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Original Article

CaMKII plays a part in the chondrogenesis of bone marrow-derived mesenchymal stem cells

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Abstract: Aims: The purpose of the study is to observe the functions of calcium/calmodulin dependent protein kinase II (CaMKII) in the induced chondrogenic differentiation of bone marrow derived mesenchymal stem cells (BMSCs). Methods: BMSCs was in vitro isolated and cultured for induced chondrogenesis. Western blot was used to ascertain the expression of CaMKII and phosphorylated CaMKII (PCaMKII, activatory CaMKII) in chondrogenic induced BMSCs. MTT method was utilized to observe the impact of CaMKII on the proliferation of BMSCs. The generation of cartilage matrix in BMSCs cells was detected by toluidine blue staining. The levels of cartilage marker genes *COL2A1, Aggrecan* and *SOX9* in BMSCs were gained by real-time fluorescence quantitative polymerase chain reaction (RT-QPCR). Finally, BMSCs proliferation, cartilage matrix generation and the changes of *COL2A1, Aggrecan* and *SOX9* were surveyed after CaMKII being blocked by CaMKII inhibitor KN93. Results: Expression of CaMKII and PCaMKII could be found in chondrogenic induced BMSCs. CaMKII had no significant influence on BMSCs proliferation, but the toluidine blue staining was obviously lighter, indicating a significant decline in the expression of *COL2A1, Aggrecan* and *SOX9*. Conclusion: As one of the factors influencing the chondrogenic capacity of BMSCs, CaMKII does not impact on BMSCs proliferation, but it can inhibit the chondrogenic ability of BMSCs by influencing its differentiation.

Keywords: CaMKII, bone marrow derived mesenchymal stem cells (BMSCs), chondrogenesis

Introduction

Joint dysfunction caused by articular cartilage injury is a common disease in departments of orthopedics. It is difficult for cartilage tissue nourished mainly by synovial fluid to regenerate after injury because of its deficiency in blood vessels, nerves and lymph supply [1]. Recently, despite pharmacological and surgical intervention on treatment of joint dysfunction, the outcome is still poor. So we focus on cure the disease according to the pathogenesis. Since the development of tissue engineering, chondrocyte is one of the first tissue treatment being studied and manipulated [2, 3]. Even though the clinical application of chondrocyte implantation has been quite popular in recent years, the bone marrow derived mesenchymal stem cells (BMSCs), became more popular [4-8].

BMSCs, featured by easy obtaining, rapid proliferation, multi-directional differentiation and excellent security [9], is one kind of non-hematopoietic stem cells existing in bone marrow and an important seed cell in medical fields of tissue engineering and regeneration [10]. But the differentiation of stem cells was affected by various factors like some protein kinases [11].

As a multi-functional serine/threonine protein kinase, calcium/calmodulin-dependent protein kinase II (CaMKII) plays an important biological role in multiple aspects such as cell metabolism, cell secretion, DNA damage repair, cell cycle regulation and gene expression [12], is a crucial factor related to the cell growth and activity [13]. Since the identified, CaMKII play an important role in regenerating skeletal muscle

[14], CML stem cells [15], heart failure [16], and myeloid leukemia cells [17]. CaMKII is increased in activation of ion channels [18], gene transcription [19], and apoptosis [20, 21]. But there are little reports about its specific functions in BMSCs proliferation and chondrocyte differentiation, so its influence on the chondrogenesis of BMSCs deserves our attention. The study explored the functions of CaMKII in the chondrogenesis of BMSCs, and provided theoretical foundation to promote the application of bone marrow derived mesenchymal stem cells in repairing cartilage.

Materials and methods

Reagents and animals

Experimental materials consisted of MTT solution and DMSO (dimethyl sulfoxide) produced by Sigma, as well as 20 SD rats of either gender, provided by the Experimental Animal Center, the Military Academy of Medical Scien-ces, China.

Separation and culture of BMSCs in rats

The tibia and femur of rats were cut off ,and the cells in medullary cavity were pushed into 50 ml centrifugal tube from one end of these long bones using complete culture solution mixed with heparin. The cells contained in tubes were centrifuged at 1500 rpm for 4 min. Supernate was discarded, and mononuclear cells in midlayer were inoculated in culture dish after being resuspended. The culture dish was put in incubator at 37°C with 5% CO_2, and the culture solution was replaced every 2-3 days. The primary culture was counted as complete when achieving 80% fusion. The cells were carried on the passage with the ratio of 1:3, noted as 1st generation (P1) and identified using flow cytometry. The 3rd generation cells were used for experiment.

Levels of PCaMKII and total CaMKII through Western blot

The third generation of BMSCs was cultured in chondrogenic medium for 72 hours. The cells were collected for Western blot analysis and divided into two groups, one group washed by icy PBS containing phosphatase and protease inhibitor (Roche), the other washed by icy PBS without the inhibitors, and then the extracted cells were transferred to nitrocellulose membrane (GE Healthcare) after polyacrylamide gel electrophoresis. CaMKII was tested by monoclonal antibody against CaMKII rats (1:1000 dilution), while phosphorylated calcium/calmodulin dependent protein kinase II (PCaMKII, activatory CaMKII) by monoclonal antibody against PCaMKII rats (1:1000 dilution) and GAPDH by polyclonal antibody against GAPDH rats (1:500 dilution). They were next incubated by second antibody containing HRP (1:2,000 dilution), and finally developed and fixed.

Influence of CaMKII on BMSCs proliferation capability

The third generation BMSCs was divided into two groups and cultured by two kinds of chondrogenesis medium respectively. Of them one kind only contained chondrogenic medium while the other was mixed with CaMKII inhibitor KN93 [22]. Cells from each group were inoculated to 96-pore plate respectively with 1×10^3 cells per pore, and then ended to be cultured after 0 hour, 24 hours, 48 hours and 72 hours successively. 20 μl MTT solution was added for incubating 4 h ago before the end of culture. 150 μl DMSO was added to each pore after the supernate being discarded by suction. The plates were vibrated for 10 min on shaking table. The light absorption value of each pore was measured using 490 nm wavelength and then recorded.

Changes in the cartilage matrix generation of BMSCs after being cultured with CaMKII inhibitor KN93

The third generation BMSCs was digested using 0.25%Trypsin-EDTA at 37°C for 1 min, collected after being centrifuged, and then divided into two groups for culture. Of them one group was put into the culture medium without KN93 while the other was with it. Cells from each group were inoculated with 200 μl per pore in 96-pore plate of "V" shaped bottom, and centrifuged with a force of 400 g for 5 min. When the supernate was discarded by suction after centrifuging, two kinds of cell deposits were added with chondrogenic medium which would be replaced every 2 days. Tiny clumps of cells could be visible to naked eye after 21 days of culture at 37°C with 5% CO_2. The micells were experienced toluidine blue staining after being made into paraffin sections.

Table 1. RT-QPCR primer sequences

Gene	primer sequences
RAT-*SOX9* F	CGTCAACGGCTCCAGCA
RAT-*SOX9* R	TGCGCCCACACCATGA
RAT-*Col-2 a1* F	CCGGACTGTGAGGTTAGGAT
RAT-*Col-2 a1* R	AACCCAAAGGACCCAAATAC
RAT-*Aggrecan* F	CTAGCTGCTTAGCAGGGATAACG
RAT-*Aggrecan* R	TGACCCGCAGAGTCACAAAG

Changes in cartilage marker genes in BMSCs after being cultured with CaMKII inhibitor KN93

Two groups of BMSCs, one with and the other without KN93, were cultured in chondrogenic medium for 21 days. Total RNA was extracted respectively from two groups using Trizol reagent and were conducted reverse transcription into cDNA. Fluorescent quantitative PCR amplification was performed using embedded fluorescent dye SYBR Green I with GAPDH as housekeeping gene to test the mRNA expression of *Co12A1, Aggrecan* and *SOX9*. Amplification system had a total volume of 50 μl, containing 25 μl 2×Sybr green I PCR MIX, each 1 μl of upstream and downstream primers, 2 μl template cDNA and 25 μl deionized water. The amplification conditions were as follows: at 94°C for 4 min, at 94°C for 20 s, at 60°C for 30 s and at 72°C for 30 s as one cycle which was proceeded for 35 times. Signal was detected at 72°C. The primers were shown in **Table 1**.

Results

Levels of PCaMKII and total CaMKII through Western blot

Through Western blot test, all GAPDH developing were distinct, indicating that the test were valid, and the developing of total CaMKII in cell lysate were clear, while the developing of PCaMKII was only visible with lower gray (**Figure 1**). All these manifested that a part of CaMKII in BMSCs had transformed into PCaMKII.

CaMKII has no significant influence on BMSCs proliferation capacity

The light absorption values of BMSCs represented slightly higher in the group with KN93 than in the other without KN93 at the points of 0 hour, 48 hours and 72 hours through the analysis of MTT results in two groups, while the

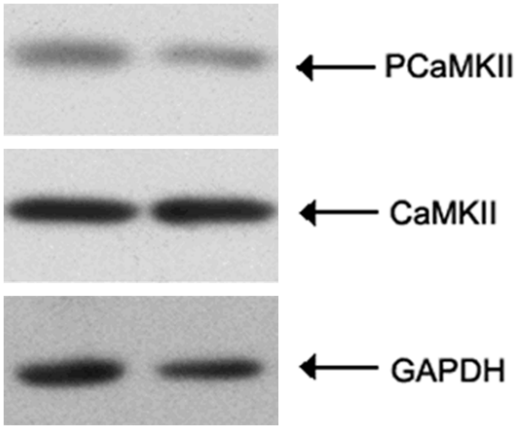

Figure 1. CaMKII expression. Western blot test showed that the visible developing of PCaMKII (activatory CaMKII) in cell lysate presented lower gray, but still indicated that a part of CaMKII in BMSCs had transformed into PCaMKII.

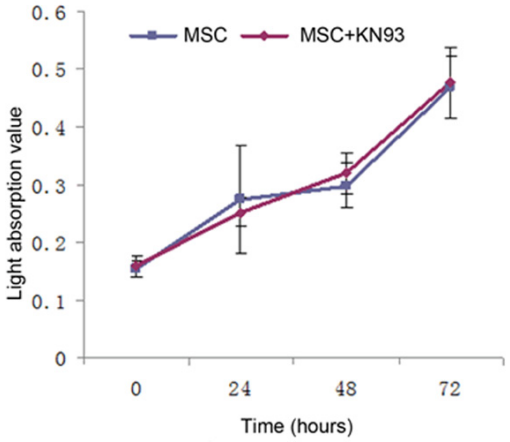

Figure 2. Correlation between CaMKII and BMSCs proliferation capacity. MTT result showed that the light absorption values of BMSCs cultured with KN93 had no statistically significant difference from those without KN93 during 0 hour to 72 hours (P>0.05).

light absorption values of BMSCs showed marginally lower in the group with KN93 than in the other without KN93, but the differences were all not statistically significant (P>0.05, **Figure 2**). Thence, there was no influence of CaMKII on BMSCs proliferation capacity.

Changes in cartilage matrix generation of BMSCs cultured by the compound of chondrogenic medium and KN93

The changes in cells volumes were not significant whether KN93 was added or not. The

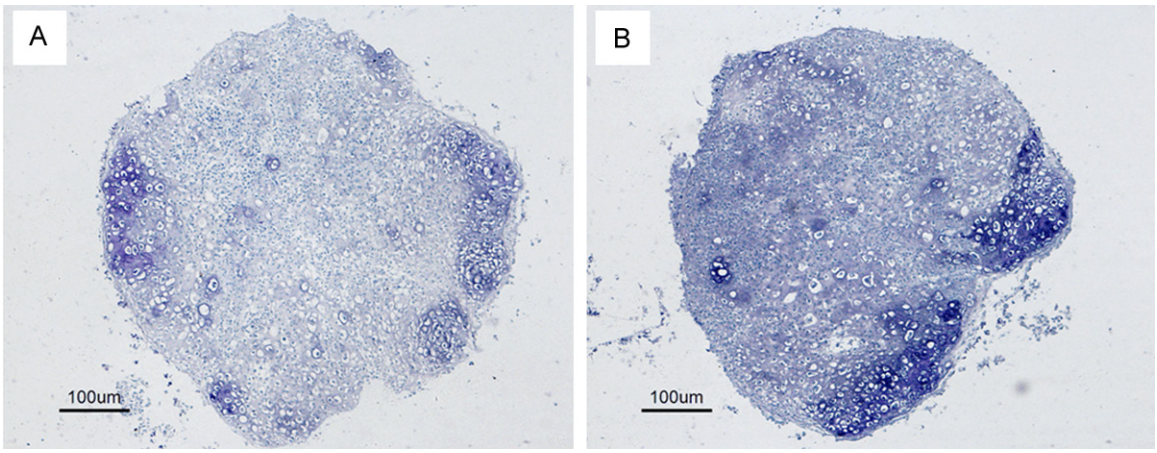

Figure 3. Changes in cartilage matrix generation of BMSCs cultured by the compound of chondrogenic medium and KN93. In different culture conditions--one without KN93 (A) and the other with KN93 (B)--the changes of cells volumes manifested little differences from in one condition to in the other. And BMSCs without KN93 presented a kind of lighter staining, while the cells with KN93 showed heavier staining.

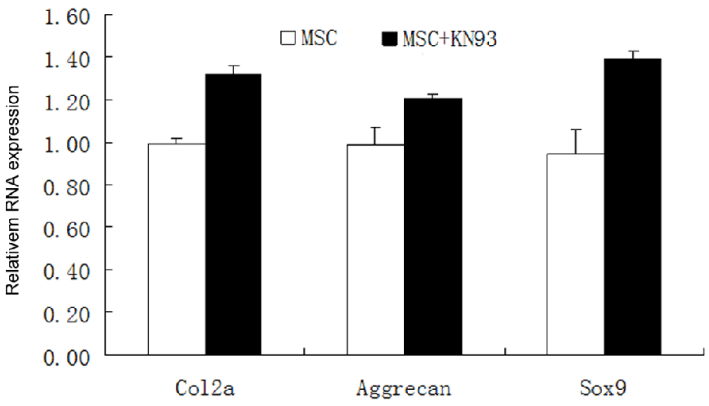

Figure 4. Changes in cartilage marker genes of BMSCs cultured by the compound of chondrogenic medium and KN93. The mRNA levels of COL2a1, Affrecan and Sox9, three major markers of BMSCs in chondrogenic differentiation, were significantly higher after the addition of KN93, and the differences in levels had statistical significance ($P<0.05$).

staining of BMSCs was lighter when KN93 was not added, while the staining became heavier when KN93 was added (**Figure 3**). So we suggested that KN93 can be effectively suppressed the activity of CaMKII in cartilage matric generation of BMSCs.

Changes in cartilage marker genes of BMSCs cultured by the compound of chondrogenic medium and KN93

The mRNA levels of *COL2A1, Aggrecan* and *SOX9*, cartilage differentiation symbols, were detected by RT-QPCR test. Ct value was obtained and conducted the comparative analysis with the GAPDH reference. Then the result showed that the mRNA levels of three major cartilage differentiation markers in BMSCs were significantly higher when KN93 was added compared to not added, and the differences were statistically significant ($P<0.05$, **Figure 4**). Thus we can conclude that the presence of CaMKII should inhibit the cartilage differentiation.

Discussion

BMSCs, one kind of non-hematopoietic stem cells existing in bone marrow, not only provides as an alternative cell candidate, but also can be used for the ideal cells for implantation in medical fields of tissue engineering and regeneration. But the differentiation of stem cells was affected by various factors like some protein kinases. Recent studies showed that CaMKII plays an important role in multiple aspects [5], and is a crucial factor related to the cell growth and activity [6]. In this study, we focused on the chondrogenesis which induced by BMSCs, and carried out an initial research for the mechanism for CaMKII in this process.

In the study, Western blot was first used to verify the fact that chondrogenic induced BMSCs contained CaMKII and that a part of CaMKII

transformed into PCaMKII, confirming that CaMKII was involved in the chondrogenic differentiation of BMSCs. Second, the light absorption value was measured at 4 points of 0 hour, 24 hours, 48 hours and 72 hours during the culture of chondroblast induction of BMSCs. All these values showed that BMSCs proliferation expressed no significant difference between whether CaMKII was inhibited or not, through 0 to 72 hours. So CaMKII could be regarded as having no influence on BMSCs proliferation. The result was different from previous studies, CaMKII exerts an important regulator of cell proliferation [23, 24]. On leukemia cells, CaMKII inhibits myeloid leukemia cell proliferation [25].

In order to get the conclusive evidence about the correlation between CaMKII and BMSCs chondrogenic, cell clusters in chondrogenic inducted BMSCs were conducted toluidine blue staining and showed that the content of cartilage matrix increased after CaMKII being inhibited. RT-QPCR result also showed that the mRNA levels of *COL2A1, Aggrecan* and *SOX9* genes [26], symbols in BMSCs chondrogenic differentiation, all rose significantly after CaMKII being inhibited, indicating that the presence of CaMKII could reduce the cartilage differentiation. Evidence indicated that Ca^{2+} signaling pathway was involved in osteoclast differentiation and activation [27], indirect showed CaMKII may be regulate cell differentiation and activation.

CaMKII mechanism for inhibiting BMSCs chondrogenic differentiation is not yet very clear, but researches have proven that CaMKII can activate nuclear factor-kappa B (NF-kB) [15, 28]. Widely existing in eukaryotic cells, NF-kB is involved in multiple biological processes from the regulation of congenital and adaptive immune responses of body, inflammation to cell growth and apoptosis. Moreover, activatory NF-kB can increase the activities of cytokines such as IL-1β and TNF-α to directly activate matrix metalloproteinases (MMPs) and cathepsin, and all these changes may inhibit the cartilage generation [29, 30]. On the other side, CaMKII can dephosphorylate nuclear factor of activated T cell (NF-AT). Dephosphorylation can not only bring about the accumulation of NF-AT of nuclear factor-kappa B [31], but also expose DNA binding domain in NF-AT through its intrinsic conformation changes to make it combine with DNA more easily [32]. The concentration combination of NF-AT and DNA is bound to produce some biological effects such as the induced expressions of cytokines like IL-1β and TNF-α [33, 34] as well as other effects unknown currently, and all these effects may exert an important influence on BMSCs when it differentiates into cartilage cells and then forms cartilage tissue.

Combined the aforementioned results, CaMKII plays an important role in the chondrogenesis of BMSCs. It has no evident impact on BMSCs proliferation, but can significantly reduce the chondrogenic capacity of BMSCs by inhibiting BMSCs chondrogenic differentiation. As for CaMKII mechanism for inhibiting BMSCs chondrogenic differentiation still needs to be explored and verified by further studies.

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Disclosure of conflict of interest

None.

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