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Identification of Differentially Expressed Genes Between Osteoblasts and Osteocytes

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Abstract

Osteocytes represent the most abundant cellular component of mammalian bones with important functions in bone mass maintenance and remodeling. To elucidate the differential gene expression between osteoblasts and osteocytes we completed a comprehensive analysis of their gene profiles. Selective identification of these two mature populations was achieved by utilization of visual markers of bone lineage cells. We have utilized dual GFP reporter mice in which osteocytes are expressing GFP (topaz) directed by the DMP1 promoter, while osteoblasts are identified by expression of GFP (cyan) driven by 2.3kb of the Col1a1 promoter. Histological analysis of 7-day-old neonatal calvaria confirmed the expression pattern of DMP1GFP in osteocytes and Col2.3 in osteoblasts and osteocytes. To isolate distinct populations of cells we utilized fluorescent activated cell sorting (FACS). Cells suspensions were subjected to RNA extraction, *in vitro* transcription and labeling of cDNA and gene expression was analyzed using the Illumina WG-6v1 BeadChip.

Following normalization of raw data from four biological replicates, 3444 genes were called present in all three sorted cell populations: *GFP negative*, *Col2.3cyan*⁺ (osteoblasts), and *DMP1topaz*⁺ (preosteocytes and osteocytes). We present the genes that showed in excess of a 2-fold change for gene expression between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cells. The selected genes were classified and grouped according to their associated gene ontology terms. Genes clustered to osteogenesis and skeletal development such as *Bmp4*, *Bmp8a*, *Dmp1*, *Enpp1*, *Phex* and *Ank* were highly expressed in *DMP1topaz*⁺ cells. Most of the genes encoding extracellular matrix components and secreted proteins had lower expression in *DMP1topaz*⁺ cells, while most of the genes encoding plasma membrane proteins were increased. Interestingly a large number of genes associated with muscle development and function and with neuronal phenotype were increased in *DMP1topaz*⁺ cells, indicating some new aspects of osteocyte biology. Although a large number of genes differentially expressed in *DMP1topaz*⁺ and *Col2.3cyan*⁺ cells in our study have already been assigned to bone

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development and physiology, for most of them we still lack any substantial data. Therefore, isolation of osteocyte and osteoblast cell populations and their subsequent microarray analysis allowed us to identify a number of genes and pathways with potential roles in regulation of bone mass.

Keywords

Col2.3; Dentin matrix protein 1; osteoblast; osteocyte; microarray; GFP

INTRODUCTION

Bone is a multifunctional, highly dynamic mineralized connective tissue that undergoes significant turnover. Osteoprogenitor lineage differentiation is one of the key processes responsible for bone formation and remodeling. During this process, a subpopulation of mesenchymal progenitors undergoes osteoblast lineage commitment and matures through a series of differentiation steps. In response to appropriate signals the progenitor cells first proliferate, then secrete extracellular matrix that will then mineralize, embedding the cells within the matrix. The osteocytes, engulfed in this mineralized matrix, represent the terminal differentiation stage of osteoblast lineage. They are the most abundant cellular component of mature mammalian bones and constitute as much as 95% of all bone cells. Osteocytes are thought to be mechanosensors and may coordinate the remodeling process carried out by osteoblasts and osteoclasts.

Comprehensive analysis of gene expression patterns and regulatory networks involved in skeletal development and remodeling is a prerequisite to completely understanding physiological bone structure, function and homeostasis. It also has a crucial role in the development of appropriate therapeutic strategies for various diseases affecting the skeleton. Still, there is limited knowledge about the underlying gene expression pattern that is responsible for the osteoblast-to-osteocyte transition and determination of the osteocyte is morphology and function. Over the years, commitment of osteoprogenitor cells, lineage progression, and differentiation into terminally differentiated bone cells/osteocytes have been studied in various cell lines and primary cultures derived from human and rodents. The most extensively studied primary cell cultures are neonatal murine derived calvarial cells obtained by enzyme digestion. Primarily, the attention has been given to the expression of specific gene products, but up to now only a small percentage of them have been fully characterized within the *in vivo* context of osteoblast lineage differentiation. Obtaining a comprehensive profile of the changes in gene expression as osteoblasts differentiate to their mature phenotype was only feasible recently with the advent of gene array technology. However, the limited number of cells that become mature osteoblasts/osteocytes after osteogenic induction of cell cultures and the heterogeneity of these cultures represent obstacles to their analysis. In addition, utilization of an *in vitro* system makes it unclear whether the observed changes in gene expression originate mainly from a population of fully differentiated osteoblasts and/or osteocytes or from other cell populations present in the samples. Furthermore, the results obtained studying cell cultures represent only an approximation of changes that are occurring in the *in vivo* setting and have to be confirmed by appropriate *in vivo* models.

In addition, the lack of appropriate molecular and cell surface markers, that can be used to isolate and characterize these cell populations prevents the isolation of homogeneous cell samples. Therefore, experiments performed on enzymatically-isolated cells still have to deal with substantial heterogeneity in these cell populations. In our previous studies we have utilized visual markers expressed by osteoblast lineage directed promoters (Col3.6GFP and Col2.3GFP) that are active in preosteoblast and osteoblast stages respectively [1].

In this report, we have characterized the global expression profile of osteoblasts and osteocytes obtained from murine neonatal calvaria. In order to selectively isolate defined populations of cells uncontaminated with other cell fractions (various pre-osteoblast and non-osteoblast cells), we used dual transgenic GFP reporter mice. In this animal model, osteoblasts and osteocytes are identified by expression of different GFP variants that allowed separation of these cells as more homogeneous populations. This approach allowed us to define the gene expression profile of the terminal stages of osteoblast lineage differentiation in a manner representing their true in vivo conditions.

MATERIALS AND METHODS

Experimental mouse model

Visual markers directed to osteoblast lineage cells—To define cells as mature osteoblasts we utilized a previously developed and characterized transgenic mouse in which a 2.3 kb collagen type I promoter directs the expression of the cyan variant of GFP to mature osteoblast lineage cells (Col2.3CFPcyan (blue)) [2,3]. To selectively label a population of preosteocytes/osteocytes we have utilized a DMP1 promoter driven GFP (DMP1GFPtopaz (yellow)) [4]. To generate experimental mice we crossed Col2.3CFPcyan with DMP1GFPtopaz mice. The mice were genotyped by epifluorescence detection using of tail snips. The procedures involving the use of animals were approved by Institutional Animal Care Committee under the protocols 2005–147 and 2007–344.

Histological evaluation of GFP expression

Neonatal mice harboring dual transgenic constructs (pOBCol2.3CFPcyan and DMP1GFPtopaz) were killed by CO₂ asphyxiation. Calvariae were dissected free from surrounding tissue and fixed in 10% formalin at 4° C for 24 hours. Following fixation, bones were decalcified in 15% EDTA (pH 7.1) for 24 hours, placed in 30% sucrose overnight, and embedded in tissue embedding medium (Cryomatrix, Thermo Shandon, Pittsburgh, USA) on dry ice. Bones were cryosectioned longitudinally in 5 µm thin sections using a CryoJane tape transfer system (Instrumedics, NJ, USA). After rehydration in 1 mM MgCl₂/physiological saline, GFP expression was observed and photographed using a Zeiss Axiovert 200M microscope and an Axiocam digital camera. The following GFP-variant specific filters were utilized: GFPtopaz/Texas Red dual filter cube for visualization of green fluorescent protein, and Cyan/Texas Red dual cube for blue fluorescent protein as described previously. The filters were obtained from Chroma (Rockingham, VT). The dual bandpass design is required to distinguish the color of the GFP signal from the autofluorescence of the bone and bone marrow.

Separation of cell populations

Calvariae were isolated from 5–8-day-old double transgenic mice (pOBCol2.3-GFPcyan and DMP1GFPtopaz) killed by CO₂ asphyxiation. After the removal of the sutures, pooled calvarial tissue (8–15 animals per biological replica) were subjected to four sequential, 30-minute long digestions in a mixture containing 0.05%/0.2mM trypsin/EDTA and 1.5U/ml collagenase-P (Roche) at 37°C. Cell fractions 2–4 were collected, pooled and resuspended in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% FBS (Hyclone) and centrifuged. Cells were resuspended in PBS, filtered through a 70-µm cell strainer, centrifuged, resuspended in the PBS/2%FBS, and filtered through a 45-µm filter. Cell sorting was performed using a FACS-Vantage BD cell sorter with a 130-µm nozzle at a speed of 3–5K cells/sec. Sorting was performed using appropriate lasers to distinguish GFPcyan from GFPtopaz expressing cells. The GFPcyan was excited at 413nm by the violet line of a krypton laser, and a 470/20 emission filter was used, while GFPtopaz was excited at 488 nm with an argon laser and a 550/30 emission filter was utilized. Sorting can identify four different populations: a) DMP1GFPtopaz[−]/pOBCol2.3-GFPcyan[−] (termed *GFPnegative*), b)

DMP1GFPtopaz⁻/pOBCol2.3-GFPcyan⁺ (termed *Col2.3cyan[±]*), c) DMP1-GFPtopaz⁺/pOBCol2.3-GFPcyan⁺ cells and d) DMP1GFPtopaz⁺/pOBCol2.3GFPcyan⁻. Populations that are DMP-1 GFPtopaz positive and identified as groups C and D were combined into one sample during the cell sorting procedure (termed *DMP1topaz[±]*). Cells were collected in DMEM media containing 20%FBS, centrifuged and washed in cold PBS. Prior to, during and following the whole sorting process cell suspensions were kept cold to minimize changes in gene expression.

RNA extraction, and array hybridization

Total RNA was isolated from sorted cell populations using TRIzol reagent (Invitrogen, Carlsband, CA, USA) according to the manufacturer's instructions. Measurement of RNA yield was performed using a NanoDrop 1000A Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and their quality was verified using Bioanalyzer RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA, USA) following manufacturer's procedure. The cRNA preparation and array hybridization were performed using Illumina microarray technology (Illumina, San Diego, CA, USA). A total of 250 ng of isolated total RNA was convert to biotinylated-cRNA following the Ambion "Illumina® TotalPrep™ RNA Amplification Kit" procedure (Applied Biosystems/Ambion, Austin, TX, USA). Briefly, reverse transcription to synthesize first strand cDNA was carried out for two hours at 42°C, primed with an oligo(dT) primer bearing a T7 promoter, and catalyzed by ArrayScript™ reverse transcriptase. Second strand cDNA was synthesized by adding DNA polymerase I, and RNase H, and incubation was carried out for 2 hours at 16°C. After cDNA purification by proprietary cDNA filter cartridge, eluted cDNA was used for *in vitro* transcription with T7 RNA polymerase (Ambion MEGAscript IVT technology). *In vitro* transcription was carried out at 37°C for 14 hours, yielding with multiple copies of biotinylated antisense RNA molecules from each mRNA in the sample. Labeled cRNA was purified by cRNA filter cartridge. Quality of eluted biotin-cRNA was verified using the Bioanalyzer RNA Nano Chips according to manufacturer's protocol. Measurement of cRNA yield was performed using a NanoDrop 1000A Spectrophotometer. A total of 1,500 ng of biotin-cRNA from each sample (sorted cell population from corresponding biological replicate) was loaded on to an individual array spot on the 6-Sample Illumina Mouse-WG6 v1 BeadChip following the Illumina hybridization protocol. The chips were hybridized at 58°C for 19 hours, washed, fluorescently labeled and scanned in the Illumina BeadArray Reader.

Microarray data analysis

The scanned data were initially analyzed using Illumina BeadStudio software. The presence/absence call was determined and intensity values derived from the hybridization signals of each gene (i.e. illumina's source IDs) to represent their raw expression level. Normalization of raw data was performed using the lumi package of the Illumina microarray analysis software to rescale gene expression intensities across all Mouse-WG6 v1 BeadChip arrays used for hybridization of cRNA samples from four analyzed biological replicas [5]. The annotations of the Illumina probe sets (source IDs) and corresponding genes were derived using the nuID part of the lumi software package.

We applied two statistical methods to select differentially expressed genes: SAM (Significance analysis of Microarray data) and LIMMA (Linear modeling of Microarray data) [6–8]. They rank genes based on q-values and adjusted p-values for multiple comparisons respectively (based on evidence of being differentially expressed). A gene is called differentially expressed by SAM (LIMMA) when its q-value (adjusted p-value) is less than 0.05. The up-regulated and down-regulated genes with a ratio above a pre-set threshold for significantly higher (≥ 2 fold change) and lower expression (≤ 0.5 fold change) intensity between *DMP1topaz[±]* and *Col2.3cyan[±]* cell populations were further analyzed according to their known biological function. We utilized functional annotation tools and bioinformatics software to arrange genes

in related groups according to associated gene ontology terms and participation in biological pathways. For that purpose we were using open-web based DAVID Bioinformatics Resources 2008 [The Database for Annotation, Visualization and Integrated Discovery/DAVID/from the National Institute of Allergy and Infectious Diseases (NIAID), NIH - <http://david.abcc.ncifcrf.gov/>].

Real timePCR data analysis

Following the extraction and quantification procedure, RNA was subjected to DNase (DNase I, Invitrogen) digestion to eliminate genomic DNA contamination. cDNA was synthesized using an Invitrogen Superscript First-strand Synthesis System for RT-PCR. For real time PCR gene expression analysis we evaluated selected genes that exhibited different patterns and levels of expression obtained by microarray analysis. TaqMan® Gene Expression Assays were purchased from ABI and real time PCR was performed on the 7500 Real-Time PCR System (assay ID:Dmp1, Mm01208365_m1; NPY, Mm00445771_m1; Reln, Mm00465200_m1; Kera, Mm00515230_m1; Oscar, Mm00558665_m1). GAPDH was used as internal control (Mm99999915_g1). Before using the $\Delta\Delta CT$ method for quantification, validation experiments were performed to demonstrate that the amplification efficiencies of target genes and the reference gene were approximately equal. For detection of Oscar expression, bone marrow mononuclear cells (BMMC) induced with M-CSF+ RANKL were used as a positive control. Real time PCR analysis was completed on RNA samples derived from three independent biological replicates (Reln, Kera, Npy and Osc). The expression of DMP1 represent the data obtained from one representative biological experiment. Data is presented with standard deviation and statistical analysis was performed utilizing Student's t-test (*DMP1topaz⁺versus Col2.3cyan⁺*).

RESULTS

Histological evaluation GFP expression in mouse neonatal calvarial tissue

We have generated double transgenic mice harboring dual transgene constructs: pOBCol2.3-GFPcyan and DMP1GFPtopaz. To contrast the patterns of expression among specific cell types in bone, decalcified frozen sections of calvariae from 7-day old double transgenic mice were prepared for histological examination. Strong expression of the pOBCol2.3GFPcyan reporter is localized within the osteoblastic layer lining bone surfaces (Figure 1A–B) and in some proportion of osteocytes (Figure 1A, 1C, arrows) [2]. Not all osteocytes express the Col2.3GFP transgene (Figure 1A, 1C, arrowhead). In contrast to the Col2.3GFPcyan expression pattern, the DMP1GFPtopaz transgene is restricted to cells partially or fully embedded in the bone matrix (Figure 1C–D). An overlay image obtained using GFPtpz and GFPcyan specific filters shows the differential expression pattern of osteoblasts and osteocytes (Figure 1E–F). A higher magnification view reveals the expression of DMP1GFP within the osteocytic processes embedded in the bone matrix and extending between osteocytes and osteoblasts on the bone surface (Figure 1F). Osteocyte and preosteocyte specific expression of GFP (topaz) driven by the DMP1 promoter has already been described in previous studies [4, 9–11]. Together with these findings, the data shown in Figure 1 confirms our ability to differentiate between osteoblast and osteocyte cell populations in neonatal calvarial bone on the basis of the dual color, GFP-transgenic approach.

Isolation and separation of cell populations

Heterogeneous cell populations obtained by sequential enzymatic digestion of calvarial tissue excised from neonatal double transgenic GFP-reporter mice were subjected to FACS in order to isolate three cell populations (GFP⁺; gated as population P3), (*Col2.3cyan⁺*; gated as population P4), and (*DMP1topaz⁺*; gated as population P2) (Figure 2A–B). Prior to separation, we evaluated the sorting selection area by analyzing the cells derived from GFP negative, and

single color GFP positive cells (topaz or cyan) (Figure 2B). As shown in Figure 2, this approach yields successful separation of the two colors (GFPcyan versus GFPtopaz expressing cells). GFP negative cells (representing various non-osteoblast cell lineages mixed with osteoprogenitor and preosteoblastic cell populations) were clearly separated from both positive populations, as shown by reanalysis after cell sorting (*GFP*⁻; Figure 2C, quadrant Q3). Cells that expressed Col2.3GFPcyan but did not express DMP1GFPtopaz were isolated as one highly enriched cell population of cells that are localized on the bone surface and named *Col2.3cyan*⁺ (Figure 2C, quadrant Q1). Cells expressing DMP1GFPtopaz; (Figure 2C, quadrant Q4) or both gene markers (DMP1GFPtopaz⁺ and Col2.3GFPcyan⁺; figure 2C, quadrant Q2) were pooled together as they histologically represent a population of cells that are fully or partially embedded within the bone matrix (osteocytes and preosteocytes) and are named (*DMP1topaz*⁺). This approach was necessary since it appears that some of the osteocytes express only DMP1GFPtopaz, but not Col2.3GFPcyan (Figure 1A, 1C).

Gene expression by microarray analysis of isolated populations

Four biological replicates of isolated cell populations obtained by FACS sorting and subsequently processed through RNA extraction, in vitro transcription, cRNA labeling, and microarray hybridization were analyzed. The 6-sample (array spot) Illumina MouseWG-6 v1 BeadChip was used to compare global gene expression profiles between the samples. Each array spot contains a total of 45,856 different oligonucleotide gene probes (i.e. Illumina source IDs,).

To determine the comprehensive gene expression pattern behind the process of osteoblast-to-osteocyte transformation we compared gene expression profiles of fluorescence sorted, (*DMP1topaz*⁺) versus (*Col2.3cyan*⁺) cell populations. After the normalization of raw data for all four biological replicates, 3444 genes (i.e. Illumina source IDs) were found to be expressed in all three populations. We analyzed the microarray data by Significance Analysis of Microarrays (SAM) and Linear Modeling of Microarray data (LIMMA) methods, both of which gave an overlapping list of 561 and 385 genes (Illumina source IDs) with statistically significant changes in gene expression between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cell populations.

We further selected those genes that exceeded a pre-set threshold for significantly higher (≥ 2 fold change) or lower (≤ 0.5 fold) expression intensity difference between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cell populations. Of the genes present on the array, 514 genes (i.e. Illumina source IDs) met this prerequisite. Since the mouse genome on the Illumina Beadchip is represented by more than one source ID per corresponding gene, this resulted in 385 differentially expressed known genes that were examined further. Among them, 136 were down regulated and 249 were up regulated in the *Dmp1topaz*⁺ population. The selected genes were classified according to the molecular function of their cognate protein and their involvement in biological processes and cellular component distribution, using web-based classification programs as described in the Methods section. Based on that approach we present differentially expressed genes in four categories: genes encoding extracellular matrix components and secreted proteins, genes encoding plasma membrane proteins, genes involved in transcription and muscle cell related genes.

Validation of microarray results by real time PCR

The expression of selected genes was confirmed by real time PCR. The data obtained with real time PCR confirmed the microarray data on gene expression for *Dmp1*, *Npy*, *Reln*, *Kera* and *Oscar* (Figure 3.). For this analysis we selected genes by the level of their expression and by differences in patterns of expression. *Dmp1*, *Npy* and *Reln* show a corresponding moderate level of expression and mirror the pattern detected by microarray analysis (increase in

expression in the *DMP1topaz*⁺ population). We have also confirmed the higher level of keratocan expression in osteoblasts (*Col2.3cyan*⁺) versus osteocytes (*DMP1topaz*⁺). Keratocan is the gene that shows the strongest difference between these cell populations with its peak in the *Col2.3cyan*⁺ population. Osteoclast associated receptor (Oscar) gene expression was selected because it exhibits an overall low level of expression in the microarray analysis and was an unexpected finding since it is thought to be expressed by osteoclast lineage cells. We have obtained a similar result by real time PCR, which shows increased Oscar levels in *Col2.3cyan*⁺ cells and very low, but detectable levels in GFP⁺ and *DMP1topaz*⁺ population.

Genes encoding extracellular matrix components and secreted proteins

Differentially expressed genes that exceeded a pre-set threshold in expression intensity between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cell populations and whose protein products constitute basement membrane and extracellular matrix or are secreted proteins are listed in Table 1. They are typically secreted by osteoblasts and represent the most abundant bone proteins. There are a total of 88 genes in this group: 67 exhibit lower expression levels in *DMP1topaz*⁺ and 21 show higher levels of expression in *DMP1topaz*⁺ versus *Col2.3cyan*⁺ cells. Among them, only 49 genes showed a statistically significant change (SAM, LIMMA). As expected, many of these genes encode collagen proteins (15 genes from the list in Table 1.). Among them, five genes have a higher level of expression (*Col15a1*, *Col18a1*, *Col4a1*, *Col22a1* and *Col4a2*), while others (*Col16a1*, *Col27a1*, *Col3a1*, *Col9a2*, *Col9a1*, *Col8a2*, *Col12a1*, *Col14a1*, *Col2a1*, and *Col8a1*) have lower expression intensity in *DMP1topaz*⁺ cells compared to *Col2.3cyan*⁺ cells. Another important constituent of the extracellular matrix are the various matrix metalloproteinases, and from that family matrix metalloproteinase 9 and matrix metalloproteinase 23 (*Mmp9*, *Mmp23*) and a gene for a disintegrin and metalloproteinase with thrombospondin motifs (*Adamts18*) all exhibit lower expression values in *DMP1topaz*⁺ cells. There are seven other genes in this list whose protein products have a peptidase activity: reelin (*Reln*), tubulointerstitial nephritis antigen like gene (*Tingal*), Htra serine peptidase 1 (*Htra1*), serine protease 12 (*Prss12*), metallocarboxypeptidase CPX-1 (*Cpxm1*) and complement factor (*Cfb*). Except for *Reln* and *Tingal*, all the others have lower expression values in *DMP1topaz*⁺ cells. Genes encoding members of the proteoglycan family; i.e. keratocan (*Kera*), aggrecan 1 (*Acan*), proteoglycan 4 (*Prgh4*), hyaluronan, proteoglycan link protein 1 (*Hapln1*), asporin (*Aspn*), chondroadherin (*Chad*), and fibromodulin (*Fmod*), are also down-regulated, with keratocan, showing the largest negative change among all genes analyzed in this study. Furthermore, genes encoding other noncollagenous structural components normally present in extracellular matrix of bone and cartilage tissue, such as fibulin 1 and fibulin 2 (*Fbln1*, *Fbln2*), thrombospondin proteins 2 and 3 (*Thsb2*, *Thsb3*), spondin 1 (*Spon1*), cartilage oligomeric matrix protein (*Comp*), proline arginine-rich and leucine-rich repeat protein (*Prelp*), and matrilin 1 (*Mat1*) also showed lowered expression intensity in the *DMP1topaz*⁺ cell population.

Beside the genes for structural constituent of extracellular matrix and its modulating enzymes, there are a number of other genes on this list with defined functions in bone cell biology. Dentin matrix protein 1 (*Dmp1*) is predominantly expressed by osteocytes and, as expected, it is the gene with the highest positive intensity ratio. Contrary to *Dmp1*, periostin (*Postn*) has a very low expression intensity in *DMP1topaz*⁺ cells [12]. Considering growth factors that affect osteoblast lineage differentiation, members of the transforming growth factor beta (TGF- β), bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs) and fibroblast growth factor families (FGF's) are perceived as main local regulators of osteogenesis. Six members of the TGF- β superfamily in our study showed significant changes in gene expression. Three members of the BMP family (*Bmp4*, *Bmp8a* and *Bmp3*) were increased, while the genes for glial cell line derived neurotrophic factor (*Gdnf*), growth differentiation factor 10 (*Gdf10*) and transforming growth factor beta 3 (*Tgfb3*) had lower expression in *DMP1topaz*⁺ positive cells.

Among members of the fibroblast growth factors (FGF) family, only fibroblast growth factor 1 (*Fgf1*) showed a significantly elevated gene expression in this study. Other growth factors such as pleiotrophin (*Ptn*), as well as genes for insulin-like growth factor binding proteins 3 and 4 (*Igfbp3* and *Igfbp4*) were decreased in *DMP1topaz*⁺ cells.

Since the protein product of the Frizzled1 (*Fzd1*) gene functions as an integral part of the plasma membrane, data for this gene are presented among downregulated genes in Table 2. The cognate proteins of six other genes associated with the Wnt signaling pathway in our data are secreted in extracellular matrix. Therefore they are all, according to the observed ratio of their expression intensities between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cell populations, listed in Table 1.

Interestingly, among them only the *Dkk1* gene showed elevated expression in *DMP1topaz*⁺ cells. We also observed elevated expression of the gene encoding neuropeptide Y (Npy). Although expression of neuropeptide Y has been previously found in nerve fibers within the bone tissue and its receptors are present in osteoblast lineage cells, the exact role of this protein in bone biology is still to be deciphered [13].

Expression of genes encoding plasma membrane proteins

Among the genes encoding plasma membrane proteins, 75 of them show differential expression between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cells. *DMP1topaz*⁺ cells had higher expression of 51 and lower levels of 24 genes than *Col2.3cyan*⁺ cells (Table 2). The largest increase (8 fold) in gene expression among them was observed for the phosphate-regulating gene with homologies to endopeptidase on the X chromosome (*Phex*), a gene expressed predominantly by terminally differentiated osteoblasts [14,15].

Other up-regulated genes associated with bone formation, metabolism and structure include progressive ankylosis (*Ank*), gene encoding the guanine nucleotide binding protein (*Gnas*) and ectonucleotide pyrophosphate/phosphodiesterase protein 1 (*Enpp1*). Another member of this protein family, *Enpp6*, showed much higher expression in *DMP1topaz*⁺ cells (4.6 fold increase), compared to *Enpp1*, whose role in bone biology remains to be further investigated. Podoplanin, the earliest osteocyte-selective protein expressed during osteoblast-osteocyte differentiation (also known as E11/gp38, T1alpha, Gp36, Gp40 or RT140, depending on the species and tissue in which it is expressed) is also among the up-regulated genes. Expression of E11 is necessary for elongation of dendritic processes of osteocytes in response to fluid flow shear stress and may be critical for normal osteocyte function and viability [16].

Notch signaling is a key mechanism in the control of cell fate determination and pattern formation during organ development. Furthermore, recent data demonstrated the dimorphic effect of Notch signaling in osteoblast differentiation and bone remodeling [17,18]. We observed that *Notch1*, *Notch3* and delta-like homolog 1 (*Dlk1*) have elevated expression in *DMP1topaz*⁺ cells. The gene for protein tyrosine phosphatase receptor, type Z (*Ptprz1*) which is preferentially expressed in the brain is also up regulated in *DMP1topaz*⁺ cells. While the expression pattern and biological function of *Ptprz1* and its existing protein isoforms are well known in neuronal tissue, the precise role of this gene in bone cell biology still requires further investigations. Contrary to neuronal cells that express all four known isoforms of this protein, studies performed on calvarial bone tissue showed specific expression of only the short transmembrane isoform of *Ptprz1* [19].

Genes encoding transcription factors and related proteins

We observed 29 differentially expressed genes whose protein products function as transcription factors and related proteins (Table 3). Among them, 23 showed increased levels of gene expression in *DMP1topaz*⁺ cells, while the remaining six had higher expression values in *Col2.3cyan*⁺ cells. An increase in expression in *DMP1topaz*⁺ cells was observed for a gene

encoding hairy related transcription factor protein 1 (*Hey1*). Hey1 is a member of the HES transcription factors superfamily that act as notch signaling mediators. Another transcription factor associated with osteoblast lineage differentiation is distal-less homeobox 3 (*Dlx3*) gene showing a 2-fold higher expression in the *DMP1topaz*⁺ population. Dlx3 is one of the homeodomain proteins that provide a key series of molecular switches that regulate expression of Runx2 throughout osteoprogenitor differentiation [20,21]. Other transcription factors that can regulate osteoblast proliferation and differentiation such as paired-like homeodomain transcription factor 2 (*Pitx2*) [22] and T-box3 (*Tbx3*) [23] protein are also increased in *DMP1topaz*⁺ cells. We also observed elevated expression for two Iroquois related homeobox genes: *Irx6* and *Irx5*. Iroquois (*Irx*) proteins comprise a family of homeodomain-containing transcription factors that are involved in patterning and regionalization of embryonic tissues. Until now, only expression of *Irx5* gene was confirmed in an osteocyte like cell line [24]. Other genes with elevated gene expression listed in Table 3 have no known function in osteoblast lineage differentiation, and confirmation of their possible importance will require additional studies.

Interestingly, the highest elevation in gene expression was observed for a gene encoding the helix-loop-helix transcription factor myosin (*Msc/MyoR*). The protein encoded by this gene is a transcriptional repressor that blocks myogenesis and activation of E-box dependent muscle genes [25]. Another gene with known function in muscle biology is the gene for SET and MYND domain containing protein 1 (*Smyd1*). Smyd1, also known as Bop acts as a transcriptional repressor in cardiac and skeletal muscle, as well as in lymphocytes and thymus, and has an essential role in the process of cardiomyocyte differentiation and cardiac morphogenesis [26]. The gene with the lowest expression in *DMP1topaz*⁺ cells is adipocyte enhancer binding protein 1 (*Aebp1*), a transcriptional repressor with carboxypeptidase activity that is expressed in vascular smooth muscle cells, and at lower levels in adipose and osteoblastic cells. Although the expression of *Aebp1* was detected in a murine osteoblastic cell line, its transcription ceased during the mineralization phase [27]. The group of genes with lower expression in *DMP1topaz*⁺ cells includes *Zic1*, Goosecoid (*Gsc*), runt related transcription factor 1 (*Runx1t1*), *Pax1*, and scleraxis (*Scx*), a cartilage specific transcription factor with a basic helix-loop-helix motif.

Genes related to muscle function, development and differentiation

Besides *Msc*, *Vgll2*, *Myocd* and *Smyd1*, whose protein product function as transcription factors and related proteins, 36 other genes that exceeded our pre-set threshold also have well known functions in muscle cell biology. They are listed in Table 4 according to their ratio in expression intensity between *DMP1topaz*⁺ and *Col2.3cyan*⁺ cell populations. Interestingly, all these genes showed elevated gene expression in *DMP1topaz*⁺ cells. One group functions as structural myofibril components (*Myh11*, *Csrp3*, *Sync*, *Acta1*, *Dmd*, *Ttn*, *Tcap*, *Tnnt2*, *Tnni1*, *Myoz2*, *Tnnt3*, *Atp2a1*, *Tnnc2*, *Pdlim3*, *Tnnt1*, *Actn2*, *Tpm2*). Others are involved in various muscle cell processes (e.g. *Casq2*, *Atp2a2*, *Gucyl1a3*, *Atp1a2*), myogenesis (e.g. *Srp3* and *Csrp3*) and skeletal muscle and heart development. Surprisingly, some of them showed the highest elevation in gene expression compared to all other differentially expressed genes in our study. The largest increase (29.6 fold) was observed for skeletal muscle actin alpha 1 (*Acta1*), followed by the genes encoding skeletal muscle protein troponin 2 (*Tnni2*), fast skeletal muscle myosin light chain (*Myh11*), troponin C2 (*Tnnc2*), myosin heavy polypeptide 11 (*Myh11*), and myosin light polypeptide 1 (*Myh11*). All showed a more than twenty-fold elevation in gene expression. Despite their well-known function in muscle cell physiology, elucidation of possible roles of these genes and their cognate proteins in bone metabolism, and elucidation of their exact functions in osteocyte cell biology requires further study.

DISCUSSION

Defining the gene expression profiles of cells within the osteoprogenitor lineage has been the focus of numerous studies. Early reports evaluated gene expression in whole bone tissue samples and in cell lines undergoing osteogenic differentiation due to the presence of different inducers [28–32]. More comprehensive analyses have been conducted on cells stimulated by BMP2 [20,33–36]. These in vitro studies revealed a substantial number of molecular players involved in the process of osteogenic differentiation and helped defined the role of some of the signaling pathways important for its overall control [20,37,38]. However, the inherent heterogeneity of biological models, both in whole tissues or primary cultures and cell lines, can present problems that obscure gene expression in a particular subset of osteoblasts. To overcome these obstacles and obtain cell type specific in vivo expression profiles we have developed an approach using bone-directed promoter-GFP transgene constructs in order to isolate homogeneous populations of cells at relatively specific stages of osteoblast lineage progression [2,4]. This procedure utilizes FACS sorting of GFP-expressing bone lineage cells that have been enzymatically released from neonatal calvarial tissue. Utilizing this approach, we initially reported on gene expression analysis in cells at the preosteoblast (*Col2.3GFP⁺*) and mature osteoblast-osteocyte stage (*Col2.3GFP⁺*) [1]. Recently, we have developed a transgenic mouse harboring a DMP1 promoter GFP reporter that allows for the identification of the terminal osteoblast lineage stage (preosteocytes/osteocytes) [4]. As can also be seen in Figure 1, the expression of GFP is localized to cells that are fully or partially embedded within the bone matrix. By combining complementary GFP variants of blue color (cyan) driven by *Col2.3* and yellow-green color (topaz) driven by DMP1 regulatory sequences, we are now able to distinguish the population of osteoblasts identified by expression of *Col2.3Cyan* from the osteocytes expressing *DMP1GFPtopaz*. Since the isolation of viable osteocytes from the adult bone is extremely difficult, in this study we have utilized cell populations derived from mouse neonatal calvarial tissue. Using the above-described combination of enzymatic digestion followed by appropriately designed cell sorting, we were able to identify and isolate a *DMP1topaz⁺* positive population that constitutes up to 10% of total cells extracted from calvarial tissue.

Furthermore, the final results gathered through microarray data analysis indicated a strong increase in expression of *Phex*, *DMP1* and *E11* in *DMP1GFPtpz* positive cells. That strengthens our initial reports and adds additional validity to the use of the DMP1 promoter to selectively identify osteocytes [4,10,16]. Additionally, in concordance with findings that osteocytes exhibit much lower overall protein production and secretion, we have also observed a decrease in expression of collagen genes and a number of noncollagenous matrix protein genes (fibulin 1, 2, trombospondin 2, 3, hyaluronan, asporin, matrilin, keratocan, aggrecan and etc. (Table 1.) in the *DMP1topaz⁺* population.

Osteocytes have a role as a mechanosensory cell whose transmission of mechanical loading signals is through secretion of specific growth factors [39–41]. This includes the recently identified osteocyte specific protein sclerostin, a product of a *Sost* gene that has the ability to bind *Lrp5/6* and antagonize Wnt signaling [41,42]. However, using the Illumina microarray platform we did not detect significant expression levels of *Sost* in our sorted populations of cells. One possible explanation of this finding could be that cells obtained from newborn calvaria tissue may have a lower level of *Sost* compared to the osteocytes in the long bones of adult mice. Another molecular player involved in this process is *Dkk1*. *Dkk1* can also bind *Lrp5/6* and limits the binding of Wnt proteins to *Lrp5/6* [43,44]. Our results show a higher expression of *Dkk1* in *DMP1topaz⁺* cells, an observation similar to previous report on *Dkk1* expression [1,45].

We also detected the presence of neuropeptide Y (NPY) gene expression, a central regulator of bone mass that exhibited higher levels in *DMP1topaz*⁺ cells. Further studies will be necessary to determine if NPY locally secreted by osteocytes could have a role in regulating osteoblast activity in vivo. In addition, *DMP1topaz*⁺ cells expressed much higher levels of genes encoding proteins that function in synaptic membranes as neurotransmitter-gated ion channels, such as cholinergic receptors *Chrna1*, *Chrb1*, *Chrng* and *Chrna4* or receptor associated proteins of synapse (*Rapsn*). There are also few genes encoding voltage gated ion channels like potassium channel 13 (*Kcnk13*). Interestingly, among all up-regulated genes listed in Table 2., *Chrna1*, which encodes the nicotinic acetylcholine receptor, (alpha polypeptide, muscle), showed the largest elevation in gene expression (15.3 fold). A specific dendritic morphology, formation of a network within mineralized matrix [46], mechanosensory ability and evidence for the expression of NPY and other ion channels, are all observations that support the notion that osteocytes can function as “neuronal” cells in bone.

Microarray analysis generates a large data set that needs to be analyzed in a number of biological replicates, and expression of the genes of interest has to be confirmed by independent techniques such as real time PCR analysis or at the protein level by antibody detection. One of the major problems in evaluating expression of particular gene by microarray arises from the affinity of the probe sequence utilized on the chip as well as the hybridization conditions used. To avoid the above-mentioned problems with probes and hybridization, an analysis utilizing two different microarray platforms would be very beneficial. Nonetheless, a major disadvantage of this approach is the expense of the methodologies utilized. In our analysis we have attempted to evaluate the genes that are known to be regulated between the populations of osteoblasts (*Col2.3cyan*⁺) and osteocytes (*Dmp1tpz*⁺), as well as some genes whose expression has not been reported yet in osteoblast lineage cells (*Kera*, *Npy*, *Reln*, and *Oscar*). Our real time PCR analysis encompassed a number of genes expressed at different levels in isolated populations, from intensities of below 100 units to above 1500 units. The levels of expression below 100 units can still be detected by real time PCR as present, but these very low levels require a more stringent analysis of the particular gene signal by measurement of RNA by real time PCR. In addition we present the data obtained from real time PCR analysis of three independent biological replicates. This analysis of selected genes shows a high level of correlation between the data obtained from microarray analysis and real time qPCR (figure 3 and supplementary figure 1).

The approach we have utilized in this study is unique and novel in the field of bone biology. Data generated in this study have the potential to become an important asset in the study of the effects of numerous factors on osteocyte biology. Our analysis identified different patterns of gene expression that direct or accompany distinct cell functions thought to occur in osteoblasts or osteocytes. The osteoblastic population (*Col2.3cyan*⁺ cells) exhibited high levels of expression of matrix protein genes (see Table 1), a well-known characteristic of osteoblasts. In contrast the osteocyte population does not express major matrix protein genes, but has relatively high levels of genes (*DMP1*, *Phex* and *ANK*) that participates in phosphate regulation. The dendritic morphology of osteocytes and their expression of voltage gated channels and cholinergic receptors, along with neural tissue related genes (*Reln*, *Npy*), is supportive of their role as mechanosensory and neuronal regulators of bone mass. The list of genes highly expressed in osteocytes includes structural myofibril components such as skeletal muscle actin alpha 1 (*Acta1*), which has the highest change in gene expression compared to all other differentially expressed genes in our study (29.6 fold increase). Recent report revealed an interesting aspect of osteocyte morphology. Using a time lapse imaging technique Dallas et al. have shown that osteocytes are not a static cell embedded within the bone matrix [47]. As time lapse imaging can detect movement of different cells types further studies will be necessary to define if the higher expression levels of muscle related genes are responsible for the osteocytes processes movement.

To summarize, we would like to indicate some conceptual difficulties with approach utilized in this study. We used intramembranous bone (calvariae) as the source of osteoblasts and osteocytes, so the data obtained may be different from the gene expression pattern exhibited by osteoblasts versus osteocytes isolated from endochondral bone. In addition, calvaria were obtained from neonatal animals which allow for easier digestion of the bone matrix. It is possible that cells derived from neonatal bones might maintain expression of certain genes (a number of muscle lineage related genes) that could represent earlier stages of the mesenchymal lineage. The calvarial bones, especially at the neonatal stage, are also not exposed to any significant level of mechanic loading stimulation, which in long bones is responsible for changes in gene expression of a number of genes that participate in bone formation. Therefore, to overcome these obstacles it remains to develop a methodology that would allow isolation of the osteocytes that are deeply embedded in the matrix of adult long bones. This would facilitate an understanding of the signaling due to mechanical loading or following treatment with different osteogenic agents. Rapid expansion of the osteocyte biology field and development of new approaches to isolate these cells will provide additional insights into osteocyte biology and regulation of bone mass.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Distinct expression of pOBCol2.3GFPcyan, and DMP1GFPtopaz in double transgenic mouse neonatal calvaria tissue

(A, C) represent sections through sagittal suture, while (B, D) are images of section of parietal bone. (A, B) Osteoblasts lining the bone surfaces of calvariae are positive for osteoblastic pOBCol2.3GFPcyan directed expression. (C, D) DMP1GFPtopaz expression is restricted to cells within the bone matrix (osteocytes and peryosteocytes).

(E, F) represents overlaid images taken under topaz and cyan GFP specific filters. Dual Col2.3GFPcyan and DMP1GFPtopaz expressing osteocytes are indicated by arrows in figure 1A and 1C, while some of the osteocytes express DMP1GFPtopaz with no signal of Col2.3cyan (Figure 1A, C, indicated by arrowheads). A higher magnification shows that the expression of DMP1topaz observed within the bone matrix is localized to osteocyte dendritic extension connecting osteocytes and extending to osteoblasts on the bone surface (F, inserted image). Images were taken at the 20x magnification.

Figure 3. Validation of microarray gene expression by real time PCR analysis

Real time PCR analysis for the expression of selected genes (Dmp1, Npy, Reln, Kera and Oscar) was obtained using the Taqman gene expression system. The analysis has been completed for all three isolated cells populations: negative (white bar), *Col2.3cyan*⁺ (blue bar) and *Dmp1tpz*⁺ (green bar). For this experiment three independent biological replicates have been utilized. The exception is the analysis of DMP1 expression that is derived from one of the three representative experiments. To evaluate the level of expression of osteoclast associated receptor (Oscar), in a positive control we have utilized a bone marrow mononuclear cells (BMMC) induced to differentiate into osteoclasts by addition of M-CSF and Rankl (grey bar), (* p<0.05).

Table 1
Genes associated with extracellular matrix and secreted proteins

GENE SYMBOL	GENE NAME	NEG	AVERAGE INTENSITY CYAN	TPZ	RATIO TPZ/CYAN
Dmp1 *#	dentin matrix protein 1	39	77	560	7.3
Col15a1	collagen, type XV	242	190	1222	6.5
Reln *#	reelin	168	84	526	6.3
Olfml2a *#	olfactomedin-like 2A	158	126	712	5.7
Lama5 *#	laminin, alpha 5	41	37	161	4.4
Col18a1 *#	collagen, type XVIII, alpha 1	829	637	2669	4.2
Npy *#	Neuropeptide Y	58	56	231	4.1
Bmp4 *#	bone morphogenetic protein 4	423	405	1439	3.6
Bmp8a *#	Bone morphogenetic protein 8a	62	305	988	3.2
Tinagl	Tubulointerstitial nephritis antigen-like	55	42	136	3.2
Col4a1 *#	collagen, type IV, alpha 1	3801	1848	5978	3.2
Sparcl1 *#	SPARC-like 1	135	104	274	2.6
Arsj *#	arylsulfatase J	147	46	121	2.6
Col22a1	collagen, type XXII, alpha 1	108	5792	14023	2.4
Col4a2 *	collagen, type IV, alpha 2	9785	4222	10059	2.4
Fgf1 *#	fibroblast growth factor 1	52	57	134	2.3
Gal *#	Galanin	65	357	820	2.3
Angpt2 *#	Angiotensinogen 2	518	515	1136	2.2
Dkk1 #	Dickkopf homolog 1	44	154	334	2.2
BC004044 #	cDNA sequence bc004044	53	67	142	2.1
Bmp3	Bone morphogenetic protein 3	192	732	1532	2.1
Col16a1 #	collagen, type XVI, alpha 1	19333	21460	10975	0.5
Col27a1 *	collagen, type XXVII, alpha 1	446	236	120	0.5
Tgfb3	transforming growth factor, beta 3	947	804	411	0.5
Loxl1	lysyl oxidase-like 1	5942	2734	1392	0.5
Cxcl14	chemokine (C-X-C motif) ligand 14	269	121	62	0.5
Il18 *	interleukin 18	278	116	58	0.5
Wnt9a *	wingless-type MMTV integration site 9A	1280	759	378	0.5
Gdnf	glial cell line derived neurotrophic factor	226	175	87	0.5

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Tac1	tachykinin 1	186	112	56	0.5
Fbln1 *	fibulin 1	262	187	92	0.5
Spock2 #	sparc/osteonectin, cwcv and kazal-like domains proteoglycan 2	73	120	58	0.5
C1qmf5	C1q and tumor necrosis factor related protein 5	335	236	114	0.5
Pla2g7	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	235	122	59	0.5
Clec11a **	C-type lectin domain family 11, member a	3619	3628	1745	0.5
Mmp23	matrix metalloproteinase 23	1704	3660	1756	0.5
Wfdc2	WAP four-disulfide core	360	196	93	0.5
Smoc2 #	SPARC related modular calcium binding 2	1838	702	325	0.5
Igfbp4	Insulin-like growth factor binding protein 4	8796	3989	1812	0.5
Hapln1	hyaluronan and proteoglycan link protein 1	314	174	78	0.5
Col3a1 **	collagen, type III, alpha 1	11552	13072	5853	0.5
Gdf10	Growth differentiation factor 10	8382	1508	666	0.5
Dkk3 **	Dickkopf homolog 3 (Xenopus laevis)	7981	7631	3329	0.5
Itih2	Inter-alpha trypsin inhibitor, heavy chain 2	411	147	64	0.5
Htra1	Htra serine peptidase 1	2828	1911	836	0.5
Thbs2 **	thrombospondin 2	16495	13684	6032	0.4
Rspo2 *	R-spondin 2 homolog (Xenopus laevis)	160	130	57	0.4
Spon1	spondin 1, (f-spondin) extracellular matrix protein	93	251	104	0.4
Comp	cartilage oligomeric matrix protein	2104	233	95	0.4
Prss12 **	Protease, serine, 12 neurotrypsin, (motopsin)	718	287	116	0.4
Islr	Immunoglobulin superfamily containing leucine-rich repeat	1797	929	384	0.4
Cpxm1	Metallocarboxypeptidase CPX-1	1810	789	310	0.4
Tgfb1#	transforming growth factor, beta induced	4767	3111	1202	0.4
Igfbp3	Insulin-like growth factor binding protein 3	2006	1329	509	0.4
Chad	chondroadherin	1345	517	195	0.4
Acan *	aggrecan	1285	520	197	0.4
Fbln2	fibulin 2	2199	921	345	0.4
Col9a2	collagen, type IX, alpha 2	1756	236	86	0.4
Lgi2 **	Leucine-rich repeat lgi family, member 2	263	251	92	0.4

GENE SYMBOL	GENE NAME	NEG	AVERAGE INTENSITY CYAN	TPZ	RATIO TPZ/CYAN
Col9a1	collagen, type IX, alpha 1	5774	531	188	0.4
Prg4*#	proteoglycan 4 (megakaryocyte stimulating factor, articular superficial zone protein)	104	132	46	0.4
Thbs3*#	Thrombospondin 3	638	589	203	0.3
Il33	interleukin 33	470	276	94	0.3
Mmp9	matrix metalloproteinase 9	6568	6808	2320	0.3
Adams18#	a disintegrin-like and metalloproteinase with thrombospondin type 1 motif, 18	119	465	158	0.3
Ogn	osteoglycin	1473	775	260	0.3
Lbp*#	Lipopolysaccharide binding protein	351	165	55	0.3
Col8a2	collagen, type VIII, alpha 2	2865	1302	425	0.3
Cfb*	Histocompatibility 2, complement comp. factor B	207	155	50	0.3
Prep#	proline arginine-rich end leucine-rich repeat	3470	2089	679	0.3
Ptn	pleiotrophin	13475	6569	2107	0.3
Stc2*#	Stanniocalcin 2	69	209	67	0.3
Angptl2	Angiotensin-like 2	459	567	177	0.3
Col12a1#	collagen, type XII, alpha 1	4068	5835	1649	0.3
Mfap4	microfibrillar-associated protein 4	4170	1604	402	0.3
Matn1	matrilin 1, cartilage matrix protein 1	8495	919	230	0.3
Col14a1	collagen, type XIV, alpha 1	1976	882	216	0.2
Angptl1*#	Angiotensin-like 1	2468	1941	469	0.2
Wnt10a	wingless related MMTV integration site 10a	42	153	36	0.2
Col2a1	collagen, type II, alpha 1	10001	1938	466	0.2
Col8a1*#	collagen, type VIII, alpha 1	1494	1479	297	0.2
Fmod*#	fibromodulin	450	579	113	0.2
Postn*#	periostin, osteoblast specific factor	1207	1225	238	0.2
Aspn*#	asporin	569	716	129	0.2
Clqmf3*#	Clq and tumor necrosis factor related protein	4089	5231	915	0.2
Sfrp2*#	Secreted frizzled-related sequence protein 2	5666	3384	587	0.2
Kera*#	keratocan	80	1480	50	0.03

TABLE 2
Expression of genes associated with Plasma membrane

GENE SYMBOL	GENE NAME	NEG	AVERAGE INTENSITY CYAN	TPZ	RATIO TPZ/CYAN
Chrna1 ##	cholinergic receptor, nicotinic, alpha polypeptide 1	40	40	613	15.3
Phex #	Phosphate regulating gene with homologies to endopeptidase on the x chromosome	94	1080	8848	8.2
Chrnbl ##	cholinergic receptor, nicotinic, beta polypeptide 1	49	43	343	8.0
Bves #	blood vessel epicardial substance	73	59	375	6.4
Chrmg ##	cholinergic receptor, nicotinic, gamma polypeptide	38	35	220	6.2
Notch3	Notch gene homolog 3 (Drosophila)	211	171	978	5.7
cdhl5	cadherin 15	44	151	807	5.3
Kcnk13 ##	potassium channel, subfamily K, member 13	81	49	246	5.0
Aoc3 #	amine oxidase, copper containing 3	528	168	839	5.0
Gja4 #	gap junction protein, alpha 4	72	60	300	5.0
Enpp6 ##	ectonucleotide pyrophosphatase/phosphodiesterase	59	816	3744	4.6
Pcdh10 *#	protocadherin 10	103	85	369	4.3
tspan12	tetraspanin 12	91	71	307	4.3
itga7 ##	integrin alpha 7	37	33	140	4.2
Mcam #	melanoma cell adhesion molecule	154	102	408	4.0
Kcnell ##	potassium voltage-gated channel, Isk-related family, member 1-like	134	86	339	4.0
Rapsn ##	receptor-associated protein of the synapse	54	39	142	3.7
adcyap1r1 ##	adenylate cyclase activating polypeptide 1 rec. 1	83	47	161	3.4
Ryr1 ##	ryanodine receptor 1, skeletal muscle	49	45	147	3.2
Dlk1	delta-like 1 homolog (Drosophila)	4656	1017	3275	3.2
Esam1 *	endothelial cell-specific adhesion molecule	453	320	1017	3.2
Pcdh7 ##	protocadherin 7	301	161	491	3.0
Pdpr ##	podoplanin	2109	1809	5449	3.0
Chrna4 ##	cholinergic receptor, nicotinic, alpha polypeptide 4	88	46	139	3.0
Slc6a17	solute carrier family 6, member 17	71	41	121	3.0
Cav1	caveolin, caveolae protein 1	1706	1351	3807	2.8
Ca_v1h #	calcium channel, voltage-dependent, alpha 1H	54	41	114	2.8
Magil	membrane associated guanylate kinase	85	94	256	2.7

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Ptprz1 #	protein tyrosine phosphatase, receptor type Z	43	77	201	2.6
ifitm1 #	interferon induced transmembrane protein 1	78	107	277	2.6
Atpla2 #	ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	52	38	96	2.5
Ednrb	endothelin receptor type B	975	159	402	2.5
Cd82 #	CD82 antigen	554	435	1087	2.5
P2ry14 *	purinergic receptor P2Y, G-protein coupled, 14	117	66	160	2.4
Gfra1 #	glial cell line derived neurotrophic factor family receptor alpha 1	175	139	337	2.4
gpr20 #	G protein-coupled receptor 20	44	39	96	2.4
Ank #	progressive ankylosis	659	2177	5226	2.4
Olfir78 #	olfactory receptor 78	42	40	96	2.4
Edg7 #	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	44	208	498	2.4
Irig1 #	leucine-rich repeats and immunoglobulin-like domains 1	332	167	399	2.4
Notch1 #	Notch gene homolog 1 (Drosophila)	448	319	755	2.4
Sgca#	sarcoglycan, alpha	37	135	317	2.3
P2ry1 #	purinergic receptor P2Y, G-protein coupled 1	80	57	133	2.3
Prkeq #	protein kinase C, theta	42	37	86	2.3
Gnas	GNAS (guanine nucleotide binding protein, alpha stimulating) complex	221	84	189	2.2
fyd6 #	FXYD domain-containing ion transport regulator 6	2664	2295	5015	2.2
Ppap2a #	phosphatidic acid phosphatase 2a	636	492	1066	2.2
Jam2#	junction adhesion molecule 2	94	263	555	2.1
Slc8a1 #	solute carrier family 8 (sodium/calcium exchanger), member 1	169	81	171	2.1
chodl	chondrolectin	248	601	1211	2.0
enpp1	ectonucleotide pyrophosphatase/phosphodiesterase 1	242	705	1379	2.0
Calcr	Calcitonin receptor	37	39	76	2.0
Agtr1a	angiotensin II receptor, type 1a	548	329	163	0.5
Csflr	colony stimulating factor 1 receptor	625	237	115	0.5
Panx3 #	pannexin 3	1377	12408	5977	0.5
Caana1g #	calcium channel, voltage-dependent, alpha 1G	610	361	173	0.5
Kcna6	potassium voltage-gated channel, member 6	434	149	71	0.5
f3#	coagulation factor III	377	419	198	0.5
Ly6a	lymphocyte antigen 6 complex, loc. A	2475	726	340	0.5

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Prm8 *	protein arginine N-methyltransferase 8	173	98	46	0.5
il11ra1	interleukin 11 receptor, alpha chain 1	12285	4889	2286	0.5
Gng2	guanine nucleotide binding protein, gamma 2	430	338	156	0.5
Cldn11	claudin 11	2452	672	308	0.5
EfnA5	ephrin A5	4639	3732	1706	0.5
Fgfr3	fibroblast growth factor receptor 3	159	222	98	0.4
Fgfr2 *#	fibroblast growth factor receptor 2	9362	7346	3052	0.4
Oscar	Osteoclast associated receptor	66	245	100	0.4
Tmeff1 *#	transmembrane protein with EGF-like and two follistatin-like domains 1	165	188	78	0.4
slc24a4 *#	solute carrier family 24, member 4	68	115	46	0.4
hs3st3a1 *#	heparan sulfate 3-O-sulfotransferase 3A1	459	325	126	0.4
Fzd1	frizzled homolog 1 (Drosophila)	617	556	219	0.4
Kcnn4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	153	338	132	0.4
Gria3 *#	glutamate receptor, ionotropic, AMPA3 (alpha 3)	417	232	82	0.3
Mst1r *#	macrophage stimulating 1 receptor	174	188	66	0.3
clic6 *#	chloride intracellular channel 6	61	141	45	0.3
Tnmd *#	tenomodulin	4500	6226	853	0.1

Table 3

Transcription factors

GENE SYMBOL	GENE NAME	NEG	AVERAGE INTENSITY CYAN	TPZ	RATIO TPZ/CYAN
Msc #	musculin	122	100	1345	13.4
Zfp536 #	zinc finger protein 536	58	41	305	7.5
Vgll2 #	vestigial like 2 homolog (Drosophila)	41	42	288	6.9
Nrarp #	Notch-regulated ankyrin repeat protein	109	92	588	6.4
Myel1 #	v-myc myelocytomatosis viral oncogene homolog1, lung carcinoma derived (avian)	68	47	195	4.2
Smyd1 #	SET and MYND domain containing 1	49	42	164	3.9
Prox1 #	prospero-related homeobox 1	57	49	145	3.0
Hey1 #	hairy/enhancer-split related with YRPW motif-like	60	61	176	2.9
Sox11 #	SRY-box containing gene 11	942	362	992	2.7
Ankrd15 #	ankyrin repeat domain 15	443	356	973	2.7
Zfp238 #	zinc finger protein 238	235	167	427	2.6
Zbtb46 #	zinc finger and BTB domain containing 46	174	108	274	2.5
Cbfa2i3	core-binding factor, runt domain, alpha subunit 2	123	157	384	2.4
Irx6	Iroquois related homeobox 6 (Drosophila)	38	34	79	2.3
Dlx3	distal-less homeobox 3	73	841	1815	2.2
Satb1 #	special AT-rich sequence binding protein 1	403	677	1461	2.2
Irf8 #	interferon regulatory factor 8	151	51	117	2.2
Pitx2 #	paired-like homeodomain transcription factor 2	62	70	147	2.1
Myocd	myocardin	54	53	107	2.1
Etv5	ets variant gene 5	84	121	246	2.0
Tbx3	T-box 3	92	98	197	2.0
Rb1 #	Retinoblastoma 1	107	186	367	2.0
Irx5 #	Iroquois related homeobox 5 (Drosophila)	807	1646	3163	1.9
Zic1 #	zinc finger protein of the cerebellum 1	2873	1557	815	0.5
Runx1t1	runt-related transcription factor 1translocated to, 1	437	323	161	0.5
Cdkn2b	cyclin-dependent kinase inhibitor 2B	140	446	210	0.5
Scx #	scleraxis	168	339	156	0.5
Gsc #	goosecoid homeobox	147	258	117	0.5

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Pax1 #	paired box gene 1	300	223	98	0.4
Rab15 #	RAB15, member RAS oncogene family	588	448	192	0.4
Aebp1 #	AE binding protein 1	11048	10469	2976	0.3

Table 4
Expression of genes related to muscle function, development and differentiation

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Acta1 #	actin, alpha 1, skeletal muscle	334	160	4727	29.6
Tnni2 #	troponin I, skeletal, fast 2	215	97	2362	24.3
Mylpf #	myosin light chain, phosphorylatable	183	126	3109	24.6
Tnnc2 #	troponin C2, fast	119	97	2359	24.2
Chrna1 #	cholinergic receptor, nicotinic, alpha polypeptide 1	46	49	1174	24.0
Myh11 #	myosin, heavy polypeptide 11, smooth muscle	63	43	1012	23.8
Myl1 #	myosin, light polypeptide 1	73	52	1123	21.5
Tpm2 #	tropomyosin 2, beta	94	76	1288	17.0
Atp2a1 #	ATPase, Ca++ transporting, cardiac muscle	56	41	622	15.3
Tnnf3 #	troponin T3, skeletal, fast	99	65	774	11.9
Actn2 #	actinin alpha 2	55	41	355	8.6
Vgll2 #	vestigial like 2 homolog (Drosophila)	41	42	289	6.9
Myoz2 #	myozenin 2	58	50	338	6.8
Tnnf2 #	troponin T2, cardiac	43	51	305	6.0
Mustn1 #	musculoskeletal, embryonic nuclear protein 1	189	114	680	6.0
pvalb	parvalbumin	79	66	388	5.9
Acta2 #	actin, alpha 2, smooth muscle, aorta	221	139	692	5.0
Tnnf1 #	troponin T1, skeletal, slow	117	97	454	4.7
Lama5 #	laminin, alpha 5	41	37	161	4.4
Csrp3 #	cysteine and glycine-rich protein 3	50	50	187	3.7
Ky #	kyphoscoliosis peptidase	51	57	205	3.6
Bmp4 #	bone morphogenetic protein 4	423	405	1439	3.6
Rtn2 #	reticulon 2	59	62	217	3.5
Tcap #	titin-cap	63	70	236	3.4
Dtna #	dystrobrevin alpha	66	84	283	3.4
Casq2 #	calsequestrin 2	38	38	123	3.3
Dmd #	dystrophin, muscular dystrophy	49	51	165	3.2
Ryr1 #	ryanodine receptor 1, skeletal muscle	49	45	147	3.2
Sync #	syncolin	47	42	129	3.1

GENE SYMBOL	GENE NAME	AVERAGE INTENSITY		TPZ	RATIO TPZ/CYAN
		NEG	CYAN		
Srp3 #	serine/arginine-rich protein specific kinase 3	52	48	144	3.0
Cav1	caveolin, caveolae protein 1	1706	1351	3807	2.8
Gucy1a3 #	guanylate cyclase 1, soluble, alpha 3	514	554	1558	2.8
Capzb #	capping protein (actin filament) muscle Z-β	63	64	179	2.8
My14 #	myosin, light polypeptide 4	308	289	748	2.6
Atpla2 #	ATPase, Na+/K+ transporting, alpha 2 polypeptide	52	38	96	2.5
Pdlim3 #	PDZ and LIM domain 3	45	45	116	2.5
My13 #	myosin, light polypeptide 3	41	58	142	2.5
Dmpk	dystrophin myotonic-protein kinase	399	644	1554	2.4
Notch1 #	Notch gene homolog 1 (Drosophila)	448	320	755	2.4
Sgca #	sarcoglycan, alpha	37	135	317	2.3
Ttn #	titin	46	50	105	2.1
Atp2a2	ATPase, Ca++ transporting, cardiac muscle	872	665	1441	2.1
Tnni1	troponin I, skeletal, slow 1	43	41	87	2.1
Pitx2 #	paired-like homeodomain transcription factor 2	62	70	147	2.1
Myocd	myocardin	54	53	107	2.1
Chodl	chondrolectin	248	601	1211	2.0
Tbx3	T-box 3	92	98	197	2.0