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The Effect of Bay 11-7082 Inhibits Rat BMSCs to Hepatocyte-Like Cells via NF-κB Signaling in Vitro

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Abbreviations:

AAT	Alpha-1-antitrypsin;
BMSCs	bone marrow stem cells;
BAY	BAY 11-7082;
β-NGF	beta-nerve growth factor;
ES	embryonic stem;
FBS	fetal bovine serum;
HGF	hepatocyte growth factor;
ICG	indocyanine green;
LTF	liver tissue fluid;
NF-κB	nuclear factor-kappa B;
PBS	phosphate buffered saline;

Abstract: Bone marrow stem cells (BMSCs) could be induced into hepatocytes by different cytokines, which has a great potential in tissue engineering. However, the differentiation was not so efficient mainly due to the less understanding of molecular mechanism. Our objective was to investigate the effect of BAY 11-7082 on the differentiation of BMSCs into hepatocyte-like cells. We observed the majority of differentiated cells were spherical or ovate in shape, indocyanine green (ICG) positive, α-1-antitrypsin (AAT) expression increased and NF-κB transferred into nuclear. Whereas the BMSCs additionally added with BAY 11-7082, which were showed fusiform or polygonal morphologies unchanged in shape, ICG uptake and AAT expression reduced, NF-κB scattered in the cytoplasm. These data suggest the activation of BAY 11-7082 may inhibit the differentiation of BMSCs into hepatocyte-like cells via NF-κB signaling.

Keywords: BMSCs; Hepatocyte; Differentiation; NF-κB; Indocyanine green; BAY 11-7082;

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1. Introduction

Bone marrow stem cells (BMSCs) are multipotent stem cell population that can be derived from bone marrow and induced to differentiate into a variety of cell types in vitro[1]. However, the differentiation of BMSCs was too complicated to demonstrate clearly the biochemical and molecular signals of these phenomenons .

In previous studies showed NF-κB participates in the differentiation of BMSCs into neurons or osteoblast. NF-κB usually stays in the cytoplasm and was a trimer. Such as P50, P60 and inhibit-protein IκBα[2]. Then IκBα will dissociate from the trimer by phosphorylation after stimulated by other signals. At the same time, NF-κB consist of P50 and P65 transfers into nuclear and combines with the common κB sequence in the enhancer domain of κB reaction gene to promote the expression of downstream genes[3].

Our work recently showed BMSCs could be induced to hepatocyte-like cells that ingested ICG. While ICG-uptake is a useful marker to identify differentiated hepatocytes in vitro, which was used to illustrate the degree of differentiation[4]. And we mainly stimulated BMSCs by BAY 11-7082 (Beyotime) to investigate the variety of BMSCs into hepatocyte-like cells.

2. Materials and methods

2.1 Reagents and animals

The DMEM-low glucose medium was from Hyclone. The fetal bovine serum (FBS) was obtained from Gibco. HGF and β-NGF were from Sigma. Adult SD rats (weight from 200~300g, six weeks) were provided from the Medical-Experimental Animal Center of Qingdao University.

2.2 Separation and culture of rat BMSCs

By whole bone marrow adherence method for separation of SD rats BMSCs, we took adult rats bone marrow aspirates from bilateral shinbones of normal donors, flushed with DMEM-low glucose medium supplemented with 10% FBS, and seeded directly in 25 cm^2 culture flasks at a density of 1×10^7 cells/cm^2[5]. The cultures were maintained at 37 ºC in a humidified atmosphere of 5% CO2, while the non-adhered cells were removed via the culture medium changing at 24th h during primary culturing. When BMSC reached to 80% confluency, we dissociated them with Accutase enzymes (Mpbio) and replated for subculture. Subsequently, the third passage cells were collected and used in this experiment.

2.3 Preparation of LTF

The neonatal rat liver homogenate was supplemented with 10% FBS DMEM-low glucose medium (100 mg:1 ml), which was centrifugalized for 20 min at 10000 rpm after standing at 4 ºC, then the supernatant fluid after 0.22 μm membrane filter reserved at -20 ºC. The effective concentration of LTF was 100 mg/ml.

2.4 Hepatocyte-like cells induction and group design

The subjects were divided into eight groups in total, each group induced by different stimuli (Table 1). The final concentration of HGF, β-NGF, LTF and BAY 11-7082 was 20 ng/ml[6], 40 ng/ml, 1 mg/ml, 10nmol/ml[7], respectively. Culture media was changed every 3 days and the induction maintained for 20 days. In addition, all of BAY groups were treated with BAY 11-7082 in the presence of 10% FBS DMEM-low glucose medium for one day before the formal induction.

2.5 ICG uptake assay

ICG was dissolved in sterile phosphate buffered saline (PBS) to produce a fresh 5 mg/ml stock solution and was diluted in 10% FBS DMEM-low glucose medium to a final concentration of 1 mg/ml[4], added to differentiated BMSCs induced for 1 day and 20 days, which were incubated at 37 ºC for 2 h for the uptake of ICG by the BMSCs. Then culture flask

was refilled with 10% FBS DMEM-low glucose medium after washed twice with PBS and observed under a phase-contrast microscope (CKX31,Olympus). Randomly selected five visual fields and count number of positive cells that ingested ICG in each field of vision, After the examination, The cells were then incubated further for six hour to allow the release of the ICG dye completely for other detection.

2.6 Immunofluorescence.

BMSCs induced for 20 days were fixed and permeabilized using 100% cold acetone for 10 min. After washing with PBS, the samples were blocked using 5% BSA and incubated overnight at 4 ºC with the primary antibody, rabbit polyclonal anti-NF-κB p65 (1:400, Boster), antibodies diluted in antibody dilution solution. The excess primary antibody was removed by washing three times in PBS, and the samples were incubated with chicken anti-rabbit TRITC conjugate (1:1000, Novex) as the secondary antibody at room temperature for 2 h. In addition, cells were counter-stained for 4 min using DAPI (Beyotime) to label the nucleus. Images were captured using a fluorescence microscope (BX63, Olympus). Positive cells, which NF-κB transferred into nucleus were counted in five visual fields randomly selected.

2.7 Western blot analysis

Nuclear proteins of BMSCs induced for 20 days were extracted by using a nuclear protein extraction kit (Epigentek), or lysed using RIPA buffer (Beyotime) containing 1 mmol/L PMSF. protein concentrations were measured using the BCA Protein Assay Kit (Beyotime). In this experiment, proteins extract were boiled with 5×SDS sample loading buffer for 5 min at 100 ºC and resolved by 10% SDS-polyacrylamide gel electrophoresis[8]. The fractionated proteins were then transferred to PVDF membranes (Roche). Blocked in 5% BSA containing TBST (0.02% Tris-HCl pH 7.6, 0.1% Tween20, and 150 mmol/L HCl) for 1h, the membranes were incubated with rabbit anti-NF-κB p65 (1:500, Boster), rabbit anti-AAT (1:600, Boster) or rabbit anti-GAPDH (1:1000; Boster), rabbit anti-TATA binding protein TBP antibodies (1:1000; Abcam) overnight at 4 ºC. The blots were washed thrice with TBST buffer and incubated with secondary goat anti-rabbit lgG phosphatase conjugated (Thermo). Peroxidase activity was visualized with the Westar Eta C Ultra (Cyanagen) according to the manufacturer' instructions. Chemiluminescent signal was captured on autoradiography by Fusion Fx (Vilber lourmat) and used to assess protein content[9]. The relative protein levels were assessed by the chemiluminescent signal compared with GAPDH or TBP protein.

2.8 Statistical analysis

The results were expressed as means ± SD. Statistical significance was determined using GraphPadPrism 5 software, Analysis followed a Student's t test was used to determine significant difference between two groups[10]. For analysis of multiple groups, the P values were adjusted using the Bonferroni method after analysis of variance (ANOVA) and P values < 0.05 were considered to be statistically significant.

3 Results

3.1 Morphologist characteristic of induced BMSCs

The primary cells of BMSCs during three times subculture presented fibroblast-like, fusiform or polygonal morphologies closely spaced growth in shape (Figures 1 A). After stimulated for 20 days. Some BMSCs were differentiated into hepatocyte-like cells and became round or oval shaped (Figures 1 B) in HGF induced group, β-NGF induced group, LTF induced group, BAY HGF group, BAY β-NGF group and BAY LTF group. Whereas the cells of control group and BAY control group remained fibroblast-like, fusiform or polygonal morphologies and were similar to the third passage cells.

3.2 ICG uptake of differentiated BMSCs

After continuously induced for 20 days. None of cells within control group and BAY control group could ingest ICG and the percentage of positive cells was 0% (Figures 1 C-D). The cells appeared green in HGF induced group, β-NGF induced group and LTF induced group (Figures 1 E-G), which ingested ICG were hepatocyte-like cells and the positive rates respectively were (34.96 ± 2.28)%, (25.44 ± 4.87)%, (56.61 ± 11.72)%. The ICG positive rates of BAY HGF group, BAY β-NGF group and BAY LTF group (Figures 1 H-J) were (9.99 ± 5.57)%, (8.53 ± 3.92)%, (29.28 ± 7.74)% and decreased significantly ($t=5.85$, $p<0.05$; $t=3.96$, $p<0.05$; $t=6.41$, $p<0.05$) compared with HGF induced group, β-NGF induced group and LTF induced group (Figures 2 D), respectively. The decline of ICG positive rate probably was due to the influence of BAY 11-7082 in the BAY group. In addition, all of BMSCs induced for 1 day were not uptake of ICG.

3.3 NF-κB transfer of differentiated BMSCs

After induced for 20 days (Figures 2 A-B), The NF-κB positive rate of BAY HGF, BAY β-NGF and BAY LTF group was (13.81 ± 3.73)%, (14.04 ± 4.75)% , (31.26 ± 8.47)% and increased significantly ($t=3.59$, $p<0.05$; $t=3.17$, $p<0.05$; $t=4.97$, $p<0.05$) respectively compared with HGF induced (28.72 ± 6.28)%, β-NGF induced (27.19 ± 4.53)% and LTF induced group (51.89 ± 9.46)%, as shown in Figures 2 C. Moreover, NF-κB signals of the control groups were mainly in the cytoplasm. The results indicate BAY 11-7082 could prohibit the NF-κB signals from transferring into nuclear.

3.4 NF-κB and AAT expression of differentiated BMSCs

The ANOVA and multiple comparisons for data by Bonferroni method ($F=125.3$, $p<0.05$). There were significantly difference ($t=9.63$, $t=9.54$, $t=14.04$, $p<0.05$) of NF-κB expression in nuclear between HGF induced group and BAY HGF group, β-NGF induced group and BAY β-NGF group, LTF induced group and BAY LTF group (Figures 2 G), which was similar to the result of immunofluorescence and implied that BAY 11-7082 suppresses NF-κB activation.

To ICG assay, the differentiation was not complete in all BMSCs, accordingly AAT (a glycoprotein synthesized chiefly in hepatocytes) expression has a good linear relationship with the degree of differentiation. As shown in Figures 2 H, The AAT expression in HGF induced, β-NGF induced and LTF induced group was increasing significantly ($F=251.7$, $p<0.05$; $t=12.94$, $t=9.41$, $t=24.28$; $p<0.05$) compared with BAY HGF, BAY β-NGF and BAY LTF group, which has demonstrated again the BAY 11-7082 will decrease the preference that differentiation of BMSCs into hepatocyte-like cells.

4. Discussion

As we know, BMSCs do not express specific surface antigen and generally are positive for CD29, CD44, CD54, CD58, CD71, CD90, CD105, CD124, CD166[11], SH2, SH3, SH4, collagensI and collagensV, negative for CD3, CD11a, CD14, CD19, CD34, CD38, CD45[12], CD50, CD68, CD80[10], CD79a, CD117, vWF, HLA-DR, collagensII and collagensIII[13]. Using the technology of flow cytometry, density gradient centrifugation and magnetic activated cell sorting to isolate cells may have influence on morphology and activity of BMSCs. Yet Li Y et al[14] showed the positive rates of CD29, CD44, CD34, CD45 respectively were 99.76%, 93.60%, 0.66%, 0.60%, which were expressed of BMSCs that isolated by whole bone marrow adherence method. Therefore, we can collect high purity, uniform and activity BMSCs by the method.

ICG is clinically used as a test substance to evaluate liver function because it is eliminated exclusively by hepatocytes. Yamada T et al[4] focused on molecular analyses that liver-specific protein genes such as AAT , CPSase I (the first enzyme of the urea cycle

pathway), and PEPCK (a key regulatory enzyme of hepatic gluconeogenesis) were highly expressed in ICG positive cells, whereas these genes were not expressed in ICG negative cells. These results indicate that ICG positive cell differentiation is specified toward hepatocytes and that these cells possess the genetic characteristics of almost terminally differentiated hepatocytes. Furthermore, [15]. But in this paper, we just observed whether NF-κB signals transfer into nuclear.

Only by altering the expression of genes or stimulating with cytokines can we induce the differentiation of BMSCs into hepatocytes. Levinson-Dushnik et al[16] examined embryonic stem (ES) cells expressed albumin which specific protein come from hepatocytes after transfected HNF3α or HNF3β genes into the ES cells. Liu X P et al[17] demonstrated albumin and AAT were found from primordial germ cells which were cultured in the presence of hepatocyte abstract, β-NGF, HGF and ingested ICG. In the present study, LTF as a positive control provided an appropriate microenvironment that supports differentiation of BMSCs into hepatocytes. However LTF is a multiple factor inducer, for this reason, HGF (an essential factor in the development and regeneration of the liver)[18] and β-NGF were used for inducing in order to explore distinctly.

NF-κB as one of the most important factor for cell proliferation and differentiation. Previous studies had indicated inhibiting the activation of NF-κB could promote the differentiation of BMSCs into neurons[19, 20]. Interestingly, studies of osteoblast emphasized the activation of NF-κB enhanced the expression of BMP2 genes, which promote the osteogenic differentiation[21, 22]. There may have something different between hepatocyte and neurons.

In fact, a number of questions require answered. For instance, NF-κB expression of LTF group in nuclear was higher than the other BAY group (Figures 2 G), even blocked by BAY 11-7082. On one hand, It is unknown whether BAY 11-7082 was ineffective due to the LTF which consist of abundant cytokines to stimulate the NF-κB signals transferring into nuclear. On the other hand, ICG positive differentiated cells has already reached (56.61 ± 11.72)% in LTF induced group, so the paracrine effect of the pre-differentiated BMSCs probably plays an important role in this process. To data, we can obviously observed that ICG positive cells and AAT expression were reduced in BAY group, which compared with induced group. However, it was still higher than control (Figures 2 H). This implied that some other factors except NF-κB signaling regulate cell differentiation.

5. Conclusions

In this paper, we showed the cells of BAY group reduced ICG uptake and AAT expression, NF-κB scattered in the cytoplasm. While the induce groups showed ICG uptake and AAT expression increased, NF-κB signals transferring into nuclear. Therefore BAY 11-7082 could prohibit the NF-κB signals from transferring into nuclear and decrease the preference that differentiation of BMSCs into hepatocyte-like cells at same time. To sum up, these data suggest that a activation of BAY 11-7082 might inhibit the differentiation of BMSCs into hepatocyte-like cells via NF-κB signaling.

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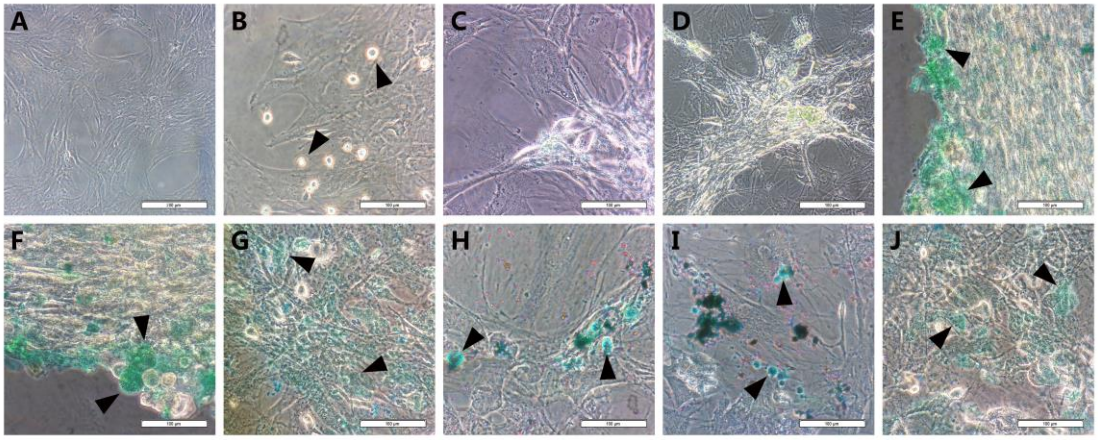
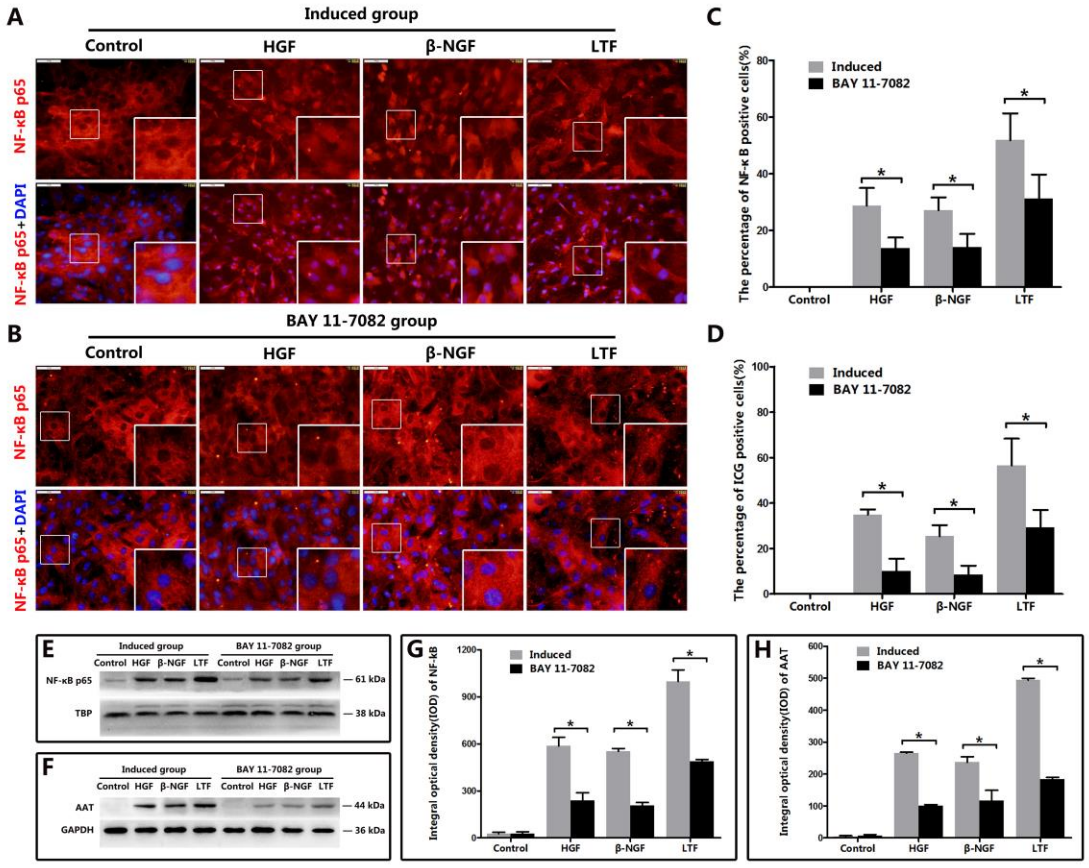
Figure legends



Figures 1 The morphologist characteristic of BMSCs and ICG uptake of differentiated BMSCs. The morphologist of the BMSCs at third passage (A) and stimulated for 20 days (B) observed under phase-contrast microscope. ICG uptake of differentiated BMSCs in each group induced for 20 days (C-J). Scale bars A = 200 μm; Scale bars B-J = 100 μm.



Figures 2 After induced for 20 days, The NF-κB position of induce group and BAY group observed under fluorescence microscope (A)- (B). And the percentage of NF-κB positive cells are shown in the column chart (C). ICG uptake levels of each group are shown in the column chart (D). The expression of NF-κB in nuclear (E) and AAT in cells (F) from different group were extracted by western blot respectively. The exact NF-κB and AAT expression levels are shown in the column chart (G)-(H). *P < 0.05. Scale bars A and B = 50 μm.