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Microarray analysis of perichondral and reserve growth plate zones identifies differential gene expressions and signal pathways

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Abstract

In the growth plate, the reserve and perichondral zones have been hypothesized to have similar functions, but their exact functions are poorly understood. Our hypothesis was that significant differential gene expression exists between perichondral and reserve chondrocytes that may differentiate the respective functions of these two zones. Normal Sprague-Dawley rat growth plate chondrocytes from the perichondral zone (PC) and reserve zone (RZ) were isolated by laser microdissection and then subjected to microarray analysis. In order to most comprehensively capture the unique features of the two zones, we analyzed both the most highly expressed genes and those that were most significantly different from the proliferative zone (PZ) as a single comparator. Confirmation of the differential expression of selected genes was done by quantitative real time RT-PCR. A total of 8 transcripts showing high expression unique to the PC included translationally-controlled tumor protein (Tpt1), connective tissue growth factor (Ctgf), mortality factor 4 (Morf4l1), growth arrest specific 6 (Gas6), type V procollagen (Col5a2), frizzled-related protein (Frzb), GDP dissociation inhibitor 2 (Gdi2) and Jun D proto-oncogene (Jund). In contrast, 8 transcripts showing unique high expression in the RZ included hyaluronan and proteoglycan link protein 1 (Hapln1), hemoglobin beta-2 subunit, type I procollagen (Col1a2), retinoblastoma binding protein 4 (LOC685491), Sparc related modular calcium binding 2 (Smoc2), and calpastatin (Cast). Other genes were highly expressed in cells from both PC and RZ zones, including collagen II, collagen IX, catenin (cadherin associated protein) beta 1, eukaryotic translation elongation factor, high mobility group, ribosomal protein, microtubule-associated protein, reticulocalbin, thrombospondin, retinoblastoma binding protein, carboxypeptidase E, carnitine palmitoyltransferase 1, cysteine rich glycoprotein, plexin B2 (Plxnb2), and gap junction membrane channel protein. Functional classification of the most highly expressed transcripts were analyzed, and the pathway analysis indicated that ossification, bone remodeling, and cartilage development were uniquely enriched in the PC whereas both the PC and RZ showed pathway enrichment for skeletal development, extracellular matrix structural constituent, proteinaceous extracellular matrix, collagen, extracellular matrix, and extracellular matrix part pathways. We conclude that differential gene expression exists between the RZ and PC

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chondrocytes and these differentially expressed genes have unique roles to play corresponding to the function of their respective zones.

Keywords

Growth plate; microarray; chondrocytes; rat; bone

Introduction

Longitudinal bone growth results from a differentiation cascade of growth plate chondrocytes through a series of morphologic changes along with provisional calcification, apoptosis, and metaphyseal bone deposition. Cells in the reserve zone give way to flattened stacks of proliferative zone cells which go through a transitional phase before ceasing cell division and going through a hypertrophic stage just before they yield to the terminal changes in the growth plate. Perichondral chondrocytes surround the periphery of the growth plate. Although perichondral and reserve chondrocytes are well characterized histomorphologically, an understanding of their contribution in regulating progression in the growth plate is not fully explained. Both endocrine effects and a local regulatory loop involving Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) have been described [1,2,3]. The local regulatory loop involves epiphyseal and perichondrial chondrocyte production of PTHrP, which maintains chondrocyte proliferation [4,5]. PTHrP promotes expression of B-cell leukemia-2 protein (Bcl-2), inhibiting apoptosis by blocking the pro-apoptotic effects of Bcl-2-associated X protein (BAX). Beginning in the transitional zone, Ihh promotes chondrocyte differentiation [3]. The regulatory role in the perichondral and reserve chondrocytes for insulin-like growth factor I (IGF-I), fibroblastic growth factor (FGF), transforming growth factor β (TGF-β) and the bone morphogenic proteins (BMP) is less well-defined [6,7,8].

Whether the pathways currently implicated in control of longitudinal growth are the primary ones responsible is unknown. Other pathways involving genes not previously described in the growth plate may also play a contributory role. Transcriptional analysis by DNA microarray can enable a more comprehensive investigation of these complex interactions. In addition, laser microdissection allows isolation of chondrocytes from individual growth plate zones for comparative analysis. In this study, we combined both approaches to identify in vivo differential gene expression between chondrocytes in the perichondral zone (PC) and reserve zone (RZ) in the tibial growth plates of the adolescent Sprague-Dawley rat. Our hypothesis was that significant differential gene expression exists between perichondral and reserve chondrocytes that would provide clues at the transcriptional level as to their functions.

Materials and Methods

Tissue Preparation

All animal procedures were reviewed and approved by the Committee for Humane Use of Animals (CHUA). Three male Sprague-Dawley rats were euthanized at 42, 46 and 51 days of age, respectively, all by carbon-dioxide asphyxiation. The tibias were dissected and halved sagittally immediately after euthanasia. Tissues were then molded in pre-chilled Tissue Tek O.C.T. compound (Sakura-finetek, Tokyo, Japan) and rapidly frozen in liquid nitrogen. Tissue samples were then stored at −70°C in sealed bags. Tissue blocks were equilibrated to −21°C for one hour in the cryostat cabinet before cryo-microtomy. Blocks were trimmed with a razor blade to remove excessive OCT embedding media and mounted for sectioning with a thin layer of OCT on the cutting head, and slowly warmed to −18°C. Subsequently, 6 um sections cut on a Leica CM3050 cryostat were collected and mounted onto RNase-free stainless steel framed PEN-foiled slides (Leica) for laser capture microdissection. All sectioned tissue was briefly

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brought to room temperature for 30 sec to enhance tissue adherence to the slides, and returned to the cryomicrotome. Slides with sectioned tissue were then stored in a desiccator at −70°C until further use or laser capture microdissection.

Laser Microdissection

The PC, RZ and PZ proximal tibial growth plate chondrocytes were laser microdissected separately by the Leica Application Solution Laser Microdissection instrument (Leica Microsystems, Bannockburn, IL) as previously described [9]. The reserve zone was defined as the region from the epiphysis (excluding all cells related to the secondary center of ossification) to the first flattened chondrocyte at the base of a cell column. The hypertrophic zone extended from the transitional zone to the chondro-osseous junction. The transitional zone was defined as extending from the first shape change away from flattened cell morphology to the first level of fully enlarged chondrocytes. The perichondral zone was referred to the ring of chondrocytes surrounding the growth plate. In the growth plate, we identified chondrocytes close to the epiphysis including regenerating columns and non-regenerating column chondrocytes as PZ and chondrocytes close to metaphyseal side as HZ chondrocytes.

RNA Extraction and Gene Chip Hybridization Procedures

The dissected piece of film with the captured tissue fell directly into 40 μl of RLT Lysis buffer and total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). The quality and concentration of the total RNA were analyzed on an Agilent Bioanalyzer as previously described [9]. Initial RNA samples used in the array study contained approximately 10 ng per sample. Based on the Bioanalyzer concentrations, we pooled approximately equal amounts of RNA from three rats samples of each cell type (PC, RZ or PZ) at three time points (42 days, 46 and 51 days of age) to create 9 pooled samples. Each pooled sample contained 30–50 ng total RNA. The RNA samples were prepared for hybridization using the Ovation™ Biotin RNA Amplification and Labeling System (NuGen). After the amplification and labeling, equal amounts (2.2 ug) of labeled single-stranded cDNA product was then added to a Rat RAE 230 2.0 GeneChip (Affymetrix) according to manufacturer instructions. After hybridization, washing, and scanning the GeneChips, the Affymetrix software (GeneChip Operating System, Santa Clara, CA) calculated the intensity of the signal from each perfect match probe relative to the signal for the mismatch probe and determined whether or not the gene was present in the sample, and also provided a measure of the expression level of the gene. The overall chip intensities for each sample were scaled by linear adjustment to the same target value (500), and subsequently normalized by the RMA algorithm The experimental series of data files has been deposited into the Gene Expression Omnibus (GEO) at NCBI (accession GSE9537).

Analyses of Gene Expression

Affymetrix GCOS/MAS 5 was used to generate a list of genes designated as “present” in PC, RZ and PZ. GeneSpring GX (Agilent Technologies, Palo Alto, CA) was used to identify differential expression and create a raw dataset using the Robust Multiarray analysis (RMA) method. Using the raw data set, the 30 probe sets with the highest expression within both RZ and PC were identified, excluding unnamed transcribed locus probes and duplicate gene names (Figure 1). To identify the significant differential gene expression between perichondral and reserve chondrocytes, the proliferative zone was used as a comparator. Normalized data was filtered on expression using a cutoff of five-fold increased in PC, RZ and PZ. A total of 471 probe sets passed this filter and were cross-referenced with the top 30 genes identified by expression level alone. Of these 471 probes, eight overlapped with the top 30 in RZ and eight overlapped with the top 30 in PC. The differential expression of these latter genes ranked among the top 0.1% of all changes (i.e., exceeded the 99.9% confidence level) when using PZ as a comparator. Differential expression between zones was further analyzed using a two-way

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ANOVA (Zone × Age), to create a list of 49 probe sets with statistically significant differences in expression. Of these 49, 44 exceeded the 99.9% confidence level of all changes when using PZ as a comparator. After eliminating the unnamed transcribed locus probes and duplicate gene names, this list of 49 was reduced to only 33 genes (Table 1). When comparing this list of 33 significantly different genes with the list containing the top 30 by expression level in both PC and RZ, there was one gene in common for each of the zones between the lists (Figure 2).

Real-time RT-PCR

Real-time quantitative RT-PCR was performed using an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, CA). The 25 μl reaction consisted of SYBR Green PCR Master Mix (PE Applied Biosystems, CA), a set of rat specific primers and template cDNA generated by reversed-transcripted PCR (Table 2). The primers were designed to genes of matrix Gla protein (Mgp), Growth arrest specific 6 (Gas6), Thrombospondin 1 (Thbs1), Frizzled-related protein (Frzb), Procollagen type IX alpha 1 (Col9a1), Procollagen type X alpha 1 (Col10a1), secreted acidic cysteine rich glycoprotein (Sparc), Cyclin-dependent kinase inhibitor 1C (Cdkn1c), hemoglobin beta-2 subunit (LOC689064), Procollagen type I alpha 2 (Col1a2), Connective tissue growth factor (Ctgf), Stearoyl-coenzyme A desaturase 2 (Scd2), Procollagen type XXVII alpha 1 (Col27a1), Sparc-related modular calcium binding 2 (Smoc2), Procollagen type V alpha 2 (Col5a2). All samples were run in triplicate along with 18S rRNA as reference gene and no template controls. The threshold cycle (C_T) was normalized to that of 18S rRNA to account for differences in cDNA loading. The 18S rRNA was chosen because there was no differential expression between samples from different days. The comparative threshold (C_T) method was utilized for relative quantitative analysis of gene expression. In addition to the melting point analysis that is routinely provided by the ABI Prism unit, the size and identity of the amplicons from the PCR reaction were then directly verified by gel electrophoresis (using a 2% agarose gel).

Pathway Analysis

For both the RZ and PC, overall functional pathway analysis was performed for the top 30 genes by expression level using Gene Ontology (GO) annotations provided by the NetAffx browser (Affymetrix). Enrichment of GO functional groups was determined to be meaningful when the number of probe sets in our list that mapped to a specific GO pathway was greater than 1, a hypergeometric p-value estimating the probability that this amount of overlap could have occurred due to chance alone based on the size of the list and the annotated content of the array was less than 0.05, and the fold enrichment of overlap with a specific pathway compared to what would be expected by chance alone exceeded 2. Because of the large number of pathways that met these criteria, however, we utilized two additional screens to narrow the field. First, we separated out those pathways which had a p-value less than 0.05, ≥5 probe sets (rather than >1 probe set) and showed a fold enrichment of ≥5.0. Second, we identified those from the narrowed list that relate to bone, cartilage, matrix, and/or skeletal development (BCMSD). The latter screen was determined based on a search utilizing AmiGO, a search engine for the GeneOntology database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi).

Results

Highly Expressed Genes According to Growth Plate Zone

Of the 31,099 probe sets arrayed on the RAE 230.2.0 chips, the 30 genes showing the greatest absolute expression levels within the PC (Table 3) and RZ (Table 4) were selected for further analysis. Twenty-one genes from the top 30 genes expressed within the PC were also present among the top 30 genes expressed within the RZ (Figure 1), so only 8 genes from each list were unique to the respective zone. The 37 total genes in both zones at 42, 46 and 51 days of

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age for the Sprague-Dawley rat were comprised of 8 genes known to be involved in chondrocyte function and 33 (9 in PC, 9 in RZ, 15 shared) not previously identified within chondrocytes.

Highly Expressed Genes in Both PC and RZ

Among the mRNAs highly expressed within both the PC and RZ were 21 genes, including Col2a1, Col9a3, Ctnnb1 (Catenin β1), Eef1a1 (eukaryotic translation elongation factor 1 α1), ND3 (NADH dehydrogenase subunit 3), Hmgb1 (high mobility group box 1), Rpl35a (ribosomal protein L35a), Plxnb2 (Plexin B2), Mtap7 (microtubule-associated protein 7), Rcn1 (reticulocalbin 1), Col9a1, Rps6 (ribosomal protein S6), Col11a1, Cpe (carboxypeptidase E), Cpt1a (carnitine palmitoyltransferase 1a), Sparc (secreted acidic cysteine rich glycoprotein), Gja1 (gap junction membrane channel protein 1), RGD 1308977 (similar to RIKEN cDNA), RGD 1306734 (similar to hypothetical protein FLJ32743), and LOC 685491 (similar to retinoblastoma binding protein 4), Mgp (matrix Gla protein). Eight of these play a known role in growth plate extracellular matrix (ECM) function: Col2a1, Ctnnb1, Eefla1, Col9a1, Col11a1, Cpt1a, Sparc, and Mgp. The most abundant ECM mRNA in both PC and RZ zones by microarray was Col2a1 at 42, 46 and 51 days, and its absolute expression level was greater in the proliferative zones at all three time points.

Differential Expression According to Growth Plate Zone

Of the top 30 most highly expressed gene transcripts within the PC, 8 transcripts (Tpt1, Ctgf, Morf4l1, Col5a2, Gas6, Frzb, Gdi2, Jund,) were uniquely highly expressed only within the PC chondrocytes (Figure 1). Five (Ctgf, Col5a2, Gas6, Frzb, Jund) of these transcripts had previously been identified in bone or cartilage.

Eight transcripts (Hapln1, LOC689064, Col1a2, Col9a1, Smoc2, Rpl37, LOC497729, Cast) were uniquely highly expressed within the RZ (Figure 1). Only 3 (Cola2, Col9a1 and Cast) of these transcripts had previously been identified in bone or cartilage.

Real time RT-PCR

To confirm the microarray result, real-time quantitative RT-PCR was performed with a set of rat specific primers and template cDNA generated by reverse-transcription PCR. The primers were designed to selected representative genes among the top 30 most highly expressed genes in each zone as determined by microarray filters described previously. These genes included matrix Gla protein (Mgp), growth arrest specific 6 (Gas6), thrombospondin 1 (Thbs1), frizzled-related protein (Frzb), procollagen type IX alpha 1 (Col9a1), procollagen type X alpha 1 (Col10a1), secreted acidic cysteine rich glycoprotein (Sparc), cyclin-dependent kinase inhibitor 1C (Cdkn1c), hemoglobin beta-2 subunit (LOC689064), procollagen type I alpha 2 (Col1a2), connective tissue growth factor (Ctgf), stearoyl-coenzyme A desaturase 2 (Scd2), procollagen type XXVII alpha 1 (Col27a1), Sparc-related modular calcium binding 2 (Smoc2), procollagen type V alpha 2 (Col5a2). All samples were run in triplicate along with 18S rRNA as reference gene and controls containing no template. The LOG2 ratios of microarray and real time PCR data for both the PC and RZ zones were analyzed, indicating that the real time PCR results highly correlated with the microarray and confirmed these gene expression levels in both the PC and RZ (Table 5).

Pathway Analysis According to Zone

Functional analysis using the 30 most highly expressed genes from each zone revealed 37 pathways that showed enrichment with a minimum of 5 probe sets per pathway from our data set and a minimum enrichment score of 5. Eighteen (49%) of the 37 pathways overlapped the two zones, but there were 13 pathways (35%) unique to the PC and 6 pathways (16%) unique to the RZ. Nine (24%) of the 37 pathways involved bone, cartilage, matrix, and/or skeletal

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development (BCMSD). Of those, three pathways were unique to the PC: GO ID 1503 ossification, 46849 bone remodeling, and 51216 cartilage development; none were unique to the RZ, the rest overlapping the two zones: 1501 skeletal development, 5201 extracellular matrix structural constituent, 5578 proteinaceous extracellular matrix, 5581 collagen, 31012 extracellular matrix, 44420 extracellular matrix part. Other overlapping and unique pathways not related to BCMSD are shown in Table 6.

Pathway analysis of the list of 33 genes which were derived from RZ and PC by filtering for an expression level of five times changed and significance by ANOVA showed 30 enriched pathways (minimum 2 probe sets, fold enrichment score 5 or greater, $p \leq 0.05$). Twelve pathways involved bone, cartilage, matrix, and/or skeletal development, 8 of which overlapped with those pathways identified by similar pathway analysis of the 30 most highly expressed genes (1503 ossification, 46849 bone remodeling, 1501 skeletal development, 5201 extracellular matrix structural constituent, 5578 proteinaceous extracellular matrix, 5581 collagen, 31012 extracellular matrix, 44420 extracellular matrix part) described earlier. Only four additional but closely related pathways (5583 fibrillar collagen, 5584 collagen type I, 8147 structural constituent of bone, 5588 collagen type V) were uncovered by this analysis (Table 7).

Discussion

Our findings support the hypothesis that significant differential gene expression exists between perichondral and reserve chondrocytes. A number of genes never reported previously to play a role in the growth plate were identified as showing differential expression in either the PC or RZ, suggesting unique functions for these two zones.

Highly Expressed Genes and Pathways in Both PC and RZ

By the presence of 8 genes known to be involved in growth plate extracellular matrix (ECM) function among the 21 most highly expressed genes in both the PC and RZ, it would appear that support of the extracellular matrix is an important function of both zones. The most highly expressed single gene in both zones was Col2a1, which is the single gene coding for collagen type II protein, the major protein of extracellular cartilage matrix. Genes for the two minor components of growth plate collagen, collagen types IX and XI, were also highly expressed in both the RZ and PC. Col9a3 may contribute to the three-dimensional integrated structure of type II collagen molecules [10]. Col9a1 is required for long term tissue stability by mediating interactions between fibrillar and extrafibrillar macromolecules [11]. Type XI collagen (Col11a1) is a component of the collagen fibrillar network found in cartilage and consists of three genetically distinct polypeptide chains: α1, α2 and α3 [12]. Matrix GLA protein (Mgp) is a mineral binding extracellular matrix protein synthesized by growth plate cartilage chondrocytes [13]. In mammalian growth plate, Mgp has been reported to be expressed by proliferative and late hypertrophic chondrocytes. Coordinately regulated levels of Mgp during chondrocyte differentiation are crucial for chondrocyte survival and mineralization [14]. Secreted Protein Acidic and Rich in Cystein/osteonectin (Sparc) is a nonstructural matricellular protein involved in cell-matrix interaction during tissue remodeling and embryonic development. Sparc modulates ECM synthesis and turnover through its effect on collagen and extracellular proteases [15]. Thrombospondin 1 (Thbs1) is an extracellular modular glycoprotein that—like Sparc—modulates cell-matrix interactions. It is pleiotropic in function and affects processes as disparate as bone growth and hemostasis. Pericellular levels of the matrix metalloproteinase, Mmp2, are controlled to a large extent by Thbs1 and Thbs2 [16]. High expression of both Sparc and Thbs1 in both zones was confirmed by RT-PCR.

Pathway analysis further supported the results from the individual gene expression. Of the six enhanced pathways shared by zones and involving bone, cartilage, matrix, or skeletal

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development (BCMSD), five of the six were directly related to some aspect of the extracellular matrix. These included extracellular matrix structural constituent, proteinaceous extracellular matrix, collagen, extracellular matrix, and extracellular matrix part pathways. The only other shared enhanced pathway involved skeletal development. Also highly expressed in both the RZ and PC was catenin beta-1 (Ctnnb1), which has a dual role in stabilizing cell-cell adhesion and transducing canonical Wnt signaling [17]. Its role of repressing chondrocytic differentiation in the Wnt/β-catenin pathway also interacts with the Ihh pathway in distinct ways to control chondrocyte proliferation, hypertrophy, and survival during enchondral bone growth [18]. Catenins have been reported previously to reside in the murine growth plate, mostly in the zone of hypertrophy, but to our knowledge this is the first report of catenins in the RZ or PC [19].

Three additional highly expressed genes in both the RZ and the PC are predominately involved in protein synthesis. Eukaryotic elongation factor 1A (Eef1A1) is a core component of the protein synthesis machinery involved at the onset of cell transformation. Ribosomal protein L35a (Rpl35a) is phosphorylated and activated by Rpsk 1, a major protein kinase involved in translation initiation, resulting in selectively increased translation of mRNA encoding for elongation factors and ribosomal proteins [20]. Carboxypeptidase E (Cpe) is a major enzyme in the biosynthesis of numerous neuroendocrine peptides that are involved in a wide variety of physiological processes [21].

The remainder of the highly expressed genes common to the two zones are more ubiquitous and not previously described specifically in growth plate chondrocytes to our knowledge. NADH dehydrogenase subunit 3 (ND3) belongs to the intricate membrane-bound enzyme family of the mitochondrial respiratory chain. High mobility group box 1 (Hmgb 1) is a nuclear DNA-binding protein that is widely distributed [22]. Although described previously in primary osteoblasts and osteoclasts, it has not yet been reported in the growth plate [23]. Reticulocalbin (Rcn1), which is distributed predominantly in endocrine and exocrine organs, is one member of the Ca^{2+}-binding proteins in the secretory pathway [24]. Carnitinine palmitoyltransferase 1A (Cpt1a) is the key regulatory enzyme of hepatic long-chain fatty acid β-oxidation [25]. Cpt1A is also known to regulate apoptotic processes [26]. Gja1, also known as connexin 43, is a member of the larger family of connexins, the subunits of gap junctions, and it's mutation has been shown to cause skeletal malformations [27].

Highly Expressed Genes and Pathways Unique to PC

Very few of the highly expressed genes unique to the PC have been previously described to have a major role in the growth plate. Of those that have are Ctgf and Frzb. Two additional genes (JunD and Gas6) are related to the Mapk pathway. All of those except JunD were examined and confirmed by RT-PCR in the current manuscript to be uniquely highly expressed in the PC.

Connective tissue growth factor (Ctgf, Ccn2) is a secreted protein that mediates interactions with growth factors, integrins and extracellular matrix components. Ctgf is suggested to mediate collagen deposition during wound healing and is important for cell proliferation and matrix remodeling during chondrogenesis. In the growth plate, Ctgf is a key regulator coupling extracellular matrix remodeling to angiogenesis. Ctgf deficiency leads to skeletal dysmorphisms as a result of impaired chondrocyte proliferation and extracellular matrix composition within the hypertrophic zone, but it has not been previously isolated to the PC [28,29]. Frzb encodes for secreted frizzled-related protein 3 (sFRP3), a glycoprotein that antagonizes the signaling of wingless (wnt) ligands through the frizzled membrane-bound receptors that are required for maintaining cartilage integrity [30]. Through its influence on Wnt signaling, Frzb is a powerful and direct modulator of chondrocyte maturation. In the

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growth plate, Frzb-1 expression was reported previously to be strong in prehypertrophic chondrocytes. [31].

Two of the genes highly expressed by the PC and not by the RZ that also are regulated in part by the Mapk pathway are JunD, and Gas6. JunD is a member of the Jun family proteins which play critical roles in cell growth and cell apoptosis, bone remodeling, and apoptotic phenomena [32]. Much is known about JunD signaling in osteoblasts, but little is known regarding its function in chondrocytes [33]. Growth arrest-specific gene 6 (Gas6) was originally identified in fibroblasts as a gene whose expression is upregulated in growth arrest. Its role in the growth plate is not established. Gas6 acts directly on mature osteoclasts through activation of Tyro 3 and p42/p44 Mapk, possibly contributing to the bone loss associated with estrogen deficiency [34]. Gas6 has been shown to negatively regulate chondrogenic differentiation through the Mapk pathway. The gene coding for the α2 chain of human type V collagen (Col5a2) accounts for a relatively minor component of tissues rich in collagen I, such as bones, blood vessels, and tendons, where collagen V copolymerizes with collagens I and III to form heterotopic I/III/V fibrils [35]. In this manuscript, Col5a2 high expression in the PC was confirmed by RT-PCR. Tpt1 encodes the Translationally Controlled Tumor Protein (TCTP), which interacts with translation factor Eef-1a, a gene that is reported in this study to be highly expressed in both the RZ and PC [36]. Mortality factor on chromosome 4 (Morf4) is a member of the MRG (Morf-related genes) protein family, which plays a vital role in embryonic development, cell proliferation and cellular senescence [37]. Rho GDP-dissociation inhibitor 2 (Gdi2) binds to various Rho proteins and regulates their function in cell adhesion and migration, as well as multiple cellular activities including proliferation, apoptosis, and transcription [38]. The three pathways identified to be unique to the perichondrium by pathway analysis included those for ossification, bone remodeling, and cartilage development.

Highly Expressed Genes Unique to RZ

Similar to the PC, the RZ has only a few of the most highly expressed genes that have been previously described in the growth plate. These include Hapln1 and Col9a1. In addition, three genes highly expressed in this zone (Col1a2, SMOC-2, Cast) have previously been described in bone. The others are reported here as previously undescribed growth plate genes which, based upon their high level of expression in the RZ, may play an important role in the growth plate. There were no pathways identified as being uniquely enriched in the RZ by our analysis filters.

Hyaluronan and proteoglycan link protein (Hapln1) is an unsulfated glycosaminoglycan consisting of a single repeating disaccharide unit that comprises up to 10% of cartilage glycosaminoglycans [39]. Col9a1 is one of the main proteoglycan components of hyaline cartilage. The major proteinaceous component of the bone matrix, collagen I, is encoded for in part by Col1a2, which has been previously identified in the HZ of the growth plate in our own work [**Error! Bookmark not defined**.]. Secreted modular calcium-binding protein (SMOC-2) is a Sparc related protein that is expressed in nearly all tissues and has been demonstrated to show an up-regulation in response to injury [40]. Calpastatin (Cast) is an endogenous inhibitor protein acting specifically on calpain that has a known role in bone in the regulation of osteoclastogenesis, pre-osteoblastic proliferation and differentiation [41].

Of the three genes shown by microarray to be most highly expressed uniquely in the RZ, one (LOC497729) was confirmed by real-time PCR in the current work. Gene 6A3-5 (Transcription factor 1, 6A3-5) (LOC497729) is a member of the ARID transcription factor family involved in control of gene expression during cell growth, cell cycle, and organism development [42]. Ribosomal protein L37 (Rpl37) is the gene for ribosomal protein, the expression of which is likely determined mainly by cellular growth and proliferation [43].

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In summary, the current work identified a number of highly expressed genes in both the RZ and PC which related to extracellular matrix and skeletal development. In the perichondrium, Ctgf is uniquely highly expressed along with components of the Mapk signaling cascade. Wnt/b-catenin signaling is a common theme among genes highly expressed within both zones and some (Frzb) uniquely expressed in the PC. While no single pathway was identified among the highly expressed genes unique to the RZ, additional matrix components and genes related to Sparc were identified. These pathways should be the targets of additional evaluation to determine their roles not only in the normal growth plate but in response to injury.

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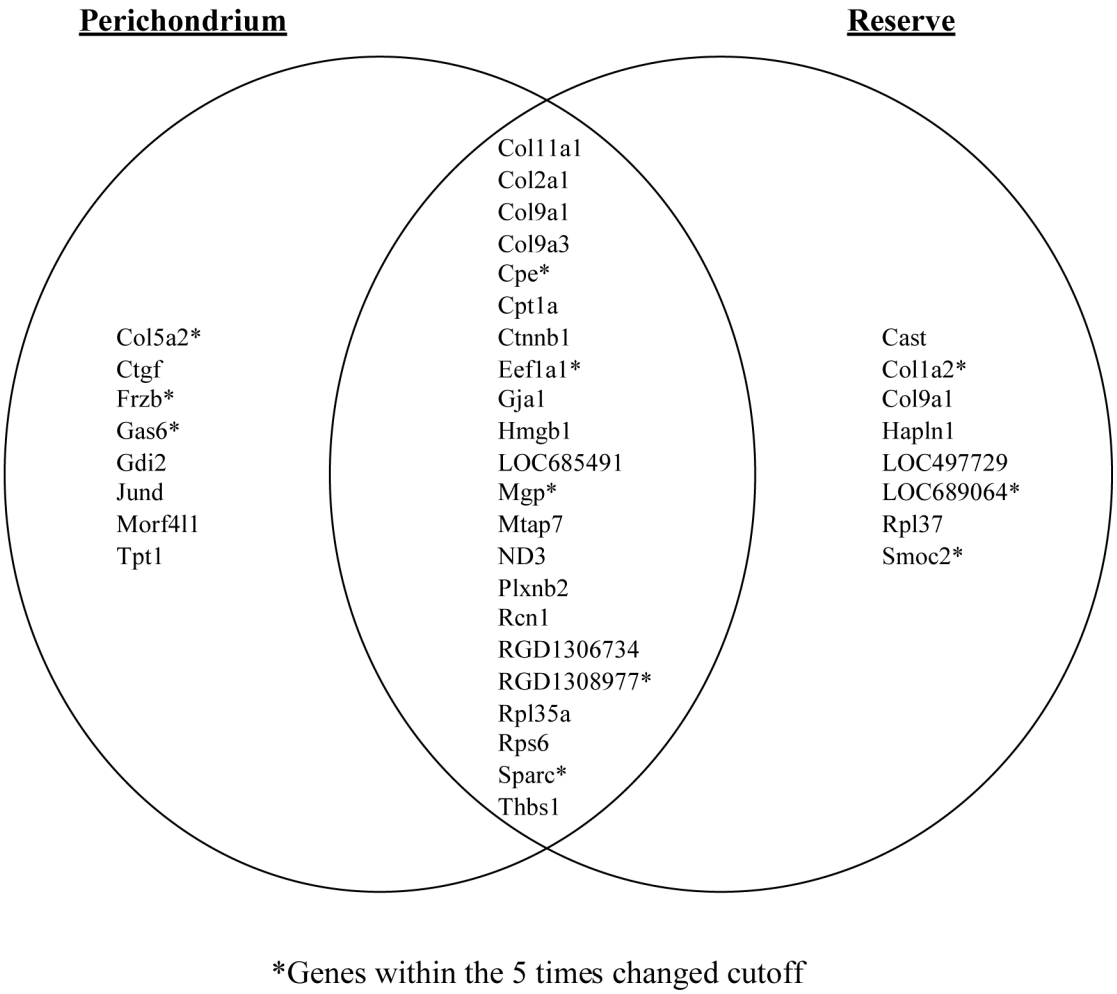

Figure 1. Top 30 genes showing the greatest expression in PC and RZ

Venn diagram of differential expression according to growth plate zone. A list of genes was designated as “present” in PC and RZ using Affymetrix GCOS/MAS 5 software. Differential gene expressions within PC and RZ were identified by GeneSpring GX and a raw dataset was created using the Robust Multiarray analysis (RMA) method. The top 30 genes by highest expression within both RZ and PC (individually) were identified, excluding unnamed transcribed locus probes and duplicate gene names. Twenty-two genes from the top 30 genes expressed within the PC were also present among the top 30 genes expressed within the RZ. Only 8 genes from each list were unique to the respective zone.

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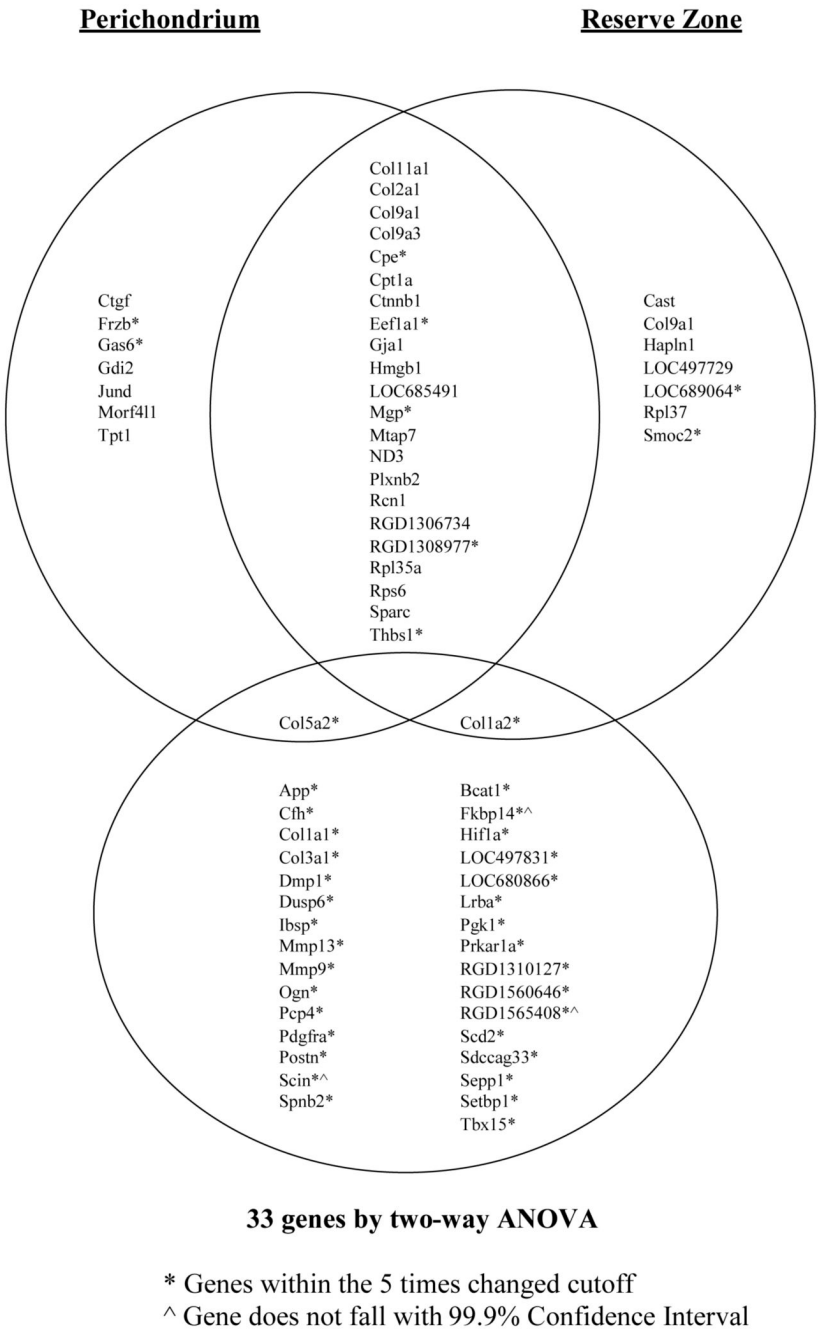

Figure 2. Top 30 genes in PC and RZ by greatest expression compared to 33 genes by two-way ANOVA and 5 times changed

To identify the significant differential gene expression between PC and RZ chondrocytes, the PZ was used as a comparator. Normalized data was filtered on expression using a cutoff of five times changed up or down in PC, RZ and PZ described as in the methods section. Differential expression between zones was analyzed using two-way ANOVA and created a gene list of 33 significant differentially expressed genes. When comparing the gene list of 33 significantly different than the PZ to the top 30 by expression in both PC and RZ, there was one gene in common for each of the zones.

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Table 1

Log_2 Ratio of 33 genes showing differential expression in PC and RZ by two-way ANOVA and Expression level cutoff of 5 times changed. Red indicates presence in positive 99.9% confidence level while green indicates presence in negative 99.9% confidence level.

33 Genes by 2-Way ANOVA Showing Differential Expression in RZ and PC								
			99.9% CI			99.9% CI		
		Mean Log2	0.01	0.02	0.03	0.00	0.01	0.01
		StDev	0.71	0.68	0.57	0.56	0.52	0.57
		+99.9 CL	2.42	2.30	1.97	1.89	1.77	1.93
		# Incr 99.9CL	384	495	122	321	355	308
		−99.9 CL	−1.38	−1.31	−1.10	−1.09	−1.02	−1.11
		# Decr 99.9CL	618	303	1112	781	496	715
			Log2 RZ/PZ			Log2 PC/PZ		
Probe ID	Gene Name	Gene Symbol	7 days	11days	16 days	7 days	11days	16 days
1388204_at	---	*Mmp13*	7.43	6.69	4.55	4.42	0.85	−1.46
1368416_at	integrin binding sialoprotein	*Ibsp*	5.88	7.37	4.62	3.95	6.43	1.59
1370864_at	procollagen, type 1, alpha 1	*Col1a1*	4.26	6.87	6.02	3.91	4.83	6.41
1398275_at	matrix metallopeptidase 9	*Mmp9*	6.32	4.89	1.97	0.87	−0.25	0.62
1370959_at	procollagen, type III, alpha 1	*Col3a1*	3.79	1.03	0.58	4.92	2.84	6.15
1393756_at	dentin matrix protein 1	*Dmp1*	3.71	5.87	3.44	−0.27	1.15	2.75
1370155_at	procollagen, type I, alpha 2	*Col1a2*	5.65	5.85	2.56	4.42	2.63	5.81
1370895_at	procollagen, type V, alpha 2	*Col5a2*	4.09	4.68	3.35	4.25	4.84	5.50
1387029_at	complement component factor H	*Cfh*	5.13	5.44	3.22	3.90	2.60	4.91
1370941_at	platelet derived growth factor receptor, alpha polypeptide	*Pdgfra*	3.02	2.99	2.07	3.02	2.81	4.48
1382778_at	Dual specificity phosphatase 6	*Dusp6*	2.27	4.28	2.66	0.65	2.38	−1.14
1373911_at	periostin, osteoblast specific factor	*Postn*	−0.22	0.63	0.33	3.09	1.56	3.96
1371571_at	amyloid beta (A4) precursor protein	*App*	2.57	1.75	−0.01	3.95	2.92	1.90
1371419_at	spectrin beta 2	*Spnb2*	3.74	3.40	1.45	1.64	0.15	0.51
1368145_at	Purkinje cell protein 4	*Pcp4*	3.41	0.40	0.59	3.66	2.33	2.64
1390450_a_at	osteoglycin (predicted)	*Ogn*	2.72	2.45	0.03	2.95	3.22	3.40
1368806_at	selenoprotein P, plasma, 1	*Sepp1*	2.84	3.28	1.49	1.98	0.87	2.33
1383916_at	T-box 15	*Tbx15*	2.09	1.85	0.40	1.77	2.33	2.86
1389562_at	SET binding protein 1	*Setbp1*	1.10	0.00	0.12	2.47	1.43	2.82
1391537_at	similar to SERTA domain containing 4	*RGD1565408*	2.22	−0.05	0.55	2.02	0.01	2.80
1373951_at	Protein kinase, cAMP dependent regulatory, type I, alpha	*Prkar1a*	0.98	2.77	0.33	1.39	2.73	1.43
1378988_at	LPS-responsive beige-like anchor	*Lrba*	1.94	0.20	0.06	2.63	0.40	0.63
1383573_at	Serologically defined colon cancer antigen 33	*Sdccag33*	−0.26	1.13	0.93	1.73	1.72	2.12
1384195_at	---	*LOC680866*	0.75	0.91	−1.73	1.45	1.87	0.70
1387076_at	hypoxia inducible factor 1, alpha subunit	*Hif1a*	0.15	1.35	−1.21	1.15	1.86	0.73
1367668_a_at	stearoyl-Coenzyme A desaturase 2	*Scd2*	0.53	1.86	−2.01	0.22	1.29	−0.90
1389330_at	similar to cDNA sequence BC017158	*RGD1310127*	1.75	0.75	0.74	0.08	0.11	−0.47
1372525_at	FK506 binding protein 14	*Fkbp14*	1.62	0.93	−0.36	1.31	0.46	1.37
1388318_at	phosphoglycerate kinase 1	*Pgk1*	1.17	1.58	−1.56	0.74	0.64	−0.52
1370869_at	branched chain aminotransferase 1, cytosolic	*Bcat1*	0.51	0.94	−1.43	0.25	0.58	1.28
1391279_at	Scinderin	*Scin*	0.90	−0.17	−0.94	−0.27	−0.20	0.80
1372897_at	hypothetical gene supported by NM_175869	*LOC497831*	−0.80	0.75	−2.12	−0.16	0.23	−0.73
1376804_at	similar to Myosin VI	*RGD1560646*	−0.60	−0.23	−1.36	−0.54	−0.89	−2.03
				Falls within +99.9% CI				
				Falls within −99.9% CI				

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Table 2

Primers and targeted genes in real time PCR

Systematic Name	Gene symbol	Gene Title	Amplicon Size (bp)	Sequences (5' – 3')	
	18 srRNA		149	Forward:	GGTCATAAGCTTGCGTTGAT
				Reverse:	TCAAGTTCGACCGTCTTCTC
1367568_a_at	Mgp	matrix Gla protein	127	Forward:	GCCTACAACCGCTACTTCAG
				Reverse:	CGCACACGAATCTGTGTATC
1383047_at	Gas6	Growth arrest specific 6	129	Forward:	GTACATAGTGGGACTGATAC
				Reverse:	TTCTTCAGATAGTCCCGCTC
1374529_at	Thbs1	Thrombospondin 1	122	Forward:	CTTAAAACTACTGTCGTGTC
				Reverse:	ACCAGAACTTTGAGGAGTTG
1375961_at	Frzb	Frizzled-related protein	127	Forward:	ATTTGGCTCGCTGTATTGAC
				Reverse:	GTGCACATTTCACTGTCCTG
1392915_at	Col11a1	Procollagen type IX alpha 1	156	Forward:	GCCAATCCATTTTATGCCAC
				Reverse:	ATGAGCCCTGTTGCCATCTC
1370944_at	Col10a1	Procollagen type X alpha 1	141	Forward:	GAAACAGGTGTCTGACTTAC
				Reverse:	TACTTCCAGTGGAATAGAAG
1367562_at	Sparc	Secreted acidic cysteine rich glycoprotein	147	Forward:	TGGGCTACAGGAAAGTGAGA
				Reverse:	TGGCTTGGAAGTTTCTCTTG
1372299_at	Cdkn1c	Cyclin-dependent kinase inhibitor 1C	125	Forward:	ACCAGCTTCAGATTACCCAC
				Reverse:	TCTAATAATGTGGAGGACAC
1371245_a_at	LOC689064	hemoglobin beta-2 subunit	108	Forward:	CCTTTCCTGCTTGTCTATGC
				Reverse:	TTTATTAGATAGAAGCTAGC
1387854_at	Col1a2	Procollagen type I alpha 2	136	Forward:	GAAGACTCGATCCTCCATAG
				Reverse:	ATGCTGAATCTAGAGAGGAG
1381925_x_at	LOC497729	hypothetical gene supported by NM_172157	125	Forward:	GAAGAAACACAACTTCAAAG
				Reverse:	GAAAAGGAGGAATTTACATC
1367631_at	CTGF	Connective tissue growth factor	154	Forward:	GACGTTTGTGCCTATTGTTC
				Reverse:	CAGACAGTCACTCAGGTTAC
1367668_a_at	Scd2	Stearoyl-coenzyme A desaturase 2	131	Forward:	GCACATTTTATAGTAACCG
				Reverse:	TTCCATAGCAATGAGTATAC
1374870_at	Col27a1	Procollagen type XXVII alpha 1	126	Forward:	AACCCTTCCCTGTATACTGC
				Reverse:	AAGGAGACAGCGCAAGACAC
1392965_a_at	Smoc2	Sparc-related modular calcium binding 2	124	Forward:	AAACTGTTCATGGTCCCCAG
				Reverse:	AGCGTTGATGTGAGCTGGTC
1373463_at	Col5a2	Procollagen type V alpha 2	109	Forward:	CCATCAATGACCAACACAAG
				Reverse:	GGTCAGGCACTTCAGATCAT
1371226_at	Col2a1	Procollagen type II alpha 1	104	Forward:	CCGGACTGTGAGGTTAGGAT
				Reverse:	AACCCAAAGGACCCAAATAC

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Table 3

Top 30 Genes in Perichondrium by highest expression

Gene Title	Gene Symbol	7 days		11 days		16 days	
		MC	PCR	MC	PCR	MC	PCR
procollagen, type II, alpha 1	Col2a1	10414	4672.6	5941	677.93	10595	7643.4
procollagen, type IX, alpha 3	Col9a3	5082		3097		2785	
Similar to RIKEN cDNA 1110017I16	RGD1308977	2951		1092		5071	
secreted acidic cysteine rich glycoprotein	Sparc	2291	4.91	927	−37.92	4972	3.25
eukaryotic translation elongation factor 1 alpha 1	Eef1a1	1659		622.3		4296	
NADH dehydrogenase subunit 3	ND3	3765		3314		2957	
high mobility group box 1	Hmgb1	3598		3739		2064	
matrix Gla protein	Mgp	1068	53.08	324.2	17.15	3665	411.57
tumor protein, translationally-controlled 1	Tpt1	2110		1748		3647	
connective tissue growth factor	Ctgf	1159	4.06	1714	6.52	3618	524.57
ribosomal protein L35a	Rpl35a	2593		3057		1513	
mortality factor 4 like 1	Morf4l1	945.2		732.1		2911	
procollagen, type V, alpha 2	Col5a2	1267	10.20	528.7	28.25	2897	92.73
growth arrest specific 6	Gas6	273	1.13	113.3	1.55	2816	151.17
Plexin B2	Plxnb2	2277		2112		2730	
Catenin (cadherin associated protein), beta 1	Ctnnb1	2600		2185		2717	
Microtubule-associated protein 7	Mtap7	2330		2640		1127	
reticulocalbin 1	Rcn1	1996		1892		2504	
Thrombospondin 1	Thbs1	763.2	22.16	328.4	36.25	2360	461.44
Similar to hypothetical protein FLJ32743	RGD1306734	1539		2359		917.8	
procollagen, type IX, alpha 1	Col9a1	1824		710.9		2358	
similar to retinoblastoma binding protein 4	LOC685491	2340		1873		1292	
ribosomal protein S6	Rps6	1725		858.7		2321	
procollagen, type XI, alpha 1	Col11a1	2183	59.71	1013	28.35	2112	269.66
carboxypeptidase E	Cpe	661		209.4		2132	
Carnitine palmitoyltransferase 1a, liver	Cpt1a	1285		2130		1699	
frizzled-related protein	Frzb	479.1	1.61	252.3	3.43	2093	54.00
gap junction membrane channel protein alpha 1	Gja1	1288		2047		822.6	
GDP dissociation inhibitor 2	Gdi2	769.4		460		2019	
Jun D proto-oncogene	Jund	1148		1969		394.5	
Correlation coefficient R		0.9773		0.9523		0.9336	

Note: Among the top 30 genes in PC by highest expression in microarray (MC) analysis, nine selected representative genes were confirmed by real time PCR (PCR) with high correlation coefficient ($R > 0.9$). The comparative threshold ($\Delta\Delta C_T$) method was utilized for relative quantitative analysis of gene expression in real time PCR. The threshold cycle (C_T) was normalized to the reference gene and the numbers represent relative quantitation for gene expression level.

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Table 4

Top 30 Genes in Reserve Zone (RZ) by highest expression

Gene Title	Gene Symbol	7 days		11 days		16 days	
		MC	PCR	MC	PCR	MC	PCR
procollagen, type II, alpha 1	Col2a1	11250	15076	4313	916.5	6432	594.3
high mobility group box 1	Hmgb1	2951		3613		4480	
procollagen, type IX, alpha 3	Col9a3	4269		3031		4477	
NADH dehydrogenase subunit 3	ND3	3689		2909		3270	
Similar to RIKEN cDNA 1110017I16	RGD1308977	3629		757.1		884.7	
Microtubule-associated protein 7	Mtap7	2038		2110		3236	
secreted acidic cysteine rich glycoprotein	Sparc	3225	13.00	619.7	1.53	1071	−22.09
Similar to hypothetical protein FLJ32743	RGD1306734	1326		1735		3007	
ribosomal protein L35a	Rpl35a	2131		2127		2708	
Plexin B2	Plxnb2	1972		2693		2219	
Catenin (cadherin associated protein), beta 1	Ctnnb1	2399		2653		2418	
reticulocalbin 1 (predicted)	Rcn1	2498		1974		2566	
Hyaluronan and proteoglycan link protein 1	Hapln1	1164		991.7		2516	
procollagen, type XI, alpha 1	Col11a1	2506	93.70	1072	66.26	1686	48.84
Carnitine palmitoyltransferase 1a, liver	Cpt1a	2307		1502		1529	
gap junction membrane channel protein alpha 1	Gja1	1178		2157		1114	
similar to Hemoglobin beta-2 subunit	LOC689064	935.8	28.05	2119	21.11	154	152.70
procollagen, type I, alpha 2	Col1a2	2019	272.48	1822	13	1207	109.50
procollagen, type IX, alpha 1	Col9a1	1969		867.1		1008	
similar to retinoblastoma binding protein 4	LOC685491	1490		1950		1639	
eukaryotic translation elongation factor 1 alpha 1	Eef1a1	1907		1140		738.5	
SPARC related modular calcium binding 2	Smoc2	1901	67.18	107.1	15.30	38.31	13.78
carboxypeptidase E	Cpe	1879		691.9		534.8	
ribosomal protein S6	Rps6	1858		772.9		567.6	
ribosomal protein L37	Rpl37	1759		803.5		578.2	
hypothetical gene supported by NM_172157	LOC497729	670.6	10.93	1759	4.96	962.8	21.48
Calpastatin	Cast	789		942.5		1756	
matrix Gla protein	Mgp	1742	129.34	162.6	36.33	82.63	3.04
similar to DnaJ homolog subfamily B member 6	LOC686213	676		1699		1473	
ribosomal protein L13A	Rpl13a	1612		656.3		428.9	
Correlationi coefficient R		0.9720		0.8228		0.9152	

Note: Among the top 30 genes in RZ by highest expression in microarray (MC) analysis, eight selected representative genes were confirmed by real time PCR (PCR) with high correlation coefficient ($R > 0.8$). The comparative threshold ($\Delta\Delta C_T$) method was utilized for relative quantitative analysis of gene expression in real time PCR. The threshoyld cycle (C_T) was normalized to the reference gene and the numbers represent relative quantitation for gene expression level..

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Table 5

LOG2 Ratio of microarray and real time PCR between PC/RZ, RZ/PZ, PC/PZ

		PC/RZ						RZ/PZ						PC/PZ					
		7days		11days		16days		7days		11days		16days		7days		11days		16days	
	Gene Symbol	MC	PCR	MC	PCR	MC	PCR	MC	PCR	MC	PCR	MC	PCR	MC	PCR	MC	PCR	MC	PCR
type II,	Col2a1	−0 11	−1.69	0.46	−0.44	0.72	3.69	0.68	4.67	−0.17		−0.40	−1.27	0.57	2.98	0.30		0.32	2.42
lic cysteine otein	Sparc	−0.49	−1.52	0.58	−2.64	2.21	6.17	2.98	3.64	0.47	2.70	−1.32	−4.64	2.49	2.12	1.05	1.30	0.90	1.53
rotein	Mgp	−0.71	−1.29	1.00	−1.10	5.47	7.08	3.36	5.13	4.26	9.10	−0.92	−1.89	2.66	3.85	5.26	8.01	4.55	5.19
ssue growth	Ctgf	−0.29	−1.70	0.89	0.07	1.51	3.53	0.06	3.42	1.20	1.60	0.14	0.41	−0.23	1.72	2.09	7.31	1.65	3.94
type V,	Col5a2	0.75	−0.36	−0.91	−0.64	2.55	2.76	2.05	4.58	5.30		3.72	2.28	2.80	4.22	4.39		6.27	5.03
t specific 6	Gas6	−0.41	−1.48	−0.13	−2.77	5.00	7.14	1.58	4.21	2.93		−0.16	−1.37	1.17	2.73	2.80		4.84	5.78
ndin 1	Thbs1	1.25	1.58	0.81	0.81	2.91	4.95	−0.37	0.45	1.49		−1.67	−2.40	0.87	2.03	2.30		1.24	2.55
type XI,	Col11a1	−0.20	−0.70	−0.08	−1.23	0.33	2.47	−0.14	−0.97	−0.07		−2.04	−3.40	−0.34	1.34	−0.15		−1.72	−0.94
ed protein	Frzb	−0.85	−0.74	2.85	0.25	6.11	5.67	4.88	7.11	0.09		−3.19	−1.63	4.03	7.11	2.94		2.92	4.05
type I, alpha	Col1a1	−0.22	−1.31	−0.96	−5.47	0.29	2.07	3.65	5.95	5.19		5.23	3.62	3.43	4.64	4.23		5.52	5.68
type a 1	Col2a1	−1.22	−2.08	−0.29	−2.86	1.44	2.58	2.71	5.35	−0.86		−1.60	−2.08	1.50	3.27	−1.15		−0.16	0.49
gene	LOC497729	0.52	2.66	−0.46	1.79	−1.52	1.36	−0.39	−0.53	2.37	3.50	0.80	−0.71	0.13	2.13	1.92	1.75	−0.73	0.65
ed modular ling 2	Smoc2	−0.80	−1.47	1.77	1.02	4.93	3.30	5.85	5.15	2.59		−0.98	−1.00	5.06	3.68	4.36		3.95	2.30
ndent kinase (P57)	Cdkn1c	0.69	0.02	0.46	0.50	0.92	0.22	0.52	3.39	−0.24		−3.31	−3.00	1.21	3.41	0.22		−2.39	−2.78
nzyme A	Scd2	−0.32	−1.47	−0.57	−1.79	1.11	2.78	0.53	2.84	1.86		−2.01	−2.51	0.22	1.37	1.29		−0.90	0.27
type X,	Col10a1	2.20	1.96	3.90	2.82	5.48	5.36	−0.49	1.52	−0.38		−8.00	−4.64	1.71	3.47	3.52		−2.52	−0.91
emoglobin it	LOC389064	−6.01	−6.93	−8.47	−11.69	−4.06	−2.86	7.66	8.72	9.20		−1.11	−0.04	1.64	1.79	0.73		−5.16	−2.90
coefficient R		0.9136		0.8792		0.8486		0.8482		0.95		0.8549		0.7471		0.7572		0.9414	

ratios of microarray (MC) and real time PCR (PCR) between zones (PC/RZ, RZ/PZ and PC/PZ) show high correlation coefficient (R = 0.7471~0.9414) and confirmed the microarray data.

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Table 6

Enriched pathways related to other than bone, cartilage, matrix and/or skeletal development comprised of 5 or more probe sets from our data and their corresponding zones

GO ID	GO Term	GP Zone(s)	
902	cell morphogenesis	PC	
3735	structural constituent of ribosome		RZ
5198	structural molecule activity	PC	RZ
5509	calcium ion binding	PC	
5576	extracellular region	PC	RZ
5615	extracellular space	PC	RZ
5829	cytosol		RZ
5840	ribosome		RZ
6412	translation		RZ
6811	ion transport	PC	RZ
6817	phosphate transport	PC	RZ
6820	anion transport	PC	RZ
7155	cell adhesion	PC	RZ
8283	cell proliferation	PC	
9059	macromolecule biosynthetic process		RZ
9887	organ morphogenesis	PC	
9888	tissue development	PC	RZ
15698	inorganic anion transport	PC	RZ
16337	cell-cell adhesion	PC	
22610	biological adhesion	PC	RZ
30529	ribonucleoprotein complex		RZ
31214	biomineral formation	PC	
32989	cellular structure morphogenesis	PC	
44421	extracellular region part	PC	RZ
48513	organ development	PC	RZ
48731	system development	PC	
48771	tissue remodeling	PC	
65008	regulation of biological quality	PC	

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Table 7

Perichondral zone (PC) and Reserve Zone (RZ) enriched pathways of 33 genes by two-way ANOVA showing hypergeometric p values ≤ 0.05, FER ≥ 5 and ≥ 2 probe sets. Gray indicates pathways association with bone, cartilage, matrix and/or skeletal development.

GO Category	GO ID	GO Term	% of Array	% of list	Fold Enrichment	P value
Biological	48513	organ development	5.52%	34.69%	6.29	0.000000
Biological	1501	skeletal development	1.09%	24.49%	22.53	0.000000
Cellular	5578	proteinaceous extracellular matrix	0.90%	24.49%	27.11	0.000000
Cellular	31012	extracellular matrix	0.92%	24.49%	26.64	0.000000
Biological	9888	tissue development	1.52%	20.41%	13.40	0.000000
Biological	1503	ossification	0.59%	16.33%	27.66	0.000000
Biological	31214	biomineral formation	0.59%	16.33%	27.66	0.000000
Biological	46849	bone remodeling	0.68%	16.33%	24.10	0.000000
Biological	48771	tissue remodeling	0.75%	16.33%	21.82	0.000000
Biological	6817	phosphate transport	0.35%	14.29%	41.01	0.000000
Biological	15698	inorganic anion transport	0.66%	14.29%	21.60	0.000000
Biological	6820	anion transport	0.80%	14.29%	17.93	0.000000
Cellular	5583	fibrillar collagen	0.06%	14.29%	246.03	0.000000
Cellular	5581	collagen	0.18%	14.29%	79.08	0.000000
Cellular	44420	extracellular matrix part	0.43%	14.29%	33.05	0.000000
Molecular	5201	extracellular matrix structural constituent	0.33%	14.29%	43.85	0.000000
Molecular	5198	structural molecule activity	2.59%	14.29%	5.51	0.000221
Biological	30198	extracellular matrix organization and biogenesis	0.25%	10.20%	41.62	0.000000
Biological	43062	extracellular structure organization and biogenesis	0.53%	10.20%	19.17	0.000006
Biological	6800	oxygen and reactive oxygen species metabolic process	0.60%	10.20%	16.92	0.000011
Cellular	5584	collagen type I	0.01%	8.16%	632.65	0.000000
Molecular	8147	structural constituent of bone	0.03%	8.16%	316.33	0.000000
Molecular	5506	iron ion binding	1.03%	6.12%	5.97	0.012311
Cellular	5588	collagen type V	0.02%	4.08%	210.88	0.000036
Molecular	8133	collagenase activity	0.02%	4.08%	253.06	0.000024
Molecular	4222	metalloendopeptidase activity	0.32%	4.08%	12.65	0.010439
Molecular	16705	oxidoreductase activity, acting on paired donors	0.45%	4.08%	8.97	0.019554
Molecular	4866	endopeptidase inhibitor activity	0.55%	4.08%	7.44	0.027229
Molecular	30414	protease inhibitor activity	0.55%	4.08%	7.40	0.027509
Molecular	8237	metallopeptidase activity	0.65%	4.08%	6.30	0.036338