Experimental study of millimeter wave-induced differentiation of bone marrow mesenchymal stem cells into chondrocytes

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Received November 14, 2008; Accepted January 9, 2009

DOI: 10.3892/ijmm_00000152

Abstract. Low power millimeter wave irradiation is widely used in clinical medicine. We describe the effects of this treatment on cultured mesenchymal stem cells (MSCs) and attempted to identify the underlying mechanism. Cells cultured using the whole marrow attachment culture method proliferated dispersedly or in clones. Flow cytometric analyses showed that the MSCs were CD90 positive, but negative for CD45. The negative control group (A) did not express detectable levels of *Cbfa1* or *Sox9* mRNA at any time point, while cells in the millimeter wave-induced groups (B and C) increasingly expressed both genes after the fourth day post-induction. Statistical analysis showed that starting on the fourth day post-induction, there were very significant differences in the expression of *Cbfa1* and *Sox9* mRNA between groups A and B as well as A and C at any given time point, between treated groups B and C after identical periods of induction, and within each treated group at different induction times. Transition electron microscopy analysis showed that the rough endoplasmic reticulum of cells in the induced groups was richer and more developed than in cells of the negative control group, and that the shape of cells shifted from long-spindle to near ellipse. Toluidine blue staining revealed heterochromia in the cytoplasm and extracellular matrix of cells in the induced groups, whereas no obvious heterochromia was observed in negative control cells. Induced cells also exhibited positive immunohistochemical staining of collagen II, in contrast to the negative controls. These results show that millimeter wave treatment successfully induced MSCs to differentiate as chondrocytes and the extent of differentiation increased with treatment duration. Our findings suggest that millimeter wave irradiation can be employed as a novel non-drug inducing method for the differentiation of MSCs into chondrocytes.

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Key words: bone marrow mesenchymal stem cells, millimeter wave, chondrocyte

Introduction

Bone marrow mesenchymal stem cells (MSCs) are adult stem cells that possess multiple differentiation potentials. MSCs differentiate into osteoblasts, chondrocytes, and adipocytes (1-3), as well as endothelial, epithelial and myocardial cells (4,5). MSCs are retrieved easily and massively proliferate *in vitro* under certain conditions. Furthermore, they are favorably suited for autotransplantation and are considered one of the optimal types of seed cells for cell therapy in tissue injury repair (6,7). Also, they have high application potential.

However, directional induction of MSC differentiation remains a significant limitation for progress in cell therapy and tissue engineering. The current general methods for inducing *in vitro* differentiation of MSCs include a series of drug treatments (1-3) or co-culture protocols (8). It is not clear whether these treatments influence the cells in other subtle ways that hinder transplant success. Development of a drug-independent, physical method to induce directional differentiation of MSCs is a new challenge for the field.

Low power millimeter wave irradiation produces many biological effects in mammals, noticeably at the individual, tissue, cellular and subcellular levels (9), and is broadly used in clinical medicine. However, the impact of low power millimeter wave irradiation on directional differentiation of MSCs has not yet been reported. In the present study, we describe the effects of this treatment on cultured MSCs and attempt to identify the underlying mechanism. We find that specific gene expression changes, induced by treatment with low power millimeter wave irradiation, correlate with morphological and functional differentiation of MSCs into chondrocytes.

Materials and methods

The irradiation treatments were performed with a KFA-100A millimeter wave instrument (Beijing Zhongcheng Kangfu Science and Technology Co., Ltd). The wavelength range of the instrument is 7.5-10.0 mm, the power density is 4 Mw/cm^2, the irradiation area is 33.2±3 mm x 45.6±4 mm = 15.1 cm^2, and the treatment times are 30±3 and 60±6 min.

Experimental animals. Forty-five 4-week-old male Sprague-Dawley (SD) rats, weights ranging from 90 to 110 g, were

Table I. RT-PCR primers.

Gene	Primer sequence	Amplicon length	Annealing temperature X (˚C)
Cbfa1	Forward 5'-AGTCCCAACTTCCTGTGCT-3'		
	Reverse 5'- GGTGAAACTCTTGCCTCGTC-3'	243 bp	58
Sox9	Forward 5'-AGCCCTGGTTTCGTTCT-3'		
	Reverse 5'-CTGCTCGTCGGTCATCTT-3'	297 bp	58
ß-actin	Forward 5'-ACTGGCATTGTGATGGACTC-3'		
	Reverse 5'-CAGCACTGTGTTGGCATAGA-3'	201 bp	55

provided by the Shanghai SLAC Laboratory Animal Co., Ltd., Certificate of Quality Number: SCXK (HU) 2003-0003.

MSC isolation, purification and culture. Fifteen SD rats were sacrificed by cervical dislocation and their bilateral femurs and tibias were retrieved under aseptic conditions. The bones were dipped in 75% alcohol and the marrow was flushed out with DMEM containing 15% fetal bovine serum (FBS). The cells were inoculated at a concentration of 2xl0^6/ml in 50 ml culture bottles and cultured in a 37˚C incubator with 5% CO_2, marked as P_0. The medium was first changed 72 h after the culture period began and then subsequently every three days. When the cells coated about 90% of the bottom of the bottle, they were digested with 0.25% trypsin, diluted and then sub-cultured, marked in turn as p1, 2, 3. Nucleated cells were counted with a hemacytometer to allow adjustments in cell concentrations for later experiments.

Positive detection of MSC surface marker antigens CD45 and 90. P3 adherent cultured cells were collected after digestion with 0.25% trypsin plus 0.02% EDTA and were then incubated with saturated FITC-conjugated CD45 or 90 monoclonal antibodies in the dark at room temperature for 45 min. After a PBS wash, positive cells were detected with flow cytometry (FCM).

Induced directional differentiation culture of MSCs to chondrocytes. P3 MSCs were digested with 0.25% trypsin in order to adjust cell concentrations to 5x10^5/ml. Cell suspension (4 ml) was inoculated in 50 ml culture bottles and divided into three groups 24 h after inoculation, group A, cultured in DMEM containing 15% FBS; group B, cultured in DMEM containing 15% FBS with one 30-min millimeter wave irradiation per day; and group C, cultured in DMEM containing 15% FBS with one 60-min millimeter wave irradiation per day. Group A was used throughout the study as a negative control for the irradiation treatment, whereas groups B and C served as induced or experimental groups. Cell shape and growth rates were observed with an inverted phase contrast microscope each day. On days 1, 4, 8, and 12 after induction, RT-PCR was used to measure the mRNA expression levels of *Cbfa1* and *Sox9* in the three groups. Twelve days after induction, transmission electron microscopy (TEM) was used to detect potential ultrastructural changes. Cells from each group were also processed as described below for toluidine blue and immunohistochemical staining in order to assess biological changes after twelve days of treatment. Throughout the twelve-day treatment period, irradiation, media changes, and passages were performed as described earlier.

RT-PCR analysis. Total RNA was extracted according to the Trizol protocol. RNA (1 μg) was used as template to generate cDNA with reverse transcriptase, which was then used as a template to amplify *Cbfa1* and *Sox9* by PCR. ß-actin was used as an internal reference gene. The primers used in these reactions are listed in Table I. The reaction conditions were 95˚C for 5 min, 35 cycles of 94˚C for 30 sec, X˚C for 40 sec, and 72˚C for 30 sec, and finally 72˚C for 10 min and 4˚C for 10 min. The amplified products were analyzed by 1.5% agarose gel electrophoresis. The optical density ratios of *Cbfa1*, *Sox9* and ß-actin were used for semi-quantitative analyses.

TEM observations. Twelve days after the irradiation treatments commenced, cells from each group were digested, collected, and fixed with 3% pentodialdehyde and 1.5% paraformaldehyde for two days at 4˚C. Following fixation, cells were treated with 1% osmium acid for 1.5 h, washed in PBS, dehydrated through an alcohol-acetone gradient and then embedded using epoxy resin 618 as the embedding medium. Ultra thin sections (80 nm) were stained with uranyl acetate and lead citrate for 8 to 10 min each and finally observed with Hitachi H7650 TEM and photographed with the accompanying CCD camera.

Toluidine blue staining. Twelve days after induction, cells from each group were digested with 0.25% trypsin to adjust the concentration to 1x10^5/ml and inoculated at 2 ml/well into 6-well plates prepared with slides in each well. After 24 h, the slides were removed and the cells were fixed with paraformaldehyde for 20 min before staining with 1% toluidine blue for 10 min. After gum fixation, cells were observed and photographed under a light microscope.

Immunohistochemical staining. As above, slides were removed from 6-well plates after 24 h of subculture and cells were fixed with paraformaldehyde for 20 min. The cells were subsequently incubated with 3% hydrogen peroxide for 10 min, followed by the primary antibody (collagen II Ab-2) and secondary antibody (enzyme-labeled sheep anti-mouse IgG polymer) at room temperature for 60 and 30 min, respectively. Positive staining was revealed by DAB, after which cells were observed under the microscope, counterstained with hematoxylin, subjected to gum fixation and finally observed and photographed under the light microscope.

Statistical methods. Each experiment was independently performed three times. SPSS 11.5 was used for data analysis and data are shown as mean ±SD. Comparisons between

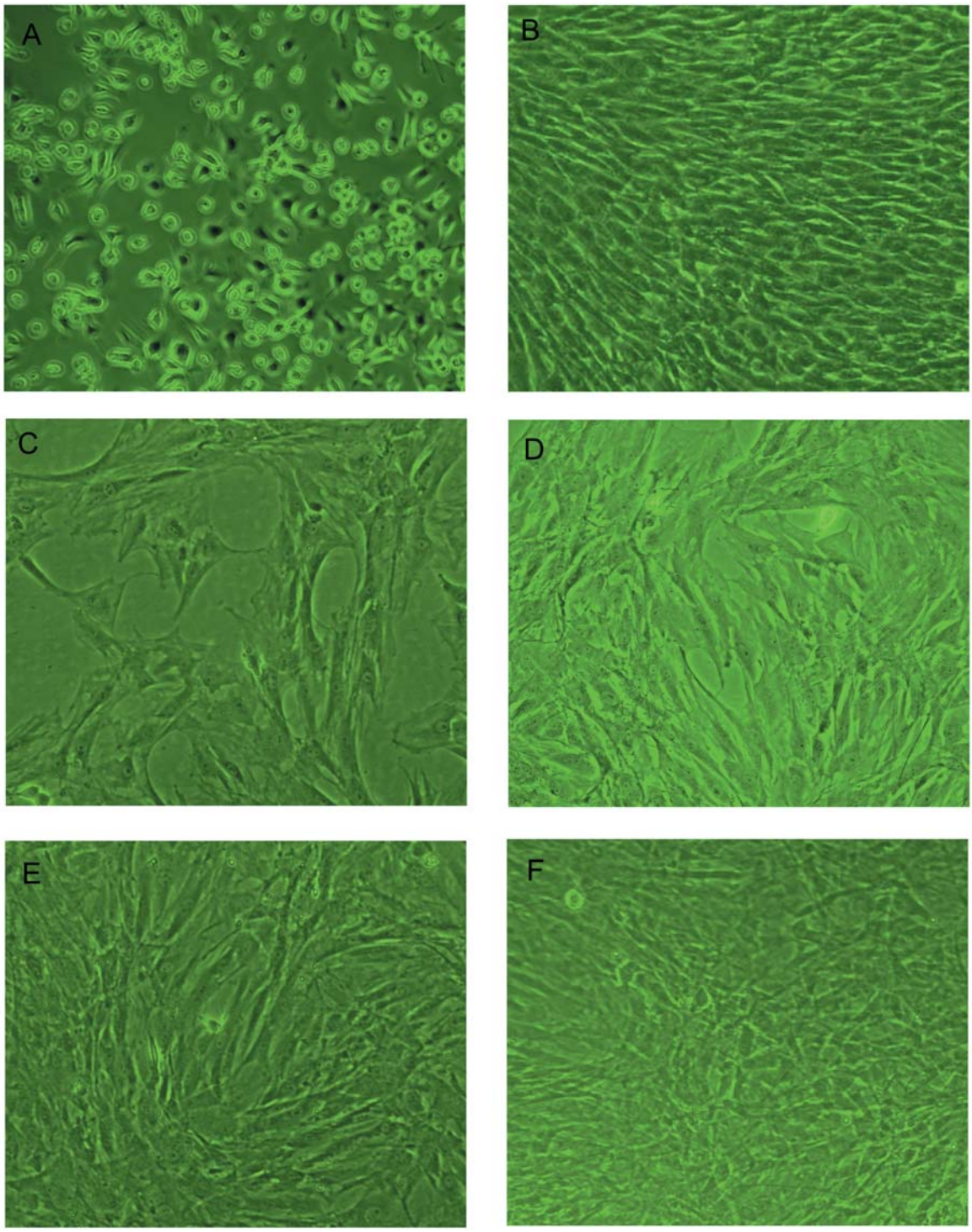

Figure 1. The shapes of P_0 MSCs 2 and 14 days after culture and P_2 MSCs 8 and 14 days after induced culture under phase contrast microscopy (x100). (A) At the second day after regular culture of P_0 MSCs, most cells were circular or ellipsoid. (B) At the 14th day after regular culture of P_0 MSCs, cells were arranged as vortices, cellular boundaries were blurred and the most common shape was long-spindle. (C) After eight days of 30 min-millimeter wave irradiation treatments of P_2 MSCs, cell shapes shifted from long-spindle to polygonal. (D) After eight days of 60 min-millimeter wave irradiation treatments of P_2 MSCs, cell shapes shifted from long-spindle to polygonal, and the number of cells was significantly increased compared to the 30-min treatment group. (E) After fourteen days of 30 min-millimeter wave irradiation treatments of P_2 MSCs, the most common shape was flat polygon, polygonal protuberance, and polygonal spindle. (F) After fourteen days of 60 min-millimeter wave irradiation treatments of P_2 MSCs, the most common shape was flat polygon, polygonal protuberance or polygonal spindle, and the number of cells significantly increased compared to the 30-min treatment group.

multiple groups were conducted with one-way ANOVA, whereas within group data were analyzed with intraclass variance analysis. P values of <0.05 and <0.01 were considered significant and very significant differences, respectively.

Results

Cell shape changes. The cells cultured with the whole marrow attachment culture method proliferated as dispersed or clonal

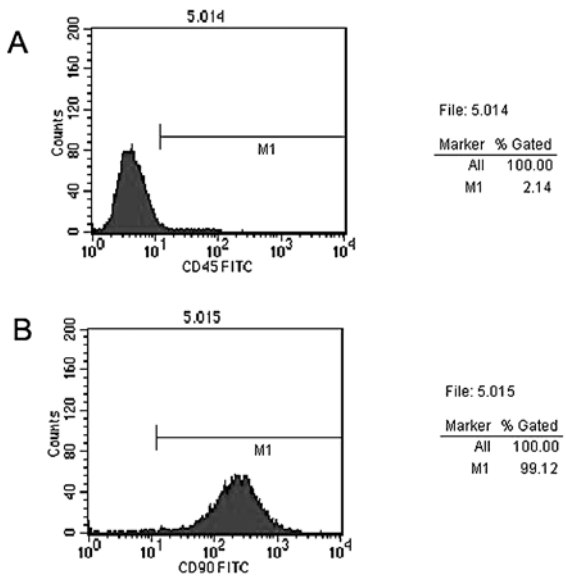

Figure 2. Positive expression rates of P_3 MSC surface marker antigens CD45 and 90. (A) The CD90-positive rate was 99.12%; (B) CD45-positive was only 2.14%.

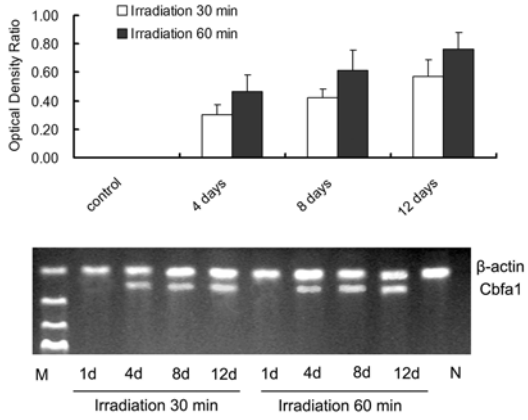

Figure 3. The effect of millimeter wave irradiation on Cbfa1 mRNA expression. The expresssion of *Cbfal* mRNA was detected on 1st, 4th, 8th and 12th day after 30-min and 60-min millimeter wave irradiation treatment respectively. N, negative control group; M, marker.

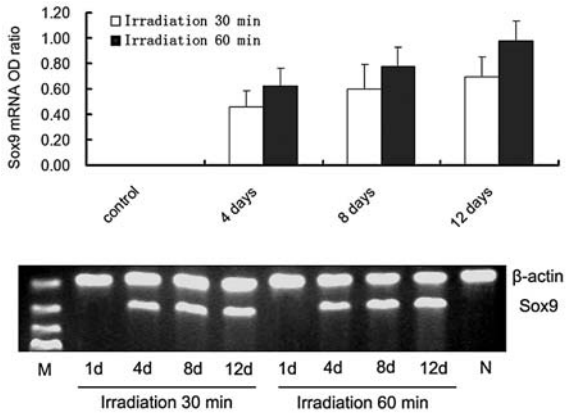

Figure 4. The effect of millimeter wave irradiation on Sox9 mRNA expression. The expresssion of *Sox9* mRNA was detected on 1st, 4th, 8th, and 12th day after 30- and 60-min millimeter wave irradiation treatment respectively. N, negative control group; M, marker.

colonies in primary culture. Between the first and third days most of the cells were circular or oval. From the third to the seventh day, the number of attached cells increased and they gradually changed into polygon, multi-spindle or single spindle shapes. Adjacent colonies fused to cover >90% of the culture dish between the seventh and fourteenth days. The cell arrangements resembled vortices, cellular boundaries were blurred, and the predominant cell shape was long-spindle. After induced culture, cells gradually transitioned from spindle to polygon shapes. Fourteen days after induction, the most common shapes were flat polygon, polygonal protuberance or polygonal spindle (Fig. 1).

Positive expression rates of P_3 MSC surface marker antigens CD45 and 90. The CD45-positive rate of adherent cells was 2.14%, while the CD90-positive rate was 99.12% (Fig. 2).

RT-PCR detection results. Expression of *Cbfa1* and *Sox9* mRNA was not detected in the negative control group (A) at any time point. Remarkable increases in *Cbfa1* and *Sox9* mRNA were observed in both induced groups (B and C) starting on the fourth day. These increases became more significant over time. *Cbfa1* and *Sox9* mRNA expression levels were significantly different between groups that experienced the same duration of inductive treatment (day 4, *Cbfa1*, F=55.590, P=0.000, *Sox9*, F=51.173, P=0.000; day 8, *Cbfa1*, F=78.267, P=0.000, *Sox9*, F=51.413, P=0.000; day 12, *Cbfa1*, F=56.748, P=0.000, *Sox9*: F=98.314, P=0.000). Expression levels were also significantly different between groups with different induction durations (*Cbfa1*, F=58.067, P=0.000; *Sox9*, F=61.964, P=0.000). Finally, significant differences were observed within each group across induction durations (*Cbfa1*, F=28.634, P=0.000; *Sox9*, F=29.042, P=0.000); these differences became more significant over time (Tables II and III; Figs. 3 and 4).

TEM ultrastructural examination. Non-induced MSCs exhibited spindle shapes, small volumes, few protrusions through the cell surface, and organelles were scarce, predominantly composed of mitochondria and endoplasmic reticulum. The nucleocytoplasmic ratio was relatively high, nuclear shape was nearly ellipsoid, more euchromatin was present in the nuclear matrix than heterochromatin, and nucleoli were typically large and eccentric (Fig. 5A).

Induced cells were mostly ellipsoid with various numbers of surface protuberances. These cells contained more cytoplasm with abundant and developed endoplasmic reticulum compared with control. Secretory vesicles, mitochondria, lysosomes and glycogen granules were present in the cytoplasm and the nucleocytoplasmic ratio decreased. The nuclei were medium-sized or smaller and the nuclear shape was mostly ellipsoid, although occasionally zonal and irregular. Euchromatin was more prevalent in the nuclear matrix, heterochromatin was distributed close to the nuclear membrane, and some nucleoli were large (Fig. 5B and C).

Table II. The effect of millimeter wave treatment on *Cbfa1* mRNA expression.

Group	*Cbfa1* mRNA			
	Day 1	Day 4	Day 8	Day 12
Group A	0	0	0	0
Group B	0	0.3057±0.0656[a]	0.4211±0.0613[a]	0.5728±0.1176[a]
Group C	0	0.4643±0.1172[a,c]	0.6170±0.1382[a,c]	0.7630±0.1212[a,c]

Group A, negative control group; group B, 30-min irradiation group; group C, 60-min irradiation group. Mean ± SD, n=6; compared to group A, [a]P<0.01, [b]P<0.05; compared to group B: [c]P<0.05, [d]P<0.01.

Table III. The effect of millimeter wave treatment on *Sox9* mRNA expression.

Group	*Sox9* mRNA			
	Day 1	Day 4	Day 8	Day 12
Group A	0	0	0	0
Group B	0	0.4593±0.1257[a]	0.5995±0.1913[a]	0.6997±0.1519[a]
Group C	0	0.6203±0.1437[a,c]	0.7797±0.1474[a,c]	0.9788±0.1532[a,c]

Group A, negative control; group B, 30-min irradiation; group C, 60-min irradiation. Mean, ±SD, n=6; compared to group A, [a]P<0.01, [b]P<0.05; compared to group B: [c]P<0.05, [d]P<0.01.

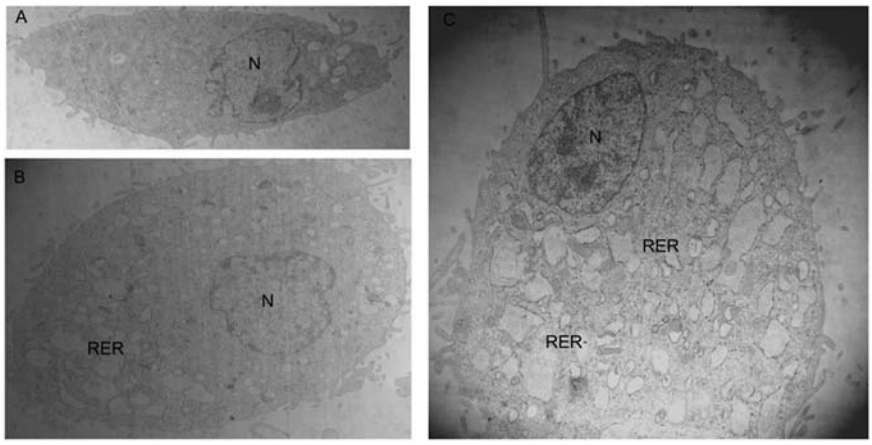

Figure 5. The effects of millimeter wave treatment on the ultrastructure of cells from each group. (A) Negative control treatment (group A), after regular culture for 12 days, cells were spindle-shaped with small volumes and a relatively high nucleocytoplasmic ratio. Magnification x8,000. (B) Thirty-min millimeter wave irradiation treatment (group B), after 12 days of treatment, the nuclear surface appeared processed, the nucleocytoplasmic ratio decreased, and the endoplasmic reticulum was more abundant and developed. Magnification x12,000. (C) Sixty-min millimeter wave irradiation treatment (group C), after 12 days of treatment, most cells were ellipsoid with a greater amount of cytoplasm, more abundant and developed endoplasmic reticulum and obvious glycogen granules. Magnification x20,000.

Toluidine blue staining. Distinct regions of the cytoplasm and extracellular matrix of the MSCs were differentially stained after induction, whereas no pronounced staining was seen in the absence of induction (Fig. 6).

Immunohistochemical staining of type II collagen. Positive staining was detected in the cytoplasm in most cells from induced groups B and C. Stronger staining was observed in group C, which was given longer irradiation treatments. Cells of the negative control group did not present a positive reaction (Fig. 7).

Discussion

Seed cells, scaffolds, and growth factors are the three major elements that must be optimized for a successful tissue engineering approach. MSCs are used as carriers in many cell replacement and gene therapies for the following reasons: rich sources, ease of sampling, separation, purification and culture, weak immunological reactions after transplant, and their self-renewal and differentiation potentials. The methods of isolation and *in vitro* culture are well established. Studies found that MSCs express specific surface marker antigens CD29, 44, 71

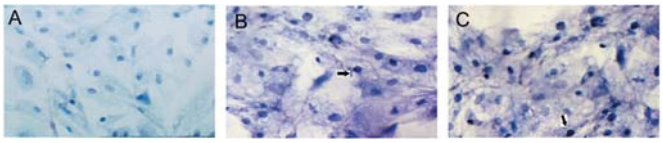

Figure 6. Toluidine blue staining results of cells from each group. (A) Negative control treatment (group A), after regular culture for 12 days, only nucleoli were stained blue and no differentially stained cells were observed. (B) Thirty-min millimeter wave irradiation treatment (group B), after 12 days of treatment, the cytoplasm was uniformly stained. (C) Sixty-min millimeter wave irradiation treatment (group C), after 12 days of treatment, the cytoplasm was uniformly stained and the number of differentially dyed cells was significantly increased compared to group B. Magnification x100; arrows indicate differentially stained cells.

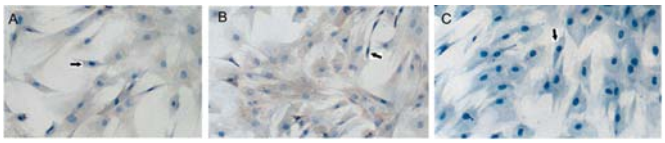

Figure 7. Immunohistochemical detection of type II collagen in each group. (A) Thirty-min millimeter wave irradiation treatment (group B), after 12 days of treatment, brown granules were detected in the cytoplasm, indicating a significant positive reaction. (B) Sixty-min millimeter wave irradiation treatment (group C), after 12 days of treatment, the number of cells presenting a positive reaction increased significantly as compared with group B. (C) Negative control treatment (group A), after regular culture for 12 days, cells presented a negative reaction. Magnification x200; arrows indicate cells with positive expression.

and 90, and do not express CD34 or 45. Therefore, the purity of an MSC population is initially determined by detecting positivity rates for CD45 and 90. In the present study, positivity rates for CD45 and 90 were 2.14 and 99.12%, respectively, permitting the conclusion that P_3 MSCs represented a pure MSC population. These cells therefore provide a suitable context to study the effects of millimeter wave on MSC differentiation into chondrocytes.

Millimeter wave irradiation refers to high-frequency electromagnetic waves with wavelengths of 1 to 10 mm and frequencies of 30 to 300 GHz. In the 1970s, millimeter wave treatments were employed in clinical medicine with beneficial effects. Research on the effects of millimeter wave treatments began in the late 1980s in China. These initial studies found that this therapy causes a variety of direct and indirect biological effects in humans and animals. Studies also noted that the oscillation experienced by living biological tissue ($0.5x10^{10}$-$3x10^{12}$ Hz) in the metabolic process falls within the frequency range of millimeter wave treatment, perhaps allowing the millimeter wave energy to be absorbed through resonance of biological tissue. After absorbing the electromagnetic energy by resonance, biological systems may then exhibit biological effects that are not temperature sensitive.

Core binding factor a1 (*Cbfa1*), also known as runt-related gene 2 (Runx2) and multi-tumor virus enhancer combining protein, is a transcription factor that belongs to the Runt domain gene family. It is specifically expressed by osteoblasts (OBs) and clearly regulates OB differentiation, as its function is essential for this process (10). *Cbfa1* not only promotes intramembranous ossification and endochondral bone formation, but also induces differentiation of other source cells into OBs and plays an important role in the maintenance of regular bone growth. Bone formation and growth during the embryonic period and after birth were associated with *Cbfa1*-dependent regulation of OB differentiation (11-14). Studies by Piccolo and others (15) found that *Cbfa1* was expressed before osteoblast-specific genes, implying that *Cbfa1* was the key switch in osteoblast differentiation. Researchers also found that *Sox9* is an important transcription factor in the regulation of chondrogenesis, as it promotes the expression of type II collagen both *in vivo* and *in vitro* (16). Ectopic expression of *Sox9* could also induce up-regulation of endogenous type α1 (II) collagen (16). Throughout the chondrocyte differentiation process, all chondroprogenitors express *Sox9* and type II collagen. *Sox9* expression is maintained until chondrocytes transition into hypertrophic chondrocytes (17). This line of research concluded that *Sox9* regulates the differentiation of cartilage by activating expression of the chondrocyte-specific molecular type II collagen and binding to enhancer elements of proteoglycan protein (15).

These two genes are therefore useful markers for different periods of chondrocyte differentiation and were used in this study to indicate the state of millimeter wave induced-differentiation of MSCs. RT-PCR was used to detect changes in the mRNA levels of both genes. The results of these experiments indicate that millimeter wave treatment induces *Cbfa1* and *Sox9* mRNA expression at increasing levels with prolonged treatment. Hypertrophic chondrocytes were not observed throughout the two weeks of induced culture, suggesting that millimeter wave treatment did not promote the differentiation of MSCs into OBs in short-term culture. This mechanism may be associated with the non-thermal effects caused by electromagnetic coherent oscillations following millimeter wave action. Further study is needed to determine the mechanism that underlies this process. Statistical analyses show that *Cbfa1* and *Sox9* mRNA levels increased to a greater extent in cells that experienced 60-min millimeter wave treatments compared to those given shorter irradiation treatments. There were significant differences ($P<0.01$) between these two groups, suggesting that energy accumulation and action induces persistent effects following millimeter wave irradiation.

Cells changed shape dramatically over the culture period. After twelve days of induction, the cells converted from spindle-shaped to polygonal. Electron microscopy revealed that organelles such as rough endoplasmic reticulum were more abundant and developed in cells of the induced group than the negative controls and that the endoplasmic reticulum cisterna further expanded in the 60-min treatment group. These results indicate that millimeter wave treatment enhances cellular metabolic function. At the same time, the observed cell shape changes suggest that millimeter wave treatment induces differentiation of MSCs into various cell types, mainly chondrocytes. This finding is consistent with the multiple differentiation potentials of MSCs.

Toluidine blue staining and immunohistochemical analysis respectively show that the cytoplasm and extracellular matrix were differentially dyed and that cells expressed type II collagen after induction. These morphological observations confirm that millimeter wave treatment induces differentiation of MSCs into

chondrocyte, as evidenced by excretion of chondrocyte-specific matrix glycosaminoglycan and type II collagen. The causative mechanism may again be the promotion of *Sox9* expression and subsequent expression of type II collagen genes, in accordance with the results of Bell *et al* (16).

In addition to the power flow density of millimeter wave irradiation, cell monolayers are affected by many factors, such as the structure and thickness of the incubation vial, wavelength of the millimeter wave, electromagnetic properties of the incubation vial and culture medium, resulting in a non-uniform distribution of cell growth following induction. A universal demand in these experiments is that the cells be irradiated with a uniformly distributed power flow. Furthermore, the frequency, power, and irradiation time of millimeter wave treatment directly impacts the success of this therapy. However, a set of optimal parameters has not yet been described and more basic research is required to determine these conditions.

In conclusion, daily millimeter wave irradiation treatments with wavelengths between 7.5 and 10.0 mm and a power density of 4 Mw/cm^2 for 30 or 60 min can successfully induce bone marrow mesenchymal stem cells to differentiate into chondrocytes that excrete chondrocyte-specific matrix glycosaminoglycan and type II collagen. Our results also indicate that the 60-min treatment is more effective than the 30-min treatment. The mechanism of this transition may be related to the notable up-regulation of *Cbfa1* and *Sox9* mRNA, which elevates glyco-saminoglycan and type II collagen production. Further research is needed to explore the complexities of MSC differentiation.

Acknowledgements

This research is supported by a grant of the Fujian Key Laboratory of Integrative Medicine on Geriatrics (No. 2008J1004-16) and National Nature Science Foundation of Fujian (No. C0110019).

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