Identification of key gene networks associated with fracture healing using αSMA-labeled progenitor cells

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Received October 31, 2015; Accepted October 27, 2016

DOI: 10.3892/mmr.2018.9029

Abstract. The aim of the present study was to investigate the key gene network in fracture healing. The dataset GSE45156 was downloaded from the Gene Expression Omnibus. Differentially expressed genes (DEGs) were identified using the linear models for microarray data package of Bioconductor. Subsequently, Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were conducted for DEGs in day 2 and 6 fractured samples via the Database for Annotation, Visualization and Integrated Discovery. Furthermore, protein-protein interactions (PPIs) of DEGs were analyzed and a PPI network was constructed. A total of 774 and 1,172 DEGs were identified in day 2 and 6 fractured samples, respectively, compared with unfractured controls. Of the DEGs in day 2 and 6 fractured samples, various upregulated DEGs, including protein kinase C α (Prkca) and B-cell lymphoma antagonist/killer 1 were significantly enriched in GO terms associated with cell death, and certain downregulated DEGs, including fms-related tyrosine kinase 1 (Flt1), nitric oxide synthase 3 (Nos3), bone morphogenetic protein 4 (Bmp4) and Notch1 were enriched in GO terms associated with angiogenesis. Furthermore, a series of downregulated DEGs were enriched in the Notch signaling pathway, including hes family bHLH transcription factor 1 and Notch1. Certain DEGs had a high degree and interacted with each other, including Flt1, Nos3, Bmp4 and Notch1, and Prkca and ras-related C3 botulinum toxin substrate 3. The up and downregulated DEGs may exert critical functions by interactively regulating angiogenesis or apoptosis.

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Key words: fracture, differentially expressed gene, pathway, network

Introduction

Fracture healing is an intricate biological process that involves numerous events that occur during embryonic skeletal development and requires the altered expression of thousands of genes (1).

There have been numerous advances in the understanding of the process of fracture healing. For example, certain proinflammatory cytokines, including interleukin (IL)-1, IL-6, IL-11, IL-18 and tumor necrosis factor-α (TNF-α), are involved in the inflammatory response, which is essential for the process of healing (2). Hypoxia inducible factor-1α has been demonstrated to be critical for bone repair, via the induction of vascular endothelial growth factor (VEGF) in the revascularization process at the fracture site (3). The deficiency of Fas activity prolongs the life span of chondrocytes and Fas synergizes with TNF-α signaling to modulate chondrocyte apoptosis, which affects fracture healing (4). The expression of α smooth muscle actin (αSMA) identifies mesenchymal progenitor cells in bone marrow stromal cell cultures *in vitro* (5), and osteoblast precursors in the periodontium and bone marrow *in vivo* (6,7). Using microarray analysis of αSMA-labeled periosteal cells in mice, Matthews *et al* (8) identified a series of differentially expressed genes (DEGs) in fractured and unfractured samples, and identified Notch signaling as an important signaling pathway during bone healing. However, the protein-protein interactions (PPIs) of DEGs, which are central to the majority of biological processes and allow associations between genes to be analyzed (9), were not investigated.

The present study used the microarray data deposited by Matthews *et al* (8) to examine DEGs in fractured and unfractured samples. Following Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, the PPIs of DEGs were analyzed, and the PPI network was constructed. The results may provide information for subsequent experimental studies, and contribute to

the understanding of the molecular mechanisms underlying fracture healing.

Materials and methods

Illumina microarray data. The raw gene expression profile dataset GSE45156 (8) was obtained from Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/). The initial study was performed on the platform of GPL6885 Illumina MouseRef-8 version 2.0 expression beadchip (Illumina, Inc., San Diego, CA, USA). A total of nine αSMA-labeled periosteal cell samples from the tibia of mice were included in this dataset, including three unfractured controls collected two days following tamoxifen injections, which labeled αSMA-expressing cells, and six samples isolated two (day 2; n=3) and six (day 6; n=3) days following fracture.

In addition, CEL files and probe annotation files were downloaded, and the gene expression data of all samples were preprocessed by background correction, quantile normalization, probe summarization and expression calculation using the linear models for microarray data (LIMMA) package of Bioconductor (bioconductor.org/packages/release/bioc/html/limma.html) (10).

DEG screening. The LIMMA package was used to identify DEGs in day 2 and 6 fractured samples, compared with unfractured controls. P-values for each gene were calculated using unpaired Student's t-test, and genes with $P<0.05$ and fold-change ≥2 were designated as DEGs.

Furthermore, the up and downregulated DEGs common to day 2 and 6 fractured samples were identified.

Enrichment analysis of DEGs. To further reveal the functions of DEGs, GO functional and KEGG pathway enrichment analyses of DEGs were performed, via the Database for Annotation, Visualization and Integrated Discovery (david.abcc.ncifcrf.gov/) (11). $P<0.05$ was set as the cut-off criterion, other parameters were set as default.

Construction of PPI network. To investigate the interactions of DEGs, the Search Tool for the Retrieval of Interacting Genes (string-db.org/), which integrates a variety of known and predicted proteins associations (12), was used to identify the PPIs of DEGs by calculating the combined score (threshold, score >0.4), and the PPI network was visualized using Cytoscape (cytoscape.org/) (13).

Results

Identification of DEGs. Based on the cut-off criteria, a total of 774 DEGs (371 upregulated and 403 downregulated) and 1,172 DEGs (636 upregulated and 536 downregulated) were identified in day 2 and 6 fractured samples, respectively, compared with unfractured controls.

Hierarchical cluster analysis of the data suggested that the DEGs may be used to accurately distinguish day 2 and 6 fractured samples from unfractured controls (Fig. 1).

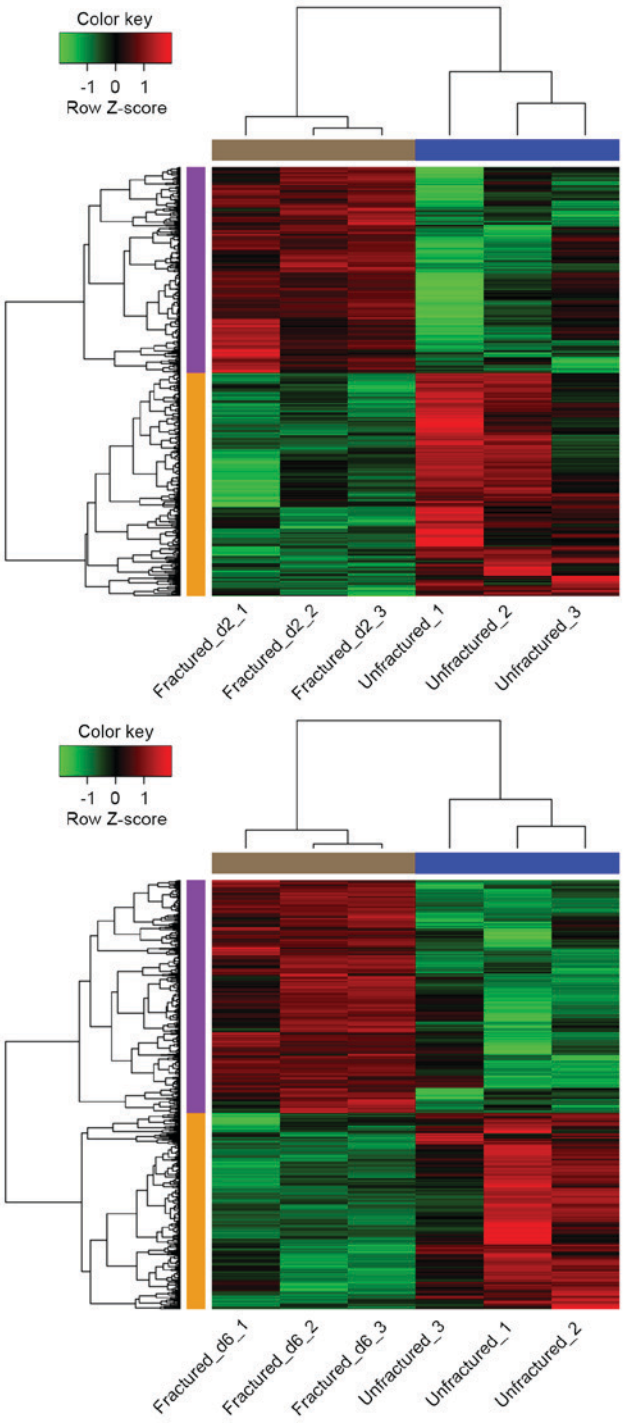

Figure 1. Cluster heatmaps for genes differentially expressed between day 2 and 6 fractured samples, and unfractured controls. There were three samples per group. Each row represents a single gene; each column represents a tissue sample. Red indicates upregulation; green indicates downregulation.

Enrichment analysis of up and downregulated DEGs. To examine the functions of DEGs, GO functional and KEGG pathway enrichment analyses of DEGs common to day 2 and 6 fractured samples were performed.

Of the upregulated DEGs, various DEGs, including protein kinase C α (Prkca), caspase 6 and B-cell lymphoma antagonist/killer 1 were significantly enriched in GO terms associated with cell death, including positive regulation of apoptosis and positive regulation of programmed cell death. Various other upregulated DEGs, including transcription factor B2, mitochondrial and transcription factor B1,

Table I. GO terms with the greatest P-values in BP, CC and MF for the upregulated genes differentially expressed by day 2 and 6 fracture samples.

Category	Term	P-value	Count	Genes
BP	GO:0043065-positive regulation of apoptosis	0.011428	7	*Prkca, Casp6, Bak1, Nudt2, Mlh1, Dapk3, Il10*
	GO:0043068-positive regulation of programmed cell death	0.011855	7	*Prkca, Casp6, Bak1, Nudt2, Mlh1, Dapk3, Il10*
	GO:0010942-positive regulation of cell death	0.012292	7	*Prkca, Casp6, Bak1, Nudt2, Mlh1, Dapk3, Il10*
	GO:0005996-monosaccharide metabolic process	0.015052	6	*Pdk1, Galk1, Ugt1A10, Pgam1, Gale, Eno1*
	GO:0006775-fat-soluble vitamin metabolic process	0.027270	3	*Rdh11, Vkorc1L1, Crabp2*
CC	GO:0005783-endoplasmic reticulum	0.013554	14	*Scd2, Rrbp1, Alg3, Tor2A, Ugt1A10, Rdh11, Bak1, Vrk2, Zdhhc16, Stx18…*
	GO:0005739-mitochondrion	0.027269	18	*Pdk1, Prkca, Usp30, Nudt2, Acat2, Spryd4, Gmppb, Bak1, Nudt8, Tfb2M…*
MF	GO:0000166-nucleotide binding	0.020428	28	*Acox2, Rac3, Tia1, Tube1, Rhof, Prkca, Pdk1, Nudt2, U2Af1L4, Tor2A…*
	GO:0016433-rRNA (adenine) methyltransferase activity	0.024853	2	*Tfb2M, Tfb1M*
	GO:0008649-rRNA methyltransferase activity	0.024853	2	*Tfb2M, Tfb1M*
	GO:0000179-rRNA (adenine-N6,N6-)-dimethyltransferase activity	0.024853	2	*Tfb2M, Tfb1M*
	GO:0017076-purine nucleotide binding	0.034672	24	*Pdk1, Acox2, Prkca, Ephb4, Tor2A, Galk1, Rac3, Dhx37, Rhof, Nek6…*

Day 2 and 6 fractured samples represent α smooth muscle actin-labeled periosteal cell samples from the tibia of mice isolated two and six days, respectively, following fracture. GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component; rRNA, ribosomal RNA.

mitochondrial were distinctly enriched in ribosomal RNA (adenine) methyltransferase activity (Table I). Of the downregulated DEGs, a set of genes, including fms-related tyrosine kinase 1 (Flt1), nitric oxide synthase 3 (Nos3), bone morphogenetic protein 4 (Bmp4) and Notch1 were markedly enriched in GO terms associated with blood vessels, including angiogenesis and blood vessel morphogenesis (Table II).

Additionally, according to the pathway enrichment analysis, the upregulated DEGs GDP-mannose pyrophosphorylase B, galactokinase 1, N-acetylneuraminate synthase and UDP-galactose-4-epimerase were primarily enriched in the pathways of amino and nucleotide sugar metabolism. The downregulated DEGs were significantly enriched in certain pathways, including the notch signaling pathway (hes family bHLH transcription factor 1, Notch1 and MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase), leukocyte transendothelial migration (F11 receptor, claudin 9 and platelet and endothelial cell adhesion molecule 1), and vascular smooth muscle contraction [protein kinase C θ, adenylate cyclase (Adcy) 4 and Adcy9; Table III].

Analysis of the PPI network. The PPI network for the up and downregulated DEGs consisted of 249 genes and 512 interactions. Prkca and Il10 interacted with Nos3; Flt1, Nos3, Bmp4 and Notch1 interacted with each other (Fig. 2).

Various genes had a high connectivity degree, including Flt1 (degree=27), Nos3 (degree=23), Bmp4 (degree=22), ras-related C3 botulinum toxin substrate 3 (Rac3; degree=21), Notch1 (degree=18) and Prkca (degree=18).

Discussion

The present study identified a total of 774 DEGs (371 upregulated and 403 downregulated) and 1,172 DEGs (636 upregulated and 536 downregulated) from day 2 and 6 fractured samples, respectively, compared with unfractured controls. According to the analysis of the PPI network, various downregulated DEGs with a high degree were revealed to interact with each other, including Flt1, Nos3, Bmp4 and Notch1. Furthermore, based on the enrichment analysis, all of these genes were significantly enriched in angiogenesis and blood vessel morphogenesis.

Flt1, is also known as vascular endothelial growth factor receptor 1 (Vegfr-1) (14). VEGF is an essential regulator during angiogenesis, which is critical for bone growth, remodeling and repair (15). A previous study observed Flt1 expression in vascular endothelial cells at the fracture site 8 h to 8 weeks following fracture (16). Endothelial nitric oxide synthase (eNOS), encoded by Nos3 in endothelial cells, is the predominant NOS isoform expressed in bone (17). A previous study has demonstrated that mice with eNOS deficiency have reduced bone mineral density, compared with wild-type controls (18). In addition, Nos3 was detected to be differentially expressed in lymph node lymphocytes and endothelial cells in patients with bone fracture (19). Nitric oxide is associated with vascular smooth muscle relaxation, and modulates VEGF-induced angiogenesis (20). Thus, Flt1 and Nos3 may be closely associated with angiogenesis during fracture healing.

Table II. GO terms with the greatest P-values in BP, CC and MF for the downregulated genes differentially expressed by day 2 and 6 fracture samples.

Category	Term	P-value	Count	Genes (n≤10)
BP	GO:0001525-angiogenesis	3.09E-07	13	*Bmp4*, *Vegfc*, *Notch1*, *Flt1*, *Epas1*, *Ovol2*, *Notch4*, *Edn1*, *Sox18*, *Nos3*…
	GO:0048514-blood vessel morphogenesis	6.42E-07	15	*Bmp4*, *Flt1*, *Epas1*, *Notch1*, *Hey1*, *Ovol2*, *Notch4*, *Tgm2*, *Nos3*, *Sox18*…
	GO:0001568-blood vessel development	1.50E-06	16	*Bmp4*, *Flt1*, *Epas1*, *Edn1*, *Notch1*, *Hey1*, *Ovol2*, *Notch4*, *Nos3*, *Sox18*…
	GO:0001944-vasculature development	2.03E-06	16	*Bmp4*, *Flt1*, *Epas1*, *Edn1*, *Notch1*, *Hey1*, *Ovol2*, *Notch4*, *Nos3*, *Sox18*…
	GO:0007242-intracellular signaling cascade	2.27E-05	30	*Adcy4*, *Rab9B*, *Edn1*, *Cdc42Ep4*, *Pik3C2G*, *Bmx*, *Rps6Ka6*, *Prkcq*, *Adcy9*, *Notch4*…
CC	GO:0005886-plasma membrane	7.67E-05	63	*Adcy4*, *Eltd1*, *Nos3*, *Prkcq*, *Gpr22*, *Flt1*, *Maob*, *Abca8A*, *Notch1*, *Adcy9*…
	GO:0005576-extracellular region	0.003702	37	*Edn1*, *Clu*, *Il15*, *Timp3*, *Dspp*, *Ifna1*, *Apod*, *Tgm2*, *Itih5*, *Bmp4*…
	GO:0044459-plasma membrane part	0.007555	35	*Enpp5*, *Nos3*, *Slc22A2*, *Gabrg1*, *F11R*, *Selp*, *Flt1*, *Prkcq*, *Notch1*, *Notch4*…
	GO:0016021-integral to membrane	0.013021	94	*Adcy4*, *Kcnc4*, *Olfr883*, *Flt1*, *Gpr33*, *Rprml*, *Notch1*, *Adcy9*, *Plscr4*, *Notch4*…
	GO:0031224-intrinsic to membrane	0.025857	95	*Adcy4*, *Kcnc4*, *Olfr883*, *Flt1*, *Notch1*, *Notch4*, *Olfr917*, *Pecam1*, *Dsc3*, *Adra1A*…
MF	GO:0030552-cAMP binding	8.23E-04	4	*Pde1C*, *Rapgef3*, *Hcn3*, *Cnga2*
	GO:0016208-AMP binding	0.002427	4	*Pde1C*, *Rapgef3*, *Hcn3*, *Cnga2*
	GO:0030551-cyclic nucleotide binding	0.003213	4	*Pde1C*, *Rapgef3*, *Hcn3*, *Cnga2*
	GO:0009975-cyclase activity	0.004139	4	*Adcy4*, *Adcy9*, *Gucy1A2*, *Npr1*
	GO:0016849-phosphorus-oxygen lyase activity	0.004139	4	*Adcy4*, *Adcy9*, *Gucy1A2*, *Npr1*

Day 2 and 6 fractured samples represent α smooth muscle actin-labeled periosteal cell samples from the tibia of mice isolated two and six days, respectively, following fracture. GO, gene ontology; BP, biological process; MF, molecular function; CC, cellular component; rRNA, ribosomal RNA.

Table III. Enriched pathways for the up and downregulated genes differentially expressed in day 2 and 6 fractured samples.

Up/downregulated	Term	P-value	Count	Genes
Upregulated	mmu00520-amino sugar and nucleotide sugar metabolism	0.011615	4	*Gmppb*, *Galk1*, *Nans*, *Gale*
	mmu05310-asthma	0.048953	3	*Fcer1A*, *Prg2*, *Il10*
Downregulated	mmu04330-Notch signaling pathway	0.010106	5	*Hes1*, *Notch1*, *Mfng*, *Notch4*, *Dll1*
	mmu04670-leukocyte transendothelial migration	0.015684	7	*F11R*, *Cldn9*, *Pecam1*, *Cldn11*, *Rapgef3*, *Jam2*, *Ctnna3*
	mmu04270-vascular smooth muscle contraction	0.016287	7	*Prkcq*, *Adcy4*, *Adcy9*, *Gucy1A2*, *Adra1A*, *Prkch*, *Npr1*
	mmu04530-tight junction	0.027350	7	*F11R*, *Prkcq*, *Cldn9*, *Prkch*, *Cldn11*, *Jam2*, *Ctnna3*
	mmu04514-cell adhesion molecules	0.047343	7	*F11R*, *Selp*, *Cldn9*, *Pecam1*, *Cldn11*, *Jam2*, *Sele*

Day 2 and 6 fractured samples represent α smooth muscle actin-labeled periosteal cell samples from the tibia of mice isolated two and six days, respectively, following fracture.

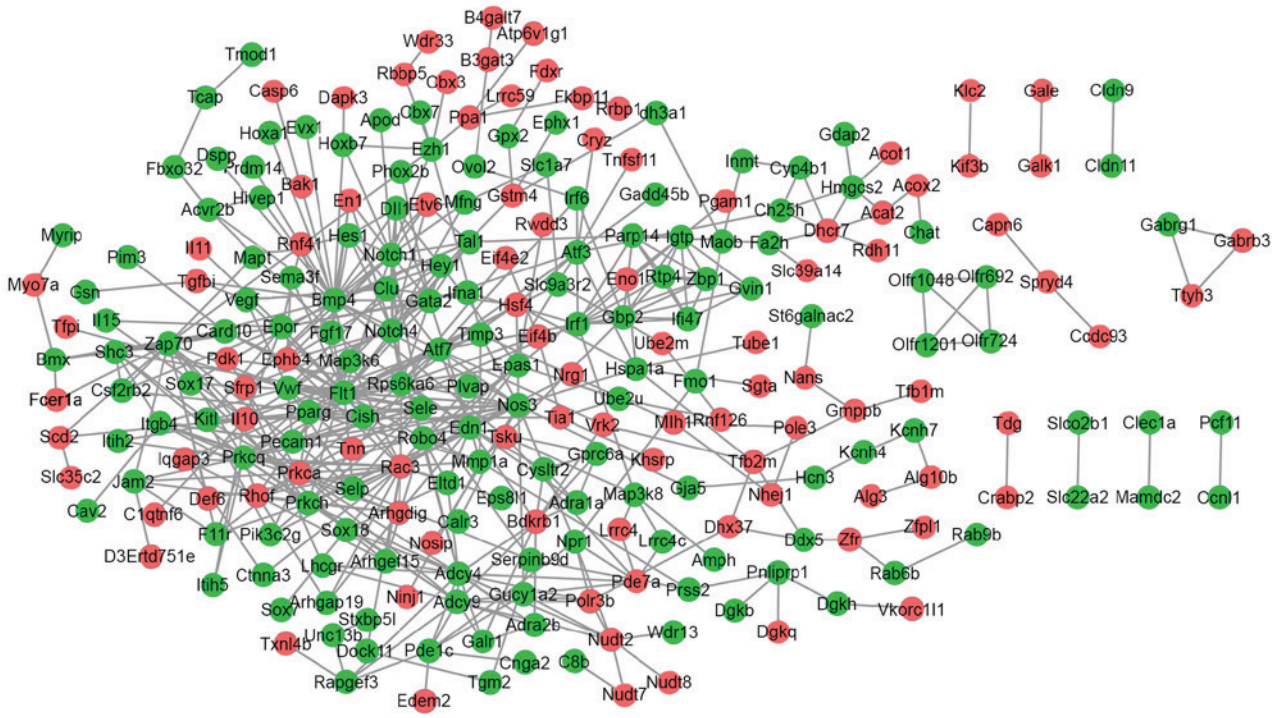

Figure 2. The protein-protein interaction network of genes differentially expressed on days 2 and 6 following fracture. Red nodes represent upregulated genes; green nodes represent downregulated genes. Lines between nodes indicate an interaction between the two nodes.

Bmp4 is a member of the transforming growth factor-β superfamily (21). Bone morphogenetic proteins (BMPs) are important in the initiation of endochondral bone formation in humans. Types I and II, the BMP receptors, bind BMPs and act in collaboration to phosphorylate mothers against decapentaplegic (SMAD) 1 and SMAD5, which translocate to the nucleus in cooperation with SMAD4 to initiate BMP responses including fracture healing (22). There is evidence that rat adipose-derived stromal cells expressing Bmp4 may induce bone formation *in vitro* and *in vivo* (23), indicating that Bmp4 may be key for bone repair. Furthermore, Notch1 was significantly enriched in the Notch signaling pathway in the present study. Genetically inducible inhibition of Notch signaling extends the inflammatory phase of fracture healing and alters cartilage formation (24). Matthews *et al* (8) reported that downregulation of Notch signaling in αSMA-labeled progenitor cells contributes to fracture callus formation. A recent study demonstrated that transient inhibition of Notch signaling and gamma secretase activity temporarily promotes osteoclastogenesis and

accelerates bone remodeling (25). In the present study, the PPI network revealed that Notch1 interacts with Flt1 and Bmp4. Notch1 may modulate angiogenesis (26,27), and functional Notch signaling is essential for BMP-induced osteoblast differentiation (28). Taken together, these results suggested that Notch1 may be crucial in fracture healing, via interactions with Flt1 and Bmp4.

Of the upregulated DEGs, Rac3 and Prkca have a high degree in the PPI network, and interacted with Nos3. Rac3 encodes a GTPase belonging to the ras superfamily of small GTP-binding proteins, which are involved in the regulation of cell growth, the activation of protein kinases and cytoskeletal reorganization (29,30). To date, there is no evidence that Rac3 is associated with bone; it does however interact with Nos3, and therefore may be involved in fracture healing via Nos3. It has been demonstrated that Rac1 deficiency increases vertebral osteoclast-mediated bone quality compared with wild-type bones in a murine ovariectomy model (31). Therefore, Rac3 may be additionally implicated in bone quality. Prkca, a serine- and threonine-specific protein kinase, was markedly enriched in positive regulation of apoptosis in the present study. Apoptosis is active during the phase of callus remodeling (32). In addition, Prkca has been observed to be upregulated during fracture repair (33). Furthermore, during fracture healing accelerated by thrombin peptide TP508, a series of genes involved in apoptosis, including Prkca, were upregulated (34). Therefore, Prkca may be important in fracture repair.

In conclusion, the present study identified 774 and 1,172 DEGs in day 2 and 6 fractured samples, respectively, compared with unfractured controls. Various upregulated DEGs (for example, Rac3 and Prkca) and downregulated DEGs (for example, Flt1, Nos3, Bmp4 and Notch1) with a high degree in the PPI network may be critical for fracture healing via involvement in angiogenesis or apoptosis regulation. These results require confirmation by further studies, which is a limitation of the present study. However, the results of the present study may provide useful information for subsequent studies, and contribute to an improved understanding of the molecular mechanisms underlying fracture healing.

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