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Hepatocyte Growth Factor-induced Differentiation of BMSCs toward Hepatocyte-like Cells via the NF-κB and P38MAPK Signaling Pathway

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Abstract: Bone marrow mesenchymal stem cells(BMSCs) have great potential ability of multi-directional differentiation and reproductive activity. Recent studys have demonstrated that BMSCs can be induced into hepatocyte-like cells. However, the molecular mechanism of hepatocellular differentiation remains unknown. In this study, we investigated the nexus between p38 MAPK and NF-κB signaling pathway in the process of the hepatocellular differentiation. We isolated BMSCs from femurs and tibias of rats. The third generation were divided into three main groups: induction group, inhibition group and negative control group. Hepatic differentiation was induced by 10% fetal bovine serum with hepatocyte growth factor(HGF). The inhibitors of p38 (SB203580) and NF-κB(BAY 11-7082) were added to the differentiation medium for inhibition of signaling molecular activities. Morphological characteristics and transferring function of the differentiated cells were examined by indocyanine green (ICG) uptake assay. Immunohistochemical staining was used to evaluate the protein expression position of NF-κB. And western blot analysis was used to detect the protein expression of several markers, including the specifc markers of hepatocytes(AAT), phosphorylated-p38(p-p38) and NF-κB. NF-κB were observed transferred into nuclear in the induction group. The respective inhibitors inhibited the expressions of NF-κB and p-p38 effectively. Compared to the induction group, expressions of specifc marker, AAT, were decreased visibly in the p38 and NF-κB inhibitor-treated groups. Notably, expression of NF-κB were significantly lowered in the p38 inhibitor-treated group. These data suggest both NF-κB pathway and p38MAPK pathway participate in the hepatocellular differentiation of BMSCs, p38MAPK can affect the regulation of NF-κB to this process of differentiation.

Keywords: Bone marrow stem cells; Hepatocyte; Differentiation; Hepatocyte growth factor; Nuclear factor-kappa B; p38MAPK

INTRODUCTION

As an ideal cell source for transplantation or liver tissue engineering, bone marrow stem cells (BMSCs) could be induced into hepatocytes under the stimulus of different cytokines[1]. However the exact differentiation mechanism remains unclear.

The mitogen-activated protein kinases(MAPKs) signaling pathways is the most important signal pathway protein kinases, which participate in the

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regulation of mediating stem cell proliferation and differentiation[2, 3]. Recent studies have demonstrated that MAPK signaling pathway[4], especially p38, is sufficient to drive differentiation of BMSCs into hepatocyte[5]. As the downstream signaling pathway of MAPKs[6, 7], NF-κB have be showed also participates in the differentiation of BMSCs into hepatocytes in previous studies. However, whether there is a link between p38MAPK and NF-κB signal pathway in regulating the hepatic differentiation still unclear.

In the present study, we demonstrated that BMSCs can be induced into the hepatocyte-like cells and express specifc markers of hepatocytes(AAT) after 20 days. We mainly inhibited NF-κB by BAY 11-7082 and inhibited p38 by SB203580 to investigate the relationship between NF-κB and p38MPAK in hepatic differentiation of BMSCs.

MATERIALS AND METHODS

Isolation and cultivation of Rat bone marrow mesenchymal stem cells

BMSCs were seperated from young male SD rat by using the whole bone marrow adherence method and cultured in DMEM-low glucose medium supplemented with 10% FBS at 37 ℃ in a humidified atmosphere of 5% CO_2. The non-adhered cells were removed from the via the culture flask by changing the medium after 24h during primary culturing. At once the BMSCs nearly reached to 80% confluency, they were dissociated with Accutase enzymes (Mpbio) and replated for subculture. Whereafter the cells were cultured to the third generation, they were collected and used in this experiment.

In vitro Hepatic induction and group design

BMSCs were inoculated on 60 mm petri dishes and divided into four groups in total, each group induced under different stimulating environments (Table 1). The final concentration of HGF,SB203580 and BAY 11-7082 was 20 ng/ml, 15nmol/ml, 10nmol/ml, respectively. Culture medium was changed every 3 days and the induction maintained for 20 days. All groups are cultured at 37 ℃ and 5% CO_2. The cells were collected at the 7th day and the 20th day, respectively.

Table 1 The group design and treatment of BMSCs

Control group	Induction group	SB group	BAY group
Equal medium	HGF	HGF SB203580	HGF BAY 11-7082

ICG uptake assay

ICG was made into 5 mg/ml stock solution by dissolved in sterile phosphate buffered saline (PBS), and then were mixed with 10% FBS DMEM-low glucose medium to a final concentration of 1 mg/ml. Cells were incubated in ICG medium at 37 ℃ for 2h . The culture solution was substituted with 10% FBS DMEM-low glucose medium after the ICG was washed off with PBS buffer solution.

Immunohistochemical

In brief, the slides of BMSCs induced for 7 day and 20 day fixed in cold acetone at room temperature for 20 min. Cells were treated with blocking solution (5% Bull Serum Albumin) for 30min and incubated overnight at 4 ℃ with the primary antibody, rabbit polyclonal anti-NF-κB p65 (1:200, Boster), antibodies diluted in antibody dilution solution. After three washes in PBS, the slides of cells were incubated secondary antibody using the SV0002 anti-rabbit IgG-HRP kit(Boster). Images were captured using a fluorescence microscope (BX63, Olympus).

Western blot analysis

The total protein of BMSCs were lysed with RIPA buffer (Beyotime) containing 1 mmol/L PMSF. Cells were cooled on ice for 15min and shaken every 5min. The lysate was centrifuged at 12000 rpm for 20min. Protein concentrations were measured using the bicinchoninic acid Protein Assay Kit (Beyotime). Proteins were fractionated by 10%

SDS-polyacrylamide gel electrophoresis [7] and then transferred to polyvinylidene fluoride membranes (Roche). The membranes were blocked in the blocking solution TBST (0.02% Tris-HCl pH 7.6, 0.1% Tween20, and 150 mmol/L HCl) containing with 5% skim milk for 1h, and then were incubated with the following primary antibodies: anti-NF-κB p65 (1:500, Boster) ,anti-phospho-p38（1：:1000 ） anti-GAPDH (1:1000; Boster) antibodies overnight at 4 ℃. After three washes with TBST buffer， membranes were incubated with secondary goat anti-rabbit lgG phosphatase conjugated (Thermo) for 1h. Immunoreactive bands were detect by western bloting using the Westar Eta C Ultra (Cyanagen) according to the manufacturer' instructions.

Statistical analysis

Statistical analysis was proformed using the GraphPadPrism 5 software.

All results of evaluation parameters were reported as means ± SD. Among the multiple groups, the P values' analysis were adjusted with the Bonferroni method after analysis of variance (ANOVA) and when the P values < 0.05 , the results were considered to be statistically significant.

RESULTS

Morphological characteristic of BMSCs during differentiation into hepatocyte-like cells and NF-κB inhibition

The BMSCs displayed typical fibroblast-like morphologies closely spaced growth in shape after three times subculture (Fig. 1A). After 7 days of culturing with different stimulating, Part of the cells in the differentiation groups grew to polygonal. After 20 days culturing , the cells in these colonies partly became round or oval shaped(Figure 1B), whereas the cells in the negative control groups remained fibroblast-like with fusiform or polygonal morphologies and as consistently as the third passage cells(Figure 1A) . The majority of the cells in the differentiation environment that contained inhibitiors displayed fibroblast-like with fusiform or polygonal morphologies, while a little showed round or oval , hepatocyte-like cell shape(Figure 1C, D).

ICG uptake of differentiated BMSCs

After 20 days culturing with different stimulating, none of cells in negative control group could ingest ICG(Figure 2A). The majority of cells in the hepatic induced group showed the ability to uptake ICG(Figure 2B), whereas less of cells in the the differentiation medium that contained p38-inhibitor(Figure 2C) or NF-κB-inhibitor(Figure 2D) ingested ICG.

NF-κB transfer of differentiated BMSCs

NF-κB sustained an inactive state in the control group, while the NF-κB positive rate of induction group was increased significantly with the prolongation of the induction time. The inhibitors of NF-κB and p38 suppressed the activation of NF-κB compared with the induction group for 7 day and 20 day(Figure 4).

Effects of p38MAPK inhibitor and NF-κB inhibitor on AAT, p-p38 and NF-κB protein expression

The hepatocyte marker, NF-κB and the phosphorylation of p38 were measured by immunoblotting with antibodies against the corresponding molecules. Compared with the negative control that was cultured in basal medium only, the AAT protein was expressed in the induction medium. When the cells were treated with the p38 inhibitor and the NF-κB inhibitor, the protein expression of the special hepatocyte marker bands became weaker, and the p38 inhibitor were more efficiency than the NF-κB inhibitor. Comprised with the induction group, the expression of NF-κB were reduced not only in the NF-κB inhibition group but also in the p38 inhibition group. While p-p38 decreased only when treated with p38 inhibitor(Figure 5).

Discussion

With the advantages of high safety, less side effect and efficient curative effect , hepatocyte transplantation has been researched widely. But the difficulty of obtaining freshly isolated hepatocytes is a current problem for its

practical application. BMSCs have been demonstrated that can be induced to differentiate into a variety of cells originate from different gren layers, such as osteoblast, neurons and cartilage cells[8-10]. BMSCs also have the potential to differentiate into liver cells[11, 12]. At present, some cytokines like HGF[13], β-NGF, FGF4, were widely used as inductor for the hepatic differentiation of BMSCs in vitro[14]. But the molecular mechanism underlying the hepatic differentiation of BMSCs are still unclear. Uptake of indocyanine green is an unique function of liver cells[15], in this experiment, we observed that part of the hepatocyte-like cells can assimilate ICG after 20 days' inducing. This result confirmed that these cells also have the transport function as liver cells.

NF-κB is one of the most important nuclear factors reside in eukaryotic organism. Repressor protein IκBα usually combined with NF-κB in the cytoplasm of the resting cells[16], thus hide the nuclear localization signal of the protein polymer. IκBα can be phosphorylated and hydrolyzed by activated protein kinase under certain situations. And this process leads the nuclear translocation of NF-κB[17]. Recent studies have shown that the activation of NF-κB signal pathway via p38MAPK[18]. In our previous study, NF-κB signal pathway may participate in the hepatocellular differentiation process of BMSCs.

As the most important signal pathway protein kinases, Mitogen-activated protein kinase(MAPKs) establish a huge kinase network that regulates a variety of physiological processes[19], like cell proliferation, differentiation, and apoptosis. MAPK signal pathway consists of three steps kinase cascade involving MAPKKK,MAPKK and MAPK, as well as ERK, JNK and p38 kinase[20, 21]. The MAPK signal pathway, especially p38, has been reported to be sufficient to drive BMSCs to form hepatocytes.

ICG-uptake is a useful marker to identify differentiated hepatocytes in vitro, which was used to illustrate the degree of differentiation. In our present study, according to the ICG uptake assay, hepatocyte-like cells are appeared in the induction groups after 20 days. Whereas the cells in the negative control group remained fibroblast-like. The result of western blot also showed that the specific maker of hepatocytes, AAT, was expressed in the induction groups at day 20, whereas not expressed in the negative control group. We added the inhibitors of p38MAPK(SB203580) and NF-κB(BAY11-7082) in the induction culture to test the relationship and the role of p38 MAPK pathway and NF-κB pathway during the hepatocellular differentiation of BMSCs. Morphology of most cells showed fibroblast-like and the expression level of AAT reduced evidently in both p38-inhibition group and NF-κB-inhibition group. ICG uptake results also validated that inhibition of p38 and NF-κB could suppressed the hepatocellular differentiation.

Immunohistochemical staining showed that NF-κB appeared in the nuclear in the induction groups whereas only expressed in the cytoplasm in the negative control group at the day seven and the day twenty. The nuclear translocation of NF-κB was suppressed visibly by adding the inhibitor of NF-κB as well as the inhibitior of p38. Expression level also showed the same results. Therefore, we demonstrated that both NF-κB pathway and p38MAPK pathway participate in the hepatocellular differentiation of BMSCs and these two pathways have a certain connection in this process.

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Figure:

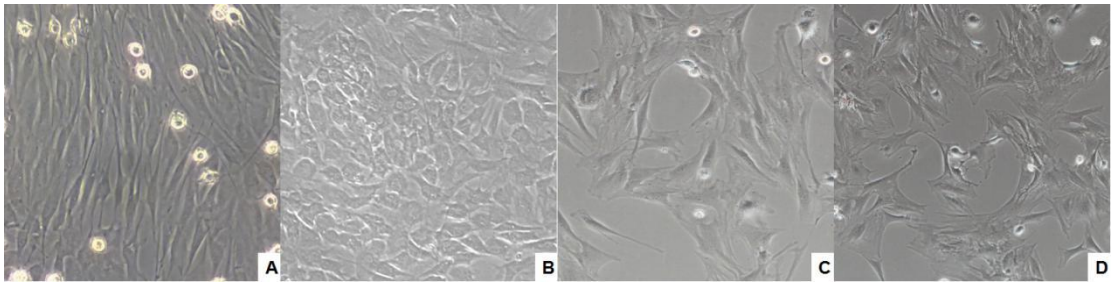

Figure 1. Morphologic changes of BMSCs among groups. **A.**Negative control cells; **B.** Induction group of the hepatic-like cells; **C.** P38 inhibitor group cells; **D.** NF-κB inhibitor group cells.

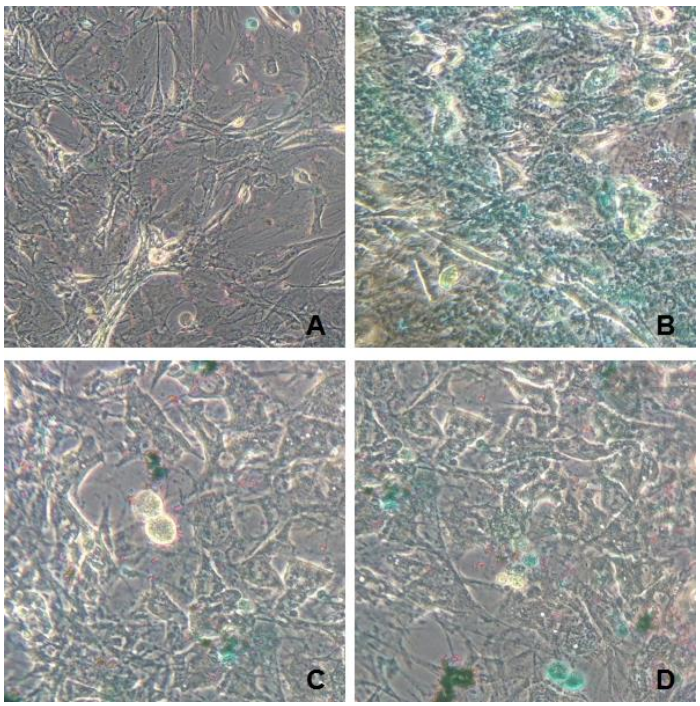

Figure 2. ICG uptake of differentiated BMSCs. **A.**Negative control cells; **B.** Induction group cells; **C.** P38 inhibitor group cells; **D.** NF-κB inhibitor group cells.

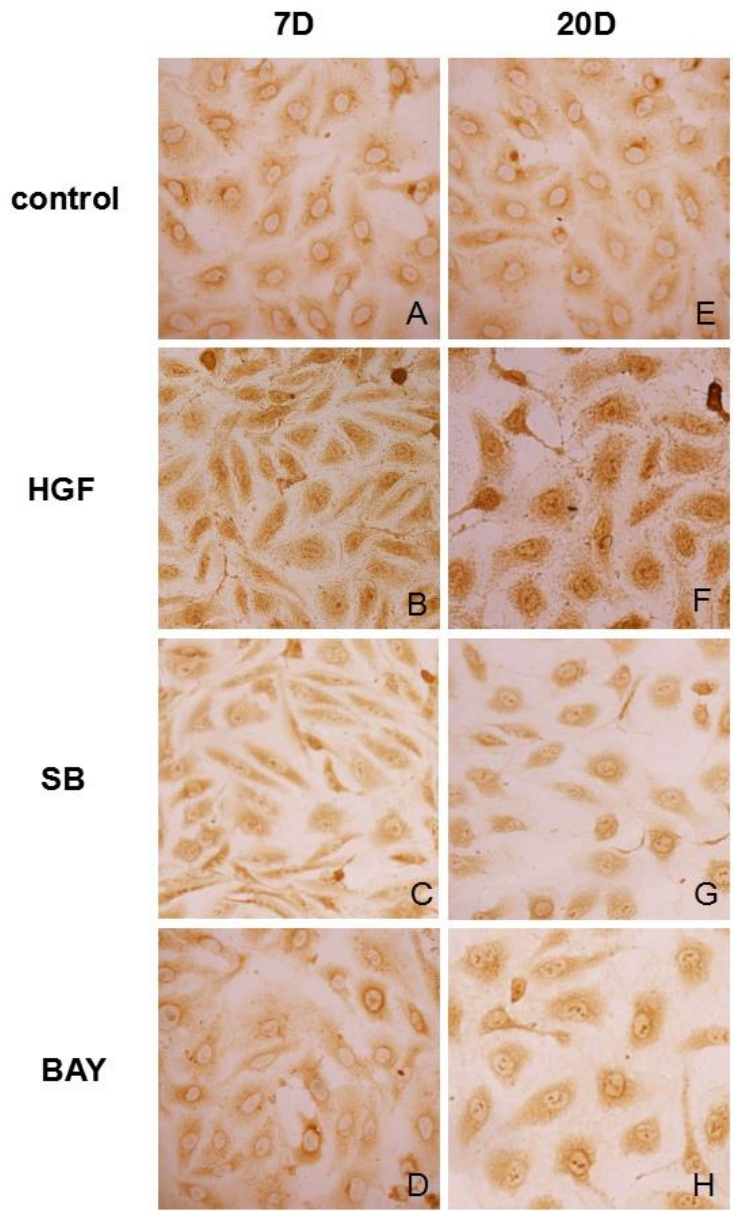

Figure 3.NF-κB transfer of differentiated BMSCs. **A.**Negative control cells in day 7; **B.** Induction group cellsin day 7; **C.** P38 inhibitor group cellsin day 7; **D.** NF-κB inhibitor group cellsin day 7; **E.**Negative control cells in day 20; **F.** Induction group cellsin day 20; **H.** P38 inhibitor group cells in day 20; **G.** NF-κB inhibitor group cellsin day20.

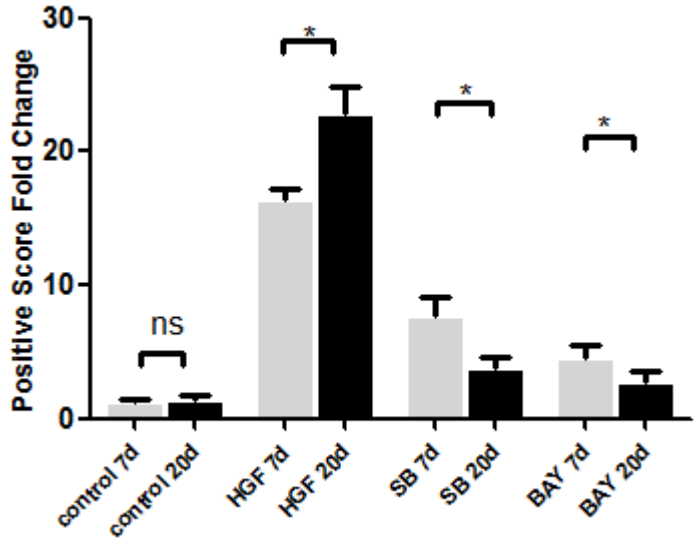

Figure 4. NF-κB transfer of differentiated BMSCs.

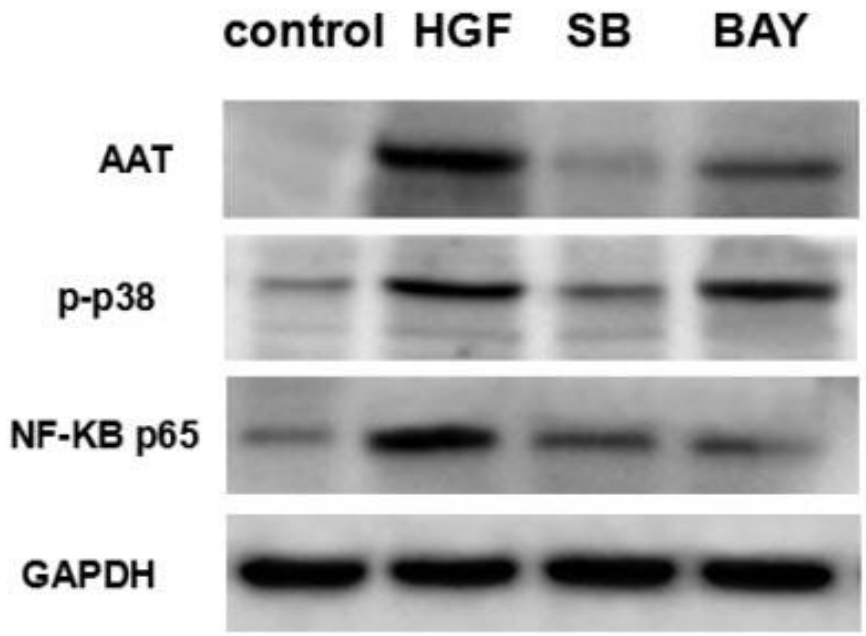

Figure 5. Effects of p38MAPK inhibitor and NF-κB inhibitor on AAT, p-p38 and NF-κB protein expression