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BONE BIOLOGY

Transcriptome analysis of osteoblasts in an ovariectomized mouse model in response to physical exercise

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Objectives

Osteoporosis is a metabolic disease resulting in progressive loss of bone mass as measured by bone mineral density (BMD). Physical exercise has a positive effect on increasing or maintaining BMD in postmenopausal women. The contribution of exercise to the regulation of osteogenesis in osteoblasts remains unclear. We therefore investigated the effect of exercise on osteoblasts in ovariectomized mice.

Methods

We compared the activity of differentially expressed genes of osteoblasts in ovariectomized mice that undertook exercise (OVX+T) with those that did not (OVX), using microarray and bioinformatics.

Results

Many inflammatory pathways were significantly downregulated in the osteoblasts after exercise. Meanwhile, IBSP and SLc13A5 gene expressions were upregulated in the OVX+T group. Furthermore, in *in vitro* assay, IBSP and SLc13A5 mRNAs were also upregulated during the osteogenic differentiation of MC3T3-E1 and 7F2 cells.

Conclusion

These findings suggest that exercise may not only reduce the inflammatory environment in ovariectomized mice, indirectly suppressing the overactivated osteoclasts, but may also directly activate osteogenesis-related genes in osteoblasts. Exercise may thus prevent the bone loss caused by oestrogen deficiency through mediating the imbalance between the bone resorptive activity of osteoclasts and the bone formation activity of osteoblasts.

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Keywords: Microarray, Osteoblast, Ovariectomized mice, Treadmill

Article focus

- To explore the effect of exercise on the osteoblast in ovariectomized mice by transcriptome analysis.

Key messages

- Exercise has a positive effect on increasing or maintaining bone mineral density in both pre-menopausal and postmenopausal women.
- In micro-CT analysis, exercise prevented bone loss in ovariectomized mice.
- After exercise, the inflammatory microenvironment of the osteoblast in the ovariectomized mice was reduced.
- IBSP and SLc13A5 genes, contributing to bone formation, were upregulated in the osteoblast after exercise.

Strengths and limitations

- The study showed that exercise prevented bone loss by stimulating the expression of IBSP and SLc13A5 genes, beneficial for bone formation, in the osteoblast and reducing the inflammatory microenvironment induced by oestrogen deficiency, to inhibit osteoclast formation.
- We were unable to determine which pathway linked exercise with the upregulation of IBSP and SLc13A5 genes in the osteoblast.

Introduction

Bone mass is determined by a balance between osteoblastic and osteoclastic activity. Osteoporosis is a silent and progressive metabolic disease causing progressive loss of

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bone as measured by bone mineral density (BMD). In postmenopausal women, oestrogen deficiency disrupts the metabolic balance owing to large increases in bone resorption through the enhanced formation and reduced apoptosis of osteoclasts.[1,2] The continuous demolition of bone increases its fragility. Patients with osteoporotic fractures have high rates of mortality and morbidity, and chronic pain reduces the quality of life and increases the cost of social care.[3,4] With the ageing population, osteoporosis has become a major global health problem.[3]

Previous studies have shown that mechanical stimuli can trigger the formation of bone by activating signal pathways and the expression of genes that contribute to its anabolism.[5-7] During physical exercise, bone has mechanical stimuli from ground reaction forces and the contraction of muscles.[8,9] Exercise increases or maintains BMD in both pre- and postmenopausal women,[10-12] and has been recommended as a preventive strategy to manage osteoporosis.[13] In 1998, Oursler found that the gene expression profiles in the osteoblast vary according to the presence or absence of oestrogen.[14] The underlying mechanisms of the effect of exercise on the osteoblasts in oestrogen deficiency are not well understood. The primary aim of this study was to analyze the transcriptome of osteoblasts in ovariectomized mice, with or without the intervention of exercise, by microarray. Comparative analysis was performed using bioinformatics tools for clustering and grouping genes.

Materials and Methods

Six-week-old C57BL/6J female mice were purchased from a commercial supplier (BioLASCO, Ilan, Taiwan). A mouse model of osteoporosis was set up as described by Ferguson et al.[15] The surgical procedures in six mice in a sham group were the same as those used in the ovariectomized mice (OVX), with the exception of leaving the ovaries intact. The sham group served as controls for micro-CT and quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis.

After a two-week postoperative recovery period, the ovariectomized mice were randomly divided into an exercise group (OVX+T, n = 6) and a non-exercise group (OVX, n = 6). As described previously,[16,17] we used a treadmill, with modifications, for the exercise. The OVX+T mice used the treadmill five days a week for eight weeks, with each session lasting 60 minutes. The treadmill was run at 10 m/min with an angle of inclination of 10°. The mice were then killed by CO_2 asphyxiation and their femurs and tibias were harvested. Serum was also collected for assay of bone metabolism biomarkers.

All procedures were approved by the Institutional Animal Care and Use Committee, and all complied with the Guide for the Care and Use of Laboratory Animals available through the National Academy of Sciences (IACUC number: 2015122311).

The harvested femurs were immediately put into Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS) (Gibco, Waltham, Massachusetts) and 1% ammonium persulphate (APS) (Gibco) and residual muscle and connective tissue were meticulously removed. All cleaned femurs were stored in 70% alcohol at -4°C until scanning. The μCT data were acquired by the SkyScan 1076 system (Bruker Microct, Kontich, Belgium) with at 48 kV, 200 μA, and a voxel resolution of 11.54 μm. The images were imported into CTAn software (Bruker) to measure four parameters of the architecture of trabecular bone: bone volume / total volume (BV/TV); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp) and the trabecular number (Tb.N) of the femurs in each group.

The serum level of osteocalcin (SEA471Mu), a marker of bone formation, and cross-linked C-telopeptide of type I collagen (CTX-1) (CEA665Mu), a marker of bone resorption, were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Wuhan USCN Business Co. Ltd, Wuhan, Hubei, China) following the manufacturer's instructions.

Working under a tissue culture hood, adherent soft tissue was removed from the harvested tibias in each group. The epiphysis was removed and the bone marrow cells flushed out using a 27-gauge needle syringe with phosphate-buffered saline (PBS). The hollowed-out bones were milled into bone chips of 1 mm^2 to 2 mm^2 and washed three times with PBS. The chips underwent two sequential 30-minute digestion runs using a mixture (DMEM with 20 ng/ml type II collagenase (Gibco)) at 37°C in an oscillating water bath. After digestion, the chips were washed three times with PBS and transferred into a 10 cm Petri dish containing 20% FBS DMEM (2 mM L-glutamine and 1% penicillin/streptomycin) at a density of about 20 to 30 chips per dish. The primary osteoblast starts to migrate from bone chips. The cells were incubated in a humidified incubator containing 5% CO_2 at 37°C. Fresh medium was replaced three times per week.[18]

RNA extraction and cDNA synthesis were performed as described previously.[19] The quality of total RNA was evaluated by the Agilent system (Agilent Technologies, Inc., Santa Clara, California) according to the manufacturer's protocol. Complementary DNA was synthesized using random hexamers as a primer, as described in the SuperScript IV Reverse Transcriptase Kit protocol for RT (Invitrogen, Waltham, Massachusetts). The cDNA was hybridized to Affymetrix GeneChip Mouse Exon 1.0 ST Array (Affymetrix, Santa Clara, California, and the arrays were normalized (Robust Multi-array Average transcript cluster level) in the Transcriptome Analysis Console (TAC) (Affymetrix, Thermo Fisher Scientific Inc., Santa Clara, California) according to the manufacturer's instructions. The microarray data were deposited at the Gene Expression Omnibus (GEO) website (GEO accession: GSE111628). These data were filtered by a set of

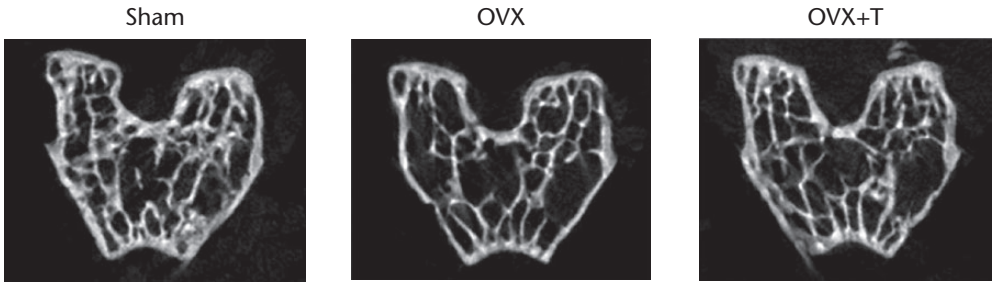

Fig. 1a

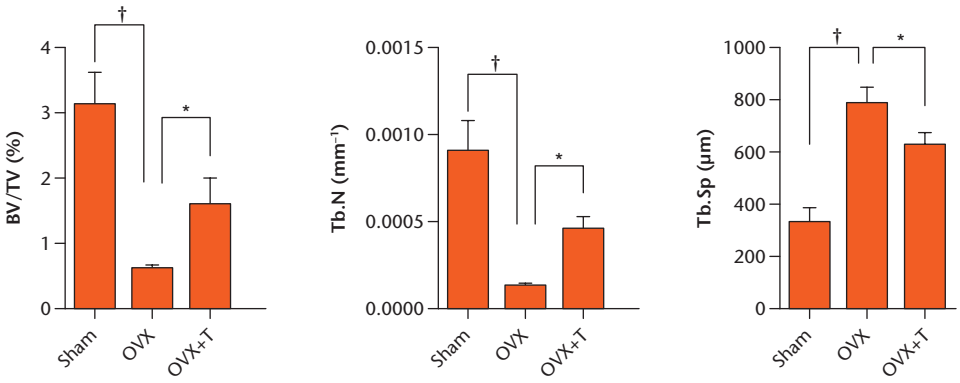

Fig. 1b

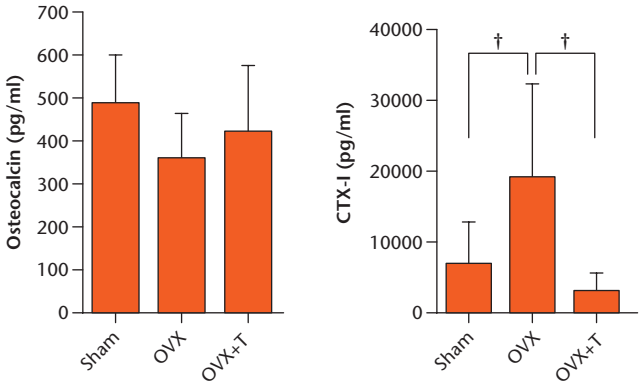

Fig. 1c

Physical exercise diminished bone loss in ovariectomized (OVX) mice. a) Images of the distal femoral epiphysis and b) quantification of bone volume fraction (BV/TV), trabecular number (Tb.N), and trabecular spacing (Tb.Sp). c) Serum bone markers osteocalcin and CTX-1 were measured by specific enzyme-linked immunosorbent assay (ELISA). *$p < 0.05$; †$p < 0.01$. OVX+T, ovariectomized mice that undertook exercise.

Table I. Top diseases and analysis of the functions of differentially expressed genes predicted by Ingenuity Pathway Analysis (IPA)

Top diseases and functions	Molecules in network	Score
Metabolic disease, inflammatory response, organismal injury and abnormalities	ARG1, Bst2, C3, CCL2, Ccl6, Ccl9, CD300LD, Collagen type I, Collagen(s), CREB3L1, CSF2RB, CXCL12, CXCR4, GZMH, ID1, IFIT1B, Ifnar, Ige, IL33, IL12 (complex), IL1R1, IL1RL1, IL1RN, Ly6a (includes others), mir-135, mir-320, MMP9, NFkB (complex), NQO1, OAS2, P38 MAPK, PI3K (complex), PTGS1, RNF213, Wfdc17	47
Metabolic disease, endocrine system disorders, gastrointestinal disease	Acp5, AKR1C3, Akt, Alpha catenin, ANTXR2, CCK, COL3A1, CTSK, CTSV, ERK, ERK1/2, F2R, GPNMB, HAS2, HDL-cholesterol, IGFBP7, IRS2, JAK1, Jnk, let-7, LIPA, LPL, p85 (pik3r), PAK1, PDGFRA, PIK3R5, Pka, RGS2, SERPINE1, Tgf beta, THBS4, TNC, TNN, VCAN, XDH	44
Inflammatory response, infectious diseases, cellular movement	AHR, CACHD1, CELA1, CLEC12A, COX8A, CXCL6, CYP1B1, EDN1, EMP2, F13A1, FADS3, FOLR2, GADD45A, GBP2, GLIS2, Gpr137bps, GSTM2, IKBKE, IL6, IL10RA, ITGA5, KEAP1, LBP, LTBP2, MGP, mir-32, miR-191 5p, Pcp4l1, PPIF, PTAFR, Rpl29, SASH1, SDC1, TLR3, TNC	20

absolute fold change cutoff of 1.5 and then analyzed using Ingenuity Pathway Analysis (IPA), a programme interpreting large-scale transcriptome data across species. (Ingenuity Systems, Redwood City, California). The

Table II. Top canonical pathways and percentage of regulated genes analyzed by Ingenuity Pathway Analysis (IPA)

Pathway	Log (p-value)	Downregulated gene, n (%)	No change, n (%)	Upregulated gene, n (%)
Atherosclerosis signalling	5.47	24/114 (*21*)	80/114 (*70*)	6/114 (*5*)
Th2 pathway	4.01	26/135 (*19*)	104/135 (*77*)	1/135 (*1*)
IL-6 signalling	3.34	31/124 (*25*)	89/124 (*72*)	4/124 (*3*)
IL-10 signalling	3.79	21/65 (*32*)	42/65 (*65*)	2/65 (*3*)
PPAR signalling	3.11	25/91 (*27*)	61/91 (*67*)	3/91 (*3*)
Role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis	2.67	51/226 (*23*)	163/226 (*72*)	12/226 (*5*)
NF-κB signalling	2.61	42/172 (*24*)	123/172 (*72*)	3/172 (*2*)
HMGB1 signalling	2.49	32/126 (*25*)	89/126 (*71*)	5/126 (*4*)
IL-17 signalling	2.45	18/79 (*23*)	58/79 (*73*)	3/79 (*4*)
PDGF signalling	2.25	26/90 (*29*)	61/90 (*68*)	3/90 (*3*)
IGF-1 signalling	2.05	27/103 (*26*)	72/103 (*70*)	4/103 (*4*)
CXCR4 signalling	2.03	36/162 (*22*)	121/162 (*75*)	5/162 (*3*)

Table III. The differentially expressed genes in role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis pathway

Gene symbol	Fold change	Full name
Downregulated		
Il1r1	-3.29	Interleukin 1 receptor, type I
Ctsk	-3.04	Cathepsin K
Il33	-2.73	Interleukin 33
Il1rl1(Il33 receptor)	-2.55	Interleukin 1 receptor-like 1
Pik3r5	-2.1	Phosphoinositide-3-kinase, regulatory subunit 5, p101
Irs2	-2.02	Insulin receptor substrate 2
Lrp6	-1.85	Low-density lipoprotein receptor-related protein 6
Nfkbia	-1.81	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
Il1rn	-1.79	Interleukin 1 receptor antagonist (IL1Ra)
Il6	-1.77	Interleukin 6
Igf1	-1.73	Insulin-like growth factor 1; insulin-like growth factor 1 (Igf1), transcript variant 2, mRNA.
Il1rl2	-1.67	Interleukin 1 receptor-like 2
Adam17	-1.65	A disintegrin and metallopeptidase domain 17 (Adam17).
Mmp3	-1.63	Matrix metallopeptidase 3
Tnfrsf11a	-1.62	Tumour necrosis factor receptor superfamily, member 11a, NFKB activator; tumour necrosis factor receptor superfamily, member 11a (RANK)
Pik3r1	-1.6	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
Birc2	-1.6	Baculoviral IAP repeat-containing 2
Pik3cb	-1.59	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide
Il1b	-1.58	Interleukin 1 beta; interleukin 1 beta (Il1b)
Cbl	-1.57	Casitas B-lineage lymphoma
Tnfrsf1b	-1.57	Tumour necrosis factor receptor superfamily, member 1b
Nfat5	-1.57	Nuclear factor of activated T cells 5
Pik3ca	-1.57	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide
Bmpr2	-1.56	Bone morphogenetic protein receptor, type II
Nfatc1	-1.56	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1
Sfrp4	-1.55	Secreted frizzled-related protein 4
Csf1r	-1.54	Colony stimulating factor 1 receptor
Map2k3	-1.52	Mitogen-activated protein kinase kinase 3
Upregulated		
Dkk4	1.62	Dickkopf homolog 4 (Xenopus laevis)
Il1f10	1.5	Interleukin 1 family, member 10

IPA diseases and functions analysis and canonical pathway tool were used to investigate phenotypes and top canonical pathways associated with the differentially expressed molecules, respectively. According to IPA protocol, data sets containing Affymetrix probeset ID identifiers and corresponding expression values were uploaded into the IPA website. Each probeset ID was mapped to a mouse splicing variant in the Ingenuity Knowledge Base.

MC3T3-E1 (CRL-2593) and 7F2 (CRL-12557) were grown in alpha Minimum Essential Medium (αMEM) (2 mM L-glutamine and 1 mM sodium pyruvate), supplemented with 10% FBS, without ribonucleosides and deoxyribonucleosides. Two cell lines were incubated in a humidified incubator containing 5% CO_2 at 37°C. For osteogenic differentiation, MC3T3E1 and 7F2 cells were plated into 48-well plates at about 80% confluence (~3X10^3) and the growth medium was replaced by differentiation medium (growth medium supplemented with 50 μg/ml L-Ascorbic acid (Sigma-Aldrich, St. Louis, Missouri) and 10 mM β–Glycerophosphate disodium salt hydrate (Sigma-Aldrich)). The mineralization of

Table IV. Top analysis-ready molecules predicted by Ingenuity Pathway Analysis (IPA)

Gene symbol	Experimental value	Full name
Downregulated		
MMP9	-7.90	Matrix metallopeptidase 9
PI15	-3.66	Peptidase inhibitor 15
LPL	-3.24	Lipoprotein lipase
THSB4	-3.15	Thrombospondin-4
ACP5	-2.76	Tartrate-resistant acid phosphatase type 5
Upregulated		
SLc13A5	2.87	Solute carrier family 13 member 5
IBSP	2.77	Integrin binding sialoprotein

MC3T3-E1 becomes evident at about 21 days after treatment with differentiation medium and 7F2 at about seven days.[20] The medium was refreshed every three to four days.

The RT-qPCR was performed as described previously.[19] A total of 500 ng cDNA was used with SYBR green PCR master mix (Bio-Rad Laboratories, Hercules, California) and 10 nM of sequence-specific primers (Supplementary Table ii). Amplification of the target sequences was detected with Bio-Rad CFX96 Real-time PCR System. The expression of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used to normalize the abundance of the test RNAs. All RT-qPCR reactions were performed in triplicate. The dentin matrix protein 1 (DMP1) is a highly expressed and critical factor during osteoblast differentiation.[21,22] Alkaline phosphatase (ALP)[23] and collagen I (Col I) are also well known markers of this differentiation. Therefore, DMP1, ALP, and Col I served as a positive control for osteoblast differentiation. As time goes by, the density of cells will increase owing to their growth, and the density will enhance differentiation. Thus, the relative expression was first normalized to that at each timepoint without differentiation medium and then the RNA level at day 0 was set equal to 1.

Statistical analysis. Data are presented as the mean and standard deviation. One-way analysis of variance (ANOVA) was used for determining the differences between the groups. A p-value of < 0.05 was considered significant. All analyses were performed using Prism (6.0; GraphPad Software Inc., La Jolla, California).

Results

The μCT data (Fig. 1) showed that a decrease in BV/TV and Tb.N, and the increase in Tb.Sp, in the OVX group was significantly diminished after exercise. The level of serum CTX-1 was significantly lower in the OVX+T group than in the OVX group, but the level of osteocalcin was not lower. These data indicate that exercise can help maintain bone mass and microarchitecture and protect against bone loss resulting from oestrogen deficiency.

We next compared the gene expression profiles of osteoblasts between OVX+T and OVX groups, by

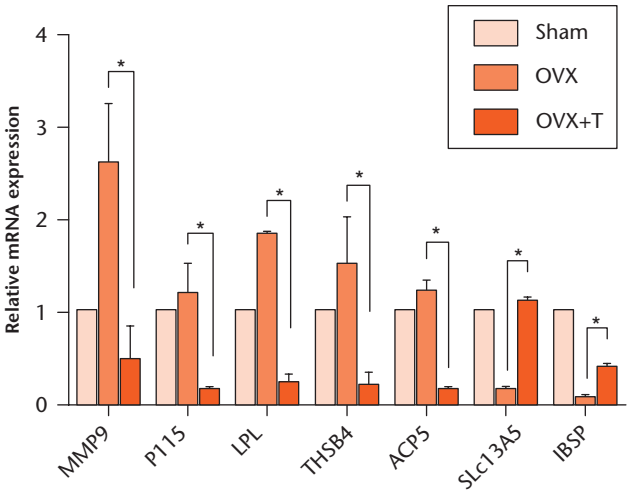

Fig. 2

The validation of microarray by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). Total RNAs of primary osteoblasts from sham, ovariectomized (OVX) and ovariectomized plus exercise (OVX+T) groups were collected and subjected to RT-qPCR analysis. The relative expression of indicated genes was calculated by setting the sham RNA level equal to 1. *$p < 0.05$.

microarray. After normalization of the gene expression, the log2 fold-change between the samples in the two groups was calculated as altered expression values. Scatter plot and hierarchical clustering was performed to visualize the differential expression of genes in the two groups with a set of absolute fold change cutoff of 1.5 (Supplementary Figs aa and ab). Overall, there were 7640 filtered genes; 3267 (42.8%) were downregulated and 4373 (57.2%) were upregulated in the OVX+T group compared with the OVX group (Supplementary Fig. ac).

In order to examine further the biological significance, the filtered genes were subjected to IPA to search the networks for the cellular functions of the differentially expressed genes. Top diseases and functions, determined by IPA, are summarized in Table I. We noted that the differentially expressed genes are primarily involved in metabolic disease and inflammatory response. In order to determine the particular pathway, analysis of the canonical pathways was performed and the results are summarized in Table II. Among these pathways, the role of osteoblasts, osteoclasts and chondrocytes in the rheumatoid arthritis pathway was intuitively relevant to our target call, the osteoblast. So, we further analyzed the molecules in the role of these cells in this pathway and the results are shown in Table III.

IPA identified the differentially expressed genes between the OVX+T and OVX groups and validated by RT-qPCR. Analysis-Ready Molecule analysis was performed to identify key genes further that are directly stimulated by exercise in the osteoblast. A list of significantly regulated genes with the highest score according to IPA is shown in Table IV. The MMP9, PI15, LPL, THBS4, and ACP5 genes were downregulated in OVX+T compared

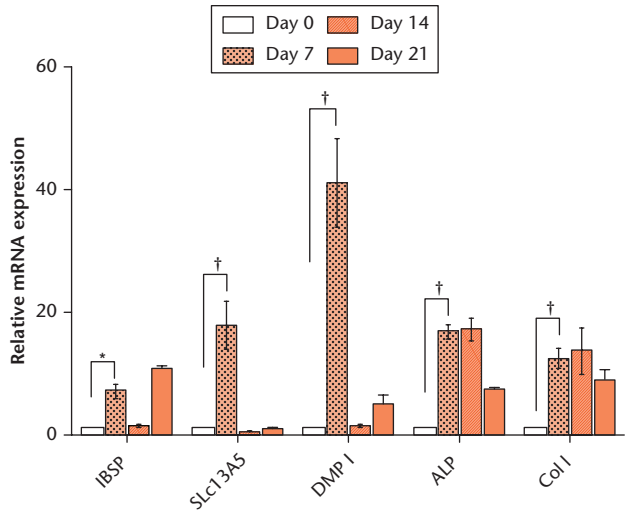

Fig. 3a

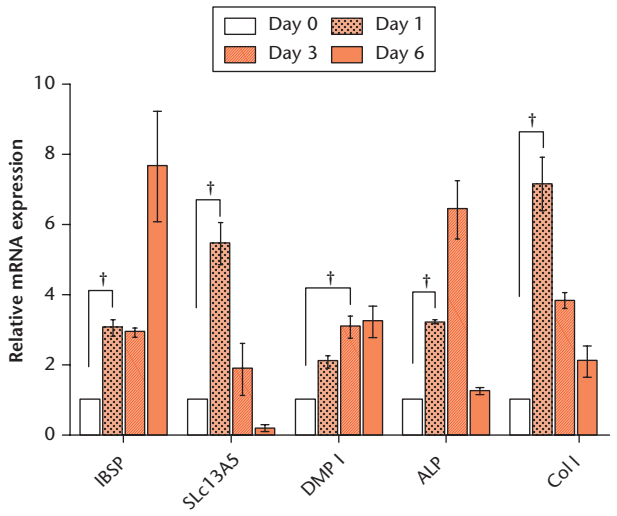

Fig. 3b

IBSP and SLc13A5 mRNA expressed during osteogenesis *in vitro*. a) MC3T3-E1 and b) 7F2 were cultured with and without differentiation medium. The total RNA was isolated at the indicated timepoint and subjected to RT-qPCR analysis. The relative expression was first normalized to that at each timepoint without differentiation medium and then the RNA level at day 0 was set equal to 1. The expression of DMP-1, ALP, and Col I were as positive controls for osteoblast (OB) differentiation. *$p < 0.05$. †$p < 0.01$.

with the OVX, while SLc13A5 and IBSP genes were upregulated. In order to further validate the gene expression profiles identified by IPA, we measured the levels of expression of these genes in the two groups using a RT-qPCR assay with the sham group as a control. The results are shown in Figure 2 and are consistent with those in the microarray.

The IBSP and SLc13A5 genes were upregulated in the osteoblasts after exercise and SLc13A5 was expressed during osteogenesis.

In the upregulated genes, IBSP is known as bone sialoprotein (BSP) and contributes to bone formation.[24,25] It has recently been shown that SLc13A5 deficiency leads to a low BMD and impairs bone formation in the young mouse but not in the older mouse. This suggested that SLc13A5 may play a critical role in the development and function of bone.[26] Likewise, we found a significantly upregulated expression of IBSP and SLc13A5 genes in the osteoblast after exercise. However, the role of SLc13A5 in bone formation is still unclear. Thus, we next examined the mRNA expression of the SLc13A5 gene during osteogenic differentiation. As shown in Figure 3, the mRNA of IBSP and SLc13A5 was markedly upregulated during both MC3T3-E1 and 7F2 osteogenic differentiation.

Discussion

Mechanical stimuli can trigger the osteoblasts to enhance the anabolism of bone.[5-7,27] Although exercise is effective in increasing or maintaining BMD, its effect on the oestrogen-deficient osteoblast has not been defined. We used a genome-wide screen and compared the transcriptomes of osteoblasts in OVX+T and OVX mice. In the WikiPathway analysis, the number of downregulated genes was markedly more than the number of upregulated genes in the inflammatory cytokines pathway. Similarly, in the IPA analysis, the results of biological network and function and significant canonical pathways showed that the most differentially expressed genes were involved in the inflammatory response. The percentage of downregulated genes was also higher than that of upregulated genes in significant canonical pathways analysis, suggesting that the inflammatory stimulation might be reduced. Previous authors have reported that oestrogen deficiency induced an inflammatory microenvironment,[28] and proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), IL-6, and IL-17 stimulate the differentiation of osteoclasts by synergizing with RANKL and upregulating RANKL expression in osteoblasts, thereby expanding the pool of osteoclast precursors.[29-31] Among these pathways, IL-1 is identified as an osteoclast-activating factor and participates in various steps of the development of osteoclasts including differentiation, multinucleation and survival in physiological and pathological conditions.[32,33] Moreover, it has been shown that excess activation of the IL-1 signalling pathway leads to the resorption of bone.[34-37] Interleukin-6 can stimulate bone marrow cells into osteoclastic differentiation,[38] and the bone loss induced by oestrogen deficiency is decreased in IL-6-deficient mice.[39] Interleukin-7 leads to bone loss by stimulating the production of osteoclastogenic cytokines in T cells to induce the formation of osteoclasts and by suppressing the production of osteoprotegerin in osteoblasts to inhibit bone formation. Neutralizing IL-7 can protect against bone loss caused by oestrogen deficiency.[40,41] Likewise, proinflammatory cytokines can result in aberrant osteoblast activity

by disturbing the Wnt and bone morphogenetic protein (BMP) signalling pathways.[42] Additionally, regular exercise induces anti-inflammatory activity.[43-45] Taken together, we propose that the inflammatory microenvironment caused by oestrogen deficiency might be reduced by exercise, resulting in a decrease in the formation of osteoclasts.

An in-depth analysis of the role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis pathways showed that a group of phosphatidylinositol 3-kinase (PI3K) family genes (Pik3r1, Pik3r5, Pik3ca, and Pik3cb) was downregulated. Gámez et al,[46] in 2016, showed that the p110α (Pik3ca) and p100β (Pik3cb), PI3K catalytic subunits, are critical for osteoblast differentiation and bone formation *in vitro* and *in vivo*. The administration of BEZ235 (a pan-class I PI3K and mTOR kinase inhibitor) or PIK75 (a p110α-specific inhibitor) in mice both resulted in loss of bone mass and strength.[47] These results conflict with our data that the Pik3ca and Pik3cb gene expressions were downregulated in osteoblasts after exercise. The disparity between their data and ours may be due to the presence or absence of oestrogen. What functional role Pik3ca and Pik3cb play in oestrogen-deficient osteoblasts requires further study.

MMP9 is predominantly expressed in osteoclasts and is required for them to invade ossification centres and remove hypertrophic cartilage or to widen the medullary cavity. It also stimulates angiogenesis. The MMP9 chemical inhibitor can block osteoclastic resorption, and similar results were shown in MMP9 antisense oligos treatment or MMP9 gene knockout.[48-50] In our data, MMP9 changes the most in the list (Table IV). The function of MMP9 in osteoblasts is not well defined and what role it plays in oestrogen-deficient osteoblasts also requires further study.

Integrin-binding sialoprotein enhances osteoblast differentiation and matrix mineralization through BSP-type I collagen interaction[51,52] and is a key factor in bone formation.[24,25] We found that exercise enhances IBSP gene expression in the oestrogen-deficient osteoblast to increase bone formation. It is similar to another significantly upregulated gene, the SLc13A5. Irizarry et al[26] reported that impaired development in teeth and bone was seen in SLc13A5-deficient mice, and SLc13A5 mRNA was strongly expressed in primary osteoblasts, osteoclasts, and dental cells, suggesting that it may play an important role in bone formation. Likewise, Mantila Roosa et al[53] showed that mechanical loads stimulated IBSP and SLc13A5 gene expression, and the SLc13A5 mRNA level was elevated during bone matrix formation in rats. Based on these findings and ours, we postulated that the upregulation of IBSP and SLc13A5 gene by exercise might be due to mechanical stimulation. Interestingly, the presence of oestrogen is the distinct difference between their findings and ours. Their findings were in the presence of oestrogen while we found that the expression of IBSP and SLc13A5 gene in osteoblasts was still upregulated by exercise in the absence of oestrogen. This suggests that oestrogen has little effect on IBSP and SLc13A5 gene expression stimulated by mechanical load in the osteoblast.

The crosslink peptide sequence of type I collagen, CTX-1, is cleaved by osteoclasts during bone resorption. The serum level of CTX-1 is recommended as a biomarker of the rate of bone turnover.[54] However, its level is also influenced by the circadian variation[55] and food intake.[56] We found the serum level of CTX-1 to be lower in the exercise group than in the sham group. This suggests that exercise might result in interactions between CTX-1 metabolism, circadian variation, and food intake. Despite this further suppression of CTX-1, other parameters of μCT data directly revealed the structure of bone.

Hormone replacement therapy (HRT) is approved for both the prevention and treatment of postmenopausal osteoporosis. Further study is required to confirm the comparison between the effects of HRT and those of exercise.

In conclusion, an overall transcriptomic comparison suggested that the inflammatory environment in the OVX group was reduced by exercise, leading to indirect suppression of overactivated osteoclasts. We also provide evidence that the expression of IBSP and SLc13A5 genes, beneficial for the differentiation of osteoblasts and bone formation, was upregulated in the osteoblast by exercise through a direct mechanical stimulus. Exercise might indirectly suppress the overactivated osteoclast and directly stimulate the osteoblast to mediate the imbalance between osteoclastic bone resorption osteoblastic bone formation.

Supplementary material

Transcriptomic comparison of osteoblasts between ovariectomized mice that undertook exercise and those that did not; mouse strain and training protocol; WikiPathway analysis of differentially expressed genes; and primer sequence.

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Author Contributions

- W-B. Hsu and W-H. Hsu contributed equally to the work.
- W-B. Hsu: Drafted, critically revised, and approved the manuscript, Analyzed and interpreted the data.
- W-H. Hsu: Drafted, critically revised, and approved the manuscript, Analyzed and interpreted the data.
- J-S. Hung: Acquired the data, Performed statistical analysis, Approved the manuscript.
- W-J. Shen: Designed the study, Approved the manuscript.
- R. W-W. Hsu: Designed the study, Approved the manuscript.

Conflict of Interest Statement

- None declared