[Frontiers in Bioscience E4, 1941-1950 , January 1, 2012]

Tracking the molecular signature of developing skeletal tissues

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1. ABSTRACT

We isolated cells from their native in vivo microenvironment using the Laser Capture Micro dissection (LCM). Bone and cartilage tissues were studied from mouse embryonic (18dpc) processed by cry sections enabled the cell isolation from anatomical complexity of skeletal tissues using the LCM technique. RNA was purified from the isolated cells and followed with amplification stage to hybridize on gene array for high through (HT) put analysis to profile the tissues gene expression. Bioinformatics profiling of the differential expression performed according to the tissue origin highlighted the common and divergent genes in the regulation of these tissues. Specifically, we identified that genes related to cell replication and cell metabolism were more prominent in bone, while organic acid metabolism was more prominent in cartilage. This study has demonstrated the utility of applying HT microarray analysis using RNA from small number of cells isolated by LCM from skeletal tissues. The bioinformatics provides insight which has not yet been explored for the developing skeletal tissues. The power of LCM application provides a platform to make a broad molecular analysis using transcriptom analysis to reveal the molecular signature of tissues in their nature environment.

2. INTRODUCTION

Skeletal tissues develop from mesenchymal stem cells (MSCs) differentiating to chondrogenic, osteogenic, hematopoietic-supporting and myogenic cells (1-5). MSCs differentiate into chondrocytes with subsequent matrix deposition followed by osteogenesis and bone matrix mineralization and form the skeleton. Skeletal tissues are active throughout life in skeletal growth and remodeling and are regulated by multiple factors, including systemic hormones and local regulatory factors affecting cellular potential to proliferate and differentiate. The MSCs differentiation is coordinated by activation or repression of regulatory genes (6). Genes important in this process include growth factors, receptors and the production of tissue specific extracellular matrix (ECM) proteins which are important for tissue patterning or matrix mineralization in the developing skeleton. Studies have shown the importance of particular gene expression as a reflection of the differentiation stage along the osteogenic differentiation pathway (5, 7-11).

To identify the molecular control of cells in the tissue context, we applied Laser Capture Micro dissection (LCM), a method that allows isolation of individual or clustered cells from tissues. LCM technology combines

histology and microscopy imaging to select cells from tissues that are difficult to study individually and enables sorting the anatomical complexity of skeletal tissues (12-15). Thus far, biochemical and molecular studies related to gene expression in the stages of differentiation have been conducted in vitro, on cell lines and primary cell cultures (5, 7-11). The work in vitro does not adequately represent the cells' metabolic state in vivo because the cells have lost contact with the ECM comprising the cell niche. Herein, the LCM application provides a platform to make a broad molecular analysis using high throughput gene array to profile tissue specific gene expression in their nature environment. LCM was used to select the cells' localized at their niche and to gain insights into the pattern of gene expression. This is especially relevant in skeletal tissues, where the cells are tightly connected and are difficult to separate and study individually. Thus, the use of LCM allows selecting the cells and profiling the gene expression of these cells in their native environment.

We have used the LCM approach which was successful in harvesting RNA from the isolated cells. In earlier study, a limited molecular analysis was used to analyze gene expressed in the context of intact skeletal tissue physiology. These studies demonstrated tissue specific markers, including osteocalcin for bone and collagen II in cartilage (12-15). Currently, we elaborated on the gene profile of LCM-isolated cells using the high through (HT) put transcriptome analysis of cartilage and bone tissues.

The current study analyzed cells isolated from 18-day-mice-embryos. The tissue sections were used for isolation of cells from cartilage and bone by microdissection laser microscopy. RNA was isolated from the micro-dissected cells and hybridized on gene arrays then the molecular information was processed by bioinformatics tools. The transcriptome analysis allowed us to identify functional genes with special expression, to define the molecular signature of bone and cartilage tissues and to highlight regulation of pathways in these tissues.

3. MATERIALS AND METHODS

3.1. Tissue preparation and histology

The following methods are described in details in our previous works (14, 15). Briefly, 18 days post coital (dpc) C57BL/6 mice embryos were frozen in liquid nitrogen and were stored at -80^{o}C until sectioning. Fresh frozen tissues were embedded in optimal cutting temperature (OCT) Tissue-Tek embedding medium (USA) and 6 µm sections were cut at -20°C using a cryostat (Jung Frigocut 2800N microtome, Leica). Serial sections were put onto glass slide, fixed in 70% alcohol for 90s and air-dried. Every ninth slide was stained with hematoxylin and eosin (H&E) and alcian blue for morphology visualization. The unstained sections were used for micro-dissection (PALM Micro-Laser system and software, Zeiss).

Laser cutting (micro dissection) followed by laser pressure catapulting (LPC) of the selected areas of each tissue was performed at 40 x magnification. The parameters were adjusted to enable cutting the specimen at normal speed and catapulting after single laser shots. The samples were collected into clear adhesive silicon caps without liquid (PALM). These tubes lower the RNases activity in the absence of water, prevent evaporation and crystal formation during extended sample harvesting, and lower the distance between the slide and the caps, thus lowering the energy needed to catapult. After micro dissection, the cells from the cap were spun down in a bench centrifuge (3 minutes, 13000 rpm) and the samples were stored in lysis buffer at -80°C.

3.2. RNA isolation and quality assay

RNA for each tissue sample was collected from series of sections harvested from group of animals, a method which has been proven to reduce variability and is recommended when using a small number of microarrays (16). RNA was isolated from samples retrieved by LCM using Trizol (Sigma, Israel) according to the provided protocol with some modifications, as described earlier (14, 15). Briefly, 20 µL Trizol was used for each sample which contained an estimated amount of 5000 catapulted cells. To increase the efficiency of RNA precipitation in isopropanol 1 µL of glycogen carrier was added for each sample and the precipitation was performed overnight at -20^{o}C. Extracted RNA was measured by nanodrop for absorbance at A_{260} and was also evaluated for quantity (concentration) and quality (integrity) by using Lab Chips Kit on Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

3.3. Microarray hybridization

cRNA was prepared according to Affymetrix small sample protocols. Gene expression was measured by hybridization to Affymetrix Mouse Genome 430 2.0 DNA chip microarray (Affymetrix, Santa Clara, CA, USA), containing 45,038 probe sets (PS) (excluding controls), comprising 20285 Unigene (21995 Entrez Gene IDs), according to the annotations downloaded from the Affymetrix website on the 14th of July 2006.

3.4. Gene expression validation

RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMV-RT) and oligo-dT to generate cDNA that served as a template for the polymerase chain reaction (PCR) (Takara Shuzo Co. Ltd., Japan) with gene specific primers (Table 1). The quality and quantity of cDNA was assessed by the amplification of Gluteraldehyde-3-Phosphate Dehydrogenase (G3PDH) (Clontech, Palo Alto, CA). Quantitative PCR (qPCR) was performed with a Stratagene MX 3000P™ real-time PCR system (Stratagene, USA). In Brief, to monitor target gene amplification we used Brilliant SYBR Green QPCR Master Mix kit (Stratagene, USA). The thermal cycling conditions comprised an initial Taq heat-activation step at 95°C for 10min and 45 cycles of 95°C for 30s, 58°C for 1min and 72°C for 1min. To differentiate between true (above background) amplification of a gene of interest from enhanced SYBR Green I fluorescence due to primer-dimmers or non-specific product formation, a melting curve was generated. Melting curve was comprised of fluorescence measurements of PCR products that are subjected to a stepwise increase in temperature from 55°C to 95°C; fluorescence is measured at every temperature

Table 1. Primers used for qPCR analysis

Gene	Primers
BMP4	ATGAGGGATCTTTACCGGCT TTTATACGGTGGAAGCCCTG
Calmodulin 1	ACAAGGCTGTCACCAAATC GCTCCGAGTCATGGTTCACT
Cathepsin D	CTCACCCTGGGTTCAGATTG CCATTTGCCTGCTTCAGAGT
Cathepsin Z	AGGCCGTTATCAACCACATC TCTGGTCCTCATGTCCACAA
Ergic 3	TGCCAGGTGTACGGCTTCT GTCAGCTTCACCATCATTGG
G3PDH	ACCACAGTCCATGCCATCA TCCACCACCCTGTTGCTGTA
GR	ACCACAGACCAAAGCACCTT AAGGGATGCTGTATTCATGTCA
RBM17	GATGAAGACTTGGAAGTTG CCGTCCACCAAAATACCTC
SFRS 1	ATTGATGTGCATCTGTAAACTG AGGGCTCCAATCGTCAAAA
TGFb2	AAAATGCCATCCCGCCCACTT CATCAATACCTGCAAATCTCG

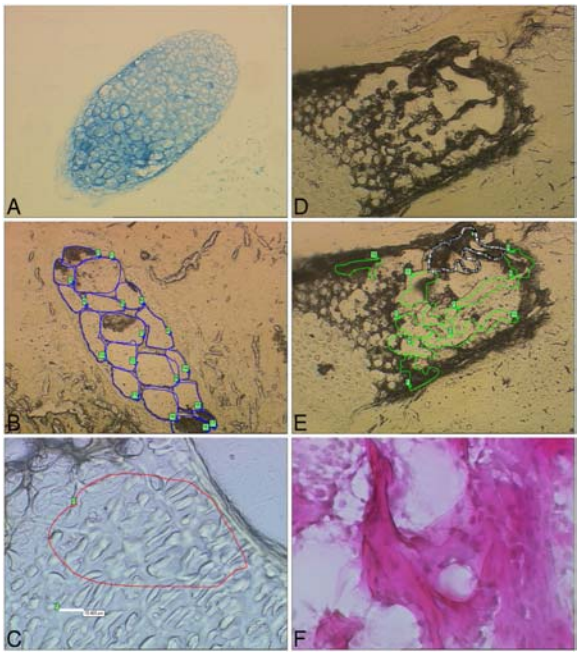

Figure 1. Frozen tissue sections of 18dpc mouse (A-C) cartilage and (D-F) bone sections were stained or unstained. Cartilage was stained with alcian blue (A), bone was stained with hematoxylin and eosin (F) for better morphology visualization. Unstained tissues were used for capturing of cells; before (cartilage - B, bone - D), after LCM (cartilage - C, bone - E).

increment. When temperature reaches 95°C, the accumulating fluorescent measurements are plotted against temperature (MxPro™ QPCR Software, Stratagene, USA). Since SYBR Green only binds double stranded DNA, the fluorescent signal decreases as the melting temperature is reached. Melting curve analysis allowed the confirmation of specific PCR products. A standard curve was then used to derive the initial template quantity. Experiments were performed with triplicates for each data point.

3.5. Computerized data analysis

GCRMA algorithm provided a baseline for the expression level for each PS. PS were retained when at least one sample had an expression level higher than 32. PS cross-reacting with more then one Entrez Gene ID were removed. This analysis was performed using the software package 'gcrma' (version 2.6.0) available as part of the Bioconductor project. For every PS, the ratio between expression levels of the compared samples was calculated. PS were included in the analysis when the expression ratio at least 2 fold. After filtering the PS were transformed so each expression pattern has a mean of 0 and a variance of 1.

3.6. Functional analysis

The two clusters were analyzed for Gene Ontology (GO) annotations appearing in a statistically significant manner in each cluster as compared to the background. For each GO term the number of genes with this term in a cluster was compared to the number of genes with this term in the background. The ratio between number of genes in the cluster and number of gene in the background was calculated, and was used to calculate a p-value. Terms with a p-value lower than 0.05, after correction using the TANGO algorithm, were listed. This analysis was done using the EXPANDER software suite (17). A second analysis was based on the GSEA algorithm (18) that analyzes ranked lists of genes, attempting to correlate gene sets (groups of known genes) with their location in the list. A p-value is estimated, based on comparing the enrichment calculated with the true ranking to random permutations of this ranking, adjusted for multiple hypothesis testing. There are multiple ways of calculating threshold, and we referred only to gene sets which had a p value, as calculated with the FWER method, of less than 0.05. While this method is a more stringent statistical method, we felt it was necessary to filter the gene sets when using an expression ranking based only on a single sample.

4. RESULTS

We used mice embryos' tissue sections to explore the molecular signature of skeletal tissues transcriptome using gene chip arrays. Figure 1 demonstrates the morphology of stained and unstained tissues from 18 dpc mice embryo. The stained sections allow morphology visualizing based on alcian blue or hematoxylin and eosin staining (Figure 1 A & D). The unstained sections (Figure 1B-F) were used for cell capturing from cartilage and bone tissues by LCM. These sections were documented before and after capturing by laser microscopy. The collected cells were used for RNA extraction - RNA quantity was 535 pg/µl and 2,085 pg/µl (with integrity of 8.1 and 9.5) for bone and cartilage, respectively. The retrieved RNA was converted to cRNA and was hybridized on Affymetrix gene chips to explore the molecular profile of the two isolated tissues. The gene analyzed represents a snapshot of genes for the biological pathways comparing between the tissues. The bioinformatics analysis based on the GCRMA algorithm provided a baseline expression of the Probe Sets

Table 2. EXPANDER functions of genes expressed higher in bone

GO Title	GO ID	p-value	Raw p-value	#Genes
Regulation of metabolism	GO:0019222	0.001	5.3088446E-8	193
Nucleic acid binding	GO:0003676	0.001	3.839724E-9	261
Transcription	GO:0006350	0.001	1.3642689E-7	181
Mrna metabolism	GO:0016071	0.008	1.0141449E-5	40
Regulation of cellular process	GO:0050794	0.001	1.382611E-6	248
Nucleobase\, nucleoside\, nucleotide and nucleic acid metabolism	GO:0006139	0.006	6.0827506E-6	259

Table 3. EXPANDER functions of genes expressed higher in cartilage

GO Title	GO ID	p-value	Raw p-value	#Genes
Organic acid metabolism	GO:0006082	0.003	3.3021743E-6	89

Table 4. Gene Set Enrichment Analysis (GSEA) ordered the genes to biologically meaningful sets significant in bone

Set	Size	ES	NES	FWER p-val
BREAST_DUCTAL_CARCINOMA_GENES	19	0.6648	2.0969	0.007
LE_MYELIN_UP	85	0.5238	2.0324	0.018
GOLDRATH_CELL CYCLE	27	0.604	2.0313	0.018
VERNELL_PRB_CLSTR1	50	0.5446	2.0079	0.022
MANALO_HYPOXIA_DN	66	0.5258	1.9879	0.032
UVB_NHEK3_C2	33	0.5669	1.9863	0.033

(PS) were retained when at least one sample had an expression level higher than 32. The expression gene profile was analyzed using two methods of functional enrichment and GSEA ranking. First we analyzed the ratio between expression levels' of the samples calculated for every PS utilized by EXPANDER software suite, when the expression ratio for PS was at least 2. The second analysis used GSEA algorithm allow to lists the genes according to biologically meaning attempting to correlate groups of known genes with their expression.

The EXPANDER analysis allows filtering and transforming the PS so that each expression pattern has a mean of 0 and a variance of 1. The analysis resulted in 5285 PS were differentially expressed between the two tissues. These genes were divided into two clusters - one includes all genes expressed at a higher level in bone when compared to cartilage (3326 PS), the second one includes genes expressed at a lower level in bone (1959 PS). The clusters were analyzed for Gene Ontology (GO) annotations appearing in a statistically significant manner in each cluster as compared to the background, which is comprised of all genes expressed in at least one sample. For each GO term the number of genes with this term in a cluster was compared to the number of genes with this term in the background. Terms with a p-value lower than 0.05, after correction using the TANGO algorithm, were listed. These GO terms represent biological functions which are enriched in the differentially expressed genes and are summarized in Tables 2 and 3. The functional enrichment elaborates on the molecular profiles of the gene expression of cells as appears *in vivo* and presents an overview of the biological activities present in these cells in their native environment. The genes expressed in the bone tissue were related to regulation of metabolism in general, as well as transcription and nucleic acid metabolism ($p < 0.001$), which indicate cell replication and metabolic activity (Table 2). The genes expressed in the cartilage tissue had a prominent function related to organic acid metabolism (Table 3).

Gene Set Enrichment Analysis (GSEA) compares ordered lists of genes to biologically meaningful sets, attempting to correlate groups of known genes with their location in the list. In other words, the algorithm points out given gene sets that appear closer to the beginning induced or repressed expression levels. The gene expression was analyzed by GSEA twice, comparing the expression level in bone and in cartilage. This analysis revealed compared the expression levels of the same PS in the tissues. The GSEA results in bone and cartilage were divided into three groups: (A) gene sets enriched only in bone (Table 4), (B) gene sets enriched only in cartilage (Table 5), (C) gene sets enriched in both bone and cartilage (Table 6). Genes with significant enrichment in the bone tissue (Group A, Table 4) included 6 gene sets which indicate cells at high metabolic state and active cell-cycle (such as in tumors); in the up-regulation we noted E2F and down-regulated for pRB and p16.

Genes that were enriched in cartilage (group B, Table 5) included 35 gene sets are related to the origin or differentiation status of these tissues and probably indicate the proliferative/less differentiated nature of cells. Some genes noted are also up-regulated in sarcomas after treated with Et-743, a DNA interacting agent or identified in undifferentiated cancer as compared to differentiated cancer. Additional gene sets are related to pyruvate metabolism and ATP generation, indicating that the cartilage is metabolically active. This impression is strengthened by the enrichment of a proteasome related gene set.

Genes enriched in both bone and cartilage (Group C, Table 6) are related to ATP synthesis (oxidative phosphorylation, glycolysis) and protein metabolism (proteasome degradation of proteins, and their synthesis via ribosome and translation factors). The gene sets enriched included genes expressed by proliferating cells such as in HSCs (RhloLin2/loSca-11c-kit1 and RhhiLin2/loSca-11c-kit1), in undifferentiated ESCs and in proliferating cells as well as in cancer tissues. The enrichment of gene sets including genes expressed in cancer might indicate tissue remodeling or only the undifferentiated status of these cells. Both cartilage and bone had enrichment of gene sets

Table 5. Gene Set Enrichment Analysis (GSEA) ordered the genes to biologically meaningful sets significant in cartilage

Set	Size	ES	NES	FWER p-val
HDACI_COLON_CUR24HRS_UP	24	0.66	2.2243	0.001
ET743_SARCOMA_6HRS_UP	22	0.6322	2.1082	0.001
PROTEASOME	17	0.6703	2.1065	0.001
BLEO_MOUSE_LYMPH_HIGH_24HRS_DN	29	0.6036	2.0962	0.001
HIF1_TARGETS	20	0.6464	2.0953	0.001
TNFALPHA_ALL_UP	59	0.5467	2.0826	0.002
TSA_CD4_UP	17	0.6566	2.0788	0.002
TARTE_PC	51	0.5454	2.0544	0.009
ET743_SARCOMA_UP	53	0.5413	2.0525	0.01
PENG_GLUTAMINE_DN	207	0.484	2.0502	0.011
DOX_RESIST_GASTRIC_UP	27	0.5931	2.0496	0.011
PYRUVATE_METABOLISM	24	0.6071	2.0434	0.011
INOS_ALL_DN	59	0.5291	2.0426	0.011
CANCER_UNDIFFERENTIATED_META_UP	53	0.54	2.039	0.011
AGED_MOUSE_HYPOTH_DN	30	0.5804	2.0368	0.011
HYPOPHYSECTOMY_RAT_UP	21	0.6145	2.026	0.012
ATP_SYNTHESIS	15	0.6538	2.0202	0.013
CALRES_MOUSE_UP	19	0.6323	2.0121	0.015
TYPE_III_SECRETION_SYSTEM	15	0.6538	2.0084	0.018
LI_FETAL_VS_WT_KIDNEY_DN	112	0.4938	2.0049	0.018
UVB_NHEK3_C0	71	0.5123	2.003	0.019
CANTHARIDIN_DN	41	0.5542	2.0021	0.019
UVB_NHEK2_DN	57	0.5209	1.9966	0.02
ADIP_DIFF_CLUSTER5	30	0.5661	1.9957	0.02
BRCA1_SW480_UP	20	0.6051	1.9901	0.021
FLAGELLAR_ASSEMBLY	15	0.6538	1.9842	0.023
ZHAN_MULTIPLE_MYELOMA_SUBCLASSES_DIFF	26	0.5766	1.9807	0.025
FALT_BCLL_IG_MUTATED_VS_WT_DN	32	0.5649	1.9805	0.026
TNFALPHA_30MIN_UP	35	0.5518	1.9726	0.03
BASSO_REGULATORY_HUBS	106	0.4801	1.9633	0.037
UVB_NHEK3_ALL	300	0.4536	1.9579	0.041
TNFALPHA_4HRS_UP	28	0.5687	1.9530	0.045
KREBS_TCA_CYCLE	26	0.5649	1.9529	0.045
CHESLER_HIGHEST_FOLD_RANGE_GENES	32	0.5449	1.9492	0.047

related to proliferation and a lower state of differentiation in normal tissues and in cancer tissues. This indicates that the tissues analyzed in the mouse embryo are proliferating, have not yet fully differentiated, and suggests the possibility of ongoing active tissue remodeling.

In order to validate the gene expression of selected genes, representing functions and differentiation pathways derived from the bioinformatic analysis of the Affymetrix array, we tested by qPCR (Figure 3). For the selected genes we used primers for qPCR which provided complementary expression information to the probes expressed on the gene array. The results show (Figure 3) that *Cathepsin D*, *Cathepsin Z*, *Ergic3* were expressed at a significantly higher level in cartilage while *Calmodulin 1*, *SFRS1*, *RBM17* were higher in bone. *TGFb2* was expressed at a higher level in bone as highlighted by the GSEA analysis but was at equal level by qPCR. *BMP4* and *GR* were expressed at comparable levels on the array and by RT-PCR in both analyzed tissues. Thus, we have shown a high rate of concordance between the array analyses that were also verified for expression by qPCR.

5. DISCUSSION

The Laser Capture Microdissection (LCM) technique allows the isolation of specific cells from histological slides. Previous work from our laboratory has shown that isolation of cells, RNA extraction and PCR amplification from skeletal tissues is possible, leading to distinctive results between the tissues (12-14). In the current study, we broaden the molecular analysis to profile gene expression from bone and cartilage using microarray technology. The bioinformatics analysis focused on enriched functions in the differentially expressed genes, using statistical tests present in the EXPANDER software suite. Examining the genes expressed at a higher level in either bone or cartilage resulted in the identification of statistically relevant GO terms, representing functions.

Genes that were expressed at a higher level in cartilage were related to the function of organic acid metabolism showing a balance of acid components in a developing cartilage but not in bone. An example is hyaluronic acid, a prominent component of cartilage, affecting cell adhesion, development and morphogenesis (19). Reviewing the genes identified with the function of organic acid metabolism, demonstrated some that are related to fat metabolism, such as *palmitoyl-coA oxidase* and *Acyl-CoA thioesterase 7*, which are involved in fatty acid oxidation (20). Fatty acids and fatty acid derived molecules such as eicosanoids have been shown to regulate bone formation and to modulate degenerative diseases of joints and cartilage including rheumatoid arthritis (21) and osteoarthritis (22, 23). Arachidonic acid is a known regulator of cartilage metabolism, and derivates of this acid are necessary for cartilage development and bone formation. The metabolism and the amount of fatty acids change as the cartilage ages (24). It is likely that expression of these fat-related genes in the cartilage sample indicates

Table 6. Gene Set Enrichment Analysis (GSEA) ordered the genes to biologically meaningful sets significant in bone and cartilage

Set	Size	ES Bone	ES Cartilage	NES Bone	NES Cartilage	FWER p-val Bone	FWER p-val Cartilage
AGED_MOUSE_CORTEX_DN	34	0.587	0.5799	2.0409	2.0601	0.017	0.007
AGED_MOUSE_HYPOTH_UP	37	0.6519	0.5875	2.3024	2.1248	0	0.001
BHATTACHARYA_ESC_UP	40	0.6079	0.5561	2.1415	2.0073	0.002	0.018
BLEO_MOUSE_LYMPH_LOW_24HRS_DN	24	0.638	0.6349	2.1048	2.1402	0.007	0.001
BRCA_PROGNOSIS_NEG	63	0.5984	0.6145	2.2443	2.3604	0	0
CANCER_NEOPLASTIC_META_UP	52	0.564	0.5948	2.0766	2.254	0.008	0
CITRATE_CYCLE_TCA_CYCLE	16	0.701	0.6966	2.1279	2.1621	0.004	0.001
ELECTRON_TRANSPORT_CHAIN	80	0.5852	0.6346	2.2539	2.5211	0	0
GLYCOLYSIS_AND_GLUCONEOGENESIS	22	0.6114	0.5974	1.9678	1.9596	0.049	0.038
GNATENKO_PLATELET	29	0.5989	0.6141	2.0123	2.1515	0.021	0.001
GNATENKO_PLATELET_UP	29	0.5989	0.6141	2.0316	2.1139	0.018	0.001
HCC_SURVIVAL_GOOD_VS_POOR_DN	100	0.5895	0.5692	2.3136	2.3165	0	0
HIPPOCAMPUS_DEVELOPMENT_PRENATAL	25	0.6407	0.6385	2.0986	2.1984	0.007	0.001
IDX_TSA_UP_CLUSTER3	72	0.5894	0.5458	2.2638	2.143	0	0.001
IFN_BETA_GLIOMA_DN	25	0.7074	0.7085	2.3362	2.4038	0	0
LEE_MYC_UP	36	0.6295	0.6497	2.1975	2.3512	0.001	0
MENSSEN_MYC_UP	24	0.6623	0.7352	2.1555	2.4544	0.001	0
MMS_MOUSE_LYMPH_HIGH_4HRS_UP	31	0.5966	0.5882	2.0424	2.0828	0.016	0.002
MOOTHA_VOXPHOS	71	0.5715	0.6007	2.147	2.349	0.001	0
NING_COPD_DN	88	0.5115	0.5233	1.9788	2.0819	0.039	0.002
OXIDATIVE_PHOSPHORYLATION	48	0.5838	0.616	2.1037	2.2864	0.007	0
PARK_MSCS_BOTH	33	0.5732	0.561	2.0053	1.9841	0.023	0.023
PARK_MSCS_DIFF	25	0.5889	0.6216	1.9919	2.108	0.026	0.001
PENG_LEUCINE_DN	125	0.5117	0.5348	2.0388	2.2076	0.017	0.001
PENG_RAPAMYCIN_DN	159	0.4897	0.5016	1.9851	2.0963	0.034	0.001
POMEROY_DESMOPLASIC_VS_CLASSIC_MD_UP	30	0.5848	0.556	1.9916	1.9488	0.026	0.047
PROTEASOME_DEGRADATION	24	0.631	0.6513	2.0876	2.234	0.008	0
PROTEASOMEPATHWAY	20	0.631	0.6793	2.0012	2.2035	0.023	0.001
RIBOSOMAL_PROTEINS	67	0.7137	0.7148	2.6789	2.7714	0	0
ROME_INSULIN_2F_UP	137	0.509	0.5159	2.0433	2.1238	0.016	0.001
SANA_IFNG_ENDOTHELIAL_DN	56	0.5268	0.5746	1.9619	2.1913	0.05	0.001
SERUM_FIBROBLAST_CELLCYCLE	95	0.5079	0.5318	1.977	2.1316	0.039	0.001
SHIPP_FL_VS_DLBCL_DN	31	0.6851	0.6778	2.3584	2.3447	0	0
TARTE_PLASMA_BLASTIC	236	0.495	0.5101	2.05	2.1872	0.014	0.001
TRANSLATION_FACTORS	43	0.5817	0.6077	2.0866	2.2477	0.008	0
UBIQUINONE_BIOSYNTHESIS	15	0.6673	0.6919	1.9967	2.1194	0.025	0.001
UVB_NHEK1_C1	29	0.6488	0.6204	2.2121	2.1773	0.001	0.001
UVB_NHEK2_UP	50	0.5389	0.6373	1.9676	2.385	0.049	0
VANTVEER_BREAST_OUTCOME_GOOD_VS_POOR_DN	44	0.5507	0.545	2.0013	2.0047	0.023	0.018
YU_CMYC_UP	37	0.5626	0.5403	1.9775	1.9627	0.039	0.037
ZELLER_MYC_UP	18	0.6706	0.6957	2.0802	2.2174	0.008	0.001
ZHAN_MMPC_SIM_BC_AND_MM	30	0.605	0.5965	2.0614	2.083	0.01	0.002
ZUCCHI_EPITHELIAL_UP	28	0.6327	0.6467	2.1061	2.22	0.007	0.001

that they play a role in the development of cartilage. This group of functionally highlighted genes included *FGFR3*, a gene which is important in the development of cartilage and bone (25). The organic acid metabolism covers a wide variety of functions, some are expressed in cartilage development (like *FGFR3*) and others are not yet known (fatty acid oxidation). It seems plausible that fatty acid derived molecules like eicosanoids have a role in cartilage development and their role needs to be further elucidated.

We tested the functional properties of the genes in another independent method, using the GSEA algorithm (Tables 4-6). Gene sets that were marked as significant by the GSEA analysis indicate that both bone and cartilage are undifferentiated and metabolically active. Embryonic stem cells have been shown to have a high level of transcripts expressed a level which decreases with differentiation to tissue specific stem cells and decreases further with final differentiation to mature tissues (26). Similarly we have shown for both bone and cartilage in embryonic tissue are transcriptional active. Samples harvested from bone, have an up regulation of E2F and repressed expression of p16 or Rb; genes that know for their opposed effect in tumors (27, 28). In the cartilage, this undifferentiated state is noted by expression of gene sets that were related to undifferentiated cancer.

Genes expressed in cartilage include a large group of H+ transporters. It contains two subunits of the mitochondrial ATP synthase - O and Epsilon subunits of the F1 complex. The F1 complex is responsible for energy production and oxidative respiration. These two genes seem to be related to the presence of oxidative phosphorylation and high metabolism in the embryonic bone and cartilage tissues analyzed. The other group of H+ transporters is all part of the vacuolar ATPase (V-ATPase), a multi subunit enzyme that mediates acidification of eukaryotic intracellular organelles (29). Inhibiting the action of this enzyme leads to a reduction in bone resorption and to a decrease in the level of indicators of cartilage damage in arthritis (30). The involvement of this enzyme in bone resorption and cartilage destruction might be related to remodeling. Samples from both cartilage and bone tissues expressed genes related to proliferation and invasiveness of cancer, which point to the proliferative nature of bone and cartilage, as well as the tissue remodeling they undergo.

Few genes were analyzed by qPCR resulted with an expressed higher level in cartilage included *Cathepsin D & Z* and *Egric3*. Cathepsin D is a proteinase expressed in many tissues which is involved in apoptosis, in regulating plasmin and may be involved in MMP regulation (31). The proteinase also plays a role in angiogenesis and cell proliferation of tumor cells (32). Cathepsin Z (also called

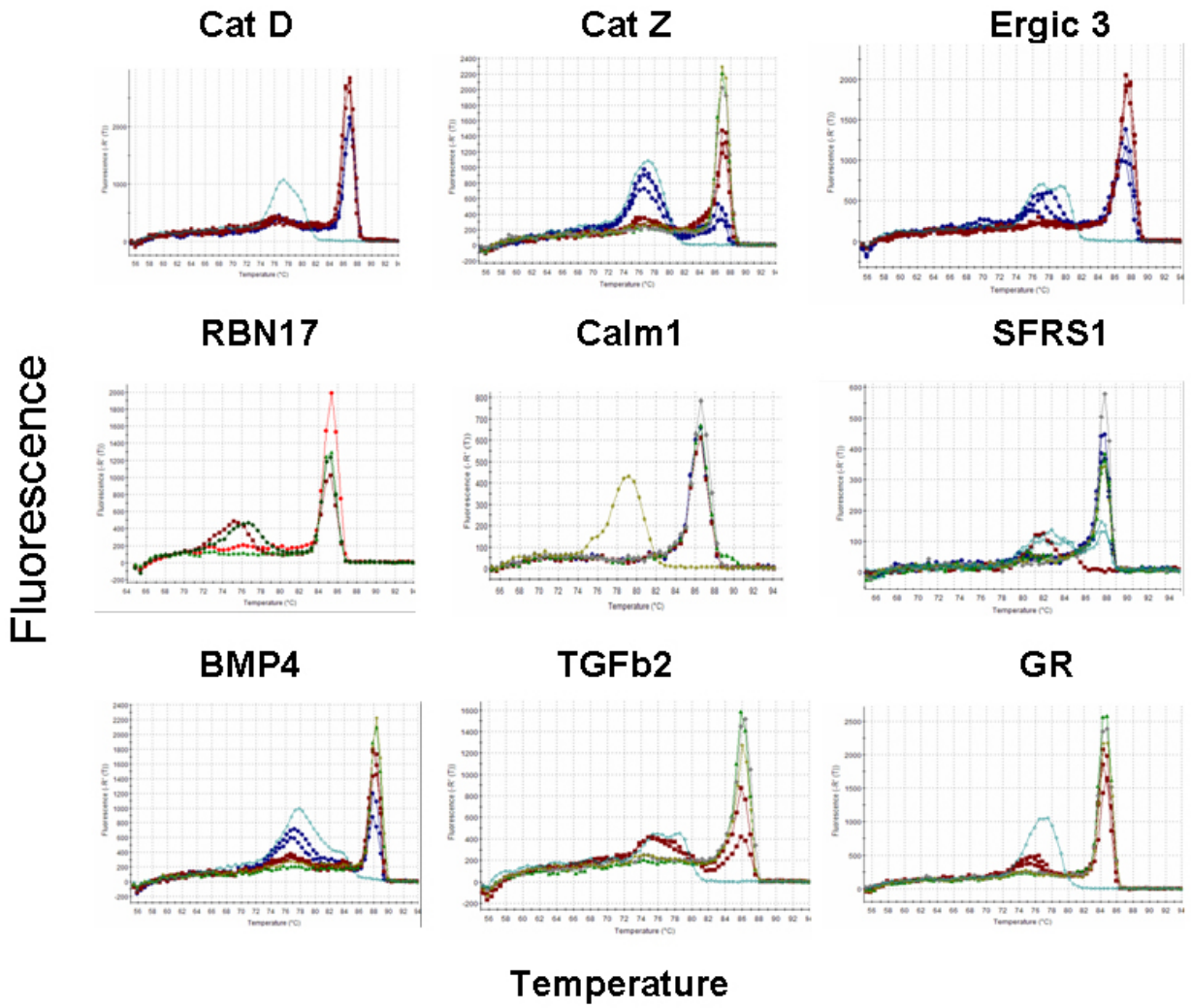

Figure 2. Melting curve analysis of qPCR amplification for the analyzed genes. Axes X represents temperature; axes Y – level of fluorescence. Major peaks signify melting points of the amplified PCR product and allow the confirmation of specificity of the PCR products.

Cathepsin P and Cathepsin X) is another member of the Cathepsin family, which is especially expressed in the liver (33). Cathepsin Z, unlike other cathepsins, has very restricted catalytic specificity (34). It is possible that the expression of Cathepsin D and cathepsin Z in cartilage is related to tissue formation and remodeling. Ergic3 is an ER protein expressed in multiple tissues, with a higher level of expression in neuronal tissues and kidneys. Over expression of this protein in cultured cells accelerated cell growth and prevented ER stress-induced cell death (35). The presence of this gene seems to be another indicator that the cartilage cells are dividing and the tissue is growing.

Genes that were expressed at a higher level in bone included *SFRS1*, *RBM17* and *Calmodulin 1*. *SFRS1* represents two functions that were significant in bone, mRNA metabolism and nucleic acid binding. SFRS1 is a splicing factor involved in constitutive and alternative splicing which is important in development (36). SFRS1 also serves as a proto-oncogene with a role in the formation of cancer (37). RBM17 protein regulates the alternative splicing and has a role in DNA repair (38, 39). Calmodulin 1 is a calcium sensing protein very important in regulating many cell processes affected by many pathways (40). The expression levels of this gene were at comparable levels when assessed on the array and by qPCR. All these genes seem to be important in the proliferation and differentiation of the developing bone tissue.

TGFbeta family plays a role in development and regulation of cellular processes in these tissues (41). TGFbeta2 has a role in the regulation of bone differentiation and remodeling. Inhibition of TGFbeta receptor signaling in osteoblasts leads to an increased bone mass (42). TGFbeta2 and BMP4 were expressed at a high and very close level in both tissues, which matches with their recognized role in regulating the development and function of both these tissues.

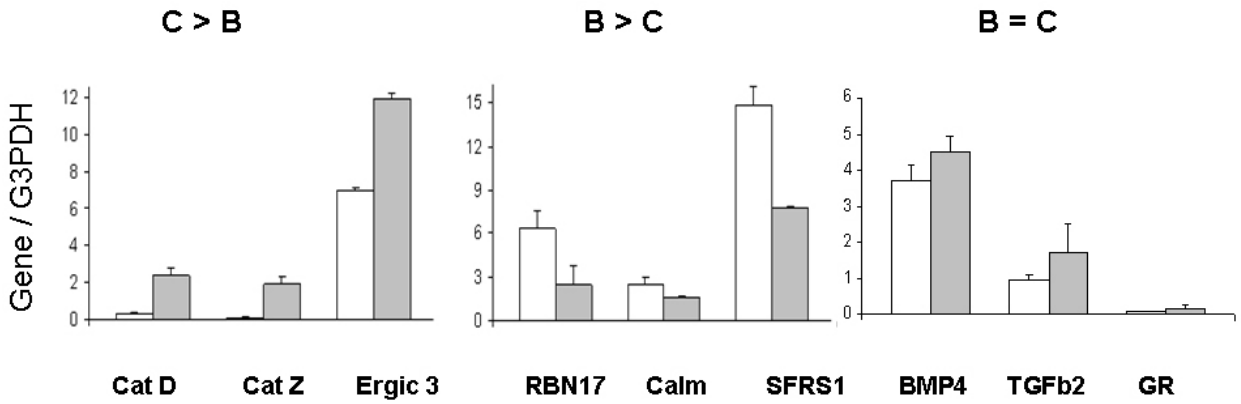

Figure 3. qPCR analysis of expression levels of selected genes. Results are presented in three graphs for genes that there expressed at higher levels in cartilage, then in bone (C>B), in bone higher then in cartilage (B>C) and genes expressed at the same level in bone and cartilage (B=C). Graphs represent the mean±SD (n=3). Grey columns represent levels of gene expression in cartilage; white – in bone.

Other studies that presented gene expression analysis of micro dissected cells from the connective tissues (43-45) analyzed mainly the chondrocytes from articular and the growth plate in mice and rats. A study on 14-day-old mice identify 107 genes that were highly expressed by the articular chondrocytes and 130 genes that expressed by the resting zone chondrocytes of growth plate (44). Another study compared the rat growth plate chondrocytes from the perichondral zone and reserve zone identified 8 unique transcripts to the perichondral included 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) and 8 transcripts that were highly expressed in the reserve zone 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) (45).

In summary, this study allows to demonstrate the utility of applying high throughput microarray analysis of RNA isolated from skeletal tissues cells by LCM. We have shown that the skeletal tissues from 18-Day-old embryos are transcriptional active and explored genes related to metabolism of fatty acids and organic acids, which play a role in regulating bone and cartilage development. The bioinformatic analysis shed light on the gene profile which have not yet been fully explored in the literature and provides a basis for future analysis of pathways in the developing skeleton.

6. ACKNOWLEDGMENT

This study was supported by a grant from the EU-FP7, Excell project 214706. Irena Shur isolated the RNA and performed the PCR analysis.

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Key Words: Laser capture microdissection, Embryo, Cartilage, Bone, Gene expression, Review

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