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Original Article

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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Background: Cyclo-oxygenase-2 (Cox-2) is an inflammatory mediator that is necessary for the tissue repair, including bone fracture healing. Although the application of Cox-2 gene therapy to a murine closed femoral fracture has accelerated bony union, but the beneficial effect was not observed until the endochondral stage of bone repair that is well after the inflammatory stage normally subsides. **Methods:** To identify the molecular pathways through which Cox-2 regulates fracture healing, we examined gene expression profile in fracture tissues in response to Cox-2 gene therapy during the endochondral bone repair phase. Cox-2 gene therapy was applied to the closed murine femur fracture model. Microarray analysis was performed at 10 days post-fracture to examine global gene expression profile in the fracture tissues during the endochondral bone repair phase. The entire repertoire of significantly expressed genes was examined by gene set enrichment analysis, and the most up-regulated individual genes were evaluated further. **Results:** The genes that normally promote inflammation were under-represented in the microarray analysis, and the expression of several inflammatory chemokines was significantly down-regulated. There was an up-regulation of two key transcription factor genes that regulate hematopoiesis and erythropoiesis. More surprisingly, there was no significant up-regulation in the genes that are normally involved in angiogenesis or bone formation. However, the expression of two tissue remodeling genes was up-regulated. **Conclusions:** The down-regulation of the inflammatory genes in response to Cox-2 gene therapy was unexpected, given the pro-inflammatory role of prostaglandins. Cox-2 gene therapy could promote bony union through hematopoietic precursor proliferation during endochondral bone repair and thereby enhances subsequently fracture callus remodeling that leads to bony union of the fracture gap.

Key Words: Cyclooxygenase 2, Fracture healing, Gene expression, Genetic therapy, Microarray analysis

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 improve bone healing impaired by age or disease are highly desirable, and experimental efforts have attempted to promote different aspects of bone repair to improve the healing process. Therapeutic studies have generally identified novel approaches to enhance bone formation during tissue development and repair and applied them to the fracture 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 highly 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 endochondral 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 rheumatoid arthritis patients under treatment with the non-steroidal 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 effects 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 triphosphate (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 formation, 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 therapy could indeed improve endochondral bone fracture repair.[12] However, the therapeutic effect was unexpectedly not seen until the endochondral stage of bone fracture repair, during fracture chondrogenesis, when the callus cartilage is being remodeled to bone and well after the inflammatory stage has subsided, during which time Cox-2 expression has been observed to initiate fracture healing.[13] We speculate that while Cox-2 functions might also mediate some aspects of post-inflammatory bone healing during endochondral bone repair. Because Cox-2 also mediates angiogenesis and tissue remodeling during certain pathogenic conditions, such as metastasis,[14,15] and because angiogenesis coincides with the ossification of fracture cartilage,[16] we hypothesized that Cox-2 gene expression might also regulate angiogenesis and extracellular matrix remodeling of the fracture callus cartilage to promote bony union of the fracture during endochondral bone repair.

To elucidate the molecular pathways regulating Cox-2 promotion of bony union during endochondral bone repair, we employed an *in vivo* gene transfer approach to express a human Cox-2 (hCox-2) transgene in murine fracture tissues, and characterized the response to hCox-2 overexpression through a microarray analysis of global gene expression at this stage of fracture healing, i.e., at 10 days post-fracture.

METHODS

Closed femoral fractures were produced in 12-week-old C57BL/6 male mice by the three-point bending technique.[17] The bone fracture model was examined because Cox-2 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 modified 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 expression 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 is modified by removing most of the 3'untranslated region (3'UTR) sequence and replacing the endogenous Kozak sequence with an enhanced Kozak sequence to increase the stability and translation efficiency of the mRNA.[12] The

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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

Table 1. Gene-specific primers for real-time reverse transcription-polymerase chain reaction measurement of fracture gene expression

Gene	Accession	Location	Sequence	Product (bp)
hCox-2	M90100	1354-1373 1690-1671	5′-GGTTGCTGGTGGTAGGAATGT-3′ 5′-CCAGTAGGCAGGAGAACATAT-3′	336
PTGER1	NM_013641	84-105 542-523	5′-CCCAGCCCCAGAGAGCAGACAT-3′ 5′-AGCAGGCTGGCGACGAACAA-3′	459
PTGER2	NM_008964	1551-1570 1761-1741	5′-AGCCGCTGCGGATTGTCTGG-3′ 5′-ACCGAAGAGCTCGGAGGTCCC-3′	211
PTGER3	NM_011196	844-863 1379-1360	5′-AAAGCCGCCGTCTCGCAGTC-3′ 5′-TGTGTCGTCTTGCCCCCGTC-3′	536
PTGER4	NM_001136079	782-801 1259-1240	5′-CACCTCGTTGGGCACGGAGC-3′ 5′-GTGGCCGGACATGGCGGAAG-3′	478
PPIA	NM_008907	314-333 596-577	5′-GCATACAGGTCCTGGCATCT-3′ 5′-GCTCTCCTGAGCTACAGAAG-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 $2^{-\Delta\Delta Ct}$ 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 PGE_2 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]

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	Microarray[b]	Real-time RT-PCR (±SD)[a]
Inflammation	CXCL9	-3.7	-6.2±1.4
	CXCL10	-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-7αr	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

[a]Fold-change in gene expression at 10 days post-fracture, cyclo-oxygenase-2 versus control transgene.
[b]Down-regulated gene expression in Table 4 was converted to negative fold-change for comparison with real-time RT-PCR.
GO, gene ontology; RT-PCR,reverse transcription-polymerase chain reaction; CXCL9, chemokine (C-X-C motif) ligand 9; CXCL10, chemokine (C-X-C motif) ligand 10; CCL8, chemokine (C-C motif) ligand 8; CCL7, chemokine (C-C motif) ligand 7; PF-4, platelet factor-4; IL-7αr, interleukin-7 alpha receptor; Ikzf3, IKAROS family zinc finger 3; IRF4, interferon regulatory factor 4; KLF1, Kruppel-like factor 1.

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

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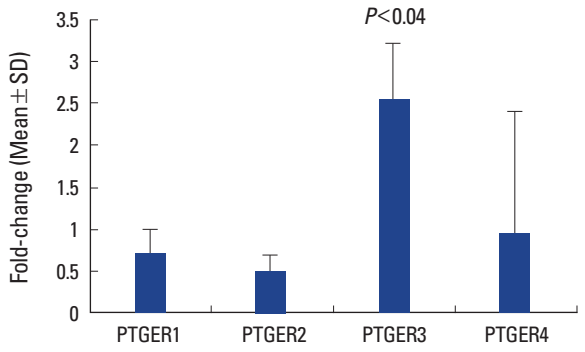

Fig. 1. Real-time reverse transcription-polymerase chain reaction determination of prostaglandin E receptor (PTGER) gene expression in response to cyclo-oxygenase-2 (Cox-2) transgene at 10 days post-fracture. The three samples in each group size were from the same individuals that underwent microarray 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-

Table 3. Leading edge analysis of gene set enrichment analysis

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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Table 3. Continued

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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Table 3. Continued

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
Suckling 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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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.

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-7αr and the different chemokines), hematopoietic and erythropoietic genes (KLF1, Ikzf3, IRF-4), and extracellular proteases (cathepsin E and cathepsin G) determined by microarray and those determined real-time RT-PCR.

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
Pdzk1ip1	PDZK1 interacting protein 1	NM_026018	2.5
Htra4	HtrA serine peptidase 4	NM_001081187	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
Pklr	Pyruvate kinase liver and red blood cell	NM_013631	2.3
Rhd	Rh blood group, D antigen	NM_011270	2.3
Spib	Spi-B transcription factor (Spi-1/PU.1 related)	NM_019866	2.3
Ank1	Ankyrin 1, erythroid	NM_001110783	2.3
Slc4a1	Solute carrier family 4 (anion exchanger), member 1	NM_011403	2.2
Mcpt8	Mast cell protease 8	NM_008572	2.2
Acmsd	Amino carboxymuconate semialdehyde decarboxylase	NM_001033041	2.2
Pkhd1l1	Polycystic kidney and hepatic disease 1-like 1	NM_138674	2.2
KLF1	Kruppel-like factor 1 (erythroid)	NM_010635	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
Bzrpl1	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
Paqr9	Progestin and adipoQ receptor family member IX	NM_198414	2.0
Butr1	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
Cd79b	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_177733	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_178874	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
Gcet2	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
Zfpm1	Zinc finger protein, multitype 1	NM_009569	1.8
Pou2af1	POU domain, class 2, associating factor 1	NM_011136	1.8
Xk	Kell blood group precursor (McLeod phenotype) homolog	NM_023500	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
Art4	ADP-ribosyltransferase 4	NM_026639	1.8
Abcb10	ATP-binding cassette, sub-family B (MDR/TAP), member 10	NM_019552	1.8
Kcnj5	Potassium inwardly-rectifying channel, subfamily J, member 5	NM_010605	1.8
Slamf1	Signaling lymphocytic activation molecule family member 1	NM_013730	1.7

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Table 4. Continued

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
Ppbp	Pro-platelet basic protein	NM_023785	1.7
Clec1b	C-type lectin domain family 1, member b	NM_019985	1.7
Kif14	Kinesin family member 14	NM_001081258	1.7
Gnaz	Guanine nucleotide binding protein, alpha z subunit	NM_010311	1.7
Atp2a3	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	Chemochine (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
IL7r	Interleukin 7 receptor	NM_008372	1.6
Blnk	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
Mns1	Meiosis-specific nuclear structural protein 1	NM_008613	1.6
Hmbs	Hydroxymethylbilane synthase	NM_013551	1.6
Slc16a10	Solute carrier family 16 (monocarboxylic acid transporters), member 10	NM_001114332	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
Pkd2l2	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

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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
Klhl6	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
Gdpd2	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
Rad54l	RAD54 like (S. cerevisiae)	NM_009015	1.5
PF-4	Platelet factor-4	NM_019932	1.5
Ufsp1	UFM1-specific peptidase 1	NM_027356	1.5
Ces2	Carboxylesterase 2	NM_145603	1.5
Gclc	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
Tnnc1	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
Omt2a	Oocyte maturation, alpha	NM_001111286	0.6
Tnni1	Troponin I, skeletal, slow 1	NM_021467	0.6
Chrna1	Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	NM_007389	0.6
Tnnt2	Troponin T2, cardiac	NM_011619	0.6
Klra5	Killer cell lectin-like receptor, subfamily A, member 5	NM_008463	0.6
CCL7	Chemokine (C-C motif) ligand 7	NM_013654	0.6

(Continued to the next page)

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
Mrgprb4	MAS-related GPR, member B4	NM_205795	0.6
Irgm1	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
Irg1	Immunoresponsive gene 1	NM_008392	0.6
Xlr	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	Gliomedin	NM_177350	0.6
Bex1	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_001099635	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_010371	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 I	NM_020498	0.4
Gzmb	Granzyme B	NM_013542	0.3
CXCL10	Chemokine (C-X-C motif) ligand 10	NM_021274	0.3
Iigp1	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

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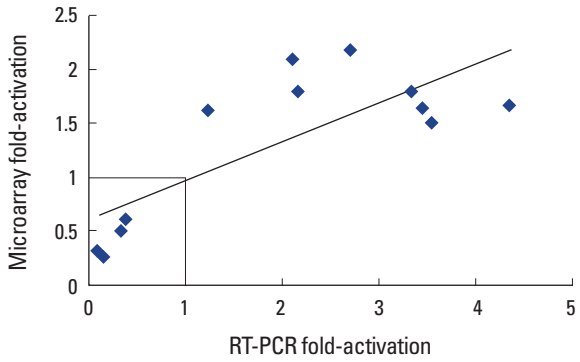

Fig. 2. Correlation of microarray and quantitative real-time reverse transcription-polymerase chain reaction measurements of gene expression for selected genes. The list of genes is shown in Table 2. The correlation coefficient was determined to be r=0.8. The genes below the 1-fold expression boundary displayed negatively regulated expression by each approach. RT-PCR, reverse transcription-polymerase chain reaction.

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 PGE_2 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 PGE_2 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 promotion of hematopoiesis might have suppressed the inflammatory reaction through the production of myeloid derived suppressor cells (MDSCs). MDSCs can be induced by PGE_2[35] and can inhibit inflammatory responses through suppressive functions on T cells in adaptive immunity and macrophages in innate immunity,[36] characteristics that might explain the reduction of inflammatory gene expression in response to a pro-inflammatory mediator such as PGE_2.

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.

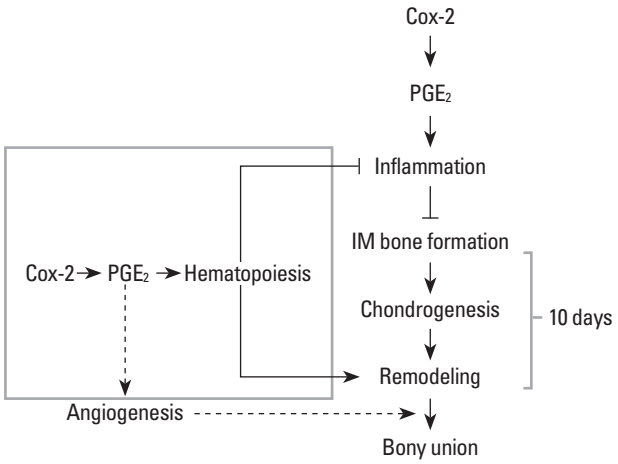

Fig. 3. A model proposed for the functions of the genes expressed during fracture callus development in response to cyclo-oxygenase-2 (Cox-2) gene therapy (box). The stages of fracture callus healing normally present at 10 days post-fracture are indicated. IM, intramembranous; Cox-2, cyclo-oxygenase-2; PGE_2, prostaglandin E_2.

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.

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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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