Regeneration in MRL mice: further genetic loci controlling the ear hole closure trait using MRL and M. m. Castaneus mice

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The MRL mouse has been shown to display an epimorphic regenerative response after ear hole punching leading to complete closure within 30 days and cartilage regrowth. The regenerative capacity of the MRL has also been seen after a severe cryoinjury to the heart leads to complete healing without scarring and functional myocardium. The wound healing ear hole closure response that occurs in MRL mice has been shown to be genetically controlled. We have previously identified 11 quantitative trait loci (QTL) that govern healing in an intercross of (MRL × C57BL/6) mice. However, it is desirable to use another poorly healing mouse strain to elucidate the full range of genetic factors that affect this important process. In the current study, we have used an inbred subspecies of the mouse, M. castaneus, and have confirmed a number of loci identified previously. In addition, we report three new healing QTL. Furthermore, in this strain combination, we note a strong sexual dimorphism also observed in the MRL × C57BL/6 cross, both in the healing trait and in the QTL that control it. (WOUND REP REG 2004;12:384–392)

The biological response to traumatic injury in higher organisms falls into two categories: wound repair and regeneration. It is generally observed that the capacity for tissue regeneration in mammals is limited or nonexistent, especially as compared to amphibians, where entire limbs can be regenerated after amputation. Several examples in mammals involve an "amphibian" type of wound healing, which can be seen in the re-growth and shedding of antlers and ear hole closure in rabbits. Ear hole closure is generally not seen in mammals, but there is one inbred strain of mouse, the MRL strain, which displays full ear hole closure. In addition to hole closure within 30 days, the MRL wound, as compared to the wound made in a control C57BL/6 (B6) mouse strain, rapidly reepithelializes, shows hair follicles in the wound site, and re-grows elastic cartilage. Both the fas-mutant (MRL/lpr) and the fas-wild-type MRL/MpJ strains display this remarkable healing ability.

In our previous studies, we showed that ear healing was a complex trait controlled by multiple genetic loci. We performed genome wide microsatellite screens of F2 and backcross mice using MRL (healer) and B6 (nonhealer) mice as founder strains. Five genetic intervals were shown to contain quantitative trait loci (QTL) that were significantly linked to the healing phenotype, and several others had suggestive linkages. Subsequently, additional wound healing QTL have been reported, detected in experiments employing MRL and a different nonhealing parental strain, SJL/J, in a similar experimental design.
Knowledge of the full range of QTL that control wound healing is dependent on, and limited by, the extent of differences between the two parental strains. For the nonhealing founders, an inbred strain that is distantly related to MRL is ideal. The *Mus musculus* group of mice consist of four subgroups including *M.m. musculus*, *M.m. domesticus* (to which the B6, MRL, and SJL/J mouse strains belong), *M.m. bactrianus*, and *M.m. castaneus*, from which the inbred CAST/Ei mouse strain is derived. It has been previously shown that the frequency of polymorphisms between wild mice and inbred laboratory mice is much greater than between different inbred strains of mice. This is especially true for *M. spreitus*; however, interbreeding *domesticus* strains with *spretus* strains is often difficult. The frequency of polymorphisms between *M. castaneus* and inbred strains is not as extensive as those seen with *M. spreitus*, but is still sufficient to provide a more useful set of polymorphisms for quantitative trait mapping.

Because genetic crosses between *M. domesticus* strains limit the genes that can be identified, a more comprehensive understanding of genes that underlie the genetic architecture of wound healing might be possible by using the CAST/Ei strain as a partner for MRL. For this reason, we have sought to verify and extend our knowledge of such loci by creating another segregating cross, mating MRL mice with CAST/Ei mice to create the F1 hybrid. We expected to increase the overall number of QTL but also to observe that certain QTL involved in wound healing would be shared between the two types of genetic crosses. Our results suggest that this is in fact a successful strategy not only for the identification of new healing loci, but for the verification of certain healing trait genes found in the original cross. The intervals containing these loci are described in this report. Further studies have revealed a highly significant difference between males and females for this trait and sex-specific QTL, as discussed below.

**MATERIALS AND METHODS**

The parental MRL/MpJ and CAST/Ei mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice from both parental strains were bred and maintained under standard conditions at The Wistar Institute (Philadelphia, PA). F1 and F2 populations were generated to conduct the genetic studies. The female parent used for generating F1 and intercross mice was the MRL mouse. All animal studies were reviewed and approved by the Wistar Institute IACUC committee.

**Phenotype determination**

A 2-mm through-and-through hole was made in the center of the cartilagenous part of both ears of 6-week-old mice using a metal ear punch (Cat. #01–337B, Fisher Scientific, Pittsburgh, PA). The holes were measured at the time of wounding and followed for wound closure using a grid-etched reticle (Bausch and Lomb 7×).

**Genetic analysis**

Genomic DNA was prepared from the liver of each animal in the (MRL/MpJ × CAST/Ei) × (MRL/MpJ × CAST/Ei) F2 population. DNA from the frozen liver was prepared using Qiagen DNA tissue extraction kit (Qiagen Inc, Valencia, CA). We identified 138 microsatellite primer pairs (Research Genetics, Huntsville, AL), that were polymorphic between MRL and CAST/Ei and relatively evenly spaced throughout the genome, and employed them to perform a genome-wide scan of the mice. Primers were radiolabeled using T4 polynucleotide kinase, or were used unlabeled. Amplification was conducted with the following reagent concentrations: 1× polymerase chain reaction buffer (New England Biolabs, Beverly, MA), 0.375 mM dNTPs, 0.5 U/µl of Taq polymerase, 0.165 µM of each primer, and 160 ng/20 µl of genomic DNA. Cycling conditions include a 1 minute at 95 °C denaturing, 35–50 cycles of 1 minute at 94 °C, 1 minute and 30 seconds at 55 °C, 2 minutes and 10 seconds at 72 °C, and a 6-minute final extension at 72 °C. Radiolabeled PCR products were resolved on 6 percent polyacrylamide gels and submitted to autoradiography as described and unlabeled products were run on 3 percent Metaphor agarose gels (FMC, Rockland, ME) and were visualized by ethidium bromide staining.

**Statistical analysis**

Genotype data was organized and analyzed through the use of the Map Manager QT program. For quantitative trait analysis, simple sequence repeat length polymorphism markers were evaluated individually based on linkage to the phenotypes, and intervals containing likely QTL were identified by interval mapping using the Zmap program in Windows QTL Cartographer v1.3, model 3 (Statistical Genetics, NCSU, Raleigh, NC). Composite interval mapping (Windows QTL Cartographer v1.3, model 6) was then performed on the dataset to allow for more precise definition of intervals containing QTL. Markers flanking the test interval are added to the regression model to control for the presence of linked QTL. Additional markers, unlabeled to the test interval but with significant effects on the trait, are added to the model to control for the genetic background. The most significant markers unlabeled to the test interval are chosen using a linear regression model with a forward/backward selection procedure in Windows QTL Cartographer v1.3, with a window size of 10 cM and the five most significant background markers. We found this necessary because we have found at least one example in the (MRL × C57BL/6)F2 where multiple distinct QTL were positioned on a...
single chromosome (in that case, chromosome 13). All analyses were performed for the combined population (males and females) as well as for males and females separately. Analysis for the combined population was performed with an additional covariate in the regression model to control for differences in lesions between the sexes in our population. Tests of significance for a QTL are reported in the form of a likelihood ratio test (LRT) statistic. Critical values for significance were determined by the permutation test routine in Windows QTL Cartographer based on a regression model developed by Churchill and Doerge. The values were based on $\alpha = 0.32$ (one standard deviation from the mean of 1000 randomly permuted LRT, as suggestive), $\alpha = 0.1$ (strongly suggestive), $\alpha = 0.05$ (significant), and $\alpha = 0.01$ (highly significant). LRT is equivalent to 4.6 × LOD (likelihood of linkage).

**RESULTS**

MRL/MpJ female mice were bred to CAST/Ei male mice and the F1 hybrids tested for ear hole closure. Considerable variance in ear hole size was observed in the (MRL × CAST/Ei) F1. Some of this variance was explained by sex. Female mice were better healers than males, with almost 50 percent being complete healers (0 mm diameter after 30 days), although the average healing among all F1 hybrid females was not significantly different from that of males (0.173 ± 0.193 vs. 0.213 ± 0.182, respectively).

The F1 mice were then intercrossed and 301 F2 mice were generated and tested for their ability to heal ear wounds (Figure 1). Again, it can be seen that the female mice were better healers with approximately 30 percent of the female mice showing complete healing. The mean residual hole size for the total (MRL × CAST/Ei) F2 population was 0.35 ± 0.33 mm, for the female F2 population was 0.25 ± 0.30 mm, and for the male F2 population was 0.47 ± 0.32 mm (difference between males and females, $p < 1 \times 10^{-9}$). In addition to the mean hole size, we analyzed the male and female F1 (not shown) and F2 histogram data for both skewness, as a measure of differences in curve symmetry, and kurtosis, as a measure of differences in data distribution (Table 1). The differences in the F2 populations between males and females are much more striking than in the F1 populations.

These F2 mice were then genotyped using microsatellite markers, at an average intermarker distance of 9.4 ± 6.2 cm. As QTL were identified, 20 more markers were added to intervals of positive linkage. As can be seen in Table 2, the wound-healing trait at 30 days could be mapped to multiple individual loci. Linkages seen for each marker were refined by submitting the dataset to composite interval mapping (CIM) (Figure 2). In this method, potential artefactual linkages are reduced by correcting for linkages existing at other loci. CIM strengthened the likelihood ratio statistic of each QTL, supporting the validity of the linkage seen at each individual microsatellite marker. In the total cohort of segregating progeny, individual markers or intervals identified by CIM showing at least suggestive linkage, using the arbitrary criterion of $\alpha = 0.32$, were on chromosomes 2, 11, 13, 14, and 17 (Figure 2). The linkage of QTL near D13Mit16, at 10 cm on chromosome 13, was seen in the initial (MRL × B6) F2 cross, and the finding of linkage in the (MRL × CAST/Ei) F2 is therefore a confirmation of the effect of that locus, heal2, on wound healing. The composite interval map of wound healing loci on chromosome 13 (Figure 3A) shows a broad peak with several subpeaks, representing what are likely to be several independent QTL on this chromosome. This is reminiscent of the multiple QTL seen on chromosome 13 in the (MRL × B6) crosses and likely represents a confirmation of at least some of those QTL. One interval in particular is located at 35 cm distal to the centromere, which showed overall significant linkage (peak LRT value by CIM = 18.0) to healing in the MRL × CAST/Ei F2; it is designated heal7.

The QTL on chromosome 11 has also been seen in a replicate study of the (MRL × B6) F2 segregating population, where it was given the designation heal10. It is located in the distal portion of the chromosome in a broad interval (60–70 cm). Another QTL was found by CIM analysis in the chromosomal region between D14Mit201 and D14Mit233 (from 12 to 20 cm) where there is an interval with highly significant linkage to wound healing (Figure 3C), and this is designated heal12. The M. m. castaneus-derived allele of heal12 is responsible for better healing and it is dominant over the MRL-derived allele at this locus.

Another highly significant linkage to the healing trait in the total population was detected on chromosome 17 (Figure 3D), in a location determined by CIM to be between 35 and 45 cm distal to the centromere. This QTL was highly significant in the (MRL × CAST/Ei) strain combination, with an LRS > 22, and is designated heal13. The allele of heal13 that is associated with better healing is from the CAST/Ei nonhealer mouse, and behaves as an additive gene, with values for wound healing in the heterozygotes intermediate between the two homozygous classes.

Analysis of the male and female populations separately (Table 2) showed that many of the QTL were sexually dimorphic. Male-associated loci included the heal2 locus on chromosome 13, and the new heal locus on chromosome 14 (Figure 3B). As in the original strain combination, heal2 is more significant to male healing than to female healing, and shows an additive effect with homozygous heal2/MM mice as the best healers. A female-associated QTL was seen on chromosome 9,
although it was of modest significance. The chromosome 9 QTL, if confirmed in subsequent crosses, comes from the MRL mouse and shows an over-dominant effect, with the heterozygotes having the most significant healing in female mice. The locus on chromosome 17 showed higher significance in the male cohort, although it is active in female mice. In general, peak LOD scores were overlapping for male and female linkages, although for the QTL on chromosome 11, maximum linkage to wound healing in female mice mapped to a more proximal position (approximately 60 cM) than that of the linkage seen in male mice.

Figure 1. Ear hole closure in mapped F2 population. Three hundred and one F2 mice were ear punched and examined 30 days after wounding. The data presented are the number of mice (y-axis) and the given hole diameter (x-axis) at 0.1 mm units measured. The three populations presented in this graph are (A) the total population; (B) the male group of the total population; and (C) the female group of the total population.

Table 1. Statistical analysis of hole diameter histograms

<table>
<thead>
<tr>
<th></th>
<th>Total F2</th>
<th>Female F2</th>
<th>Male F2</th>
<th>Total F1</th>
<th>Female F1</th>
<th>Male F1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.369</td>
<td>0.269</td>
<td>0.49</td>
<td>0.191</td>
<td>0.173</td>
<td>0.213</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>0.327</td>
<td>0.302</td>
<td>0.316</td>
<td>0.187</td>
<td>0.193</td>
<td>0.182</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.019</td>
<td>0.023</td>
<td>0.027</td>
<td>0.028</td>
<td>0.039</td>
<td>0.042</td>
</tr>
<tr>
<td>Skewness*</td>
<td>0.707</td>
<td>1.36</td>
<td>0.187</td>
<td>0.912</td>
<td>1.023</td>
<td>0.831</td>
</tr>
<tr>
<td>Kurtosis*</td>
<td>−0.366</td>
<td>1.483</td>
<td>−0.852</td>
<td>0.587</td>
<td>0.611</td>
<td>0.759</td>
</tr>
</tbody>
</table>

*Skewness and kurtosis describe the histograms seen in Figure 1. Skewness is a measure of symmetry. Kurtosis is a measure of whether the data is peaked or flat relative to a normal distribution. The female data with high kurtosis has a distinct peak near the mean and declines rapidly. The male data with low kurtosis has a flat top near the mean.
DISCUSSION

Broad-based molecular and genetic dissection of regeneration has recently come into its own. This is due to the ease of producing cDNA libraries, and techniques such as differential display and microarray analysis along with genetic mapping studies. Such analyses have been carried out in animal models of regeneration such as planaria,24 newt,25 xenopus,26,27 and the mouse.12,13,17 The potential for cross species comparison among diverse phyla should allow central pathways to be defined in the future.

Our previous studies using 101 (MRL/lpr × B6) F2 mice and 42 BC mice showed QTL linked to the wound healing and regeneration phenotype on chromosomes 7, 8, 12, 13, and 15. The cross in the present report, which used the Fas-normal healing strain, MRL/MpJ, and a different partner strain, CAST/Ei, confirmed the linkage of heal2, and gave support to previously suggestive loci12,17 on chromosomes 11 and 13 (heal10 and heal7). CIM of the (MRL × CAST/Ei) F2 cross documented the presence of at least two significant new QTL on chromosome 14 and 17 (LRT = 19.8 and 19.3, respectively). These are designated heal12 and heal13. Therefore, MRL mice and CAST/Ei mice represent a useful, genetically different combination for this trait and illuminate new loci involved in the healing and regeneration process.

We have discovered that wound healing is controlled by different genes in male and female mice. Both of the QTL on chromosome 13 are more significant in male than in female mice, whereas the QTL on chromosome 9 is significant only in females, and the pooled cohort of males and females showed no linkage of this marker. This phenomenon has also been observed in the MRL × B6 cross17 and in other QTL analyses of different complex traits. For example, in the mouse model of multiple sclerosis, experimental allergic encephalomyelitis, multiple sex-specific QTL were found that control susceptibility and severity of disease in large segregating populations of (SJL/J × B10.S) F2 and backcross animals.28–30 This effect has been seen in multiple settings, including other autoimmune models,31 immune responses to infectious agents,32 drug dependency,33 bone mass,34 and pain tolerance.35 The broad array of different quantitative trait models that exhibit sexual dimorphism for their genetic control suggests that this effect must be tested in any search for quantitative genetic factors and could be influenced by the presence of estrogen or androgens.

Interestingly, recent studies by Ashcroft and Roberts36,37 directly relate to this issue and to our results. We had previously demonstrated that MRL male mice close ear holes less well than MRL female mice and that male castration led to full healing;
whereas ovariectomy had no effect.\textsuperscript{17} In their wound healing studies examining the role of smad3, a mediator of transforming growth factor-β (TGF-β) signaling, Ashcroft et al.\textsuperscript{36,37} showed that removal of estrogen by ovariectomy resulted in poorer wound healing in both normal and smad3 \textsuperscript{−−} mice; on the other hand, removal of androgens by castration resulted in better healing but only in normal and not in smad3 \textsuperscript{−−} mice.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Depiction of the QTL identified in the (MRL × CAST/Ei) F2. Each mouse chromosome is displayed in order from its proximal to distal termini on the x-axis and the LRT on the y-axis. LRT for these composite interval map locations were generated by Windows QTL Cartographer (version 1.3). The cutoff of 11.5 is a general suggestive significance indicator and seen are the LRT for the (A) total F2; (B) LRT for the male F2; and (C) LRT for the female F2.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Analysis of data by CIM. Using this method, potential artefactual linkages are reduced by correcting for linkages existing at other loci. CIM strengthened the likelihood ratio statistic of each QTL, supporting the validity of the linkage seen at each individual microsatellite marker. Plots of (A) chromosome 13 QTL; (B) chromosome 14 QTL for males and females and (C) for the total population; and for (D) chromosome 17 QTL can be seen.}
\end{figure}
This indicated that smad3 plays a role in androgen-mediated healing. Could this be a candidate gene? Actually, smad3 is located on chromosome 9 at 34 cM and loci mapped by both ourselves and Masinde et al.13 (see Table 2) at this location support this molecule as a possible candidate gene. Furthermore, smad3−/− mice display enhanced reepithelialization, a phenomenon seen in the MRL mouse.

We also compared results from the present cross (MRL/MpJ × CAST/Ei) F2 with previous studies using MRL/lpr × CAST/Ei crosses used to map genes that modify the autoimmune phenotypes seen in the homozygous Fas-deficient cohort.38 None of the autoimmunity modifying loci colocalize with QTL that control the healing phenotype. This lack of correspondence is also true of the MRL/lpr × B6 crosses12 and follow-up studies with this strain combination.17 Comparing our results with those of the (MRLxSJL) F2 cross reported recently,13 we find that several QTL might be shared by both crosses: one on chromosome 4 in the (MRLx CAST/Ei) F2, at approximately 50 cM with the closest marker, D4Mit306, shows modest linkage in the point-wise marker analysis (not shown), but CIM shows the interval has suggestive level of linkage. This is the equivalent interval to that containing Sth4 in the MRLxSJL/J cross;13 it was weakly significant in the published report with MRLxB612 and in subsequent work we have done with this strain combination. There is a broad peak on chromosome 9 with suggestive linkage in the female cohort of MRLxCAST/Ei F2 that might align with Sth9, on chromosome 9 at 43 cM, as mentioned above. A list of all currently mapped healing and regeneration quantitative trait loci is given in Table 3, which shows the potential colocalizations of the healing trait QTL.

The intervals bearing the healing QTL discovered in this study are too broad to allow positionally informed selection of the many candidate genes contained within them. However, the most significant QTL found on chromosome 17 at ~ 44 cM does have a very interesting candidate gene, latent transforming growth factor beta binding protein 1 (LTBP-1).39–42 This molecule is critical in TGF-β regulation and is particularly interesting considering a previous study showing the MRL T cells overproduce TGF-β.43 TGF-β is stored as a latent complex for future use and the LTBP is considered a member of an extracellular matrix protein superfamily that is subject to cleavage by matrix metalloproteinase molecules.44,45 The large latent complex consists of

<table>
<thead>
<tr>
<th>Chr</th>
<th>Name of healing QTL</th>
<th>Peak marker</th>
<th>cM</th>
<th>Origin of healer allele in MRLxB6</th>
<th>Origin of healer allele in MRLxCAST</th>
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<tr>
<td>8</td>
<td>Heal1</td>
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<td>13</td>
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<tr>
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<td>15</td>
<td>Heal4</td>
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<td>Heal5</td>
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<td>Heal6 (Sth6/7)</td>
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<td>Heal7</td>
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<tr>
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<td>Heal8 (Sth4)</td>
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<td>9</td>
<td>Sth9 (Heal14)</td>
<td>D9Mit270</td>
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</table>

Heal designations as in McBrearty et al.12 Blankenhorn et al.17 and this study. Sth designations as in Masinde et al.13 Where possible, the heal nomenclature has been combined with the Sth nomenclature in intervals where there is possible overlap.
mature TGF-β1 homodimer, noncovalently associated with a disulfide-bonded complex of the N-terminal propeptide dimer of the TGF-β1 precursor and a third component, LTBP. Cleavage can occur via the activity of matrix metalloproteinase-2, -3, and -9, molecules shown to be activated during MRL ear hole during closure.46

Another group of molecules of interest on chromosome 14 includes angiogenin, found at 18 cm, as well as angiogenin-related protein, which may be an inactive analog of true angiogenin.47,48 This is especially interesting considering our demonstration that heart regeneration can be seen in the MRL mouse as well as enhanced angiogenesis.49,50 Finally, bone morphogenetic protein 4, found on chromosome 4 at 15 cm, is also a candidate gene for regulating healing. The identity of each gene underlying the QTL that control healing will require the production of congenic animals that bear the supported intervals and show a phenotypic shift in wound closure. Such studies are in progress.

ACKNOWLEDGMENTS

This work was supported by grant AI 42395 from the National Institute of Allergy and Infectious Disease, National Institutes of Health (EHK and EPB), and the National Institute of Allergy and Infectious Disease, and is a candidate gene for regulating healing. The identity of each gene underlying the QTL that control healing will require the production of congenic animals that bear the supported intervals and show a phenotypic shift in wound closure. Such studies are in progress.

REFERENCES


