COVER SHEET

Gene Expression Profiling of Bone Marrow Stromal Cells from Juvenile, Adult, Aged and Osteoporotic Rats: With an Emphasis on Osteoporosis

Yin Xiao [1*], Huihua Fu [2], Indira Prasadam [1], Yaw-Ching Yang [3], Jeffrey O. Hollinger [2]

[1] Institute of Health and Biomedical Innovation,
Queensland University of Technology, Brisbane, Australia

[2] Bone Tissue Engineering Center, Department of Biomedical Engineering,
Carnegie Mellon University, Pittsburgh, USA

[3] Windber Research Institute, Windber, Pennsylvania, USA

RUNNING TITLE: Gene expression profiling of osteoporosis
Key words: Osteoporosis, Bone marrow stromal cells, cDNA microarray.

CORRESPONDING AUTHOR
Dr. Yin Xiao
Associate Professor,
Bone Tissue Engineering
Institute of Health and Biomedical Innovation
School of Engineering Systems
Queensland University of Technology
Kelvin Grove, Qld 4059, Australia
Ph: Tel: 61-7-31386240
Fax: 61-7-31386030
Email: yin.xiao@qut.edu.au

ABSTRACT:

PURPOSE: Osteoporosis is a multi-factorial, age-related disease with a complex etiology and mode of regulation involving a large numbers of genes. To better understand the possible relationships among genes, we fingerprinted genes in a rat model **induced by ovariectomy** to determine differences among osteoporotic, non-osteoporotic, aged and juvenile rats.
METHODS: We applied genome wide cDNA microarray technology to analyze genes expressed in bone marrow mesenchymal stromal cells (BMSC) and compared non-osteoporotic adult vs osteoporotic, non-osteoporotic adult vs aged, and non-osteoporotic adult vs juvenile. Rigorous statistical analysis of functional annotation (EASE program) identified over-represented biological and molecular functions with significant group wide changes ($p \leq 0.05$). Some of the expressed genes were further confirmed by quantitative RT-PCR (reverse transcription-polymerase chain reaction)
RESULTS: Differences in gene expression were observed by identifying transcripts selected by t-test that were consistently changed by a minimum of two-fold. There were 195 transcripts that showed an increased expression and 109 transcripts that showed decreased expression relative to the osteoporotic condition. Of these, 75% transcripts were unknown gene products or ESTs (expressed sequence tag). A number of genes found in the aged and juvenile groups were not present in the osteoporotic rats. Functional clustering of the genes using the EASE bioinformatics program revealed that transcripts in osteoporosis were associated with signal transduction, lipid metabolism, protein metabolism, ionic and protein transport, neuropeptide and G-protein signaling pathways. Although some of the genes have previously been shown to play a key role in osteoporosis, several genes were uniquely identified in this study and likely play a role in developing aged related osteoporosis that could have compelling implications in the development of new diagnostic strategies and therapeutics for osteoporosis.
CONCLUSIONS: These data suggest that osteoporosis is associated with changes of multiple novel gene expression and that numerous pathways could play important roles in osteoporosis pathogenesis.

INTRODUCTION:

Aging of the human skeleton is characterized by decreased bone formation and bone mass. These changes are more pronounced in patients with osteoporosis. Osteoporosis is a silent epidemic, characterized by low bone density, leading to low trauma fractures (i.e., fragility fractures) among the elderly [1-3]. Fragility is due to intrinsic skeletal factors such as low bone mass, diminished cancellous bone, trabecular fenestrations and an imbalance in bone formation and resorption [4-6].

Osteoporosis is a complex disease with multifactorial determinants that include life style and hormonal influences as well as genetics. **Estrogen plays a fundamental role in skeletal turnover and bone homeostasis. Many animal studies on osteoporosis are using ovariectomy to represent an optimal osteoporotic model to investigate the effects of estrogen deficiency.** It has been noted that osteoporosis and its associated phenotypes are under strong genetic control [7-10]. Identification and characterization of either specific loci or genes involved in determining osteoporosis will contribute to a greater understanding of the pathogenesis of osteoporosis and could lead to the development of innovative diagnostic and treatment strategies.

Consequently, the objective of the study was to use microarray technology to identify gene expression differences in BMSC among juvenile, adult, osteoporotic **associated with estrogen deficiency** and aged rats. Adult bone marrow stroma contains a subset of non-hematopoietic cells referred to as mesenchymal stem cells (MSC) or mesenchymal progenitors. These cells have the capacity to differentiate to osteoblasts. The osteoporotic condition involves osteoblast dysfunction. Consequently, since MSCs can differentiate into osteoblasts, it is logical to study gene expression in the MSC: an osteoblast pre-cursor cell.

Gene expression and early microarray studies have shown that there are at least 200 genes directly or indirectly involved in bone metabolism which may contribute to either development or prevention of osteoporosis [11]. Recently, the first *in vivo* microarray study directly measuring osteoporosis in human circulating monocytes was reported by Liu *et al*. [12]. Further studies using microarrays described gene expression in the human osteoclast differentiation [13-16]. Therefore, we decided that microarray techniques would be suitable to study gene expression in osteoblast pre-cursor cells (i.e., MSCs) to determine if different gene expression patterns occurred among non-osteoporotic, osteoporotic, geriatric and juvenile rats.

MATERIALS AND METHODS:

Experimental design, tissue culture and RNA isolation:

The study was approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Pittsburgh and Carnegie Mellon University and complies with the Animal Care and Use Guidelines of the NIH. The surgical facilities at the University of Pittsburgh where surgeries were performed are AAALAC approved (American Association for Accreditation of Laboratory Animal Care).

Briefly, four types of physiological conditions were emphasized in female Lewis rats. These included: geriatric rats (more than two years old), osteoporotic rats (seven months old), non-osteoporotic adult rats (seven months old) and juvenile rats (seven weeks old). **Each group contained three animals for individual BMSC isolation and subsequent microarray studies. There were three microarray data generated for each group. The RNA samples left from microarray study were used for real time PCR to confirm microarray result. Thus, each group generated three set of**

data for statistics. The fold of changes of interested gene expression was presented by mean value.

Osteoporosis was induced by ovariectomy of three-month old rats, followed by a 30% caloric reduced diet and four months to develop osteopenia [5, 17]. Histological validation of these animal models demonstrated significant loss of bone density in the rats' tibiae and femurs (Figure 1), with typical fenestrated trabeculae.

Isolation and culture of rat BMSCs

Three bone marrow samples from each group of rats were isolated by flushing the femurs with 10 ml mesenchymal stem cell culture media (HyClone, Logan, UT), supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin/100 μg/ml streptomycin, 2 mM glutamine. Clumps of bone marrow were gently minced with a pipette. Cells were centrifuged for 10 min at 1000 rpm, washed by the addition of fresh medium, centrifuged again, re-suspended and plated out in the same stem cell culture media mentioned above at a density of ~$2x10^6$ cells/cm^2 in 25 cm^2 plastic culture dishes. The cells were incubated at 37°C in 5% CO_2.

Non-adherent cells were removed by replacing the medium after 7 days. Cells were grown for a further seven days to confluency, then washed with phosphate-buffered saline (PBS) and lifted by incubation with 0.25% trypsin/2 mM EDTA for 5 min. **Non-detached cells were discarded and the remaining cells were regarded as passage 1 of the BMSC culture and further cultured for seven days before RNA extraction. In our previous study we have demonstrated that all adherent cells do not express haematopoietic markers such as CD34 and CD45, and are positive for CD29, CD 73, CD90, CD105 and CD166 (data not shown).**

RNA isolation

Total RNA was isolated using TRIZOL Reagent (Invitrogen, CA, USA). Assessment of the concentration and quality of the total RNA samples were carried out by spectrophotometry and Agilent Bioanalyzer.

Microarray procedure:

CodeLink Rat Whole Genome Bioarrays (GE HealthCare, Piscataway, NJ, USA) were used for this study. Biotin-labeled cRNA target was prepared by linear amplification methods as described in CodeLink Protocols. The poly (A) + RNA subpopulation (within the total RNA population) was primed for reverse transcription by a DNA oligonucleotide containing the T7 RNA polymerase promoter 5' to a d (T) 24 sequences. After second-strand cDNA synthesis, the cDNA served as the template for an *in vitro* transcription (IVT) reaction to produce the target cRNA. The IVT was performed in the presence of biotinylated nucleotides to label the target cRNA. This method produced approximately 1000-fold to 5000-fold linear amplification of the input poly (A) + RNA.

To monitor each key step, a set of bacterial control mRNAs was included in the cDNA synthesis and the IVT reactions. In addition, these bacterial control mRNAs were used to estimate the sensitivity of RNA detection. An aliquot of the labeled cRNA was run on Agilent's Bioanalyzer for qualification and quantization. Only high quality cRNA with yield of more than 10 μg were fragmented and hybridized to CodeLink Bioarrays. Hybridization was performed overnight in a temperature-controlled incubator shaker. Most of the labeling and hybridization reagents were included in GE CodeLink Expression Assay Reagent Kit.

Post hybridization processing

Post-hybridization processing included a stringent wash to remove unbound and non-specifically hybridized target molecules, a staining step with CyTM5-Streptavidin

conjugate, and several non-stringent washing steps to remove unbound conjugate. Following a final rinse, the Bioarrays were spun dry and ready to be scanned.

Image and data analysis:

All CodeLink Bioarrays were scanned using ScanArray 5000 (PerkinElmer, Wellesley, MA, USA). Scan images were analyzed using CodeLink Expression software to generate raw data. Raw data were imported into GeneSpring software 7.2 (Agilent, Palo Alto, CA, USA). Log transform of raw data were normalized to: first, the median intensity per chip; and second, the median intensity per gene. T-tests were performed on normalized data to identify differentially expressed genes between different treatment groups ($p \leq 0.05$). These genes were filtered by fold change and only genes with at least a two-fold change between treatment groups were selected for further analysis.

Quantitative RT-PCR confirmation:

According to the representative expression from microarray data among four different physiological groups, nine genes were selected for quantitative RT-PCR confirmations of the hybridized results. Total RNA was isolated using Trizol reagent as described above. After treatment with DNase I to eliminate possible contamination of genomic DNA, RNA was quantified using RiboGreen fluorescent dye (Molecular Probes, Inc. Eugene, OR). Quantitative real-time RT-PCR (q-RT-PCR) was performed using TaqMan Reverse Transcription Reagents and SYBR Green PCR Master Mix (Applied Biosystems Foster City, CA) in an ABI Prism 7000 sequence detection system. In brief, 30-50 ng of total RNA, 6 units of TaqMan reverse transcriptase, 25 pmole of gene specific primers and 12.5 μl of 2x Master Mix were used in a 25 μl reaction volume. Quantitative RT-PCR reactions were set up in triplicates. The thermocycling conditions were as follows: 1 cycle of 30 minutes at 48 ºC for reverse transcription, 1 cycle of 10 minutes at 95 ºC for activation of the polymerase, 40 cycles of 15s at 95 ºC and 1 minute at 60 ºC for amplification. After the final cycle of the amplification, the dissociation curve analysis was carried out to verify that no primer dimer and/or non-specific amplified products were produced. The primer sequences of the testing genes were listed in table 1. The housekeeping gene glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) was used as an endogenous reference gene to normalize the calculation by Comparative Ct (Cycle of threshold) value method [18]. The ΔC_T value was obtained by subtracting the GAPDH Ct value from the test gene Ct value of the same samples. The $\Delta\Delta C_T$ was determined by subtracting the ΔC_T of target sample from the ΔC_T of the control sample. The relative mRNA quantification of the target gene was given by $2^{-\Delta\Delta CT}$.

Analysis of functional clustering and over representation of differentially expressed genes.

Bioinformatics (EASE program, see http://apps1.niaid.nih.gov/david) and literature analyses were performed to identify functional implications of differentially expressed genes by 2 fold. For the experiments described here, we used only the "biological process" and "Molecular function" Ontologies. Gene ontology classes with fewer than four or more than 30 genes represented in the data were not considered.

RESULTS:

Overview of differentially expressed genes

A total of 34,000 genes were spotted on CodeLink Rat Whole Genome Bioarrays, of which 29,000 were well-substantiated rat genes. Differences in gene expression were

observed by identifying transcripts selected by t-test that were consistently changed by a minimum of two-fold. A balanced differential expression of 2 fold or higher has been shown previously to be significant [19-21]. Of the identified genes among osteoporotic vs seven-month old non-osteoporotic, 195 transcripts showed increased expression and 109 transcripts showed decreased expression, with 75% transcripts being ESTs or unknown gene products.

Of the seven-month old non-osteoporotic vs aged rats, 62 genes were up-regulated and 86 genes were down-regulated. The remaining 86% genes that differentially expressed were either unknown or ESTs. In addition, there were 120 genes up-regulated and 80 genes down-regulated in the comparison between the juvenile rat and the seven-month old non-osteoporotic.

A list of differentially expressed genes in osteoporosis, assigned with the gene name and symbol are shown in Table 2.

Functional gene grouping:

Using the Gene Ontology (GO) tool EASE [22], we grouped the transcripts based on the Biological process and Molecular function. This tool is useful to determine the over-represented categories in the list of differentially expressed genes. Because of the small number of down-regulated genes identified by gene spring in osteoporosis, statistical analysis for significance of GO group changes with this gene list was considered not valid.

The individual annotation of the genes suggested most of the genes that were down-regulated were involved in hormone activity, for example neuropeptide y (Npy), prolactin like protein C, prolactin like protein B and cell growth and maintenance, such as Npy, Ramp3, Kcnk1, MlP and RIM 2.

The main gene groups over-represented in the comparison between seven-month old non-osteoporotic vs the osteoporotic involved genes associated with signal transduction, protein and lipid metabolism, G-protein and neuropeptide signaling pathways.

When comparing up-regulated genes among aged vs. seven-month old non-osteoporotic, the predominant gene groups included apoptosis, G-protein coupled receptor protein signaling pathway, cell growth and maintains and transport. Where as catabolism (protein and macromolecule), protein and peptidolysis and immune response were predominant over represented groups among the down-regulated genes between aged vs seven month old non-osteoporotic. Furthermore, relatively large gene group changes were observed between juvenile vs. seven-month old non-osteoporotic, compared to other age groups. These differences included biosynthesis, cell proliferation, organogenesis, metabolism (nucleobase, nucleotide, nucleic acid metabolism, phosphate, carboxylic acid, and lipid metabolisms), ion transport, macromolecule catabolism, immune response and signaling pathways.

In terms of the molecular function, oxido reductase activity, structural molecule activity, metal ion binding, and catalytic activities were over-represented in up-regulated osteoporotic rat vs non-osteoporotic gene expression profiles of BMSCs. However, most of the up-regulated genes among aged vs seven month old non-osteoporotic were involved in the signal transducer activity, receptor activity (rhodopsin and G-protein coupled and transmembrane). Moreover, the endopeptidase activity, calcium ion binding and kinase activity were over represented groups among the down regulated genes between aged vs seven month non-osteoporotic. Overall, juvenile vs seven months old non-osteoporotic gene expression profiles were enriched with categories like hormone activity, cell adhesion molecule activity and receptor activity.

Gene classification of non-osteoporotic adult vs osteoporotic

The genes were grouped according to development, metabolism, catabolism, transport, biosynthesis, apoptosis, cell growth and differentiation.

Skeletal development:

The genes Slit3, Nog, Comp are structural constituents important for skeletal development. Up-regulation of these genes was determined in the juvenile and osteoporosis groups and **was** down-regulated in the aged group. **The genes like Tnnt2, Csrp2 and Lama3 Which are important for the muscle development were upregulated in osteoporosis profile. Similarly, the genes Alpl and Zic1 important for collagen synthesis, were greater in the osteoporotic vs the adult seven-month old non-osteoporotic.**

Metabolism:

This group includes diverse metabolisms like protein, lipid, carboxylic acid, organic acid, nucleoside, nucleotide and nucleic acid metabolisms and bone mineral metabolism. All these processes are essential for tissue regeneration and bone mineral density (BMD) maintenance. They are also important for synthesis of structural constituents, and many of these genes are important for osteoclast and osteoblast activity. Genes encoding metabolisms especially protein, lipid, carboxylic and nucleobase/nucleoside/nucleotide were the largest group of genes regulated in the osteoporosis compared to normal. Our study showed that genes in the above group were up regulated in the osteoporosis and were predominant during osteoporotic state. Functionally, these processes are essential for tissue regeneration and BMD maintenance (Alpl). They are also important for synthesis of structural constituents (Uchl1), and many of these genes are important for osteoclast and osteoblast activity (Crabp2, Mmp8).

Signaling Pathways:

Among the signal transduction related genes affected by the osteoporosis included mainly G-protein coupled receptor protein signaling pathway (Hcrtr1, Htr2b, Ucn, Trrp6, Cirl3) and neuropeptide signaling pathways (Hcrtr1, Ucn, Cirl3). Both of these pathways were up regulated in osteoporosis which implies that these pathways are likely to interfere in diseased state.

Transcription:

Despite the fact that transcription factors were generally weakly expressed, we detected three regulated transcription factor genes with reliable hybridization signals. These are functionally important for osteoblast activity (Crabp2). Furthermore we confirmed regulation patterns for one of these genes by real-time RT-PCR (Crabp2). The regulation of a number of transcription factors suggested that poorly orchestrated regulation with a very low number of genes were playing crucial role in developing disease pathogenesis.

Cell growth and maintenance:

One of the striking findings in our study was the regulation of large number of genes encoding for cell growth and maintains. G-protein and neuropeptides were up-regulated in the osteoporotic and aged rat gene profiles and were down-regulated in the juvenile. The transcriptional process genes were up-regulated in both osteoporotic and juvenile rats and were down-regulated in the aged. Moreover a number of genes present in this category are important for osteoblasts (**C**rabp2, ALPL) and osteoclast activity (Csrp2) as well as for maintaining BMD (Cdkn2b). The up regulation of these genes in osteoporosis was identified.

Hormone activity:

Among the down-regulated annotated transcripts the important phenomena we observed was the decreased expression of hormones like Npy, Prlpb, Plpc-b. It was noted that the imbalance in hormone activity was directly related to osteoporosis.

Biosynthesis and apoptosis:

Apoptosis related genes were highly up-regulated in the aged profile compared to the osteoporotic and juvenile groups.

Quantitative RT-PCR

Selected marker genes were validated by real time RT-PCR. Some slight variations were detected in the expression of selected genes between microarray and quantitative PCR methods. In general, there was a correlation between the real time PCR analysis and microarray results, thus validating the microarray results (Figure 2).

DISCUSSION

We have compared the relative expression levels of more than half of the genes predicted to comprise the rat genome in osteoporotic, normal, juvenile, and aged cohorts. We confirmed the validity of the dataset by quantitative RT-PCR and clustered the differentially expressed genes into the functional categories.

Our emphasis was primarily on genes involved in **estrogen related** osteoporosis. Consequently, we will focus our discussion on osteoporotic profiles **in comparison with** the juvenile, non-osteoporotic and aged groups.

The microarray analysis identified over 308 genes that were altered in the non-osteoporotic (seven-month old rat) compared to osteoporotic rats. There were 195 genes that were increased and 109 genes that decreased more than 2 folds between the non-osteoporotic and osteoporotic. Ease analysis was used to correlate genes and groups of genes with GO biological processes, which were further analyzed and cross-referenced with the literature. Furthermore the osteoporotic genes we identified may include novel candidate biomarkers for pathogenesis of osteoporosis.

Data indicated multiple novel differences in gene expression among the designated study populations of osteoporotic and non-osteoporotic seven-month old, aged (> two years) and juvenile groups. Although descriptions of all the individual genes that exhibited altered expression were too cumbersome to report, many of the detected gene expression differences involved ESTs with no known function. **All the comparison results including osteoporotic vs adult non-osteoporotic and adult non-osteoporotic vs aged were listed in table 2.**

The majority of genes whose expression levels were altered in osteoporosis exhibited an increased pattern. These genes function in diverse processes, including signal transduction, protein metabolism, cell growth or maintenance, lipid metabolism, structural component synthesis and G-Protein. Many of these processes represent metabolic systems designed to maintain bone homeostasis and tissue turnover and their increased expression represents compensation for stresses related to bone loss and bone formation. Some examples of individual genes are lipo protein lipase, which plays a role in adipogenic differentiation in the bone marrow stroma [23] and cellular retinoic acid binding protein 2 (Crabp-2). Crabp2 is presumed to modulate the level of retinoic acid available to bind to the receptor in cells. Expression of Crabp-2 has been observed in osteoblasts [24]. G-protein coupled receptor activation is vital to the functional viability of both osteoblasts and osteoclasts, which in part involves in the successful communication between cell types [25, 26]. Moreover, the presence of nerve fibers in skeletal tissue and presence of receptors for several neurotransmitters

on both osteoblasts and osteoclasts have suggested a possible role for neuropeptides in the regulation of skeletal metabolism [27].
Another gene we identified was ALOX. A study reported that over-expression of ALOX gene kept mice from reaching peak bone mass [28]. We also found up-regulation of matrix metallo proteins 8. This factor is involved in osteoblast differentiation and mineralization [29]. Further, cytochrome p450 hormonal function and its dysfunction will lead to osteoporosis [8].
Fewer genes exhibited decreased expression in osteoporosis. Because of the relatively small number of down-regulated genes, over-representation analysis is unlikely to be informative. It could be either too specific or too general [30]. The logic for this statement is that the annotated down-regulated genes function in bone anabolism, and their decreased expression may reflect a possible cause for either decreased bone mass or osteoblast activity.
There were several genes identified in the osteoporotic rats that had a decrease in expression. These genes were: Calcitonin, a peptide hormone, involved in calcium homeostasis through their actions on osteoblast and osteoclast [31]; Wingless-type MMTV integration site family member 4, which plays a role in osteoblast and adipocytes differentiation and function [32]; Neuropeptide Y, that controls bone growth [33]; and calmodium, which influences osteoblast ion transport, growth and differentiation [34]. Additional down-regulated genes in the osteoporotic rat included: Interleukin 1 receptor, associated with osteoclast and osteoblast metabolism [34]; F-spondin2, which promotes nerve precursor differentiation [35]; and prolactin like protein c gene, reported to have an inhibitory effect on osteoblast formation. [36].
Some of the genes were uniquely identified between aged and adult non-osteoporotic groups. Relatively broad arrays of genes were down-regulated in the aged group compared to up-regulated genes. Among the down-regulated genes, the predominant **functional** processes include oxido-reductase activity, endopeptidase activity, metal ion binding, structural molecule synthesis and metabolism. **There was no overlapping gene identified in the known up-regulated or down regulated genes both in aged and osteoporotic rats in comparison with adult non-osteoporotic rats. Interestingly we noted 14 genes that were up regulated in osteoporosis but down regulated in aged and six genes that were down regulated in osteoporosis but up regulated in aged. As the aged rats were not osteoporotic in our animal model, these differential expression genes in osteoporotic and aged rats may indicate a more relevant role in the development of osteoporosis. Among 14 genes that were up regulated in osteoporotic and down in aged rats were MMP8, Sponf1, Csrp2, Ivl, Fol r1, Crabp2, Loc64305, Nac-1, Gludins, Braf, InhBp, Pgr, Slc26a1, and sulphate Sp. The six genes which were down regulated in osteoporotic but up regulated in aged were Prlpb, Ii1rn, PlpcB, Loc171569, Ramp3, Mip.**

There were specific genes down-regulated in the aged groups that included insulin like growth factor **binding protein 3 and 6 (IGFBP3 and IGFBP6),,** which regulate osteoclastogenesis. Previous studies [8] reported that the decreased expression of IGFs predisposes to osteoporosis. **However, IGF-1 was not significantly changed in comparing osteoporotic vs non osteoporotic in our microarray data.** Further, tumor necrosis factor receptor superfamily member 6 was decreased and is important for bone remodeling and repair. Moreover, interleukin 6 signal transducer, another down-regulated gene, is noteworthy for osteoprogenitor differentiation and bone formation.

The majority of the up-regulated genes contrasting the aged and adult non-osteoporotic groups emphasized cell growth and differentiation, transcription, and apoptosis related gene categories. These genes are related to the processes for tissue turnover and decrease during bone anabolism. Some examples of genes up-regulating in the aged profile were Kruppel-like factor 5, which is important for osteoblast differentiation and maturation [34] ; Apo E receptor -2, which facilitates the intravascular transport of blood lipids [37, 38]; and Vitamin K [39-41], interleukin 8 receptor beta and interleukin 9, which modulate bone homeostasis. Overall, the juvenile expression profile had an over-representation of growth and differentiation related genes important for building bone mineral density. **Through our experiments we demonstrated that age is not only the factor triggering osteoporosis, genetic factors play a predominant role in onset of the disease.**

Conclusions: Some global changes of gene expression were reported that were associated with age (> two year old rats vs juvenile rats), as well as osteoporosis vs normal adult (seven month old rats), non-osteoporotic. Several gene expression patterns emerged and several protein correlations were determined.
This report is the first we know of that uses a gene microarray profile and RT-PCR to correlate gene expression in different physiological population cohorts in the rat. The outcome underscores the opportunity to pursue more comprehensive and far-reaching studies to determine relationships between the gene-protein expression levels and the physiological conditions.
Especially compelling is the opportunity to use the techniques described to identify patterns of gene expression in the juvenile and non-osteoporotic adult that may predict the susceptibility to the osteoporotic condition, and further, to use that information to design and develop therapeutic intervention to preclude osteoporosis from occurring in the susceptible individual. However, further confirmation at the protein level and functional analysis will be necessary.

ACKNOWLEDGMENT: This study was supported by National Health and Medical Research Council of Australia (NHMRC 199925 and NHMRC 290537) and partially supported from the BTEC and NIH (JOH RO1 DE15392.).

LEGENDS:

Figure 1: Histological validation of animal models. Significant loss of bone density in tibia and femur was noted in osteoporotic rats with typical fenestrated trabeculae. **All pictures were taken at 40 times magnification from growth plate in epiphysis part of tibia and epiphyseal disk of femur head.**

Figure 2: Validation of selected gene expression quantified by microarray and real time PCR. **Three animals from each different physiological group were analyzed in triplicate by real-time PCR**. **The unit of Y-axis in each graph is the expression level measured in mocroarray and Q-PCR Abbreviations: Igfbp3 (insulin like growth factor binding protein 3); C4a (complement component 4a); Spp1 (secreted phosphoprotein 1); Ctsc (cathepsin C); Lpl (lipoprotein lipase); Cyr61 (cysteine rich protein 61); Crabp2 (cellular retinoic acid binding protein 2), Lgals (lectin, galactose binding, soluble); Pspla1 (phosphatidylserine-specific phospholipase A1)**

REFERENCES:

1. Seeman E. Osteoporosis in men: epidemiology, pathophysiology, and treatment possibilities. Am J Med 1993; 95: 22S-28S.
2. Ryan PJ, Blake GM, Fogelman I. Postmenopausal screening for osteopenia. Br J Rheumatol 1992; 31: 823-828.
3. Fernandes MS. [Fourth International Symposium on Osteoporosis and the Consensus Development Conference. Hong Kong 1993]. Acta Med Port 1993; 6: 168-170.
4. Borojevic R, Roela RA, Rodarte RS, Thiago LS, Pasini FS, Conti FM, Rossi MI, Reis LF, Lopes LF, Brentani MM. Bone marrow stroma in childhood myelodysplastic syndrome: composition, ability to sustain hematopoiesis in vitro, and altered gene expression. Leuk Res 2004; 28: 831-844.
5. Kimmel D, Moran E, Bogoch E. Animal Models of Osteopenia or Osteoporosis. Boca Raton: CRC Press; 1999.
6. Uzan B, de Vernejoul MC, Cressent M. RAMPs and CRLR expressions in osteoblastic cells after dexamethasone treatment. Biochem Biophys Res Commun 2004; 321: 802-808.
7. Huang QY, Recker RR, Deng HW. Searching for osteoporosis genes in the post-genome era: progress and challenges. Osteoporos Int 2003; 14: 701-715.
8. Niu T, Xu X. Candidate genes for osteoporosis. Therapeutic implications. Am J Pharmacogenomics 2001; 1: 11-19.
9. Dvornyk V, Recker RR, Deng HW. Gene expression studies of osteoporosis: implications for microarray research. Osteoporos Int 2003; 14: 451-461.
10. Rizzoli R, Bonjour JP, Ferrari SL. Osteoporosis, genetics and hormones. J Mol Endocrinol 2001; 26: 79-94.
11. Ho NC, Jia L, Driscoll CC, Gutter EM, Francomano CA. A skeletal gene database. J Bone Miner Res 2000; 15: 2095-2122.
12. Liu YZ, Dvornyk V, Lu Y, Shen H, Lappe JM, Recker RR, Deng HW. A novel pathophysiological mechanism for osteoporosis suggested by an in vivo gene expression study of circulating monocytes. J Biol Chem 2005; 280: 29011-29016.
13. Cappellen D, Luong-Nguyen NH, Bongiovanni S, Grenet O, Wanke C, Susa M. Transcriptional program of mouse osteoclast differentiation governed by the macrophage colony-stimulating factor and the ligand for the receptor activator of NFkappa B. J Biol Chem 2002; 277: 21971-21982.
14. Rho J, Altmann CR, Socci ND, Merkov L, Kim N, So H, Lee O, Takami M, Brivanlou AH, Choi Y. Gene expression profiling of osteoclast differentiation by combined suppression subtractive hybridization (SSH) and cDNA microarray analysis. DNA Cell Biol 2002; 21: 541-549.
15. Ishida N, Hayashi K, Hoshijima M, Ogawa T, Koga S, Miyatake Y, Kumegawa M, Kimura T, Takeya T. Large scale gene expression analysis of osteoclastogenesis in vitro and elucidation of NFAT2 as a key regulator. J Biol Chem 2002; 277: 41147-41156.
16. Hishiya A, Ikeda K, Watanabe K. A RANKL-inducible gene Znf216 in osteoclast differentiation. J Recept Signal Transduct Res 2005; 25: 199-216.
17. Lane NE, Haupt D, Kimmel DB, Modin G, Kinney JH. Early estrogen replacement therapy reverses the rapid loss of trabecular bone volume and prevents further deterioration of connectivity in the rat. J Bone Miner Res 1999; 14: 206-214.

18. Biosystems A. User Bulletin#2, ABI Prism 7700 Sequence Detection System. 2001.
19. Chang BD, Watanabe K, Broude EV, Fang J, Poole JC, Kalinichenko TV, Roninson IB. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. Proc Natl Acad Sci U S A 2000; 97: 4291-4296.
20. Guo X, Liao K. Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation. Gene 2000; 251: 45-53.
21. Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, Woolley DE, Davis RW. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. Proc Natl Acad Sci U S A 1997; 94: 2150-2155.
22. Hosack DA, Dennis G, Jr., Sherman BT, Lane HC, Lempicki RA. Identifying biological themes within lists of genes with EASE. Genome Biol 2003; 4: R70.
23. Ahdjoudj S, Lasmoles F, Holy X, Zerath E, Marie PJ. Transforming growth factor beta2 inhibits adipocyte differentiation induced by skeletal unloading in rat bone marrow stroma. J Bone Miner Res 2002; 17: 668-677.
24. Kindmark A, Torma H, Johansson AG, Ljunghall S, Melhus H. Cellular retinoic acid-binding protein type II is expressed in adult human osteoblasts and in adult liver. Biochem Biophys Res Commun 1992; 189: 1397-1403.
25. Brown EM, Chattopadhyay N, Yano S. Calcium-sensing receptors in bone cells. J Musculoskelet Neuronal Interact 2004; 4: 412-413.
26. Blair HC, Robinson LJ, Zaidi M. Osteoclast signalling pathways. Biochem Biophys Res Commun 2005; 328: 728-738.
27. Jones KB, Mollano AV, Morcuende JA, Cooper RR, Saltzman CL. Bone and brain: a review of neural, hormonal, and musculoskeletal connections. Iowa Orthop J 2004; 24: 123-132.
28. Rosen CJ, Ackert-Bicknell CL, Adamo ML, Shultz KL, Rubin J, Donahue LR, Horton LG, Delahunty KM, Beamer WG, Sipos J, Clemmons D, Nelson T, Bouxsein ML, Horowitz M. Congenic mice with low serum IGF-I have increased body fat, reduced bone mineral density, and an altered osteoblast differentiation program. Bone 2004; 35: 1046-1058.
29. Parikka V, Vaananen A, Risteli J, Salo T, Sorsa T, Vaananen HK, Lehenkari P. Human mesenchymal stem cell derived osteoblasts degrade organic bone matrix in vitro by matrix metalloproteinases. Matrix Biol 2005; 24: 438-447.
30. Pavlidis P, Qin J, Arango V, Mann JJ, Sibille E. Using the gene ontology for microarray data mining: a comparison of methods and application to age effects in human prefrontal cortex. Neurochem Res 2004; 29: 1213-1222.
31. Carter PH, Schipani E. The roles of parathyroid hormone and calcitonin in bone remodeling: prospects for novel therapeutics. Endocr Metab Immune Disord Drug Targets 2006; 6: 59-76.
32. Jackson A, Vayssiere B, Garcia T, Newell W, Baron R, Roman-Roman S, Rawadi G. Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells. Bone 2005; 36: 585-598.
33. Baldock PA, Sainsbury A, Couzens M, Enriquez RF, Thomas GP, Gardiner EM, Herzog H. Hypothalamic Y2 receptors regulate bone formation. J Clin Invest 2002; 109: 915-921.
34. Roman-Roman S, Garcia T, Jackson A, Theilhaber J, Rawadi G, Connolly T, Spinella-Jaegle S, Kawai S, Courtois B, Bushnell S, Auberval M, Call K, Baron R. Identification of genes regulated during osteoblastic differentiation

by genome-wide expression analysis of mouse calvaria primary osteoblasts in vitro. Bone 2003; 32: 474-482.
35. Schubert D, Klar A, Park M, Dargusch R, Fischer WH. F-spondin promotes nerve precursor differentiation. J Neurochem 2006; 96: 444-453.
36. Coss D, Yang L, Kuo CB, Xu X, Luben RA, Walker AM. Effects of prolactin on osteoblast alkaline phosphatase and bone formation in the developing rat. Am J Physiol Endocrinol Metab 2000; 279: E1216-1225.
37. Cauley JA, Zmuda JM, Yaffe K, Kuller LH, Ferrell RE, Wisniewski SR, Cummings SR. Apolipoprotein E polymorphism: A new genetic marker of hip fracture risk--The Study of Osteoporotic Fractures. J Bone Miner Res 1999; 14: 1175-1181.
38. Stewart WF, Schwartz BS, Simon D, Kelsey K, Todd AC. ApoE genotype, past adult lead exposure, and neurobehavioral function. Environ Health Perspect 2002; 110: 501-505.
39. Schoon EJ, Muller MC, Vermeer C, Schurgers LJ, Brummer RJ, Stockbrugger RW. Low serum and bone vitamin K status in patients with longstanding Crohn's disease: another pathogenetic factor of osteoporosis in Crohn's disease? Gut 2001; 48: 473-477.
40. Jie KS, Knapen MH, Hellemons BS, Hamulyak K, Vermeer C. [Vitamin K: current viewpoints in connection with bone metabolism]. Ned Tijdschr Geneeskd 1995; 139: 875-880.
41. Vermeer C, Jie KS, Knapen MH. Role of vitamin K in bone metabolism. Annu Rev Nutr 1995; 15: 1-22.

Table 1: Primer sequences for real-time PCR

Sequence Name	Sequence forward	Sequence reverse
Rat Lpl	GACAATGTCCACCTCTTAG	CGAAATCCGCATCATCAG
Rat Crabp2	GAGACCCTGTAAGAGTTTG	ACAACGTCGTCTGCTGTCAT
Rat Ogc	GGAGCGCATGGAAGAGAT	GGTGAGCATTCAGTCAGAG
Rat Loc	GACATTCAGCCAGGACTTC	GTGTTGTCTGTCCACTTC
Rat Npy	CTGCGACACTACATCAATCTC	GGAAAAGTCAGGAGAGCAAG
Rat Lgals9	CAGCACCCAGCCTCCATAC	GCAATGTCATTTCCACTGAAG
Rat Pspla1	CCTCAAAGTCCAGTTTCTC	GTTCCTAATGCCCTGAATC
Rat Igfbp3	CTGAAGGCGCTGCTGAATG	GAATGGAGTGGATGGAACTTG
Rat C4a	CACGGTCACAGTGGAG	CTCGAGGCCATCTGAGAAC
Rat Spp1	GGTTTGCCTTTGCCTGTTC	CGTTTCTTCAGAGGACACAG
Rat Ctsc	GGTAATGGAACCAACAGAAG	CTGCCTTTGACTTCATACTTG
Rat Hpse	CTCCTCAACTACTGCTCTTC	CCAATGTCAGGACCATAGAG
Rat Cyr61	CAGTTCCACCGCTCTGAAAG	CCCACAGCACCGTCAATAC
Rat GAPDH	GTCGGTGTGAACGGATTTG	GAACATGTAGACCATGTAGTTG

Table 2: A list of differentially expressed genes in osteoporosis

UP REGULATED GENES: OSTEOPOROSIS vs NORMAL

Accession number	Fold Change	Gene name	Gene symbol
NM_172067	7.206500715	f-spondin 1	Sponf1
NM_017244	5.925456922	cellular retinoic acid binding protein 2	Crabp2
NM_012488	5.58071026	alpha-2-macroglobulin	A2m
NM_017300	5.232851118	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_012676	4.873456913	troponin T2	Tnnt2
NM_022195	4.302501416	involucrin gene , mRNA	IvI
NM_138547	4.280290075	3-alpha-hydroxysteroid dehydrogenase	LOC191574
XM_342661	3.986285988	similar to RIKEN cDNA B130055L09	LOC362341
NM_019150	3.902632624	urocortin	Ucn
NM_019234	3.823798707	dynein, cytoplasmic, intermediate chain 1	Dncic1
NM_022513	3.44841335	dopa/tyrosine sulfotransferase	(LOC64305)
NM_021585	3.318636041	surface protein MCA-32	Mca32
NM_022221	3.284426686	neutrophil collagenease	Mmp8
M92916	3.86281E+13	glucose-dependent insulinotropic peptide mRNA exons 1-5	
NM_031732	3.2823E+13	sulfotransferase family 1A, member 2	Sult1a2
NM_147206	3.041065227	cytochrome P450 3A9	Cyp3a9
RAFB	2.986459081	transient receptor protein 6	Trrp6
NM_175766	2.942583035	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3
CB765685	2.934456722	cytochrome P450 monooxygenase	Cyp2J3
NM_032056	2.676779303	NAC-1 protein	(Nac-1)
BQ202830	2.662309904	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2
AI406334	2.607565472	alpha 2 integrin	
CB780602	2.563877392	transmembrane 4 superfamily member 4	Tm4sf4
AW524553	2.530026522	progesterone receptor	Pgr
CF111358	2.518558099	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
BF397285	2.394665348	alkaline phosphatase, tissue-nonspecific	Alpl
H34885	2.378235274	retinal pigment epithelium, 65 kDa	Rpe65
AF217593	2.318932107	camello-like 1	Cml1
AA891834	2.289758835	hypocretin receptor 1	Hcrt1
NM_130812	2.245097788	(Solute carrier family 25, member 13) (Citrin)	LOC362322
AI579087	2.242869821	cyclin dependent kinase inhibitor 2B	Cdkn2b
BQ205131	2.221184775	uterine sensitization-associated gene 1 protein	Usag1
BF394474	2.212824744	BMP/retinoic acid-inducible neural-specific protein 3	(Brinp3)
CK356986	2.86405E+12	zic protein member 1	Zic1
NM_031641	2.78896E+13	similar to sulfatase FP	LOC311642
BI288264	2.72533E+13	sulfotransferase family 4A, member 1	Sult4a1
BM388060	2.40131E+13	similar to stem cell adaptor protein STAP-1	(LOC305269)
CB731861	2.32216E+13	solute carrier family 26 (sulfate transporter), member 1	Slc26a1
AB046449	2.17217E+13	similar to kaiso protein; zinc finger transcription factor	(LOC315936)
BF419434	2.14942E+12	putative bHLH transcription factor	
BG666688	2.066735546	arachidonate 5-lipoxygenase	Alox5
NM_173306	2.05855647	inhibin binding protein	Inhbp
AA819179	2.057360408	laminin 5 alpha 3	Lama3
CK470759	2.049397622	enoyl-Coenzyme A,	Ehhadh
NM_031114	2.030111871	protein phospatase 3, regulatory subunit B, alpha isoform	Ppp3r2
BQ193682	2.029116572	S-100 related protein, clone 42C	S100a10
BM385409	2.025136744	cysteine rich protein 2	Csrp2)
NM_031242	2.014123401	ubiquitin carboxy-terminal hydrolase L1	Uchl1

NM_031242	2.008129129	CDP-diacylglycerol synthase 1	Cds1

INDIVIDUAL FUNCTIONALLY CLUSTERED GENES: INCREASED IN OSTEOPOROSIS:

BIOLOGICAL PROCESS

1. Response to external stimulus

NM_012488	**alpha-2-macroglobulin**	**A2m**
NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_017237	ubiquitin carboxy-terminal hydrolase L1	Uchl1
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_019150	urocortin	Ucn
NM_032056	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2
NM_053562	retinal pigment epithelium, 65 kDa	Rpe65
NM_147206	cytochrome P450 3A9	CYP3A9

2. Cell communication:

NM_013064	hypocretin receptor 1	Hcrtr1
NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_017250	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
NM_019150	urocortin	Ucn
NM_031114	S-100 related protein, clone 42C	S100a10
NM_053559	transient receptor protein 6	Trrp6
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3
NM_172067	f-spondin	Sponf

3. Cell growth and Maintenance

NM_012488	alpha-2-macroglobulin	A2m
NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_019234	dynein, cytoplasmic, intermediate chain 1	Dncic1
NM_022287	solute carrier family 26 (sulfate transporter), member 1	Slc26a1
NM_032056	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2
NM_053559	transient receptor protein 6	Trrp6
NM_130812	cyclin dependent kinase inhibitor 2B	Cdkn2b
NM_133527	folate receptor 1 (adult)	Folr1
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh
NM_177425	cysteine rich protein 2	Csrp2

4. Signal transduction

NM_013064	hypocretin receptor 1	Hcrtr1
NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_017250	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
NM_019150	urocortin	Ucn
NM_031114	S-100 related protein, clone 42C	S100a10
NM_053559	transient receptor protein 6	Trrp6
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3

5. Organogenesis

NM_012676	troponin T2	Tnnt2
NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_017250	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
NM_022677	zic protein member 1	Zic1

NM_173306	laminin 5 alpha 3	Lama3
NM_177425	cysteine rich protein 2	Csrp2

6. Metabolism

A.Protein metabolism

NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_017237	ubiquitin carboxy-terminal hydrolase L1	Uchl1
NM_022221	neutrophil collagenease	Mmp8
NM_053785	transmembrane 4 superfamily member 4	Tm4sf4
NM_133527	folate receptor 1 (adult)	Folr1

B. Lipid metabolism

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_031641	sulfotransferase family 4A, member 1	Sult4a1
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh
NM_138547	3-alpha-hydroxysteroid dehydrogenase	LOC191574

C. Carboxylic acid metabolism

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh

D. Organic acid metabolism

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh

E. Nucleobase\ nucleoside\ nucleotide and nucleic acid metabolism

NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_022847	progesterone receptor	Pgr
NM_134413	NAC-1 protein	Nac-1

7. Transcription

NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_022847	progesterone receptor	Pgr
NM_134413	NAC-1 protein	Nac-1

8. Neuropeptide signaling pathway

NM_013064	hypocretin receptor 1	Hcrtr1
NM_019150	urocortin	Ucn
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3

9. G-protein coupled receptor protein signaling pathway.

NM_013064	hypocretin receptor 1	Hcrtr1
NM_017250	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
NM_019150	urocortin	Ucn
NM_053559	transient receptor protein 6	Trrp6
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3

10. Electron transport

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_147206	cytochrome P450 3A9	CYP3A9

NM_175766	cytochrome P450 monooxygenase	Cyp2J3

11. Hormone activity

M92916	Glucose-dependent insulinotropic peptide	Gludins
NM_134385	prolactin-like protein C beta	Plpcbeta

MOLECULAR FUNCTION

1. Oxidoreductase activity

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh
NM_138547	3-alpha-hydroxysteroid dehydrogenase	LOC191574
NM_147206	cytochrome P450 3A9	CYP3A9
NM_175766	cytochrome P450 monooxygenase	Cyp2J3

2. Hydrolase activity

NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_017237	ubiquitin carboxy-terminal hydrolase L1	Uchl1
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_022221	neutrophil collagenease	Mmp8
NM_032056	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2
NM_175763	inhibin binding protein	InhBP

3. Structural molecule activity

NM_012676	troponin T2	Tnnt2
NM_022195	involucrin gene	Ivl
NM_173306	laminin 5 alpha 3	Lama3

4. Receptor activity

NM_012488	alpha-2-macroglobulin	A2m
NM_013064	hypocretin receptor 1	Hcrtr1
NM_017250	5-hydroxytryptamine (serotonin) receptor 2B	Htr2b
NM_022847	progesterone receptor	Pgr
NM_053559	transient receptor protein 6	Trrp6
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3

5. Metal ion binding

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_021701	protein phospatase 3, regulatory subunit B, alpha isoform,type 2	Ppp3r2
NM_022221	neutrophil collagenease	Mmp8
NM_031114	S-100 related protein, clone 42C	S100a10
NM_172067	f-spondin	Sponf

6. Catalytic activity

NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_017237	ubiquitin carboxy-terminal hydrolase L1	Uchl1
NM_017300	bile acid-Coenzyme A: amino acid N-acyltransferase	Baat
NM_022221	neutrophil collagenease	Mmp8
NM_022513	dopa/tyrosine sulfotransferase	LOC64305
NM_031641	sulfotransferase family 4A, member 1	Sult4a1
NM_031732	sulfotransferase family 1A, member 2	Sult1a2
NM_032056	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2

NM_053785	transmembrane 4 superfamily member 4	Tm4sf4
NM_130812	cyclin dependent kinase inhibitor 2B	Cdkn2b
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh
NM_138547	3-alpha-hydroxysteroid dehydrogenase	LOC191574
NM_147206	cytochrome P450 3A9	CYP3A9
NM_175763	inhibin binding protein	InhBP
NM_175766	cytochrome P450 monooxygenase	Cyp2J3

7. Binding

M92916	Glucose-dependent insulinotropic peptide	Gludins
NM_012822	arachidonate 5-lipoxygenase	Alox5
NM_013059	alkaline phosphatase, tissue-nonspecific	Alpl
NM_013064	hypocretin receptor 1	Hcrtr1
NM_017244	cellular retinoic acid binding protein 2	Crabp2
NM_019150	urocortin	Ucn
NM_021701	protein phospatase 3, regulatory subunit B, alpha isoform,type 2	Ppp3r2
NM_022221	neutrophil collagenease	Mmp8
NM_022847	progesterone receptor	Pgr
NM_031114	S-100 related protein, clone 42C	S100a10
NM_032056	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	Tap2
NM_130822	calcium-independent alpha-latrotoxin receptor homolog 3	Cirl3
NM_133527	folate receptor 1 (adult)	Folr1
NM_133606	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh
NM_134413	NAC-1 protein	Nac-1
NM_172067	f-spondin	Sponf
NM_177425	cysteine rich protein 2	Csrp2

DOWN REGULATED GENES: OSTEOPOROSIS *vs.* NORMAL

Accession number	Fold change	Gene name	Gene symbol
M31155	8.968466	prolactin-like protein B mRNA	
NM_012614	4.046593	neuropeptide Y	Npy
NM_012752	2.987254	CD24 antigen	Cd24
NM_022194	2.899609	interleukin 1 receptor antagonist gene	Il1rn
XM_237334	2.815044	similar to sphingosine kinase type 1-interacting protein	LOC316561
NM_134385	2.74138	prolactin-like protein C beta	Plpcbeta
NM_138533	2.683087	F-spondin 2	LOC171569
AY488087	2.585315	aristaless-related ALX3 mRNA	Alx3
NM_020100	2.483957	receptor (calcitonin) activity modifying protein 3	Ramp3
NM_030862	2.419674	MARCKS-like protein	Mlp
NM_053402	2.353076	wingless-type MMTV integration site family, member 4	Wnt4
NM_022862	2.311108	Munc13-2 protein	Unc13h2
NM_021688	2.22821	putative potassium channel TWIK	Kcnk1
NM_013108	2.214405	adrenergic receptor, beta 3	Adrb3
NM_133306	2.146935	oxidised low density lipoprotein (lectin-like) receptor 1	Olr1
NM_053945	2.131525	Rim2 protein	Rim2
NM_133562	2.082343	phosphatidylinositol (4,5) bisphosphate 5-phosphatase, A	Pib5pa
XM_341442	2.005784	similar to FSHD region gene 1	LOC361156

Genes Exhibiting Decreased Expression in Aged Relative to Normal

Accessions	Fold change	Gene Name	Gene Symbol
NM_022221	18.41215724	neutrophil collagenease	Mmp8

NM_031504	11.14082356	complement component 4a	C4a
NM_031766	10.5305542	carboxypeptidase Z	Cpz
NM_012598	10.41695742	lipoprotein lipase	Lpl
NM_012725	8.52940266	kallikrein B, plasma 1	Klk3
NM_013104	8.129852891	insulin-like growth factor binding protein 6	Igfbp6
NM_145682	8.111516679	filamin-interacting protein L-Filip	Filip
NM_172067	6.884874942	f-spondin	Sponf
NM_031327	6.392179933	cysteine rich protein 61	Cyr61
XM_342661	6.288972334	similar to RIKEN cDNA B130055L09	
NM_012834	6.092287298	cartilage oligomeric matrix protein	Comp
NM_031327	5.503773498	cysteine rich protein 61	Cyr61
M92340	5.22594573	interleukin 6 signal transducer	Il6st
NM_012999	4.766778356	Subtilisin - like endoprotease	Pace4
NM_022195	4.639052181	involucrin gene	Ivl
XM_343566	4.136514868	similar to TMEFF2	
NM_133527	4.092178205	folate receptor 1 (adult)	Folr1
XM_341561	3.923599813	similar to ARMET protein precursor	
NM_031337	3.898125126	sialyltransferase 9	Siat9
NM_199502	3.820039377	Rincken CDNA	
NM_017244	3.819100593	cellular retinoic acid binding protein 2	Crabp2
NM_022513	3.757624885	dopa/tyrosine sulfotransferase	LOC64305
NM_053896	3.745812365	aldehyde dehydrogenase family 1, subfamily A2	Aldh1a2
NM_181085	3.697560604	lipoma HMGIC fusion partner	LOC300286
NM_134413	3.533039532	NAC-1 protein	Nac-1
M92916	3.396550034	Glucose-dependent insulinotropic peptide	Gludins
NM_012786	3.381219734	Cytochrom c oxidase subunit VIII-H	Cox8h
NM_053492	3.194498258	transporter-like protein	Ctl1
NM_019258	3.093258301	cystatin 8	Cst8
NM_057100	3.060279853	growth arrest specific 6	Gas6
NM_012551	3.053079346	early growth response 1	Egr1
XM_231692	3.042899218	v-raf murine sarcoma viral oncogene homolog B1	Braf
NM_130744	2.92375756	stellate cell activation associated protein	Staap
NM_031315	2.91132139	cytosolic acyl-CoA thioesterase 1	Cte1
NM_053594	2.895560158	protein tyrosine phosphatase, receptor type, R	Ptprr
NM_031509	2.794028707	glutathione S-transferase, alpha 1	Gsta1
BC061739	2.767732092	similar to interleukin 17 receptor E isoform 1	LOC362417
NM_175763	2.724292643	inhibin binding protein	InhBP
NM_012947	2.721995956	eukaryotic elongation factor-2 kinase	Eef2k
XM_226743	2.698081474	similar to neuronal apoptosis inhibitory protein	
NM_133284	2.688565816	progastricsin	Pgc
NM_012595	2.675605917	lactate dehydrogenase B	Ldhb
NM_031321	2.663514518	slit homolog 3 (Drosophila)	Slit3
NM_198784	2.641748408	alpha-2u globulin	LOC362527
XM_342774	2.623367942	cAMP responsive element binding protein-like 2	
NM_017094	2.618919911	growth hormone receptor	Ghr
NM_012608	2.612595059	membrane metallo endopeptidase	Mme
NM_053865	2.60918678	reticulon 1	Rtn1
NM_022396	2.591562496	guanine nucleotide binding protein	Gng11
NM_144755	2.56909102	kinase	LOC246273
NM_012886	2.42383804	tissue inhibitor of metalloproteinase 3	Timp3
AF436847	2.420454854	complement component factor h	Cfh
NM_017330	2.399409953	cytolysin	RATCYTA
NM_031154	2.363893507	glutathione S-transferase, mu type 3 (Yb3)	Gstm3
NM_022847	2.361935379	progesterone receptor	Pgr

NM_012969	2.348917063	insulin receptor substrate 1	Irs1
U31203	2.343348617	noggin	Nog
NM_012688	2.330077263	cholecystokinin A receptor	Cckar
NM_031756	2.326138201	gamma-glutamyl carboxylase	Ggcx
NM_182816	2.323318292	transmembrane protein AMIGO2	Amigo2
NM_022287	2.317539872	solute carrier family 26 ,member 1	Slc26a1
XM_230861	2.306281162	similar to sulfatase FP	
NM_022198	2.284702838	putative chloride channel (Mm Clcn4-2)	Clcn4-2
NM_145774	2.261976757	Rab38, member of RAS oncogene family	Rab38
NM_170787	2.254906209	cytomatrix protein p110	CAST
NM_021750	2.247839858	cysteine-sulfinate decarboxylase	Csad
NM_021774	2.247515633	fragile histidine triad protein	Fhit
XM_219273	2.243373249	similar to suppression of tumorigenicity 5	
NM_012595	2.2206714	lactate dehydrogenase B	Ldhb
NM_030873	2.219242157	profilin II	Pfn2
NM_031782	2.218298173	vesicular inhibitory amino acid transporter	Viaat
NM_031027	2.186409666	dihydropyrimidine dehydrogenase	Dpyd
NM_183327	2.182484943	gamma-aminobutyric acid A receptor,	Gabrg2
NM_031357	2.180900594	ceroid-lipofuscinosis, neuronal 2	Cln2
NM_139194	2.177506417	Tumor necrosis factor receptor superfamily, member 6	Tnfrsf6
NM_019190	2.169461334	membrane cofactor protein	Mcp
NM_031635	2.086687674	fucosyltransferase 2	Fut2
NM_013042	2.058421684	trefoil factor 3	Tff3
U20796	2.054655053	nuclear receptor subfamily 1, group D, member 2	Nr1d2
NM_022936	2.04950956	cytosolic epoxide hydrolase	Ephx2
NM_031973	2.024602405	dipeptidylpeptidase 7	Dpp7
NM_053736	2.024524547	caspase 11	Casp11
AF260258	2.018291209	synaptojanin 2 binding protein	Synj2bp
NM_013082	2.000681384	syndecan 2	Sdc2

Genes Exhibiting Increased Expression in Aged Relative to Normal

Accessions	Fold Change	Gene Name	Symbol
AF110025	8.39708	solute carrier family 22 (organic cation transporter)	Slc22al2
NM_147205	14.16675337	sialyltransferase 1	Siat1
NM_019280	12.36117798	gap junction membrane channel protein alpha 5	Gja5
NM_053394	9.629100673	Kruppel-like factor 5 (intestinal)	Klf5
NM_031123	6.969684363	stanniocalcin 1	Stc1
NM_178093	6.598956025	transcription factor MTSG1	Mtsg1
NM_138533	6.089992872	F-spondin	LOC171569
NM_012696	5.904433228	T-kininogen	Kng
NM_022194	5.689746873	interleukin 1 receptor antagonist gene	Il1rn
M31155	5.165758654	prolactin-like protein B	Prlpb
AF311886	4.762383741	cytochrome P450-like protein	Loc266761
NM_022605	4.511140594	heparanase	Hpse
XM_226731	4.463675756	similar to RP105	
NM_053322	4.098994277	nuclear pore membrane glycoprotein 210	Pom210
NM_020100	3.817596082	receptor (calcitonin) activity modifying protein 3	Ramp3
NM_022297	3.76132761	dimethylarginine dimethylaminohydrolase 1	Ddah1
NM_022935	3.634004178	amiloride binding protein 1	Abp1
NM_139097	3.543705629	sodium channel beta 3 subunit	Scnb3
NM_138882	3.340577252	phosphatidylserine-specific phospholipase A1	Pspla1
NM_057191	3.32792454	sarcomeric muscle protein	Sarcosin

NM_133390	3.251234187	gonadotropin inducible ovarian transcription factor 2	Giot2
NM_021762	3.23860944	translin	Tsn
XM_342925	3.215263728	similar to syncoilin	
NM_173293	3.205105064	olfactory receptor 78	Olfr78
NM_053322	3.108771127	nuclear pore membrane glycoprotein 210	Pom210
L36460	3.005095539	interleukin 9	Il9
XM_235264	2.938917403	similar to atypical GATA protein TRPS1	
NM_053995	2.9124726	3-hydroxybutyrate dehydrogenase (heart, mitochondrial)	Bdh
XM_344233	2.894449469	similar to genomic screen homeobox protein 2	
NM_053567	2.890940194	formiminotransferase cyclodeaminase	Ftcd
XM_342877	2.694384942	similar to ApoE receptor-2	
CF107337	2.694021119	Trace amine receptor 8	Ta8
NM_172047	2.646037775	testosterone regulated apoptosis inducer and tumor suppressor	Traits
NM_181822	2.645697727	NK receptor 1c7	1C7
NM_012548	2.632329164	endothelin 1	Edn1
NM_031747	2.614717249	Calponin 1	Cnn1
NM_021868	2.611135419	cortactin isoform B	Cttnb
NM_013194	2.539760999	myosin, heavy polypeptide 9	Myh9
NM_032062	2.531236041	huntingtin-associated protein interacting protein (duo)	Hapip
NM_145788	2.474993438	TRAF family member-associated Nf-kappa B activator	Tank
NM_138511	2.471946567	cerebroglycan	Gpc2
U09229	2.426201787	cut (Drosophila)-like 1	Cutl1
NM_024349	2.396971131	adenylate kinase 1	Ak1
NM_030862	2.371534669	MARCKS-like protein	Mlp
NM_017180	2.360473563	T-cell death associated gene	Tdag
NM_017183	2.353826984	interleukin 8 receptor, beta	Il8rb
NM_134385	2.334932043	prolactin-like protein C beta	Plpcbeta
NM_013177	2.287094843	glutamate oxaloacetate transaminase 2	Got2
NM_023021	2.286703856	intermediate conductance calcium-activated potassium channel	Kcnn4
M24353	2.256595956	mannosidase 2, alpha 1	Man2a1
XM_237258	2.254008207	similar to CocoaCrisp	
NM_053992	2.239360401	neuroligin 2	Nlgn2
NM_053336	2.227507931	advanced glycosylation end product-specific receptor	Ager
NM_031240	2.22686027	testis specific protein 1	Tpx1
NM_013036	2.197839903	somatostatin receptor 4	Sstr4
NM_022686	2.1900343	Germinal histone H4 gene	Hist4
NM_017076	2.14387992	tumor-associated antigen 1	Taa1
NM_024484	2.129784441	aminolevulinic acid synthase 1	Alas1
NM_133534	2.120399439	Rbs11 protein	Rbs11
XM_236948	2.105379368	similar to taube nuss	
NM_053601	2.08965132	neuronatin	Nnat
NM_134412	2.053212752	PMF32 protein	Pmf31