The key role of PML in IFN-α induced cellular senescence of human mesenchymal stromal cells

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Abstract. Recent developments and re-emergence of interferon α (IFN-α) have renewed interest in the therapy for patients with chronic myeloid leukemia (CML). Related molecular mechanism may be the direct effect of IFN-α on CML stem cells. Human mesenchymal stromal cells (hMSCs) are important to protect CML stem cells, and IFN-α was described as a potential inhibitor of hMSCs. However, the exact mechanism remains obscure. PML as a known tumor suppressor locates downstream of the IFN-α pathway, and little is known about the PML gene regulation in hMSCs. The aim of this study was to investigate the effects of IFN-α on hMSCs and defined the role of PML involved in this process. Our results suggested that hMSCs incurred senescence upon IFN-α stimulation, while PML levels were observed significant increased. The recombinant lentiviral vector, which encodes shRNA against PML or full-length PML cDNA, was constructed. By knocking-down and overexpressing PML, we found that PML was indispensable to IFN-α mediated hMSC senescence. The molecular mechanism underlying this process may be an increased co-localization of PML and p53 induced by IFN-α. Our data demonstrated that IFN-α can induce cellular senescence of hMSCs and PML plays a key role in this process. These findings provided novel insight into the effect of IFN-α on hMSCs.

Introduction

As immunomodulatory cytokines, type 1 interferons (IFNs), have a long history of efficacy in treating hematological malignancies, especially in chronic myeloid leukemia (CML) (1). Before imatinib, IFN-α based regimens were the most important treatments for early CML patients. Recently, IFN-α was renewed to be a vital candidate for CML treatment. Many studies exist reporting that the combination of IFN-α and imatinib significantly increased the rates of molecular responses, comparing to single imatinib treatment (2-4). Molecular mechanisms underlying this phenomenon may be the inhibition of CML progenitors or the recruitment of quiescent CML stem cells into cell cycle by IFN-α (2,4). However, those mechanisms have not been clearly demonstrated. Bone marrow mesenchymal stromal cells, which may also be defined as mesenchymal stem cells, are important in regulating cell cycle arrest and survival of dormant CML cells (5,6). To ascertain the effect of IFN-α on hMSCs will provide insights into the mechanism of action of IFN-α therapy.

Patient tolerability is the main obstacles for long-term IFN-α treatment (2). As one of the most serious side-effects caused by IFN-α therapy, myelosuppression (including anemia, lymphopenia and thrombocytopenia) is IFN-α dose-dependent (7), and the growth inhibition on bone marrow cells induced by IFN-α is suggested to be one of the most possible mechanisms (8,9). As a negative regulator of cell apoptosis and cell cycle (9-11), IFN-α was described as a potential inhibitor of hMSCs in several studies (12). However, the exact mechanism involved in IFN-α and hMSCs remains unclear.

Promyelocytic leukemia (PML) gene is known as a tumor suppressor, which locates downstream of IFN-α pathway (13,14). Multiple isoforms of PML have been identified, and all of them are characterized by the presence of RBCC, or the recently termed, TRIM motif. The RBCC domain mediates the subnuclear localization and protein-protein interaction of PML (15,16). Thus, PML protein localizes in discrete, speckled subnuclear structures named PML-NBs, where it co-localizes with many other proteins. Through the interaction with other proteins, PML regulates numerous fundamental processes, such as transcription, cell apoptosis, cell cycle regulation, senescence and others (15,17). To our knowledge, although PML has been extensively studied in tumor cells, little is known about PML gene regulation in hMSCs. In our previous research, we proved that PML stably expressed in hMSCs, which was important in maintaining the normal proliferation capacity of hMSCs (18). However, the biological function and regulatory mechanism of PML induced by IFN-α in hMSCs remains undefined. In this study, we investigated the effects of IFN-α on hMSCs and defined the role of PML involved in this process.

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Materials and methods

Cell culture. The present study was approved by the Ethics Committee of the First Affiliated Hospital, Medical School of Zhejiang University (2012-l01). Additionally, an informed consent was obtained from each healthy donor prior to obtaining bone marrow samples. hMSCs were isolated by density gradient centrifugation and cultured in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Corning, Danville, VA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, Australia), which was changed every 3 days. Passage 3-6 hMSCs were used in the following experiments. Every experiment was repeated using 3 different hMSCs strains isolated from 3 different randomly selected donors.

Colony formation assay. For colony formation assay *in vitro*, hMSCs were plated at a density of 1,000 cells per well into a 6-well plate. Cells were cultured in the presence of different concentrations of IFN-α (Peprotech, Rocky Hill, NJ, USA) or PBS as vehicle control. After 14 days, the cultures were fixed and stained with 0.1% crystal violet for 30 min, and the number of colonies (≥50 cells) was counted using a microscope (Nikon, Tokyo, Japan).

Senescence associated β-galactosidase (SA-β-gal) staining. SA-β-gal staining was performed by senescence β-galactosidase staining kit according to the manufacturer's instructions (Cell Signaling Technology, Inc. Danvers, MA, USA). Briefly, cells in different treatments were grown on 6-well culture plates, washed, and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, cells were washed with PBS twice and incubated with β-galactosidase staining solution for 16 h at 37˚C (pH 6.0). Image files were collected under a light microscope and the percentage of blue cells per 400 cells was calculated using Imagepro-Plus software (IPP, Media Cybernetics, Inc. Rockville, MD, USA).

Reverse transcription and real-time PCR analysis. PCR analysis was performed as described in our previous studies. Briefly, total cellular RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, MD, USA) and 1 μg RNA was reverse-transcribed to cDNA using PrimeScript™ 1st Strand cDNA Synthesis kit (Takara Bio, Dalian, China). cDNA was amplified in a final reaction volume of 20 μl using a Takara Taq™ kit or SYBR® Premix Ex Taq™ kit. The forward and reverse primers for amplification of PML and β-actin are as follows: PML forward, 5'-TGTACCGGCAGATTGTGGAT-3'; and reverse, 5'-AGATGTTGTTGGTCTTGCGG-3'. β-actin forward, 5'-AGCGAGCATCCCCCAAAGTT-3'; and reverse, 5'-GGGCACGAAGGCTCATCATT-3'.

Western blot analysis. hMSCs proteins were lysed with radioimmunoprecipitation assay (RIPA) buffer and proteinase inhibitors (both from Beyotime, Jiangsu, China). Equal amounts of cell proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% phosphatase-free powdered milk in Tris-buffered saline with 0.1% Tween (TBST) for 2 h. Then, the membranes were incubated overnight at 4˚C with primary antibodies consisting of PML, P21, P53 (all from Abcam, Hong Kong, China) and β-actin (Cell Signaling Technology, Inc.). The membrane was then rinsed with TBST and incubated with an IRDye secondary antibody (LI-COR Biosciences, Lincoln, NE, USA) for 1.5 h at room temperature. The membranes were visualized using Odyssey infrared imaging system (LI-COR Biosciences).

Immunofluorescence. Cells were cultured in 12-well-plates and treated at different conditions. After the treatment, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 10 min, and then blocked for 1 h in 5% BSA at room temperature. Primary antibodies diluted 1:500 were incubated overnight at 4˚C. After incubation with the secondary antibodies (1:500) for 1 h at room temperature, cells were counterstained with DAPI to visualize cell nuclei. The primary antibodies used include rabitt monoclonal anti-PML and mouse or rabbit monoclonal anti-P53 (Abcam). The secondary antibody was Alexa fluor-488 conjugated goat anti-mouse and Alexa fluor-555 conjugated goat anti-rabbit (Invitrogen, Life Technologies). Cells were examined under flurescence microscope and images were collected (Nikon).

Flow cytometry analysis of cell apoptosis. After the treatments, the adherent and supernatant cells were harvested. Cells were washed with phosphate-buffered saline (PBS) and re-suspended in 500 μl of binding buffer, then incubated with 5 μl of Annexin V-PE and 5 μl of 7-amino-actinomycin D (7-AAD) solution for 15 min in the dark at room temperature (both from BD Biosciences, San Jose, CA, USA). Cell apoptosis were then analyzed by FC500 Flow Cytometer (Beckman Coulter, Inc. Brea, CA, USA).

Gene transfection. Infections of hMSCs were performed by lentiviruses. As described previously, recombinant lentiviral vector plenti6.3-PML-EGFP which encodes the full length human PML-cDNA (NM_002675) and the enhanced green fluorescent protein (EGFP) was synthesized by Life Technologies (Shanghai, China). The empty vector, plenti6.3-EGFP, was used as control. Short hairpin RNAs (shRNA) against PML in a lentiviral vector with green fluorescent protein (GFP) and control vector were designed and synthesized by GenePharma Inc. (Shanghai, China). Packaging vectors used in our experiments was pLP1, pLP2, and pLP/VSVG (Invitrogen, Life Technologies). Human embryonic kidney 293T cells were used as packaging cells. High-titer lentivirus was produced in 293T and was used to infect hMSCs which had reached 50-60% confluence. After transfection of 72 h, the efficiency was estimated by evaluation of GFP expression via fluorescence microscopy, and PML gene expression was verified by quantitative real-time PCR and western blot analysis. The DNA sequence of the PML shRNA was 5'-TGGAGGAGGAG TTCCAGTTTCTTTCAAGAGAAGAAACTGGAACTCCT CCTCCTTTTTTC-3' and 5'-TCGAGAAAAAAGGAGGAG GAGTTCCAGTTTCTTCTCTTGAAAGAAACTGGAACT CCTCCTCCA-3'. The control shRNA was 5'-TGTTCTCCG AACGTGTCACGTTTCAAGAGAACATGACACGTTCGG AGAACTTTTTTC-3' and 5'-TCGAGAAAAAAGTTCTCC GAACGTGTCACGTTCTCTTGAAACGTGACACGTTCG GAGAACA-3'.

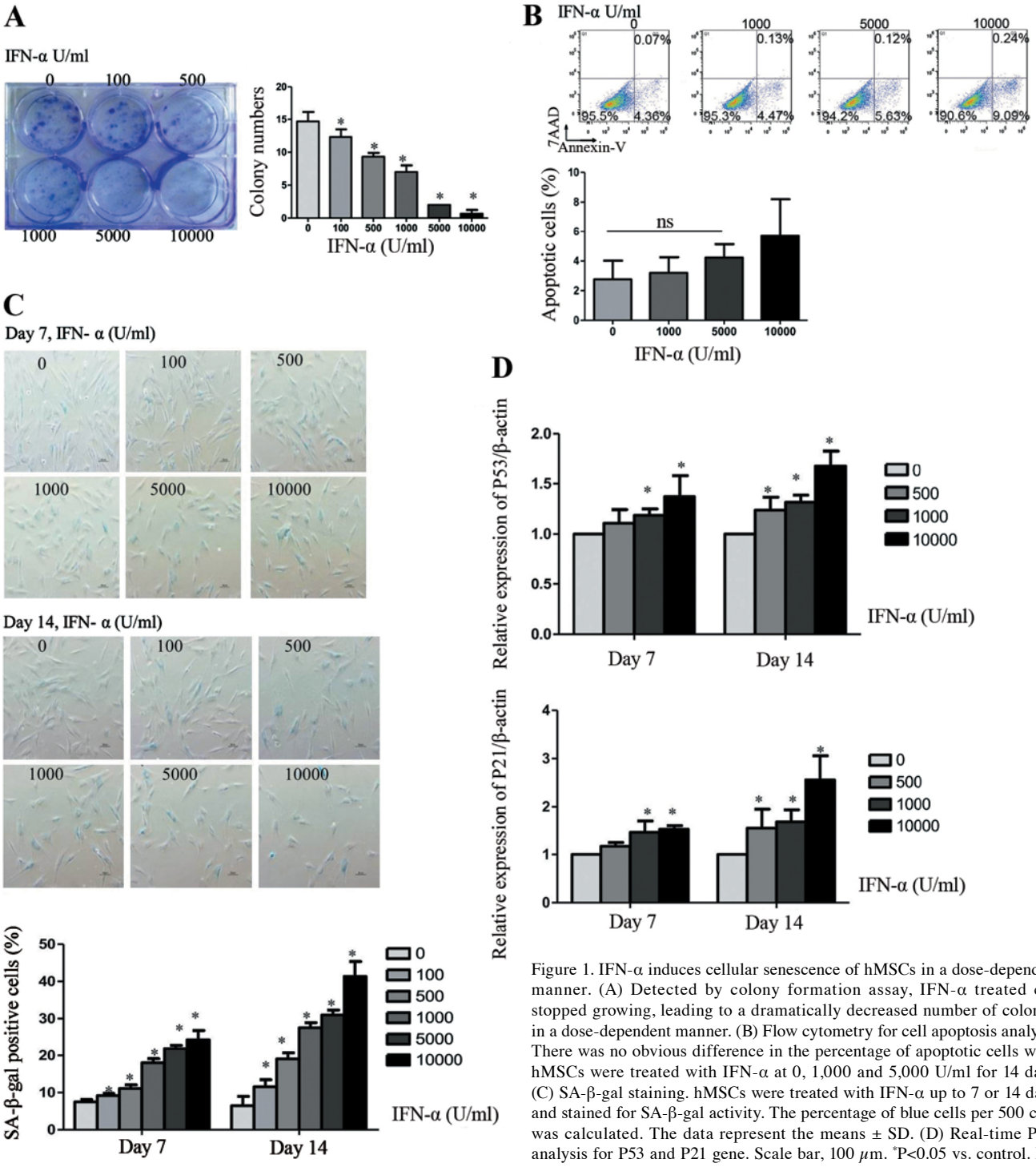

Figure 1. IFN-α induces cellular senescence of hMSCs in a dose-dependent manner. (A) Detected by colony formation assay, IFN-α treated cell stopped growing, leading to a dramatically decreased number of colonies in a dose-dependent manner. (B) Flow cytometry for cell apoptosis analysis. There was no obvious difference in the percentage of apoptotic cells when hMSCs were treated with IFN-α at 0, 1,000 and 5,000 U/ml for 14 days. (C) SA-β-gal staining. hMSCs were treated with IFN-α up to 7 or 14 days, and stained for SA-β-gal activity. The percentage of blue cells per 500 cells was calculated. The data represent the means ± SD. (D) Real-time PCR analysis for P53 and P21 gene. Scale bar, 100 μm. *P<0.05 vs. control. NS, non-significant with control.

Statistical analysis. All statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA) and all data were expressed as means ± standard deviation (SD). Differences between groups were evaluated using one-way analyses of variance or non-parametric tests. A P-value <0.05 was considered statistically significant.

Results

IFN-α induces cellular senescence of hMSCs in a dose-dependent manner. IFN-α is an important inhibitor of tumor cells, thus we investigated the biological function of IFN-α in hMSCs. Cells were treated with 0, 100, 500, 1,000, 5,000, or 10,000 U/ml IFN-α every 3 days up to 14 days. Detected by colony formation assay, IFN-α treated cell stopped growing, leading to a dramatically decreased number of colonies in a dose-dependent manner (Fig. 1A). The number of colonies was reduced to nearly half when IFN-α was 1,000 U/ml as compared with no IFN-α. However, IFN-α did not induce significant cell apoptosis. We did not find obvious difference in the percentage of apoptotic cells when hMSCs were treated with IFN-α at 0, 1,000 and 5,000 U/ml for 14 days (Fig. 1B).

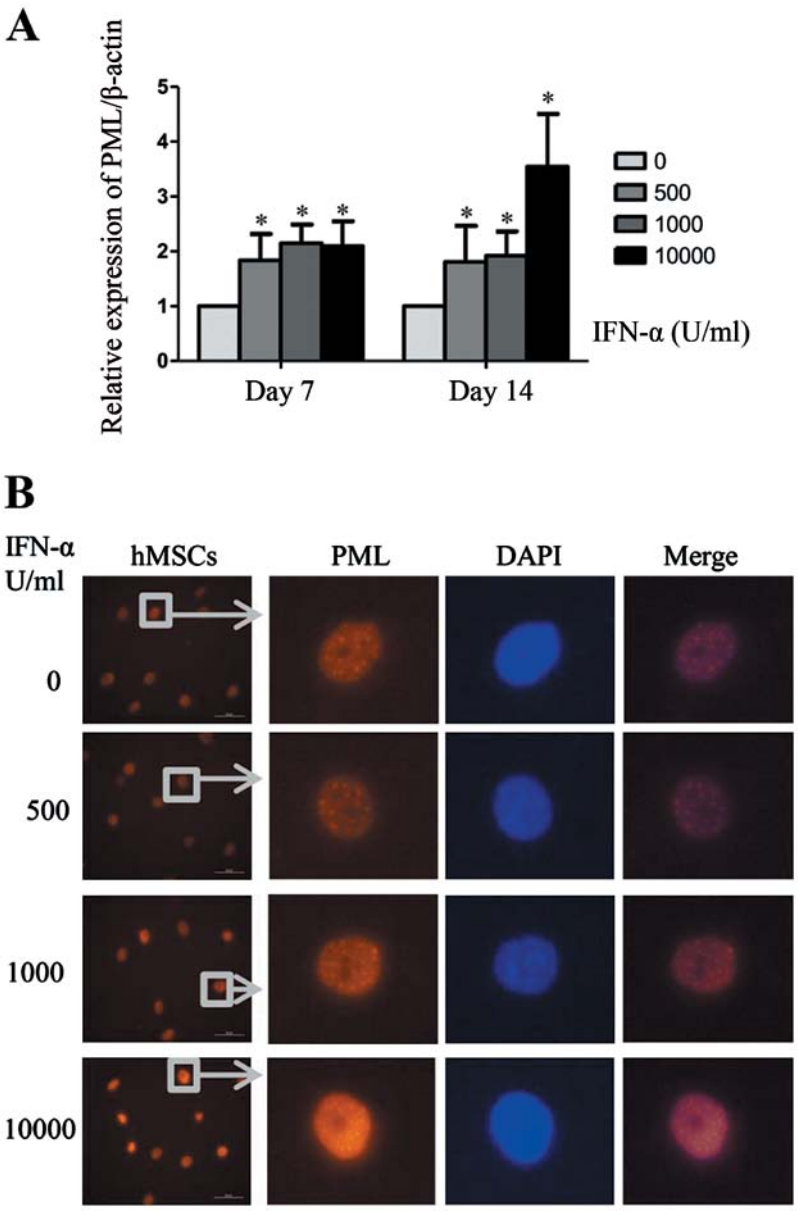

Figure 2. PML expression is upregulated in IFN-α induced senescence of hMSCs. (A) Real-time PCR analysis for PML gene. When cells were treated with IFN-α at 1,000 U/ml for 7 or 14 days, PML gene expression in hMSCs was increased >2-fold. (B) Immunofluorescence analysis of hMSCs. Treated with IFN-α for 7 days, both the number and size of PML-NBs were increased markedly in hMSCs in a concentration-dependent manner. Scale bar, 50 μm. *P<0.05 vs. control.

Apoptotic cells showed a minor increase when the concentration of IFN-α reached 10,000 U/ml. These results suggest that cell apoptosis was not the main reason for the inhibition of IFN-α. Then we examine whether the IFN-α induced inhibition of cell proliferation resulted from cellular senescence, a variety of senescence-associated detection was measured. The cellular senescence was confirmed by an increase in SA-β-gal-positive cells in IFN-α treated as compared to the untreated cells (Fig. 1C). The hMSC senescence induced by IFN-α was dose- and time-dependent. After treated with IFN-α at 1,000 U/ml for 7 or 14 days, we found that up to 18±1.1 or 27.56±1.33% of hMSCs became SA-β-gal-positive as compared with 7.53±0.55 or 6.47±2.5% of untreated cells. Real-time PCR analysis proved this process by an increase in production of the senescence marker p53 and p21 (Fig. 1D).

PML expression is upregulated in IFN-α induced senescence of hMSCs. PML is one of the most interesting upregulated genes induced by IFN-α, and PML gene has been proved to be associated with cellular senescence in other cell types. The present study was undertaken to evaluate the role of PML in IFN-α induced senescence of hMSCs. Consistent with other studies, mRNA expression of PML can be upregulated by IFN-α in hMSCs (Fig. 2A). When cells were treated with IFN-α at 1,000 U/ml for 7 or 14 days, PML gene expression in hMSCs was increased by >2-fold. Then we analyzed PML protein level by western blot analysis. However, we did not observed significant changes of PML protein during the process of IFN-α treatment. The possible reason may be that western blot analysis was not sufficiently sensitive to detect PML proteins which were mainly distributed in the

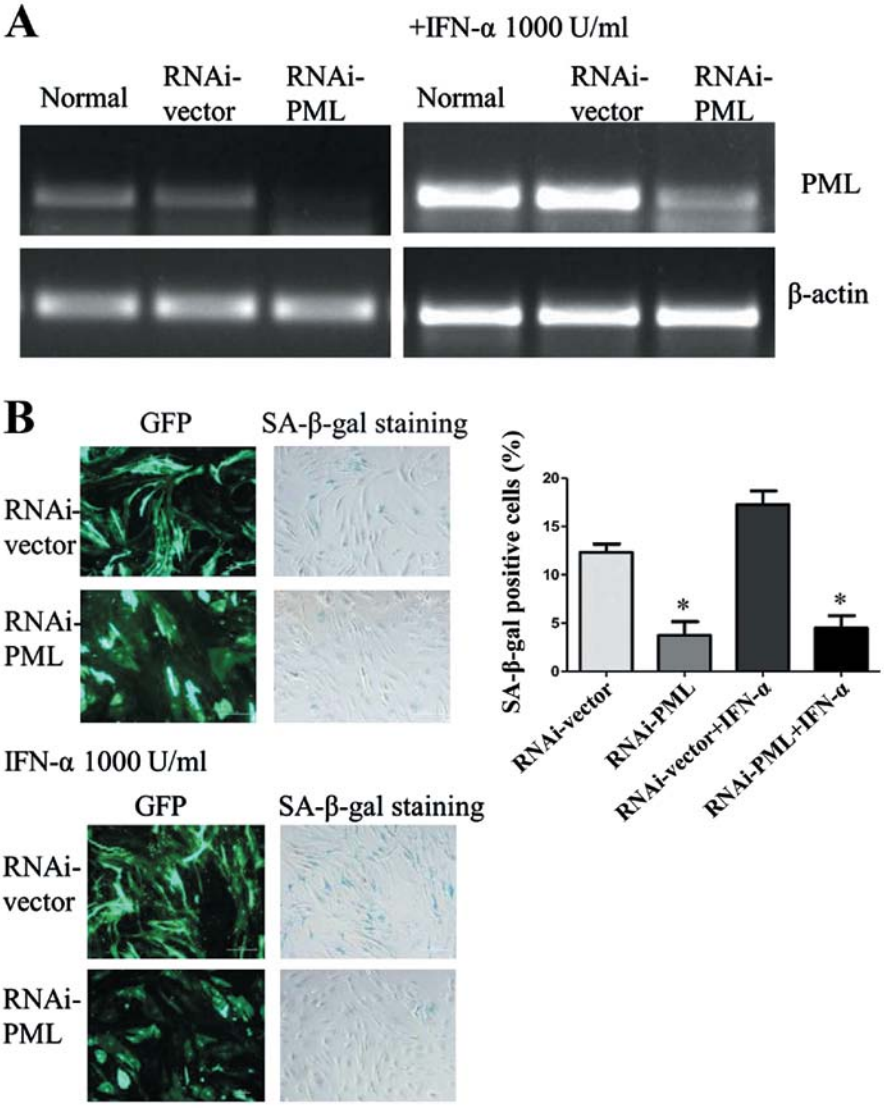

Figure 3. PML plays a key role in IFN-α induced cellular senescence of hMSCs. (A) Knocking down of PML gene. PML gene was significantly downregulated in hMSCs, as compared with normal and cells transfected with empty plasmid. When hMSCs were treated with IFN-α at 1,000 U/ml for 7 days, PML gene has no obvious upregulation in PML knockdown cells. (B) SA-β-gal staining. Under normal culture, there was a lower percentage of SA-β-gal-positive cells in PML knockdown hMSCs than the control RNAi. After treatment with IFN-α for 7 days, hMSCs senescence can be rescued by the knockdown of PML. Quantitation of data showed the percentage of SA-β-gal-positive cells. The data represent the mean ± SD. Scale bar, 100 μm. *P<0.05 vs. control.

cell nucleus. Immunofluorescence can reflect the changes of nuclear protein expression more directly. As shown in Fig. 2B, when hMSCs were treated with IFN-α for 7 days, both the number and size of PML-NBs were increased markedly in a concentration-dependent manner. These results indicate that PML protein was upregulated by IFN-α in hMSCs.

PML plays a key role in IFN-α induced cellular senescence of hMSCs. In order to investigate the role of PML in IFN-α induced cellular senescence of hMSCs, PML expression was inhibited using an RNAi-mediated PML knockdown system. As shown in Fig. 3A, PML gene significantly downregulated in hMSCs, as compared with normal and cells transfected with empty plasmid. Even though hMSCs were treated with IFN-α at 1,000 U/ml for 7 days, PML gene showed no obvious upregulation in PML knockdown cells. Then the expression of SA-β-gal was analyzed. Compared with normal, cells transduced with lentivirus became easier senescent. However, there were a lower percentage of SA-β-gal-positive cells in PML knockdown hMSCs than the control RNAi (3.74±1.42 vs. 12.32±0.87%, P<0.05). After treated with IFN-α at 1,000 U/ml for 7 days, hMSCs senescence can be rescued by the knockdown of PML. The percentage of SA-β-gal-positive cells in PML knockdown hMSCs showed a significant decrease as compared with cells transfected with control-shRNA (4.49 ±1.27 vs. 17.26±1.44%, P<0.05) (Fig. 3B). Therefore, these results suggested that PML may associate with IFN-α induced cellular senescence.

Effect of PML on cellular senescence in hMSCs. To further characterize the effect of PML on cellular senescence in hMSCs, PML-overexpressed hMSCs were analyzed, while normal and empty vector transfected cells were used as controls. As shown in Fig. 4A, PML-overexpressing hMSCs remained viable for two weeks. However, these cells became flat and enlarged. Detected 7 days post-transfection, cells

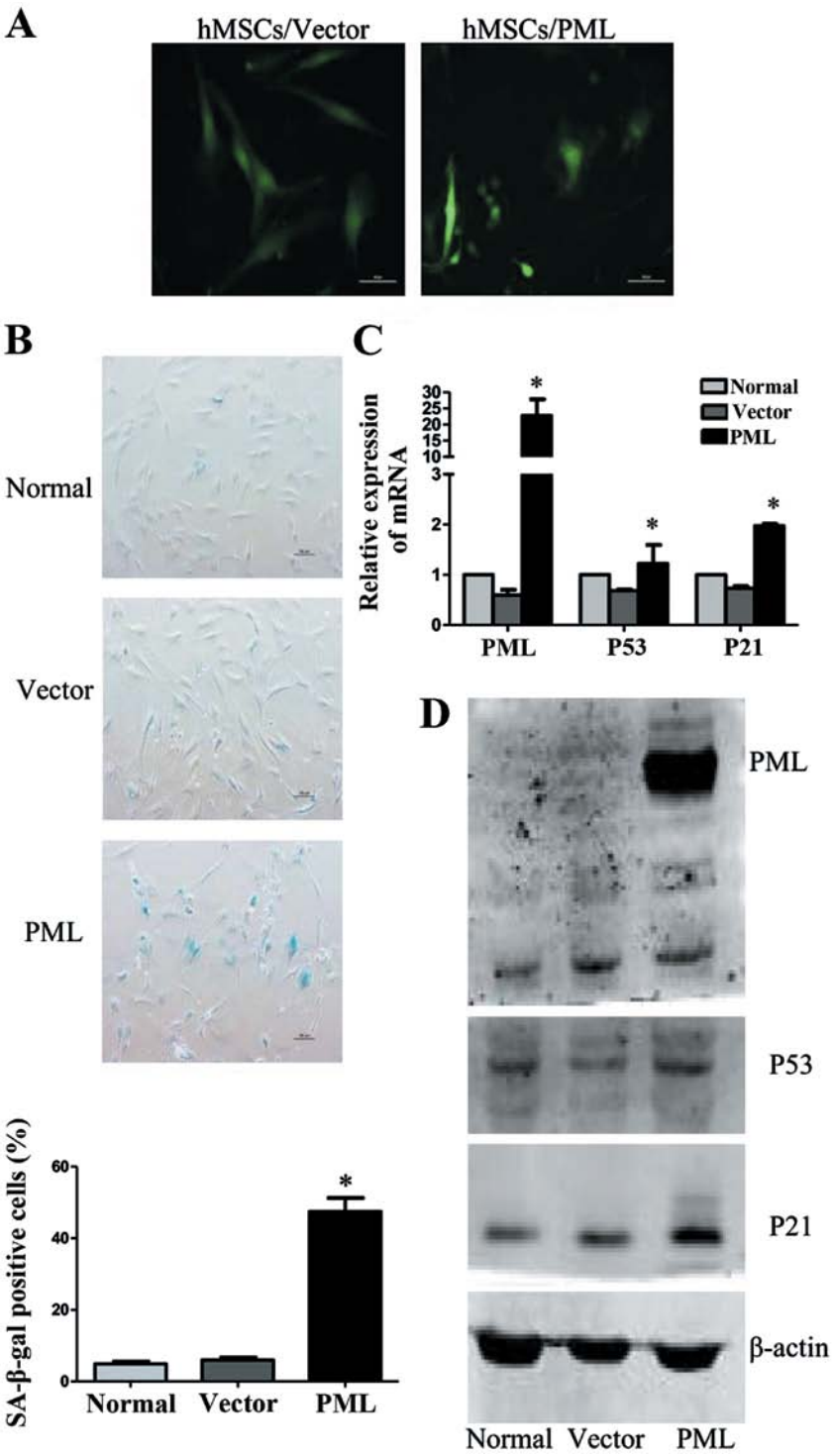

Figure 4. Effect of PML on cellular senescence in hMSCs. (A) Transfected-hMSCs were detected by fluorescence microscopy at day 14. PML-overexpressing cells became flat and enlarged. (B) SA-β-gal staining. Seven days post-transfection, cells were strongly positive for SA-β-gal activity, as compared with normal and empty vector transfected cells. The quantitation of data represents the mean ± SD of three independent counts of 500 cells. Real-time PCR (C) and western blot analysis (D) for PML, p53 and p21. β-actin was used as a protein loading control. Scale bar, 100 μm. *P<0.05 vs. control.

were strongly positive for SA-β-gal activity (47.43±3.8%), as compared with normal and empty vector transfected cells (4.9±0.7 and 5.97±0.75%). As important senescence markers, mRNA levels of P53 and P21 were also enhanced in PML-overexpressed hMSCs. The upregulation of P21 protein was confirmed by western blot analysis. However, we did not observe obvious changes of P53 protein expression. These results collectively proved that PML alone is sufficient to promote cellular senescence of hMSCs.

IFN-α promotes the co-localization of P53 and PML in hMSCs. P21 as an important downstream effector of P53 was enhanced in PML-transfected hMSCs. Then, we wondered whether upregulation of PML induced by IFN-α has a rela-

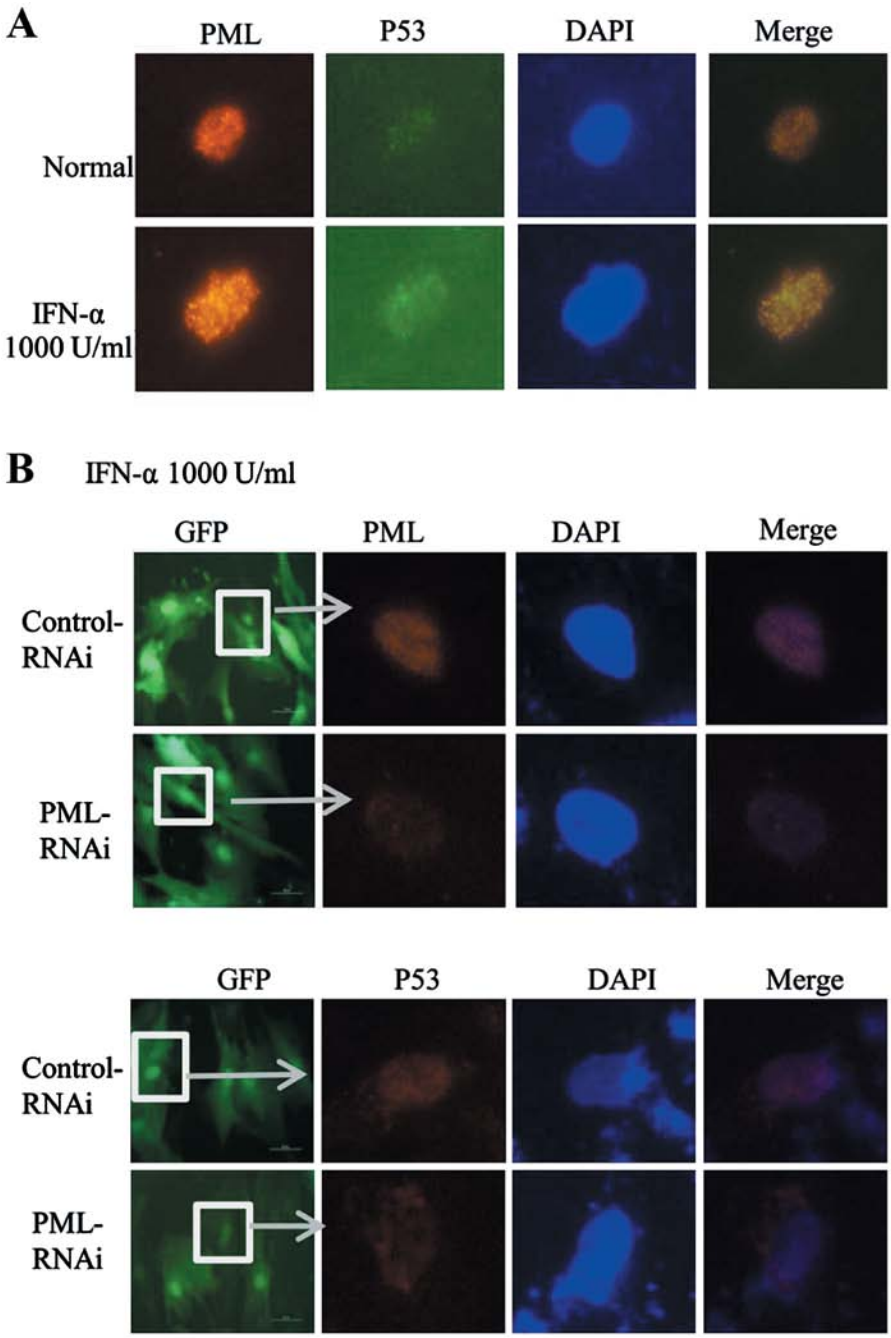

Figure 5. IFN-α promotes the co-localization of P53 and PML in hMSCs. (A) Colocalization of P53 with PML. hMSCs were treated with IFN-α (1,000 U/ml) for 7 days, and colocalization of PML (red) and P53 (green) was increased in IFN-α treated cells as compared with control. (B) Treatment of IFN-α in the PML knockdown hMSCs at 1,000 U/ml for 7 days, no significant change of PML and P53 location was observed as compared with control. Scale bar, 50 μm.

tionship with P53 pathway in hMSCs. Previous studies have proved that P53 can be recruited to the PML-NBs, leading to enhancement of its transcriptional activity. In the process of IFN-α induced hMSCs senescence, the co-localization of PML and P53 was observed by immunofluorescence assay. As shown in Fig. 5A, an increasing co-localization of PML (red) and P53 (green) was observed in IFN-α treated cells (1,000 U/ml, 7 days) as compared with untreated cells. To further confirm whether or not the change of P53 location was mediated through the upregulation of PML, we knocked down the expression of PML in hMSCs. Then, cells were treated with IFN-α (1,000 U/ml, 7 days), however, we did not find significant location of P53 (red) in PML knockdown cells as compared with control (Fig. 5B). Taken together, these results delineate that upregulated expression of PML induced by IFN-α can recruit P53 to PML-NBs, and to participate in the process of promoting cell senescence.

Discussion

IFN-α has been used widely in treatment of many diseases. Recent developments have renewed interest in IFN-α for CML therapy (4). However the precise mechanism remains unclear. In the present study, we investigated this problem starting

from hMSCs. Our study showed that IFN-α induced cellular senescence of hMSCs in a dose-dependent manner and PML was involved in this process.

Type 1 interferons have been described as negative regulator of the cell cycle and potent proliferation inhibitor of several cells, such as human uterine cells, and osteoprogenitor cells (8-10). We also found that IFN-α can significantly inhibit the proliferation of hMSCs in a concentration-dependent manner. In general, the inhibition of cell proliferation is mostly associated with cell cycle arrest and cell apoptosis. However, when hMSCs were treated with IFN-α, cell apoptosis was not obvious until IFN-α concentration of 10,000 U/ml. Therefore, cell apoptosis was not the main reason for the inhibition effect of IFN-α on hMSCs. The cell cycle was evaluated by flow cytometry, but we did not find significant cell cycle changes in hMSCs (data not shown). The possible mechanism may be that the major proportion of hMSCs was in G0/G1 phase under normal culture (19) and it is difficult to detect cell cycle arrest in hMSCs. So, as another important form of proliferative potential inhibition *in vitro*, cellular senescence was assessed.

Cellular senescence is a program activated by normal cells in response to various types of stress, such as shortening of telomere, DNA damage, oxidative stress and others (20,21). Once cells have entered senescence, they cease to divide and undergo a series of morphologic and metabolic changes (22,23). In previous research, IFN pathway involved in regulating cellular senescence has been proved in endothelial cells and fibroblasts (24,25). In our research, hMSC senescence induced by IFN-α was measured by a variety of senescence-associated detection, and it was found that IFN-α can induce cellular senescence of hMSCs in a dose-dependent manner. *In vitro*, 1,000-1,500 U/ml was considered the effective concentration of IFN-α (26). The hMSCs studied here exhibited an increased senescence phenotype and upregulation of P53 and P21 expression upon treatment with IFN-α at 1,000 U/ml for 7 or 14 days. Then, we wonder which downstream gene of IFN pathway might participate in regulating cellular senescence of hMSCs.

PML, which is known as a tumor suppressor, can be directly induced by IFN-α (13). Previously, PML attracted our interest due to its role in regulating stem cells function. We have proved that PML is stably expressed in hMSCs and plays a vital role in maintaining the normal proliferation capacity of hMSCs. At the same time, several studies have proven that PML is an important regulator in cellular senescence (27,28). Therefore, the role of PML in IFN-α induced cellular senescence was studied.

Our results here showed that PML expression was upregulated in IFN-α treated hMSCs, and was concentration-dependent. To better understand the role of PML in senescence of hMSCs, we changed the expression of PML in hMSCs. When PML was downregulated, cellular senescence induced by IFN-α can be inhibited. While PML was overexpressed, hMSCs showed changes in morphology, and were strongly positive for SA-β-gal activity. Our conclusion was that PML was required for the IFN-α induced cellular senescence of hMSCs.

P53 is a key regulator of the senescence response and PML can regulate P53 activity via direct interaction (29-31). We did not find significant changes of P53 protein expression. However, PML overexpression induced upregulation of p21, a transcriptional target of p53. Then we considered whether the activity, but not the expression of P53, was changed in this process.

In normal cells, P53 protein is expressed at low levels and has a short half-life due to rapid turnover mediated by ubiquitination or proteolysis (32,33). Phosphorylation and acetylation was the most important post-translation modification form of P53 and determined the activity and function of P53 (34). PML-NBs play an important role in this process. P53 can be recruited to the PML-NBs, leading to enhancement of its transcriptional activity (30,35). In the process of IFN-α induced hMSCs senescence, we found increased colocalization of PML and P53. Treatment of IFN-α in the PML-knockdown cells, showed no significant changes of P53 location as compared with control. These results indicated that upregulated expression of PML induced by IFN-α can recruit P53 to PML-NBs, and may thus participate in the process of promoting cell senescence.

In conclusion, our present study showed the effect of IFN-α on cellular senescence of hMSCs, and revealed a previously unknown role of PML in this process. Briefly, IFN-α can induce cellular senescence of hMSCs in a dose-dependent manner, which is accompanied by an upregulation of PML. Downregulation of PML can rescue cellular senescence induced by IFN-α in hMSCs. Upregulation of PML alone can also promote cellular senescence. One of the mechanisms may be that the co-localization of P53 and PML was increased in IFN-α treated hMSCs. However, the interconnections of PML and P53 in hMSCs need further investigation. Our results offer new insights into the role of IFN-α in hMSCs and may translate into a clinical strategy for wider use of IFN-α with fewer adverse effects.

Acknowledgements

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