Microarray Analysis of Gene Expression Reveals that Cyclo-oxygenase-2 Gene Therapy Up-regulates Hematopoiesis and Down-regulates Inflammation During Endochondral Bone Fracture Healing

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INTRODUCTION

Bone fracture repair involves the coordinated expression of growth factors and signaling molecules that regulate the ordered development of the fracture callus. Following the initial inflammatory response, fracture callus soft tissues proliferate...
and ossify to bridge the injury with woven bone that is
eventually remodeled to cortical bone, a sequence of healing
stages that facilitates the evaluation of bone repair and the
efficacy of therapeutic approaches.[1] Therapies that im-
prove bone healing impaired by age or disease are highly
desirable, and experimental efforts have attempted to pro-
mote different aspects of bone repair to improve the heal-
ing process. Therapeutic studies have generally identified
novel approaches to enhance bone formation during tis-
sue development and repair and applied them to the frac-
ture tissues at a critical point in healing when they might
be beneficial to repair. Several gene therapy and protein
therapy approaches have successfully augmented growth
factor and signaling molecule expression in bone fractures
to improve impaired healing.[2] The application of therapy
to enhance an early phase of tissue repair would seem high-
ly beneficial to impaired bone healing.

The inducible inflammatory mediator cyclo-oxygenase-2
(Cox-2) has a critical role regulating tissue homeostasis.
Cox-2 expression is up-regulated well into the endochon-
dral stage of fracture repair.[3,4] The expression of Cox-2
has been demonstrated to be necessary for bone repair,
clinically through impaired bone fracture repair in rheuma-
toid arthritis patients under treatment with the non-steroi-
dal anti-inflammatory inhibitor drugs, and experimentally,
in mice deficient in Cox-2 expression that exhibit impaired
healing of endochondral bone fractures.[5-7]

Cox-2 functions through the production of prostaglandins
(PGs), especially PG E _2_ (PGE _2_), which modulates Cox-2 ef-

tects through four PGE _2_ receptors (PTGER). The PTGERs are
expressed on a wide variety of cells and act through cyclic
adenosine monophosphate (cAMP) and inositol triphos-
phate (IP3) intracellular signaling pathways to differentially
regulate the cell response to PGE _2_ and to regulate tissue
development and repair.[8] PTGER effects on bone repair
are diverse; PTGER1 expression inhibits bone repair,[9] but
agonists for PTGER2 and PTGER4 stimulate bone forma-
tion, and promote endochondral bone repair.[10,11] These
observations indicate that Cox-2 production of PGE _2_ initiates
a complex regulation of different aspects of bone repair.

We have previously demonstrated that Cox-2 gene ther-

apy could indeed improve endochondral bone fracture re-

pair.[12] However, the therapeutic effect was unexpectedly
not seen until the endochondral stage of bone fracture re-

pair, during fracture chondrogenesis, when the callus car-

tilage is being remodeled to bone and well after the infla-
mmary stage has subsided, during which time Cox-2 ex-
pression has been observed to initiate fracture healing.[13]
We speculate that while Cox-2 functions might also medi-
ate some aspects of post-inflammatory bone healing dur-
ing endochondral bone repair. Because Cox-2 also medi-
ates angiogenesis and tissue remodeling during certain
pathogenic conditions, such as metastasis,[14,15] and be-
cause angiogenesis coincides with the ossification of frac-
ture cartilage,[16] we hypothesized that Cox-2 gene ex-
pression might also regulate angiogenesis and extracellu-
lar matrix remodeling of the fracture callus cartilage to pro-
mote bony union of the fracture during endochondral bone
repair.

To elucidate the molecular pathways regulating Cox-2
expression at this stage of fracture healing, i.e., at 10 days
post-fracture.

METHODS

Closed femoral fractures were produced in 12-week-old
CS7BL/6 male mice by the three-point bending technique.
[17] The bone fracture model was examined because Cox-
expression is normally limited to injured tissues. Male mice
were used because Cox-2 deficiency has been observed to
affect males more than females, suggesting that Cox-2 gene
therapy might have a greater effect on fracture repair in
males.[18]

An in vivo gene transfer approach that expressed a mod-
ified hCox-2 transgene in fracture tissues had previously
been highly effective in promoting bony union of fracture
gaps in a rodent femur fracture model.[12,19] The use of
hCox-2 gene is to allow us to distinguish transgene expres-
sion from endogenous Cox-2 gene expression by real-time
reverse transcription-polymerase chain reaction (RT-PCR)
with species-specific Cox-2 primers. The hCox-2 gene
was modified by removing most of the 3 untranslated
region (3’UTR) sequence and replacing the endogenous Kozak se-
quence with an enhanced Kozak sequence to increase the
stability and translation efficiency of the mRNA.[12] The
current study has confirmed significant expression of the hCox-2 transgene in the Cox-2-treated fracture tissues used in this study by microarray and real-time RT-PCR (data not shown).

On the day after fracture, a murine leukemia virus (MLV)-hCox-2 expressing vector was delivered to the lateral and medial aspects of the periosteum at the fracture site.[12] Three fracture samples injected with the hCox-2 transgene were compared with three fracture samples injected with the β-galactosidase control transgene. Fracture tissues were harvested at 10 days post-fracture, a time chosen for microarray analysis because Cox-2 gene expression is elevated at this time,[4,20] but most importantly because it is just prior to Cox-2 promotion of endochondral bony union[12] when we should expect the genes mediating this function to be expressed in response to Cox-2. To evaluate the complete repertoire of genes that might coordinate bony union among the various fracture tissues, the entire fracture callus was harvested, separated from the femoral epiphyses, pulverized in Trizol (Life Technologies, Grand Island, NY, USA) and the total RNA isolated. The quality of RNA was confirmed by Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA, USA). All animal procedures were approved by the local Institutional Animal Care and Use Committee.

To confirm gene expression of the hCox-2 transgene in the fracture tissues, the RNA from these fracture samples that underwent microarray analysis was reversed transcribed to cDNA, and hCox-2 transgene expression was confirmed by real-time RT-PCR of the fracture callus RNA with the SYBR green master mix (Applied Biosystems, Foster City, CA, USA) in an Opticon Chromo4™ system (Bio-Rad, Hercules, CA, USA) with Opticon Monitor 3.1 software (Bio-Rad), using hCox-2 gene-specific primers (Integrated DNA Technologies, Coralville, IA, USA) (Table 1). The real-time RT-PCR approach was also used to determine the expression of the four PTGER genes in response to hCox-2 transgene expression relative to the expression of the housekeeping gene, peptidylprolyl isomerase A (PPIA, cyclophilin A). Statistical analysis was performed by t-test.

The Affymetrix Mouse Gene 1.0 ST array was used for hybridization (Affymetrix, Santa Clara, CA, USA). This array represents 28,853 genes, with each target gene queried by a median number of 27 individual 25-mer oligonucleotide probe sequences. Image analysis and signal normalization was performed at the University of California at Irvine using the “Probe Logarithmic Error Intensity Estimate” (PLIER) algorithm, a normalization that improves the signal for genes with smaller changes in expression.

The gene expression and literature search software “Pathway Studio” (version 9, Elsevier, Philadelphia, PA, USA) were used for the analysis of gene regulation in response to Cox-2 transgene expression. Genes with significant changes in expression were furthered analyzed by “gene set enrichment analysis” (GSEA)[21] and were classified into the “Gene Ontology” (GO) “Biological Function” categories (statistically significant at $P<0.05$ by Kolnogorov-Smirnov test, 400 gene permutations). For this analysis, the entire repertoire of genes that displayed significant differences in expression in the initial PLIER normalization was examined. GSEA provided the enrichment score (ES) by classifying the genes with significant changes in expression into the GO Biological Function category relative to the total number of genes

| Gene     | Accession | Location | Sequence                          | Product (bp) |
|----------|-----------|----------|-----------------------------------|--------------|
| hCox-2   | M90100    | 1354-1373| 5'-GGTTGCTGGTGTAGGAATGT-3' 5'-CCAGTAGGCAGGAGAACATAT-3' | 336          |
| PTGER1   | NM_013641 | 84-105   | 5'-CCCCAGCCCAAGAGAGCAGAT-3' 5'-AGCAGGCTGGCAGAGAACC-3' | 459          |
| PTGER2   | NM_008964 | 1551-1570| 5'-ACGGCCCTGCGGATGTTCTG-3' 5'-ACGGGAAGCTCGGAGAGGCCC-3' | 211          |
| PTGER3   | NM_011196 | 844-863  | 5'-AAAGCCCGGCTCTCGACGTC-3' 5'-TGATGCTGCTTGGGCCCTG-3' | 536          |
| PTGER4   | NM_00113607 | 782-801 | 5'-CACCTGGTGGGAGCCAGGACG-3' 5'-GGCCGGAGACATGCCGAGG-3' | 478          |
| PPIA     | NM_008907 | 314-333  | 5'-GCATACAGGTTGTGGCCATCT-3' 5'-GGTCCCGACAGGAAGAAG-3' | 190          |

bp, base pairs; hCox-2, human cyclo-oxygenase-2; PTGER, prostaglandin E2 receptors; PPIA, peptidylprolyl isomerase A (housekeeping gene).
from that GO category on the chip. "Leading Edge Analysis" further identified the gene sets with the greatest representation in the repertoire of expressed genes. Those gene sets predicted to mediate the greatest regulation of Cox-2 transgene effects were then identified. Accordingly, the "leading edge" of the GSEA of the ES of these gene sets was identified as those ranked categories preceding maximum positive ES for positively enriched gene sets, and those sets following the minimum negative ES for negatively enriched gene sets. The positive and negative "leading edge" GO gene sets were then ranked by the normalized ES (NES) and presented with their median change in expression.

Further analysis examined gene expression among the individual genes with the greatest changes in magnitude of expression. This analysis of the microarray results was performed using Biometric Research Branch (BRB)-ArrayTools Version 4.1.0 (Biometric Research Branch, National Cancer Institute, Rockville, MD, USA). An arbitrary threshold of a 1.5-fold positive or negative change in expression was used to identify the affected genes. A gene was excluded when less than 20% of its expression data displayed the minimum 1.5-fold change in either direction from its median expression value. We identified genes that were differentially expressed between the two classes using a random-variance t-test. Genes were considered statistically significant if the P-value of the log-ratio variation was less than 0.01 (P<0.01), but were excluded if the minimum value of the spot intensity was less than a threshold of 10.

To confirm the microarray gene expression profile results, the expression of several key inflammatory and hematopoietic genes (Table 2) was also determined by real-time RT-PCR, using the same RNA samples that were used in the microarray analysis. The fold-increase in expression was determined by the 2^ΔΔCT method. The expression of these inflammatory, hematopoietic, and remodeling genes determined by real-time RT-PCR was then correlated with that determined by the microarray.

RESULTS

The expression of the hCox-2 transgene in these fracture callus tissues was verified by real-time RT-PCR with hCox-2-specific primers to be at least 7 cycles, or more than 128-fold, above background levels, confirming that the in vivo gene transfer approach did indeed augment Cox-2 gene expression in the fracture tissues.[12,19] We previously established that Cox-2 gene therapy augmented PGE2 expression in the rodent fracture model,[12] confirming that the hCox-2 product was functional. That study also established that hCox-2 transgene expression did not alter the expression of the endogenous murine Cox-2 gene (data not shown), and suggested that there was no feedback regulation of the endogenous murine Cox-2 gene in response to the hCox-2 gene therapy.[12]

An initial examination identified individual genes that displayed significant changes in expression. Of the 28,853 genes represented on the chip, 1,031 individual genes displayed significant differences (P<0.01) in expression between the Cox-2 and β-galactosidase transgene-injected fractures. Of these genes, 433 were up-regulated and 598 were down-regulated, indicating that Cox-2 regulated many genes, probably functioning in several different pathways.

Only one of the PTGER exhibited significant changes in expression in response to Cox-2 transgene expression. PTGER3 was up-regulated 1.4-fold by microarray analysis, and confirmed by real-time RT-PCR to be up-regulated 2.6-fold (P<0.04) at 10 days post-fracture (Fig. 1). None of the other PTGER exhibited significant changes in gene expression.

### Table 2. Comparison of relative fold-changes in gene expression of selected genes determined by microarray and by real-time reverse transcription-polymerase chain reaction

| GO category          | Gene       | MicroarrayΔ| Real-time RT-PCR (±SD)Δ |
|----------------------|------------|------------|--------------------------|
| Inflammation         | CCL9       | -3.7       | -6.2±1.4                 |
|                      | CCL10      | -3.2       | -9.9±2.6                 |
|                      | CCL8       | -2.0       | -2.9±1.1                 |
|                      | CCL7       | -1.6       | -2.6±1.0                 |
|                      | PF-4       | 1.5        | 3.6±0.9                  |
|                      | IL-7α      | 1.6        | 1.2±1.0                  |
|                      | CXCL5      | 1.7        | 4.4±1.7                  |
| Hematopoiesis        | Ikzf3      | 1.8        | 3.3±1.7                  |
|                      | IRF4       | 1.8        | 2.2±1.0                  |
|                      | KLF1       | 2.2        | 2.7±1.7                  |
| Tissue Remodeling    | Cathepsin G| 1.6        | 3.5±1.8                  |
|                      | Cathepsin E| 2.1        | 2.1±0.6                  |

ΔFold-change in gene expression at 10 days post-fracture, cyclo-oxygenase-2 versus control transgene.

ΔDown-regulated gene expression in Table 4 was converted to negative fold-change for comparison with real-time RT-PCR.
Fig. 1. Real-time reverse transcription-polymerase chain reaction determination of prostaglandin E receptor (PTGER) gene expression in response to cyclo-oxygenase-2 (Cox-2) transgene at 10 days post-fracture. The three samples in each group size were from the same individuals that underwent microarray analysis. Statistics were performed by t-Test. PTGER, prostaglandin E2 receptors.

at 10 days post-fracture between the Cox-2 and control gene samples, either by microarray or by real-time RT-PCR analysis. Real-time RT-PCR confirmed that PTGER3 expression was up-regulated 8-fold ($P<0.04$) in response to Cox-2 transgene expression at 5 days post-fracture, indicating that its expression might have also been induced during inflammation in bone repair but persisted to at least 10 days post-fracture, at which time when the benefits of Cox-2 gene therapy on bony bridging were seen in fracture repair.[12]

GSEA (Table 3)

A "leading edge" analysis of the GSEA classified genes with different magnitudes of changes in expression into various functional "GO" categories, providing a more comprehensive method for identifying molecular pathways associated with transgene expression. By this approach, the gene sets containing regulators of cell proliferation were very highly represented. These gene sets included several categories of genes that have functions in the regulation of mitosis and cell proliferation.

Among the genes with significant changes in expression, the growth factors traditionally assigned angiogenic functions that would be expected to mediate this stage of bone fracture repair were notably absent. Specifically, the members of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) gene families were absent.

Several inflammatory genes, notably the inflammatory chemokines, were reduced in the "leading edge" analysis, suggesting that inflammation was negatively regulated. As a group, the genes with inflammatory functions were some of the most frequently represented in the entire microarray analysis in terms of numbers of genes, but also some of the most down-regulated in terms of expression.

In contrast, the hematopoietic and erythropoietic genes were the most abundant gene sets in the GSEA, with several GO categories represented. This observation indicates that a significant consequence of Cox-2 transgene expression at this stage of fracture repair involved blood cell proliferation and development. Many of these gene sets also displayed the greatest increases in expression among all gene sets, while several others displayed smaller but still significant changes in expression.

Individual Gene Analysis (Table 4)

Further analysis examined the individual genes with the greatest changes in expression. Among the genes that exhibited at least 1.5-fold changes in expression in the fracture tissues in response to Cox-2 transgene expression, 172 genes were up-regulated and 99 genes were down-regulated more than 1.5-fold ($P<0.01$).

The inflammatory genes were the most dramatically down-regulated in the individual gene analysis (Table 4). The 1.5-fold down-regulated genes included several chemokines, such as the monocyte chemoattractants chemokine ligand (CCL)-7, CCL-8, CXC chemokine ligand (CXCL)-9 and CXCL-10. The sole chemokine receptor up-regulated 1.5-fold was CXC chemokine receptor (CXCR)-5. In addition, platelet factor-4 (PF-4) and interleukin-7 alpha (IL-7α) receptor (IL-7αr) were among the few inflammatory regulators with expression up-regulated 1.5-fold. Thus, the inflammatory genes were not only well-represented in the microarray analysis in terms of numbers of individual genes, but also displayed some of the greatest decreases in expression in response to hCox-2 fracture therapy.

As in the GSEA, the hematopoietic and erythropoietic genes were also well-represented among the genes that were up-regulated more than 1.5-fold (Table 4). Many of the most up-regulated gene products are expressed during blood cell development. It also included a repertoire of transcription factors related to these pathways. For example, Kruppel-like factor 1 (KLF1, 2.2-fold) and IKAROS family zinc finger 3 (Ikzf3, 1.8-fold) were significantly up-regulated in this analysis, and these transcription factors regu-
| Positively enriched gene sets                                      | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|------------------------------------------------------------------|----------------|------------------------|---------------|--------------|---------|
| Mitotic prometaphase                                             | 90             | 80                     | 3.3           | 1.2          | 0.00    |
| Mitotic cell cycle                                               | 316            | 287                    | 3.2           | 1.1          | 0.00    |
| M phase of mitotic cell cycle                                    | 96             | 91                     | 3.2           | 1.2          | 0.00    |
| Cell division                                                    | 336            | 277                    | 2.9           | 1.1          | 0.00    |
| Mitosis                                                          | 252            | 212                    | 2.9           | 1.1          | 0.00    |
| Cell cycle                                                       | 604            | 518                    | 2.7           | 1.1          | 0.00    |
| Chromosome segregation                                           | 75             | 55                     | 2.7           | 1.1          | 0.00    |
| G1-S transition of mitotic cell cycle                            | 154            | 138                    | 2.6           | 1.1          | 0.00    |
| Cell cycle checkpoint                                            | 141            | 122                    | 2.6           | 1.1          | 0.00    |
| S phase of mitotic cell cycle                                    | 120            | 106                    | 2.6           | 1.1          | 0.00    |
| Regulation of transcription involved in G1-S phase of mitotic cell cycle | 23             | 19                     | 2.5           | 1.2          | 0.00    |
| Porphyrin biosynthetic process                                   | 19             | 12                     | 2.5           | 1.6          | 0.00    |
| G2-M transition of mitotic cell cycle                            | 116            | 101                    | 2.4           | 1.1          | 0.00    |
| M-G1 transition of mitotic cell cycle                            | 79             | 72                     | 2.4           | 1.1          | 0.00    |
| B cell differentiation                                           | 54             | 40                     | 2.3           | 1.1          | 0.00    |
| B cell receptor signaling pathway                                 | 27             | 22                     | 2.3           | 1.1          | 0.00    |
| Erythrocyte development                                          | 14             | 13                     | 2.3           | 1.2          | 0.00    |
| Regulation of small GTPase mediated signal transduction          | 200            | 151                    | 2.3           | 1.1          | 0.00    |
| Erythrocyte differentiation                                      | 39             | 29                     | 2.2           | 1.1          | 0.00    |
| Mitotic cell cycle spindle assembly checkpoint                    | 34             | 29                     | 2.2           | 1.1          | 0.00    |
| Regulation of ARF GTPase activity                                | 39             | 22                     | 2.2           | 1.2          | 0.00    |
| DNA replication checkpoint                                       | 12             | 8                      | 2.2           | 1.3          | 0.00    |
| Cytokinesis                                                      | 60             | 48                     | 2.2           | 1.1          | 0.00    |
| Positive regulation of GTPase activity                           | 188            | 179                    | 2.1           | 1.1          | 0.00    |
| Interspecies interaction between organisms                        | 325            | 292                    | 2.1           | 1.1          | 0.00    |
| Platelet activation                                              | 242            | 226                    | 2.1           | 1.1          | 0.00    |
| Germ cell migration                                              | 10             | 10                     | 2.1           | 1.2          | 0.00    |
| Phosphatidylinositol-mediated signaling                          | 77             | 67                     | 2.1           | 1.1          | 0.00    |
| T cell differentiation                                           | 42             | 30                     | 2.0           | 1.1          | 0.00    |
| Negative regulation of macrophage derived foam cell differentiation| 14             | 11                     | 2.0           | 1.2          | 0.00    |
| Mitotic cell cycle checkpoint                                    | 17             | 13                     | 2.0           | 1.2          | 0.00    |
| Mitotic spindle organization                                     | 21             | 18                     | 2.0           | 1.1          | 0.00    |
| Ras protein signal transduction                                  | 81             | 70                     | 2.0           | 1.1          | 0.00    |
| Estrogen receptor signaling pathway                              | 19             | 14                     | 2.0           | 1.1          | 0.00    |
| Regulation of cell cycle                                        | 99             | 84                     | 2.0           | 1.1          | 0.00    |
| Mitotic metaphase                                                | 7              | 7                      | 2.0           | 1.2          | 0.00    |
| One-carbon metabolic process                                     | 38             | 29                     | 2.0           | 1.0          | 0.00    |
| Regulation of peptidyl-tyrosine phosphorylation                  | 16             | 11                     | 2.0           | 1.1          | 0.00    |
| Barbed-end actin filament capping                                | 8              | 8                      | 2.0           | 1.2          | 0.00    |
| Positive regulation of Rab GTPase activity                       | 40             | 39                     | 1.9           | 1.1          | 0.00    |
| Positive regulation of endothelial cell migration                | 22             | 18                     | 1.9           | 1.1          | 0.00    |
| Oocyte maturation                                                | 16             | 15                     | 1.9           | 1.1          | 0.00    |
| Toll-like receptor 2 signaling pathway                           | 74             | 65                     | 1.9           | 1.1          | 0.00    |
| Positive regulation of mitotic cell cycle                        | 32             | 24                     | 1.9           | 1.0          | 0.00    |

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| Positively enriched gene sets                                      | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|------------------------------------------------------------------|----------------|------------------------|---------------|---------------|---------|
| Lymphocyte differentiation                                       | 13             | 7                      | 1.9           | 1.2           | 0.00    |
| Purine ribonucleoside monophosphate biosynthetic process         | 21             | 15                     | 1.9           | 1.1           | 0.00    |
| Positive regulation of protein serine-threonine kinase activity  | 16             | 11                     | 1.9           | 1.1           | 0.00    |
| Cell aging                                                      | 41             | 32                     | 1.9           | 1.1           | 0.00    |
| Toll-like receptor 1 signaling pathway                           | 71             | 65                     | 1.9           | 1.1           | 0.00    |
| Nerve growth factor receptor signaling pathway                   | 224            | 201                    | 1.9           | 1.1           | 0.00    |
| Mitotic metaphase-anaphase transition                            | 16             | 13                     | 1.9           | 1.1           | 0.00    |
| MyD88-dependent toll-like receptor signaling pathway             | 80             | 70                     | 1.9           | 1.1           | 0.00    |
| Actin filament capping                                           | 23             | 18                     | 1.9           | 1.0           | 0.00    |
| Attachment of spindle microtubules to kinetochore                | 9              | 6                      | 1.8           | 1.3           | 0.00    |
| Microtubule nucleation                                           | 18             | 11                     | 1.8           | 1.1           | 0.00    |
| G2-M transition DNA damage checkpoint                            | 19             | 17                     | 1.8           | 1.1           | 0.00    |
| Toll-like receptor 4 signaling pathway                           | 83             | 72                     | 1.8           | 1.1           | 0.00    |
| Stress-activated MAPK cascade                                     | 54             | 47                     | 1.8           | 1.1           | 0.00    |
| ATP biosynthetic process                                         | 74             | 56                     | 1.8           | 1.0           | 0.00    |
| Toll-like receptor signaling pathway                             | 92             | 72                     | 1.8           | 1.1           | 0.00    |
| Small GTPase mediated signal transduction                        | 375            | 290                    | 1.7           | 1.1           | 0.00    |
| MyD88-independent toll-like receptor signaling pathway           | 69             | 63                     | 1.7           | 1.1           | 0.00    |
| Pyrimidine nucleotide biosynthetic process                       | 13             | 8                      | 1.7           | 1.1           | 0.00    |
| Intracellular signal transduction                                 | 388            | 309                    | 1.7           | 1.1           | 0.00    |
| Toll-like receptor 3 signaling pathway                           | 66             | 59                     | 1.7           | 1.1           | 0.00    |
| Alpha-beta T cell differentiation                                 | 16             | 7                      | 1.7           | 1.0           | 0.00    |
| Induction of apoptosis by extracellular signals                  | 118            | 94                     | 1.7           | 1.1           | 0.00    |
| Microtubule-based movement                                       | 121            | 82                     | 1.6           | 1.0           | 0.00    |
| Cell morphogenesis                                               | 64             | 54                     | 1.6           | 1.0           | 0.00    |
| Toll signaling pathway                                           | 80             | 72                     | 1.6           | 1.1           | 0.00    |
| Regulation of cell shape                                         | 76             | 67                     | 1.6           | 1.1           | 0.00    |
| Metabolic process                                                | 2,421          | 2,156                  | 1.6           | 1.0           | 0.00    |
| Integrin-mediated signaling pathway                              | 103            | 93                     | 1.6           | 1.0           | 0.00    |
| Platelet degranulation                                           | 80             | 74                     | 1.6           | 1.0           | 0.00    |
| Cell proliferation                                               | 429            | 359                    | 1.6           | 1.0           | 0.00    |
| Intracellular protein kinase cascade                              | 125            | 105                    | 1.5           | 1.1           | 0.00    |
| Cell surface receptor linked signaling pathway                    | 259            | 218                    | 1.4           | -1.0          | 0.00    |
| Anti-apoptosis                                                   | 243            | 207                    | 1.4           | 1.0           | 0.00    |
| Viral reproduction                                                | 362            | 267                    | 1.4           | 1.0           | 0.00    |
| Transmembrane transport                                          | 831            | 695                    | 1.3           | -1.0          | 0.00    |
| Regulation of transcription, DNA-dependent                       | 2,872          | 1,888                  | 1.1           | 1.0           | 0.00    |

| Negatively enriched gene sets                                     | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|------------------------------------------------------------------|----------------|------------------------|---------------|---------------|---------|
| Proteolysis                                                      | 657            | 470                    | -1.3          | -1.0          | 0.03    |
| Detection of chemical stimulus involved in sensory perception of smell | 1,100          | 773                    | -2.3          | -1.1          | 0.00    |
| Spermatogenesis                                                  | 423            | 336                    | -1.3          | -1.0          | 0.03    |
| Multicellular organismal development                             | 1,146          | 985                    | -1.3          | -1.0          | 0.00    |
| Ion transport                                                    | 693            | 595                    | -1.3          | -1.0          | 0.01    |
| Cell differentiation                                             | 735            | 585                    | -1.3          | -1.0          | 0.01    |

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Table 3. Continued

| Negatively enriched gene sets                                      | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|-------------------------------------------------------------------|----------------|------------------------|---------------|--------------|---------|
| Elevation of cytosolic calcium ion concentration                  | 132            | 115                    | -1.4          | -1.1         | 0.04    |
| Regulation of sequence-specific DNA binding transcription factor activity | 68             | 56                     | -1.4          | -1.0         | 0.05    |
| Sodium ion transport                                              | 154            | 123                    | -1.4          | -1.1         | 0.03    |
| Negative regulation of endopeptidase activity                     | 166            | 144                    | -1.4          | -1.1         | 0.03    |
| Anterior-posterior pattern formation                               | 130            | 114                    | -1.4          | -1.1         | 0.04    |
| Ion transmembrane transport                                       | 540            | 487                    | -1.4          | -1.1         | 0.00    |
| Negative regulation of peptidase activity                         | 114            | 99                     | -1.4          | -1.1         | 0.02    |
| Neuron migration                                                   | 95             | 81                     | -1.4          | -1.0         | 0.04    |
| Negative regulation of angiogenesis                               | 53             | 44                     | -1.4          | -1.0         | 0.05    |
| Phototransduction                                                  | 36             | 30                     | -1.4          | -1.1         | 0.05    |
| Hyperosmotic salinity response                                     | 11             | 9                      | -1.5          | -1.2         | 0.03    |
| Response to calcium ion                                            | 78             | 65                     | -1.5          | -1.0         | 0.03    |
| Neuron differentiation                                              | 136            | 116                    | -1.5          | -1.0         | 0.01    |
| Triglyceride mobilization                                          | 7              | 6                      | -1.5          | 1.0          | 0.04    |
| Skeletal muscle tissue development                                 | 68             | 55                     | -1.5          | -1.0         | 0.03    |
| Melanin biosynthetic process                                       | 13             | 11                     | -1.5          | -1.1         | 0.04    |
| Potassium ion transmembrane transport                              | 143            | 131                    | -1.5          | -1.1         | 0.01    |
| Long-term memory                                                   | 24             | 21                     | -1.5          | -1.1         | 0.04    |
| Axon extension involved in development                             | 6              | 5                      | -1.5          | -1.2         | 0.05    |
| Defense response                                                   | 204            | 122                    | -1.5          | -1.1         | 0.01    |
| Bone trabecula formation                                           | 8              | 7                      | -1.5          | -1.1         | 0.03    |
| Regulation of synaptic transmission                               | 54             | 25                     | -1.5          | -1.1         | 0.04    |
| Tissue regeneration                                                | 35             | 31                     | -1.5          | -1.1         | 0.04    |
| L-phenylalanine catabolic process                                  | 9              | 8                      | -1.5          | -1.1         | 0.05    |
| Locomotory behavior                                                | 91             | 77                     | -1.5          | -1.1         | 0.02    |
| Pituitary gland development                                        | 31             | 30                     | -1.5          | -1.1         | 0.03    |
| Positive regulation of type 2 immune response                      | 6              | 6                      | -1.5          | -1.2         | 0.04    |
| Circadian rhythm                                                   | 72             | 58                     | -1.5          | -1.1         | 0.03    |
| Bile acid metabolic process                                        | 38             | 35                     | -1.5          | -1.1         | 0.02    |
| Atrial cardiac muscle tissue morphogenesis                         | 6              | 6                      | -1.5          | -1.2         | 0.05    |
| Regulation of smooth muscle contraction                            | 20             | 14                     | -1.5          | -1.2         | 0.04    |
| Negative regulation of insulin secretion                           | 31             | 27                     | -1.5          | -1.0         | 0.03    |
| Adrenal gland development                                          | 28             | 26                     | -1.5          | -1.1         | 0.04    |
| Digestive system development                                       | 6              | 6                      | -1.5          | -1.2         | 0.04    |
| Gap junction assembly                                              | 7              | 7                      | -1.5          | -1.0         | 0.05    |
| Regulation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate selective glutamate receptor activity | 9              | 7                      | -1.5          | -1.2         | 0.04    |
| Striated muscle contraction                                        | 24             | 16                     | -1.5          | -1.1         | 0.05    |
| Positive regulation of neuroblast proliferation                    | 15             | 13                     | -1.5          | -1.2         | 0.04    |
| Positive regulation of epithelial cell differentiation              | 11             | 8                      | -1.5          | -1.0         | 0.04    |
| Response to pheromone                                              | 113            | 97                     | -1.7          | -1.1         | 0.00    |
| Positive regulation of glycogen biosynthetic process               | 12             | 12                     | -1.5          | -1.0         | 0.03    |
| Startle response                                                   | 13             | 13                     | -1.5          | -1.1         | 0.03    |
| Negative regulation of adenylate cyclase activity                  | 22             | 20                     | -1.5          | -1.1         | 0.02    |
| Binding of sperm to zona pellucida                                 | 25             | 20                     | -1.5          | -1.1         | 0.03    |

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Table 3. Continued

| Negatively enriched gene sets                                           | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|------------------------------------------------------------------------|----------------|------------------------|---------------|--------------|---------|
| Inhibition of adenylate cyclase activity by metabotropic glutamate receptor signaling pathway | 9              | 6                      | -1.5          | -1.2         | 0.03    |
| Fertilization                                                          | 36             | 30                     | -1.5          | -1.0         | 0.03    |
| Positive regulation of leukocyte chemotaxis                            | 17             | 8                      | -1.5          | -1.1         | 0.05    |
| Thyroid gland development                                              | 20             | 19                     | -1.5          | -1.1         | 0.05    |
| Forebrain dorsal-ventral pattern formation                             | 6              | 5                      | -1.5          | -1.3         | 0.03    |
| Chemotaxis                                                             | 153            | 113                    | -1.5          | -1.1         | 0.02    |
| Insulin secretion                                                      | 43             | 36                     | -1.5          | -1.1         | 0.03    |
| Positive regulation of tissue remodeling                              | 8              | 6                      | -1.6          | -1.2         | 0.01    |
| Mating behavior                                                        | 10             | 9                      | -1.6          | -1.1         | 0.03    |
| Thyroid hormone generation                                             | 10             | 10                     | -1.6          | -1.1         | 0.04    |
| Cellular response to gonadotropin stimulus                             | 11             | 9                      | -1.6          | -1.2         | 0.05    |
| Organic acid metabolic process                                         | 14             | 9                      | -1.6          | -1.1         | 0.04    |
| Behavioral response to ethanol                                         | 6              | 6                      | -1.6          | -1.1         | 0.05    |
| Phenol-containing compound metabolic process                           | 11             | 6                      | -1.6          | -1.2         | 0.04    |
| Retinoid metabolic process                                             | 19             | 14                     | -1.6          | -1.1         | 0.03    |
| Bile acid biosynthetic process                                         | 25             | 22                     | -1.6          | -1.1         | 0.03    |
| Myoblast differentiation                                               | 18             | 13                     | -1.6          | -1.1         | 0.04    |
| Calcium ion-dependent exocytosis                                       | 25             | 18                     | -1.6          | -1.1         | 0.03    |
| Positive regulation of immunoglobulin secretion                        | 7              | 7                      | -1.6          | -1.2         | 0.02    |
| Cardiac muscle cell differentiation                                    | 20             | 16                     | -1.6          | -1.0         | 0.05    |
| Positive regulation of circadian sleep-wake cycle, non-REM sleep       | 6              | 5                      | -1.6          | -1.2         | 0.02    |
| Calcium-independent cell-cell adhesion                                 | 27             | 24                     | -1.6          | -1.1         | 0.02    |
| Steroid metabolic process                                              | 120            | 91                     | -1.6          | -1.0         | 0.01    |
| Gamma-aminobutyric acid signaling pathway                              | 25             | 24                     | -1.6          | -1.1         | 0.03    |
| Forebrain anterior-posterior pattern formation                          | 6              | 5                      | -1.6          | -1.2         | 0.00    |
| Response to vitamin A                                                  | 27             | 20                     | -1.6          | -1.0         | 0.03    |
| Retrograde axon cargo transport                                        | 6              | 6                      | -1.6          | -1.0         | 0.03    |
| Sensory perception of sound                                            | 126            | 105                    | -1.6          | -1.1         | 0.00    |
| Peptide hormone processing                                             | 26             | 16                     | -1.6          | -1.1         | 0.02    |
| Biphenyl metabolic process                                             | 10             | 8                      | -1.6          | -1.1         | 0.02    |
| Glucocorticoid metabolic process                                       | 14             | 7                      | -1.6          | -1.2         | 0.03    |
| Maternal process involved in parturition                               | 7              | 5                      | -1.6          | -1.0         | 0.03    |
| Sensory perception of taste                                            | 106            | 51                     | -1.6          | -1.1         | 0.01    |
| Rhodopsin mediated phototransduction                                   | 6              | 6                      | -1.6          | -1.2         | 0.02    |
| Maintenance of gastrointestinal epithelium                             | 8              | 5                      | -1.6          | -1.2         | 0.02    |
| Maternal behavior                                                      | 8              | 6                      | -1.6          | -1.2         | 0.03    |
| Regulation of cytoskeleton organization                               | 13             | 7                      | -1.6          | 1.0          | 0.03    |
| Negative regulation of secretion                                       | 19             | 5                      | -1.6          | -1.2         | 0.03    |
| Axis specification                                                     | 22             | 15                     | -1.6          | -1.2         | 0.02    |
| Adult heart development                                                | 15             | 15                     | -1.6          | -1.2         | 0.02    |
| Intermediate filament cytoskeleton organization                        | 13             | 9                      | -1.6          | -1.1         | 0.02    |
| Phthalate metabolic process                                            | 7              | 7                      | -1.6          | -1.2         | 0.03    |
| Adenohypophysis development                                            | 11             | 11                     | -1.6          | -1.2         | 0.02    |
| Estrogen biosynthetic process                                          | 10             | 10                     | -1.6          | -1.1         | 0.03    |

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| Negatively enriched gene sets                                      | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|-------------------------------------------------------------------|----------------|------------------------|---------------|--------------|---------|
| Pattern specification process                                     | 115            | 93                     | -1.6          | -1.0         | 0.00    |
| Sensory perception of chemical stimulus                           | 33             | 27                     | -1.6          | -1.1         | 0.02    |
| Positive regulation of glycolysis                                 | 9              | 9                      | -1.6          | -1.2         | 0.01    |
| Central nervous system development                                | 137            | 126                    | -1.6          | -1.1         | 0.00    |
| Neuron fate commitment                                             | 36             | 29                     | -1.6          | -1.2         | 0.01    |
| Inner ear development                                             | 34             | 32                     | -1.6          | -1.0         | 0.00    |
| Response to bacterium                                              | 42             | 36                     | -1.6          | -1.0         | 0.01    |
| piRNA metabolic process                                           | 10             | 8                      | -1.6          | -1.2         | 0.01    |
| Sucking behavior                                                   | 13             | 11                     | -1.6          | -1.0         | 0.04    |
| Regulation of heart contraction                                   | 48             | 39                     | -1.6          | -1.1         | 0.02    |
| G-protein signaling, coupled to cyclic nucleotide second messenger  | 43             | 40                     | -1.6          | -1.1         | 0.01    |
| Positive regulation of receptor internalization                   | 9              | 8                      | -1.6          | -1.2         | 0.03    |
| Sarcomere organization                                            | 22             | 15                     | -1.6          | -1.2         | 0.02    |
| Glucocorticoid biosynthetic process                               | 13             | 9                      | -1.6          | -1.1         | 0.03    |
| Regulation of alternative nuclear mRNA splicing, via spliceosome   | 11             | 9                      | -1.6          | -1.2         | 0.04    |
| Behavioral response to nicotine                                   | 8              | 7                      | -1.6          | -1.2         | 0.02    |
| Intermediate filament organization                                | 15             | 11                     | -1.6          | -1.2         | 0.00    |
| Regulation of calcium ion-dependent exocytosis                    | 14             | 11                     | -1.6          | -1.2         | 0.03    |
| Sensory perception of light stimulus                              | 14             | 11                     | -1.6          | -1.2         | 0.01    |
| Cellular response to transforming growth factor beta stimulus      | 21             | 18                     | -1.7          | -1.1         | 0.03    |
| Neutrophil chemotaxis                                             | 37             | 30                     | -1.7          | -1.0         | 0.01    |
| Regulation of ion transmembrane transport                         | 177            | 165                    | -1.7          | -1.1         | 0.00    |
| Spinal cord association neuron differentiation                     | 10             | 9                      | -1.7          | -1.2         | 0.01    |
| Cerebral cortex GABAergic interneuron migration                    | 5              | 5                      | -1.7          | -1.2         | 0.02    |
| Positive regulation of tyrosine phosphorylation of Stat5 protein   | 18             | 15                     | -1.7          | -1.1         | 0.02    |
| Intermediate filament-based process                               | 10             | 8                      | -1.7          | -1.2         | 0.02    |
| Positive regulation of neuron differentiation                     | 60             | 56                     | -1.7          | -1.1         | 0.00    |
| Muscle organ development                                          | 115            | 102                    | -1.7          | -1.0         | 0.00    |
| Response to stilbenoid                                            | 14             | 13                     | -1.7          | -1.2         | 0.01    |
| Phototransduction, visible light                                  | 9              | 8                      | -1.7          | -1.2         | 0.02    |
| Excretion                                                         | 46             | 38                     | -1.7          | -1.1         | 0.00    |
| Plasma membrane repair                                            | 6              | 5                      | -1.7          | -1.3         | 0.00    |
| Behavior                                                          | 67             | 40                     | -1.7          | -1.1         | 0.01    |
| Anatomical structure morphogenesis                                 | 166            | 96                     | -1.7          | -1.1         | 0.00    |
| Sodium ion transmembrane transport                                | 29             | 28                     | -1.7          | -1.1         | 0.00    |
| Visual perception                                                 | 230            | 198                    | -1.7          | -1.1         | 0.00    |
| Bile acid and bile salt transport                                 | 24             | 22                     | -1.7          | -1.1         | 0.02    |
| Cartilage condensation                                            | 24             | 21                     | -1.7          | -1.0         | 0.00    |
| Dibenzo-p-dioxin metabolic process                                | 12             | 11                     | -1.7          | -1.2         | 0.01    |
| Forebrain neuron differentiation                                   | 11             | 10                     | -1.7          | -1.2         | 0.01    |
| Cytolysis                                                         | 31             | 22                     | -1.7          | -1.1         | 0.02    |
| Positive regulation of heart rate                                 | 12             | 9                      | -1.7          | -1.2         | 0.01    |
| Inner ear morphogenesis                                           | 68             | 65                     | -1.8          | -1.1         | 0.00    |
| Grooming behavior                                                 | 14             | 13                     | -1.8          | -1.2         | 0.02    |

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late erythropoiesis, and lymphocyte development, respectively.[22-24] Interferon regulatory factor-4 (IRF-4), which functions in the development of T-lymphocytes, was up-regulated 1.8-fold.

The proliferation regulating genes were not nearly as well represented among the 1.5-fold regulated genes as they were in the GSEA. There were selected genes implicated in cell cycle progression, such as cyclin F and yippee-like-4 (Ypel-4),[25] but it appears that the individual cell cycle regulators were not highly up-regulated in expression.

The genes regulating the extracellular matrix organization and cell adherence were less abundant than in the control fracture tissues. However, Cox-2 transgene expression increased the expression of osteoclast-associated tissue remodeling gene expression. The expression of cathepsin E, cathepsin G and carbonic anhydrases 1 and 2 was up-regulated more than 1.5-fold. On the other hand, only two of the matrix metalloproteinase (mmp) genes, mmp-3 and mmp-12, exhibited increases in expression, and this up-regulation was below the 1.5-fold threshold.

To confirm the microarray gene profiling results, we also performed real-time RT-PCR analysis of expression of selected genes of several GO categories using the same RNA samples that were used in the microarray analysis (Table 2). Fig. 2 shows a strong positive correlation between the relative expression levels of several pro-inflammatory cytokine genes (PF-4, IL-7α and the different chemokines), hematopoietic and erythropoietic genes (KLF1, Ikkf3, IRF-4), and extracellular proteases (cathepsin E and cathepsin G) determined by microarray and those determined real-time RT-PCR.

**Table 3. Continued**

| Negatively enriched gene sets                              | Total entities | # of measured entities | Normalized ES | Median change | P-value |
|------------------------------------------------------------|----------------|------------------------|---------------|--------------|---------|
| Neuron fate specification                                 | 11             | 8                      | -1.8          | -1.1         | 0.00    |
| Camera-type eye development                               | 65             | 51                     | -1.8          | -1.1         | 0.01    |
| Positive regulation of insulin-like growth factor receptor signaling pathway | 13             | 11                     | -1.8          | -1.2         | 0.01    |
| Detection of light stimulus involved in visual perception  | 8              | 7                      | -1.8          | -1.2         | 0.00    |
| Digestion                                                 | 69             | 43                     | -1.8          | -1.2         | 0.00    |
| Cytosolic calcium ion homeostasis                         | 20             | 14                     | -1.8          | -1.1         | 0.01    |
| Peripheral nervous system development                     | 43             | 34                     | -1.8          | -1.1         | 0.00    |
| Complement activation, classical pathway                   | 59             | 26                     | -1.8          | -1.1         | 0.00    |
| Positive regulation of T cell chemotaxis                  | 8              | 7                      | -1.8          | -1.0         | 0.00    |
| Feeding behavior                                           | 48             | 37                     | -1.8          | -1.1         | 0.00    |
| Synaptic transmission, cholinergic                         | 23             | 17                     | -1.8          | -1.2         | 0.00    |
| Positive regulation of phagocytosis                       | 37             | 27                     | -1.8          | -1.2         | 0.00    |
| Regulation of muscle contraction                           | 32             | 24                     | -1.8          | -1.1         | 0.01    |
| Hormone biosynthetic process                               | 72             | 61                     | -1.9          | -1.1         | 0.00    |
| Keratinocyte proliferation                                 | 17             | 14                     | -1.9          | -1.1         | 0.00    |
| Macrophage chemotaxis                                      | 16             | 11                     | -1.9          | -1.2         | 0.00    |
| Aromatic amino acid family metabolic process               | 18             | 9                      | -1.9          | -1.2         | 0.00    |
| Neurotransmitter secretion                                 | 73             | 61                     | -2.0          | -1.1         | 0.00    |
| Positive regulation of nitric oxide biosynthetic process   | 36             | 33                     | -2.0          | -1.0         | 0.00    |
| Regulation of membrane potential                           | 58             | 44                     | -2.0          | -1.2         | 0.00    |
| Chemokine-mediated signaling pathway                       | 7              | 5                      | -2.0          | -1.4         | 0.00    |
| Epidermis development                                      | 102            | 77                     | -2.0          | -1.1         | 0.00    |
| Lymphocyte chemotaxis                                      | 14             | 10                     | -2.1          | -1.3         | 0.00    |
| Cellular response to interferon-beta                       | 15             | 11                     | -2.2          | -1.8         | 0.00    |
| Muscle contraction                                         | 126            | 105                    | -2.2          | -1.1         | 0.00    |
| Muscle filament sliding                                    | 41             | 35                     | -2.3          | -1.2         | 0.00    |
| Cellular response to interferon-gamma                      | 25             | 23                     | -2.3          | -1.2         | 0.00    |

ES, enrichment score; GTPase, guanosine triphosphatase; ARF, ADP-ribosylation factor; MAPK, mitogen-activated protein kinase; ATP, adenosine triphosphate; REM, rapid eye movement; GABA, gamma-aminobutyric acid.
Table 4. 1.5-fold gene expression changes

| 1.5-Fold up-regulated gene symbol | Description | Genbank accession | Fold-change |
|-----------------------------------|-------------|------------------|-------------|
| Mela                              | Melanoma antigen | D10049           | 3.2         |
| Slc25a21                          | Solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21 | NM_172577     | 2.9         |
| Kel                               | Kell blood group, metallo-endopeptidase | NM_032540     | 2.7         |
| Epb4.2                            | Erythrocyte protein band 4.2 | NM_013513     | 2.6         |
| Rhag                              | Rhesus blood group-associated A glycoprotein | NM_011269     | 2.6         |
| Pdk1ip1                           | PDZK1 interacting protein 1 | NM_026018     | 2.5         |
| Htra4                             | Htra serine peptidase 4 | NM_001681187   | 2.5         |
| Slc38a5                           | Solute carrier family 38, member 5 | NM_172479     | 2.5         |
| Spna1                             | Spectrin alpha 1 | NM_011465     | 2.4         |
| Tspan33                           | Tetraspanin 33 | NM_146173     | 2.4         |
| Trim10                            | Tripartite motif-containing 10 | NM_011280     | 2.4         |
| Hemgn                             | Hemogen | NM_053149     | 2.4         |
| Ermap                             | Erythroblast membrane-associated protein | NM_013848     | 2.3         |
| Cldn13                            | Claudin 13 | NM_020504     | 2.3         |
| Vpreb3                            | Pre-B lymphocyte gene 3 | NM_009514     | 2.3         |
| Tspan8                            | Tetraspanin 8 | NM_146010     | 2.3         |
| Ankrd43                           | Ankyrin repeat domain 43 | NM_183173     | 2.3         |
| Pikr                              | Pyruvate kinase liver and red blood cell | NM_013631     | 2.3         |
| Rhd                               | Rh blood group, D antigen | NM_011270     | 2.3         |
| Spi8                             | Spi-B transcription factor (Spi-1/PU.1 related) | NM_019886     | 2.3         |
| Ank1                             | Ankyrin 1, erythroid | NM_001110783   | 2.3         |
| Slc4a1                            | Solute carrier family 4 (anion exchanger), member 1 | NM_011403     | 2.2         |
| Mctp8                             | Mast cell protease 8 | NM_008572     | 2.2         |
| Acmsd                             | Amino carboxymuconate semialdehyde decarboxylase | NM_00163041   | 2.2         |
| Pkd1l1                            | Polycystic kidney and hepatic disease 1-like 1 | NM_138674     | 2.2         |
| Klfl                              | Kruppel-like factor 1 (erythroid) | NM_01635      | 2.2         |
| Car1                              | Carbonic anhydrase 1 | NM_009799     | 2.1         |
| Pax5                              | Paired box gene 5 | NM_008782     | 2.1         |
| Add2                              | Adducin 2 (beta) | NM_013458     | 2.1         |
| Slc6a20a                          | Solute carrier family 6 (neurotransmitter transporter), member 20A | NM_139142     | 2.1         |
| Ctse                              | Cathepsin E | NM_007799     | 2.1         |
| Bzpl1                             | Benzodiazepine receptor, peripheral-like 1 | NM_027292     | 2.1         |
| Rag1                              | Recombination activating gene 1 | NM_009019     | 2.1         |
| Cd19                              | CD19 antigen | NM_009844     | 2.1         |
| Gp1ba                             | Glycoprotein 1b, alpha polypeptide | NM_010326     | 2.0         |
| Gypa                              | Glycophorin A | NM_010369     | 2.0         |
| Pag9                              | Progestin and adipoQ receptor family member IX | NM_198414     | 2.0         |
| But1                              | Butyrophilin related 1 | NM_138678     | 2.0         |
| Ms4a1                             | Membrane-spanning 4-domains, subfamily A, member 1 | NM_007641     | 2.0         |
| Cd79a                             | CD79A antigen (immunoglobulin-associated alpha) | NM_007655     | 2.0         |
| Myb                               | Myeloblastosis oncogene | NM_010848     | 2.0         |
| Prss34                            | Protease, serine, 34 | NM_178372     | 2.0         |
| Gfi1b                             | Growth factor independent 1B | NM_008114     | 2.0         |
| Bach2                             | BTB and CNC homology 2 | NM_007521     | 2.0         |

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### Table 4. Continued

| 1.5-Fold up-regulated gene symbol | Description                                                                 | Genbank accession | Fold-change |
|-----------------------------------|-----------------------------------------------------------------------------|-------------------|-------------|
| Orc1l                             | Origin recognition complex, subunit 1-like (S.cerevisiae)                    | NM_011015         | 2.0         |
| Epor                              | Erythropoietin receptor                                                      | NM_010149         | 2.0         |
| Gata1                             | GATA binding protein 1                                                       | NM_008089         | 2.0         |
| Fcrla                             | Fc receptor-like A                                                           | NM_145141         | 1.9         |
| F2rl2                             | Coagulation factor II (thrombin) receptor-like 2                             | NM_010170         | 1.9         |
| Fcna                              | Ficolin A                                                                   | NM_007995         | 1.9         |
| Gp9                               | Glycoprotein 9 (platelet)                                                   | NM_018762         | 1.9         |
| Prtn3                             | Proteinase 3                                                                | NM_011178         | 1.9         |
| Cdfb                              | CD79B antigen                                                               | NM_008339         | 1.9         |
| Spnb1                             | Spectrin beta 1                                                             | NM_013675         | 1.9         |
| Hist1h1a                          | Histone cluster 1, H1a                                                       | NM_030609         | 1.9         |
| IL-1a                             | Interleukin-1 alpha                                                         | NM_010554         | 1.9         |
| Car2                              | Carbonic anhydrase 2                                                        | NM_009801         | 1.9         |
| Trim58                            | Tripartite motif-containing 58                                               | NM_001039047      | 1.9         |
| Nfe2                              | Nuclear factor, erythroid derived 2                                         | NM_008685         | 1.9         |
| Acss1                             | Acyl-CoA synthetase short-chain family member 1                             | NM_080575         | 1.9         |
| Bcl11a                            | B-cell CLL/lymphoma 11A (zinc finger protein)                               | NM_016707         | 1.8         |
| Hbq1                              | Hemoglobin, theta 1                                                         | NM_175000         | 1.8         |
| Gp5                               | Glycoprotein 5 (platelet)                                                   | NM_008148         | 1.8         |
| E2f2                              | E2F transcription factor 2                                                   | NM_17733          | 1.8         |
| Lrmp                              | Lymphoid-restricted membrane protein                                         | NM_008511         | 1.8         |
| Nup210                            | Nucleoporin 210                                                             | NM_018815         | 1.8         |
| Alas2                             | Aminolevulinic acid synthase 2, erythroid                                  | NM_009653         | 1.8         |
| Tmcc2                             | Transmembrane and coiled-coil domains 2                                     | NM_178674         | 1.8         |
| Tac2                              | Tachykinin 2                                                                | NM_009312         | 1.8         |
| Ikzf3                             | IKAROS family zinc finger 3                                                  | NM_011771         | 1.8         |
| Itga2b                            | Integrin alpha 2b                                                           | NM_010575         | 1.8         |
| Irf4                              | Interferon regulatory factor 4                                               | NM_013674         | 1.8         |
| Slc25a37                          | Solute carrier family 25, member 37                                         | NM_026331         | 1.8         |
| Gct2                              | Germinal center expressed transcript 2                                      | NM_008099         | 1.8         |
| Dntt                              | Deoxynucleotidyltransferase, terminal                                       | NM_009345         | 1.8         |
| Bpgm                              | 2,3-bisphosphoglycerate mutase                                              | NM_007563         | 1.8         |
| Cecr2                             | Cat eye syndrome chromosome region, candidate 2 homolog (human)             | NM_001128151      | 1.8         |
| Siglecg                           | Sialic acid binding Ig-like lectin G                                        | NM_172900         | 1.8         |
| Zfp41                             | Zinc finger protein, multitype 1                                            | NM_009669         | 1.8         |
| Pou2af1                           | POU domain, class 2, associating factor 1                                   | NM_011136         | 1.8         |
| Xk                                | Kell blood group precursor (McLeod phenotype) homolog                       | NM_023600         | 1.8         |
| Chst3                             | Carbohydrate (chondroitin 6/keratan) sulfotransferase 3                     | NM_016803         | 1.8         |
| Epb4.9                            | Erythrocyte protein band 4.9                                                | NM_013514         | 1.8         |
| Mup2                              | Major urinary protein 2                                                     | NM_008647         | 1.8         |
| Ant4                              | ADP-ribosyltransferase 4                                                    | NM_026639         | 1.8         |
| Abcb10                            | ATP-binding cassette, sub-family B (MDR/TAP), member 10                     | NM_019652         | 1.8         |
| Kcnj5                             | Potassium inwardly-rectifying channel, subfamily J, member 5               | NM_016065         | 1.8         |
| Slamp1                            | Signaling lymphocytic activation molecule family member 1                  | NM_013730         | 1.7         |

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| 1.5-Fold up-regulated gene symbol | Description                                           | Genbank accession | Fold-change |
|-----------------------------------|-------------------------------------------------------|-------------------|-------------|
| Cdc6                              | Cell division cycle 6 homolog (S. cerevisiae)         | NM_011799         | 1.7         |
| Slc43a1                           | Solute carrier family 43, member 1                    | NM_001081349      | 1.7         |
| Fhdc1                             | FH2 domain containing 1                               | NM_001033301      | 1.7         |
| Snca                              | Synuclein, alpha                                      | NM_001042451      | 1.7         |
| Bard1                             | BRCA1 associated RING domain 1                        | NM_007525         | 1.7         |
| Prg4                              | Proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein) | NM_021400         | 1.7         |
| Stab2                             | Stabilin 2                                            | NM_138673         | 1.7         |
| Mpo                               | Myeloperoxidase                                       | NM_010824         | 1.7         |
| Uhrf1                             | Ubiquitin-like, containing PHD and RING finger domains, 1 | NM_010931         | 1.7         |
| Rasgrp2                           | RAS, guanyl releasing protein 2                        | NM_011242         | 1.7         |
| Ms4a3                             | Membrane-spanning 4-domains, subfamily A, member 3    | NM_133246         | 1.7         |
| Cd5l                              | CD5 antigen-like                                      | NM_009690         | 1.7         |
| Pppb                              | Pro-platelet basic protein                            | NM_023785         | 1.7         |
| Clec1b                            | C-type lectin domain family 1, member b                | NM_019885         | 1.7         |
| Kif14                             | Kinesin family member 14                              | NM_001081258      | 1.7         |
| Gnaz                              | Guanine nucleotide binding protein, alpha z subunit    | NM_010311         | 1.7         |
| Atg2a3                            | ATPase, Ca++ transporting, ubiquitous                 | NM_016745         | 1.7         |
| Tmc8                              | Transmembrane channel-like gene family 8              | NM_181856         | 1.7         |
| Abcb4                             | ATP-binding cassette, sub-family B (MDR/TAP), member 4 | NM_008830         | 1.7         |
| Abcg4                             | ATP-binding cassette, sub-family G (WHITE), member 4   | NM_138955         | 1.7         |
| Cxcr5                             | Chemokine (C-X-C motif) receptor 5                    | NM_007551         | 1.7         |
| Slc6a4                            | Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4 | NM_010484         | 1.6         |
| Ctsg                              | Cathepsin G                                           | NM_007800         | 1.6         |
| Ly6d                              | Lymphocyte antigen 6 complex, locus D                 | NM_010742         | 1.6         |
| Fbxo5                             | F-box protein 5                                       | NM_025995         | 1.6         |
| Depdc1b                           | DEP domain containing 1B                              | NM_178683         | 1.6         |
| Itil                              | Interleukin 7 receptor                                | NM_008372         | 1.6         |
| Spl                              | B-cell linker                                         | NM_008528         | 1.6         |
| Cdc25b                            | Cell division cycle 25 homolog B (S. pombe)           | NM_023117         | 1.6         |
| Alad                              | Aminolevulinate, delta-, dehydratase                  | NM_008525         | 1.6         |
| Kcnn4                             | Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 | NM_008433         | 1.6         |
| Hmbs                              | Hydroxymethylbilane synthase                          | NM_008613         | 1.6         |
| Slc16a10                          | Solute carrier family 16 (monocarboxylic acid transporters), member 10 | NM_00114332       | 1.6         |
| Cpox                              | Coproporphyrinogen oxidase                            | NM_007757         | 1.6         |
| Slc15a2                           | Solute carrier family 15 (H+peptide transporter), member 2 | NM_021301         | 1.6         |
| Pdia2                             | Protein disulfide isomerase associated 2              | NM_001081070      | 1.6         |
| Pkd2i2                            | Polycystic kidney disease 2-like 2                    | NM_016927         | 1.6         |
| Slc14a1                           | Solute carrier family 14 (urea transporter), member 1  | NM_028122         | 1.6         |
| Ypel4                             | Yippee-like 4 (Drosophila)                            | NM_001005342      | 1.6         |
| Rbm38                             | RNA binding motif protein 38                          | NM_019547         | 1.6         |
| Tspan32                           | Tetraspanin 32                                        | NM_020286         | 1.6         |
| Btk                               | Bruton agammaglobulinemia tyrosine kinase             | NM_013482         | 1.6         |
| Ela2                              | Elastase 2, neutrophil                                | NM_015779         | 1.6         |

(Continued to the next page)
### Table 4. Continued

| 1.5-Fold up-regulated gene symbol | Description | Genbank accession | Fold-change |
|-----------------------------------|-------------|------------------|------------|
| Grap2                             | GRB2-related adaptor protein 2 | NM_010815 | 1.6 |
| Muc13                             | Mucin 13, epithelial transmembrane | NM_010739 | 1.6 |
| Khl16                             | Kelch-like 6 (Drosophila) | NM_183390 | 1.6 |
| Treml1                            | Triggering receptor expressed on myeloid cells-like 1 | NM_027763 | 1.6 |
| Ccnf                              | Cyclin F | NM_007634 | 1.6 |
| Mkrn1                             | Makorin, ring finger protein, 1 | NM_018810 | 1.6 |
| E2f8                              | E2F transcription factor 8 | NM_001013368 | 1.6 |
| Fcho1                             | FCH domain only 1 | NM_028715 | 1.6 |
| Gpd2                              | Glycerophosphodiester phosphodiesterase domain containing 2 | NM_023608 | 1.6 |
| Cenpk                             | Centromere protein K | NM_021790 | 1.5 |
| Gch1                              | GTP cyclohydrolase 1 | NM_008102 | 1.5 |
| Pip5k1b                           | Phosphatidylinositol-4-phosphate 5-kinase, type 1 beta | NM_008846 | 1.5 |
| Prg2                              | Proteoglycan 2, bone marrow | NM_008920 | 1.5 |
| Mup5                              | Major urinary protein 5 | NM_008649 | 1.5 |
| Kif22                             | Kinesin family member 22 | NM_145588 | 1.5 |
| Slc9a7                            | Solute carrier family 9 (sodium/hydrogen exchanger), member 7 | NM_177353 | 1.5 |
| Dyrk3                             | Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3 | NM_145508 | 1.5 |
| Fn3k                              | Fructosamine 3 kinase | NM_001038699 | 1.5 |
| Prkar2b                           | Protein kinase, cAMP dependent regulatory, type II beta | NM_011158 | 1.5 |
| Slc22a23                          | Solute carrier family 22, member 23 | NM_001033167 | 1.5 |
| Casc5                             | Cancer susceptibility candidate 5 | NM_029617 | 1.5 |
| Tal1                              | T-cell acute lymphocytic leukemia 1 | NM_011527 | 1.5 |
| Mup2                              | Major urinary protein 2 | NM_001045550 | 1.5 |
| Rad54i                            | RAD54 like (S. cerevisiae) | NM_009015 | 1.5 |
| PF-4                              | Platelet factor 4 | NM_019832 | 1.5 |
| Ufsp1                             | UFM1-specific peptidase 1 | NM_027356 | 1.5 |
| Ces2                              | Carboxylesterase 2 | NM_145603 | 1.5 |
| Gldc                              | Glutamate-cysteine ligase, catalytic subunit | NM_010295 | 1.5 |

| 1.5-Fold down-regulated gene symbol | Description | Genbank accession | Fold-change |
|-------------------------------------|-------------|------------------|------------|
| Pcp4                                | Purkinje cell protein 4 | NM_008791 | 0.7 |
| IL-33                               | Interleukin-33 | NM_133775 | 0.7 |
| Phf11                               | PHD finger protein 11 | NM_172603 | 0.6 |
| Lmod2                               | Leiomodin 2 (cardiac) | NM_053098 | 0.6 |
| EG620915                            | Predicted gene, EG620915 | XR_030718 | 0.6 |
| Tnc1                                | Troponin C, cardiac/slow skeletal | NM_009393 | 0.6 |
| EG408196                            | Predicted gene, EG408196 | NM_001082542 | 0.6 |
| Nudt10                              | Nudix [nucleoside diphosphate linked moiety X]-type motif 10 | NM_001031664 | 0.6 |
| Scn7a                               | Sodium channel, voltage-gated, type VII, alpha | NM_009135 | 0.6 |
| Omr2a                               | Oocyte maturation, alpha | NM_001111286 | 0.6 |
| Tnn1i                               | Troponin I, skeletal, slow 1 | NM_021467 | 0.6 |
| Chma1                               | Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle) | NM_007389 | 0.6 |
| Tnnt2                               | Troponin T2, cardiac | NM_011619 | 0.6 |
| Kira5                               | Killer cell lectin-like receptor, subfamily A, member 5 | NM_008463 | 0.6 |
| CCL7                                | Chemokine (C-C motif) ligand 7 | NM_013654 | 0.6 |

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**Table 4.** Continued

| 1.5-Fold down-regulated gene symbol | Description                                                   | Genbank accession | Fold-change |
|-------------------------------------|---------------------------------------------------------------|-------------------|-------------|
| C1qtnf3                             | C1q and tumor necrosis factor related protein 3               | NM_030888         | 0.6         |
| Mrgrp4                              | MAS-related GPR, member B4                                    | NM_205795         | 0.6         |
| Igm1                                | Immunity-related GTPase family M member 1                     | NM_008326         | 0.6         |
| Mphosph6                            | M phase phosphoprotein 6                                      | NM_026758         | 0.6         |
| Cd3g                                | CD3 antigen, gamma polypeptide                                 | NM_009850         | 0.6         |
| Ig1                                 | Immunoresponsive gene 1                                       | NM_008382         | 0.6         |
| Xir                                 | X-linked lymphocyte-regulated complex                         | NM_011725         | 0.6         |
| Csrp3                               | Cysteine and glycine-rich protein 3                           | NM_013808         | 0.6         |
| Snord116                            | Small nucleolar RNA, C/D box 116                              | AF241256          | 0.6         |
| Zfp459                              | Zinc finger protein 459                                       | NM_177811         | 0.6         |
| Magea5                              | Melanoma antigen, family A, 5                                 | NM_020018         | 0.6         |
| Tnmd                                | Tenomodulin                                                   | NM_022322         | 0.6         |
| Gldn                                | Giomedinin                                                    | NM_177350         | 0.6         |
| Rex1                                | Brain expressed gene 1                                        | NM_009052         | 0.6         |
| Igtp                                | Interferon gamma induced GTPase                               | NM_018738         | 0.6         |
| Myoz2                               | Myozenin 2                                                    | NM_021503         | 0.6         |
| Ddah1                               | Dimethylarginine dimethylaminohydrolase 1                     | NM_026993         | 0.6         |
| EG215974                            | Predicted gene, EG215974                                      | XM_894477         | 0.5         |
| Gbp3                                | Guanylate binding protein 3                                   | NM_018734         | 0.5         |
| Myh3                                | Myosin, heavy polypeptide 3, skeletal muscle, embryonic       | NM_001089635      | 0.5         |
| Dnahc3                              | Dynein, axonemal, heavy chain 3                               | XM_355934         | 0.5         |
| Gbp5                                | Guanylate binding protein 5                                   | NM_153564         | 0.5         |
| Gzmc                                | Granzyme C                                                    | NM_013071         | 0.5         |
| Gbp1                                | Guanylate binding protein 1                                   | NM_010259         | 0.5         |
| CCL8                                | Chemokine (C-C motif) ligand 8                                | NM_021443         | 0.5         |
| Dleu2                               | Deleted in lymphocytic leukemia 2                             | AF380423          | 0.5         |
| Fcgr4                               | Fc receptor, IgG, low affinity IV                             | NM_144559         | 0.5         |
| Gbp4                                | Guanylate binding protein 4                                   | NM_008620         | 0.5         |
| Tgtp                                | T-cell specific GTPase                                        | NM_011579         | 0.4         |
| Mpa2l                               | Macrophage activation 2 like                                  | NM_194336         | 0.4         |
| Gzme                                | Granzyme E                                                    | NM_010373         | 0.4         |
| Gbp2                                | Guanylate binding protein 2                                   | NM_010260         | 0.4         |
| Gzmd                                | Granzyme D                                                    | NM_010372         | 0.4         |
| Saa3                                | Serum amyloid A 3                                              | NM_011315         | 0.4         |
| Ly6i                                | Lymphocyte antigen 6 complex, locus 1                         | NM_020498         | 0.4         |
| Gzmb                                | Granzyme B                                                    | NM_013542         | 0.3         |
| CXCL10                              | Chemokine (C-X-C motif) ligand 10                             | NM_021274         | 0.3         |
| Ilgip1                              | Interferon inducible GTPase 1                                 | NM_021792         | 0.3         |
| CXCL9                               | Chemokine (C-X-C motif) ligand 9                              | NM_008599         | 0.3         |

**DISCUSSION**

Initially, a GSEA organized according to the gene GO “Biological Function” category examined the changes in gene expression in response to Cox-2 transgene expression. However, this analysis immediately suggested that Cox-2 inhibits inflammation but promotes blood cell development at this stage of fracture repair (Table 3).

In a further analysis the expression of individual 1.5-fold regulated genes, a limited number of pro-inflammatory
genes displayed increased expression in response to Cox-2 transgene expression, notably PF-4 (Table 4). The chemokine receptor CXCR-5, a regulator of B cell trafficking[26] was up-regulated, as was IL-7αr, a regulator of T cell development.[27] However, most inflammatory genes were down-regulated. The monocyte attractant chemokines CXCL-9 and CXCL-10[28,29] were more than 2-fold down-regulated, and were among the most down-regulated genes on the microarray. Other inflammatory chemokines were also down-regulated more than 1.5-fold, notably the monocyte trafficking chemokines CCL-7 and CCL-8.[30] These results were intriguing and somewhat unexpected, since Cox-2 has been traditionally assigned pro-inflammatory functions, at least during the initial stages of tissue repair. These findings suggest that Cox-2-derived PG products can promote bony union by inhibiting inflammatory gene expression, and raises the interesting possibility that the inflammatory response must subside before bony union can occur.

In addition to several up-regulated antigen genes associated with hematopoietic development observed in the GSEA, intracellular signaling pathways that have been described in hematopoietic cell development were up-regulated (Table 4), including pathways for the genes pyruvate kinase, liver and red blood cell (Pklr, liver and red blood cell), the Bruton gammaglobulinemia tyrosine kinase (Btk) and CXCR-5. In particular, the genes for IRF-4, a lymphocyte regulator, and the transcription factor KLF1, a regulator of stem cell contributions to erythropoiesis,[24] exhibited significant increases in expression of 1.8-fold and 2.2-fold, respectively. These results correlated well between the real-time microarray and RT-PCR approaches.

It is possible that the erythropoietic and hematopoietic gene expression was actually secondary to blood vessel development from the expression of angiogenic growth factors, such as VEGF, prior to the healing time that we examined. However, because 1) hematopoietic stem cell genes are induced during fracture repair,[31] 2) hematopoietic stem cell expansion has been demonstrated to be dependent on PGE₂ production,[32] and 3) the PGs promote diverse aspects of erythropoietic and hematopoietic progenitor cell proliferation, survival and development,[33] it is plausible that Cox-2 transgene expression at 10 days post-fracture enhances bony union through hematopoiesis. The absence of changes in CXCL-12 and CXCR-4 expression in this analysis also suggests that angiogenic effects of PGE₂ are not mediated through endothelial cells,[34] although our gene therapy approach was effective in promoting hematopoiesis by targeting Cox-2 transgene expression to periosteal cells.

The results of the GSEA were also surprising because the genes traditionally assigned angiogenic roles that we expected to be expressed were not represented. In this respect, our microarray analysis agrees with that of Hadjiargyrou et al.[3] On the other hand, the members of the FGF axis were expressed in a microarray analysis of early fracture repair.[4] Other fracture repair studies have described the response of healing to VEGF therapy in the rabbit radius[37] and in the multiple tibial fracture model.[19] In the latter case, increased expression of VEGF genes was observed, but slightly after our harvest time of 10 days post-fracture. Additionally, the multiple fracture approach used in this study might have exposed more marrow cell targets than the periosteal cells targeted in this study and promoted angiogenic growth factor expression.[8]
The expression of the Cox-2 transgene has been established to up-regulate PGE_2 production,[12] whose effects are mediated through the 4 PTGER receptors. PTGER3 expression was up-regulated in response to Cox-2 transgene expression by 1.4-fold in the microarray analysis, and confirmed as 2.6-fold up-regulated by real-time RT-PCR analysis, suggesting that PTGER3 was important in mediating PGE_2 effects in fracture repair at this time (Fig. 1). This receptor can generate different responses to PGE_2 signaling through three different isoforms of its receptor.[38] PTGER3 has been associated with angiogenesis in acute and tumor-related chronic inflammation[39-41] and with VEGF functions in wound healing angiogenesis,[42] although the regulation described in those studies was post-transcriptional and would not have been observed by a microarray approach.

There were remarkably few bone formation genes represented in the microarray analysis by the GSEA analysis (Table 3) or the individual gene analysis (Table 4), despite observations that PGE_2 can regulate bone morphogenetic protein-2 (BMP-2) expression.[43] Sex determining region Y-box 9 (Sox-9), an important regulator of chondrocyte commitment, was down-regulated, but only by 1.4-fold. Nevertheless, this result is consistent with our previous proposal that Cox-2 gene therapy enhances bony union by suppressing cartilage formation and promoting cartilage degradation.[37]

The sets of genes regulating osteoclast-related genes were enriched and displayed increased expression (Table 4), consistent with a Cox-2-mediated increase in bone resorption during healing. The up-regulation of the osteoclast-related genes cathepsin E, cathepsin G and the carbonic anhydrases 1 and 2 support this argument. However, the absence of changes in expression among the mmp genes in this analysis was unexpected, as mmp-9 is an established regulator of fracture callus remodeling,[44] and PTGER3 up-regulates the expression of both mmp-9 and VEGF.[45] Because this study was designed to identify possible regulatory pathways that mediate Cox-2 functions during endochondral bone repair, 1) Cox-2 expression was enhanced by gene therapy, 2) gene expression was examined at a single time, and 3) the samples examined were limited in number. The gene expression results should therefore be further characterized at other times and with additional fracture samples. Additionally, although the high Cox-2 gene expression in fracture tissues treated with the Cox-2 in vivo gene transfer approach was certainly not physiological, Cox-2 gene therapy did promote fracture healing in the fracture model, and gene expression identified by this approach might identify molecular pathways of fracture repair for further investigation.

A model for Cox-2 gene therapy for endochondral bone fracture repair is presented in Fig. 3. In this model, endogenous Cox-2 normally promotes inflammation in early bone healing, but inhibits inflammation and enhances hematopoiesis later in healing. The connection to fracture angiogenesis illustrated by dotted arrows is inferred from another study.[19] We conclude that the expression of Cox-2 gene therapy promotes bony union by up-regulating erythropoiesis- and hematopoiesis-related gene expression, but also surprisingly inhibits inflammation.

In conclusion, Cox-2 transgene expression promoted the expression of genes regulating the proliferation and development of hematopoietic blood cell precursors, but surprisingly did not up-regulate the expression of angiogenic growth factor genes. The inflammatory genes were down-regulated, which was unexpected, given the proinflammatory role of PGs. Cox-2 gene therapy could promote bony union through hematopoietic precursor proliferation and development during endochondral bone repair.
CONFLICT OF INTEREST

KHWL and CHR are co-inventors on a U.S. patent application filed for Cox-2 gene therapy for bone repair. NLP declares no conflict of interest.

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