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14. ABSTRACT

Effective gene therapy for musculoskeletal injuries requires the optimization of the components and techniques for the accurate assessment of therapeutic benefits. We have identified a murine leukemia virus (MLV) vector that provides robust transgene expression in fracture tissues, and applied it to the rat femur fracture model to express therapeutic transgenes. With these techniques, the MLV vector has been used to simultaneously express engineered bone morphogenetic protein (BMP)-4 and fibroblast growth factor (FGF)-2 transgenes in the healing fracture. Initial results indicate that this combination of FGF-2 proliferative and BMP-4 osteogenic functions does indeed enhance fracture repair. To characterize the molecular pathways of fracture healing, we have used microarray technology to examine normally healing fracture tissues during the inflammatory and endochondral bone formation stages of fracture repair. Inflammatory gene expression, so important to the initiation of healing, has been described. Molecular pathways have been characterized that 1) could mediate the regeneration of healing bone, and 2) involve intracellular FGF regulation of fracture repair. Independent techniques have confirmed microarray-based identification of genes expressed in fracture repair. Unknown genes expressed in fracture repair have been identified and the effects of inhibiting their expression in bone cell lines in vitro are under investigation.

15. SUBJECT TERMS
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INTRODUCTION

Bone fracture is one of the most frequent injuries occurring during military training and in battle. Recent conflicts have suggested that while survival rates among wounded personnel have increased, battlefield injuries to the extremities have tended to be quite severe, often requiring extensive therapy for the repair of the wounded musculoskeletal tissues (Blanck, 1999). However, even relatively minor but frequent minor fractures to bone can result in a loss of function with significant reductions in military preparedness. These injuries would also benefit from a therapeutic approach that produces rapid tissue repair. Any improvement in fracture therapy could yield substantial military benefits, as well as significant humanitarian and economic benefits in civilian applications (Einhorn, 1995).

Fracture repair requires a complex series of molecular events that coordinate the proliferation and differentiation of diverse periosteal tissues and bridge the injury with bone that is identical to the native bone (Bolander, 1992). Bone healing is unique in that bone normally heals without the production of a scar so common in the healing of most tissues, and is therefore more characteristic of a regenerative process. Growth factors are involved in all of these regenerative processes. To identify the molecular pathways that mediate the repair of the diverse tissues that develop in the fracture callus, the expression of growth factors and their receptors during fracture repair must first be characterized. Accordingly, different families of growth factors have been implicated that mediate diverse pathways of cell chemotaxis and tissue proliferation and differentiation during fracture repair (reviewed by Bolander, 1992, Andrew et al., 1993, Andrew et al., 1995, Barnes et al., 1999, Beasley and Einhorn, 2000). The physiological and morphological effects following local or systemic administration of exogenous therapeutic genes can then be tested and novel therapies developed to enhance normal and impaired bone healing.

Gene therapy has tremendous potential to optimize healing in skeletal tissue injuries by delivering and expressing bone growth-promoting therapeutic genes identified in fracture and soft tissue healing expression studies. However, approaches employing gene therapy have only started to develop systems that deliver growth factor genes to injured tissues and efficiently regulate their expression to augment in those tissues. We propose to develop approaches to gene therapy that maximize the efficiency of expression of selected transgenes and thereby enhance fracture healing.

Currently, our gene therapy approach has the potential to regenerate large skeletal defects, certainly an advantage for the repair of severe battlefield injuries of the musculoskeletal system. In our current studies, we are optimizing gene therapy for fracture repair. Our initial studies have utilized an exogenous bone morphogenetic protein (BMP)-2/4 hybrid transgene, expressed in a constitutive manner by a Murine leukemia (MLV)-based vector, to augment bone formation during repair in the rat femur fracture model (Rundle et al., 2003). However, we anticipate that to truly optimize gene therapy for fracture healing, multiple therapeutic genes with different activities during healing will be required, and the expression of those therapeutic genes will need to be regulated in a gene-specific manner. We have undertaken microarray studies of the fracture callus of multiple individual animal subjects to understand gene expression in the healing response to bone injury and identify the molecular factors that might delay the healing of

such injuries. Several hundred genes that exhibit changes in expression at early and later healing times have been identified, and those with therapeutic potential considered for inclusion in our gene therapy system. We propose that the efficient delivery and expression of a selected growth factor gene in combination with our BMP-2/4 hybrid gene will enhance fracture healing. In addition, it is essential to have both an appropriate viral vector to deliver and an effective promoter to drive expression of therapeutic genes at the fracture site. In the first year of this study, we compared the constitutive expression from a MLV-based viral vector with the gene-specific regulation from a lentiviral-based vector for marker transgene expression in periosteal cells *in vitro*. This approach established the relative levels of transgene expression from each viral vector and identified the best promoters for gene-specific regulation from the lentiviral-based vector. We have also used each vector to compare both marker and therapeutic genes for expression and fracture healing, respectively, in the rat femur fracture model. Most of these findings have been included in the Progress Report for the first and second years.

Surgical techniques have also been developed to optimize the delivery of viral vectors to the periosteal tissues of healing fractures in small animals, and thereby more accurately assess the effects of the fracture therapy (Rundle et al., 2003). During the second year of funding, we compared a combination gene therapy of two therapeutic genes compared with single gene therapy in the healing fracture. The combination of optimal surgical delivery and efficiently regulated expression of multiple therapeutic genes in the fracture by the appropriate viral vector will maximize the therapeutic effect for study. Ultimately, these approaches could not only generally enhance fracture repair, but also help to develop genetic algorithms to predict the response of individual military personnel to battlefield injury and subsequently individualize their therapy.

In our previous progress report for the first year of funding, we identified gene promoters that most efficiently regulate gene expression from our viral vectors (Technical Objective 1, Specific Objective 1), developed a surgical technique for efficient delivery of our viral vectors to the rat femur fracture model (Technical Objective 1, Specific Objective 2) and initiated microarray studies of gene expression during normal fracture repair (Technical Objective 2, Specific Objective 1). During the second year of funding, we used the surgical technique to apply the murine leukemia virus (MLV)-based vector to the fracture tissues and express a combination of therapeutic genes. Accordingly, this progress report will focus only on our goals for the third year of the funding period. During the prior reporting periods (yr 1 and 2), we completed Technical Objective 1, Specific Objectives 1,2 and 3a, as well as Technical Objective 2, Specific Objectives 1 and 2a. The following are the Technical Objectives and Specific Objectives for this continuation period:

1. <u>TECHNICAL OBJECTIVE 1: TO OPTIMIZE A GENE THERAPY FOR FRACTURE HEALING</u>. [Specific Objectives 1, 2 and 3a of this Technical Objective have been completed during the first and second years.]
   a. Specific Objective 4: To Compare the Efficacy of the BMP-2/4 Transgene in the Optimized Vector System with that of the Combination of BMP-2/4 Transgene Plus Another Growth Factor Candidate Gene Identified by Microarray (Technical Objective 2) or another bone growth factor.

2. TECHNICAL OBJECTIVE 2: TO APPLY MICROARRAY TO STUDY FRACTURE HEALING. [Specific Objectives 1 and 2a of this technical objective have been accomplished during the first and second years.]
   b. Specific Objective 3: To Evaluate the Reproducibility and To Analyze the Data from the Extended Micro-array.

   c. Additional Specific Objective Proposed for the Final Year of this Study: To evaluate the functional role of one or more ESTs with altered expression during fracture healing by inhibition or augmentation of its expression *in vitro,* followed by identification of the resulting changes cellular phenotype and gene expression.

A majority of the proposed work in these Specific Objectives has been accomplished during the current year of funding or is currently ongoing. The following section summarizes our overall progress toward each of the aforementioned Specific Objectives.

BODY

1. TECHNICAL OBJECTIVE 1: TO OPTIMIZE A GENE THERAPY FOR FRACTURE HEALING

a) Specific Objective 3: To Compare the Superiority of the MLV-based Versus the Lentiviral-based Vector Systems for the BMP-2/4 Transgene.

1) Objective

This Specific Objective compares the efficacy of the lentiviral-based and MLV-based vectors for fracture healing and was initiated in the previous year. We focused on these two viral vector systems because each of these two vector systems has the capacity to accommodate large inserts that might be necessary for larger genes-of-interest or the inclusion of regulatory elements to control expression. More importantly, both stably integrate into the host genome and can provide more prolonged transgene expression as compared to the transient transduction mediated by adenoviral and adeno-associated viral vectors. MLV-based vectors have been used extensively in gene transfer studies, and have the advantages of: 1) high viral titers that can be obtained due to their ability to withstand shearing forces encountered during ultracentrifugation; and 2) a broadened host-cell range. Their most obvious limitation is their requirement for actively proliferating cells for transduction. Expression from MLV-based vectors is controlled by the viral long terminal repeat (LTR); it provides very robust expression of gene inserts, but is too powerful to include any gene-specific regulatory promoter elements in the same vector, as they are simply overwhelmed by LTR-driven gene expression. The more recently developed lentiviral vectors have three distinct advantages over the MLV-based vectors: 1) they are capable of transducing both proliferating and non-proliferating cell types and are therefore not limited to injuries with proliferating cells; 2) because lentiviral LTRs are inherently less transcriptionally active than MLV LTRs, they allow regulation of the transgene expression using gene-specific regulatory promoters; and 3) third-generation lentiviral vector LTRs are far less susceptible to transcriptional silencing as MLV LTRs. The obvious disadvantage of lentiviral-based vectors compared to MLV-based vectors is that the promoters of lentiviral-based vectors are much less potent compared to the LTRs of MLV-based vectors. Consequently, we sought to compare the efficacy of MLV-based vectors in gene therapy of fracture repair with that of lentiviral-based vectors.

This study was initiated in the first year of the project. We examined and compared the expression of a marker transgene from a lentiviral-based vector in periosteal cells when regulated by bone-specific and non-bone-specific gene promoters. We found that more periosteal cells demonstrated the marker transgene expression when regulated by non-bone-specific promoters EF-1α and the cytomegalovirus (CMV) promoter in the lentiviral-based vector than the bone-specific promoters, suggesting that more non-osteoblastic cells than osteoblastic cells were transduced by the lentiviral-based vectors. Moreover, even with the EF-1α or CMV promoter, the extent and intensity of marker transgene expression in the fracture site transduced by lentiviral-based vectors was much lower than that in the fracture site transduced by MLV-based vectors. For these reasons, we compared the MLV-based vector with LTR-driven transgene expression and the lentiviral-based vector with CMV-driven expression for both marker and therapeutic transgene expression during the previous year of funding. However, the return to pre-

fracture mechanical strength is the definitive test of improved fracture healing. For this report, we completed a comparison of the torsional mechanical strength produced by BMP-2/4 therapeutic transgene expression from each vector. Because we have been testing these applications with the MLV-based vector, which requires proliferating tissue targets for transduction, we have injected the fracture tissues at one day post-fracture, when cell proliferation in the wound has started. The lentiviral-based vector was also injected at one day post-fracture for a direct comparison with the MLV-based vector, although it does not require the actively proliferating tissues present at this time that facilitate MLV-based vector transduction.

During the first two years we have established an intramedullary delivery method to deliver viral vectors to the entire fracture site. This intramedullary delivery method allowed transduction of cells and induced bone formation around the entire fracture site, and it was used to compare the efficacy of MLV-based vectors with lentiviral vectors in fracture repair. Accordingly, each vector expressed the BMP-2/4 transgene following intramedullary delivery. The BMP-2/4 gene was chosen as the transgene to develop the therapeutic delivery because of its documented ability to efficiently differentiate osteogenic precursors to bone (Peng et al., 2001). Accurate delivery of the transgene can be easily established by its induction of cartilage and bone in any tissues transduced with the viral vectors. The vector system that exhibits the greatest therapeutic benefits as determined by a return to pre-fracture torsional mechanical strength is used in subsequent combination therapy with multiple transgenes.

2) Materials and Methods

i) Fracture Surgery and Intramedullary Injection

The fracture surgery for exterior injections is in the rat femur fracture model as previously described (Bonnerans and Einhorn, 1984). A stainless steel Kirschner (K)-wire (1.14-mm in diameter) was inserted into the femoral medullary canal to stabilize the fracture, which was produced immediately after surgery using the three-point bending technique. However, the surgery was adapted to aseptically insert and secure a 20G catheter into the medullary canal of the femur alongside the stabilizing pin. This catheter protrudes from the medullary canal to the exterior and permits the post-fracture injection by the anterograde insertion of a needle directly into the medullary canal through the greater trochanter.

ii) Fracture Injection

The therapeutic gene chosen was the BMP-2/4 hybrid gene. To most accurately establish the expression of the marker gene or therapeutic gene, the MLV-based and lentiviral-based vectors were adjusted to equal concentrations for intramedullary application, a procedure in which the more concentrated MLV-based vector was diluted to 1/3 of its original concentration. The delivery of vectors expressing growth factor genes to the interior of the fracture retained the therapy in the subperiosteal tissues that proliferate and differentiate while mediating fracture healing. Moreover, as reported in the first year of this study (Technical Objective 1, Specific Objective 2), the intramedullary injection distributed the β-galactosidase marker and BMP-2/4 transgene expression around the fracture circumference, indicating that the therapeutic transgene expression would be evenly distributed around the fracture and provide the greatest therapeutic effect. This approach confined transgene transduction to the periosteal cell layer and avoided gene expression in the supra-periosteal layers that do not participate in fracture healing.

a) MLV-based vector with BMP-2/4 therapeutic transgene.

b) Lentiviral-based vector with BMP-2/4 therapeutic transgene.

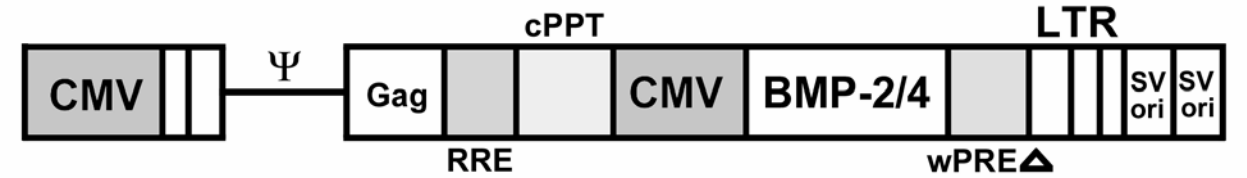


Figure 1. **BMP-2/4 therapeutic gene vector constructs used in the torsional mechanical strength testing comparison between the MLV-based and lentiviral-based vector systems.** All MLV and lentiviral elements present in the vector constructs are included in this diagram. The MLV-based vector utilized the viral LTR to express the BMP-2/4 therapeutic gene (a). In this vector, the CMV promoter functions only for vector production and is excised upon viral integration into the host genome. The lentiviral-based vector utilized the 3' CMV promoter to express the BMP-2/4 therapeutic gene (b). In this vector, the 5' CMV promoter functions only for vector production and is excised upon viral integration into the host genome.

iii. Fracture Tissue Analysis

As reported previously, the fracture tissues were examined by Faxitron X-ray analysis for mineralized tissues, measured for bone mineral content by pQCT, and examined for bone formation and BMP-4 expression by histology. X-ray analysis of the fractures suggested that each viral vector was approximately equivalent in producing bone within the periosteal fracture tissues. pQCT measurements of the bone mineral content of the fracture callus revealed no significant difference in the bone mineral content or cross-sectional areas of the fracture callus tissues produced by each vector during fracture healing, suggesting that both viral vector systems are equally effective in gene therapy of fracture repair.

In this report, we present the comparison of the healing fracture strength produced by each vector as determined by torsional mechanical testing. The femoral epiphyses were cast into dental cement that allowed them to be securely fastened and the diaphysis (with the fracture) aligned in the testing device for rotation around its vertical axis. Testing was conducted at 28 days healing on an Instron Dynamite 8100 hydraulic tester using a rotational speed of 2.5^{o} per second; 28 days is approximately one week before the normal bridging of the fracture gap with bony tissue. This time point was substituted for the originally proposed 21-day fracture strength analysis based upon the mineralized tissue and bone formation produced by BMP-2/4 transgene expression at this time in the earlier studies in this Specific Objective. The ultimate load (force in Newton-meters) to bone failure was determined as a measurement of the return to mechanical strength provided by each vector.

3) Results

Torsional Mechanical Testing

An example of the mechanical testing is shown below for one animal that received the lentiviral-BMP-2/4 vector-gene combination (Figure 2). The objective of fracture therapy is to enhance the return to the pre-fracture strength (ultimate load) displayed by the unfractured contralateral femur. The ultimate load of the fractured bone (with therapy) is compared to the ultimate load of the unfractured bone as a measure of its return to prefracture strength. The elastic value of the fracture represents the torsion to higher degrees of rotation on soft tissues that have limited stiffness to resist such loads.

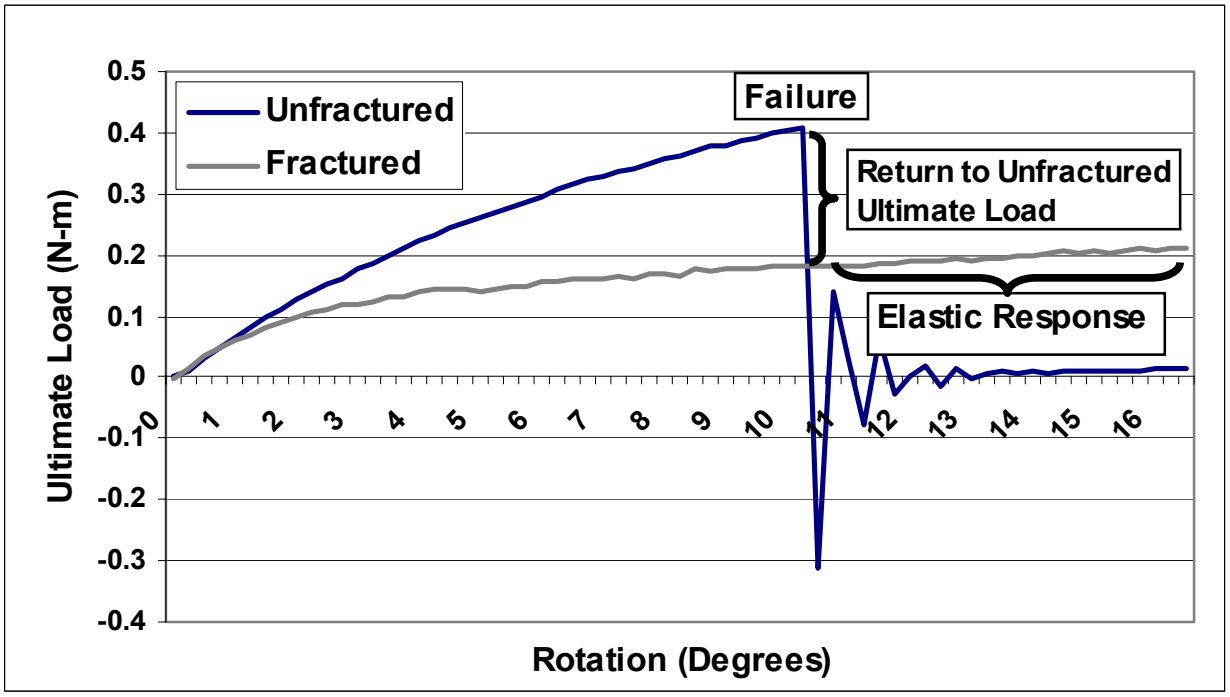


Figure 2. Illustration of the torsional mechanical testing conducted to evaluate the therapeutic value of the vectors. The intramedullary space of the fracture was injected at one day post-fracture with 0.1 ml of 1 X 10^7 transforming units (tfu) of lentiviral-based vector expressing the BMP-2/4 therapeutic transgene. The femurs were harvested at 28 days post-fracture and the fractured femur receiving the therapy compared to the unfractured contralateral femur.

Three to four animals were evaluated for the return to prefracture (unfractured contralateral) torsional strength following injection of either the MLV-based or the lentiviral-based vectors expressing the BMP-2/4 transgene (Figure 3). All tissues were harvested at 28 days healing. In agreement with the previously reported X-ray and pQCT results, there was no significant difference in the ultimate load (force) produced by the expression of the therapeutic BMP-2/4 transgene by either vector. Each produced a 30% to 50% return to contralateral ultimate load, though the lentiviral-BMP-2/4 vector might have been required at higher concentrations and the dilution of the MLV-BMP-2/4 vector to comparable levels might have reduced the effectiveness of MLV as a vector.

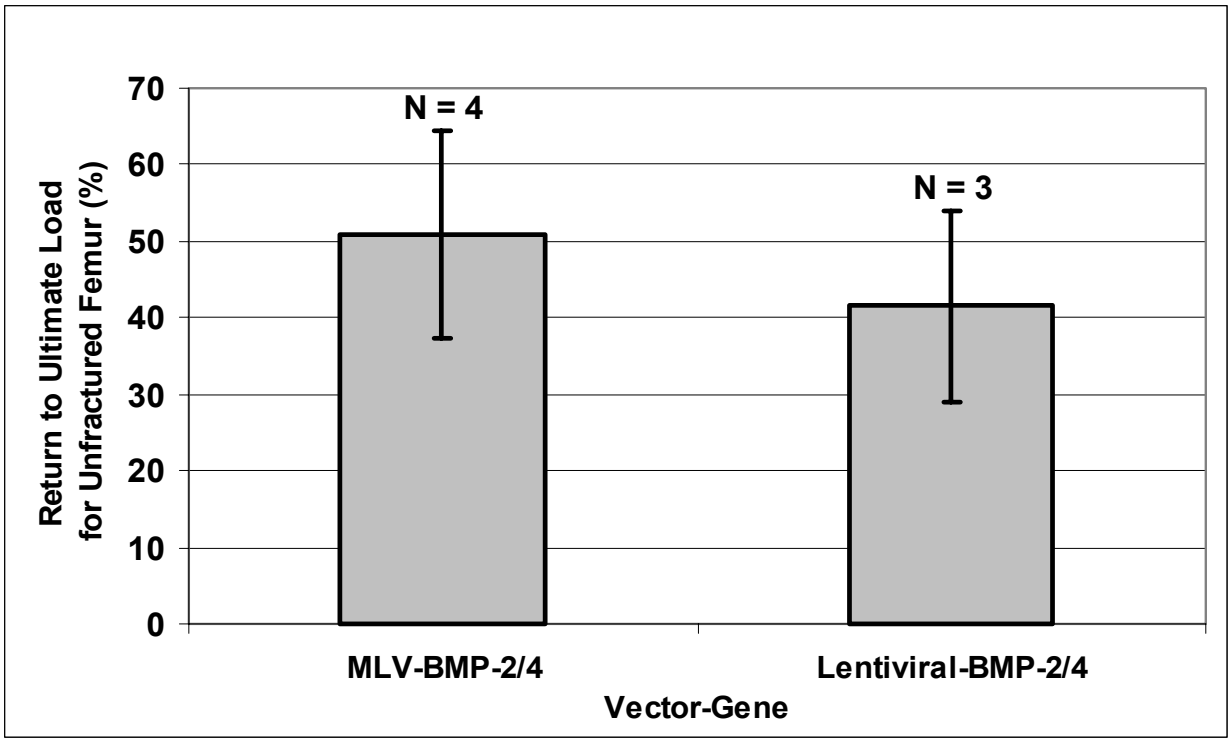


Figure 3. Comparison of MLV-based vector expressing the BMP-2/4 therapeutic transgene with lentiviral-based vector expressing BMP-2/4 transgene to promote the return to prefracture (contralateral) mechanical strength during fracture healing. The intramedullary space of the fracture was injected at one day post-fracture with 0.1 ml of 1 X 10^7 transforming units (tfu) MLV-based or lentiviral-based vectors expressing the BMP-2/4 transgene. The femurs were harvested at 28 days post-fracture. Data is presented as the mean ± standard deviation for the difference in torsional ultimate load between fracture therapy and unfractured bone. There was no significant difference between the two groups.

4) Conclusions

From the second year's progress report, there was no significant difference in the ability of the BMP-2/4 transgene in promoting mineralized fracture tissue production between MLV-based and lentiviral-based vector systems. There was a difference in β-galactosidase marker gene expression, but the reason is not clear at this time and this discrepancy remains unresolved. We are still completing histology analysis and mechanical testing of the repaired bone at this time.

Because the MLV-based vectors are much easier to produce in higher concentrations than the lentiviral-based vectors, the MLV-based vector system was chosen for our subsequent comparison between combination therapy with two different growth factor transgenes and the single gene therapy (Technical Objective 1, Specific Objective 4, below).

b) Specific Objective 4: To Compare the Efficacy of the BMP-2/4 Transgene in the Optimized Vector System with that of the Combination of BMP-2/4 Transgene Plus Another Growth Factor Candidate Gene Identified by Micro-array (Technical Objective 2) or Another Potent Bone Growth Factor.

1) Objective

To identify therapeutic gene candidates for combination therapy with the BMP-2/4 gene, we analyzed whole genome gene expression in the healing fracture tissues at 3 and 11 days post-fracture (Technical Objective #2 below). Analysis of the microarray data suggested several gene candidates whose expression was confirmed by real-time PCR analysis and that could be applied to combination therapy with our BMP-2/4 gene and MLV-based vector, but we have also developed a highly active mutant fibroblast growth factor (FGF)-2 gene and successfully expressed it in our MLV-based vector. Several previous studies have applied exogenous FGF-2 to animal bone healing models to identify its functions in fracture repair, where it appears to increase proliferation of several cell types involved in fracture healing (Nakajima et al., 2001). Three of the four FGF receptors are expressed during fracture repair (Rundle et al., 2002), suggesting that FGF gene family members regulate healing. FGF-2 does not have a classical secretion signal sequence. Its extracellular secretion is mediated by a highly inefficient, energy-dependent, non-ER/golgi pathway (Florkiewicz et al., 1995) and, as a result, the amount of secreted FGF-2 by this mechanism is low and inherently inconsistent (Moscatelli et al., 1986). The FGF-2 protein in mammalian cells can also exist in various molecular forms through intra- and inter-molecular disulfide formation. The disulfide formation reduces its biological activity and protein stability (Iwane et al., 1987). These problems are largely responsible for the inconsistent efficacy of past FGF-2 gene therapy studies (Spencer et al., 2001; Hijjawi et al., 2004; Ishii et al., 2004). Accordingly, the human FGF-2 gene within our MLV-based vectors has been modified by: 1) adding the BMP2/4 hybrid secretion signaling sequence to the 5' end of the gene to enhance the transgene secretion; 2) adding an optimized Kozak sequence to promote protein translation; and 3) mutating two key cysteines (cys-70 and cys-88) to serine and asparagine respectively, to enhance protein stability. These modifications led to an increase in secretion of functionally active FGF-2 protein in rat skin fibroblasts and marrow stromal cells by more than 200-fold (data not shown). We have applied the MLV-based vector expressing our FGF-2 mutant gene to our rat femur fracture model.

As presented in the previous progress report, FGF-2 produced a very large fracture callus without accelerated chondrocyte maturation and endochondral bone conversion. Subsequent examination of the histology of this large fracture callus contained vessels highly suggestive of increased angiogenesis (Figures 4 and 5). However, when fractures were allowed to completely heal, healing proceeded even in the presence of these large amounts of tissue and the fracture calluses resolved normally. These results suggest that this FGF-2 transgene increased periosteal mesenchymal cell proliferation and angiogensis, and it would be an excellent candidate for combination gene therapy with an osteogenic transgene partner such as our BMP-2/4 gene. Because osteogenesis acts upon the soft callus tissues, BMP-2/4 gene expression would be expected to more quickly augment bone formation in the soft callus tissues that had rapidly proliferated in response to FGF-2 gene expression. Such a combination of growth factors was hypothesized to enhance fracture healing in this way.

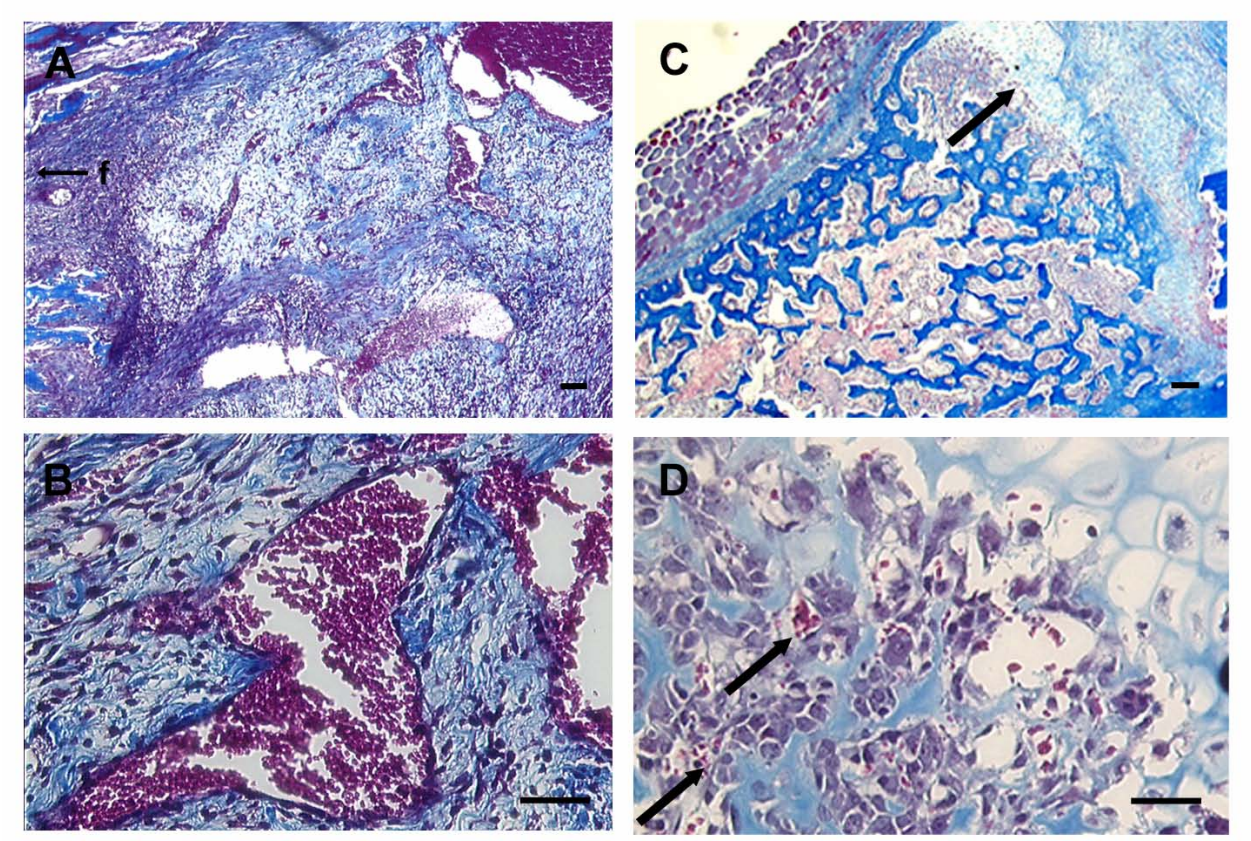


Figure 4. Bone formation in fractures expressing mutated variant of the FGF-2 gene (A, B) as compared to a wild-type FGF-2 gene (C, D) expressed from the MLV-based vector. (A) A trichrome stain of fracture tissues at 11 days post-fracture reveals several large vessels (A, upper right) with large numbers of red blood cells (B). In (C) wild-type FGF-2 gene expression provides a more normal infiltration of osteoblasts in the hypertrophic cartilage (arrow), with considerably smaller blood vessels (D, arrow). Scale bar = 100 μm. f, fracture.

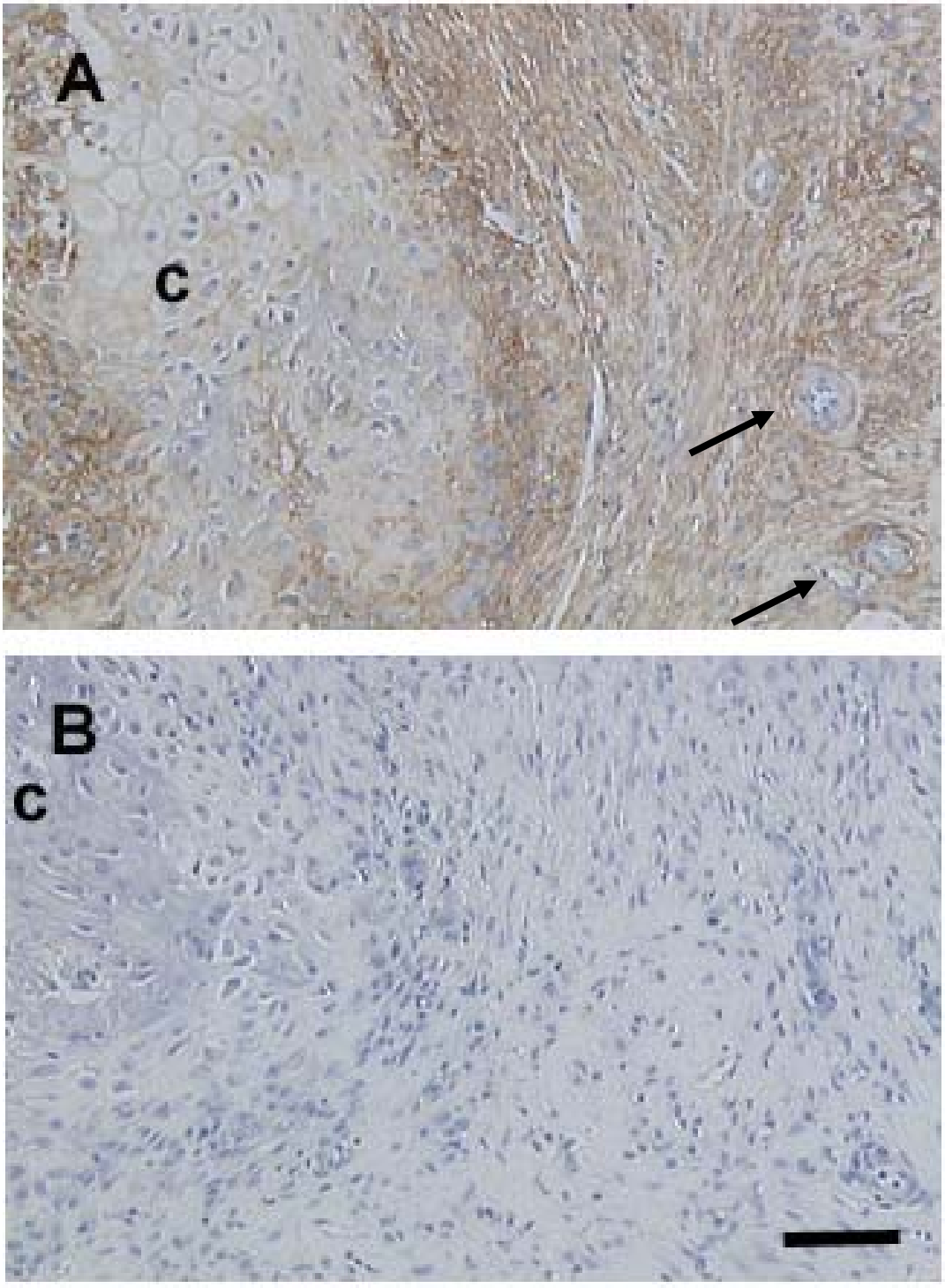
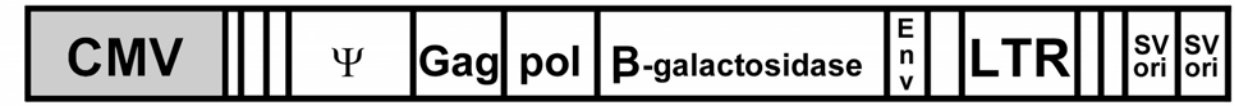


Figure 5. FGF-2 expression in fractures injected with expressing the mutated variant of the FGF-2 gene expressed from the MLV-based vector. Scale bar = 500 μm. (A) FGF-2 expression localized to the proliferating soft callus and mineralizing hard callus tissues at 11 days post-fracture, absent in only the hypertrophic chondrocytes of the cartilage (c). Sinusoid-like structures suggestive of angiogenesis are visible (arrows). (B) Omitting the anti-FGF-2 primary antibody eliminated the immunostaining.

In this work, we hypothesize that the BMP-2/4 will accelerate the osteogenic differentiation of osteoblastic precursor cells that have proliferated in response to FGF-2 gene expression and thereby accelerate endochondral bone formation and fracture repair. Studies were initiated that compared the effects of BMP-2/4 gene therapy with FGF-2 gene therapy when applied by intramedullary injection to the fracture tissues; we also plan to compare healing following a combined intramedullary injection of both genes expressed from their own MLV-based vector. The design of our constructs is shown schematically in Figure 6.

a) MLV-based vector with β-galactosidase marker transgene.



b) MLV-based vector with BMP-2/4 therapeutic transgene.

c) MLV-based vector with FGF-2 therapeutic transgene.

Figure 6. Control and therapeutic gene MLV-based vector constructs for combination gene therapy. As in Technical Objective 1, Specific Objective 2 (Figure 1), the MLV-based vector utilizes the viral LTR to express either (a) the β-galactosidase marker gene as a non-therapeutic control gene, (b) the BMP-2/4 therapeutic gene, or (c) the FGF-2 therapeutic gene. The viral backbone and vector production is identical to that of the MLV-based vectors of Figure 1.

2) Materials and Methods

i) *Fracture Surgery*

All surgical procedures were performed as described above in Technical Objective 1, Specific Objective 3.

ii) *Fracture Injection*

Following fracture surgery, groups of 4 animals each were injected with mixtures of the MLV-based vector expressing either the β-galactosidase (non-therapeutic control) gene, the BMP-2/4 gene, the modified FGF-2 gene, or a combination of the of the BMP-2/4 and modified FGF-2 genes, each application at a total concentration of 3×10^8 tfu. Because of the potency of FGF-2 gene expression that produced the very large fracture callus we previously observed in injections of undiluted MLV-FGF-2 preparations, a dose response was performed in which the fractures received an undiluted MLV-BMP-2/4 mixed with dilutions of MLV-FGF-2; the diluent for each MLV-FGF-2 dilution was the MLV-β-galactosidase (non-therapeutic control) gene in order to keep the concentration of MLV-based vector constant.

Briefly, MLV-FGF-2 was diluted:

1) 1:1 in MLV-BMP-2/4, and 100 µl injected into the intramedullary space using the surgically implanted catheter, or
2) diluted 1:4 in MLV-β-galactosidase, then 50 µl of this mixture mixed with 50 µl MLV-BMP-2/4, and the 100 µl total volume injected into the intramedullary space using the surgically implanted catheter, or
3) diluted 1:8 in MLV-β-galactosidase, then 50 µl of this mixture mixed with 50 µl MLV-BMP-2/4, and the 100 µl total volume injected into the intramedullary space using the surgically implanted catheter, or
4) diluted 1:16 in MLV-β-galactosidase, then 50 µl of this mixture mixed with 50 µl MLV-BMP-2/4, and the 100 µl total volume injected into the intramedullary space using the surgically implanted catheter; and
5) 100 µl of each of the 3 single MLV-gene preparations was also injected into the intramedullary space using the surgically implanted catheter. The MLV-β-galactosidase marker gene preparation served as the non-therapeutic control.

Each group of animals was allowed to heal for 14 days to evaluate histology and mineralized tissue formation by X-ray and pQCT analysis, or for 28 days to evaluate the torsional mechanical strength. These times were chosen for the highly characteristic fracture callus morphology present at 14 days post-fracture.

iii) Fracture Tissue Analysis

Fracture tissues are currently being examined by X-ray analysis for mineralized tissues, measured for bone mineral content by pQCT, and the histology examined for bone formation by Trichrome staining and for BMP-4 expression by immunohistochemistry. The healing fracture strength is determined by torsional mechanical testing. Samples for tissue analysis have just recently been harvested and available. Tissues for mechanical testing have been available for a few months, but stored and not analyzed because of programming problems with the torsional system of the Instron tester. Maintenance was performed on this instrument in November, 2005, and we are now ready to proceed with the torsional mechanical strength testing measurements.

3) Results

As of this date, the studies that applied combinations of MLV-(mutant)FGF-2 dilutions with MLV-BMP-2/4 to the intramedullary space using the surgically implanted catheter are being analyzed. Examination of the gross anatomy of the healing fractures revealed that the highest concentration of MLV-FGF-2, 50 µl (undiluted) mixed and injected with 50 µl MLV-BMP-2/4, provided what appear to be highly advanced healing at 14 days, with only a very small fracture callus visible in the femur (Figure 7A) and a very faint fracture line visible in the X-ray (Figure 7B). This observation is extremely encouraging, and we will confirm it with additional fractures. The remaining groups with different MLV-FGF-2 dilutions (i.e., 50 µl 1/4 MLV-FGF-2 + 50 µl MLV-BMP-2/4, 50 µl 1/8 MLV-FGF-2 + 50 µl MLV-BMP-2/4, and 50 µl 1/16 MLV-FGF-2 + 50 µl MLV-BMP-2/4) appeared to produce the bone in the fracture gap, but the callus size and the increased mineralized tissue in the fracture gap did not appear to facilitate healing beyond that of the MLV-β-galactosidase-injected control fractures at this time.

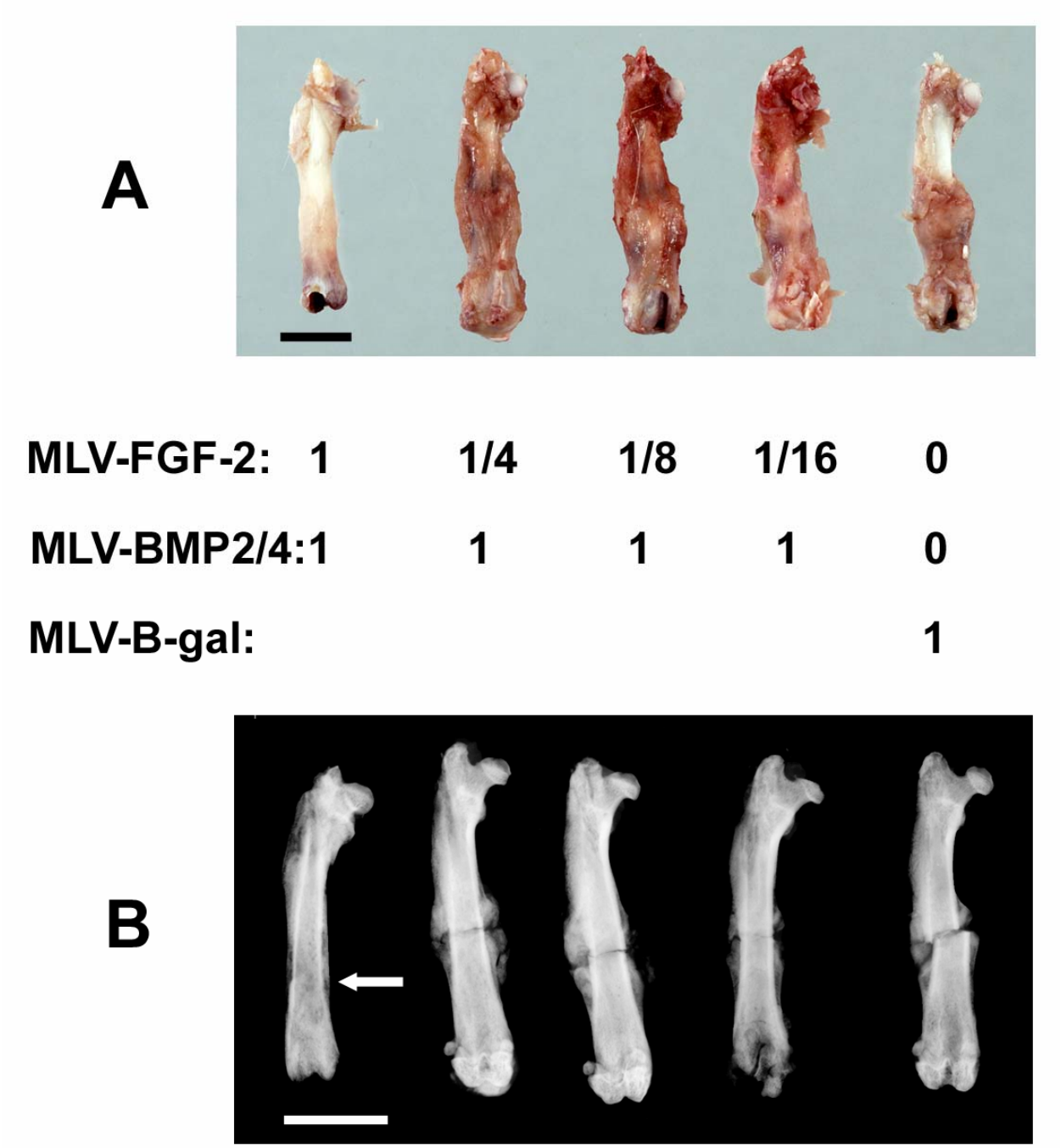


Figure 7. Combination fracture therapy using FGF-2 and BMP-2/4 transgenes at 14 days healing. (A) Gross anatomy and (B) X-ray analysis of healing fractures was performed 14 days following fracture and the application of different dilutions of MLV-(mutant)FGF-2 with a standard concentration of MLV-BMP-2/4. The fracture line of the most concentrated MLV-FGF-2 injection is barely visible and indicated by an arrow. The MLV-β-galactosidase marker vector-gene served as a non-therapeutic control comparison. Scale bar = 1 cm.

These results indicate that the FGF-2 expression was critical in bridging the fracture gap, and when FGF-2 concentrations were reduced, BMP-2/4 expression merely augmented the

fracture callus bone without accelerating healing. This study is proceeding well, but has taken considerable time, and we have not yet been able to finish the histological, immunohistochemical, and mechanical strength comparisons between the BMP2/4 single gene therapy and the BMP2/4 and FGF-2 combination gene therapy. The rotary programming problems that we experienced with the Instron mechanical testing instrument have delayed the results considerably. However, the tissues necessary for these analyses have been acquired and stored, and these comparisons are now proceeding.

4) Conclusions

In conclusion, the studies utilizing FGF-2 gene therapy in combination with BMP-2/4 gene therapy have provided exciting initial results that we are attempting to confirm and more thoroughly characterize. The remaining histological analysis and mechanical strength measurements, which are ongoing, should provide definitive descriptions of the efficacy of this therapy. Nonetheless, these preliminary studies support the approach of using combinations of FGF-2 and BMP-2/4 to promote fracture repair.

2. TECHNICAL OBJECTIVE 2: TO APPLY MICROARRAY TO STUDY FRACTURE HEALING

Our goals for this Technical Objective during this year were to: 1) expand the analysis to include additional individual fracture samples; 2) confirm genes whose expression was changed in response to fracture repair by an independent method of expression measurement, namely real-time PCR; and 3) to expand our analysis of gene expression to elucidate additional potential pathways of growth factors involved in fracture repair.

a) Specific Objective 2: To Apply Our Extended In-house Microarray to Study Gene Expression in the Fracture Callus at 3 Days After Fracture

1) Objectives

The microarray determination of whole genome gene expression in the normal healing fracture was initiated during the first year of this study. Microarray analysis has been especially valuable in identifying and characterizing the molecular pathways of fracture repair, as well as identifying potential therapeutic gene candidates for fracture gene therapy. To accomplish these goals, tissues were harvested for analysis at an early and a later time point in healing: 3 days and 11 days. The early time (3-day) is characteristic of the transition of the inflammatory phase and intramembranous bone formation phases and was the stated goal for this objective. We also studied the latter time point (11-day), which is characteristic of the maturation of the cartilage intermediate to endochondral bone. The early time point was to suggest gene candidates for early clinical intervention, and the addition of the 11-day data to this Technical Objective provided an additional measurement of gene expression during fracture healing.

Microarray evaluations of healing fractures have also been studied by other investigators (Hadjiargyrou et al., 2002; Meyer et al., 2003; Li et al., 2005). Our approach to the analysis of gene expression in fracture repair differed from previous microarray studies in two very important aspects: 1) Previous studies used intact bone without the Kirschner (K)-wire as control for healing fractures, which has a K-wire in the marrow space. Accordingly, the control tissues included marrow, while the fractured tissues lacked marrow. Marrow ablation could induce

inflammatory reactions that alter the expression of fracture-related genes. In our study, we used the appropriate marrow-ablated control diaphyses for comparison with the fractures (see Materials and Methods); and 2) Previous studies arbitrarily used two-fold changes in expression as significant effects. Our studies included sufficient individual replicate samples that allow appropriate application of statistics to determine significant changes. Accordingly, we believe that our experimental design is superior to those used in previous studies.

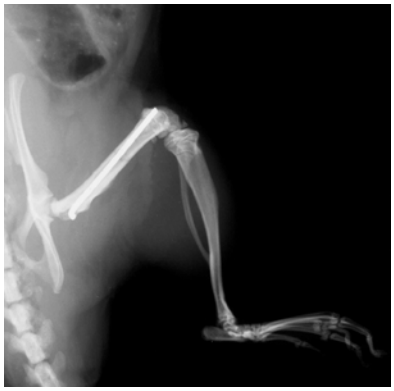
2) Materials and Methods

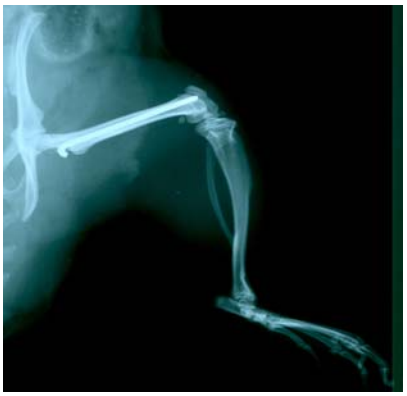
Fracture surgery was performed using the three-point bending technique (Bonnerens and Einhorn, 1984) as described in the previous progress report of the study (Technical Objective 1, Specific Objective 2). As indicated above, in contrast to other fracture microarray studies conducted in the past, our unfractured control femurs included an intramedullary K-wire as normally used in the fractured femurs. These controls also normalized the analysis for intramedullary bone formation induced by the K-wire in fractured femurs, which is expected by 11 days healing. This unfractured control comparison allowed us to obtain a more accurate determination of gene expression in the periosteal tissues that mediate fracture healing.

RNA was isolated from individual fractured femurs at 3 days and individual fractures at 11 days healing, with the fractures compared to equal numbers of individual unfractured (control) femurs at each time point. RNA isolation was performed on pulverized fracture tissues by Trizol^{TM} purification, following the manufacturer's (Invitrogen) instructions. The purity and integrity of each RNA sample was confirmed using the Agilent Bioanalyzer.

Our approach to fracture gene expression analysis by microarray technology (Figure 8) was first presented in one of the previous progress reports. We had adopted the Agilent gene chip for these studies, which had become available in the first year of this study, and continued to use it for our expanded microarray analysis. This chip contains 60-mer oligos that represent 20,000 genes either derived from the rat or homologous to rat gene sequences. The Cy3 and Cy5 labeling was performed as described in the Agilent "low input" labeling system, and the hybridization performed using equipment and procedures specified for the Agilent rat gene chip. We compared each group of fractured RNA and unfractured control RNA isolates at each time point, 3 days [corresponding to the healing phase that is immediately after the inflammatory phase but prior to the initiation of bone formation phase (Bolander 1992)] or 11 days post-fracture [corresponding to the healing phase when intramembranous and endochondral bone formation overlap (Bolander 1992)]. Because the Agilent RNA dye labeling system allowed us to analyze fracture tissues and unfractured controls for gene expression with very low amounts of RNA, we were able to avoid pooling of individual samples normally necessary to obtain sufficient RNA. In this way, we were able to use individual samples to identify individual biological variations concealed in other microarray studies where the samples are pooled.

Figure 8. Fracture Microarray Approach





K-wire, fractured femoral diaphysis

K-wire, unfractured femoral diaphysis

Healing
3 Days or 11 Days

RNA Isolation

RNA Isolation

Cy5 Label

Cy3 Label

Comparison of individual pair:
Fractured vs Unfractured

Micoarray
1 **20K Rat Oligo Chip**
2 **Low-input Hybridization**

Analysis
1 **Genespring Image Analysis**
2 **Lowess Normalization**
3 **Gene Ontology**

3 Day Analysis
(n = 5 Individual Pairs)

11 Day Analysis
(n = 8 Individual Pairs)

Microarray image analysis was also performed in-house, using ScanArray image analysis and Genespring expression analysis software. Lowess normalization was performed to identify differences in the Cy3 or Cy5 dye labeling efficiencies. One-way analysis of variance (ANOVA) established significant changes in expression of up-regulated genes and down-regulated genes for each group of fractured (as compared to the unfractured control) animals at 3 days healing and 11 days healing. Cluster analysis performed to classify the genes into Gene Ontology (GO) categories for further examination. With the expanded numbers of replicates in our study, we were able to rigorously analyze the changes in gene expression for statistical significance. Changes in gene expression were deemed statistically significant at $p<0.05$.

3) Results

The RNA recovery from fractured and unfractured animals was routinely of sufficient quantity and quality for analysis by the Agilent low-input labeling and hybridization system on the 20,000-gene chip. Our approach successfully identified several hundred known and unknown genes, as reported for the first year of the study. Inclusion of the additional samples has improved the statistical calculations, and in the current analysis 6,555 genes displayed significant changes in expression at 3 days, 11 days or both; of these genes, 4,873 genes were known and 1,682 were unknown (Table 1). The proportions of known genes (2/3) and unknown genes (1/3) are very close to our initial analysis with fewer animals. Our fracture microarray study therefore demonstrated increased sensitivity yet remained consistent. The numbers of unknown genes with expression changes during fracture healing is especially interesting, and suggests that the molecular regulatory pathways of bone repair are indeed complex and many remain to be characterized. A comparison of our analysis with those of previous fracture studies again reveals that several common genes previously associated with fracture repair also displayed significant changes in expression in our study (Table 2). These results support the accuracy of our approach.

Table 1. Summary of Fracture Microarray Gene Expression Changes

Expression Change (P<0.05)	**Known Genes**	**Unknown Genes**	**Total**
3 Days			
Up at 3 days, no change at 11 days	889	215	1104
Down at 3 days, no change at 11 days	1013	388	1401
11 Days			
Up at 11 days, no change at 3 days	904	345	1249
Down at 11 days, no change at 3 days	1206	450	1656
3 and 11 Days			
Up at both 3 and 11 days	354	96	450
Down at both 3 and 11 days	474	181	655
Biphasic			
Up at 3 days, down at 11 days	20	1	21
Down at 3 days, up at 11 days	13	6	19
Total	**4873**	**1682**	**6555**

Table 2. Comparison of selected genes with up-regulated expression in this study with previous fracture studies					
This Study					Previous Fracture Studies
Gene	Accession	Function	Fold-Change (P<0.05)		Similar Change in Expression
			3 Days	11 Days	
transforming growth factor β-2	BF420705	growth factor	**1.3**	**1.6**	[12]
transforming growth factor β-3	NM_013174	growth factor	**2.4**	**2.0**	[12, 21, 31]
fibroblast growth factor 7	NM_022182	growth factor	**1.3**	NS	[21, 31]
interleukin 6	NM_012589	inflammation	**3.5**	NS	[12, 31]
angiopoietin-2 (like)	NM_133569	angiogenesis	NS	**1.4**	[36]
mesenchymal homeobox-2	NM_017149	transcription factor	**2.5**	**2.8**	[21]
pleiotrophin/OSF-1	NM_017066	several	**2.3**	NS	[21, 31]
frizzled	NM_021266	wnt signaling	**1.5**	**1.5**	[21]
cysteine-rich protein 61	NM_031327	extracellular matrix signaling	**2.6**	**2.8**	[21, 31]
fibronectin	NM_019143	extracellular matrix	**2.4**	**2.1**	[21, 31]
tenascin	BE126741	extracellular matrix	**2.1**	**1.5**	[21, 31]
thrombospondin-2	BF408413	extracellular matrix	**1.7**	**1.8**	[21, 31]
osteonectin/SPARC	NM_012656	extracellular matrix	**1.7**	NS	[21, 31]
aggrecan	NM_022190	extrcellular matrix	NS	**5.1**	[21, 31, 36]
collagen 2α1	AA899303	cartilage maturation	**0.8**	**1.5**	[21, 31, 36]
integrin binding sialoprotein	NM_012587	mineralization	**4.0**	NS	[21, 31]
collagen 5α1	NM_134452	extracellular matrix	**2.4**	**2.1**	[21, 31]
osteocalcin/Gla	NM_012862	mineralization	NS	**2.4**	**[12, 21, 31, 36]**
protease nexin-1	X89963.1	extracellular matrix protease	**2.1**	**2.1**	[21, 31]

NS: Not Significant

Classification of all genes with highly significant changes in expression ($p<0.0002$) during fracture repair into Gene Ontology (GO) categories facilitated the analysis at both 3 days and 11 days healing (Table 3). Not surprisingly, several metabolic and signaling gene categories were up-regulated at 3 days healing, when these events would be expected to be important for healing. At 11 days healing, developmental and adhesion-related genes were expressed, consistent with the tissue differentiation of the maturing fracture callus.

Table 3. Known genes with highly significant (P<0.0002) changes in the expression during fracture healing				
Accession	**Fold-Change**		**Gene Description**	**Gene Ontology Category [4]**
	3 Days	**11 Days**		
BQ209997	**5.02**	**7.80**	similar to Mouse collagenous repeat-containing 26kDa protein (CORS26).	protein metabolism
AA858962	**4.36**	**2.15**	Rat retinol-binding protein (RBP) mRNA, partial cds.	vitamin A metabolism
NM_012587	**3.97**		Rattus norvegicus integrin binding sialoprotein (Ibsp).	extracellular space
BQ211765	**3.49**		Rattus norvegicus DEXRAS1 (Dexras1) mRNA.	signal transduction
BF415205	**2.78**	**6.19**	Rat mRNA fragment for cardiac actin.	actin cytoskeleton
NM_133566	**2.29**	**1.21**	Rattus norvegicus cystatin N (LOC171096).	organogenesis and histogenesis
NM_013104	**1.97**	**4.58**	Rattus norvegicus Insulin-like growth factor binding protein 6 (Igfbp6).	extracellular space
BQ209870	**1.80**	**3.88**	similar secreted modular calcium-binding protein 2 [Mus musculus].	calcium ion binding
CA510266	**1.71**	**1.32**	similar to prefoldin 5; myc modulator-1; c-myc binding protein [Homo sapiens].	regulation of transcription, DNA dependent
NM_012488	**1.55**	**2.53**	Rattus norvegicus α-2-macroglobulin (A2m).	protease inhibitor activity/IL-1, IL-8 binding
BE329208	**1.52**	**1.43**	similar to Cricetulus griseus SREBP cleavage activating protein (SCAP), complete cds.	steroid metabolism
NM_012816	**1.41**		Rattus norvegicus α-methylacyl-CoA racemase (Amacr).	metabolism/peroxisome
NM_057197	**1.40**		Rattus norvegicus 2,4-dienoyl CoA reductase 1, mitochondrial (Decr1).	oxidoreductase
NM_031646	**1.39**		Rattus norvegicus receptor (calcitonin) activity modifying protein 2 (Ramp2).	G-protein coupled receptor signaling
NM_031050	**1.38**		Rattus norvegicus lumican (Lum).	extracellular matrix
NM_017355	**1.27**	**1.24**	Rattus norvegicus ras-related GTP-binding protein 4b (Rab4b).	vesicle-mediated transport
U56859.1	0.90	0.79	Rattus norvegicus heparan sulfate proteoglycan, perlecan domain I (RPF-I), partial cds.	cell adhesion
BF281804	0.85	0.84	similar to solute carrier family 7 member 12; isc-type amino acid transporter 2 [Mus musculus].	amino acid transport
NM_017140	0.85		Rattus norvegicus dopamine receptor D3 (Drd3).	dopamine receptor signaling pathway
BF548886	0.85	0.78	similar to Mouse T-cell antigen receptor α-chain (TCR-ATF2), partial cds.	regulation of transcription, DNA dependent
NM_013029	0.84	0.79	Rattus norvegicus Sialyltransferase 8 (GT3 α 2,8-sialyltransferase) C (Siat8c).	amino acid glycosylation
NM_012997	0.82	0.74	Rattus norvegicus Purinergic receptor P2X, ligand-gated ion channel, 1 (P2rx1).	amino acid transport
NM_031725	0.82		Rattus norvegicus secretory carrier membrane protein 4 (Scamp4).	protein transport
AA900738	0.80	0.81	similar to Rat DNA for serine dehydratase.	amino acid metabolism/gluconeogenesis

NM_133322	0.79	0.77	Rattus norvegicus potassium voltage-gated channel, KQT-like subfamily, member 2 (Kcnq2).	synaptic transmission
NM_052801	0.78	0.76	Rattus norvegicus von Hippel-Lindau syndrome (Vhl).	regulation of transcription, DNA dependent/proteolysis & peptidolysis
CB546252	0.78	0.83	similar to zinc finger protein 261; DXHXS6673E [Mus musculus].	nucleus/zinc ion binding
NM_144730	0.78	0.80	Rattus norvegicus GATA-binding protein 4 (Gata4).	regulation of transcription, DNA dependent
NM_030854		**21.97**	Rattus norvegicus chondromodulin-1 (Chm-1).	cell growth and maintenance/proteoglycan metabolism
BF560915		**17.46**	Rattus norvegicus mRNA for collagen α 1 type X, partial.	skeletal development
NM_019189		**13.77**	Rattus norvegicus cartilage link protein 1 (Crtl1).	hyaluronic acid binding
NM_012929		**11.38**	Rattus norvegicus Procollagen II α 1 (Col2α1).	skeletal development
NM_031511		**6.72**	Rattus norvegicus Insulin-like growth factor II (somatomedin A) (Igf2).	development
BQ210664		**5.73**	similar to cartilage intermediate layer protein	unknown
BQ191772		**5.37**	similar to mouse annexin A8.	phospholipid binding
NM_022290		**5.28**	Rattus norvegicus tenomodulin (Tnmd).	collagen maturation
AI576621		**3.73**	similar to Mouse carboxypeptidase X2, complete cds.	protein binding
AA963765		**2.89**	similar to osteoglycin [Mus musculus].	regulation of DNA transcription
BQ200482		**1.41**	similar to Mouse mRNA for acetylglucosaminyltransferase-like protein.	lipopolycaccharide biosynthesis
CB547946		**1.35**	similar to Mus musculus (clone pVZmSin3B) mSin3B, complete cds.	regulation of transcription, DNA dependent
AI059288		0.83	similar to Mouse B-cell activating factor (TNFSF13b, Baff), complete cds.	positive regulation of cell proliferation
CB547491		0.83	similar to Mus musculus very large G protein-coupled receptor 1 (Vlgr1, Mass1), complete cds.	G-protein coupled receptor signaling
CB545755		0.82	similar to RAD54 like (S. cerevisiae) [Mus musculus].	DNA recombination, repair
CB544611		0.82	similar to BACR7A4.19 gene product [Drosophila melanogaster].	G-protein coupled receptor signaling
CB545661		0.81	similar to BC026845_1 Mus musculus, Similar to nucleoporin 133kD, complete cds.	RNA metabolism
AW920271		0.81	similar to mouse cat eye syndrome chromosome region, candidate 5 (Cecr5), complete cds.	metabolim
BQ196556		0.80	similar to nudix (nucleoside diphosphate linked moiety X)-type motif 5 [Mus musculus].	oxidative stress response/DNA repair
AA874884		0.60	Rat heme oxygenase gene, complete cds.	oxidoreductase activity
NM_031740		0.59	Rattus norvegicus UDP-Gal:betaGlcNAc β 1,4-galactosyltransferase, polypeptide 6	glyosphingolipid biosynthesis

			(B4galt6).	
NM_053843		0.49	Rattus norvegicus Fc receptor, IgG, low affinity III (Fcgr3).	Immune response

Bold: Up-regulated

While a detailed examination of the individual genes with inflammatory functions at 3 days healing revealed several of the inflammatory genes previously observed in fracture microarray studies, there were fewer representatives among the inflammatory cytokines and the immune response genes (Table 4). Significantly, very few of these genes were up-regulated more than 2-fold, suggesting that inflammatory gene regulation is critical for bone healing. Several of these genes were also up-regulated at 11 days healing; they might perform non-inflammatory functions, because the reduction in expression of acute phase proteins between 3 days and 11 days suggests a regulated reduction in the inflammatory response. Even within the complement activation pathway, which is normally associated with inflammation, genes with negative regulatory influences on the cascade were up-regulated in expression at both 3 and 11 days healing. Several of these results are in accordance with our hypothesis that the K-wire controls normalized the analysis for the intramedullary inflammatory response. Such inflammatory gene regulation is critical for tissue repair, and an accurate description of the inflammatory gene repertoire is essential for the design of effective fracture healing therapies.

Table 4. Up-Regulated Expression of Inflammation and Immune Function Genes in Fracture Healing

Gene		**Functions**	**Fold-Change in Expression (p<0.05)**	
Description [Reference]	**Accession**		**3 Days**	**11 Days**
Growth Factors				
Platelet-derived growth factor receptor [34]	AA925099	chemotaxis	**2.7**	**1.5**
Monocyte chemotactic protein 3 [35]	BF419899	chemotaxis	**3.3**	**1.6**
Mast cell growth factor/kit ligand [40]	AI102098	stem cell factor, hematopoietic & mast cell growth	**1.3**	**NS**
TNFα/TNFβ [15]	AA819277	inflammation	**NS**	**1.2**
TRAF2	BI282097	TNF inflammation	**1.1**	**NS**
TRAF4	CB546212	TNF inflammation	**1.6**	**NS**
TNF-stimulated gene 6	AF159103.1	TNF inflammation	**1.8**	**1.7**
TGFβ2 [12]	BF420705	inflammation	**1.3**	**1.6**
LTBP1	NM_021587	TGF regulation	**1.9**	**1.5**
TGFβli4	NM_013043	TGF regulation	**1.8**	**1.9**
Interleukins and Related Cytokines (23: www.copewithcytokines.de)				
IL1 receptor	NM_012968	IL1 inflammation	**1.6**	**NS**

accessory protein [16]				
IL3 regulated nuclear factor	NM_053727	IL3 MHC, eosinphil, basophil stimulation, apoptosis inhibition	**1.4**	**NS**
IL6 [6]	NM_012589	acute phase protein induction, proliferation	**3.5**	**NS**
IL6 gp130	298242_Rn	IL6 acute phase protein induction	**1.7**	**1.4**
IL6 signal transduction protein	BF398277	IL6 acute phase protein induction	**1.5**	**1.4**
IL11 receptor α 1	221254_Rn	IL11 progenitor growth factor, acute phase protein induction	**NS**	**1.3**
IL12 p40 precursor	NM_022611	IL12 hematopoeitic response, adhesion	**NS**	**1.3**
IL18	284329_Rn	T cell activation, hematopoiesis	**1.3**	**NS**
Interferon-γ	NM_138880	immune response	**NS**	**1.4**
Interferon inducible p27-like	NM_130743	immune response	**1.4**	**1.4**
ATP dependent interferon responsive	BG373987	immune response	**NS**	**1.4**
Complement Pathway (29: users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Complement.html)				
Complement 1Q binding protein	NM_019259	Complement 4 activation	**1.7**	**NS**
Complement 1R	AA799803	Complement 4 activation	**1.7**	**1.4**
Complement 1S	NM_138900	Complement 4 activation	**2**	**2.6**
Complement 2	NM_172222	Complement 3 activation	**NS**	**1.4**
Complement 4	AI412156	Complement 2 activation	**NS**	**2.3**
Complement H	NM_130409	Complement 3 inhibition	**1.6**	**NS**
Complement I	NM_024157	Complement 3 inhibition	**NS**	**1.1**
CDs (26, www.immunologylink.com)				
CD14	NM_021744	LPS receptor	**1.4**	**NS**
CD39-like 3	AI070096	ecto-nucleoside triphosphate diphosphohydrolase	**1.5**	**NS**
CD34	AI102873	adhesion, stem cell marker	**1.6**	**1.9**
CD36 [18]	NM_054001	scavenger receptor, inflammation, angiogenesis	**1.6**	**NS**
CD81	NM_013087	T cell stimulation	**1.8**	**2.1**
CD151	NM_022523	adhesion, signaling	**1.4**	**1.3**
CD164	NM_031812	hematopoeitic-stromal interaction	**1.6**	**1.2**

NS: Not Significant

4) Conclusions

We have accomplished the proposed work on microarray studies of fracture repair. Microarray analysis of fracture healing by our approach has identified a number of genes or ESTs with either significant up-regulation or down-regulation in expression at days 3 and 11 days of healing. Some of the genes that we identified had been previously described by other investigators, but many had not. The expressed inflammatory gene repertoire was altered compared to previous studies, an important observation in early fracture repair, when healing is initiated. Our use of a K-wire-stabilized unfractured control bone allowed ablated the marrow, and with our statistical analysis of individual samples, allows for more sensitive detection of gene expression in the fracture tissues. The analysis of our microarray data is still on-going, and we anticipate that further examination of the data will produce additional information.

c) Specific Objective 3: To Evaluate the Reproducibility and To Analyze the Data from the Extended Microarray

1) Objectives

During the current funding period, we extended the microarray analysis of fracture healing to include additional animals in the analysis from the first year. The microarray analysis was expanded to achieve a final sample size of 5 individuals (fractured and unfractured controls) at 3 days healing and 8 individuals (fractured and unfractured controls) at 11 days healing. The more extensive analysis was used to rigorously identify and confirm differences in fracture gene expression, characterize different gene pathways that participate in fracture healing, and identify potential therapeutic gene candidates. The expression of several genes of interest determined by microarray analysis was also verified by an independent method.

2) Materials and Methods

All surgical procedures and RNA isolation were performed as stated above (Technical Objective 2, Specific Objective 2). RNA labeling and hybridization to the Agilent 20,000 gene chip using the Agilent low-input system, data analysis and normalization were also performed as described above.

Changes in gene expression as determined by microarray analysis were independently confirmed by real-time RT-PCR for selected genes of interest. This confirmation was performed on some of the same fracture tissues that underwent microarray analysis, as well as additional fracture tissues at 3 and 11 days healing. Total RNA was treated with DNAse I and reverse transcribed using the Superscript III kit (Invitrogen) according to the manufacturer's specifications. Real-time RT-PCR was performed on 50 ng of cDNA using gene-specific primers and the Quantitect SYBR Green detection (Qiagen) as specified by the manufacturer. Real-time PCR was performed on a DNA Engine Opticon thermal cycler (BioRad Laboratories) at 45 seconds per step for 35 cycles at a specific annealing temperature optimized to amplify both the gene of interest and the cyclophilin housekeeping gene with the greatest efficiencies. Each gene of interest was normalized to expression of the housekeeping gene cyclophilin for each fracture tissue, and the difference between fractured and unfractured real-time PCR cycle numbers used to calculate the fold-change in expression in the fracture for comparison to the microarray values. Table 5 lists the primers and annealing temperatures used for real-time PCR confirmation of the cyclophilin gene, the genes associated with our initial "scarless wound healing genes" analysis, and the FGF family genes of our subsequent microarray analysis.

Table 5. Real-time PCR primers and conditions for the confirmation of fracture microarray gene expression.

Target Gene		Primers				
Gene	Accession	Position	Direction	Product	Sequence	Annealing Temp[1]
Cyclophilin	BC059141	320	Forward	192	5'-GCATACAGGTCCTGGCATCT-3'	Footnote 1
		511	Reverse		5'-TCTTGCTGGTCTTGCCATTC-3'	
"Scarless Healing" Gene Expression Confirmation						
Prx-2	NM_238327	432	Forward	227	5'-CTCGCTGCTCAAGTCTTACG-3'	56.2
		658	Reverse		5'-GGCTGTGGTGTAAGCTGAAC-3'	
TGF-β3	BC092195	1067	Forward	194	5'-CAGCATCCACTGTCCATGTC-3'	56.4
		1260	Reverse		5'-GTCGGTGTGGAGGAATCATC-3'	
Fibromodulin	NM_080698	901	Forward	232	5'-ATGGCCTTGCTACCAACACC-3'	55.2
		1132	Reverse		5'-ATAGCGCTGCGCTTGATCTC-3'	
PN-1	NM_012620	2656	Forward	267	5'-CTCCTGGTCAACCACCTTAG-3'	55.4
		2922	Reverse		5'-CCTGTGGTACACGGTGTATG-3'	
Mmp-14	NM_031056	966	Forward	330	5'-ACTTCGTGTTGCCTGATGAC-3'	56.5
		1295	Reverse		5'-TGCCATCCTTCCTCTCATAG-3'	
FGF Gene Family Expression Confirmation						
FGF-13		290	Forward	249	5'-TCTTCGAGTCGTGGCTATTC-3'	54.3
		538	Reverse		5'-GCAGGCTTGTTCTTCTTGAC-3'	
JIP-2a		216	Forward	262	5'-CCATGCAGCTGGTACTGAAG-3'	60.0
		477	Reverse		5'-AGGTCCATCTGCAGCATCTC-3'	
FGFR-5		1404	Forward	216	5'-AACGCAGTGGTGACAAGGAC-3'	54.3
		1619	Reverse		5'-GACATGCTGGTGCTGATGAG-3'	

1: Each annealing temperature produces the most efficient amplification of cyclophilin when compared to the gene of interest.

3) Results

A microarray analysis that combined the 3-day and 11-day fracture calluses into one group identified 6,555 genes with significant changes in expression ($p<0.05$). The increased power of the statistical analysis improved the sensitivity of the approach, and allowed a better examination of the molecular pathways involved in fracture repair.

Because, as we have indicated in the Introduction, fracture repair leads to bridging the injury with bone that is identical to the native bone but lacks scar tissue (Bolander, 1992), there might be similarities between fracture repair and scarless tissue healing. To further characterize the regenerative molecular pathways operating in fracture repair and scarless tissue healing, we used real-time PCR (Table 6) to confirm the expression of several of the "scarless wound healing genes" that we described in the in the previous progress report. These genes included members of the transforming growth factor β (TGFβ) family, and other genes previously linked with fracture expression. Novel genes included Prx-2, fibromodulin and mmp-14, which have not

been previously reported in microarray studies of fracture repair. The application of real-time PCR to the fracture RNA samples succeeded in confirming the expression of several of the more important scarless wound healing genes, both in the same samples analyzed by microarray and in additional samples not examined by microarray. More than 5 samples were analyzed for selected genes. Real-time PCR was reproducible to a standard deviation of approximately 0.5 cycles, and was usually effective at confirming changes in expression in excess of 2-fold (1 cycle), though the magnitudes often varied from the microarray values of fold-activation. Fold-activation values of less than 2 were difficult to confirm with the sample numbers examined. Combined with the statistical approach to the analysis of several samples by microarray, however, the real-time PCR confirmation provided a more reliable characterization of the regulatory pathways of fracture repair than previous studies.

Table 6. Fracture Microarray Genes Associated With Scarless Fetal Wound Healing

		3 Day Expression		11 Day Expression	
		Microarray (P<0.05)	**Real-Time PCR**	**Microarray (P<0.05)**	**Real-Time PCR**
Gene (Function) [Reference]	**Accession**	**Fold-Change**	**Fold-Change[1] (n)**	**Fold-Change**	**Fold-Change[1] (n)**
Homeodomain					
Prx-2 (TGF-β3, PN-1 regulation) [48, 53]	BE118447	**4.4**	**2.2 ± 1.9 (7)**	**2.7**	**2.6 ± 1.6 (6)**
Meox-2 (cell migration) [33, 54]	NM_017149	**2.5**	**1.7 ± 0.6 (9)**	**2.8**	**2.5 ± 1.8 (5)**
TGF-β3-Related [13, 51]					
TGF-β3 (proliferation, differentiation) [30]	NM_013174	**2.4**	**1.7 ± 0.9 (7)**	**2**	**4.3 ± 2.0 (8)**
LTBP-1 (TGF-β3 binding) [43]	NM_021587	**1.9**	**ND**	**1.5**	ND
Fibromodulin [50]	NM_080698	**2.3**	**2.1 ± 1.2 (8)**	**5.2**	**21.0 ± 13.2 (6)**
Other Growth Factors					
VEGF-C (angiogenesis) [36]	NM_053653	**1.2**	ND	NS	ND
Hepatocyte Growth Factor (anti-apoptosis) [41]	NM_017017	NS	ND	**1.4**	ND
Extracellular Matrix (ECM) [11]					
Fibronectin-1	NM_019143	**2.4**	ND	**2.7**	ND
Collagen V (α1) (cell spreading)	NM_134452	**2.4**	ND	**2.1**	ND
ECM Matricellular (Adhesion) [38, 45]					
Tenascin [27, 32]	BE126741	**2.1**	ND	**1.5**	ND
Calpactin I Heavy Chain (Ten receptor)	NM_019905	**1.9**	ND	NS	ND
Thrombospondin-2	BF408413	**1.7**	ND	**1.8**	ND
Thrombospondin-4	X89963.1	**1.9**	ND	**3.6**	ND
Calreticulin (TSP-receptor)	NM_022399	**1.6**	ND	NS	ND
SPARC	NM_012656	**1.7**	ND	NS	ND
ECM Remodeling					
Protease Nexin-1 (ECM regulation) [44]	X89963.1	**2.1**	**1.1 ± 0.5 (8)**	**2.1**	**12.3 ± 6.4 (8)**
Mmp-14 [14]	NM_031056	NS	**1.1 ± 0.5 (10)**	**2.1**	**4.2 ± 1.8 (9)**

TIMP-2 (Mmp-14 regulation) [3]	NM_021989	**2.3**	ND	**1.8**	ND
NS: Not Significant; ND: Not Determined; n: number of fractured vs unfractured pairs of tissues in real-time RT-PCR; [1] mean +/- SD.					

Consequently, our microarray data strongly suggest, and our real-time PCR data support, a similarity between fetal repair and scarless healing, since both processes shared developmental gene expression pathways (Ferguson et al., 1999). Because adult bone is a tissue unique in its ability to heal without a scar, these genes immediately suggest a regulatory pathway for the regenerative characteristics of bone repair.

Additional growth factor pathways were also examined to further elucidate the molecular pathways that regulate fracture healing. The fibroblast growth factor (FGF) family is of particular interest, because the members of this family are generally thought to be highly potent mediators of cell proliferation, a critical early step in tissue repair. While we found FGF7 expression to be up-regulated (1.3-fold at 3 days), in agreement with other studies (Hadjiargyrou et al., 2002; Li et al., 2005), there was an unexpected absence of other FGFs. An exception was FGF-13, which was up-regulated 2.3-fold at 11 days healing; lacking a signal sequence, FGF-13 might act intracellularly or upon release from damaged cells. Subsequent real-time PCR analysis with gene-specific primers (Table 5) confirmed the microarray study and established that FGF-13 and its intracellular link to the JNK signaling pathway, JNK interacting protein (JIP)-2a were significantly up-regulated in expression during endochondral bone formation (FGF-13: 6.7, $p<0.0005$; JIP-2a: 6.4-fold, $p<0.0004$). Other members of this pathway are regulated through phosphorylation, rather than transcriptionally, and would not be detected in gene expression studies. Interestingly, FGF receptor 5 (FGFR5), a truncated FGF receptor variant that lacks a kinase signaling domain, was also significantly up-regulated in expression at 11 days healing (2.1-fold), an observation confirmed by real-time PCR (3.1-fold up-regulated in expression, $p<0.0001$). FGFR5 might act as a dominant negative receptor, binding extracellular FGFs and maximizing the effect of intracellular FGF signaling mediated by FGF13 through JIP-2a. We will more fully investigate these findings.

At the present time, we continue to analyze the microarray gene expression data to functionally classify the genes with changes in expression, characterize gene pathways important in fracture healing and identify gene candidate(s) for our fracture therapy.

4) Conclusions

Using a 20,000 rat gene chip and appropriate controls that normalized marrow RNA input among multiple replicates at two healing times, we were able to analyze whole genome expression in fracture tissues among multiple replicate animals using unfractured controls that normalized marrow RNA input. This approach allowed us to more accurately identify 6,555 genes with significant changes during fracture healing. We have identified and confirmed growth factor, structural, and transcription factor genes that participate in developmentally related (scarless wound healing) pathways and must also contribute to the complex regulation of bone repair. These results demonstrate that fracture repair is similar to fetal tissue development and repair, and that the regenerative qualities of bone repair can be used to elucidate therapies for improved wound healing of skeletal and nonskeletal tissues. We have also expanded the microarray analysis and confirmation to a subset of the FGF gene family that might modulate fracture healing through intracellular pathways.

Currently, analysis of our microarray data and potential pathways involved are on-going. The high number of unknown genes and ESTs that displayed changes in expression during fracture healing suggests remaining pathways that are undiscovered as of this time are important in bone repair. Further characterization of such gene expression pathways should facilitate the molecular understanding of normal and impaired fracture repair.

An additional specific objective proposed for the final year of this study: To evaluate the functional role of one or more ESTs with altered expression during fracture healing by inhibition or augmentation of its expression *in vitro,* followed by identification of the resulting changes cellular phenotype and gene expression.

1) Objectives

To further investigate the possible functions of the large numbers of unknown genes and ESTs in the regulation of fracture repair, we are inhibiting the expression of at least one of those ESTs in rat bone cells *in vitro*. We will then determine the response of those cells to the inhibition of EST expression by measuring the expression of bone formation marker genes in those cells. EST expression will inhibited through short inhibitory (si)RNA expression, a new and powerful in which oligonucleotides homologous to a target gene mediate transcriptional inhibition post-transcriptional digestion of their target sequences (Kim et al., 2005; Solias et al., 2005). These studies will most effectively characterize the effects of expression of an unknown gene on bone cells in a homogeneous system.

2) Materials and Methods

Several ESTs that displayed significant changes in expression in fracture tissues at 3 days and 11 days were identified in the microarray analysis. RNA was purified from several primary and transformed cell lines that represented mesenchymal, chondrocytic and osteoblastic stages of bone cell development (Table 7).

Table 7. PCR Confirmation of Fracture EST Expression in Cell Lines for siRNA Studies

	PCR Expression						Microarray Expression	
EST	**Product**	**Normal Cells**		**Transformed Lines**			**Fracture Tissues**	
		RMS	**RCOB**	**Rat-1**	**RCS**	**ROS17.2/8**	**3 Days**	**11 Days**
BQ209715	**253**	+	+	+	+	+	**1.87[a]**	**0.73[a]**
BF283714	299	-	-	-	-	-	NS	2.29
CORS26	137	-	-	+	-	+	5.02	7.88
AP2M1	117	-	-	-	-	-	NS	0.8
BGLAP1	147	+	+	+	+	+	0.36	NS
CHM-1	153	+	+	-	-	-	NS	21.97
AW528046	168	-	+	-	+	+	2.81	NS
CB546087	322	+	-	+	-	+	4.6	4.83
CB545954	**381**	+	+	+	+	+	**3.33**	**2.66**
CB547532	153	-	-	-	-	-	3.51	1.93
BU758349	146	+	+	+	+	+	2.57	NS
BM390058	483	-	-	-	-	-	2.31	3.32

RMS: rat marrow stromal cells; RCOB: rat calvarial osteoblasts; Rat-1: rat fibroblasts; RCS: rat chondrosarcoma;

ROS: rat osteosarcoma
Product: +; No product: -
Bold: Candidate for siRNA inhibition; a: Confirmed by Real-Time PCR in fracture tissues

Total RNA was isolated from cultures of these cells by the Trizol^{TM} method, and cDNA prepared as previously described for the microarray analysis. PCR primers were designed to the selected ESTs and (non-real-time) PCR performed on the cDNAs for 35 cycles at a 55^{o}C annealing temperature. Amplicons were visualized by agarose gel elctrophoresis and the products evaluated for the number of cell lines expressing each, as compared to their change in expression in the fracture microarray at 3 days, 11 days or both.

At least one of the ESTs that show the greatest changes in expression in the fracture microarray and are expressed in the most cell lines will have its expression confirmed by real-time PCR, as previously described. The bone cell lines will then be analyzed for the effect of its inhibition by siRNA technology. A 27 base blunt-end hairpin oligomer duplex (Kim et al., 2005) will be designed from the EST and this duplex cloned into a proprietary, neomycin-selectable, MLV-based vector (Imgenex). Cultures of these cell lines (Table 7), as well as rat primary periosteal cells, will be transduced in vitro and cultured under selection with neomycin. Near confluence, total RNA will be isolated from each cell culture and the cDNA analyzed by real-time PCR (as previously described) for changes in the expression of bone cell marker genes such as alkaline phosphatase and osteocalcin, as well as chondrocyte cell marker genes such as collagens 2 and 10, and sox-9.

3) Results

Approximately 10 ESTs that displayed significant changes in expression in the fracture microarray were analyzed for expression in a number of different primary and transformed bone cell lines (Table 7). Two (BQ209715, CB545954) were expressed in all lines and displayed significant changes during fracture repair. One of those (BQ209715) has had its fracture expression confirmed by real-time PCR, and the other (CB545954) will also have its expression confirmed. siRNA oligomer duplexes will be designed to one or both of these ESTs, transduced into bone cell cultures and the resulting effects on gene expression determined by real-time PCR. A random oligomer duplex with no homology to any gene will serve as the negative control in the transduction and gene expression studies.

4) Conclusions

This study is underway. The determination of the best EST candidates for siRNA inhibition studies is complete and we are currently designing the siRNA construct and assembling the MLV-based siRNA vector(s).

KEY RESEARCH ACCOMPLISHMENTS

1. We have completed the comparison of MLV-based and lentiviral-based vector systems the expression of therapeutic transgenes for fracture repair. Mechanical testing confirmed no differences between vectors.

2. We are completing comparison studies of single and combination gene therapy in fracture repair using two therapeutic genes (BMP-2/4 and FGF-2) expressed from the MLV-based vector and applied using our novel surgical techniques.

3. We have performed an extensive and rigorously controlled microarray analysis on the RNA from multiple individual animal subjects during early (inflammatory) and late (endochondral bone formation) stages of fracture repair.
   a) Several hundred known and unknown genes are being classified into functional categories to better understand the molecular pathways of fracture repair.
   b) The expression of genes with possible therapeutic value is being independently confirmed by real-time PCR. This confirmation included genes expressed in scarless fetal wound healing that could be adopted for novel wound therapies.
   c) This analysis continues to identify and confirm genes expression in novel pathways of gene regulation for fracture repair, one of which involves the members of the FGF family of growth factors.

4. Studies are underway that will utilize siRNA technology to study the effects of inhibition expression in bone cell lines of one or two ESTs that were expressed in the healing fracture tissues.

REPORTABLE OUTCOMES

1. Rundle CH, Wang H, Yu H, Chadwick RB, Wergedal JE, Lau K-HW, Mohan S, Ryaby JT, Baylink DJ. (2006) Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair. *Bone: In Press*.

CONCLUSIONS

The development of highly effective gene therapy approaches to musculoskeletal injuries requires the optimization of the components and techniques for the accurate assessment of therapeutic benefits. We have optimized conditions for the accurate evaluation of therapeutic transgene candidates by modifying a standard *in vivo* fracture model to compare viral vector and therapeutic gene combinations. We have also performed microarray analysis of global gene expression in the normal healing fracture callus to characterize the molecular pathways of fracture healing at early (3 days) and later (11 days) healing times and we have identified potential therapeutic gene candidates among the several hundred known and unknown genes with significant increases or decreases in expression. The application of combinations of therapeutic genes to enhance fracture healing is underway.

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Appendices

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Microarray analysis of gene expression during the inflammation and endochondral bone formation stages of rat femur fracture repair

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Abstract

Microarray analysis of gene expression was performed in the healing femur fractures of 13-week-old male rats during the inflammatory stage of repair, at 3 days post-fracture, and the endochondral bone formation stage of repair, at 11 days post-fracture. Multiple replicate pairs of fracture tissues paired with unfractured tissues, and unfractured control bones that had the stabilizing K-wire were introduced. This approach normalized the marrow contributions to the RNA repertoire. We identified 6555 genes with significant changes in expression in fracture tissues at 3 days and 11 days healing. The repertoire of growth factor genes expressed was also surprisingly restricted at both post-fracture intervals. The large number of Expressed Sequence Tags (ESTs) expressed at both post-fracture times indicates that several molecular pathways yet to be identified regulate fracture repair. The number of genes expressed during immune responses and inflammatory processes was restricted with higher expression largely during the early post-fracture analysis. Several of the genes identified in this study have been associated with regulation of cell and extracellular matrix interactions during scarless healing of fetal skin wounds. These observations suggest that these genes might also regulate the scarless healing characteristic of bone regeneration by similar mechanisms.

Keywords: Fracture healing; Microarray; Inflammation; Endochondral; Scarless

Introduction

Several million long bone fractures occur annually in the United States, approximately 10% of which display impaired healing [17]. Traditional surgical and non-surgical interventions can facilitate healing, but society would achieve considerable humanitarian and economic benefits from any improvements in fracture treatments. Such improvements would be realized through an understanding of bone repair.

Bone repair requires the regulated expression of diverse families of genes that coordinate complex interactions among various cell types. Endochondral bone repair proceeds through ordered stages of inflammation, intramembranous bone formation, chondrogenesis, endochondral bone formation and finally remodeling [8]. Fracture callus formation eventually results in the bridging of the fracture and the restoration of skeletal integrity. However, bone is unique in that it is one of the very few adult tissues normally capable of healing without a scar, and in this respect, bone repair is a truly regenerative process. Although the molecular pathways that regulate bone repair remain largely unknown, studies of gene expression in endochondral bone repair have established that several extracellular matrix components and growth factor gene families that play significant roles in tissue development are also expressed during the different stages of fracture repair ([1,2,12,22], reviewed by [5,7]). These observations suggest that the molecular regulation of fracture

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repair is complex but probably recapitulates some aspects of skeletal morphogenesis [19]. If developmental genes regulate both fracture repair and bone regeneration, then these pathways can be characterized by identification of the genes expressed during fracture repair.

Microarray analysis of gene expression offers the opportunity for a global survey of gene expression during fracture repair and the elucidation of the molecular pathways of bone regeneration. Previous examinations of genome-wide gene expression in rodent fracture healing have demonstrated that gene expression in fracture healing is indeed complex and have also been very helpful in identifying known and novel genes that are expressed in fracture healing and elucidating the molecular pathways of bone repair. These studies have generally examined up-regulated genes from a cDNA-subtracted library [20] throughout normal fracture repair or up-regulated and down-regulated genes during very restricted periods in normal fracture repair [28] or in restricted repertoires of genes in the normal fracture model [35]. Models of impaired fracture healing [21,33], determinations of response to fracture therapy [46] or alternative bone healing models [38] have also been utilized. Previous studies have generally analyzed pooled RNA samples, an approach that homogenizes individual biological variations. Additionally, microarray analysis usually uses an arbitrary threshold of 1.5- or 2-fold to identify significant changes in gene expression relative to control tissues. Their design has also utilized unfractured control bones without the stabilizing pin, which incorporates the substantial contributions of the marrow component into the RNA repertoire for examination of gene expression from the control bone, but not from the fractured bone. Each of these variables can affect the interpretation of the results.

The objective of this study was to use whole genome microarray gene analysis to identify the genes expressed in fracture repair of the rat femur at two stages: (1) at 3 days, immediately after the inflammatory phase but prior to bone formation, and (2) at 11 days, when intramembranous and endochondral bone formation overlap [8]. However, two important factors distinguish our approach from previous microarray studies using the rodent fracture model. The unfractured femurs used for the control comparison had the stabilizing Kirschner wire introduced into the intramedullary cavity, which controlled for bone formation induced by the pin in the absence of a fracture, as well as for marrow contributions to the RNA repertoire. We also utilized the Agilent Technologies (Palo Alto, CA) 20,000 gene chip and low-input hybridization system that allowed the examination of gene expression during healing in individual animals without sample pooling and took biological variation into account. This approach allowed us to apply statistical analysis to corroborate the arbitrary thresholds normally used to define significant changes in gene expression between fractured and unfractured bone. The identification of gene expression changes under these conditions of analysis should provide more accurate insights on the molecular pathways that regulate the bone repair and regeneration and suggest potential therapeutic pathways to enhance the healing of bone injuries.

Materials and methods

Femur fractures were produced in 13-week-old male Sprague–Dawley rats (Harlan, Indianapolis, IN) by the three-point bending technique [9]. Thirteen-week-old rats were used in this study because (a) as young adults, these animals had passed the period of most rapid adolescent bone growth that might affect interpretations of gene expression in fracture healing, and (b) the fracture healing ability of younger animals is expected to be more effective than older animals. A 1.14-mm diameter stabilizing Kirschner (K)-wire was inserted in both fractured and unfractured control femurs to ablate the marrow equally in each bone. This approach normalized the substantial contributions of the marrow to the fracture RNA repertoire and controlled for marrow gene expression unrelated to fracture healing and gene expression due to K-wire induction of bone formation in the marrow.

RNA was purified from the fracture diaphyses at two post-fracture healing times: 3 days, between the inflammation and intramembranous bone formation stages, and 11 days, during the endochondral bone formation stage. These post-fracture times were sufficiently separated to provide an examination of very different stages of bone healing and identify the molecular pathways that regulate each stage. Briefly, the diaphysis was isolated from the fractured femur and from an unfractured control femur with a K-wire at each post-fracture time. The bone was pulverized while cooled with liquid nitrogen, and the RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Total RNA was further purified by RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's specifications, quantified by Nanodrop (Agilent) and its integrity confirmed by Bioanalyzer (Agilent).

Microarray analysis was performed using the Agilent low-input labeling system and the Cy-5 or Cy-3 labeled RNA applied to the rat 20,000 oligomer microarray chip (Agilent). The low-input system allowed the use of low RNA recoveries from the control tissues and avoided pooling samples that could conceal individual variations in gene expression. Individual fracture tissues were randomly paired with individual K-wire stabilized but unfractured tissues: 5 pairs of tissues were compared at 3 days healing, and 8 pairs of tissues were compared at 11 days healing. Microarray image segmentation analysis was performed using ImaGene software (BioDiscovery, El Segundo, CA), that used an internal statistical analysis of the signal intensity of the spot and immediate surrounding area to flag each spot as present, empty, negative or marginal. Gene expression results were based upon spots flagged as present as well as those flagged as present or marginal. Lowess normalization and statistical analysis were performed using the Genespring software package (Agilent). Changes in gene expression at $P < 0.05$ were deemed significant.

Changes in gene expression as determined by microarray analysis were independently confirmed by real-time RT-PCR for selected genes of interest. This confirmation was performed on some of the same fracture tissues that underwent microarray analysis, as well as additional fracture tissues at 3 and 11 days healing. Total RNA was treated with DNAse I (Invitrogen) and reverse transcribed using the Superscript III kit (Invitrogen) according to the manufacturer's specifications. Real-time RT-PCR was performed on 50 ng of cDNA using gene-specific primers (Integrated DNA Technologies, Coralville, IA) and the Quantitect sybr green detection (Qiagen) as specified by the manufacturer. Real-time PCR was performed on a DNA Engine Opticon thermal cycler (Biorad Laboratories, Hercules, CA) at 45 s per step for 35 cycles at annealing temperatures optimized to amplify the gene of interest and the housekeeping gene (Table 1). Each gene of interest was normalized to expression of the housekeeping gene cyclophilin for each fracture tissue.

Results

Microarray analysis of fracture calluses at 3 days and 11 days post-fracture identified 6555 genes with significant changes in expression ($P < 0.05$), 67% (4873) of which were known genes and 33% (1682) of which were unidentified genes and ESTs (Table 2).

Table 1
Real-time PCR primers and conditions for the detection of scarless wound healing gene expression

Target gene		Primers				
Name	Accession	Position	Direction	Product	Sequence	Annealing temperature [a]
Cyclophilin	BC059141	320	Forward	192	5′-GCATACAGGTCCTGGCATCT-3′	
		511	Reverse		5′-TCTTGCTGGTCTTGCCATTC-3′	
Fibromodulin	NM_080698	901	Forward	232	5′-ATGGCCTTGCTACCAACACC-3′	55.2
		1132	Reverse		5′-ATAGCGCTGCGCTTGATCTC-3′	
Meox-2	NM_017149	569	Forward	301	5′-GTCCTGTGCTCCAACTCTTC-3′	53.7
		869	Reverse		5′-GGTCTAGGTTCACCGCTATC-3′	
Mmp-14	NM_031056	966	Forward	330	5′-ACTTCGTGTTGCCTGATGAC-3′	56.5
		1295	Reverse		5′-TGCCATCCTTCCTCTCATAG-3′	
PN-1	NM_012620	2656	Forward	267	5′-CTCCTGGTCAACCACCTTAG-3′	55.4
		2922	Reverse		5′-CCTGTGGTACACGGTGTATG-3′	
Prx-2	NM_238327	432	Forward	227	5′-CTCGCTGCTCAAGTCTTACG-3′	56.2
		658	Reverse		5′-GGCTGTGGTGTAAGCTGAAC-3′	
TGF-β3	BC092195	1067	Forward	194	5′-CAGCATCCACTGTCCATGTC-3′	56.4
		1260	Reverse		5′-GTCGGTGTGGAGGAATCATC-3′	
HAS-1 [b]	NM_172323	1543	Forward	219	5′-CTGGCTGCTAACTATGTACC-3′	54.5
		1761	Reverse		5′-TCTGCACAGTCTCCTTACAC-3′	

[a] Each annealing temperature produces the most efficient amplification of cyclophilin and in the gene of interest.
[b] Hyaluronic acid synthetase-1.

An examination of the known genes that were significantly up-regulated or down-regulated revealed that several are associated with fracture healing in previous fracture expression analyses, including several extracellular molecules and members of selected growth factor gene families (Table 3). The increases in expression of these genes corresponded generally to those gene expression changes in previous studies at similar post-fracture healing times, though there were differences in magnitude of expression between studies. The changes in gene expression among other genes did vary between studies; different genes compared well with one study but not with another (data not shown).

A "Gene Ontology" classification [4] of the genes with statistically significant changes in expression was performed for 3 days healing, 11 days healing and both 3 and 11 days healing (Table 4). Because there were 4873 known genes with significantly altered expression at $P < 0.05$, only those genes with changes in expression at $P < 0.0002$ were included. Even at this very high level of statistical significance, this list revealed some TGFβ-related genes, developmental transcription factors, extracellular matrix and adhesion genes, and provided an initial analysis from which to further examine and functionally associate genes with less significant levels of expression.

Table 2
Summary of fracture microarray gene expression changes

Expression change ($P < 0.05$)	Known Genes	Unknown Genes	Total
3 days			
Up at 3 days, no change at 11 days	889	215	1104
Down at 3 days, no change at 11 days	1013	388	1401
11 days			
Up at 11 days, no change at 3 days	904	345	1249
Down at 11 days, no change at 3 days	1206	450	1656
3 and 11 days			
Up at both 3 and 11 days	354	96	450
Down at both 3 and 11 days	474	181	655
Biphasic			
Up at 3 days, down at 11 days	20	1	21
Down at 3 days, up at 11 days	13	6	19
Total	4873	1682	6555

The up-regulated inflammatory and immune genes were further examined (Table 5). Relatively few inflammatory genes were significantly up-regulated in expression, those that were observed at 3 days post-fracture, corresponding to the established inflammatory phase of fracture repair [8]. B cell, T cell and major histocompatibility genes were notably absent. Members of the complement pathway were present, but the cascade ended before C3a activation, suggesting regulation of innate immunity and the inflammatory response. Relatively few interleukins and Cluster of Differentiation (CD) antigens were represented, and some of those could have non-inflammatory or non-immune functions [24].

To determine whether regenerative molecular pathways are common in fracture repair and tissue development, we examined growth factor genes and transcription factors involved in skeletal development at 3 and 11 days healing (Table 6). Developmental factor and homeodomain transcription factor genes showed statistically significant and greater fold-changes in expression during fracture repair. This analysis included TGF-β3, and the paired box transcription factor Prx-2, not previously described in fracture healing. A further examination of genes related to TGF-β3 and Prx-2 revealed several growth factors and extracellular matrix genes previously described in studies of scarless wound healing in fetal skin. Real-time PCR measurements of changes in expression of some of these genes generally confirmed their up-regulation in expression during fracture repair, although there was a

Table 3
Comparison of selected genes with up-regulated expression in this study with previous fracture studies

This study					Previous fracture studies
Gene	Accession	Function	Fold-change ($P < 0.05$)		Similar change in expression
			3 days	11 days	
Transforming growth factor β-2	BF420705	Growth factor	1.3	1.6	[12]
Transforming growth factor β-3	NM_013174	Growth factor	2.4	2.0	[12,20,28]
Fibroblast growth factor 7	NM_022182	Growth factor	1.3	NS	[20,28]
Interleukin 6	NM_012589	Inflammation	3.5	NS	[12,28]
Angiopoietin-2 (like)	NM_133569	Angiogenesis	NS	1.4	[33]
Mesenchymal homeobox-2	NM_017149	Transcription factor	2.5	2.8	[20]
Pleiotrophin/OSF-1	NM_017066	Several	2.3	NS	[20,28]
Frizzled	NM_021266	wnt signaling	1.5	1.5	[20]
Cysteine-rich protein 61	NM_031327	Extracellular matrix signaling	2.6	2.8	[20,28]
Fibronectin	NM_019143	Extracellular matrix	2.4	2.1	[20,28]
Tenascin	BE126741	Extracellular matrix	2.1	1.5	[20,28]
Thrombospondin-2	BF408413	Extracellular matrix	1.7	1.8	[20,28]
Osteonectin/SPARC	NM_012656	Extracellular matrix	1.7	NS	[20,28]
Aggrecan	NM_022190	Extracellular matrix	NS	5.1	[20,28,33]
Collagen 2α1	AA899303	Cartilage maturation	0.8	1.5	[20,28,33]
Integrin binding sialoprotein	NM_012587	Mineralization	4.0	NS	[20,28]
Collagen 5α1	NM_134452	Extracellular matrix	2.4	2.1	[20,28]
Osteocalcin/Gla	NM_012862	Mineralization	NS	2.4	[12,20,28,33]
Protease nexin-1	X89963.1	Extracellular matrix protease	2.1	2.1	[20,28]

NS—not significant.

difference in the magnitude of expression noted for some genes (Table 6).

Discussion

Our global analysis of fracture tissue gene expression recognized many genes important in fracture repair, underscoring the complexity of the fracture repair process (Table 2). The numbers of genes with significant changes in expression at 3 or 11 days indicated that relatively few genes were common to both the inflammatory–intramembranous bone formation and endochondral bone formation stages of fracture repair, and different molecular pathways of gene expression regulate different phases of bone healing. The large number of unknown genes and ESTs identified by our analysis also implies that novel, yet-to-be identified molecular pathways play significant roles in the regulation of fracture repair, and that bone regeneration will need to be characterized by a detailed examination of gene expression in bone healing.

An examination of the known genes that displayed significant changes in expression either up-regulated or down-regulated at 3 and 11 days healing revealed representatives of several growth factor gene families observed in previous studies of gene expression in fracture repair (Table 2). With respect to the collagens and growth factors, we generally found agreement with previous studies [12] and in comparable clusters by Hadjiargyrou et al. [20] and at 3 days post-fracture with the later time point examined by Li et al. [28]. Among the growth factors observed at the earliest time point in healing fractures in younger rats [33], only one vascular endothelial growth factor (VEGF) isoform was represented, though the same extracellular matrix molecules were expressed. Differences in fracture gene expression between studies were common, and it appears that variations in experimental approaches, in addition to biological variation in the regulation of fracture healing, can affect interpretations of gene expression changes.

Several Gene Ontology categories were represented that suggested important regulatory pathways at each time, even at a high level of significance (Table 4). The cell proliferation and protein metabolism categories were well represented at 3 days healing, as required for the subsequent proliferation of periosteal mesenchymal cells of the early soft callus. Several members of the skeletal development, cell adhesion and extracellular matrix categories were present at 11 days healing, consistent with the maturation of the various callus tissues during endochondral bone formation. This classification provided an insight into important molecular pathways of fracture repair that could be further characterized by an analysis of functionally related genes with less significant changes in expression.

Despite the importance of the inflammatory reaction early in fracture healing, the expression of inflammatory genes in fracture healing has not been well characterized. The marrow can be a major source of RNA and inflammatory gene expression resulting from damage produced by K-wire stabilization of the fractured bone. Our fracture controls included the K-wire that ablated the marrow to the same degree as the fracture, normalizing the marrow repertoire to allow for more sensitive detection of inflammatory gene expression in the periosteum, whose vessels constitute the major blood supply to the fracture [11]. Several inflammatory and immune-related genes were observed to be down-regulated in expression at 3 days post-fracture by Li et al. [28]. However, conflicting results were presented showing an up-regulation of inflammatory and immune genes in the cDNA-subtracted library microarray analysis performed by Hadjiargyrou et al. [20], suggesting that

Table 4
Known genes with highly significant ($P < 0.0002$) changes in the expression during fracture healing

Accession	Fold-change		Gene description	Gene ontology category [4]
	3 days	11 days		
BQ209997	**5.02**	**7.80**	Similar to mouse collagenous repeat-containing 26-kDa protein (CORS26)	Protein metabolism
AA858962	**4.36**	**2.15**	Rat retinol-binding protein (RBP) mRNA, partial cds	Vitamin A metabolism
NM_012587	**3.97**		*Rattus norvegicus* integrin binding sialoprotein (Ibsp)	Extracellular space
BQ211765	**3.49**		*Rattus norvegicus* DEXRAS1 (Dexras1) mRNA	Signal transduction
BF415205	**2.78**	**6.19**	Rat mRNA fragment for cardiac actin	Actin cytoskeleton
NM_133566	**2.29**	**1.21**	*Rattus norvegicus* cystatin N (LOC171096)	Organogenesis and histogenesis
NM_013104	**1.97**	**4.58**	*Rattus norvegicus* Insulin-like growth factor binding protein 6 (Igfbp6)	Extracellular space
BQ209870	**1.80**	**3.88**	Similar secreted modular calcium-binding protein 2 [*Mus musculus*]	Calcium ion binding
CA510266	**1.71**	**1.32**	Similar to prefoldin 5; myc modulator-1; c-myc binding protein [*Homo sapiens*]	Regulation of transcription, DNA dependent
NM_012488	**1.55**	**2.53**	*Rattus norvegicus* α-2-macroglobulin (A2m)	Protease inhibitor activity/IL-1, IL-8 binding
BE329208	**1.52**	**1.43**	similar to *Cricetulus griseus* SREBP cleavage activating protein (SCAP), complete cds	Steroid metabolism
NM_012816	**1.41**		*Rattus norvegicus* α-methylacyl-CoA racemase (Amacr)	Metabolism/peroxisome
NM_057197	**1.40**		*Rattus norvegicus* 2,4-dienoyl CoA reductase 1, mitochondrial (Decr1)	Oxidoreductase
NM_031646	**1.39**		*Rattus norvegicus* receptor (calcitonin) activity modifying protein 2 (Ramp2)	G-protein-coupled receptor signaling
NM_031050	**1.38**		*Rattus norvegicus* lumican (Lum)	Extracellular matrix
NM_017355	**1.27**	**1.24**	*Rattus norvegicus* ras-related GTP-binding protein 4b (Rab4b)	Vesicle-mediated transport
U56859.1	0.90	0.79	*Rattus norvegicus* heparan sulfate proteoglycan, perlecan domain I (RPF-I), partial cds	Cell adhesion
BF281804	0.85	0.84	Similar to solute carrier family 7 member 12; isc-type amino acid transporter 2 [*Mus musculus*]	Amino acid transport
NM_017140	0.85		*Rattus norvegicus* dopamine receptor D3 (Drd3)	Dopamine receptor signaling pathway
BF548886	0.85	0.78	Similar to mouse T cell antigen receptor α-chain (TCR-ATF2), partial cds	Regulation of transcription, DNA dependent
NM_013029	0.84	0.79	*Rattus norvegicus* sialyltransferase 8 (GT3 alpha 2,8-sialyltransferase) C (Siat8c)	Amino acid glycosylation
NM_012997	0.82	0.74	*Rattus norvegicus* Purinergic receptor P2X, ligand-gated ion channel, 1 (P2rx1)	Amino acid transport
NM_031725	0.82		*Rattus norvegicus* secretory carrier membrane protein 4 (Scamp4)	Protein transport
AA900738	0.80	0.81	Similar to rat DNA for serine dehydratase	Amino acid metabolism/gluconeogenesis
NM_133322	0.79	0.77	*Rattus norvegicus* potassium voltage-gated channel, KQT-like subfamily, member 2 (Kcnq2)	Synaptic transmission
NM_052801	0.78	0.76	*Rattus norvegicus* von Hippel-Lindau syndrome (Vhl)	Regulation of transcription, DNA dependent/proteolysis and peptidolysis
CB546252	0.78	0.83	Similar to zinc finger protein 261; DXHXS6673E [*Mus musculus*]	Nucleus/zinc ion binding
NM_144730	0.78	0.80	*Rattus norvegicus* GATA-binding protein 4 (Gata4)	Regulation of transcription, DNA dependent
NM_030854		**21.97**	*Rattus norvegicus* chondromodulin-1 (Chm-1)	Cell growth and maintenance/proteoglycan metabolism
BF560915		**17.46**	*Rattus norvegicus* mRNA for collagen α 1 type X, partial	Skeletal development
NM_019189		**13.77**	*Rattus norvegicus* cartilage link protein 1 (Crtl1)	Hyaluronic acid binding
NM_012929		**11.38**	*Rattus norvegicus* Procollagen II α 1 (Col2a1)	Skeletal development
NM_031511		**6.72**	*Rattus norvegicus* insulin-like growth factor II (somatomedin A) (Igf2)	Development
BQ210664		**5.73**	Similar to cartilage intermediate layer protein	Unknown
BQ191772		**5.37**	Similar to mouse annexin A8	Phospholipid binding
NM_022290		**5.28**	*Rattus norvegicus* tenomodulin (Tnmd)	Collagen maturation
AI576621		**3.73**	Similar to mouse carboxypeptidase X2, complete cds	Protein binding
AA963765		**2.89**	Similar to osteoglycin [*Mus musculus*]	Regulation of DNA transcription
BQ200482		**1.41**	Similar to mouse mRNA for acetylglucosaminyltransferase-like protein	Lipopolysaccharide biosynthesis
CB547946		**1.35**	Similar to *Mus musculus* (clone pVZmSin3B) mSin3B, complete cds	Regulation of transcription, DNA dependent
AI059288		0.83	Similar to mouse B cell activating factor (TNFSF13b, Baff), complete cds	Positive regulation of cell proliferation
CB547491		0.83	Similar to *Mus musculus* very large G-protein-coupled receptor 1 (Vlgr1, Mass1), complete cds	G-protein-coupled receptor signaling
CB545755		0.82	Similar to RAD54 like (*S. cerevisiae*) [*Mus musculus*]	DNA recombination, repair
CB544611		0.82	Similar to BACR7A4.19 gene product [*Drosophila melanogaster*]	G-protein-coupled receptor signaling
CB545661		0.81	Similar to BC026845_1 *Mus musculus*, similar to nucleoporin 133kD, complete cds	RNA metabolism
AW920271		0.81	Similar to mouse cat eye syndrome chromosome region, candidate 5 (Cecr5), complete cds	Metabolism
BQ196556		0.80	Similar to nudix (nucleoside diphosphate linked moiety X)-type motif 5 [*Mus musculus*]	Oxidative stress response/DNA repair
AA874884		0.60	Rat heme oxygenase gene, complete cds	Oxidoreductase activity
NM_031740		0.59	*Rattus norvegicus* UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 6 (B4galt6)	Glyosphingolipid biosynthesis
NM_053843		0.49	*Rattus norvegicus* Fc receptor, IgG, low affinity III (Fcgr3)	Immune response

Bold: up-regulated.

Table 5
Up-regulated expression in fracture in inflammation and immune function genes

Gene		Functions	Fold-Change in expression ($P < 0.05$)	
Description [Reference]	Accession		3 days	11 days
Growth factors				
Platelet-derived growth factor receptor [31]	AA925099	Chemotaxis	2.7	1.5
Monocyte chemotactic protein 3 [32]	BF419899	Chemotaxis	3.3	1.6
Mast cell growth factor/kit ligand [36]	AI102098	Stem cell factor, hematopoietic and mast cell growth	1.3	NS
TNFα/TNFβ [15]	AA819277	Inflammation	NS	1.2
TRAF2	BI282097	TNF inflammation	1.1	NS
TRAF4	CB546212	TNF inflammation	1.6	NS
TNF-stimulated gene 6	AF159103.1	TNF inflammation	1.8	1.7
TGFβ2 [13]	BF420705	Inflammation	1.3	1.6
LTBP1	NM_021587	TGF regulation	1.9	1.5
TGFβli4	NM_013043	TGF regulation	1.8	1.9
Interleukins and related cytokines (www.copewithcytokines.de) [23]				
IL1 receptor accessory protein [16]	NM_012968	IL1 inflammation	1.6	NS
IL3 regulated nuclear factor	NM_053727	IL3 MHC, eosinphil, basophil stimulation, apoptosis inhibition	1.4	NS
IL6 [6]	NM_012589	Acute phase protein induction, proliferation	3.5	NS
IL6 gp130	298242_Rn	IL6 acute phase protein induction	1.7	1.4
IL6 signal transduction protein	BF398277	IL6 acute phase protein induction	1.5	1.4
IL11 receptor alpha 1	221254_Rn	IL11 progenitor growth factor, acute phase protein induction	NS	1.3
IL12 p40 precursor	NM_022611	IL12 hematopoeitic response, adhesion	NS	1.3
IL18	284329_Rn	T cell activation, hematopoiesis	1.3	NS
Interferon-γ	NM_138880	Immune response	NS	1.4
Interferon inducible p27-like	NM_130743	Immune response	1.4	1.4
ATP-dependent interferon responsive	BG373987	Immune response	NS	1.4
Complement pathway (users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/Complement.html) [26]				
Complement 1Q binding protein	NM_019259	Complement 4 activation	1.7	NS
Complement 1R	AA799803	Complement 4 activation	1.7	1.4
Complement 1S	NM_138900	Complement 4 activation	2	2.6
Complement 2	NM_172222	Complement 3 activation	NS	1.4
Complement 4	AI412156	Complement 2 activation	NS	2.3
Complement H	NM_130409	Complement 3 inhibition	1.6	NS
Complement I	NM_024157	Complement 3 inhibition	NS	1.1
CDs [24]				
CD14	NM_021744	LPS receptor	1.4	NS
CD39-like 3	AI070096	Ecto-nucleoside triphosphate diphosphohydrolase	1.5	NS
CD34	AI102873	Adhesion, stem cell marker	1.6	1.9
CD36 [18]	NM_054001	Scavenger receptor, inflammation, angiogenesis	1.6	NS
CD81	NM_013087	T cell stimulation	1.8	2.1
CD151	NM_022523	Adhesion, signaling	1.4	1.3
CD164	NM_031812	Hematopoeitic-stromal interaction	1.6	1.2

NS—not significant.

the repertoire of expressed genes can be affected by marrow contributions to the fracture model.

In this study, the up-regulation of the platelet-derived growth factor (PDGF) receptor gene implicated PDGF genes during inflammation. The mast cell growth factor, monocyte chemotactic protein 3 [32] and members of the tumor necrosis factor family [15] displayed up-regulated expression, which was higher at 3 days post-fracture (Table 5) and consistent with inflammatory functions in healing. Most notably, in comparison to previous microarray studies [20,28], the T cell receptor, immunoglobulin genes and major histocompatibility genes displayed no significant changes during early and later fracture healing. Though 3 days is probably too early for the adaptive immune response, if immune genes were functional in fracture repair, their expression would be observed by 11 days post-fracture. In agreement with previous studies [20], the innate immunity complement genes were up-regulated; however, the complement cascade ceased expression at C3a, the initial immune effector complement component, probably through expression of C3 inhibitors (Table 5). As with growth factor expression, the repertoire of interleukins and CD antigens were similar to other studies with some variations in individual members. The inflammatory mediators interleukin IL-1 [16] and IL-6 [6] and their related components, also observed in other studies, were up-regulated in early fracture healing. Other interleukins and CD antigens were up-regulated later in healing and could be assigned non-inflammatory and non-immune functions. We conclude that marrow gene expression could affect interpretations of microarray analysis in fracture repair.

Bone is the only adult tissue that is capable of healing without scar formation [10], and an examination of fracture gene expression from previous studies (Table 3) in combination with a more detailed analysis of our Gene Ontology list (Table 4) identified growth factors and developmental genes previously associated with scarless fetal skin repair that might also regulate bone regeneration during fracture healing (Table 6). The genes previously associated with scarless fetal skin wound

Table 6
Fracture microarray genes associated with scarless fetal wound healing

Gene (function) [Reference]	Accession	3-day expression		11-day expression	
		Microarray ($P < 0.05$)	Real-time PCR	Microarray ($P < 0.05$)	Real-time PCR
		Fold-change	Fold-change[a] (n)	Fold-change	Fold-change[a] (n)
Homeodomain					
Prx-2 (TGF-β3, PN-1 regulation) [10,43]	BE118447	4.4	2.2 ± 1.9 (7)	2.7	2.6 ± 1.6 (6)
Meox-2 (cell migration) [48]	NM_017149	2.5	1.7 ± 0.6 (9)	2.8	2.5 ± 1.8 (5)
TGF-β3-related					
TGF-β3 (proliferation, differentiation) [13]	NM_013174	2.4	1.7 ± 0.9 (7)	2	4.3 ± 2.0 (8)
LTBP-1 (TGF-β3 binding) [39]	NM_021587	1.9	ND	1.5	ND
Fibromodulin [23,44]	NM_080698	2.3	2.1 ± 1.2 (8)	5.2	21.0 ± 13.2 (6)
Other growth factors					
VEGF-C (angiogenesis) [10]	NM_053653	1.2	ND	NS	ND
Hepatocyte Growth Factor (anti-apoptosis) [37]	NM_017017	NS	ND	1.4	ND
Extracellular matrix (ECM) [10]					
Fibronectin-1	NM_019143	2.4	ND	2.7	ND
Collagen V (α1) (cell spreading)	NM_134452	2.4	ND	2.1	ND
ECM matricellular (adhesion) [34]					
Tenascin [13,25,29]	BE126741	2.1	ND	1.5	ND
Calpactin I Heavy Chain (Ten receptor)	NM_019905	1.9	ND	NS	ND
Thrombospondin-2	BF408413	1.7	ND	1.8	ND
Thrombospondin-4	X89963.1	1.9	ND	3.6	ND
Calreticulin (TSP-receptor)	NM_022399	1.6	ND	NS	ND
SPARC	NM_012656	1.7	ND	NS	ND
ECM remodeling					
Protease Nexin-1 (ECM regulation) [41]	X89963.1	2.1	1.1 ± 0.5 (8)	2.1	12.3 ± 6.4 (8)
Mmp-14 [14]	NM_031056	NS	1.1 ± 0.5 (10)	2.1	4.2 ± 1.8 (9)
TIMP-2 (Mmp-14 regulation) [3]	NM_021989	2.3	ND	1.8	ND

NS—not significant; ND—not determined; n—number of fractured vs. unfractured pairs of tissues in real-time RT-PCR.
[a] mean ± SD.

healing are diverse in function, but those identified to date influence the turnover or adhesion of cells and extracellular matrix components [10]. The genes associated with scarless wound healing and not observed in previous studies might have displayed less dramatic changes in expression that we were able to detect with the statistical approach afforded by the multiple replicates in our study.

Due to their extensively documented regulation during tissue development, the homeodomain transcription factors are obvious candidates for the regulation of bone regeneration. The paired-related homeodomain transcription factor Prx-2 has been previously associated with scarless wound healing [47] and displayed significant increases in expression in fracture healing. Tenascin-C [25] and the plasmin inhibitor protease nexin (PN)-1 [43] have been identified as possible target genes of Prx-2 expression; they also displayed significant increases in expression in fracture healing. Prx-2-mediated changes in the extracellular matrix components through tenascin and PN-1 expression could bind and alter TGF-β3 availability. Consequently, our findings support the involvement of Prx-2 in fracture healing, suggesting a similarity of molecular pathways in both fracture healing and scarless healing in fetal tissues. The expression of the mesenchymal homeodomain transcription factor Meox-2 was also up-regulated during fracture healing. Its role is not well defined, but it has been shown to affect cell migration during developmental somitogenesis [30,48]. Meox-2 expression might therefore regulate bone regeneration by balancing cell adhesion and migration.

Growth factor genes involved in skeletal development and previously associated with scarless fetal wound healing were identified. TGF-β3 gene expression was especially notable in this respect [27,45], as it was increased 2-fold throughout fracture healing. The TGFs are pleiotropic growth factors that could exert varied effects on inflammation, proliferation, differentiation and apoptosis in the healing fracture [13]. The TGFs could also be differentially regulated post-transcriptionally by specific extracellular matrix components, such as latent TGF binding protein (LTBP)-1 [39] and fibromodulin [44]. Other growth factor genes expressed in scarless fetal wound healing that were also up-regulated in fracture healing included the angiogenic VEGF-C gene [10] and the apoptosis inhibitor hepatocyte growth factor [37].

The up-regulation of tenascin expression immediately suggested that additional matricellular genes could modulate cell–matrix adhesion and de-adhesion [34]. The expression of the matricellular genes and their respective receptors was up-

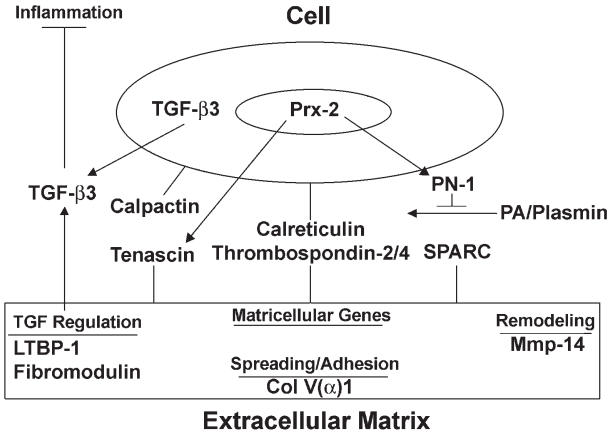

Fig. 1. A model for bone regeneration regulation by scarless fetal wound healing genes. TGF-β3 exerts pleiotropic effects, including inhibition of inflammation. Cell motility and cell–matrix adhesion are mediated by the matricellular genes and their receptors, whose digestion by plasmin is inhibited by PN-1.

regulated throughout fracture healing (Table 6). Although only thrombospondin-1 has been associated with fetal wound healing [42], these genes are critical in regulating the cell–matrix interactions implicated in scarless wound healing by other genes expressed in fetal skin repair. The extracellular matrix composition might also affect expression of the remodeling genes, such as matrix metalloproteinase (mmp)-14, observed in both scarless wound healing [14] and during endochondral bone formation in this study.

These results suggest that a similar set of genes that regulate normal tissue healing and scarless wound healing are differentially expressed during the inflammatory and endochondral stages of fracture repair. These include genes that modulate cell–matrix interactions and extracellular matrix organization at different times during healing, such as the matricellular genes. Other genes regulate these genes transcriptionally or post-transcriptionally; examples are Prx-2 and PN-1 inhibition of plasmin [41] and the pleiotropic effects TGF-β3, which might include the inhibition of inflammation (Fig. 1). The expression of these common genes implies that the molecular pathways of fracture repair mediate bone regeneration by mechanisms similar to scarless fetal wound healing.

We examined gene expression during the normal repair of a simple femur fracture with the elimination of scar tissue from the healing bone. This model does not address impaired healing, such as fracture non-unions, in which fibrous tissue is retained within the fracture gap without healing of the injured bone. Fracture non-unions can result from several causes beyond the scope of this study, including severe trauma to the bone, often with extensive comminution or a large interfragmentary gap, interruption of the periosteal blood supply and nerves, as well as infection associated with trauma (reviewed in [40]). Nevertheless, even in the absence of very severe trauma, a fracture non-union that results simply from a large interfragmentary gap or excessive motion of the fracture tissues implicates the extracellular matrix as an important mediator of tissue repair.

In conclusion, we identified 6555 genes with significant changes in expression in fracture tissues at 3 days and 11 days healing using the Agilent rat 20,000 gene chip. Our approach took advantage of multiple replicates of fracture tissues paired with unfractured tissues with the K-wire introduced into the bone to examine gene expression at these critical times of fracture healing. The induction and resolution of the inflammatory phase of early fracture healing are important for the transition from inflammation to repair; it immediately affects the deposition and resolution of the extracellular matrix and ultimately affects osteogenesis in bone and scar production in injured tissues. A profile of inflammatory gene expression during the early stages of fracture repair identified fewer inflammatory mediators of fracture healing than in previous microarray studies. The intramedullary K-wire also causes intramedullary damage to the bone that might increase osteogenic activity outside of the fracture callus and affect femoral gene expression during endochondral bone repair. Several of the genes identified during early and later fracture healing have been associated with regulation of the extracellular matrix during scarless healing of fetal skin wounds. A comparison of gene expression in fracture repair by microarray analysis with fetal scarless wound healing would present an ideal opportunity to ascertain additional genes that regulate bone regeneration. The expression of genes that regulate the regenerative qualities of bone repair can be used to elucidate therapies for improved wound healing of both skeletal and non-skeletal tissues.

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