[Frontiers in Bioscience, Landmark, 22, 783-794, January 1, 2017]

Blockage of hemichannels alters gene expression in osteocytes in a high magneto-gravitational environment

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1. ABSTRACT

Osteocytes, the most abundant cells in bone, are highly responsive to external environmental changes. We tested how Cx43 hemichannels which mediate the exchange of small molecules between cells and extracellular environment impact genome wide gene expression under conditions of abnormal gravity and magnetic field. To this end, we subjected osteocytic MLO-Y4 cells to a high magneto-gravitational environment and used microarray to examine global gene expression and a specific blocking antibody was used to assess the role of Cx43 hemichannels. While 3 hr exposure to abnormal gravity and magnetic field had relatively minor effects on global gene expression, blocking hemichannels significantly impacted the expression of a number of genes which are involved in cell viability, apoptosis, mineral absorption, protein absorption and digestion, and focal adhesion. Also, blocking of hemichannels enriched genes in multiple signaling pathways which are enaged by TGF-beta, Jak-STAT and VEGF. These results show the role of connexin hemichannels in bone cells in high magneto-gravitational environments.

2. INTRODUCTION

During space flight, astronauts experience a variety of physiological changes. Among them, the reduction of bone remodeling is commonly observed (1). Previous studies have been undertaken to investigate the causes of the bone loss induced by microgravity in space; however the underlying mechanism still remains largely elusive (2).

In more recent years, numerous evidence suggest that osteocyte is a primary responser to the changes of mechanical environment in bone tissue (3-5). Several cellular components such as cytoskeleton and membrane channels are shown to be involved in biological responses of osteocytes to mechanical loading (6-8). As the most abundant connexin subtype in bone tissue in forming gap junctions and hemichannels, connexin 43 (Cx43) plays a key role in sensing and transmitting the mechanical signal to chemical signals in bone cells (9,10). Gap junctions and hemichannels permit the passage of small molecules below 1.2. kDa between adjacent cells and the cell and the extracellular environment, respectively (11). Earlier studies have

shown that Cx43-deficiency mice desensitize bone to the effects of mechanical disuse induced by hindlimb suspension (12,13). Also, our previous results have shown that parabolic flight decreases the expression of Cx43 (14). However, there is limited knowledge regarding how weightlessness and hemichanels influence global gene expression pattern and regulatory networks in osteocytes. Due to the technical challenges associated with conducting experiments in the outer space, many studies have been conducted using simulated equipment on the ground. In our labortoary, high magneto-gravitational environment (HMGE) has been used to simulate the effects of weightlessness on mammalian cells, plant callus, silkworm eggs and microorganism (15). The data generated from our previous studies have demonstrated that the simulator is an invaluable tool which can be used to mimic weightless environment in the outer space (16-21).

In this study, microarray analysis was conducted to identify the alterations of global gene expression profile of osteocytic MLO-Y4 cells under HMGE. Furthermore, the effects of Cx43 hemichannels on gene expression were investigated by using a potent, Cx43 hemichannel-blocking antibody Cx43(E2) (22). The specificity ad potency of this antibody on inhibition of Cx43 hemichannels has been demonstrated by us and other groups (23-27).

3. MATERIALS AND METHODS

3.1. Exposure to HMGE

The superconducting magnet used in this study was manufactured by Japan Superconductor Technology, Inc (JASTEC, Seishin, Japan), and several accessories used for cell experiments were constructed in our laboratory, including temperature control system, object stage, gas control system, and observation system. The diamagnetic materials were placed into the bore of the superconducting magnet. In the center of the bore the magnetic field strength is maximal (16 Tesla (T)). Here the magnetic field gradient is zero and the magnetic force is also zero. The diamagnetic materials are only affected by gravity and corresponding position called 1g (16T). At a position where the direction of magnet force and gravity are opposite and the magnitude is equal, the magnet force can balance out the gravity, leading to stable levitation. This position is called μg (hypo-gravity, diamagnetic levitation, 12T). For the same reason, there is a position called 2g (hyper-gravity, 12T) where the direction and magnitude of magnet force and gravity are the same. Figure 1. Diagram illustrates three positions of different magnetic force and gravity in the center of superconducting magnet. For external control (C), the cells were cultured under the identical culture condition in an incubator, but away from the magnet to avoid the influence of magnetic field. We obtained pure gravity effects by comparing the results at 2g and μg, because the magnetic fields of the two positions were identical. Magnetic effects were obtained by comparing the results at 1g and external control, by which the gravity was identical.

3.2. Cell culture

The MLO-Y4 cell line was provided by Dr. Lynda Bonewald (University of Missouri, Kansas City, MO, USA) (28). The cells were cultured in MEM (Gibco, Paisley, UK) with 2.5 percent fetal bovine serum (FBS) and 2.5 percent calf serum including 1 percent penicillin/streptomycin. Cells (5×10^5 cells/dish) in the logarithmic phase were seeded in 35 mm culture dish (Nunc, Inc. Roskilde, Danemark) for 24 hrs, and then treated by irrelevant rabbit IgG antibody (CWBIO, Beijing, China, 1:50 dilution) in an incubator at 37°C with 5 percent CO_2 for 30 mins, then separated into 4 groups: control (C), 1g, 2g and μg groups (Table 1). The cells of C group were cultured in the same incubator for another 3 hrs, and at the meantime the cells of 1g, 2g and μg groups were placed on sample holder and cultured on the HMGE platform with temperature controlled at 37±0.5.°C and 5 percent CO_2 for 3 hrs (15). For antibody treated group, Cx43(E2) antibody (1:50 dilution, 28 μg/ml) was added into the media in 35 mm culture dish for 30 min, the identical culture condition with previous study (22), and then separated into 4 groups: E2-C, E2-1g, E2-2g and E2-μg groups (Table 1). Cells of E2-1g, E2-2g and E2-μg groups were cultured in the given positions on HMGE platform, while cells of E2-C group were cultured in the same incubator with C group.

3.3. RNA extraction and labeling

Total RNA was extracted using TRIzol Reagent (Life technologies, Carlsbad, CA, USA) following the manufacturer's instructions, integrity of isolated RNA was examined by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA). Total RNA was further purified by RNeasy micro kit and RAase-Free DNase Set (QIAGEN, GmBH, Germany). Then total RNA was amplified, labeled and purified using Ambion WT Expression kit (Ambion, USA) and Gene chip WT Terminal labeling Kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions to obtain biotin-labeled cDNA.

3.4. Array hybridization and scanning

The whole genome transcriptional profile was determined by 2 repeats of independent hybridization assay using Affymetrix Mouse Gene 1.0 ST Microarrays. Array hybridization and wash were performed using GeneChip Hybridization, Wash and Stain Kit in Hybridization Oven 645 and Fluidics Station 450 based on the manufacturer's instructions (Affymetrix). Slides were scanned by GeneChip Scanner 3000 (Affymetrix) operated by Command Console Software 3.1 (Affymetrix) with default settings. Raw data were

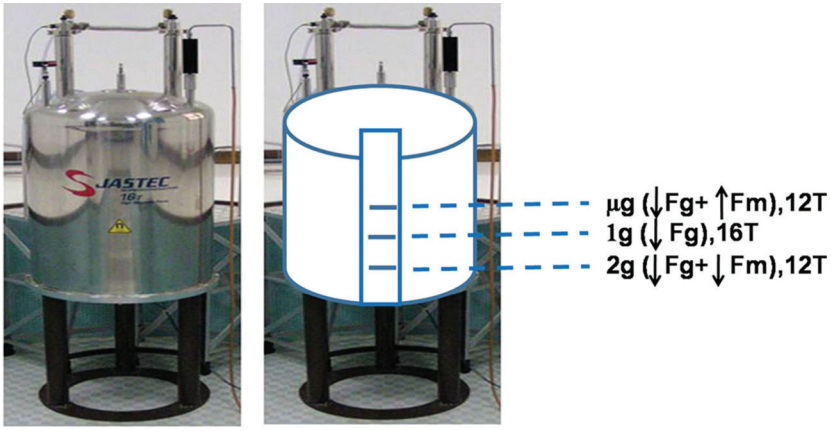

Figure 1. Diagram illustrates three positions of different magnetic force and gravity in the center of superconducting magnet. From top to bottom, μg position, where the direction of magnet force and gravity are opposite and the magnitude is equal, and the magnetic field strength is 12T; 1g, where the magnetic force is zero with only the existence of gravity, and the magnetic field strength is 16T; 2g, where the direction and magnitude of magnet force and gravity are the same, and the magnetic field strength is 12T.

Table 1. Experimental groups

Cx43(E2) treatment	Control groups	E2 groups
Control	- (C)	+ (E2-C)
1g	- (1g)	+ (E2-1g)
2g	- (2g)	+ (E2-2g)
μg	- (μg)	+ (E2-μg)

normalized by Gene Spring Software 11.0 (Agilent Technologies).

3.5. Microarray data analysis

SBC Analysis System (SAS, Shanghai Biotechnology Corporation, shanghai, CN) was performed to filter differentially expressed genes (DEGs) under different conditions, and the fold changes of any two groups more than 1.5 or less than 0.65 were selected. The Gene Ontology (GO) project was used to provide controlled codes to describe gene and gene product attributes in any organism (http://www.geneontology.org). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (http://www.genome.jp/kegg/) analysis was performed to cluster the DEGs according to biological processes. The p-value (EASE-score, Fisher p value or Hypergeometric p value) was used to denot the significance of the pathway correlated to the conditions. The network of pathway (Path-Act-Net) was built according to the relationship among the changed pathways from KEGG database, and the network of gene (Gene-Act-Network) was built according to the relationship among the DEGs. The clustered heatmap was presented using gplots package of R programming tools.

3.6. Real time PCR

Primers were designed and synthesized (Sangon, Shanghai, China) based on the gene sequences available in GenBank. These primer sequences are shown in Table 2. The total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). Real-time relative quantitative RT-PCR analysis was performed with SYBR® Premix Ex Taq™ II based on the manufacturer's protocol (TaKaRa, Dalian, China). Each sample was measured in triplicate and normalized to GAPDH. The $2^{-\Delta\Delta Ct}$ method was used to estimate the relative change in gene expression. The differences between μg (microgravity) *vs* (versus) control, μg *vs* 2g, 1g *vs* control, and antibody-treated groups *vs* control groups were statistically analyzed by one-way ANOVA of GraphPad Prism. *P* value of less than 0.1 was regarded as being significant.

4. RESULTS

4.1. HMGE and hemichannel inhibition affected gene expression profile of MLO-Y4 cells

The genome-wide gene expression analysis using microarray (GEO databases online: GSE85968) was performed to obtain a list of DEGs after exposure to HMGE and hemichannel inhibition for 3 hrs in MLO-Y4 cells (Table 3). Among a total of 35556 detected genes, only 4 genes showed significant alteration in the 2g position, compared with μg position (Figure 2A). These genes were *Slc30a1*, *Zfp942*, *AA987161* and an unknown gene. The results showed that gravity exposure for 3 hrs did not significantly affect overall gene expression profile. However, incubation with Cx43(E2) antibody (E2-2g *vs* E2-μg) altered 56 genes (46 upregulated and 10 downregulated). Interestingly, *Slc30a1, Zfp942* and *AA987161* and unknown genes altered under HMGE condition were not changed by Cx43(E2) antibody. These data indicate that the responsive genes to HMGE are unlikely regulated by Cx43 hemichannels. In 1g position, 49 genes were significantly changed (4 upregulated and 45 downregulated) compared with the external control position with normal gravity. After

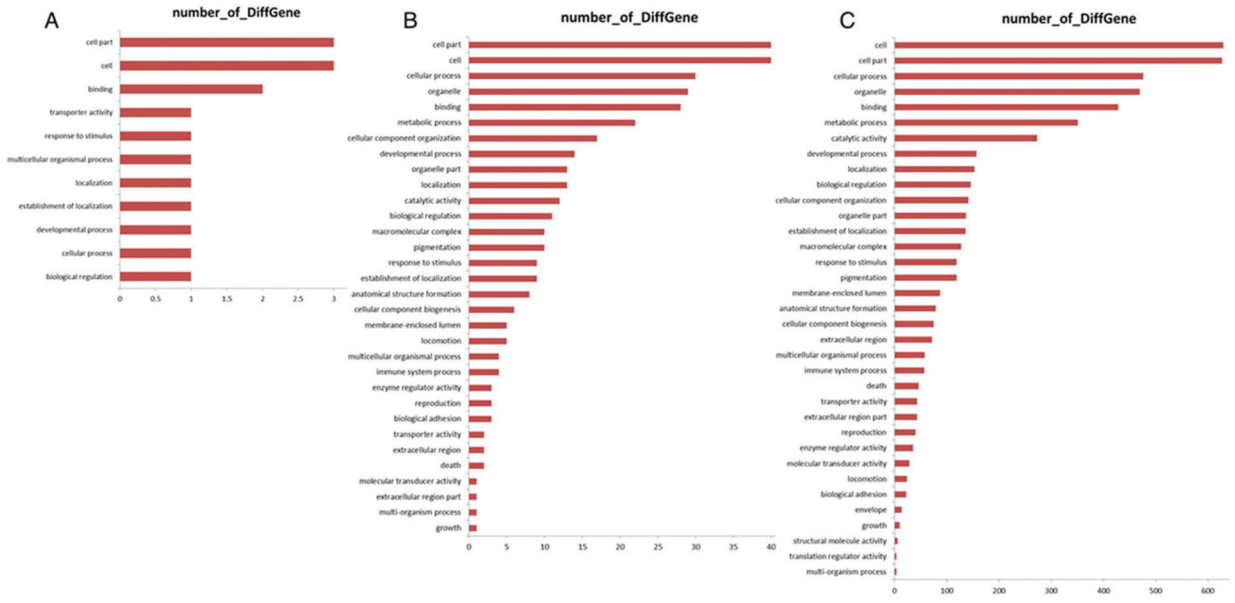

Figure 2. Gene ontology analysis of differentially expressed genes. The subsets of genes that were 1.5-fold upregulated or downregulated were analyzed after exposure of HMGE for 3 hrs and incubating with or without Cx43(E2) antibody in MLO-Y4 cells. (A) μg group *vs* 2g group (gravity effects); (B) 1g group *vs* control group (magnetic effects); (C) E2 groups *vs* control groups (effects of hemichannel inibition).

Table 2. Primer sequences

Gene name	Primer sequences-F	Primer sequences-R
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC
Arhgap5	TGACTTTGGAGGCCGAGTAGT	TGCTGACTGCAATTTTGAGGC
Ccl3	TGCCCTTGCTGTTCTTCTCT	GATGAATTGGCGTGGAATCT
IL-6	CTGCAAGTGCATCATCGTTGTT	CCGGAGAGGAGACTTCACAGAG
Rock1	ATGCCATGTTAAGTGCCACA	TCTTGTTGACAGCGTTCGAG
Col3a1	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
Csf2rb	AAAAACAGCCAGTGTCCTGTG	GATGCTGACGTTCTTGGGAAG
Csf3	GCAGGCTCTATCGGGTATTTC	CTGGAAGGCAGAAGTGAAGG
Figf	GCCCGAGTTAGTGCCTGTTA	TTCTTCTGGGGTCTGAATGG
IL12rb1	AGTTGCGAATGGACTGGAAT	AGCACACCTGAGCCAGAGTT
IL-8	TCGAGACCATTTACTGCAACAG	CATTGCCGGTGGAAATTCCTT
Jak2	AATGAGTGAAACCGAAAGG	ACTACAGTACCAATGAGGGAAG
Kras	TGAACACTGATGGGAAGCAG	CCTACGCCTCTGAAAGATGA
Pdgfra	CACCAAGTCAGGTCCCATTT	TCTTTCTTCGGCTTCTCTGG
Tgfbr1	TCTGCATTGCACTTATGCTGA	AAAGGGCGATCTAGTGATGGA
Xiap	GAAGGCGATAAAGTGAAGTGC	TTCCCAAAGATTCCTCAAGTG

the treatment with Cx43(E2) antibody (E2-1g *vs* E2-c), 36 genes changed (3 upregulated and 33 downregulated). As described in detail, magnetic effect is more predominant at 1g position. Among the DEGs affected by magnetic field, 9 genes changed independent of the antibody treatment (*Zfp942*, *Zfp943*, *Dzip3*, *4932438A13Rik*, *Rock1*, *Dennd4a*, *Stag2*, *Setd2* and *Myo9a*), while the other 40 genes were not changed when compared E2-1g to E2-c, therefore the changes of the 40 genes were eliminated by antibody treatment. GO analysis showed that the 40 genes were

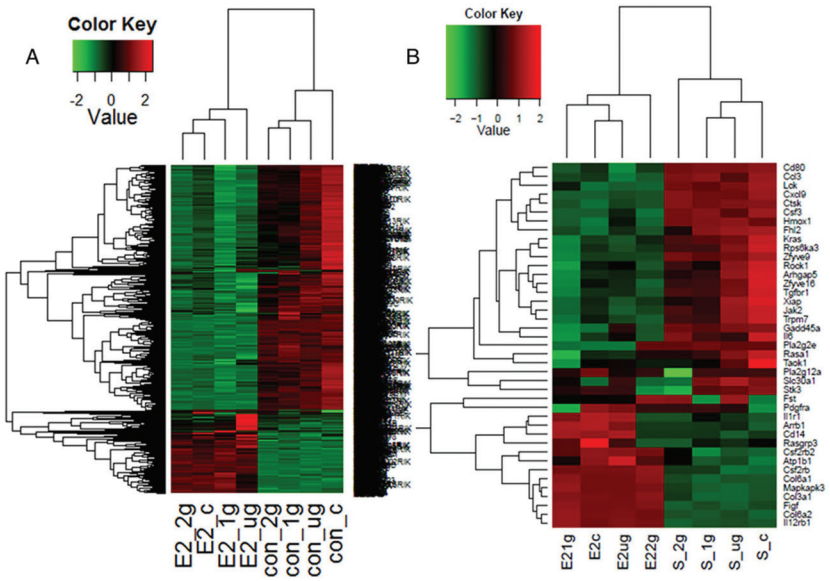

Figure 3. Hierarchical clustering shown by heatmap analysis. (A) the whole gene expression profile; (B) bone metabolism related genes differentially expressed. Red represents up-regulated genes while green represents down-regulated genes.

Table 3. List of differentially expressed genes (DEGs)

	Con-c	Con-μg	Con-1g	Con-2g	E2-c	E2-μg
Con-c						
Con-μg	4(3↑;1↓)					
Con-1g	49(4↑;45↓)					
Con-2g	76(11↑;65↓)	4(3↑;1↓)				
E2-c	801(156↑;645↓)	N/A				
E2-μg	N/A	701(208↑;593↓)	N/A	N/A	63(9↑;57↓)	
E2-1g	N/A	N/A	667(169↑;498↓)	N/A	36(3↑;33↓)	
E2-2g	N/A	N/A	N/A	347((176↑;171↓)	4(1↑;3↓)	56(46↑;10↓)
a(b↑;c↓): total DEGs (up-regulation genes↑; down-regulation genes↓). N/A: not applicable						

mainly involved in various cellular functions including RNA and protein synthesis/degradation (*Twistnb*, *Cul4b*, *Setd2*, *Thoc2*, *Eea1*), bone metabolism (*Arhgap5*, *Trpm7*, *Angpt1*), mitogen-activated protein kinase (MAPK) signaling pathway (*Taok1*) and inositol phosphate metabolism (*Pik3c2a*). For the 49 DEGs affected by magnetic effect, GO analysis showed that there were 40 genes pertained to cellular structural components, and 30 and 22 genes were related to cellular and metabolic processes, respectively. Also, 28 and 13 genes involved in the categories of binding and localization, respectively (Figure 2B).

Interestingly, 801 genes were significantly changed, when comparing Cx43(E2) antibody-treated groups with the control groups (E2-c *vs* Con-c), of which 156 genes were up-regulated and 654 genes were down-regulated. GO analysis showed 629 DEGs enriched in cellular components and 428 genes in binding, 351 and 157 genes were related to metabolic and developmental processes, respectively. Also 273, 153 and 146 genes involved in catalytic activity, localization and biological regulation, respectively (Figure 2C).

The gene expression heatmap and cluster analysis on all of DEGs also confirmed that Cx43 hemichannel was a principal factor in regulating gene expression patterns, more DEGs were detected after blockage of hemichannel (comparing E2 groups with control groups), while gravity and magnetic play relatively minor roles here (Figure 3A).

4.2. Osteocytic hemichannels regulate signaling pathways and biological processes

KEGG pathway analysis was performed to cluster the DEGs to signaling pathways and biological processes (*P* value < 0.5). Because only 4 or 49 genes changed

Table 4. Functional annotation cluster of DEGs (E2 groups vs control groups)

Pathway	P value	DEGs
Ribosome biogenesis	0.0000	Nop58, Utp18, Nmd3, Mphosph10, Rpp40, Riok2, Gnl2, Dkc1, Riok1, Xrn1, Mdn1, Gtpbp4
RNA transport	0.0003	Upf3b, Nmd3, Eif2b3, Eif3a, Xpot, Rpp40, Tgs1, Tpr, Fmr1, Eif2s2, Thoc1, Trnt1, Upf2, Thoc2, Ranbp2, Nup43
Cell cycle	0.0017	Mdm2, Ccnh, Chek1, Smc3, Ccne2, Plk1, Orc2, Stag2, Atr, Gadd45a, Orc4, Atm
Rheumatoid arthritis	0.0024	Ccl3, Atp6v1h, Ctsk, Cd80, Il6, Cxcl5, Atp6v1g1, Cxcl2, Il18
Aminoacyl-tRNA biosynthesis	0.0048	Wars, Nars, Lars, Gars, Eprs, Rars2
Terpenoid backbone biosynthesis	0.0067	Mvd, Pmvk, Pdss1, Acat2
Proximal tubule bicarbonate reclamation	0.0096	Slc4a4, Car6, Pck2, Car5b, Atp1b1
Cytokine-cytokine receptor interaction	0.0125	Csf2rb2, Eda2r, Il1r1, Figf, Pdgfra, Ccl3, Tnfsf15, Csf3, Kitl, Il12rb1, Il6, Csf2rb, Cxcl9, Cxcl5, Cxcl2, Tgfbr1, Il18
Ether lipid metabolism	0.0222	Pla2g12a, Pla2g2e, Cept1, Agps
MAPK signaling pathway	0.0319	Pla2g12a, Il1r1, Pdgfra, Taok1, Rasa1, Pla2g2e, Rasgrp3, Cd14, Arrb1, Cacnb3, Kras, Stk3, Mapkapk3, Gadd45a, Rps6ka3, Tgfbr1
Peroxisome	0.0320	Pecr, Far1, Nudt12, Pmvk, Agps, Abcd1, Pxmp4
Amino sugar and nucleotide sugar metabolism	0.0353	Gnpnat1, Gnpda2, Cyb5r1, Renbp, Uap1l1
One carbon pool by folate	0.0359	Aldh1l1, Aldh1l2, Mtr
PPAR signaling pathway	0.0360	Olr1, Fabp5, Apoc3, Cpt1b, Gyk, Pck2, Fabp7
Ubiquitin mediated proteolysis	0.0364	Mdm2, Uba6, Trim37, Cul2, Cul4b, Ube2q2, Cul5, Ube3a, Xiap, Ube2e1
Hematopoietic cell lineage	0.0451	Il1r1, Cd24a, Csf3, Kitl, Tfrc, Cd14, Il6
Glycine, serine and threonine metabolism	0.0547	Psph, Phgdh, Cth, Dld
Valine, leucine and isoleucine degradation	0.0547	Aldh6a1, 4930438A08Rik, Hibch, Dld, Acat2
p53 signaling pathway	0.0580	Mdm2, Chek1, Ccne2, Atr, Gadd45a, Atm
RNA degradation	0.0613	Hspd1, Exosc9, Skiv2l2, Dhx36, Pan2, Xrn1

the expression after gravity or magnetic treatment, we focused more on the genes altered by Cx43(E2) antibody treatment. Table 4 showed 20 biological processes and pathways with the minimum *P* value influenced by blocking Cx43 hemichannels. Among them, protein/RNA synthesis and digestion were clustered, including ribosome biogenesis, aminoacyl-tRNA biosynthesis, ubiquitin mediated proteolysis, amino acid metabolism (glycine, serine and threonine metabolism and valine, leucine and isoleucine degradation), RNA transport and degradation, etc. Also, we found enriched KEGG pathways related to bone metabolism, such as mineral absorption, osteoclast differentiation and other related signal pathways including MAPK, transforming growth factor beta (TGF-beta), vascular endothelial growth factor (VEGF), Janus kinase-signal transducer and activator of transcription (Jak-STAT) and certain cellular processes including apoptosis, protein digestion and absorption and focal adhesion, *etc*.

The heatmap (Figure 3B) also showed the alterations concerning bone metabolism-related genes after blocking Cx43 hemichannels compared with the control groups including *Tgfbr1*, *IL6*, *Jak2*, *Ctsk*, *Csf*, *Xiap* and *Mapkapk3*, etc.

4.3. Impairment of Cx43 hemichannels greatly affected MAPK signaling, apoptosis, cell cycle and ubiquitin-mediated proteolysis pathways

The network of genes (Gene-Act-Network) and pathways (Path-Act-Net) was built according to the relationship among the DEGs and pathways from the KEGG database. There were direct (i.e. activation, binding or inhibition) or indirect interaction between changed genes related with bone metabolism in response to the inhibition of Cx43 hemichannels. The more one gene interacts with others, the more important the gene is in the Gene-Act network. That means this gene regulate or are regulated by more other genes. From Figure 4, the most central 15 genes were *Pdgfra, Kras, Jak2, Figf, Arhgap5, Csf2rb, IL6, Xiap, Col3a1, Rock1, Csf3, Tgfbr1, IL8, Ccl3* and *IL12rb1*.

Path-Act-Net analysis showed that MAPK signaling pathway, apoptosis, cell cycle and

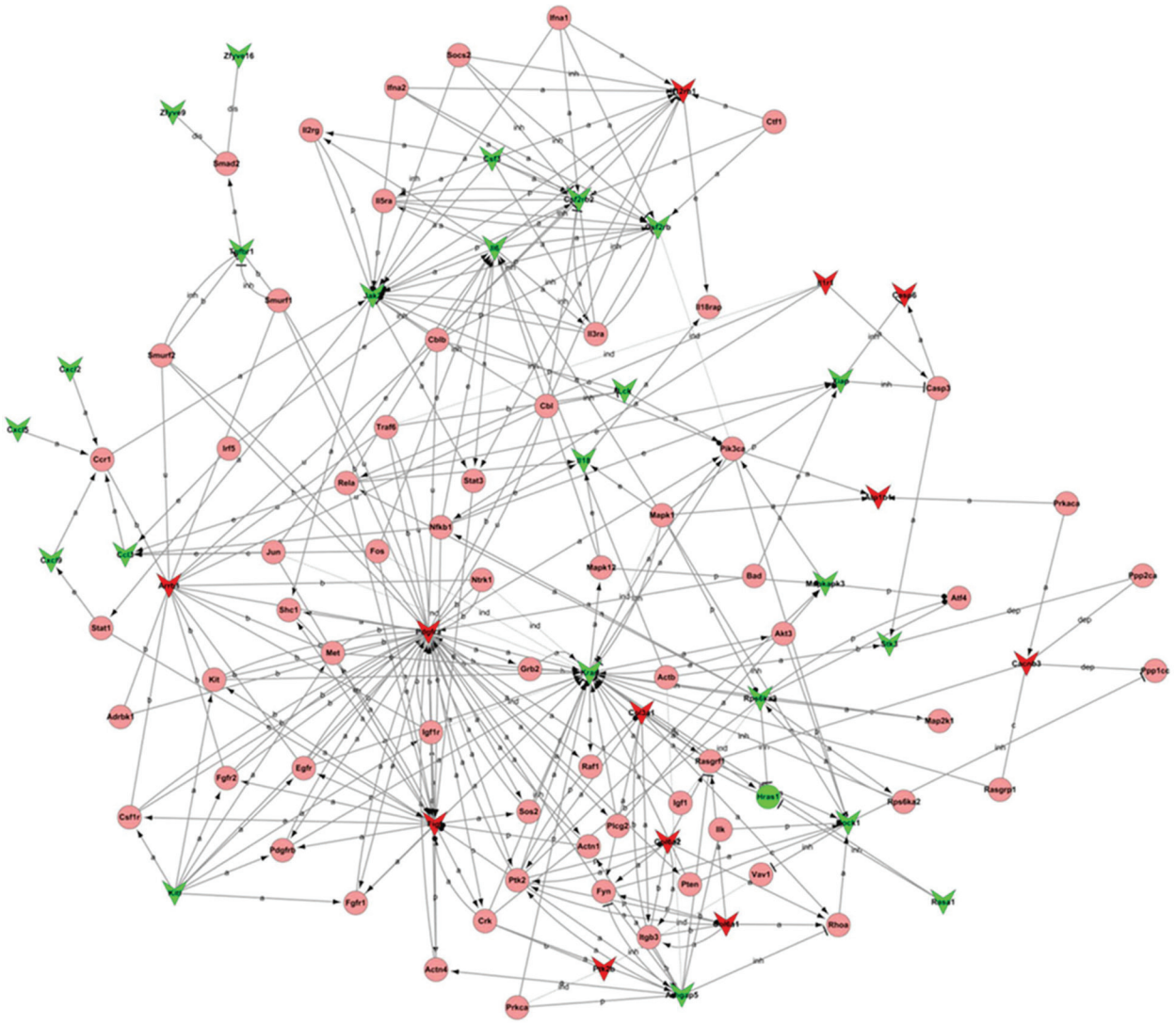

Figure 4. Relationship of differentially expressed genes related with bone metabolism and their responses to hemichannel inhibition by Gene-Act-Net analysis. In the network, red arrowheads are upregulated genes and green arrowheads are downregulated genes. a: activation; b: binding; c: compound; dep: dephosphorylation; dis: dissociation; e: expression; ind: indirect effect; inh: inhibition; p: phosphorylation; u: ubiquination.

ubiquitin-mediated proteolysis were the mostly affected cellular processes by Cx43 hemichannel inhibition (Figure 5).

4.4. Real-time PCR validated the genes responsive to Cx43 hemichannel inhibition

Real-time PCR was used to verify the results of the gene microarray. Figure 6 showed the relative expression of 15 central genes in Gene-Act-Net analysis, including *Pdgfra, Kras, Jak2, Figf, Arhgap5, Csf2rb, IL6, Xiap, Col3a1, Rock1, Csf3, Tgfbr1, IL8, Ccl3* and *IL12rb1*. These genes showed significant difference after Cx43(E2) treatment, which were consistent with the changes of gene expression pattern obtained by gene microarray analysis.

5. DISCUSSION

Our previous results have shown that HMGE treatment for 24 hrs affects osteoblast gene expression profile and cytoskeleton-related gene expression (16, 20). 2595 genes were altered in 1g position *vs* normal gravity control (magnetic effect), while 1729 genes were altered in 2g position *vs* μg position (gravity effect) (29). Also, Hammer *et al*. (2009) have shown that 48 hrs of gravitational stress lead to up- or down-regulation of hundreds of genes in MC3T3-E1 osteoblast cells (30). In osteocytic MLO-Y4 cells, we have shown earlier that 48 hrs of HMGE exposure affect the expression of energy metabolism related genes, including enzyme, peptide hormone, G-protein coupled receptors and

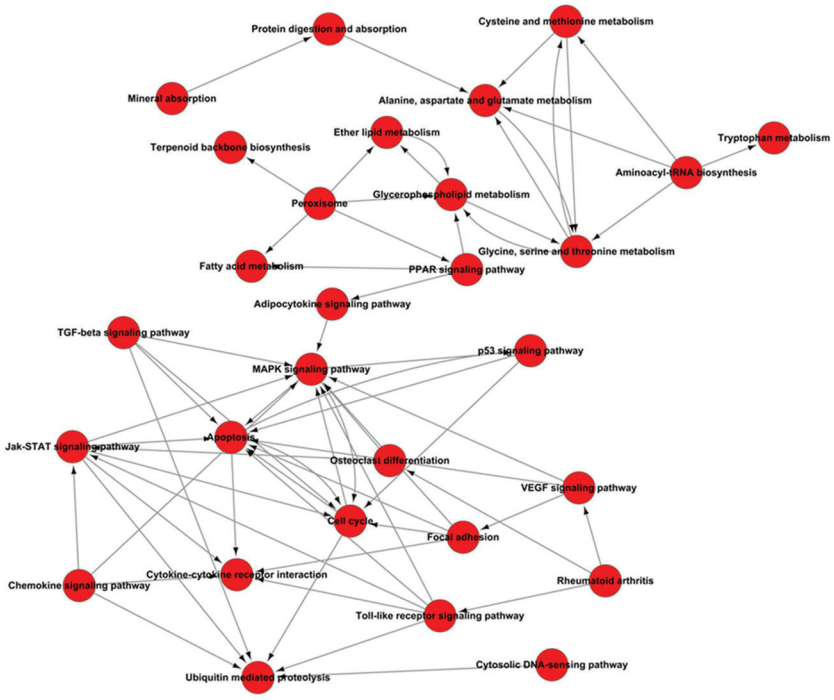

Figure 5. Relationship of changed pathways after hemichannel inbition by Path-Act-Net analysis. Genes in MAPK signaling pathway, apoptosis, cell cycle and ubiquitin-mediated proteolysis were affected by the inhibition of Cx43 hemichannels.

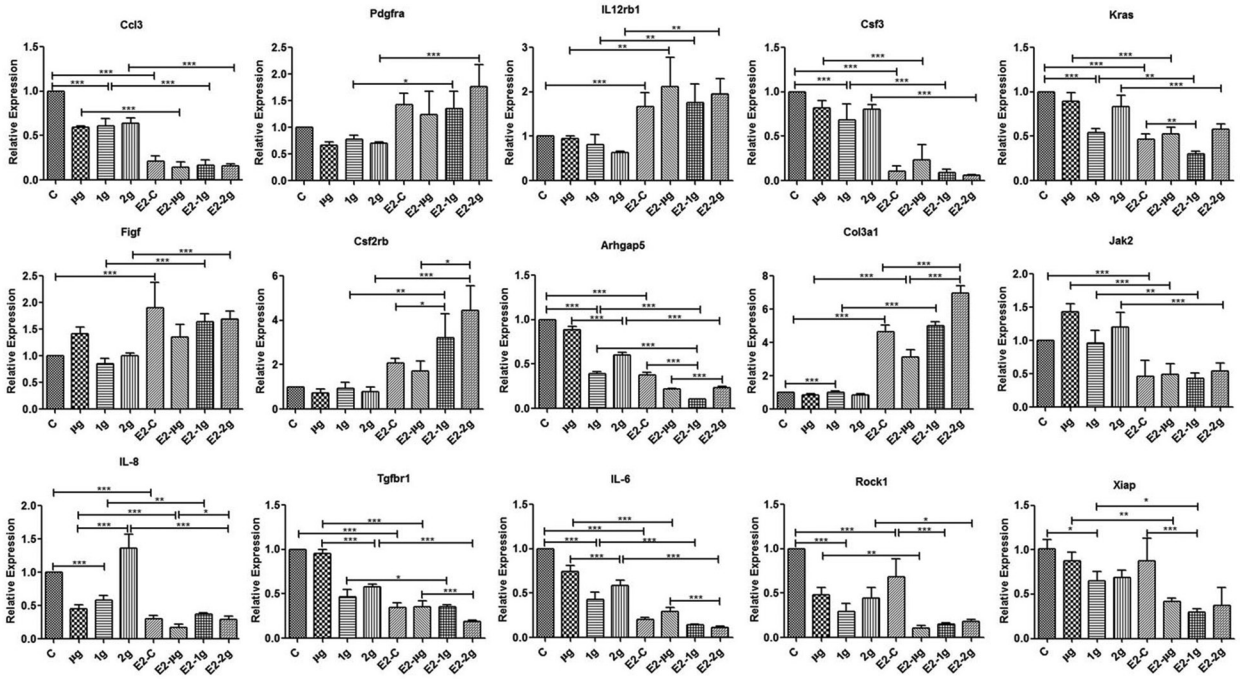

Figure 6. Validation of relative expression levels of representative genes by Real time PCR. Total RNA was extracted and real time PCR assay was used to further identify for 15 selected central genes, including *Pdgfra, Kras, Jak2, Figf, Arhgap5, Csf2rb, IL6, Xiap, Col3a1, Rock1, Csf3, Tgfbr1, IL8, Ccl3* and *IL12rb1*. The expression levels of corresponding genes were normalized by GAPDH. The results were means ± SD. The differences between μg *vs* control, μg *vs* 2g, 1g *vs* control and antibody-treated groups *vs* control groups were statistically analyzed by one-way ANOVA. ***, $P< 0.001$; **, $P < 0.01$; *, $P < 0.1$.

glucose metabolic process (31). To further understand the global gene expression profile of osteocytes under short duration of abnormal magnetic/gravity conditions, in this study, we subjected MLO-Y4 cells to HMGE for 3 hrs. Our results showed that only 4 genes expression was altered in 2g *vs* μg position (gravity effect). It is likely that 3 hrs of exposure to abnormal gravity is not long enough to cause major changes in gene expression of MLO-Y4 cells. Also, 3 hrs of exposure to high magnetic field (16T) only affect the expression of 49 genes (1g *vs* external 1g control) with down-reguation of 45 genes, which suggests a minor influence of magnetic field on cellular and metabolic processes. These results suggest that 3 hrs of HMGE treatment do not exert a significant effect on cellular function of MLO-Y4 cells.

The treatment of Cx43(E2) antibodies which specifically inhibits the opening of hemichannels (22,32) has a great impact on the gene expression of MLO-Y4 cells (E2 groups *vs* control groups, 801 DEGs). This suggests a quick response of the cell to the inhibition of hemichannels regardless of HGME treatment. Cx43 hemichannels in osteocytes have been shown to mediate the release of metabolic molecules in response to stress stimulation, including prostaglandins (PGs), ATP, etc (33-35). Blocking of hemichannels inhibits the exchange of molecules between cells and extracellular matrix, and the response of intracellular signal transduction, which may ultimately affect the gene expression and cellular process. Interestingly, except 2g group with similar up- and down-regulated genes, the other three groups (E2-C *vs* C, E2-1g *vs* 1g, E2-μg *vs* μg) consistently showed significantly more down-regulation genes than up-regulation. This data suggests that hemichannels primarily sever as a positive regulator by enhancing gene expression, and inhibition of the hemichannels has an adverse effect on gene expression in MLO-Y4 cells.

In this study KEGG pathways analysis showed that blocking of hemichannels led to the enrichment of DEGs on signaling pathways of TGF-beta, Jak-STAT and VEGF, and cellular processes of apoptosis, mineral absorption, protein digestion and absorption, and focal adhesion. These pathways and processes have been thought to involve in bone metabolism, especially osteocyte viability and osteoclast formation/differentiation.

Published studies have shown that osteocyte apoptosis is a prelude of osteoclast recruitment and followed by bone loss (36,37). Mechanical stimulation increases the production of factors required for cell survival, and thus inhibits osteocytes apoptosis (38). Our previous studies have demonstrated that Cx43 hemichannels play a predominant role in protecting osteocytes against cell apoptosis and death. We show that the apoptotic osteocytes increased in Cx43(Δ130-136) transgenic mice with the impairment of both hemichannels and gap junctions in osteocytes, but not in Cx43(R76W) mice with the impairment of only gap junctions (39). Also, Cx43 hemichannels protect osteocytes against oxidative stress-induced cell death (25). The mechanism is likely a result of the release of small molecules and/or the activation of other signaling pathways (40). In this study, based on enriched KEGG pathways, we identified several DEGs involved in cellular apoptosis (*Csf2rb2, Il1r1, Csf2rb, Casp6, Xiap* and *Atm*) after Cx43 hemichannel blockage. Moreover, Gene-Act-Net analysis showed the down-regulation of some apoptosis related genes, such as *Jak2*, *IL6*, *Csf2rb2*, *Csf2rb, Csf2* and *Csf3*, etc (41,42). Furthermore, Gene-Act-Net analysis showed up-regulation of genes related to collagen production (*Col6a2*, *Col6a1* and *Col3a1*), which may help increase cell-matrix adhesion. And Path-Act-Net analysis also showed that cell viability related pathways were greatly altered after Cx43 hemichannel inhibition, such as MAPK signaling pathway, apoptosis, cell cycle and ubiquitin-mediated proteolysis. All these gene-related changes support the notion that functional Cx43 hemichannels are crucial to survival and apoptosis status of osteocytes. Furthermore, the gene profile data provided by this study point to potential, related downstream pathways for further investigation.

Taken together, this study showed that short exposure of osteocytes to abnormal gravity and high magnetic field had minor effects on gene expression profile of osteocytic MLO-Y4 cells. Also, the alterations of 40 out of 49 genes under high magnetic conditions were eliminated by Cx43 hemichannel inibition. This result suggests that Cx43 hemichannels mediate biological responses of osteocytes to short term exposure of high magnetic field. Importantly, inhibition of Cx43 hemichannels significantly changed the expression of over 800 genes in MLO-Y4 cells. Our data provide further evidence that Cx43 hemichannels play an important role in regulating global gene expression profile of osteocytes, in particular the genes involved in viability and apoptosis of osteocytes. These results provide important information and guidance for our further understanding the role of connexin hemichannels in bone cells in future.

6. ACKNOWLEDGEMENTS

We thank the grants from the National Natural Science Foundation of China (81472090 & 31328016 to HX, JXJ and PS), Natural Science Basic Research Plan in Shaanxi Province (2015JM8443 to HX), the Fundamental Research Funds for the Central Universities (3102014JKY15012 & 3102016ZY036 to HX), and National Institute of Health grant CA196214 and Welch Foundation grant AQ-1507 to JXJ.

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Abbreviations: HMGE: high magneto-gravitational environment; Cx43: connexin 43; T: Tesla; DEGs: differentially expressed genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK: mitogen-activated protein kinase; TGF-beta: transforming growth factor beta; VEGF: vascular endothelial growth factor; Jak-STAT: Janus kinase-signal transducer and activator of transcription; PGs: prostaglandins

Key Words: Gene Expression Profile, HMGE, Connexin 43, Hemichannel, MLO-Y4 cells

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