

ORIGINAL ARTICLE

Genetically engineered bone marrow-derived mesenchymal stem cells co-expressing IFN- γ and IL-10 inhibit hepatocellular carcinoma by modulating MAPK pathway

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Summary

Purpose: One of the major challenges in delivering cytokines for the treatment of hepatocellular carcinoma (HCC) is the mode of delivery. This study hypothesized that genetically engineered bone marrow derived mesenchymal stem cells (BMSCs) co-expressing IFN- γ and IL-10 can serve as a potential therapeutic strategy in the treatment of HCC by inhibiting cell proliferation.

Methods: Male Sprague-Dawley rats ($n=5$, 200-250 g) for BMSCs isolation and Nude/SCID mice ($n=35$, 12-20g) to develop liver cancer xenograft model were used. Mice were subcutaneously injected HepG2 cell suspension on left flank. BMSCs were genetically engineered with the recombinant lentiviral vectors expressing IFN- γ and IL-10. The experiments were performed in 5 groups (phosphate buffered saline/PBS, BMSCs, BMSC-IFN- γ , BMSC-IL-10 and BMSC-IFN- γ -IL-10) and the genetically engineered BMSCs were transplanted into HCC mice. Cell viability was measured by MTT assay followed by the evaluation of the effect of cell-cycle regulators (p21, p27, cyclin D1 and Rb). Protein expression of p38, ERK and JNK was as-

essed by immunohistochemistry using the cell proliferation marker Ki67.

Results: The combination of two cytokines (IFN- γ and IL-10) engineered into BMSCs resulted in a significant reduction in HepG2 cell viability ($*p<0.05$ vs PBS-treated and $^{\#}p<0.05$ vs BMSC-treated group). Significantly increased expression of cell cycle inhibitors p21 and p27 in parallel with reduced cyclin D1 expression were observed. Reduced phosphorylation of Rb demonstrated the repression of G1/S progression. BMSC-IFN- γ -IL-10 treatment significantly reduced the tumor growth at the end of 36 days compared to the group treated with PBS or BMSCs alone. This effect was accompanied with the modulation of MAPK pathway with the activation of p38 and JNK, and inactivation of ERK.

Conclusion: The co-expression of IFN- γ and IL-10 in BMSCs inhibits HCC in vitro and in vivo by modulating cell cycle regulators and MAPK pathway.

Key words: BMSC, carcinoma, cytokines, hepatocellular, MAPK

Introduction

HCC is one of the major causes of cancer-associated death around the world because of the lack of effective treatment strategies and late diagnosis [1]. The pleiotropic nature of cytokines and their complex interactions enabled their use in the treatment of cancer by delivering cytokines either exogenously or in combination with other

therapeutic agents. The intricate nature of tumor microenvironment and its vasculature led to a series of clinical trial failures in implementing cytokine therapy [2]. Nevertheless, this enhanced the knowledge on the cascade of mechanisms involved in tumor growth and abnormal cell proliferation.

Interferons and interleukins are the two major cytokines that trigger the immune system to act against tumor progression [3,4]. Interferons provide host defense against infections and also take part in tumor surveillance. There are two major types of interferons namely type I (IFN- α and IFN- β) and type II (IFN- γ). IFN- γ receptor consists of two subunits IFN- γ R1 and IFN- γ R2, where the activated IFN- γ binds to extracellular domain of IFN- γ R1 and subsequently activates IFN- γ R2 for the intracellular transmission of the signal [5,6]. IFN- γ is known to be pro-apoptotic, inhibits cell proliferation and prevents tumor angiogenesis, which showed IFN- γ to be a potent antitumor agent [7]. IFN- γ was tested clinically to treat chronic myelogenous leukemia, but apparently failed to show a significant effect [8,9]. However, the activity of IFN- γ in promoting or eliminating tumors depends on the tumor microenvironment, specificity of tumors and the cascade of signaling events activated in response to therapy [10]. The synergistic activity of interferons and interleukins has been reported to be efficient in inhibiting tumor progression [11-13].

The co-expression of interferon- γ (IFN- γ) and interleukin (IL-10) in the HCC treatment has not been studied so far. The antitumorigenic role of IFN- γ and the mediation of humoral response by IL-10 is hypothesised to balance the immune response, thereby inhibiting the cellular proliferation. In this study, we showed the effect of synergism of IFN- γ and IL-10 in the treatment of HCC both *in vitro* and *in vivo* via modulating the expression of cell-cycle regulators, which in turn activate the MAPK pathway. BMSCs served as the safe and reliable vehicle for the delivery of cytokines into the tumor microenvironment.

Methods

Animals

Male Sprague-Dawley rats (n=5, 200-250 g) for BMSC isolation and Nude/SCID mice (n=35, 12-20 g) to develop liver cancer xenograft model were obtained from SLAC company, Shanghai, China. The experiments involving animals were performed according to NIH guidelines and were approved by the local ethics committee.

Hepatocellular carcinoma cell line culture and BMSC isolation

HepG2 (ATCC[®] HB-8065[™]) hepatocellular carcinoma cell line was obtained from American Type Culture Collection (ATCC[®]), Manassas, VA, USA. The cells were cultured in Dulbecco's modified Eagle's medium

(Gibco[™] DMEM, ThermoFisher Scientific, Chaoyang District, Beijing, China) with 10% fetal bovine serum (ThermoFisher Scientific, Chaoyang District, Beijing, China), L-glutamine (2mM) and 1% penicillin and streptomycin in 5% CO₂ at 37°C.

Primary BMSCs were isolated from the femur and tibia of male Sprague-Dawley rats. Five ml of low glucose DMEM was flushed through the shafts of sacrificed rats to collect cell suspension. The cells were centrifuged using single density gradient centrifugation at 2000 rpm/min to separate the mononuclear cells. The isolated cells were cultured in low glucose DMEM, with 20% fetal bovine serum (ThermoFisher Scientific, Chaoyang District, Beijing, China), L-glutamine (2mM) and 1% penicillin and streptomycin in 5% CO₂ at 37°C. The isolated BMSCs were passaged thrice and the medium was changed every two days.

Plasmids and transfection of recombinant lentiviral construction

cDNA fragments of IFN- γ and IL-10 were synthesised using polymerase chain reaction (PCR) and cloned into the lentiviral vector pLenti6/V5-DEST (ThermoFisher Scientific, China) to generate recombinant pLenti6/V5-DEST-IFN- γ , pLenti6/V5-DEST-IL-10, and pLenti6/V5-DEST-IFN- γ -IL-10. To ensure the delivery of lentiviral vectors, ViraPower[™] Lentiviral Expression System (ThermoFisher Scientific) was used for lentiviral packaging according to the protocol described by the manufacturer. The recombinant lentiviruses were named LV-IFN- γ , LV-IL-10 and LV-IFN- γ -IL-10 respectively. BMSCs were seeded on 24-well plates for 24 hrs. Further, the supernatant of these recombinant lentiviruses were incubated for 24 hrs in plates containing BMSCs. The transfection success was determined by selecting stable clones with 400 μ g/mL of G418 (ThermoFisher Scientific) for two weeks. BMSCs were genetically engineered with the recombinant lentiviral vectors and were further expanded for two weeks and maintained as described above. Conditioned media (CM) were obtained by culturing BMSCs and genetically engineered BMSCs in normal growth media for 24 hrs. These CMs were later incorporated in HepG2 cell culture to perform ELISA and MTT assay.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of IFN- γ and IL-10 in the transfected BMSC culture was determined using commercial ELISA kits (Enzyme Immunoassay kits; Enzo life sciences, Chang Shou, Shanghai, China). The experiment was performed according to the manufacturer's protocol.

MTT assay to evaluate cell viability

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which required HepG2 cell seeding in a 96-well plate for 24 hrs. CMs were applied to the cultured cells and grown for 24 hrs. Following this, the medium was replaced and 20 μ l of MTT (0.5 mg/ml in PBS) were

added and was then incubated for 4 hrs at 37°C. The culture medium was changed and 150 μ l of DMSO was added to each well. This melted formazan crystals and absorbance was measured at 450 nm after 15 min using spectrophotometer.

Liver cancer animal model and BMSC transplantation

Nude/SCID mice were divided into 5 groups (n=35, n=7 in each group) and were subcutaneously injected on left flank with HepG2 cell suspension in PBS (1x10⁷/100 ml PBS). Three days following HepG2 inoculation, each mice group was injected with PBS, BMSCs, BMSCs- IFN- γ , BMSCs-IL-10 and BMSCs- IFN- γ -IL-10 respectively into the tail vein. Tumor growth was monitored every three days and the tumor volume was evaluated using the formula: length x width² x 0.52. The animals were sacrificed following 36 days and the tumor from each group was excised for further analysis.

Quantitative real-time PCR (qRT-PCR)

Total RNA from the excised tissue was isolated using Trizol reagent and the RNA purity was checked using Nanodrop1000 system (ThermoFisher Scientific). SuperScript VILO cDNA Synthesis Kit was used to synthesize cDNA and qPCR was performed on CFX96 Touch™ Real-Time PCR Detection System (Biorad) using SYBR® green qPCR supermix (ThermoFisher Scientific). Specific primers (Quantitect primer assay, Qiagen) to evaluate mRNA expression was obtained and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control to normalise mean Ct values using 2^{- $\Delta\Delta$ Ct} method.

The primers used were as follows:

IFN- γ : Forward: 5'-TGAACGCTACACACTGCATCTTGG-3'
Reverse: 5'-CGACTCCTTTTCCGCTTCTGAG-3'
IL-10: Forward: 5'-GCCTAACATGCTTCG AGA TC-3'
Reverse: 5'-TGATGTCTGGGTCTTGGTTC-3'
p21: Forward: 5'-GACAAGAGGCCAGTACTTC-3'
Reverse: 5'-GCTTGGAGTGATAGAAATCTGTC-3'
p27: Forward: 5'-GGGCAGATACGAATGGCAGGA-3'
Reverse: 5'-CAGAGTTTGCCTGAGACCCAATTA-3'
CyclinD1: Forward: 5'-GCTGCGAAGTGGAACCATC-3'
Reverse: 5'-CCTCCTTCTGCACACATTTGAA-3'

Western blot analysis

Protein samples (20 μ g) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. The blot was blocked with non-fat dry milk in PBS and incubated overnight with primary antibodies at 4°C. The blots were washed thrice with PBS/0.1% tween and then incubated for 2 hrs at room temperature with goat anti-rabbit secondary antibody labelled with horseradish peroxidase (Santa Cruz, Beijing, China). The proteins were detected using a chemiluminescence system by Odyssey system (Li-cor, USA) and densitometric analysis was performed. The primary antibodies used were polyclonal rabbit anti-mouse p21, p27, cyclin D1, Rb, p38, JNK, ERK1/2 (1:100) and β -actin (1:500).

Immunohistochemistry

Tumor tissue samples were embedded in paraffin and sections (5 μ m) were cut. The slices were analyzed 50 μ m apart. Polyclonal rabbit anti-mouse Ki67 (1:100, Abcam) was incubated with the samples at 4°C overnight. It was then incubated with secondary antibody (goat anti-rabbit antibody labelled with biotin; 1:200, Abcam) at 37°C for 15 min. The sections were incubated with horseradish peroxidase-labeled streptavidin at 37°C for 15 min and counterstained with hematoxylin. The staining was visualized at 200x magnification and 10 different fields were analysed under a light Olympus microscope. Quantification was done using Image-Pro plus v 6.0 software.

Statistics

The statistical analyses were carried out using the SPSS version 13.0 software package. One-way ANOVA followed by Bonferroni test were used to evaluate statistical significance. All values were presented as mean+SEM and considered significant at *p<0.05 vs PBS-treated group and #p<0.05 vs normal BMSC-treated group.

Results

Genetically engineered BMSCs produce high levels of IFN- γ and IL-10 in HepG2

To confirm the effective transduction of IFN- γ and IL-10 into BMSCs, ELISA was performed to determine their concentration in cultured HepG2 hepatocellular carcinoma cell line after one week (Figure 1A). Further, qPCR analyses showed an increased expression of IFN- γ and IL-10 in cells treated with genetically engineered BMSCs when compared with the normal BMSCs (*p<0.05 vs PBS-treated group of cells, #p<0.05 vs normal BMSCs-treated group of cells). The results are shown in Figure 1B,C.

HepG2 cell viability following the transplantation of genetically engineered BMSCs

MTT assay demonstrated the HepG2 cell viability following the transplantation of genetically engineered BMSCs. IFN- γ and IL-10 synergistically induced HepG2 cell death. The combination of these two cytokines resulted in a significant reduction in HepG2 cell viability, which emphasised their efficiency to decrease tumor cell survival (Figure 2).

Effect of recombinant BMSC transplantation on cell cycle regulators

Tumor growth is largely dependent on the expression of cell cycle regulators. To determine the effect of BMSC transplantation on the cell cycle, we

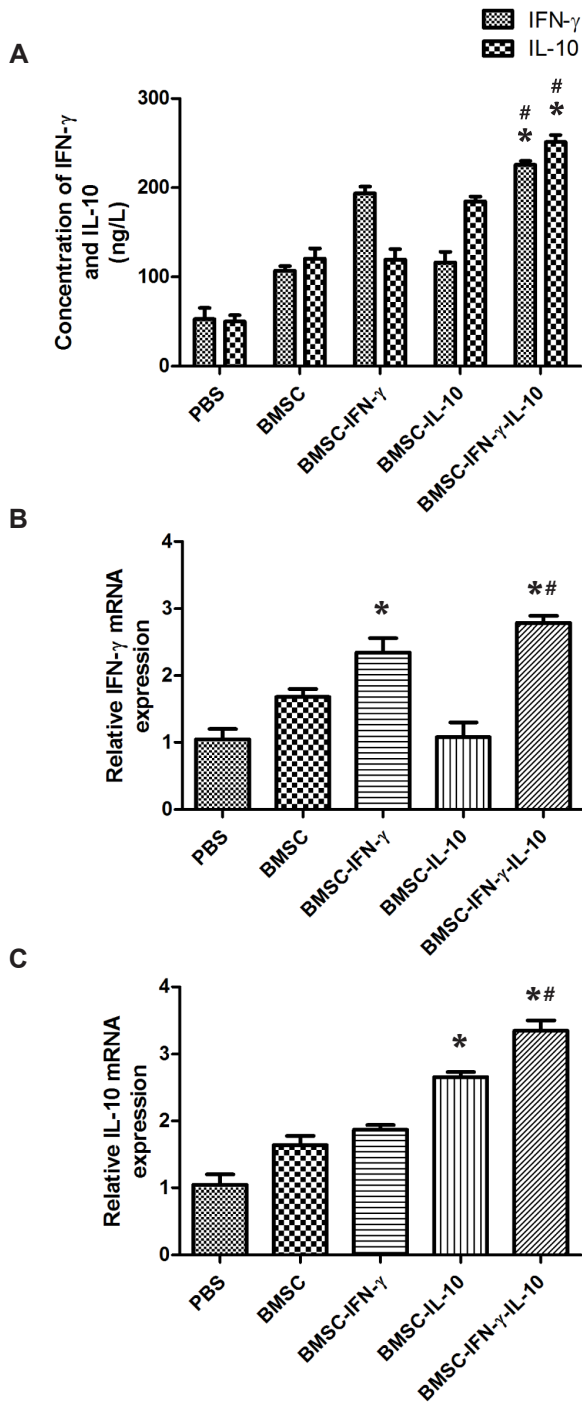


Figure 1. Production of IFN- γ and IL-10 in HepG2 cells treated with recombinant BMSCs engineered with the cytokines IFN- γ and IL-10. **(A)** The concentration of IFN- γ and IL-10 produced in the cells was evaluated using ELISA. BMSC-IFN- γ -IL-10 treated cells produced approximately equal amount of both the cytokines and the concentration was increased significantly compared to the cells treated with PBS (* p <0.05) and cells treated with normal BMSCs (# p <0.05). **(B)** Relative mRNA expression of IFN- γ was found to be significantly higher in BMSC-IFN- γ and BMSC-IFN- γ -IL-10 treated cells (* p <0.05 vs PBS and # p <0.05 vs normal BMSC treated cells). **(C)** Relative mRNA expression of IL-10 was significantly increased in cells treated with BMSCs engineered with IL-10 and both IFN- γ and IL-10. Values were expressed as mean+SEM and considered significant at * p <0.05 vs PBS treatment and # p <0.05 vs normal BMSC treatment. The experiments were performed in triplicate.

tested the expression of cell cycle regulators p21, p27 and cyclin D1. The mRNA and protein expression of these cell-cycle regulators was evaluated (Figure 3 A-D) and the results showed that there was an increased expression of cell cycle inhibitors p21 and p27 in parallel with the reduced cyclin D1 expression. Also these changes were accompanied with reduced phosphorylation of Rb which represses the G1/S progression of the cell cycle.

Effect of recombinant BMSC transplantation on the tumor growth in vivo

Transplantation of BMSCs engineered with cytokines IFN- γ and IL-10 in nude/SCID mice had significant impact on the tumor growth. HepG2 cells were inoculated into the mice to generate a liver cancer model followed by the transplantation of BMSCs. Out of the five experimental groups, the group transplanted with BMSC-IFN- γ -IL-10 significantly reduced the tumor growth when compared to the group treated with PBS and BMSCs alone. The synergistic activity of these cytokines resulted in considerable reduction of tumor growth when compared to the activity of BMSCs with one cytokine. Tumor volume was evaluated using the formula length \times width² \times 0.52 (Figure 4).

Activation of MAPK pathway checks cell proliferation

MAPK signaling plays a major role in the development and progression of cancer. The important downstream targets of MAPK signaling

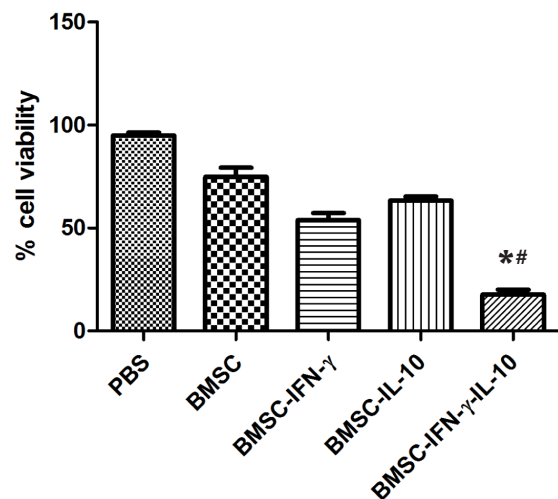


Figure 2. MTT assay to evaluate percentage of HepG2 cell viability. The combination of IFN- γ and IL-10 recombinant BMSCs in HepG2 hepatocellular carcinoma cell line resulted in an approximately two-fold decrease in cell viability compared to the cells treated with PBS. The single cytokine treatment did not produce significant reduction in cell viability (* p <0.05 vs PBS treated HepG2 cells, # p <0.05 vs BMSC treated cells). The values are mean+SEM and experiments were performed in triplicate.

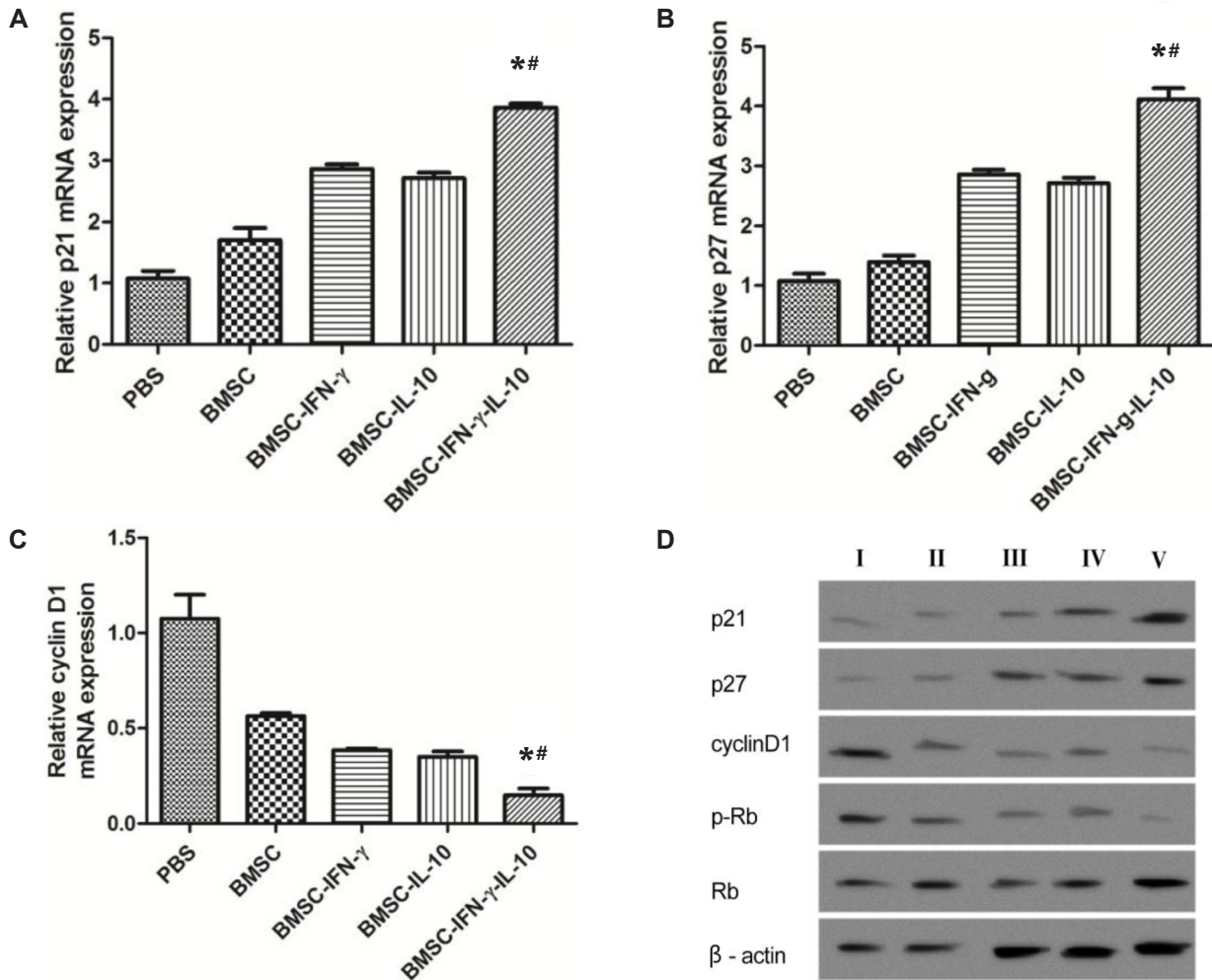


Figure 3. Varying expression of cell cycle regulators following recombinant BMSC transplantation. **(A), (B), (C)** Relative mRNA expression of p21, p27 and cyclin D1. p21 and p27 mRNA expression significantly increased, whereas cyclin D1 expression significantly reduced in the BMSC-IFN- γ -IL-10 treated excised tissue sample compared to the PBS treated sample. The synergistic action of the cytokines evidently reduced the cell cycle progression, further validated by the Western blot analyses. **(D)** Western blot analyses of p21, p27, cyclin D1, Rb and p-Rb. **I** represents PBS, **II** BMSCs alone, **III** BMSCs- IFN- γ , **IV** BMSCs-IL-10 and **V** BMSCs- IFN- γ -IL-10 treated samples. Values were expressed as mean \pm SEM and considered significant at * p <0.05 vs PBS treatment and # p <0.05 vs normal BMSC treatment. The experiments were performed in triplicate.

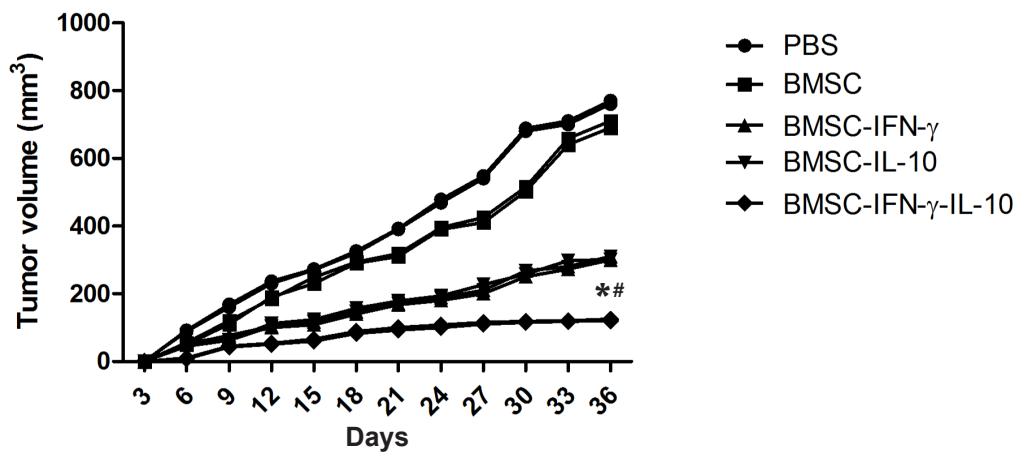


Figure 4. Graphical representation of tumor volume evaluation following BSMC transplantation. Tumor volume was evaluated for 36 days in the mice injected with HepG2 followed by the treatment with PBS, BMSCs, recombinant BMSCs with IFN- γ , IL-10 and both IFN- γ and IL-10. The results showed that BMSC-IFN- γ -IL-10 treatment significantly reduced the tumor growth at the end of 36 days compared to the group treated with PBS or BMSCs alone. Values were expressed as mean \pm SEM and considered significant a * p <0.05 vs PBS treatment and # p <0.05 vs normal BMSC treatment. The experiments were performed in triplicate.

pathway (p38, JNK, ERK) were evaluated for their protein expression following the transplantation of BMSCs. The expression of their phosphorylated proteins was also evaluated. A greater expression of phosphorylated/activated proteins, p38 and JNK, was observed in the BMSC-IFN- γ -IL-10 treated group. However, a low expression of phosphorylated ERK was observed. Immunohistochemical staining further substantiated our results that the synergistic activity of IFN- γ and IL-10 significantly decreased Ki67 expression and stopped the cell cycle progression decreasing thus the tumor growth (Figure 5).

Discussion

This study demonstrated the critical role of systemic administration of recombinant BMSCs engineered with cytokines (IFN- γ and IL-10) in the treatment of HCC. One of the major findings of this study was the synergistic action of IFN- γ and IL-10 engineered into BMSCs in inhibiting liver cancer progression *in vitro* and *in vivo*. Transplantation of BMSCs for the treatment of cancer has been largely investigated in the recent past. Autologous transplantation of bone marrow stromal cells was shown to treat secondary arm lymphedema in women with breast cancer [14]. Another study showed that autologous transplantation of human BMSCs in breast cancer patients resulted in haematopoietic recovery and the method was characterized as feasible and safe [15]. With the failure of radiation therapies in targeting specific tumor sites and the accumulating side effects, ad-

ministration of BMSC was found to improve the hepatic function [16]. BMSCs were also tested for their ability to act as excellent vehicles to induce therapeutic agents as they could readily migrate into tumors. Malignant glioma was treated *in vivo* by the transfer of Cx43 combined with HSV-TK/GCV gene therapy through BMSCs as delivery vehicles [17].

Cytokine therapy inhibits tumor development and progression, where the interferons and interleukins are the two major cytokines that stimulate the immune system to prevent the progression of tumor [2-4]. Interferons act as pro-apoptotic agents and combined with chemotherapeutic agents were reported to be efficient in preventing tumor recurrence and improving survival [3]. 5-fluorouracil/interferon combination improved survival by reducing the progression in patients with advanced HCC [18]. Treatment with IFN- α leads to apoptosis of multiple myeloma cell lines by interfering with IL-6 signaling and reducing STAT3 activity [19]. Another study reported that IFN- α targeted the death ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) *via* promyelocytic leukemia (PML) gene to treat HCC [20].

Different interleukins and their combinations with other cytokines have been approved for the treatment of cancer [4]. MSCs secreting IL-18 inhibited glioma growth and prolonged antitumor immunogenic effects provoking high T-cell infiltration [21]. BMSCs co-expressing IL-18 and IFN- β had a significant impact in inhibiting the glioma cell growth compared to the BMSCs engineered with IL-18 or IFN- β alone [22]. The synergistic

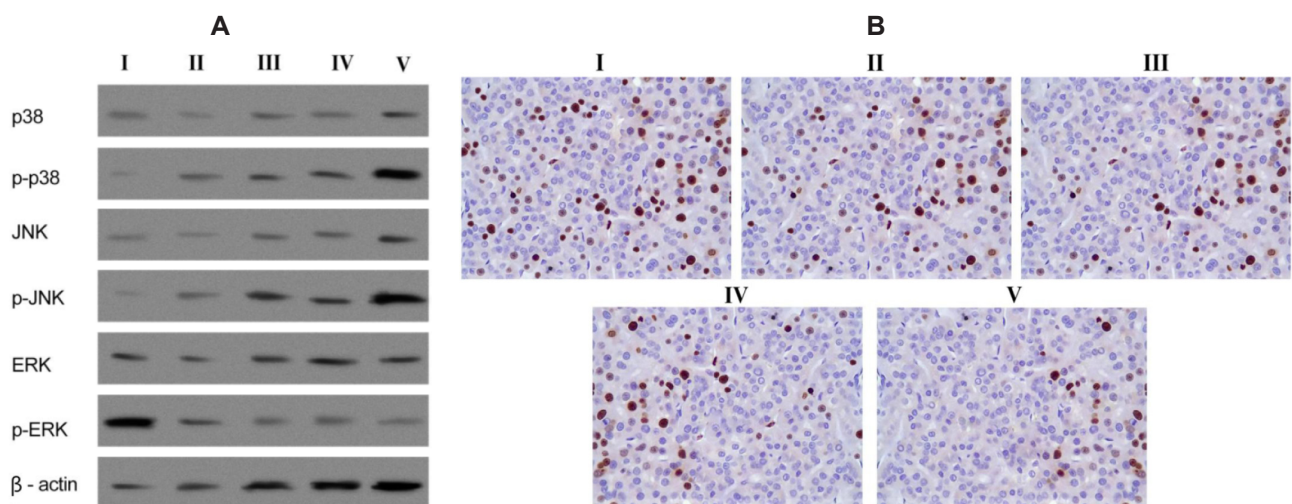


Figure 5. MAPK protein expression and cell proliferation following the transplantation. **(A)** Western blot analyses of proteins p38, JNK and ERK performed from the excised tissue sample. p38 and JNK proteins were activated, whereas ERK protein was inactivated. The expression of phosphorylated MAPK proteins was also detected. **(B)** Immunohistochemical staining using Ki67, a marker for cell proliferation. Ki67 expression was significantly reduced in the sample treated with BMSC-IFN- γ -IL-10 compared to PBS and BMSCs treated alone. The synergistic action of these cytokines decreased cell proliferation compared to the BMSCs with IFN- γ or IL-10 alone. **I** represents PBS, **II** BMSCs alone, **III** BMSCs-IFN- γ , **IV** BMSCs-IL-10 and **V** BMSCs-IFN- γ -IL-10 treated samples.

activity of cytokines (IFN- γ and TNF- α) was also proven to be efficient in inducing apoptosis in a cervical cancer cell line. This effect was attributed to the IRF1/STAT1 activation, which inhibited the cytoprotection by NF- κ B and was independent of caspase activation [23]. However, there has been no study linking IFN- γ and IL-10 in the treatment of HCC. IFN- γ is considered to exhibit antitumor immunological responses *via* Th1 T lymphocyte differentiation. On the other hand, IL-10 causes Th2 T lymphocyte differentiation which results in the activation of antibody-mediated immune response [24]. Hence, it was hypothesised that the combination of these cytokines (IFN- γ and IL-10) might be beneficial in the treatment of HCC.

One of the major challenges in introducing cytokines is that they have a shorter half-life and they might not be efficient as a therapeutic agents unless they are delivered *via* a system that enables their prolonged expression. In this study, we isolated rat BMSCs to serve as a vehicle for the delivery of these cytokines into the tumor microenvironment. BMSCs were genetically engineered to express IFN- γ , IL-10 and IFN- γ -IL-10. The co-expression of IFN- γ -IL-10 using BMSCs significantly reduced the HepG2 cell viability compared to PBS and BMSC treated group as shown by MTT assay and this served as the foundation for further experiments. The antitumor activity induced by recombinant BMSCs was further studied by evaluating the expression of cell cycle regulators. The expression of cell cycle inhibitors p21 and p27 significantly increased in the samples treated with BMSC-IFN- γ -IL-10, whereas cyclin D1 expression reduced in parallel. These changes were accompanied with reduced phosphorylation of Rb

which is known to repress G1/S progression of the cell cycle.

MAPK signaling is one of the prominent signaling pathways involved in the development and progression of cancer. MAPK pathway modulates the cell cycle progression, which in turn is dependent on the growth factors and stress cytokines in the tumor microenvironment [25]. p38 is activated under cellular stress and is activated by the pro-inflammatory cytokines, especially interferons. It has been previously reported that the IFN α or γ could activate p38 of MAPK pathway with the consequent phosphorylation of STAT1 [26]. The activation of JNK following the transplantation of BMSC-IFN- γ -IL-10 in the current study emphasises that the HCC inhibition could be *via* TNF- α induced apoptosis, and this observation requires further investigation. Previous studies have reported the role of ERK in promoting cell proliferation and cancer metastasis [27], which was further supported in our study by the significantly lower expression of p-ERK following BMSC-IFN- γ -IL-10 treatment. Hence, the co-expression of IFN- γ and IL-10 in BMSCs could prove to be a potential therapeutic strategy in the treatment of HCC by inhibiting cell proliferation and modulating MAPK pathway.

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Conflict of interests

The authors declare no conflict of interests.

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