Introduction

Recently, we published a study demonstrating that a deletion of the gene \( p21^{CIP1/WAF} \) converts a non-regenerating strain of mouse to one capable of epimorphic regeneration and has provided a unique opportunity to uncover some of the unknowns of this process in mammals. Since \( p21 \) is involved intricately in so many cellular processes, it is not clear at this time how deletion of this gene results in such a healing phenotype. This review will discuss our results, how our findings relate to other studies, and speculation as to the role of \( p21 \) in regeneration.

A mammalian model of regeneration, the MRL mouse

In 1998, the MRL (Murphy Roths Large) mouse, generated from cross-breeding AKR, C3H, C57BL/6(B6), and LG strains of mice [1], was shown to be able to close ear punches without showing residual signs of injury or scarring [2]. Multiple tissues were perfectly replaced, cartilage re-grew, and hair follicles reappeared. Furthermore, this type of perfect multi-tissue healing, known as epimorphic regeneration, occurred with the formation of a blastema-like structure that had been shown to be key to amphibian limb regeneration [3-5]. This phenomenon had earlier been seen in rabbit ear holes [6-8], and furthermore, a blastema-derived structure had also been described during antler re-growth [9]. The amphibian and mammalian ear hole regeneration processes have many features in common, including rapid re-epithelialization of the wound [2], elimination of the basement membrane between the epidermal and dermal tissue layers [10,11], blastema formation, re-growth of cartilage and hair follicles, and scarless healing [2,12,13]. However, the existence of an inbred mouse model allowed this process to be genetically approachable. It was also determined that one of the strains used to generate the MRL mouse, the LG/J mouse, contributed the regeneration phenotype [14]. Ear hole closure has lent itself exceedingly well to genetic studies as this is a wound that is easy to access and measure and has proven to be a highly quantitative trait [15-17]. Recently, making use of an advanced intercross line (LG, SM F34 AIL) employing 1,200 mice and 3,600 single nucleotide polymorphisms [18], 18 quantitative trait loci were identified for ear hole closure with small intervals from 0.661 to 7.141 Mb in length, which essentially reduced the healing intervals 10- to 50-fold from studies using F2 mice [15] (JM Cheverud et al., manuscript in preparation). This has allowed a more focused analysis of candidate genes. Further narrowing of
these loci and testing of candidates using gene knockouts should lead to the final identification of these genes.

Besides ear hole closure, multiple organ and injury systems have extended the MRL mouse’s unusual healing properties. They include regenerative studies in the heart [19-21], central nervous system stem cells and tissue [22-24], cartilage [25], cornea [26], digit [27,28] and myometrial healing [29]. Dorsal skin wound healing, which involves skin contracture, has been reported to be no different or even worse in the MRL compared to controls [30,31]. However, a recent study shows that if the wound has a syngeneic or allogenic skin transplant, the MRL shows far better healing than the control [32]. One possible explanation for the healing differences in different systems is that wound contracture, involving myofibroblasts or cells expressing Sma-1 (smooth muscle actin), known to be responsible for scarring, is different in the MRL. Preliminary studies suggest this [33] (D Gourevitch, K Bedelbaeva, unpublished data). Thus, the wound site and type of wound need to be considered in the MRL’s healing properties.

G2/M cell cycle accumulation of regenerating cells

The cells derived from the ear of regenerating and non-regenerating mice also show significant differences from each other and represent what is seen in vivo. MRL fibroblast-like cells from uninjured ears display an uncommon metabolic profile characteristic of an embryonic-type aerobic glycolysis, a feature of the adult MRL mouse itself, versus the more common metabolic state - oxidative phosphorylation - as seen in the B6 mouse [34]. These cells express stem cell markers similar to adult MRL tissue that expresses these markers [34]. In a separate study, cells derived from the injured MRL ear blastema expressed stem cell markers as found in vivo [35] and displayed highly proliferative and migratory responses in vitro similar to human multipotential progenitor cells in this study [36].

The rapid growth rate of fibroblast-like cells from the uninjured MRL ear was noted early on and examination of cell cycle regulation comparing healer MRL to non-healer B6 cells showed that the healer cells had an unusual accumulation of cells in G2/M [33]. A likely explanation of such G2/M accumulation or potential arrest was a DNA damage response and this was supported by an increased p53 response in the MRL [33] and confirmed with data showing that foci of γH2AX and TopBP1, a phosphorylated histone and a protein recruited to sites of DNA damage, respectively, were highly increased in MRL cells and tissue [33]. DNA damage itself was tested using the comet assay and found in nearly 90% of healer cells compared to 5% of non-healer cells, showing both single-strand and double-strand breaks. Furthermore, the DNA repair protein RAD51 was increased in healer cells, suggesting that error-free homologous recombination was being used [33]. The cause of the DNA damage is still unclear, but the lack of the cell cycle protein p21Cip1/Waf1 discussed below suggests a replicative stress mechanism.

These results agree with many reports in the literature that G2/M accumulation is associated with regeneration in examples ranging from hydra [37] to amphibian [38] to mammalian liver [39,40]. The literature also shows that cells undergoing blastema formation synthesize DNA but have a low mitotic index, indicating an accumulation between S and M and implicating G2 [41-47]. Multiple in vitro studies have carefully explored cell cycle arrest and the factors involved in the re-entry of cells into S phase of the cell cycle and accumulation in G2, as seen in multinucleated muscle myotubes and myofibers from regenerating amphibian limbs [48], in multinucleated mammalian myotubes generated from rat C2C12 cell line myoblasts, and in primary mouse myoblasts [49-51].

In MRL ear-derived cells, the fact that DNA damage was so widespread made one question why an accumulation of cells was seen in G2/M and not in G1/S. This led to an examination of G1 cell cycle regulatory proteins. The first to be examined, the CDKN1A or p21Cip1/Waf1 protein [52], was found to be repressed in these cultured cells. Examination of similar ear-derived cells from a CDKN1A-deficient mouse [33] showed the same phenotype as MRL cells with increased DNA damage, γH2AX expression, and G2/M accumulation. But most striking was the fact that this mouse could fully close ear-hole injuries at least as well as the MRL mouse [33]. There have been other mice that possess the ability to partially heal ear holes, including nude mice [53], mice expressing the transgene AGF (angiopoietin-related growth factor) in keratinocytes [54], and mice selected for inflammatory potential [55]. However, what was surprising to us was that deletion of this single gene, as predicted from our in vitro ear dermal cell model, could actually result in the full MRL epimorphic regeneration phenotype.

The role of p21<sup>Cip1/Waf1</sup>, regeneration, and the retinoblastoma protein

Earlier studies have examined the role of p21 in regeneration of the mammalian liver. Gene expression of p21 plays a role in hepatic regeneration by both p53-dependent and p53-independent control mechanisms [56]. Transgenic mice that over-express p21 produced large polypliod nuclei in a portion of the hepatocytes and the regenerative capacity of the livers was halted [57]. Over-expression of STAT-3 with resulting p21 upregulation impairs regeneration in fatty livers [58]. Consistent with this picture, repression of the p53/p21 pathway was shown to enhance liver regeneration [59]. Such studies parallel our recent findings [33].

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Arthur and Heber-Katz Stem Cell Research & Therapy 2011, 2:30

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The overall understanding of the functions of p21 can be quite overwhelming considering the complexity of functions in which this protein has been implicated. p21 is involved in the response to cellular stresses, such as DNA damage, oxidative stress, cytokines, mitogens, tumor viruses, and anti-cancer agents, and can have tumor suppressive activities and oncogenic capabilities depending on the cell type and context [60,61]. For example, p21 is transcriptionally regulated by p53 for tumor suppressor activity and as an inhibitor of cell cycle progression through the inhibition of cyclin-dependent kinase (CDK)-cyclin complexes and proliferating cell nuclear antigen, which can lead to differentiation, apoptosis, or senescence. Increasing this complexity is the fact that p21 can regulate gene expression and other cellular events, such as autophagy and a DNA damage repair response, through protein-protein interactions that depend on the cell type, subcellular localization, expression levels, protein stability, and post-translational modifications [62-66].

So which of these functions are involved in the regeneration phenotype seen in the p21-/- mice? Some indication may come from in vitro studies in other regenerating systems. For example, adult urodele amphibians can regenerate limbs through a process that involves loss of differentiation markers, cell cycle re-entry, proliferation, formation of a blastema, and differentiation into adult tissue [12]. In an amphibian in vitro model of skeletal muscle regeneration, retinoblastoma (Rb) protein plays a predominant role in cell cycle re-entry through phosphorylation by CDK4/6 [67]. This process requires serum to stimulate entry of the quiescent nuclei of multinuclear myotubes into S-phase with a serum-derived thrombin-activated factor being necessary for Rb hyperphosphorylation, resulting in its ‘inactivation’ [48,68]. These cells enter S phase but arrest and do not separate into single cells, which would allow further progression of the cell cycle through mitosis. However, there are conflicting reports about mammalian cells. Myotubes from an Rb-/- mouse are capable of cell cycle re-entry and show DNA synthesis upon serum stimulation but no mitosis in one study [50] but no cell cycle re-entry in another [51]. In a separate study using mammalian myotubes generated from the rat C2C12 myoblast line, newt regeneration blastema extract led to myotube cellularization to smaller myotubes and proliferating mononucleate cells, suggesting de-differentiation with reduced expression of mature muscle cell markers [49]. In addition, a recent report using primary myoblasts [69] suggests that another factor in addition to Rb, p19arf, must be inactivated for cell cycle re-entry and de-differentiation in postmitotic mammalian muscle. The tumor suppressor protein p19arf acts as a regeneration suppressor and is not found in regenerative vertebrates, suggesting that it has interesting potential as a key to mammalian regeneration. Thus, Rb inactivation has been shown to be important in both amphibian and mammalian regeneration in vitro.

The p21 protein, its major role being a CDK inhibitor found on chromosome 17 in the mouse, is known to block proliferation by preventing the phosphorylation of Rb and the transcription of cell cycle-regulated pro-proliferative proteins. The p21 protein binds to cyclin-CDK (2/4) complexes, not allowing them to function as kinases. They in turn cannot phosphorylate Rb, which remains bound to E2F, a transcription factor responsible for proliferation, effectively blocking E2F function. Thus, p21 activity directly leads to suppression of cell cycle transit and the loss of p21 should promote E2F activity, lead to enhanced DNA synthesis and potentially to de-differentiation. Rb function, then, in the studies above should be directly affected by p21 activity.

Not surprisingly, p53 and p21 have been shown to prevent the transition from fibroblasts to induced pluripotent stem cells [70-72]. The level of de-differentiation in the p21-/- mouse is being further explored, although we have previously reported that stem cell markers are over-expressed in MRL tissue [34].

The role of p53, senescence, and transforming growth factor-β in regeneration

As mentioned above, we found that p53 was up-regulated in MRL mouse ears, though p21 was absent. Is there a role for p53 in regeneration? Unlike the p21-/- mouse, which is a complete regenerator, p53-/- mice show no regenerative capacity [73]. This finding established a p53-independent function of p21 that is important for regeneration. However, MRL.p53-/- crosses showed not only healing rates similar to or better than the MRL itself but also showed enhanced differentiation in the form of increased chondrogenesis and adipogenesis [73]. The major role played by p53 as the ‘guardian’ of the genome is due to its ability to respond to DNA damage and cellular stress by inhibiting cell cycle progression and then regulating DNA repair, cell cycle control, apoptosis, differentiation, autophagy induction, and senescence. It is not clear which of these functions or lack thereof could be responsible for the enhanced differentiation observed in MRL.p53-/- mice [64,71,74-79]. One study suggests that removal of p53 allows for an accumulation of cells with elevated levels of DNA damage (on a repair-deficient background mouse), which delays hair follicle renewal and regeneration [80,81]. However, we observed hair follicle formation in our MRL/p53-/- mice [73]. Further regeneration studies on different tissue types need to be performed in order to determine the role of p53 in regeneration.

One potential area of interest are the roles of p21 and p53 in both differentiation and cellular senescence at
wound sites. It has been shown that elimination of p21 in mouse stem cells with dysfunctional telomeres, a marker for senescence induction, increases stem cell function and the life span of these mice without an increase in cancer formation, providing a direct role for p21 in both stem cell differentiation and senescence [82]. One direct link for p21 in differentiation and senescence is suppression by the Twist proteins, major regulators of embryogenesis [83]. The Twist proteins inhibit p21 in a p53-independent manner and promote epithelial-mesenchymal transition and suppress cellular senescence [84].

The two major pathways for inducing senescence in cells of multiple tissues are p53/p21 [85-91] and p16INK4a [75,92-95]. In an earlier paper, we suggested that senescence was not a factor in MRL regeneration because of the lack of p53 requirement [73]. However, there is, in fact, evidence that p21 can induce senescence in the absence of p53 [87,96-98] as well as p53-mediated p21-independent activation of senescence [99-101]. It has been suggested that reactive oxygen species are necessary to maintain the senescence phenotype and that both p16 and p21 are involved [99,102,103]. Actually, we previously reported that reactive oxygen species levels are decreased in the MRL mouse [34], consistent with an aerobic glycolytic metabolism, which argues against senescence playing a functional role. In addition, the protein RhoD, which is required for transformation by the oncogenic protein Ras, is responsible for suppressing p21 induction and subsequent senescence [104,105]. The gene ID1 has been shown to repress HRAS-mediated senescence in the presence of increased amounts of p21 [106], arguing the other way. Recently, a publication showed that the matricellular protein CCN1, which is expressed at the sites of wounds, induces senescence through p53 and actually helps to prevent fibrosis during tissue repair [107]. In this case, however, the healing is tissue repair with scarring and not blastema-induced scarless regeneration. Thus, the connection between senescence and regeneration, and its difference compared to oncogenesis, is yet to be determined.

Another major regulator of p21 is transforming growth factor (TGF)-β1, which is involved in anti-proliferation and differentiation [108]. TGF-β1 controls proliferation, differentiation, migration, and apoptosis in embryonic and adult tissue through the Smad3 pathway [109-113]. Multiple studies in mutant mice lacking the TGF-β1/Smad3 pathway have implicated a regeneration phenotype in mice: mice lacking TGF-β1 show an increase in wound closure and epithelialization [114]; transgenic mice null for Smad3 show increased re-epithelialization and tissue renewal [115]; and Smad7 over-expression leads to Smad3 down-regulation and to enhanced liver regeneration through the TGF-β1/Smad3/p21 pathway [116]. Smad3 has been implicated as a candidate gene in our genetic mapping studies of healer MRL and parental LG mice [15]. Contrary to these results, other transgenic studies on TGF-β1-null mice showed malfunctions in the repair of excisional back skin wounds due to altered inflammatory responses [117-119]. Our studies have shown that a TGF-β1/Rag1 double knockout mouse is a partial healer [73]. An interesting fact is that TGF-β1 enhances Sma-1 production and myofibroblasts associated with scarring [120] and reduces regenerative healing, whereas the TGF-β isoform TGF-β3 enhances scar-free healing [121].

Conclusions
The MRL mouse is the first genetically dissectible and molecularly tractable mammalian model of regeneration of multiple tissues in a single organism. It establishes the fact that regenerative capacity has not been lost to mammals through evolution but remains as a cryptic trait, which can be activated by the deletion of a single gene, p21. Thus, the p21-null mouse now should become a ‘single gene’ standard model for mammalian regenerative studies.

The lack of p21 may act to enhance the regenerative response in various ways. It could alter DNA damage and checkpoint responses, leading to enhanced proliferation. It could reduce TGF-β signaling, leading to reduced scar formation, and alter differentiation patterns. It could lead to lack of senescence and reduced cytokine responses. It could support progenitor cell stability as seen in induced pluripotent stem cell formation.

Besides determining exactly which function of p21 and its absence is responsible for enhanced ear hole closure, it will also be important to define the critical pathways in the MRL mouse that actually lead to p21 down-regulation and regeneration.

This article is part of a review series on Epigenetics and regulation. Other articles in the series can be found online at http://stemcellres.com/series/epigenetics

Abbreviations
CDK, cyclin-dependent kinase; MRL, Murphy Roths Large; Rb, retinoblastoma; Sma-1, smooth muscle actin; TGF, transforming growth factor.

Competing interests
The authors declare that they have no competing interests.

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