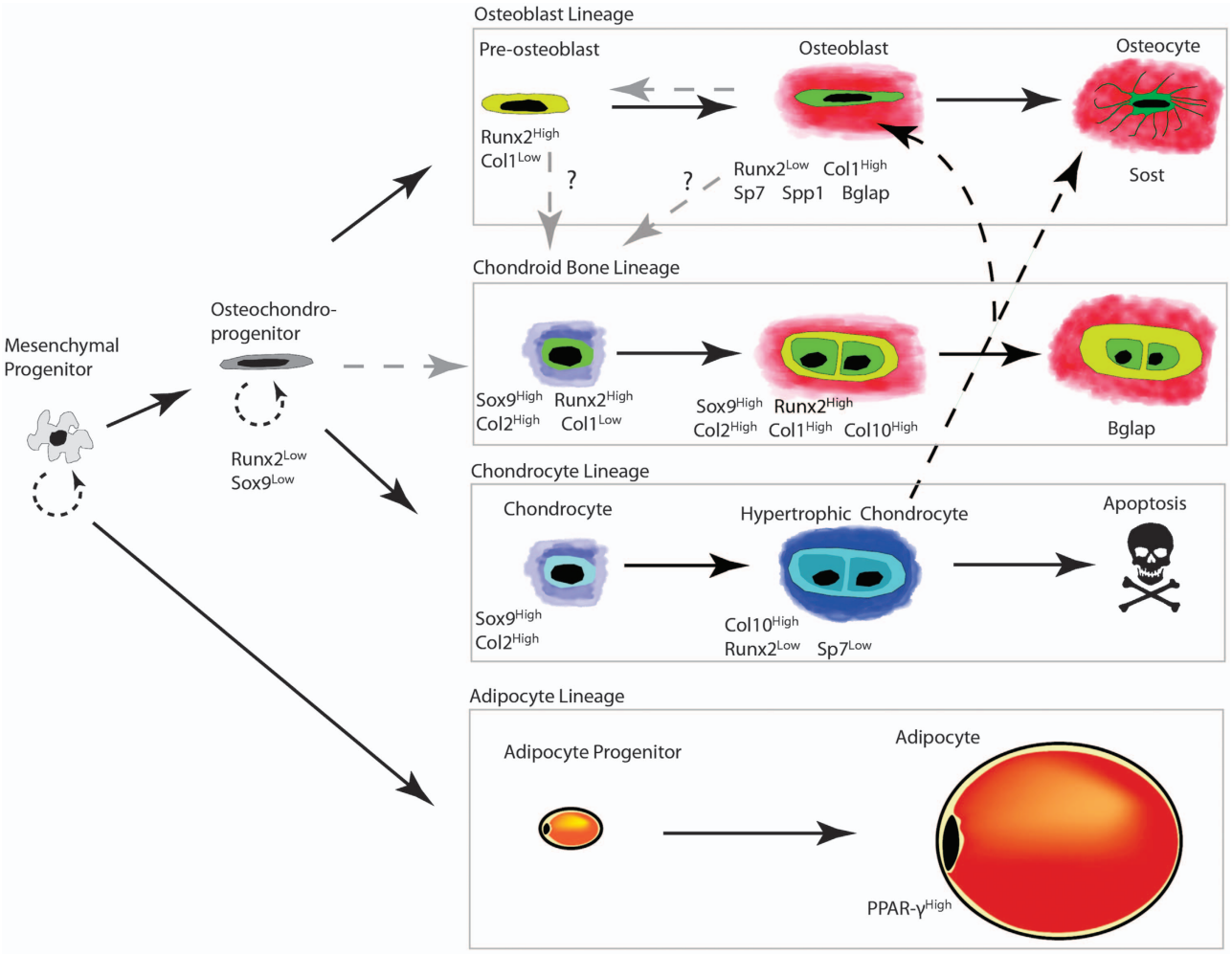

Figure 1 Plasticity in mesenchymal lineages. Mesenchymal progenitors give rise to osteocytes, chondrocytes and adipocytes, as well as a mixed cartilage–bone cell type called chondroid bone. Osteoblasts and chondrocytes are thought to originate from a common osteochondroprogenitor that expresses both *Sox9* and *Runx2*. Although some hypertrophic chondrocytes undergo apoptosis, others may transdifferentiate into osteoblasts and/or osteocytes. Studies of regenerating fin rays in zebrafish have shown that post-mitotic osteoblasts can dedifferentiate into proliferative pre-osteoblasts. During jaw regeneration in zebrafish, osteoblasts and/or pre-osteoblasts may transdifferentiate into a chondroid bone cell type expressing markers of both osteoblasts and chondrocytes.

chondrocytes weakly express many genes in common with osteoblasts, including *Runx2*, *Sp7* and *Spp1*, and undergo extensive mineralization (that is, calcification)[7] (**Figure 1**). The requirement for *Runx2* in the mineralization of both bone and hypertrophic chondrocytes suggests a common genetic program for mineralization in both cell types, despite the matrix of hypertrophic chondrocytes being relatively poor in Col1a1.[16] Similarly, hypertrophic chondrocytes express PPAR-γ in common with adipocytes, with loss of PPAR-γ in chondrocytes resulting in decreased bone growth.[17] An open question is whether the expression in hypertrophic chondrocytes of genes more commonly associated with bone and fat represents retained potential of these gene programs from a multi-potent mesenchymal progenitor, versus reinitiation during later phases of chondrocyte differentiation.

There are also examples of mesenchymal cell types that cannot be easily classified into one of the canonical lineages. One such prominent mixed skeletal tissue is chondroid bone, which is characterized by cells of chondrocyte morphology embedded in mineralized matrix[18–21] (**Figure 1**). Although a rare cell type developmentally, chondroid bone has been described in vertebrates from fish to mammals and can be found in diverse locations as the baculum of the rodent and bat penis[22,23] and the mandibular condyle of the jaw,[24,25] as well as during fracture repair.[26–29] Chondroid bone is avascular and may arise in part due to mechanical strain, as with secondary cartilage.[19] Consistent with a mixed osteoblast/chondrocyte identity, chondroid bone cells simultaneously produce cartilage-associated proteins (Col2a1 and Col10a1) and bone-associated proteins (Col1a1 and Bglap).[30] Given that osteoblasts have been postulated to have evolved from chondrocytes,[31] it may be that chondrocytes and osteoblasts represent two ends of a spectrum, with intermediate cell types such as chondroid bone in the middle.[32] Indeed, others have described at least eight classes of cartilage in teleost fishes based on cell morphology and the abundance and type of skeletal matrix,[33,34] as well as both cellular and acellular bone.[35] Further, chondrocytes in the pinna of the mammalian ear have been found to have lipid droplets reminiscent of fat tissue, suggestive of cells intermediate between chondrocytes and

adipocytes (termed lipochondrocytes).[36] Clearly, the repertoire of mesenchymal cells is much more complex than the three cell types typically diagrammed.

In addition to mesenchymal cell types of mixed identity, there is growing evidence that differentiated cells may be able to change their identities. Since at least the 1970s, it has been recognized that cultured chondrocytes can turn into osteoblasts.[37–39] This observation had led to the suggestion that hypertrophic chondrocytes in the mammalian growth plate may change into osteoblasts as the cartilage template is converted into bone.[40–42] This idea was then largely supplanted by the notion that most hypertrophic chondrocytes undergo apoptosis, with a new source of osteoblasts generating the majority of bone.[43] However, modern lineage-tracing studies have begun to revisit the idea of chondrocyte to osteoblast transdifferentiation during growth plate development. Using a conditional Cre transgene driven by *Col10a1* regulatory elements, two groups have shown that hypertrophic chondrocytes give rise to long-lived *Col1a1a* + osteoblasts and *Sclerostin* + osteocytes, mostly in primary spongiosa and trabecular bone but occasionally also in the bone collar.[44,45] One concern of these experiments is whether the Cre lines used are entirely specific for hypertrophic chondrocytes, especially given expression of *col10a1* in intramembranous osteoblasts of zebrafish.[14] However, similar results have been obtained using an *Aggrecan*-based Cre.[45,46] If such transdifferentiation is true, an interesting question is how the large hypertrophic chondrocytes change their morphology into the more slender osteoblasts. Possibilities include only the smaller hypertrophic chondrocytes near the bone collar (that is, 'borderline chondrocytes') transdifferentiating into osteoblasts,[40,43] or reductive cell divisions generating a smaller osteoblast and a larger apoptotic cell.[47,48] Chondrocytes might also dedifferentiate and then redifferentiate into osteoblasts, or adopt an osteoblast-like gene expression profile while maintaining a chondrocyte-like morphology, a process termed 'metaplasia'.[19]

A common assumption is that the skeletal differentiation programs active during development are re-employed during adult skeletal repair. In most mammalian fractures, especially when the broken bone is not rigidly stabilized, an early response is the formation of a bridging cartilage callus.[49] Col10a1-based lineage-tracing studies in mice have shown that hypertrophic chondrocytes within the repair callus contribute to long-lived osteoblasts and osteocytes within the healed bone, suggesting that a similar transdifferentiation process occurs during fracture repair as during growth plate development.[45] However, other studies have found cells of chondrocyte morphology embedded in mineralized matrix and expressing mature osteoblast genes such as *Bglap*, which suggests that cells in the repair callus retain aspects of chondrocyte identity while producing bone matrix. This suggests that repair chondrocytes may be more similar to those in chondroid bone, rather than to hypertrophic chondrocytes of the growth plate that lose their chondrocyte identity before transdifferentiating into osteocytes.[50]

A Hybrid Cartilage–Bone Cell Type Drives Zebrafish Jawbone Regeneration

A recent study in zebrafish provides more evidence that the cartilage-like cells during bone repair are similar to the mixed identity cells of chondroid bone.[51] Previous work in amphibians and zebrafish had shown that distal amputation of the adult lower jaw is followed by robust regeneration, with the lower jawbone repairing through a cartilage intermediate[21,52–54] (**Figure 2**). As the lower jaw forms largely by intramembranous ossification (that is, absence of a cartilage intermediate, with the exception of the mandibular condyle near the jaw joint and the distal tips at the midline), involvement of a cartilage intermediate during repair shows a prominent difference from lower jawbone development. During zebrafish jawbone repair, cells of chondrocyte morphology concurrently produce cartilage-associated proteins such as Sox9a and Col2a1a and bone-associated proteins such as Runx2b and Col1a1a.[51] These mixed identity cells also extensively mineralize (**Figure 2e**), which is unusual given that developmental bones in zebrafish are largely perichondral and not endochondral (that is, shell-like as opposed to displaying internal mineralization as in mammals). The expression of *col1a1a* before *col10a1* is also markedly different from what occurs during mammalian endochondral ossification, in which Col10a1-positive hypertrophic chondrocytes lose cartilage identity before transitioning into Col1a1-positive osteoblasts.[44,45] Although repair cells in the zebrafish jaw eventually stop producing Col2a1a as they turn into mature Bglap-positive osteoblasts,[51] this process reflects mixed cartilage–bone cells progressing into pure bone cells, rather than initial cartilage cells transdifferentiating into bone cells as suggested for mammalian growth plates.

An interesting question is why zebrafish and likely other vertebrates utilize chondroid bone for adult repair. As opposed to intramembranous bone formation that occurs in close association with blood vessels,[43] the avascular nature of chondroid bone may allow it to thrive in the hypoxic environment of an adult wound.[19] Chondroid bone cells have also been observed to be highly proliferative, for example, driving the rapid seasonal growth of the jaw in male Atlantic salmon.[55] This rapid growth potential may aid in filling large gaps in bone, with direct mineralization of the cartilage template, as opposed to secondary replacement by new bone-forming cells, accelerating structural recovery of the damaged body part. Further investigation is needed to determine the extent to which chondroid bone is also involved in mammalian bone repair.

Dedifferentiation of Osteoblasts During Zebrafish Bone Regeneration

The source of hybrid cartilage–bone cells for zebrafish lower jawbone regeneration appears to be the periosteum and/or osteoblasts lining the bone.[51] Bone remodeling involves the concerted removal of bone by osteoclasts and the addition of new bone by osteoblasts. The periosteum is a dense connective tissue layer on the outside of bone that is an important source of osteoblasts for this process.[5] Upon jawbone resection in zebrafish, the remaining periosteum next to the cut ends undergoes a rapid proliferative expansion to fill in the missing piece of bone.[51] Although definitive lineage-tracing analysis has not been performed, this observation suggests that the cartilage-like callus derives from periosteal cells or nearby early osteoblasts. In mice, lineage-tracing studies using a conditional *Prrx1*-CreER line, as well as older periosteal grafting

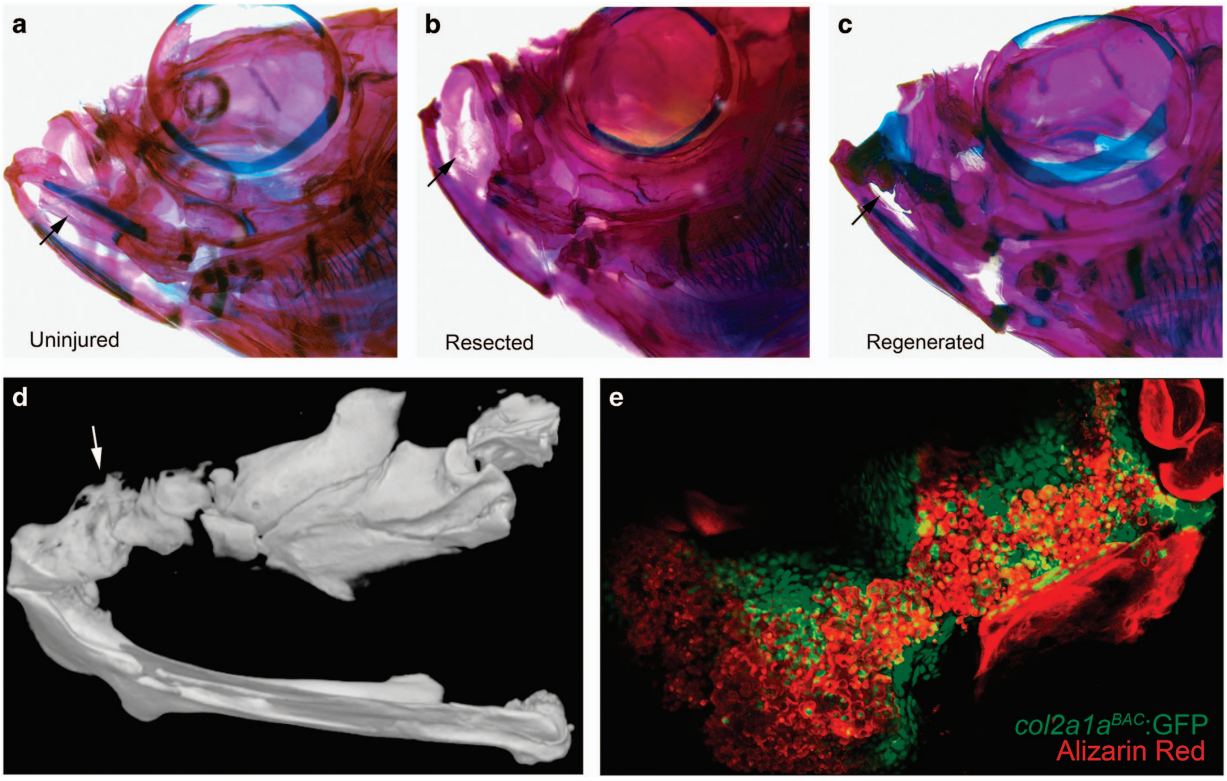

Figure 2 Large-scale bone regeneration in the zebrafish jaw employs a hybrid cartilage–bone tissue. (**a**–**c**) Adult zebrafish heads are stained with Alizarin Red and Alcian Blue to show bone and cartilage, respectively. Arrows show the lower jawbone in uninjured controls (**a**), loss of part of the jawbone immediately after resection (**b**), and regeneration of the jawbone one month later (**c**). (**d**) Bone μCT of the dissected lower jaw shows regeneration of the jawbone (arrow). (**e**) Jawbone regeneration in a *col2a1a*^{BAC}:GFP transgenic zebrafish shows that repair chondrocytes (green) bridging the wound produce extensive Alizarin + bone matrix (red) one month following resection.

experiments, similarly indicate the periosteum as a source of the cartilage callus during fracture repair.[56]

In the zebrafish fin and skull, bone regeneration generally occurs in the absence of a cartilage intermediate (but see a study in medaka showing transient cartilage during fracture repair in the fin[57]). It has been shown that mature osteoblasts dedifferentiate in response to injury in the fins and calvaria, which is accompanied by cell morphology changes, loss of mature osteoblast markers (for example, *sp7* and *bglap*), and upregulation of pre-osteoblast genes (for example, *runx2b*)[58,59] (**Figure 3**). These dedifferentiated cells then proliferate and redifferentiate into new osteoblasts that regenerate bone. However, fin bone can still regenerate even if pre-existing osteoblasts are genetically ablated before injury, suggesting another source of new osteoblasts, such as pre-existing progenitors in the periosteum.[60] During jawbone regeneration, a similar upregulation of *runx2b* is seen in *sp7*- cells that give rise to the hybrid cartilage–bone cells.[51] As the majority of bone-lining cells are *sp7*:GFP + and *Runx2*:GFP − before jaw resection, one possibility is that a similar dedifferentiation of osteoblasts occurs during jawbone regeneration, with the difference that these cells then generate cartilage-like tissue. Alternatively, or in parallel, rare *Runx2*:GFP + periosteal cells may also undergo proliferative expansion in response to injury to generate hybrid cartilage–bone cells. In the future, it will be interesting to determine whether mammalian bone healing also involves the dedifferentiation of mature osteoblasts. Indeed, this theme of dedifferentiation in response to injury is becoming increasingly common, for example, cardiomyocyte dedifferentiation drives heart regeneration in both zebrafish and neonatal mice.[61–63]

Regeneration-Specific Role of Ihh Signaling in Cartilage Callus Formation

An intriguing question is why periosteal cells and/or dedifferentiated osteoblasts generate intramembranous bone in certain contexts (for example, calvarial repair and zebrafish fin regeneration) and a cartilage callus in others (for example, mammalian fracture repair and zebrafish jawbone regeneration). Mechanical forces seem to have an important role as rigid stabilization of fractures prevents cartilage callus formation.[49] In mice, BMP2 is sufficient to induce periosteal cells to adopt a chondrogenic fate.[64] The vasculature also likely has a role in cartilage-dependent repair, particularly in the conversion of the cartilage callus to bone.[43,65] Recently, a study of zebrafish jawbone regeneration uncovered a role for Hedgehog signaling in induction of the cartilage callus. Zebrafish lacking *indian hedgehog a* (*ihha*) make very little cartilage callus in response to jaw resection, which correlates with a decreased ability to regenerate the missing jawbone.[51] Ihha appears to control the differentiation of progenitors into repair chondrocytes, as the proliferation of both the progenitors and the few repair chondrocytes that did form were normal. As the developmental role of Ihh signaling is to promote the proliferation and not the specification of growth plate chondrocytes in mammals,[66] the role of Ihh in the differentiation of regenerative chondrocytes is a distinct function of adult bone repair. A fruitful line of

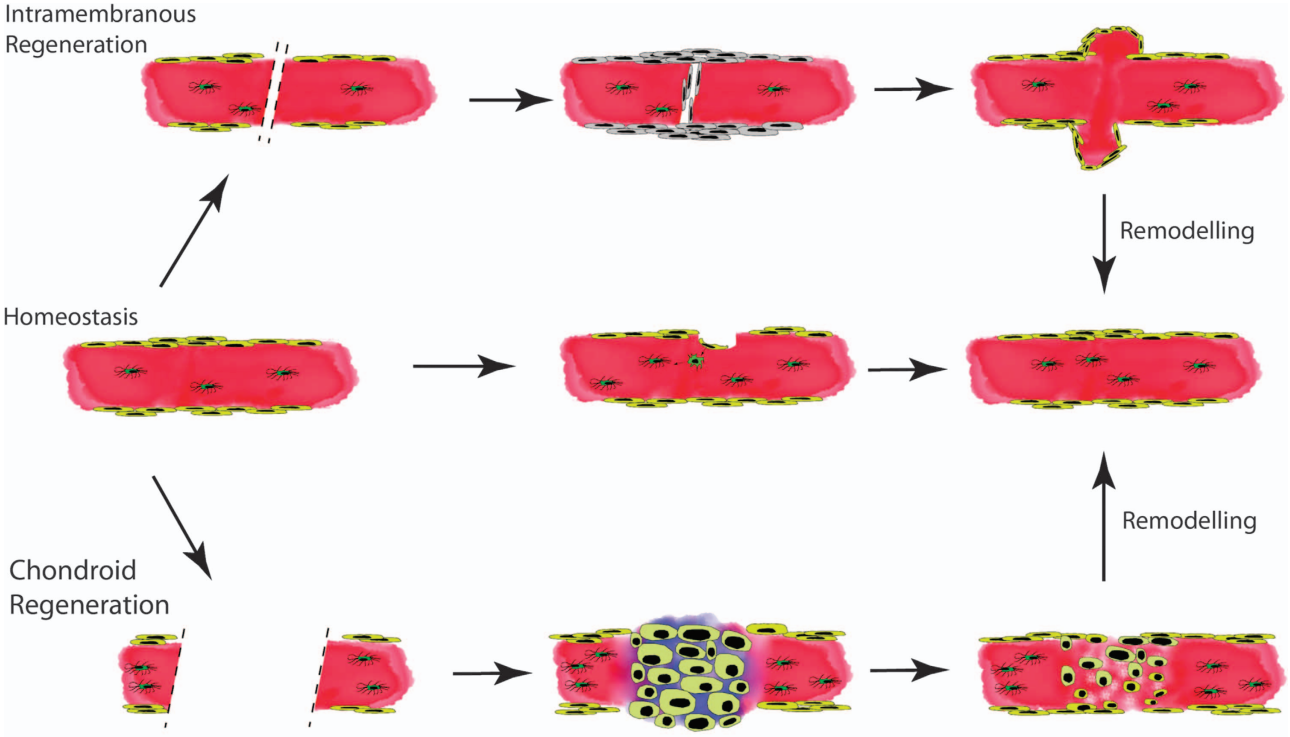

Figure 3 Distinct models of bone homeostasis and regeneration. During bone homeostasis, progenitors and early osteoblasts in the periosteum (yellow) give rise to new osteoblasts to replace bone matrix (red) degraded by osteoclasts (not shown). In the amputated fin or injured skull of zebrafish, intramembranous regeneration can involve dedifferentiation of osteoblasts, proliferation and redifferentiation directly into new osteoblasts. In the resected zebrafish jaw, chondroid bone regeneration involves production of a mixed cartilage–bone cell type from bone-lining cells, with these cells producing first cartilage matrix (blue) and then mineralized matrix (red). In both types of regeneration, gradual remodeling restores normal bone architecture. Intramembranous and chondroid bone repair processes can also co-exist during bone healing.

investigation will be to understand how mechanosensation and Ihh signaling are integrated to shift the periosteal cells involved in bone homeostasis towards making cartilage-like tissue during repair.

In addition to its role in inducing the cartilage callus during jawbone repair, there is also evidence from developmental studies that Ihh signaling helps to determine the extent of osteoblast gene expression within skeletal cells. In mice, loss of *Ihh* results in decreased osteoblast differentiation within the periosteum.[6] Further, work in zebrafish has shown that Ihha is required for transient and weak expression of osteoblast genes in developmental chondrocytes.[15] Conversely elevation of Hh activity by loss of the inhibitory co-receptors *patched1* and *patched2* or by treatment with the Hh agonist purmorphamine transformed chondrocytes into osteoblast-like cells. However, this low level of osteoblast gene expression in normal developmental chondrocytes seems different from the chondroid bone seen during adult repair, as other groups have failed to observe mineralization of developmental chondrocytes in either normal or patched mutant zebrafish.[51,67] Nonetheless, it is tempting to speculate that elevated Ihh signaling during jawbone regeneration could also contribute to the high-level osteoblast gene expression observed in the cartilage callus. As several studies have shown a role for Hh signaling in mammalian fracture repair, it will be interesting to investigate whether these effects are through induction of the cartilage callus, promotion of osteoblast-like character in the callus, and/or some other mechanism.[68–70]

Conclusion

It is becoming increasingly apparent that there are myriad flavors of chondrocytes, osteoblasts, and adipocytes. These cell types can display distinct properties (for example, elastic versus hyaline cartilage), occupy intermediate states between cell types (for example, chondroid bone), transdifferentiate between cell types, and dedifferentiate into progenitors. It will be important to determine how cell types become locked into a particular identity, and how these identities can be overridden in developmental contexts (for example, transdifferentiation of growth plate chondrocytes into osteoblasts) and repair contexts (for example, dedifferentiation of osteoblasts). It must also be acknowledged that the very different environments and cellular histories of embryonic development and adult repair will result in skeletal cells occupying distinct spaces in the continuum of cartilage to bone, which may underlie differences in building versus rebuilding the skeleton. By learning how skeletal cell identity and plasticity is controlled in the animal, we will be better able to repair skeletal injuries with long-lasting cells of the right type.

Conflict of Interest

The authors declare no conflict of interest.

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