J Huazhong Univ Sci Technol [Med Sci]
DOI 10.1007/s11596-008-0209-3
28 (2): 152-155, 2008

Electromagnetic Field Change the Expression of Osteogenesis Genes in Murine Bone Marrow Mesenchymal Stem Cells*

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Summary: In order to identify the differentially expressing gene of bone marrow mesenchymal stem cells (MSCs) stimulated by electromagnetic field (EMF) with osteogenesis microarray analysis, the bone marrow MSCs of SD rats were isolated and cultured *in vitro*. The third-passage cells were stimulated by EMFs and total RNA was extracted, purified and then used for the synthesis of cDNA and cRNA. The cRNA of stimulated group and the control group was hybridized with the rat oligo osteogenesis microarray respectively. The hybridization signals were acquired by using X-ray film after chemiluminescent detection and the data obtained were analyzed by employing the web-based completely integrated GEArray Expression Analysis Suite. RT-PCR was used to identify the target genes: Bmp1, Bmp7, Egf and Egfr. The results showed that 19 differentially expressing genes were found between the stimulated group and the control group. There were 6 up-regulated genes and 13 down-regulated genes in the stimulated group. Semi-quantitative RT-PCR confirmed that the expressions of Bmp1, Bmp7 mRNA of the stimulated group were up-regulated ($P<0.05$) and those of Egf, Egfr were down-regulated ($P<0.05$). It was suggested that the gene expression profiles of osteogenesis of the bone marrow MSCs were changed after EMF treatment. It is concluded that the genes are involved in skeletal development, bone mineral metabolism, cell growth and differentiation, cell adhesion etc.
Key words: electromagnetic fields; osteogenesis microarray analysis; bone marrow mesenchymal stem cells

Some investigations reported that electromagnetic field (EMF) can facilitate cell proliferation and osteogenesis of MSCs[1,2], while the underlying mechanism remains unclear. Osteoanagenesis is complicated and it can be regulated by various intracellular factors. Genes involved in the regulation include growth factor gene and intracellular signaling molecular gene, as well as pro- or post-differentiation genes. Oligo osteogenesis microarray can be used to detect the expression of related genes which only occur during the process of cell differentiation. In this study, we employed oligo osteogenesis microarray to investigate the effect of EMF on osteogenesis of MSCs.

1 MATERIALS AND METHODS

1.1 Reagents and Equipment

The reagents included DMEM-F12 (Hyclone, USA), fetal bovine serum (Hyclone, USA), trypsin (Amersco, USA), Trizol reagent (Invitrogen, USA), oligo osteogenesis microarray, TrueLabeling-AMP^{TM} linear RNA amplification kit, SuperArray ArrayGrade cRNA Cleanup kit, GEAhyb hybridization solution, chemiluminescent detection kit (SuperArray Bioscience, USA). The instruments used included EMF appratus (15Hz, sine wave form, with intensity adjustable, designed and manufactured by Navy University of Engineering, China), inverted phase contrast microscope (OLYMPUS, Japan), desk-top micro-centrifuge, freezing centrifuge (Eppendorf, Germany), PCR reaction system (Beijing Biodev-tech Scientific & Technical Co., Ltd., China) etc.

1.2 Animals

Sprague-Dawley (SD) rats of clean grade, aged 4–5 weeks, of either sex, were provided by the Center of Experimental Animals of Tongji Medical College, Huazhong University of Science and Technology, China.

1.3 Cells Culture

The rats were sacrificed by the dislocation of the cervical vertebra. Then the rats were immerged into 75% alcohol for 10 min. The bilateral femur and tibia were cut and the attached soft tissues were removed on a super clean bench. The medullary cavity of the bone was washed by DMEM-F12 medium containing 10% fetal bovine serum. The medium full of cells was centrifuged for 5 min at 1000 r/min and the radius of centrifugation was 8 cm. The supernatant was abandoned and the cells were suspended by adding them into the medium. The cells were counted and adjusted to a concentration of about 5×10^6 cells/mL. Then the cells were inoculated to a 50 mL culture flask, and cultured in saturation humidity incubator at 37°C, in 5 % CO_2. The culture medium

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*This project was supported by a grant from the National Natural Sciences Foundation of China (No. 50477043).

was changed on the 3rd day. After about 7–10 days，the cells were digested with trypsin and transferred at the ratio of 1:2 when they grew to confluence.

1.4 Grouping and EMF Exposure

The MSCs from the fourth passage in good condition were digested and adjusted to a concentration of about 1×10^5 cells/mL and then were transferred into two 9-cm disposable culture capsules marked A and B. The stimulated groups (group B) were exposed to EMF (15 Hz, 1 mT, 8 h/d) for 2 days.

1.5 Isolation and Assessment of Total Ribonucleic Acid (RNA)

After last exposure of group B to EMF, 1 mL of Trizol reagent was added to each culture capsule. Then the total RNA was extracted and purified by following the instructions. The samples A and B were respectively designated 1 and 2. UV spectrophotometer and Denatured agarose gel electrophoresis were used to determine the concentration, purity and integrity of RNA.

1.6 Probe Synthesis and Hybridization

cRNA labeling and synthesis were done by following the kit instructions. After pre-hybridization for about 2 h, the pre-hybridization solution was removed from the hybridization tube. The target hybridization mix containing the labeled cRNA target was added to the tube. Hybridization was performed overnight at 60°C with continuous agitation at 5 to 10 r/min.

1.7 Chemiluminescent Detection

Chemiluminescent detection was conducted by following the kit instructions after the last washing. Then the membrane was exposed on the X-ray film.

1.8 Data Transformation and Analysis

The image was scanned on the film and saved as electronic file in gray scale (8 or 16 bit) and TIFF format. The web-based completely integrated GEArray expression analysis suite was employed for data analysis.

1.9 RT-PCR Identifying the Differential Expression Genes

On the basis of the result of oligo osteogenesis microarray, RT-PCR was used to identify the typical genes: Bmp1, Bmp7, Egf and Egfr. The primer sequences are in table 1.

Table 1 Primers for PCR assays

Genes	Oligo nucleotides	Cycle (*n*)	Lenth (bp)
GAPDH	5′-GTGCTGAGTATGTCGTGGAG-3′ 5′-GTCTTCTGAGTGGCAGTGAT-3′	30	301
Bmp1	5′-CCTGTGCTGGTATGACTATGTG-3′ 5′-CGGAGACGAACTTGAGCC-3	30	476
Bmp7	5′-AGTCCGACCTCTTCTTGCT-3′ 5′-CATACGGCTCGTGTTTCTT-3′	30	396
Egf	5′-AGCCACGGTTACATTCAC-3′ 5′-GACATCGTTTCCCATCAG-3′	30	517
Egfr	5′-GTATAAGGGTCTCTGGATCC-3′ 5′-GGCCAGTCCAAAATCTGTGA-3′	30	400

1.10 Statistical Analysis

Statistical analyses were conducted by employing *t*-test. For all statistical tests, a $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 RNA Assessment

The total RNA from samples 1 and 2 was extracted by Trizol reagent and examined for UV absorbance and denatured agarose gel electrophoresis. A_{260}/A_{280} of the RNA was between 1.9 to 2.0 (table 2). Electrophoresis revealed two fairly sharp and intense ribosomal RNA bands (28S and 18S) (fig. 1).

Table 2 RNA assessment

Samples	A_{260}	A_{280}	A_{260}/A_{280}	RNA concentration (μg/μL)
1	35.027	17.673	1.98	1.4
2	28.546	14.471	1.97	1.14

2.2 Oligo Osteogenesis Microarray

The images after hybridization are showed in fig. 2 and fig. 3. On the basis of the analysis of the image data, the following criterion was used for identification of differentially expressing genes: The ratio of certified value 2 to certified value 1 is more than 2.0 or less than 0.5. Compared with the control group, there were 19 differentially expressing genes in group B. Among these 19 genes, 6 genes were up-regulated and 13 genes down-regulated substantially (tables 3 and 4).

2.3 RT-PCR

Compared with the control group, the mRNA levels of Bmp1 and Bmp7 in the exposure group were higher while Egf and Egfr were lower ($P<0.05$) (fig. 4). The results were coincident with those of the oligo osteogenesis microarray.

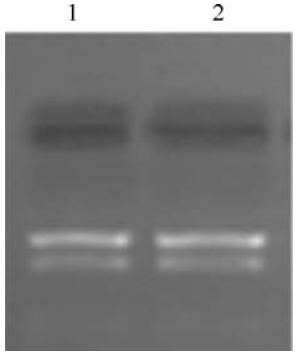

Fig. 1 RNA denatured agarose gel electrophoresis
1: Total RNA from sample 1；2: Total RNA from sample 2

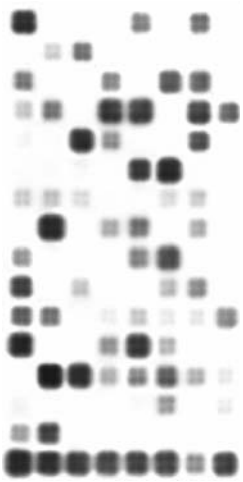

Fig. 2 Sample 1

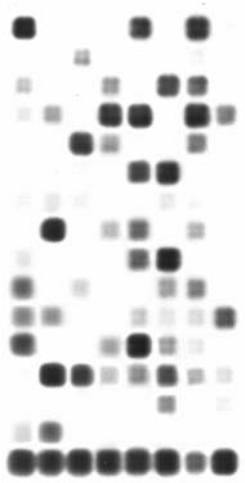

Fig. 3 Sample 2

Table 3 Remarkably up-regulated genes after exposure to EMF

Position	GeneBank	Symbol	Description
8	XM_573814	Bmp1	Bone morphogenetic protein 1
13	NM_013107	Bmp6	Bone morphogenetic protein 6
14	XM_342591	Bmp7	Bone morphogenetic protein 7
15	NM_030849	Bmpr1a	Bone morphogenetic protein receptor, type 1A
95	XM_345947	Smad6_predicted	MAD homolog 6 (Drosophila) (predicted)
111	NM_017058	Vdr	Vitamin D receptor

Table 4 Remarkably down-regulated genes after exposure to EMF

Position	GeneBank	Symbol	Description
10	NM_017105	Bmp3	Bone morphogenetic protein 3
17	XM_217409	Bmpr2	Bone morphogenic protein receptor, type II (serine/threonine kinase)
25	XM_216399	Col15a1	Procollagen, type XV
33	XM_225043	Col4a2_predicted	Procollagen, type IV, alpha 2 (predicted)
41	XM_342599	Col9a3_predicted	Procollagen, type IX, alpha 3 (predicted)
42	NM_012834	Comp	Cartilage oligomeric matrix protein
49	NM_012842	Egf	Epidermal growth factor
50	NM_031507	Egfr	Epidermal growth factor receptor
51	NM_012846	Fgf1	Fibroblast growth factor 1
55	XM_341940	Fgfr2	Fibroblast growth factor receptor 2
65	NM_030994	Itga1	Integrin alpha 1
105	NM_017256	Tgfbr3	Transforming growth factor, beta receptor III
113	NM_053549	Vegfb	Vascular endothelial growth factor B

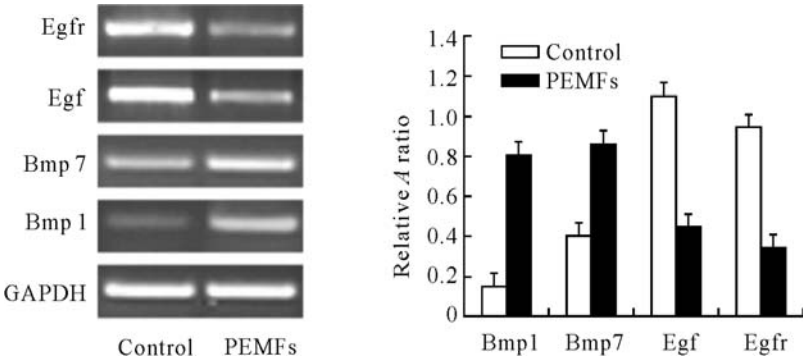

Fig. 4 DNA gel electrophoresis and data analysis

3 DISCUSSION

EMF can induce MSCs to differentiate into osteoblasts[2] and facilitate synthesis and secretion of several kinds of bone growth factor. The effects of EMF mentioned above are now believed to be one of the mechanisms by which EMF promote bone healing[3]. The identification of differentially expressing genes of BMSCs exposed to EMF has become increasingly important. Several methods are now available for the detection of the gene expression, such as Northern blotting, RT-PCR, hybridization in situ *etc*. These techniques, however, can deal with only several genes at one time. Gene array can be used for the study of nucleic acid expression from a variety of tissues and cells at the genome level with high-flux and parallelisation. And it requires little sample and is automatic and inexpensive[4].

In this study, we used oligo osteogenesis microarray to investigate the effect of EMF on osteogenesis of MSCs. There are altogether 113 genes concerining osteogenesis in the oligo osteogenesis microarray. The analysis of microarray showed that there were 19 differentially expressing genes in the EMF exposure group. Among these 19 genes, 6 genes were up-regulated and 13 genes down-regulated substantially. Bmp1 is the prototype of a family of metalloproteases involved in morphogenesis in a broad range of species. It is a regulatory factor of bone growth. Bmp1 also plays an important role in embryogenesis, chondrogenesis, osteogenesis and the deposition of nonfibrillar ECM[5]. However, Bmp7 is the first identified member of BMP superfamily which was characterized by its ability to induce osteogenesis[6], and Bmp7 combined with scaffold can induce the formation of new bone after being implanted into animals[7,8]. Egf binding with Egfr can facilitate cell proliferation and differentiation. In order to confirm the results of the oligo osteogenesis microarray, we used RT-PCR to detect the two up-regulaed genes (Bmp1 and Bmp7) and two down-regulated gene (Egf and Egfr). The result showed that, compared with the control group, the mRNA levels of Bmp1 and Bmp7 in exposure group were higher while those of Egf and Egfr were lower.

To sum up, we used the oligo osteogenesis microarray which can detect 113 genes about osteogenesis at the same time and found that the expression of 19 genes concerning osteogenesis of BMSCs was changed greatly after exposure to EMF (15 Hz, 1 mT). Further study is needed to explore the underlying mechanisms of the change.

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(Received Nov. 1, 2007)