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Activation of Nervous System Development Genes in Bone Marrow Derived Mesenchymal Stem Cells Following Spaceflight Exposure

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ABSTRACT

Stalled cell division in precursor bone cells and reduced osteoblast function are considered responsible for the microgravity-induced bone loss observed during spaceflight. However, underlying molecular mechanisms remain unraveled. Having overcome technological difficulties associated with flying cells in a space mission, we present the first report on the behavior of the potentially osteogenic murine bone marrow stromal cells (BMSC) in a 3D culture system, flown inside the KUBIK aboard space mission ISS 12S (Soyuz TMA-8 + Increment 13) from March 30 to April 8, 2006 (experiment "Stroma-2"). Flight 1*g* control cultures were performed in a centrifuge located within the payload. Ground controls were maintained on Earth in another KUBIK payload and in Petri dishes. Half of the cultures were stimulated with osteo-inductive medium. Differences in total RNA extracted suggested that cell proliferation was inhibited in flight samples. Affymetrix technology revealed that 1,599 genes changed expression after spaceflight exposure. A decreased expression of cell-cycle genes confirmed the inhibition of cell proliferation in space. Unexpectedly, most of the modulated expression was found in genes related to various processes of neural development, neuron morphogenesis, transmission of nerve impulse and synapse, raising the question on the lineage restriction in BMSC. J. Cell. Biochem. 111: 442–452, 2010.

KEY WORDS: MSC; AFFYMETRIX; GENE-CHIP ANALYSIS; MICROGRAVITY; SPACEFLIGHT

Exposure to microgravity results in a bone loss of approximately 1% per month [Rabin et al., 1993]. Studies of Skylab and Salyut-6 long-term space missions (28–184 days) found that astronauts not only lost bone density during their missions, but also that 5 years later they had failed to recover to prelaunch bone density levels [Schneider et al., 1995]. Results obtained from humans and rodents after spaceflights indicate that bone mass changes are site specific rather than evenly distributed throughout the skeleton, with the weight-bearing bones more affected than the non-weight-bearing bones [Carmeliet and Bouillon, 1999]. Microgravity induces an uncoupling of bone remodeling equilibrium between bone formation and resorption, which could count for the bone loss [Caillot-Augusseau et al., 1998]. These changes result in weakened and brittle bones prone to fracture on re-entry and in accelerated osteoporosis, making bone deterioration a major problem obstructing the prospects of long-duration manned spaceflight [Baldwin et al., 1996].

A reciprocal relationship is postulated between specific gene expression and bone matrix remodeling. Histological evidence for decreased bone formation obtained in early spaceflight researches was preceded by a detectable altered in vivo expression of genes coding for bone-matrix proteins, such as osteocalcin and prepro-α2(I) chain of the type I procollagen [Backup et al., 1994], and for growth factors, such as transforming growth factor β1 [Westerlind and Turner, 1995] and insulin-like growth factor 1 [Cavolina et al., 1997]. Upon exposure to microgravity, altered gene expression of cytokines, growth factors and matrix proteins was detected in primary cell cultures derived from marrow stroma, as well as in more

Massimiliano Monticone and Yi Liu contributed equally to this study.

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committed osteoblast precursor cell lines [Carmeliet and Bouillon, 1999].

Due to infrequent accessibility of spaceflights, ground-based simulators have become useful venues to create hypogravity conditions. Recently, using powerful high-throughput technology such as microarray, simulated microgravity by random positioning machine (RPM) was reported to inhibit proliferation and osteogenesis in primary cell cultures from rat bone marrow [Dai et al., 2007]. Modeled gravity (10^{-3} g) simulated by the rotating wall vessel (RWV) inhibited osteoblastogenesis and increased adipocyte differentiation in human mesenchymal stem cells [Saxena et al., 2007; Zheng et al., 2007]; in primary mouse calvarial osteoblasts up-regulated expression of genes involved in apoptosis induction and down-regulated expression of genes involved in extracellular matrix components and osteoblast differentiation and function [Capulli et al., 2009]. Diamagnetic levitation alters the expression of cytoskeleton-related genes in human osteoblast-like cell line [Qian et al., 2009]. Each simulator differs from the others mainly in terms of physical principle and duration of weightlessness. A systematical comparison was made between RWV and RPM by microarray analysis of a preosteoblast murine cell line, in which the two simulators of microgravity produced similar but not identical results with regard to bone cell differentiation and osteoblast function [Patel et al., 2007]. Spaceflight remains as the preferred means to study weightlessness effects.

Bone marrow is a physiological niche rich of bone precursor cells. The bone marrow derived mesenchymal stem cells (BMSC) are commonly used as precursor cells source for bone repair [Derubeis and Cancedda, 2004]. They can be readily isolated, expanded, and differentiated towards osteogenic lineage in Petri dishes. When seeded onto porous biomaterials and implanted in immunocompromised mice, autologous BMSC form bone ectopically, providing a valuable mean for critical-size bone repair [Kon et al., 2000]. Porous biomaterials, in comparison to the routine Petri dish 2D surface, provide a more physiological microenvironment for BMSC to proliferate and differentiate in vitro [Tortelli et al., 2009]. In this study murine BMSC were seeded on a silicon-stabilized tricalcium phosphate bioceramic (Skelite™), which is known to favor survival of bone marrow-derived mesenchymal stem cells in transplantation model [Wang et al., 2007]. An additional advantage of Skelite over hydroxyapatite-based scaffold lies in its resorbability [Mastrogiacomo et al., 2007]. BMSC grown throughout the Skelite thin disks were incubated in fully automated cell-incubation units inside the KUBIK payload aboard space mission ISS 12S (Soyuz TMA-8 + Increment 13) from March 30 to April 8, 2006 (experiment "Stroma-2"). Besides static exposure of BMSC/Skelite constructs to spaceflight microgravity, on board centrifuge created an artificial 1*g* conditions. At the end of the 8-day exposure to microgravity, cells were washed with and conserved in RNAlater stabilization reagent for post-flight processes. The effects of microgravity on BMSC were investigated at global gene expression level by microarray. Our data suggest that microgravity and spaceflight exposure alters gene expression profile, favoring neural-associated processes, while inhibiting cell-cycle-related genes. Our analysis also identified cell adhesion as the most affected process by spaceflight during the early stage of osteogenic stimulation.

MATERIALS AND METHODS

ETHICAL APPROVAL

This study did not require the use of humans or animals, but for mice from which tissues were collected in order to establish the cell cultures. These mice were bred and maintained at the Institution's animal facility. The care and use of the animals were in compliance with laws of the Italian Ministry of Health and the guidelines of the European Community. All experimental protocols involving the use of mice were approved by the "Comitato Etico per la Sperimentazione Animale dell'Istituto Nazionale per la Ricerca sul Cancro (Istituto Nazionale per la Ricerca sul Cancro Animal Experimentation Ethical Committee)."

SPACEFLIGHT EQUIPMENT

The spaceflight experiment was performed in fully automated bioreactors (Kayser-Italia, Livorno, Italy) containing one culture chamber connected with five cylindrical plunger compartments. Liquid in the culture chamber was substituted by liquid from storage compartments at preprogrammed times on command of the experiment control unit. The transfer of liquid from each storage compartment to the culture chamber was performed by means of a plunger, driven by a preloaded spring, and activated by a heater-wire system (Fig. 1A,B). Ten bioreactors were used for flight experiment, of which eight were integrated into a static rack (FE, flight experiment) and two on a 1*g* centrifuge in the KUBIK incubator (FC, flight control). The 1*g* centrifuge was used as an in-flight unit-gravity reference. The ground control (GC) was conducted in eight manually operated bioreactors on Earth in another KUBIK payload. Additional eight disks were incubated under routine laboratory conditions (LC) in Petri dishes.

CELL CULTURE

C57BL/J6 mice were purchased from Charles River Laboratories (Calco, LC, Italy). Mice were bred and maintained at the animal facility of the Istituto Nazionale per la Ricerca sul Cancro (Genova, Italy). The care and use of animals were in compliance with laws of the Italian Ministry of Health and the guidelines of the European Community. Six- to 8-week-old mice were sacrificed, by CO_2 asphyxiation, and bone marrow cells were collected by flushing nucleate cells out of the femurs and tibiae with cold PBS. Cells were cultured (2×10^6 nucleated cells/10-cm Petri dish) in a growth medium [Coon's modified Ham's F12 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (GIBCO, Italy), 1% glutamine, 1% penicillin–streptomycin]. No cytokines were added at any stage. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. After 3 days, non-adherent cells were removed. When 80% confluent, the adherent cells were trypsinized (0.05% trypsin/EDTA at 37°C for 10 min) and expanded (P1 stage). Additional quality controls were previously published [Augello et al., 2005].

EXPERIMENTAL DESIGN

Ten days before the Soyuz launch, 2×10^5 BMSC were transferred by capillarity onto a porous bioceramic Skelite disks (Φ 9 mm × T 1.2 mm, Millenium Biologix, Ontario, Canada) and incubated in normal growth medium, that is, alpha-modified minimum essential

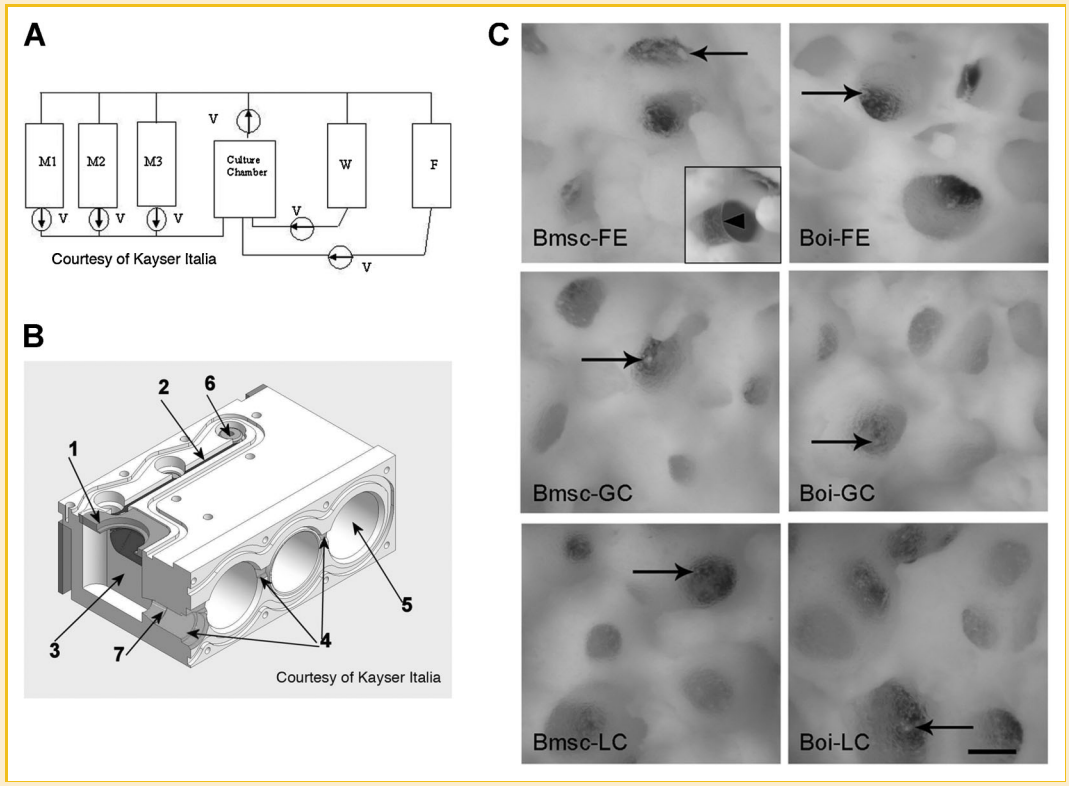

Fig. 1. Experimental bioreactor and the 3D BMSC/scaffold constructs exposed to microgravity. A: The hydraulic scheme foresees three inner circuits, the 1st one for the three times of culture medium (M) refreshment, the 2nd and 3rd ones for washing (W) and fixative (F) solutions, respectively. Each cylinder is separated from the others by a custom designed check valve (V) as well as the culture chamber exhausting channel is separated from the waste repositories by a dedicated check valve. The exhausting circuit after the culture chamber is unique for all the five reservoirs. The waste liquid goes out from the culture chamber flowing in the backside of the activated piston; the scheme does not foresee a preferential cylinder for the waste liquid, but, due to the pressure balance in the circuit, the waste fluid could flow only in the cylinder space left free by the activated piston. B: Half section of the experiment hardware where the Skelite support is indicated (1); the cavity underneath the Skelite support is the so-called culture chamber (3) where the culture medium, the washing and the fixative solution flow via the inner circuit (2) at the plungers activation. Each of the five plungers have a inner valve (6) kept in close position by a spring, that, thanks to the pressure of the fluid due to the plunger activation, is released open until the fluid is completed flown; the consequently overpressure into the culture chamber opens the outer valve (7) (valve not shown into the design) and let the waste fluid flows out of the culture chamber to the backside of the activated plunger (5) via the exhausting circuit (4). C: Cell morphology on Skelite scaffold at sample retrieval. BMSC grew into a veil-like network (arrow head). Arrows indicate snowflake-like aggregates inside pores. Size bar 500 μm.

medium Eagle (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen Ltd, Paisley, UK). Seven days before launch, the cells/Skelite constructs were transported to the launch site at the Baikonur Cosmodrome, Kazakhstan inside a Thermocase B5 (COMAT SA, Flourens, France) at 37°C. Five days before launch, half of the disks were transferred to osteogenic medium, that is, normal growth medium supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate and 10^{-8} M dexamethasone (Sigma, Italy). Two days before launch, disks were integrated individually into bioreactors, then into the KUBIK payload. Following launch, disks were exposed to microgravity for 200 h with three times of medium refreshment at an interval of 50–51 h, then conserved in RNAlater (Qiagen, Valencia, CA) by two consecutive flushing at an interval of 10 min. All bioreactors were stored at 12°C till landing. Two days post-landing they were brought to the laboratory for disassembling to extract disks for further processing. The GC disks were treated in the same manner as the flight disks (FE and FC). The LC disks were processed immediately after the addition of RNAlater solution.

POST-FLIGHT RNA EXTRACTION AND QUALITY ANALYSIS

The cells/Skelite constructs were extracted from the FE, FC, and GC bioreactors, and photographed with a stereo-microscope. Constructs cultured in the same condition were pooled for total RNA isolation using RNeasy® MinElute columns (Qiagen). RNA concentration, purity and integrity were determined by measuring absorbance at 260 and 280 nm; 2 μg of total RNA were separated on a 1% denaturing gel and 100 ng were loaded on the 2100 Bioanalyzer (Agilent, Palo Alto, CA) to verify RNA integrity.

GENECHIP MICROARRAY ANALYSIS AND DATA NORMALIZATION

According to the recommendations of the manufacturer, 100 ng of total RNA was used in the first-round synthesis of double-stranded cDNA. The RNA was reverse transcribed using a Whole Transcript cDNA synthesis and amplification kit (Affymetrix UK Ltd, High Wycombe, UK). The resulting biotin-labeled cRNA was purified using an IVT clean-up kit (Affymetrix) and quantified using a UV spectrophotometer (A260/280; Beckman, Palo Alto, CA). An aliquot

(15 μg) of cRNA was fragmented by heat and ion-mediated hydrolysis at 94°C for 35 min. Fragmented cRNA, run on the Bioanalyzer (Agilent Technologies, Santa Clara, CA) to verify the correct RNA profile, was hybridized in a hybridization oven (16 h, 45°C) to a Mouse Gene 1.0 ST array (Affymetrix) representing whole-transcript coverage. Each one of the 28,853 genes is represented on the array by approximately 26 probes spread across the full length of the gene, providing a more complete and more accurate picture of gene expression than the 3′ based expression array design. The washing and staining procedures of the arrays with phycoerythrin-conjugated streptavidin (Invitrogen Ltd) was completed in the Fluidics Station 450 (Affymetrix). The arrays were subsequently scanned using a confocal laser GeneChip Scanner 3000 7G and the GeneChip Command Console (Affymetrix).

PCA of variance and KMC, after mean scaling and log 2 transformation, with Euclidean distance were performed with the software tool TIGR MeV [Saeed et al., 2003] http://www.tigr.org/software/tm4/mev.html. We used PCA to reduce the complexity of high-dimensional data and to simplify the task of identifying patterns and sources of variability in these large data sets. Several different methods were used for clustering, and we found empirically that the four or five waves of expression were visually apparent regardless of the method used. Prior to using divisive methods like K-median, in which the user supplies the overall number of clusters/nodes at the outset, we performed a figure-of-merit (FOM) calculation to gauge the fit of the data to various numbers of clusters [Yeung et al., 2001]. This calculation confirmed that further divisions beyond five yielded only marginal improvements to how the data fit into the cluster framework and suggested that our visual observation that there were five major clusters was correct (data not shown). All the microarray information has been submitted to the National Center for Biotechnology Information Gene Expression Omnibus web site (www.ncbi.nlm.nih.gov/geo/) with experiment series number: GSE17696.

PATHWAY IDENTIFICATION BY EXPRESSION ANALYSIS SYSTEMIC EXPLORE (EASE)

Gene lists (Affymetrix ID) from affymetrix results were submitted to the EASE, accessible via http://david.abcc.ncifcrf.gov/. EASE takes into account the frequencies of genes belonging to particular Gene Ontology (GO, http://www.geneontology.org/index.shtml) terms among the regulated genes and all genes addressed in a given experiment. Based on this, EASE performs a statistical analysis to detect over-represented functional gene categories in the data set compared with all genes on the arrays. GO terms are reported with corresponding EASE scores, a modified Fisher Exact *P*-values, for gene-enrichment analysis. It ranges from 0 to 1. Fisher Exact *P*-value = 0 represents perfect enrichment. Usually *P*-value is equal or smaller than 0.05 to be considered strongly enriched in the annotation categories. A more detailed analysis of the genes' association with physiological pathways was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/pathway.html). Each identified process was confirmed through PubMed/Medline.

RT-PCR ANALYSIS

Starting from about 1 μg of total RNA, cDNA was synthesized by using an Oligo(dT)20 primer, random hexamers and a Superscript III first-strand synthesis system for RT-PCR (Invitrogen Ltd). Relative quantification was performed by real-time quantitative RT-PCR sybrgreen using the Mastercycler ep Realplex (Eppendorf AG, Hamburg, Germany) following manufacturer's instructions. The housekeeping gene Gapdh was used as the endogenous control for normalization. To avoid possible signal production from potential contaminating genomic DNA, specific primers for each gene (Supplemental Table I) were designed across a common exon–exon splice junction by the Primer Express software (Applied Biosystems). Dissociation curve analysis defined the specificity of the products by the presence of a single dissociation peak on the thermal melting curve.

RESULTS

GENERAL PROPERTIES OF THE 3D BMSC CULTURES EXPOSED TO MICROGRAVITY

The study involved four groups of cells/Skelite constructs: FE (spaceflight, static on orbit, in bioreactors), FC (spaceflight, artificial 1*g* on orbit, in bioreactors), GC (ground 1*g*, static, in bioreactors), and LC (ground 1*g*, static in Petri dishes). Half of the constructs of each group were maintained in normal growth medium (BMSC cultures), the other half was stimulated with osteo-inductive medium (BOI cultures). Inspection by stereo-microscope showed vigorous cell growth in all BMSC/Skelite constructs recovered at the end of the experiment; cells grew into a veil-like network, filling pores and frequently forming snowflake-like aggregates. No major difference in morphology was seen between BMSC and BOI cultures in all groups (Fig. 1C). Since cells maintained in RNAlater solution tended to detach from the scaffold (data not shown), to maximize yield we extracted RNA from the BMSC/Skelite constructs and from the solution recovered from the bioreactor cell chamber. Half amount of total RNA was obtained from the FE respective to the GC samples, and comparable amount was obtained from BMSC and BOI cultures (Supplemental Table II).

SPACEFLIGHT ALTERS THE GENE EXPRESSION PROFILE OF BMSC

The effects of microgravity and spaceflight on gene expression were investigated by performing affymetrix analysis studies. Data from probed and scanned arrays were normalized, filtered by removing probe sets that were regarded as not expressed and analyzed by principal component analysis (PCA). PCA reduces the complexity of high-dimensional data and simplifies the task of identifying patterns and sources of variability in a large data set. Samples were represented by points in a two-dimensional plot. The distance between any pair of points is related to the similarity between the two observations in high-dimensional space. Samples that are near each other in the plot are similar in a large number of variables, that is, expression level of individual genes. Conversely, samples that are far apart in the plot are different in a large number of variables [Raychaudhuri et al., 2000]. By performing PCA, ground controls (BMSC-GC, BOI-GC) and the laboratory controls (BMSC-LC, BOI-LC) partitioned away from the spaceflight samples (BMSC-FE, BOI-FE,

BMSC-FC, and BOI-FC), and BMSC cultures portioned away from the osteo-induced BOI cultures (Fig. 2A). Differential expression within the experimental conditions was demonstrated by K-means cluster (KMC) analysis according to Euclidean distance (Fig. 2B). Six percent of the 19,628 transcripts were found to be up-regulated in response to spaceflight: cluster 1 comprising of 175 probe sets that were highly up-regulated with the peak average log 2 ratio of the cluster being greater than 3, corresponding to eightfold induction;

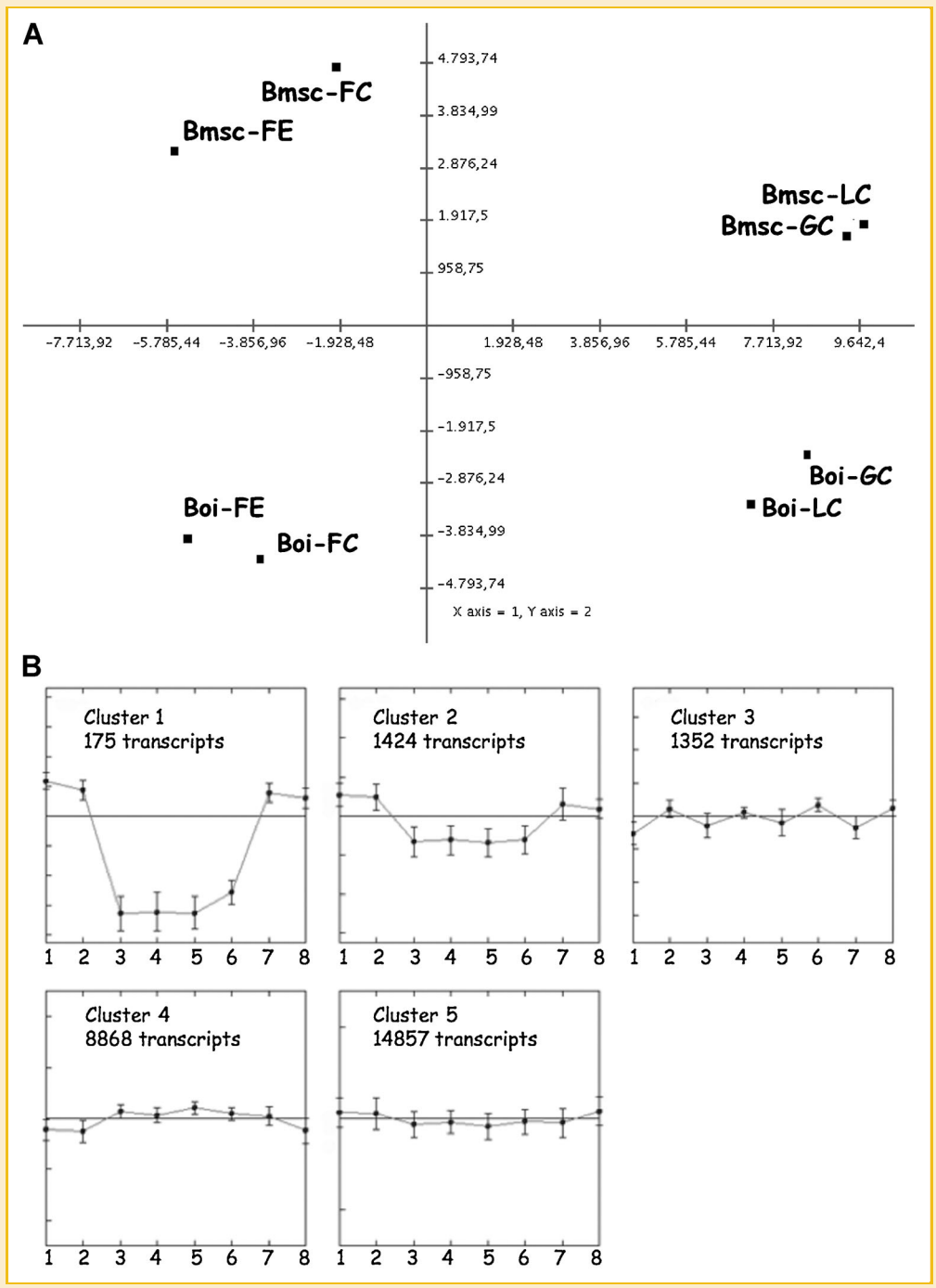

Fig. 2. Microarray analysis of BMSC samples. A: Principal component analysis (PCA) clustering of BMSC samples. Depicted are the 2D projections onto the plane spanned by two principal components for the profiling data set. B: Sequences, mean-filtered, that passed intensity filter criteria in at least one sample were further analyzed by K-means clustering to assess differential expression. Cluster number and the number of genes are denoted at the top of each cluster. The log 2 (sample/mean) ratio is on the *y*-axis. The eight microarrays analyzed (1: BMSC-FE, 2: BOI-FE, 3: BMSC-GC, 4: BOI-GC, 5: BMSC-LC, 6: BOI-LC, 7: BMSC-FC, 8: BOI-FC) are represented along the *x*-axis, error indicators show SD. C: Clusters derived from the analysis of gene expression profiles of 1: BMSC-FE, 2: BMSC-GC, 3: BMSC-LC, and 4: BMSC-FC. D: Clusters of most regulated probe sets from analysis performed with TIGR MeV program. Ratios of each gene relative to mean were used to rearrange the gene list on the basis of their expression pattern. Genes with similar regulation trend were placed close to each other. Magnitudes of ratios are reflected from their color intensity comparable to the color-ratio bar.

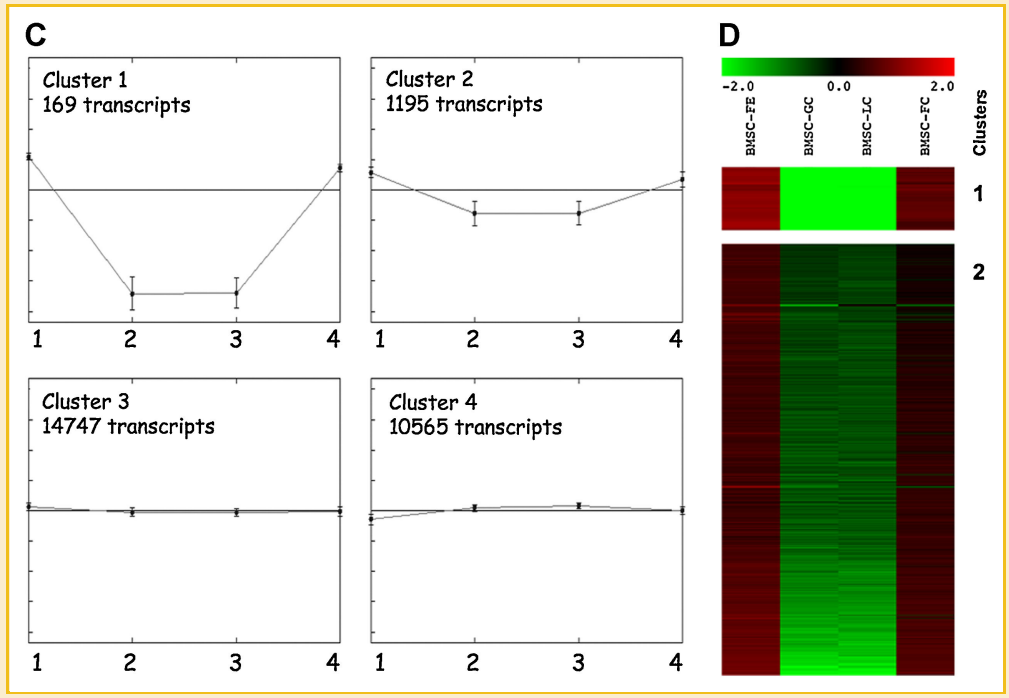

Fig. 2. (*Continued*)

the expression of 1,424 probe sets in cluster 2 was also increased, but to a lesser extent. Individual transcripts and their expression patterns are listed in Supplemental Table III.

CLUSTERING ANALYSIS OF BMSC MAINTAINED IN NORMAL GROWTH MEDIUM

To better manifest alterations of the global gene expression observed in BMSC after exposure to the space environment, we separately analyzed the cultures maintained always in normal growth medium (BMSC-FE, BMSC-FC, BMSC-GC, and BMSC-LC) and the osteo-induced cultures (BOI-FE, BOI-FC, BOI-GC, and BOI-LC) by PCA and KMC. The PCA confirmed that the BMSC-GC and the BMSC-LC were far more similar to each other than to the BMSC-FE or BMSC-FC samples (Supplemental Fig. 1). KMC grouped probe sets into four clusters: clusters 1 and 2 representing probe sets that were, respectively, strongly or moderately up-regulated during space-flight (BMSC-FE and BMSC-FC); cluster 3 representing probe sets without significant change during spaceflight; cluster 4 including those moderately down-regulated (Fig. 2C,D). The list of genes differentially regulated was shown in Supplemental Table IV. Altogether, among 26,676 expressed transcripts 95% (clusters 3 and 4) were similarly expressed in all experimental conditions, except 5% (clusters 1 and 2) were definitely modulated by spaceflight voyage.

EXPRESSION PATTERNS AND BIOLOGICAL PATHWAYS SPECIFICALLY IDENTIFIED BY EASE IN GROUND-BMSC AND SPACEFLIGHT EXPOSED-BMSC

To gain a more mechanistic understanding of the main processes affected by microgravity and spaceflight exposure, the EASE score [Hosack et al., 2003] was used to identify GO database functional categories, which were significantly over-represented in the defined K-means clusters 1 (169 probe sets) and 2 (1,195 probe sets). The results were filtered to avoid redundant and/or generic categories, statistically significant GO terms associated with spaceflight-up-regulated genes were found (Table I and, for a complete list of all gene symbols, Supplemental Table V). The two clusters showed very similar category pattern. Many transcripts were assigned to categories related to synapse transmission, synapse transporter activity. Furthermore, when concerned transcripts were investigated by the analysis of KEGG pathways, the "Neuroactive ligand–receptor interaction" and the "axon guidance" resulted as the highest-ranked categories (Table II and Supplemental Table VI). Therefore, both ontological categorization and KEGG pathway analysis assigned the spaceflight-induced genes in BMSC to those encoding for neural-associated processes. The absence of specific bioreactor-influenced genes confirmed the specificity of neurogen-esis genes found in BMSC-FE and BMSC-FC samples.

CLUSTERING ANALYSIS OF BMSC-OSTEO-INDUCED (BOI) SAMPLES

The BOI cultures (BOI-FE, BOI-FC, BOI-GC, and BOI-LC) were cells treated with the dexamethasone-based osteo-inductive medium. PCA analysis (Supplemental Fig. 2A) separated the ground samples (BOI-GC and BOI-LC) from the flight ones (BOI-FE and BOI-FC). KMC procedure identified two clusters of differently up-regulated genes in BOI-FE and BOI-FC (Supplemental Fig. 2B and Supplemental Table VII). These results are in agreement with those obtained from BMSC samples maintained in normal medium (Fig. 2C, Supple-mental Fig. 1 and Supplemental Table IV). To verify this similarity of

TABLE I. Gene Ontology Analysis of BMSC

System gene category–term	Count	%	*P*-value
Cluster 1–BMSC			
GO biological process			
Synaptic transmission	4	16.7	1.3E–03
Localization	10	41.7	1.6E–03
Transport	8	33.3	1.3E–02
GO cellular component			
Synapse part	3	12.5	1.1E–02
Cell projection	4	16.7	1.4E–02
Cell surface	3	12.5	3.7E–02
GO molecular function			
Transporter activity	6	25	6.0E–03
Gated channel activity	3	12.5	2.6E–02
Lipid binding	3	12.5	5.3E–02
Cluster 2–BMSC			
GO biological process			
Synaptic transmission	63	7.5	2.5E–34
Nervous system development	98	11.6	7.5E–26
Localization	237	28.1	1.1E–21
GO cellular component			
Synapse	78	9.3	5.0E–45
Neuron projection	45	5.3	7.1E–27
Cell projection	58	6.9	1.6E–14
GO molecular function			
Substrate-specific transmembrane transporter activity	103	12.2	1.2E–22
Gated channel activity	55	6.5	1.2E–20
Extracellular ligand-gated ion channel activity	23	2.7	1.8E–13

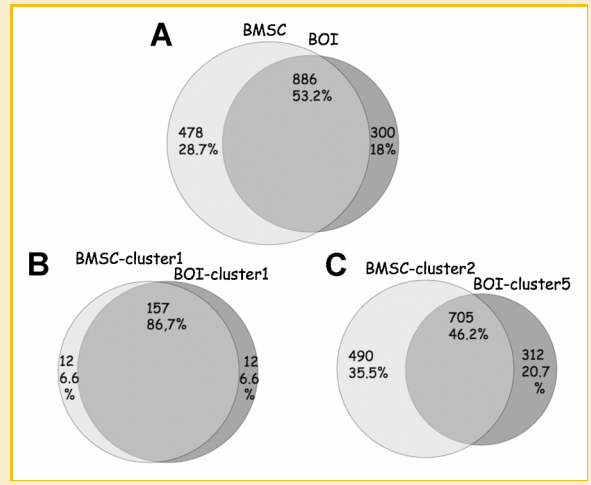

Fig. 3. Venn diagrams of overlapping genes with altered expression in clusters from KMC analysis. A: Global comparison between BMSC and BOI microgravity-regulated genes. B: Comparison of most regulated genes (BMSC-cluster 1 vs. BOI-cluster 1). C: Comparison of less regulated genes (BMSC-cluster 2 vs. BOI-cluster 5). Numbers in the overlaps show co-regulated genes.

results obtained from BMSC and BOI cultures, a cluster comparison analysis was performed between the genes of the up-regulated clusters in BMSC and BOI cultures. Venn diagrams showed that flight conditions regulated largely overlapping sets of genes; 886 up-regulated genes were found in the overlap, corresponding to 53.24% of the considered genes (Fig. 3A). Majority of genes were regulated in a similar manner in the two sample types: 86.74% when comparing the clusters of highly up-regulated genes (BMSC cluster 1 versus BOI cluster 1, Fig. 3B) and 46.78% when comparing the clusters of moderately up-regulated genes (BMSC cluster 2 vs. BOI cluster 5, Fig. 3C). Furthermore, the presence of a significant number of genes, ranging from 12 to 478, in the non-overlapping area suggested the existence of distinct spatial transcriptional profiles between BMSC and BOI conditions (Fig. 3).

GENES MOSTLY AFFECTED BY SPACEFLIGHT DURING OSTEO-INDUCTION

The experimental conditions differ by two major variables: spaceflight and culture conditions. Assuming that spaceflight exposure and osteo-induction synergically yield the expression patterns found in BOI samples, we then tried to separate these two experimental factors by comparing the 300 probe sets (Supplemental Table VIII) from Venn diagram analysis (Fig. 3A) as genes specifically osteo-induced, to the clusters 1 and 2 of BMSC as genes clearly microgravity-induced. We used the EASE score to identify GO functional categories and performed a KEGG pathway analysis. These analysis identified cell adhesion genes as the genes most affected by spaceflight exposure during the osteo-stimulation (Table III and Supplemental Table IX).

TABLE II. KEGG Pathway Analysis of BMSC

KEGG pathways	Count	%	*P*-value
Cluster 1–BMSC			
Neuroactive ligand–receptor interaction	2	8.3	1.8E–01
Cluster 2–BMSC			
Axon guidance	26	3.1	1.1E–07
Neuroactive ligand–receptor interaction	36	4.3	4.3E–06
Gap junction	16	1.9	3.3E–04

SPACEFLIGHT-MEDIATED TRANSCRIPTIONAL REPRESSION OF CELL-CYCLE GENES

The KMC procedure identified a number of genes (cluster 4, Fig. 2B) that could be down-regulated in flight samples. Functional over-representation analysis was performed to objectively identify biological processes potentially affected by spaceflight-mediated transcriptional repression. First, using MS Excel software we highlighted seven transcripts (Supplemental Table X) whose expression was inhibited more then twofold in all flight samples (BMSC-FE, BOI-FE, BMSC-FC, and BOI-FC); then we searched for enriched biological categories of genes in this list by using the functional annotation tool Database for Annotation, Visualization and Integrated Discovery (DAVID). The GO annotation "mitosis" showed the most significant enrichment (Table IV and Supplemental Table XI). Other specific GO annotations and pathway analyses suggested that "cell-cycle progression" was affected by the spaceflight-mediated transcriptional repression (Table IV and Supplemental Table XI).

QUANTITATIVE RT-PCR VALIDATION OF THE MICROARRAY DATA

To verify and validate the affymetrix data, we performed real-time PCR (Fig. 4) on a subset of spaceflight-modulated genes. RNA samples subjected to RT-PCR were identical to those used for the affymetrix analysis. In particular, we confirmed the enhanced expression of the genes known to be involved in neural processes, for example, L1 cell adhesion molecule (L1cam), solute carrier

TABLE III. DAVID Analysis of BOI

System gene category–term	Count	%	*P*-value
GO biological process			
Cell adhesion	18	14.4	9.5E−07
Blood vessel development	11	8.8	1.4E−06
GO cellular component			
Extracellular space	34	27.2	6.6E−08
Membrane	68	54.4	4.8E−05
GO molecular function			
Transporter activity	19	15.2	1.3E−03
Calcium ion binding	14	11.2	2.5E−03
KEGG pathways			
Cell communication	4	3.2	8.8E−02
Cell adhesion molecules (CAMs)	4	3.2	1.1E−01

EASE identified statistically significantly over-represented GO terms, non-redundant functional categories are shown, and KEGG pathways associated with the probe sets specifically altered by spaceflight during osteo-induction.

family 6 (Slc6a1). Genes involved in the cell adhesion, for example, Cd36 antigen (Cd36) and ATPase, Na+/K+ transporting, beta 2 polypeptide (Atp1b2), were also proved to be regulated coherently with the affymetrix data (Fig. 4). In summary, two methodologies (real-time PCR and microarray) produced highly consistent results, which provided a good level of assurance regarding the validity of the microarray data.

DISCUSSION

In this study, we used murine BMSC seeded onto porous Skelite to study their response to microgravity and spaceflight at gene level. Skelite is a resorbable bioceramic based on silicon stabilized tricalcium phosphate (Si-TCP) known to favor survival of BMSC in transplantation models [Wang et al., 2007] and to facilitate the repair of large-sized defects in long bones [Mastrogiacomo et al., 2006]. Compared to other routine in vitro culture methods, the culture system we adopted has the advantage of being three-dimensional (3D), therefore, resembling better the physiological niche of BMSC. In this 3D culture system, osteoblasts can differentiate to form a mineralized matrix within 1 month, whereas, when cultured in the classical 2D Petri dish, they differentiate only till the stage of matrix maturation [Tortelli et al., 2009].

Osteoblasts and osteocytes are known to be mechano-sensor, and their differentiation was reported to be repressed by microgravity [Carmeliet et al., 1997, 1998]. Also osteoblast precursors such as BMSC were shown to be sensitive to mechanical loading. When a clinostat or a rotating cell culture vessel was used to simulate microgravity conditions, an inhibition of proliferation and osteogenesis was observed in both human and rat BMSC [Dai et al., 2007; Saxena et al., 2007; Zheng et al., 2007]. In agreement with these data in our present study the yield of total RNA extracted from the spaceflight samples was about half of those obtained from ground samples, suggesting an inhibition of BMSC proliferation also in a ''real'' microgravity environment. This indication was further strengthened by the identification of seven down-regulated genes that normally regulate cell-cycle progression. These two data together suggest that microgravity represses BMSC proliferation by down-regulating cell-cycle genes.

TABLE IV. DAVID Analysis of Microgravity-Repressed Genes as Described in Table III

System gene category–term	Count	%	*P*-value
GO biological process			
Mitosis	3	50	9.6E−04
GO cellular component			
Intracellular non-membrane-bound organelle	4	66.7	3.9E−03
GO molecular function			
ATP binding	4	66.7	2.4E−03
KEGG pathways			
Cell cycle	3	50	2.1E−03

A variety of cells have been tested under real or simulated microgravity conditions. The response of these cells to changes of gravitational fields was dependent on cell type and length of exposure. Most cells exhibited cytoskeleton changes within the first 3 days after microgravity exposure [Crawford-Young, 2006]. In this study we also observed a down-regulation of several cytoskeleton genes in the flight sample cells.

BMSC is a heterogeneous population of stem/progenitor cells that can give rise to mesenchymal lineage phenotypes both in vitro and in vivo. Their mesenchymal cell fate includes chondrocytes, osteocytes and adipocytes. Main objective of this study was to examine the microgravity-induced global expression changes in cells with osteogenic potential. An unexpected strong expression was found in several subsets of genes related to neural patterns and pathways. These genes play a role in nervous system development, neuron morphogenesis during differentiation, transmission of nerve impulse and synapse.

When transferred in a defined in vitro microenvironment, murine BMSC can be induced to develop neurite-like morphological processes accompanied by the rapid expression of some neuronal and glial markers [Egusa et al., 2005]. In this article we report that neural marker genes, such as beta tubulin 3, Mapt, and Nefm were preferentially expressed in space, in particular Nefm was highly expressed in Bmsc-FE cultures. A similar specificity of the expression profile was also seen for the neuroectodermic gene aldolase C, as well as for axon guidance gene markers, such as several ephrin receptors, plexins, DCC, and Ablim. When cells were induced toward osteogenic differentiation in microgravity, some reduced expression of these genes was observed. The culture condition-dependent specific expression of many neurogenesis markers led us to postulate the activation of a neurogenesis process in BMSC. Activation of these genes also in cells cultured inside the 1*g* centrifuge on orbit led us to hypothesize a significant role, at least in part if not all, played in activating these genes by the stresses encountered by the cells during the different phases of the flight such as launch vibrations and exposure to radiations during the permanence in the space microenvironment.

Several spaceflight experiments demonstrated the deleterious effects of microgravity and spaceflight on neurogenesis in embryos [Crawford-Young, 2006]. It is possible that the observed impairment of neurogenesis in microgravity-exposed embryos could be ascribed more to an impairment of the progenitor/neural cell migration than to the intrinsic cell neural differentiation. Interestingly, cDNA microarray showed that hindlimb-unloading treatment up-regulated key steps of synaptic plasticity and learning process in mouse brain [Frigeri et al., 2008].

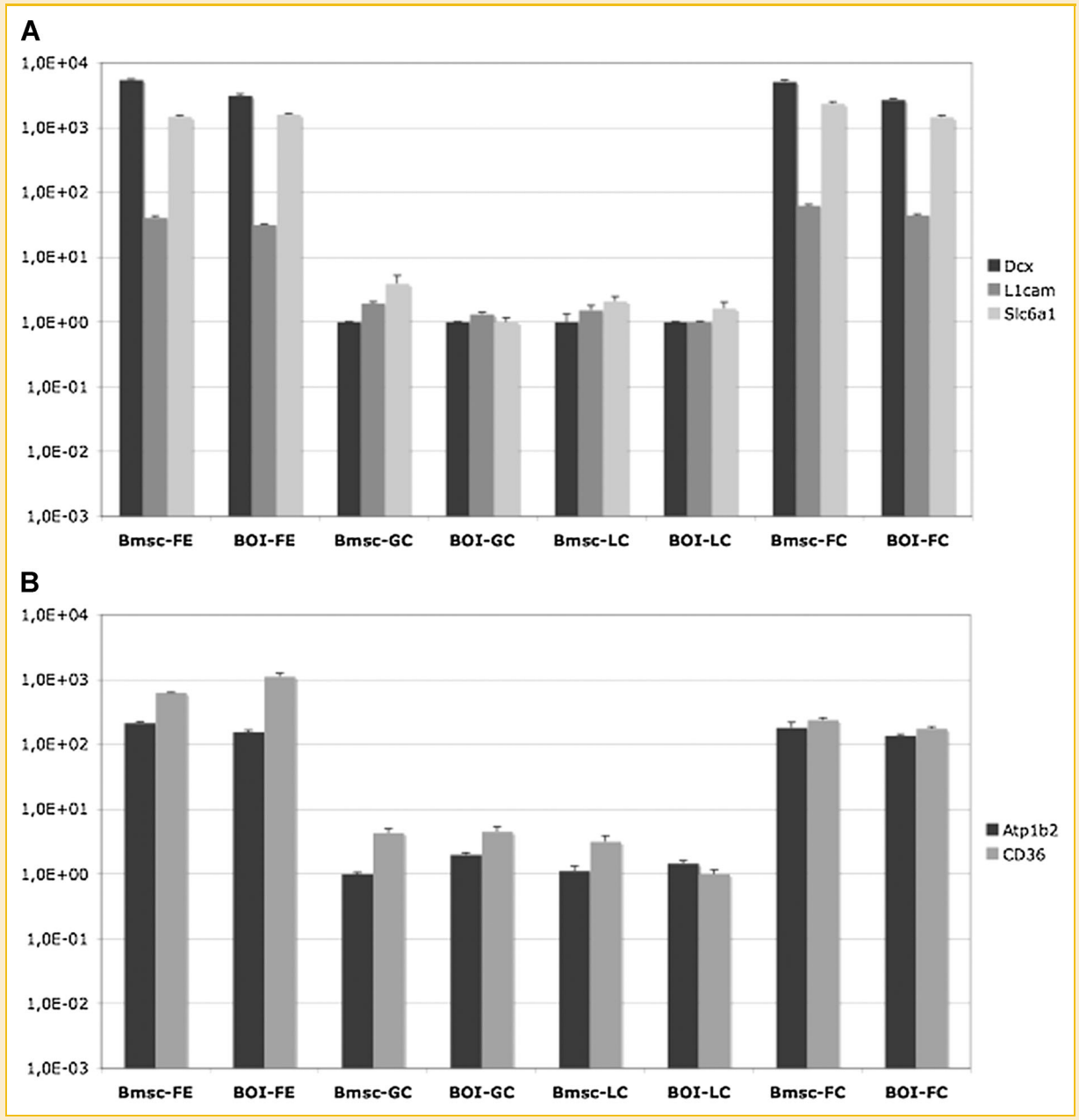

Fig. 4. Confirmation of microarray data by RT-PCR. Real-time RT-PCR analysis showing in arbitrary units (log scale) (A) microgravity-dependent up-regulation of doublecortin (Dcx, black bars), L1 cell adhesion molecule (L1cam, dark-gray bars) and solute carrier family 6 (Slc6a1, light-gray bars) as neural-differentiation associated genes and (B) microgravity-dependent repression of ATPase, Na+/K+ transporting, beta 2 polypeptide (Atp1b2, black bars), and Cd36 antigen (Cd36, gray bars) transcripts as cell adhesion genes. Expression levels are relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

Adult BMSC are considered not to be totipotent nor pluripotent stem cells but instead are regarded as lineage-restricted multipotent mesenchymal stem cells. Pluripotency requires a set of genes not expressed in other cell types, whereas lineage-restricted stem cells express genes predictive of their differentiated lineage [Tanaka et al., 2002]. During embryogenesis, neuronal differentiation occurs before mesenchymal lineage differentiation. The expression of a wide range of neurogenesis genes in BMSC raised questions on their lineage restriction. If this lineage restriction is well respected in nature, does our finding add another evidence to the unpublished but common idea that murine BMSC are less committed progenitor cells than their counterparts in human? It would be very interesting to conduct similar experiments on more committed mesenchyme derived murine cells such as the bone-forming cells, the osteoblasts.

This neuronal differentiation involves suppression of discordant phenotypes through gene silencing [Egusa et al., 2005]. Our finding that BMSC expressed neural genes only during spaceflight was in agreement with the idea that their phenotype restriction might

not be as narrow as previously thought [Tondreau et al., 2004]. Why murine BMSC are more capable to undergo a neurogenic differentiation in space than on Earth? A clear answer is very difficult to give at this stage, but the disruption of actin cytoskeleton in a less committed cell population [Neuhuber et al., 2004] might very well be the initial cue of the neuronal phenotype.

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REFERENCES

Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R, Pennesi G. 2005. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. Eur J Immunol 35:1482–1490.

Backup P, Westerlind K, Harris S, Spelsberg T, Kline B, Turner R. 1994. Spaceflight results in reduced mRNA levels for tissue-specific proteins in the musculoskeletal system. Am J Physiol 266:E567–E573.

Baldwin KM, White TP, Arnaud SB, Edgerton VR, Kraemer WJ, Kram R, Raab-Cullen D, Snow CM. 1996. Musculoskeletal adaptations to weightlessness and development of effective countermeasures. Med Sci Sports Exerc 28:1247–1253.

Caillot-Augusseau A, Lafage-Proust MH, Soler C, Pernod J, Dubois F, Alexandre C. 1998. Bone formation and resorption biological markers in cosmonauts during and after a 180-day space flight (Euromir 95). Clin Chem 44:578–585.

Capulli M, Rufo A, Teti A, Rucci N. 2009. Global transcriptome analysis in mouse calvarial osteoblasts highlights sets of genes regulated by modeled microgravity and identifies a ''mechanoresponsive osteoblast gene signature''. J Cell Biochem 107:240–252.

Carmeliet G, Bouillon R. 1999. The effect of microgravity on morphology and gene expression of osteoblasts in vitro. FASEB J 13(Suppl):S129–S134.

Carmeliet G, Nys G, Bouillon R. 1997. Microgravity reduces the differentiation of human osteoblastic MG-63 cells. J Bone Miner Res 12:786–794.

Carmeliet G, Nys G, Stockmans I, Bouillon R. 1998. Gene expression related to the differentiation of osteoblastic cells is altered by microgravity. Bone 22:139S–143S.

Cavolina JM, Evans GL, Harris SA, Zhang M, Westerlind KC, Turner RT. 1997. The effects of orbital spaceflight on bone histomorphometry and messenger ribonucleic acid levels for bone matrix proteins and skeletal signaling peptides in ovariectomized growing rats. Endocrinology 138:1567–1576.

Crawford-Young SJ. 2006. Effects of microgravity on cell cytoskeleton and embryogenesis. Int J Dev Biol 50:183–191.

Dai ZQ, Wang R, Ling SK, Wan YM, Li YH. 2007. Simulated microgravity inhibits the proliferation and osteogenesis of rat bone marrow mesenchymal stem cells. Cell Prolif 40:671–684.

Derubeis AR, Cancedda R. 2004. Bone marrow stromal cells (BMSCs) in bone engineering: Limitations and recent advances. Ann Biomed Eng 32:160–165.

Egusa H, Schweizer FE, Wang CC, Matsuka Y, Nishimura I. 2005. Neuronal differentiation of bone marrow-derived stromal stem cells involves suppression of discordant phenotypes through gene silencing. J Biol Chem 280: 23691–23697.

Frigeri A, Iacobas DA, Iacobas S, Nicchia GP, Desaphy JF, Camerino DC, Svelto M, Spray DC. 2008. Effect of microgravity on gene expression in mouse brain. Exp Brain Res 191:289–300.

Hosack DA, Dennis G, Jr., Sherman BT, Lane HC, Lempicki RA. 2003. Identifying biological themes within lists of genes with EASE. Genome Biol 4:R70.

Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R, Quarto R. 2000. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. J Biomed Mater Res 49:328–337.

Mastrogiacomo M, Corsi A, Francioso E, Di Comite M, Monetti F, Scaglione S, Favia A, Crovace A, Bianco P, Cancedda R. 2006. Reconstruction of extensive long bone defects in sheep using resorbable bioceramics based on silicon stabilized tricalcium phosphate. Tissue Eng 12:1261–1273.

Mastrogiacomo M, Papadimitropoulos A, Cedola A, Peyrin F, Giannoni P, Pearce SG, Alini M, Giannini C, Guagliardi A, Cancedda R. 2007. Engineering of bone using bone marrow stromal cells and a silicon-stabilized tricalcium phosphate bioceramic: Evidence for a coupling between bone formation and scaffold resorption. Biomaterials 28:1376–1384.

Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I. 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: Disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192–204.

Patel MJ, Liu W, Sykes MC, Ward NE, Risin SA, Risin D, Jo H. 2007. Identification of mechanosensitive genes in osteoblasts by comparative microarray studies using the rotating wall vessel and the random positioning machine. J Cell Biochem 101:587–599.

Qian A, Di S, Gao X, Zhang W, Tian Z, Li J, Hu L, Yang P, Yin D, Shang P. 2009. cDNA microarray reveals the alterations of cytoskeleton-related genes in osteoblast under high magneto-gravitational environment. Acta Biochim Biophys Sin (Shanghai) 41:561–577.

Rabin R, Gordon SL, Lymn RW, Todd PW, Frey MA, Sulzman FM. 1993. Effects of spaceflight on the musculoskeletal system: NIH and NASA future directions. FASEB J 7:396–398.

Raychaudhuri S, Stuart JM, Altman RB. 2000. Principal components analysis to summarize microarray experiments: Application to sporulation time series. Pac Symp Biocomput 5:452–463.

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J. 2003. TM4: A free, open-source system for microarray data management and analysis. Biotechniques 34:374–378.

Saxena R, Pan G, McDonald JM. 2007. Osteoblast and osteoclast differentiation in modeled microgravity. Ann NY Acad Sci 1116:494–498.

Schneider V, Oganov V, LeBlanc A, Rakmonov A, Taggart L, Bakulin A, Huntoon C, Grigoriev A, Varonin L. 1995. Bone and body mass changes during space flight. Acta Astronaut 36:463–466.

Tanaka TS, Kunath T, Kimber WL, Jaradat SA, Stagg CA, Usuda M, Yokota T, Niwa H, Rossant J, Ko MS. 2002. Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity. Genome Res 12:1921–1928.

Tondreau T, Lagneaux L, Dejeneffe M, Massy M, Mortier C, Delforge A, Bron D. 2004. Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. Differentiation 72:319–326.

Tortelli F, Pujic N, Liu Y, Laroche N, Vico L, Cancedda R. 2009. Osteoblast and osteoclast differentiation in an in vitro three-dimensional model of bone. Tissue Eng Part A 15:2373–2383.

Wang Y, Chen X, Armstrong MA, Li G. 2007. Survival of bone marrow-derived mesenchymal stem cells in a xenotransplantation model. J Orthop Res 25:926–932.

Westerlind KC, Turner RT. 1995. The skeletal effects of spaceflight in growing rats: Tissue-specific alterations in mRNA levels for TGF-beta. J Bone Miner Res 10:843–848.

Yeung KY, Haynor DR, Ruzzo WL. 2001. Validating clustering for gene expression data. Bioinformatics 17:309–318.

Zheng Q, Huang G, Yang J, Xu Y, Guo C, Xi Y, Pan Z, Wang J. 2007. Could the effect of modeled microgravity on osteogenic differentiation of human mesenchymal stem cells be reversed by regulation of signaling pathways? Biol Chem 388:755–763.