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Enhanced cartilage repair in ‘healer’ mice—New leads in the search for better clinical options for cartilage repair

Jamie Fitzgerald*

Bone and Joint Center, Department of Orthopedic Surgery, Henry Ford Hospital System, Detroit, MI 48202, USA

Abstract

Adult articular cartilage has a poor capacity to undergo intrinsic repair. Current strategies for the repair of large cartilage defects are generally unsatisfactory because the restored cartilage does not have the same resistance to biomechanical loading as authentic articular cartilage and degrades over time. Recently, an exciting new research direction, focused on intrinsic cartilage regeneration rather than fibrous repair by external means, has emerged. This review explores the new findings in this rapidly moving field as they relate to the clinical goal of restoration of structurally robust, stable and non-fibrous articular cartilage following injury.

Keywords

Cartilage; Epimorphic regeneration; MRL/MpJ mice; Healer mice; Osteoarthritis

1. Introduction

At its simplest, articular cartilage, located at the ends of long bones and in articulating joints, is a meshwork of highly organized and cross-linked collagen fibrils that immobilize the hydrophilic proteoglycan aggrecan. The molecular properties of these components allow the joint cartilage to resist both compressive and tensile forces associated with locomotion and daily activities. In humans, full-thickness cartilage lesions that penetrate the subchondral bone heal poorly and cartilage lacerations do not heal, probably because of the lack of resident blood vessels, lymphatic vessels, and nerves, and access to reparative cells. Traumatic articular cartilage damage typically leads to a cascade of events that result in further cartilage damage, cartilage erosion and later, osteoarthritis (OA) [1,2]. To date, clinical options for patients with traumatic cartilage defects remain limited to symptomatic treatment until patients become candidates for total joint replacement. In recent years, treatments that attempt to repair or restore the cartilage lesion have started to be developed to slow or stop the progression towards OA. These include the microfracture technique, osteochondral auto/allografts, autologous chondrocyte implantation, and osteochondral transplant systems. In general, these approaches have provided variable and unpredictable results [3–10]. The microfracture and related techniques mostly produce fibrocartilage and

*Correspondence to: Jamie Fitzgerald Head, Musculoskeletal Genetics Bone and Joint Center Henry Ford Hospital Integrative Biosciences Center 6135 Woodward Avenue Detroit, MI 48202 USA. jfitzge2@hfhs.org.

thus do not offer a long-term solution. Autologous chondrocyte implantation is expensive and technically difficult and the long-term functional properties of the repaired defect have yet to be assessed. Other more experimental approaches that have yet to reach the clinic involve using tissue engineered or artificial scaffolds [11,12]. While tissue-engineering approaches are promising, they are still some time away from clinical use. Hence, there is a great clinical need for new research directions and novel treatment modalities for the repair of full-thickness cartilage defects. One such approach that has emerged in recent years focuses on the body's ability to regenerate tissue instead of effecting repair. This process has been studied mostly in invertebrates and amphibians but recent findings of limited epimorphic regeneration in certain strains of mice raises the real possibility of inducing a response to injury that involves authentic tissue regeneration, rather than fibrous repair.

2. Emerging evidence for mammalian tissue regeneration

Wound repair in the tissues of higher organisms is dominated by a fibroproliferative response where the injured organ is patched (repaired) rather than restored structurally and functionally to its original condition (regenerated). It is important to make the distinction between 'repair' and 'regeneration'. Normal wound **repair** is a highly dynamic process that involves blood vessel disruption, clotting and the initiation of inflammatory processes [13]. The initial inflammatory stage is characterized by local activation of the innate immune system, with the subsequent influx of neutrophilic granulocytes and macrophages. Cells present in the wound secrete a variety of proteases, growth factors, and inflammatory mediators, the balance of which is partially responsible for modulating the repair process. Factors released by arriving monocytes and macrophages activate fibroblasts to produce proteoglycans and collagens leading to the formation of granulation tissue and later a fibrous tissue rich in collagens [14].

Tissue **regeneration**, on the other hand, is the architectural and functional restoration of damaged tissues and organs. Mammalian regeneration, such as that achieved by the liver and a limited number of other tissues, is thought to be mediated by activation of resident stem cells, or by proliferation of quiescent cells. 'Authentic' regeneration seen in the restoration of complete limbs in amphibians, for example, is known as segmental or epimorphic regeneration. While less is known about cellular and molecular mechanisms involved in epimorphic regeneration compared to fibrous repair, the major phases have been described from observations and experiments in lower vertebrates. With tissue damage, a wound epidermis is established, followed by the formation of a blastema composed of mesenchymal progenitor cells. These cells are necessary for the proliferation and patterning of the regenerating structure leading to the next phase of regenerative outgrowth and re-patterning [15,16]. Tissue 'repair' is the dominant tissue injury response in mammals while regeneration is more common in some invertebrate species, such as planaria and hydra, which can regenerate complete body plans from small pieces of tissue (reviewed by Li et al. [16]). Limited regeneration has been reported in a number of adult vertebrate species including the appendages and tissues of some fish, urodeles (amphibians and newts) and larval anurans (frogs and toads) [17,18]. Deer and moose replace antlers annually [19], and rabbits [20] and the African spiny mouse (*Acomys*) [21] close ear wounds. Cutaneous wound healing in early-to-mid-gestation human embryonic skin occurs with the complete

restoration of normal skin (reviewed by Harty et al. [22]). In some mammalian tissues such as cornea [23], liver [24] and skeletal muscle [25], regeneration has been reported although this is likely mediated by stem or tissue-resident undifferentiated cells, rather than by epimorphic mechanisms.

In a serendipitous discovery reported in 1998, Clark et al. described a new example of epimorphic tissue regeneration in mammals [26,27]. A particular strain of Murphy Roths Large mice used for systemic lupus erythematosus research (MRL/MpJ-Fas^{lpr}; deficient for the FAS gene) seamlessly closed 2 mm wounds made in their ears with a biopsy punch within four weeks. In other mouse strains tested, scar tissue formed around the wound edge, and the holes failed to close. The MRL/MpJ ear wounds showed progression towards full closure with rapid re-epithelialization, formation of new hair follicles and sebaceous glands in the new growth areas—that is, architectural and functional restoration of damaged tissues. Significantly for those interested in cartilage repair, cartilage islands had formed by 12 weeks and complete restoration of elastic cartilage occurred soon after [26]. Subsequent experiments demonstrated that the parental MRL/MpJ strain (containing a wild-type *Fas* allele) also possessed this regenerative ability. The ear wound closure model in MRL/MpJ mice is of great interest because it shares similarities with the regeneration seen in lower vertebrates in that a blastema-like structure with a transient basement membrane forms, leading to the re-growth of multiple tissues without scar formation. These features distinguish the epimorphic healing response from the ‘repair’ healing response and from the stem cell-mediated response seen in some mammalian tissues. The MRL/MpJ strain was derived by selective breeding from the LG/J strain and the two strains share 75% of their genomes [28]. Notably, ear hole tissue regeneration has also been demonstrated in the parental LG/J strain [29,30].

The discovery of seamless ear wound closure stimulated further research into the superior healing ability of the MRL/MpJ strain. Regeneration was subsequently demonstrated to be complete in several other tissues and cells, including cornea [31], heart [32–35], central nervous system stem cells [36–38], neurons [39,40], transplanted skin [41] and partially amputated neonatal digits [42,43]. Studies that showed regeneration of cartilage and other tissues in healer mice are listed in Table 1. Consistent with a role in scarless healing, the MRL/MpJ strain is resistant to skeletal muscle fibrosis associated with muscular dystrophy, suggesting that healer mice may be a new avenue of research for disorders of increased fibrosis [44]. Collectively, these studies suggest that **MRL/MpJ mice have an intrinsic ability to bypass the normal fibrotic ‘repair’ response and instead implement a more ‘regenerative’ program of tissue restoration.** Enhanced healing has been extended beyond directed tissue damage with the recent finding that MRL/MpJ mice are also resistant to high fat diet-induced metabolic changes, including type-2 diabetes [45] and cardiac remodeling [46]. However, it is important to note that not all injuries heal better in the MRL/MpJ strain. Examples of poor, or lack of, regeneration have been demonstrated with certain skin wound regimes [39,47–49] and dopaminergic neuron lesions [50] suggesting that there may be limits to regeneration (reviewed by Heydemann [51]). It is also noteworthy that, while neonatal amputations demonstrated some ability to regenerate [42], there was no evidence of regeneration in an adult amputation model [43]. Further studies are required to define the

cellular, molecular and developmental boundaries for tissue regeneration in the MRL/MpJ and related healer strains.

3. Articular cartilage is capable of regeneration

In 2008, a trio of papers reported enhanced articular cartilage healing in two strains of mice: the MRL/MpJ ‘healer’ mice, described above, and the DBA/1 strain. Since the MRL/MpJ strain has been shown to undergo scarless healing in several tissues, including auricular cartilage following ear lesion, we assessed whether articular cartilage lesions, which normally induce a fibrocartilaginous response, could ‘regenerate’ in the MRL/MpJ strain [52]. Full-thickness trochlear groove lesions that penetrated the underlying bone were made in adult mice. After 12 weeks, the majority of C57BL/6 control mice failed to elaborate a proteoglycan-positive extracellular matrix at the articular cartilage wound site. In contrast, the male MRL/MpJ mice deposited a proteoglycan-rich matrix populated with small round chondrocyte-like cells. The resulting tissue was rich in the articular cartilage markers collagen II and collagen VI, strongly indicating that authentic articular cartilage had been restored at the lesion site. A partial-thickness laceration did not heal in any experimental group suggesting that in order for regeneration to occur, the bone marrow space has to be breached and extrinsic factors such as cells or circulating molecules delivered to the wound site [52].

Eltawil et al. examined articular cartilage healing in DBA/1 mice subjected to a patellar groove lesion [53]. The control C57BL/6 mice repaired poorly and developed early signs of osteoarthritis (OA). By 8 weeks-post surgery the defects were partially filled with cancellous bone, and with a surface fibrous layer that extended to areas outside of the injury site and included a cellular areas, surface fibrillation and poor safranin-O staining. Negligible collagen II staining was present, even after 8 weeks of healing. In contrast, DBA/1 mice displayed consistent articular cartilage healing as evidenced by histological staining and good repair and new collagen II deposition. Immunohistochemical staining using neo-epitope antibodies demonstrated that the non-healing C57BL/6 mice had more aggrecanase activity and less matrix metalloproteinase activity than the cartilage healing DBA strain, suggesting a difference in response to injury between the two strains in extracellular matrix remodeling.

Ward et al. reported resistance to post-traumatic OA in the MRL/MpJ strain [54]. For this study a model of closed articular fracture of the tibial plateau was developed and the resulting pathology included articular cartilage surface impaction and osteochondral fracture. C57BL/6 mice showed more cartilage degeneration post-fracture compared to MRL/MpJ mice. This was indicated by loss of cartilage and proteoglycan, and development of thickened fibro-cartilage. A variety of circulating cytokines were measured at 4 and 8 weeks post fracture. Of the pro-inflammatory cytokines, IL1 α was lower in MRL/MpJ compared to C57BL/6 mice. Conversely, the anti-inflammatory cytokines IL-4 and IL-10 were present at a higher level in MRL/MpJ versus C57BL/6 mice, suggesting that the balance of pro-and anti-inflammatory cytokines are involved in regenerative bone and cartilage healing in the MRL/MpJs.

The studies showed for the first time that a subset of mouse strains have a superior ability to heal articular cartilage lesions and are resistant to injury-induced OA. The challenge ahead is to define a mechanism of articular cartilage regeneration and to decipher how the enhanced healing differs from standard wound repair. The hope is that these studies can then be leveraged to develop new clinical strategies for the improved treatment of articular cartilage lesions in humans.

4. Mapping the articular cartilage healing trait

It is clear that, although the MRL/MpJ and DBA/1 strains healed better overall compared to the control strains, there were good and poor healers within both the healer and non-healer strains suggesting that genetic variability was influencing the articular cartilage healing trait. This is consistent with earlier studies establishing that enhanced ear wound closure in MRL/MpJ mice is a complex genetic trait involving the interaction of multiple genetic factors [55–58]. Recent fine-mapping studies on LG/J (the parental strain of MRL/MpJ) and SM/J intercross mice narrowed down the loci intervals for the ear wound closure and provided candidate genes for further analysis [29,30]. For heritability studies, the use of inbred strains is preferred because any phenotypic variation detected is due to different combinations of each parental genome without the confounding influence of different genetic backgrounds that outbred strains would have [59]. These LG/J and SM/J inbred strains have been studied for a range of complex musculoskeletal phenotypes including ectopic calcification [60], long bone growth, mechanical properties [61] and muscle weight [62].

The first study to specifically investigate the heritability of articular cartilage regeneration in healer strains of mice in segregating populations was published in 2012 [63]. Several common lines (MRL/MpJ, DBA/1J, DBA/2J, C57BL/6, LG/J and SM/J) and inbred lines generated from an intercross of healer LG/J and non-healer SM/J mice were subjected to a trochlear groove full-thickness injury (and ear biopsy punch) and evaluated for articular cartilage regeneration. Of the common strains, only the MRL/MpJ and LG/J mice showed histological evidence of articular cartilage regeneration 16 weeks post-surgery, consistent with earlier reports [52,54]. On the other hand, C57BL/6, DBA/1J, DBA/2J and SM/J mice failed to heal articular cartilage defects. The reason for the poor ear wound and cartilage healing response in the DBA/1J strain, which was shown to undergo articular healing previously [53], is unclear but it may be related to difference in wounding protocols or differences between DBA/1 and DBA/1J strains, which have been separated for >20 years and may, therefore, have experienced some genetic drift [63].

Two recombinant inbred lines derived from the LG/J and SM/J intercross were identified at the extremes of healing, with LGXSM-6 being a good cartilage healer, similar to the LG/J strain, and LGXSM-33 being a poor healer [63]. All other LGXSM lines had an intermediate healing response to injury. This finding of a range of healing phenotypes in a recombinant inbred line of mice suggests that this variability is due to genetic factors alone. This is because the inbred strains represent different mixtures of the same parental genomes, and therefore, phenotypic differences between the inbred strains are due to genetic factors and not extrinsic factors.

An additional series of experiments in the same series of inbred mice assessed whether the inbred healer (LGXSM-6) and non-healer (LGXSM-33) lines developed OA and whether the tendency towards OA correlated with cartilage healing using the destabilization of medial meniscus (DMM) model to initiate OA [64]. It was established that the poor cartilage healer strain (LGXSM-33) developed a higher grade of OA compared to the good cartilage healer strain (LGXSM-6). This finding is consistent with Ward et al. who showed that the MRL/MpJ healer strain of mice was relatively protected from post-traumatic OA following intraarticular fracture [54].

Conclusions from these studies that have implications for future regeneration-gene hunting efforts are that the articular cartilage regeneration trait is heritable, and that **ear tissue regeneration, articular cartilage healing and protection from OA following trauma are correlated**. These findings suggest that the responses to these pathologies are likely to have the same underlying genetic basis and are not tissue-or location-specific. Pooling these results, the mouse strains can be divided into good healers (MRL/MpJ, LG/J and LGXSM-6), intermediate healers (LGXSM-5 and LGXSM-35) and poor healers (C57BL/6, SM/J, and LGXSM-33) [65]. These mice are a valuable experimental resource for the next phase of research into mammalian epimorphic regeneration: gene identification.

5. Progress towards identifying cartilage regeneration-promoting genes

With the assumption that a common set of genes are involved in ear hole closure and articular cartilage regeneration, Rai et al. [66] investigated gene expression differences in ear wound tissue and articular cartilage from the common inbred and recombinant inbred strains described above [63,64]. A key finding from cluster analysis of the gene expression data was that the strains clustered with their healing ability [63]. One cluster contained the healers (MRL/MpJ, LG/J, LGxSM-6) and the other the non-healers and intermediate healers (C56BL/6, SM/J, LGXSM/5 and LGXSM-35). The implication is that functional differences between alleles derive from sequence differences and/or genetic background. The exception is the non-healing LGXSM-33 line whose gene expression profile clusters with the healer strains. It is unclear why the LGXSM-33 gene expression profile mirrors that of a healing strain but the investigators noted that this strain appeared to be a good healer (similar to LGXSM-6) early on, but that later on healing was halted and cartilage degraded, leading to the poor healer classification [66]. LGXSM33 should therefore be considered an intermediate healer.

Are there specific gene: healing correlations, and what can they tell us about mechanisms of articular cartilage regeneration? When gene expression levels were plotted against healing scores, six gene expression signatures were correlated with articular cartilage healing scores; *Pcna*, *Wnt16*, *Xrcc2*, *Axin2*, *Cebpb* and *Ulk1* [66]. Both *Pcna* and *Xrcc2* are involved in cell proliferation and maintenance of chromosome stability at wound sites and have been implicated in DNA repair [67]. *Ulk1* codes for a kinase involved in autophagy following nutrient deprivation and DNA damage [68]. Autophagy is suggested to be protective in cartilage and autophagy genes are downregulated in pathologies of cartilage, including OA [69]. *Axin2* and *Wnt16* are involved in Wnt pathways, which are known to be implicated in regeneration in different species [70–72]. *Cebpb* encodes a transcription factor involved in

the inflammatory response. It is upregulated following macrophage activation and implicated in liver regeneration [73] and skeletal muscle wound repair [74].

Interestingly, four of these genes were also correlated with ear wound closure; *Xrcc2* and *Pcna*, involved in DNA repair and cell cycle control, and *Axin2* and *Wnt16*, involved in Wnt signaling, suggesting that these two functional classes of genes are part of a universal regeneration mechanism in the MRL/MpJ and related healer strains [66].

6. Several distinct mechanisms appear to promote articular cartilage healing

Genetic, cellular, and metabolic comparisons between MRL/MpJ and non-healer strains have revealed many striking differences in a range of processes, leading to speculation on mechanisms for the enhanced regeneration phenotype. The reader is directed to an excellent review by Heydemann, where these are covered in detail [51]. The discussion here focuses on a subset of possible mechanisms as they relate to articular cartilage healing.

6.1. Cell cycle and DNA repair in regeneration

Since *Xrcc2* and *Pcna* are associated with articular cartilage healing, is there evidence for DNA repair and/or cell cycle regulation in regeneration? One of the consistent features of tissue regeneration in a wide range of settings, including the Hydra and mammalian liver and stem cells, is G2/M cell cycle arrest [18,24,75,76]. Based on these observations, Bedelbaeva et al. asked whether regeneration in MRL/MpJ mice involved cell cycle control [77]. All healer mouse strains (including MRL/MpJ, LG/J and recombinant inbred strain LGXSM-6) had more ear pinna fibroblasts in the G2/M phase compared to the non-healer control strains (C57BL/6, SM/J and LGXSM33). Surprisingly, DNA damage markers were higher in healer strains. Since cell cycle abnormalities and DNA damage have also been reported in mouse embryonic stem cells that lack p21 induction [78] the healer strains were assessed for p21 expression and it was established that the MRL/MpJ strain lacked p21 expression. The loss of p21 leads to skipping of the G1 checkpoint and arrest at G2/M. Further studies showed that p21-null mice closed ear lesions within four weeks compared to the background strain of this genetic model, which did not close ear lesions [77,79]. Cells isolated from the pinna of p21-null mice exhibited increased DNA damage, cell cycle arrest and other biochemical features common to healer mice. Further experiments in mice null for several other cell cycle checkpoint components, including p53, p16 and GADD45, failed to induce a healing phenotype [80], suggesting that p21 may have additional functions beyond cell cycle arrest that suppress regeneration.

Further support for cell cycle involvement in the generalized healer phenotype comes from fine-mapping studies using ear lesion closure as a phenotypic readout. Cheverud et al. [30], using an advanced intercross (AI) line for healing, confirmed previously identified healing loci on chromosomes 9, 10, 11, 14 and 18 [29,81] and identified new loci on chromosomes 1, 7, 12 and 13 [30]. Overall, 19 intervals were associated with healing. When pathway analysis was conducted on genes present within these intervals, the overrepresented molecular processes included 'cell cycle' and 'DNA damage'. Of the 19 QTL loci, many

contained genes involved in cell cycle regulation and DNA damage and repair including *Blm*, *Brca1*, *Cdk2*, *Fgf1*, *Fn1*, *Ptprc*, *Xrcc5*, *Atr*, *Becn1*, *Kif23*. One of these, *Kif23*, a gene overexpressed in LG/J healer mice, was studied further. *Kif23* which encodes the kinesin-like protein KIF23, is a motor molecule involved in cytokinesis and developmental processes important for regeneration in amphibians and fish. It localizes to the midzone during cytokinesis and was present in the regeneration blastema and epidermis of healer mice, but was absent in non-healer SM/J tissues [30].

It is unknown why high levels of cell cycle arrest promote tissue regeneration, but they may be related to the need to synthesize a new extracellular milieu during certain regenerative phases. Also, the role of increased DNA lesions in regeneration is unclear and healer mice do not appear to present with higher rates of cancer as might be expected with increased DNA damage. Further studies are needed to clarify whether cell cycle and DNA damage alterations persist throughout the regeneration process or just during certain programs within the regeneration process, i.e., during blastemal production, the proliferation and patterning of the regenerating structure, or regenerative outgrowth phases.

6.2. Wnt pathway and regeneration

Two additional genes identified in the Rai et al. transcriptome study that had expression profiles correlating with articular cartilage (and ear wound) healing encode the Wnt signaling pathway molecules Axin2 and Wnt16. The Wnt pathway is no stranger to regeneration, as it was previously involved in regeneration of adult skeletal muscle [70], and amphibian limb [71] and zebrafish fin [72]. Fine mapping identified *Wnt3a* as a candidate gene for ear-wound regeneration and follow-up immunostaining demonstrated higher levels of its protein product in the regenerating ear epidermis in LG/J healer compared to SM/J non-healer mice, suggesting that enhanced Wnt activity leads to enhanced healing response, possibly due to the proliferative actions of beta-catenin [30]. In the same study, a non-coding RNA (miRNA-344) known to modulate Wnt/beta-catenin signaling [82] was also found to be differentially expressed and may function to regulate *Wnt3a* expression [30]. However, inhibition of Wnt pathway signaling by topical application of intracellular Wnt signaling inhibitors resulted in improved healing in a non-healer strain including a robust restoration of auricular cartilage [83]. While clearly implicating Wnt signaling in healing, this finding suggests that Wnt may have a more complex relationship with regeneration than previously believed and additional studies are required to tease out the role of Wnt in regeneration.

6.3. Dampened inflammatory response

The immunomodulatory transcription factor Cebpb was identified as having its gene expression signature associated with articular cartilage healing [66]. Altered levels of immunomodulatory factors have been associated with scarless healing in a number of contexts (reviewed by Harty et al. [22]). In the well-studied embryo/early fetus cutaneous wound healing model, suppression of inflammation due to alterations in the balance of pro- and anti-inflammatory cytokines has been implicated in scarless wound healing with restoration of normal ECM architecture [84,85].

There is ample evidence for a reduced inflammatory response in epimorphic healer mice [54,86,87]. Transcriptomic studies on tissue from healing ear wounds support the notion that the regenerative response in MRL/MpJ mice is associated with an altered inflammatory response [88,89]. A comparison of mRNA expression profiles between MRL/MpJ-Fas^{lpr} (containing a Fas ligand mutation) and C57BL/6 mice revealed that wound repair in the MRL/MpJ mice is associated with a metabolic shift towards a low inflammatory response and increased tissue repair [90]. To gain further insights into tissue-specific differences in gene expression, Podolak-Popinigis et al. conducted a transcriptomic analysis on ears and four other tissues from 8-week-old MRL/MpJ and the C57BL/6 and BALB/c reference strains [89]. Pathways analyses indicated MRL/MpJ-specific suppression of immune system genes in all tissues, supporting the hypothesis that immune system regulation is a mechanistically relevant part of the regeneration response.

The importance of the inflammatory response is supported by the observation of scarless skin wound healing in macrophage-deficient PU.1-null mice and several other inflammatory gene knockout mice [91,92]. Gourevitch et al. confirmed that the immune response was altered in such mice following ear injury and showed that inhibiting the immune response with the use of a Cox inhibitor partially impaired regeneration, the opposite of what many studies predict if higher inflammation inhibits regeneration. The effect was modest but since the drug was only given for the first three days, the authors speculated that a more aggressive treatment regime may have resulted in a greater effect on healing. The investigators also identified a mast cell population that was not present in the wounds of the non-healer control mice and that may play a role in the enhanced healing phenotype [88]. As noted by Heydemann, the extent of inflammation may be a simplistic explanation [51]. While the immune system reaction to wounding appears to be important, the presence or absence of specific subsets of immune system cells and the order in which they are delivered to the wound site, are likely to be critical in promoting or suppressing regeneration.

What about joint tissues? Enhanced healing in the MRL/MpJ strain was associated with lower systemic levels of pro-inflammatory IL-1 α and IL-1 β and higher levels of anti-inflammatory cytokines compared with controls in an intraarticular fracture model [54,87]. Pro-inflammatory TNF α mRNA was upregulated 13-fold in synovial tissue isolated from C57BL/6 immediately following articular fracture and remained high for 7 days whereas TNF α levels were unchanged in the MRL/MpJ strain [87]. IL-1 β mRNA levels increased in both strains but were 10-fold higher in C57BL/6 mice compared to MRL/MpJ mice and persisted for twice as long. Gene expression levels for macrophage cytokines were higher in C57BL/6 mice than for MRL/MpJ mice, although levels were increased above baseline in both strains following injury. These factors are important for the recruitment of macrophages, monocytes, neutrophils and lymphocytes to sites of tissue injury and have been implicated in the pathophysiology of arthritis. C57BL/6 mice had markedly increased infiltration of activated macrophages in synovial tissue. The authors concluded that a robust local immune response initiates within 4 h of intraarticular fracture in C57BL/6 mice and that attenuation of this response in the MRL/MpJ strain might explain why the latter strain is relatively protected from post-traumatic arthritis. While the role of inflammation in cartilage restoration is still under debate, anti-inflammatory therapies for rheumatoid arthritis, such as

specific TNF α and IL-1 inhibitors, are in clinical use and could be considered for use in post-traumatic osteoarthritis.

6.4. Other mechanistic observations

There is clear evidence for a role for circulating factors in MRL/MpJ ear biopsy regeneration. When two ear hole punches were made 30 days apart, the rate of ear hole closure of the second hole was accelerated, even when the second hole was made on the opposite ear [93]. While this observation is consistent with a role for systemic immunological components in healing, it also raises the possibility for involvement of circulating stem cells. To assess whether bone marrow-resident mesenchymal stem cells (MSCs) can transfer healing properties from non-healer C57BL/6 mice to MRL/MpJs, Leonard et al. conducted an allogenic bone marrow transplantation experiment [94]. If MSCs or immune system cells and precursors present in the bone marrow of MRL/MpJs are important for the superior healing phenotype of MRL/MpJs then a bone marrow transplant from MRL/MpJ cells into C57BL/6 recipients should have better ear wound and articular cartilage healing. Rather surprisingly, the authors found that neither ear biopsy punch nor full-thickness articular cartilage injuries healed in C57BL/6 containing MRL/MpJ bone marrow. Furthermore, MRL/MpJ mice that received C57BL/6 marrow healed as well as control MRL/MpJ mice. Rarely has a negative result been so informative. MRL/MpJ-derived cells, believed to derive from the donor hematopoietic system, are present in the synovial niche. However, there is a complete lack of MRL/MpJ-derived cells in the injured cartilage of the C57BL/6 mice suggesting that MRL/MpJ MSCs are not contributing to cartilage regeneration. This study argues that immune system cells, in this case hematopoietic stem cells, present in the donor C57BL/6 marrow were not sufficient to drive articular cartilage and ear wound injury healing and the authors speculate that cell types outside of the bone marrow cavity are important to enhance repair in MRL/MpJ mice. The lack of recipient ear-wound healing in a bone marrow transfer experiment was reported in several other studies [86,95]. It is unclear, however, what effect irradiation had on the injury response [95].

One possible source of stem cells is the perichondrium, the dense connective tissue adjacent to cartilage that contains chondrogenic cells. Srour et al. developed a novel animal model for costal cartilage repair where the central cartilage is removed with and without the perichondrium and tested the ability of MRL/MpJ and CD-1 mice to restore costal cartilage [96]. Both strains regenerated costal cartilage within one to two months post-surgery when the perichondrium was left intact, whereas no healing occurred when the perichondrium was removed thus highlighting the importance of the perichondrium in cartilage healing. On the other hand, stem cells may not be essential since a cell-free extract of regenerating blastemal cells enhanced ear-wound healing in a non-healer strain [97]. This suggests that the blastemal extracellular matrix with its bound constituents, such as cytokines and growth factors, are sufficient to direct regeneration.

7. Outlook for cartilage regeneration research

The finding that mammals have retained a higher capacity for cartilage regeneration than previously assumed is an unexpected observation and has stimulated interest in using

regenerative medicine to isolate molecular pathways that could promote enhanced tissue repair. The healer mice identified in recent years appear to possess an intrinsic ability to bypass the normal fibrotic 'repair' response and, instead, execute a more 'regenerative' program of tissue restoration, suggesting that ancient genetic networks for tissue regeneration exist in latent form in mammals. The challenge ahead is to understand mechanisms that are responsible for the regenerative tissue response and to translate these insights into new treatments for articular cartilage lesions. The data so far have not conclusively defined such mechanisms and although many important questions remain, significant progress made in the pioneering studies reported here help us get a better idea of directions to take in future research investigations.

Studies that have focused on articular cartilage regeneration have identified multiple genetic loci and gene expression profiles that distinguish a cartilage healer from a non-healer. There is evidence for DNA damage/cell cycle, immuno-regulation, Wnt pathway and autophagy involvement in articular cartilage regeneration [54,66,87]. The complexity of epimorphic regeneration, with its distinct phases from the establishment of a wound epidermis to formation of a mesenchymal cell blastema to regrowth and repatterning, may mean that different classes of molecules/processes are important at different stages of regeneration. The discovery of intermediate healers in the recombinant inbred and advanced intercross lines [29,30,59,63,64] suggest that this is indeed the case, with these lines having some, but not all the gene variants necessary for complete regeneration. One approach to tackle this genetic complexity is to test multiple genome variants simultaneously using CRISPR-Cas9 gene editing technologies. The CRISPR-Cas9 method is well-suited for investigating complex traits because of the relative ease and speed of editing multiple genes. This could take the form of re-creating a specific collection of SNPs enriched in healer strains or altering the regulation of key players by manipulating promoter/enhancer elements, and looking for effects on healing.

With the association of certain gene expression 'states' with regeneration in cartilage, it is likely that non coding RNAs, which play major roles in gene regulation, are involved and this has begun to be investigated [66]. The role of epigenetic changes, which can decisively alter gene expression, have not been explored in healing yet, but certainly represent a valuable line of inquiry.

In addition, as awareness of epimorphic regeneration in mammals grows, other genetic models with regenerative capacity are likely to surface, especially since the ear biopsy punch wound is easily made and scored. It will be fascinating to see whether articular cartilage regenerates in these strains and in specific genetic mouse models including the p21-null mice, or in wild populations such as the African spiny mouse where ear wound healing has been demonstrated.

The poor quality cartilage that results from fibrous repair of large cartilage defects is a biological barrier that needs to be overcome if clinical outcomes are to be improved. While a specific mechanism has not yet been fully described, several broad pathways and a few candidate genes have been identified and we can begin to consider specific pharmacological interventions to steer the healing process towards authentic restoration. From a drug

treatment perspective, there already exists immuno-modulatory drugs and Wnt pathway activators/inhibitors that could be tested for enhanced cartilage repair in animal models. One can imagine a time not too far off when drugs or biologicals, introduced at the time of surgery, activate latent ‘regeneration’ pathways to induce rapid, high quality, structurally robust cartilage restoration.

Abbreviations

MRL	Murphy Roths Large
OA	osteoarthritis
DMM	destabilization of medial meniscus MSC mesenchymal stem cell
IL	interleukin
TNF	tumor necrosis factor

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Table 1

Cartilage-containing tissues with enhanced healing in MRL/MpJ and related strains. Healer strains in bold.

Tissue	Lesion	Strains Tested	Reference
Articular Cartilage	full thickness and laceration	MRL/MpJ , C57BL/6	[52]
	trochlear lesion	DBA1 , C57BL/6	[53]
	intraarticular fracture	MRL/MpJ , C57BL/6	[54,87]
	surgical osteoarthritis model	LGXSM-6 , LGXSM-33	[64]
	full thickness lesion -loci mapping	MRL/MpJ , DBA/1J, DBA2/J, C57BL/6, LG/J , SM/J and LGXSM RI lines	[63]
	full thickness lesion -candidate gene analysis	MRL/MpJ , DBA/1J, DBA2/J, C57BL/6, LG/J , SM/J and LGXSM RI lines	[66]
	full thickness lesion -bone marrow transplant	MRL/MpJ , C57BL/6	[94]
Costal cartilage	costal cartilage removal	MRL/MpJ , CD-1	[96]
Elastic cartilage (ear hole lesion)	ear hole biopsy punch	MRL/MpJ-Fas-lpr, C57BL/6	[26] [57] [103]
	ear hole biopsy punch	MRL/MpJ-Fas-lpr , B10.BR	[86]
	ear hole biopsy punch	MRL/MpJ-Fas-lpr , C57BL/6NTac	[81]
	ear hole biopsy punch	LG/J , SM/J	[81]
	ear hole biopsy punch	MRL/MpJ , SJL/J	[56]
	ear hole biopsy punch	MRL/MpJ , C57BL/6	[34,39,47,48,49,52,88,102,104]
	ear hole punch in tg mice expressing AGF	tgK14-AGF in BABL/c	[105]
	ear hole biopsy punch	MRL/MpJ , CAST/Ei	[95,58]
	ear hole biopsy punch	MRL/MpJ	[93]
	ear hole biopsy punch	AIRmax , AIRmin	[106]
	ear hole biopsy punch	LG/J , SM/J	[29]
	ear hole biopsy punch	8wk-old C57BL/6 and 40-wk-old C57BL/6, 8-wk old BALB/c	[108]
	ear hole punch in p21-null mice	CDKN1a (p21) -/- , B6129SF2/J	[77]
	ear hole punch in TGFBRI1-null mice	TGFBRI1R244Q mutant on C57BL/6, BALB/c backgrounds	[107]
	ear hole biopsy punch	MRL/MpJ , DBA/1J, DBA2/J, C57BL/6, LG/J , SM/J and LGXSM RI lines	[63]
	ear hole punch in a wild population	African spiny mouse , Swiss Webster mus	[21]
	ear hole biopsy punch	LG/J , SM/J advanced intercross	[30]
	ear hole biopsy punch	prolyl hydroxylase inhibitor-Swiss Webster	[99]
other tissues	digital tip amputation	MRL/MpJ , DBA/2, C57BL/6	[42,109]
	heart	MRL/MpJ , C57BL/6	[32,34,103]
	alkali-burned cornea	MRL/MpJ , C57BL/6	[31]
	skin transplantation	MRL/MpJ , B ₁₀ -BR	[41]
	peripheral nerve injury	MRL/MpJ , C57BL/6	[39]
	muscular dystrophy model	MRL/MpJ-DBA/2J-Sgec admixture	[44]