Author's response to reviews

Title: Delayed Union of Femoral Fractures in Older Rats: Decreased Gene Expression

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Carole Mongin,

We have submitted a revised version of our manuscript entitled "Delayed Union of Femoral Fractures in Older Rats: Decreased Gene Expression" (MS # 48989785083710) by R. A. Meyer, Jr., M.H. Meyer, L.S. Phieffer, and D.M. Banks.

Please consider this for publication in BMC Musculoskeletal Disorders.

We have made the corrections suggested by the two referees. We thank them for their helpful and insightful comments. The manuscript has been improved by the review process.

Our response to the referees is shown below.

We thank you for your attention to our manuscript and hope that this revised version will be acceptable to the editorial board. Please let us know if anything further can be done to expedite its consideration.

Ralph Meyer

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Response to Jason Spector:

1. Introduction:
   a. We have enlarged the introduction and included other citations to research on the role of BMPs in fracture healing.
   b. The goal of the project has been restated to more closely relate it to the actual experiment.

2. Materials and Methods
   a. We have enlarged the description of the collection of the bone sample at tissue harvest. All of the fracture callus that was visible on the external surface of the bone was collected.
b. We agree that the data are semi-quantitative measurements and that there are too few observations to test for quantitative differences in concentration between time points. We comment on this on page 12 in the last paragraph of the Discussion. The main goal of this report is to emphasize the decrease in gene expression in older rats prior to the healing of the femoral fractures. We have adequate numbers of rats to report with confidence that this delayed union in the older rats is associated with decreased gene expression and not with negative-feedback driven up-regulation in an attempt to elicit a response from the skeleton.

c. We agree on the need to use caution in the interpretation of RT-PCR data. There is variability in the amplification of the oligonucleotides. The traditional approach is to use control, housekeeping genes to assure uniform amplification in all samples. We have found this to be impractical because we have not yet found a gene whose expression is unaffected by fracture. The increase in cell metabolism caused by fracture repair and the change in cell population as other cell types invade the site of injury combine to alter the expression level of all housekeeping genes so far measured. Thus, we have adopted the more traditional physiological solution to variation. This strategy is to increase sample size numbers until the desired effects can be demonstrated statistically. In this case, we have done 20 older rats at 4 and 6 weeks of age and have found undetectable amplimers for the BMP-related genes at these time points. In contrast, an equivalent numbers of rats at the same age show up-regulation of gene expression at 1-3 weeks after fracture. These samples are done side-by-side in adjacent wells of our electrophoresis gels and are in adjacent lanes of our Southern blots. These data lead to the conclusion that gene expression decreases in these rats, even though their fractures are not healed. The suggestion to use in situ hybridization is valuable. Unfortunately, we are not equipped to undertake that technique.

3. Results and Figures.
   a. We thank the referee for his kind remark on the graphs.
   b. The sentence on page 5, paragraph 3, beginning: "In the 15-month-old rats reported?", has been reworded to eliminate the confusion.
   c. The interpretation of the osteocalcin and type I collagen data has been moved to the discussion.
   d. We have revised the description of the housekeeping genes. It is not that they were not seen. Rather, the expression level varied with time after fracture. Expression of these genes also increased after fracture and decreased at 4 to 6 weeks after fracture. This variation was not as great as for the BMP data, but it was present. We felt that we could not normalize to an unstable reference gene. We organized our blots as a "complete block" design and were prepared to use analysis of variance with complete blocks to reduce the variability between blots. However, the hybridization of all three blots at the same time with the same lot of oligonucleotide probe gave quite uniform levels of radioactivity. The data are mean ? SEM for the level of radioactivity without further normalization. However this was done on samples run side-by-side on the same gels and hybridized at the same time to the same lot of labeled probe. Thus, much of the normalization was done by our experimental design which minimized the variation between samples.

4. Discussion
   a. An introductory paragraph has been added and the section beginning "the major conclusion?" has been revised and reworded.
   b. We have done limited histological study of fracture healing in these older rats. The pictures are unremarkable except that the process progresses more slowly in the older animals. Our TRIzol extraction discriminates against DNA, so we cannot get accurate measurements of the DNA collected in each sample. Thus, we cannot comment on the total number of cells present. However, we do monitor the RNA content of our samples, which does increase after fracture in the older rats to levels comparable to the younger rats. We run all RT-PCR reactions with a constant amount, 0.8 ug, of RNA. We hope to have the resources to perform a more elaborate histological study of this process in rats of
the two different ages in the future. While there are literature references to support a more limited number of bone forming cells in older rats, this does not impede the ability of the older rats to show early up-regulation of all genes so far studied. The similar rise in osteocalcin, type I collagen and type II collagen suggests that bone matrix forming cells are present in the callus of the older rats and make an appropriate amount of mRNA for the matrix proteins. Our working hypothesis is that this is a failure of stimulation of the skeletal cells rather than an absence or resistance of them than prevents skeletal healing. Further work will be needed to explain this interesting and unexpected finding. This manuscript is the initial report that fracture healing in older individuals is different than in younger individuals.

c. We agree that there are too few samples to compare one time point to another. However, we do have adequate sample size to report early subsiding of gene expression in the older rats prior to fracture healing. The results from this study have stimulated a larger study to make these comparisons.

Overall
We have tried to address the areas of concern and have increased the introduction and discussion to include additional references and greater interpretation of the results.

Response to Tony Freemont

1. More detail on sample size has been added to the statistics section in Materials and Methods. We have given more detail on the loss of animals in the 6 week older rat group. We started with an overall goal of three rats per age per time point. We allotted four rats to this group so as to assure three survivors. After the early time points had been collected, we lost two of the four to displacement of the intramedullary pin and one to infection that was not realized until the sample was harvested. At that time, it was too late to reapporion the rats as these were the last ones left. We do have a total of four rats at 4 and 6 weeks combined. The two time points are similar in being in the late stage of this study in which gene expression has decreased to baseline, yet the fractures remain unhealed. Moreover, we have done the additional rats presented in the Results section that confirm these findings.

2. We agree. This author is an experienced endocrinologist who is used to seeing negative-feedback control over biological processes. These results are most unsatisfactory! We have enlarged the Discussion to develop some of these ideas in greater detail. There are several competing hypotheses:
(a) end organ resistance (but then there is normal up-regulation of bone matrix genes and a failure of up-regulation in the stimulatory cytokines),
(b) constant time of stimulation with no negative feedback control (rats don't live this long in the wild and fracture may be a sufficiently rare event for them that having a good repair process for older animals may not be that important), or
(c) these are the wrong cytokines (perhaps the BMPs are needed for soft callus formation by chondrocytes and fibroblasts, and other cytokines stimulate bridging callus formation by the osteoblasts).
We are now engaged in additional experimentation to differentiate between these ideas.