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Overexpression of microRNA-29b induces apoptosis of multiple myeloma cells through down regulating Mcl-1

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ABSTRACT

MicroRNAs (miRNAs) are small, noncoding ribonucleic acids (ncRNAs), which regulate gene expression by targeting mRNAs for translational repression and degradation. Several lines of evidences have indicated that miRNAs act as tumor suppressors and oncogenes. However, the role of miRNAs in pathogenesis of multiple myeloma (MM) remains unclear. In this study, we examined the profile of miRNA expression of primary MM cells, using miRNA microarray and quantitative real-time polymerase chain reaction (qPCR) techniques. These results showed that in the bone marrow specimens analyzed, miRNA-29b was significantly downregulated. Similar results were also observed in human myeloma cell lines (HMCLs). Adenovirus-mediated overexpression of miR-29b induced apoptosis and elevated caspase-3 activation in HMCLs. Using a bioinformatics approach, we found a perfect complementarity between miRNA-29b and the 3′UTR of myeloid-cell-leukemia 1(Mcl-1). It is further confirmed that miRNA-29b downregulated the level of Mcl-1 without effect on the mRNA level using both qRT-PCR assays and Western blot analyses. Moreover, we observed that enforced miR-29b expression by using a retarget miRNA-29b expression vector (Ad5F11p-miR-29b) could induce apoptosis and elevate caspase-3 activation in HMCLs. Our results also indicated that miRNA-29b-induced apoptosis acted antagonistically with IL-6 in HMCLs. These findings suggest that miRNA-29b may play an important role in MM as a tumor suppressor.

1. Introduction

Multiple myeloma (MM) is a postgerminal center B cell malignancy, characterized by clonal plasma cell expansion within the bone marrow. MM is one of the most common type of plasma cell cancer. Although conventional chemotherapy, followed by autologous stem-cell transplantation, can modestly prolong patient survival to 4–5 years, MM remains largely incurable.

MicroRNAs (miRNAs) are short endogenous nonprotein-coding RNA (ncRNA) molecules, typically 19–25 nucleotides in length, which can mediate the posttranscriptional downregulation of target genes [1]. They have been shown to play a fundamental role in diverse physiological and pathological processes, including proliferation, apoptosis, differentiation, and cell fate determination [2]. Recent studies have revealed that approximately 50% of human miRNAs are located in areas of the genome, known as fragile sites, which are associated with cancer [3,4]. Increasing evidence indicates that miRNAs might have a crucial function as both tumor suppressors and oncogenes in the pathogenesis of human malignancies [5,6]. Specifically, a recent study showed that increased aggressiveness in B cell chronic leukemia correlated with considerable reduction of miR-29b and miR-181 levels and increased expression of their common target, the B and T cell malignancy-specific oncogene, TCL1 [7,8]. Furthermore, in a recent report on cholangiocytes in cholangiocarcinoma, miR-29b was shown to regulate the expression of the Mcl-1 protein by inhibiting its translation, without affecting Mcl-1 mRNA levels [9]. Mcl-1 is an anti-apoptotic member of the Bcl-2 family, whose proteins and mRNA have a short half-life.

In multiple myeloma, IL-6 is known to protect malignant plasma cells from apoptosis induced by different stimuli and to promote proliferation [10–12]. IL-6 is also known to rapidly upregulate Mcl-1 by the JAK/STAT pathway [11]. IL-6 induces Mcl-1 upregulation and provides an inhibitory effect on the proliferation

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of myeloma cells. Thus Mcl-1 is considered as a target for multiple myeloma therapy.

Significant effort has therefore been devoted to using genetic and gene expression microarray approaches to define MM molecular subtypes and to predict susceptibility to various treatment regimens [13,14]. In the present study, we investigated the miRNA expression profile in primary multiple myeloma (MM) cells taken from bone marrow samples of MM patients. By using a retargeting adenovector carrying hsa-miR-29b pre-mRNA to transduce multiple myeloma cells, we investigated that miR-29b could induce apoptosis of MM cells through interrupting Mcl-1 upregulation.

2. Materials and methods

2.1. Clinical samples and purification of primary multiple myeloma cells

Specimens of primary MM cells, including IgG and IgM isoforms, were obtained from the bone marrow samples of patients from the Beijing Chao Yang Hospital (Beijing, China), with approval of the Local Research Ethics Committee. None of the patients in the current study had received chemotherapy or radiation therapy prior to paracentesis. Myeloma plasma cells were purified to >85% using CD138 magnetic microbeads (MACS system, Miltenyi Biotec., Bergisch Gladbach, Germany).

2.2. Cell lines and cultures

Two human myeloma cell lines (HMCLs), SKO-007 and U266, were maintained in RPMI 1640 (Sigma, St. Louis, MO) and supplemented with 10% fetal bovine serum (FBS; Hyclone, USA, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. XG7 cells were cultured in RPMI-1640 containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 50 mM 2-mercaptoethanol, and 3 ng/ml of recombinant human IL-6 (rhIL-6) (Peprotech, Rocky Hill, NJ).

HEK 293T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCOBRL, Grand Island, NY) containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL/Invitrogen, Carlsbad, CA, USA). All other chemicals and reagents were purchased from Sigma unless otherwise specified.

2.3. Quantitative analysis of Mcl-1 mRNA – real-time PCR

Total RNA was isolated from treated, untreated and infected myeloma cells using TRIzol reagent (Invitrogen) per the manufacturer's instructions and was purified with RNA kit (Ambion, Austin, TX). 1 μg RNA was used to make cDNA through a reverse transcription reaction with oligo (dT) primers (Invitrogen). The cDNA was then used for real-time PCR with SYBR chemistry (Applied Biosystems, Inc., Foster City, CA). mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. Primers used for amplification of Mcl-1 and GAPDH were purchased from Invitrogen and Integrated DNA Technologies, respectively. Sequences were as follows. Mcl-1: 5′-CGCCAAGGACACAAAGCC-3′ (forward), 5′-GTCTCGTGGTTGCGCTGC-3′ (reverse); GAPDH: 5′-AGAAGGCTGGGGCTG-3′ (forward), 5′-AGGGGCCATCCACAGTCTTC-3′ (reverse).

2.4. qRT-PCR for miRNAs

For miRNA detection, total RNA was first polyadenylated by poly(A) polymerase (Ambion, Austin, TX). The reaction was carried out with 10 μg total RNA and 1 μl (2 U) poly(A) polymerase, according to the manufacturer's protocol, in a total reaction mixture of 50 μl. The reaction was incubated at 37 °C for 60 min. After incubation, poly(A)-tailed total RNA was recovered by phenol/chloroform extraction and ethanol precipitation. Reverse transcription was performed using 1 μg poly(A)-tailed total RNA and 1 μg reverse transcription primer (5′-gcgagcacagaattaatacgactcactatagg(t) 18VN-3′) with 1 μl ImPro-II Reverse Transcriptase (Promega), according to the manufacturer's protocol. Quantitative PCR was performed as described in Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) using the Mx3000p system (Stratagene, La Jolla, CA). One primer of miRNA amplification was microRNA-specific miR-29a-forward: 5′-gggggTACCCTTAzzATAgATAgTACAgACTC-3′; miR-29a-reverse: ggggATATCCCTTTgCATTATTgCTzTTgC; miR-29b-forward: 5′-gggggTACCCTTCAggAAgCTggTTTC-3′; miR-29b-reverse: ggggATATCTACATgTgAggCAggTTCTCAC; miR-29c-forward; 5′-gggggTACCgAggATgCCCTggAgTATTC-3′; miR-29c-reverse: ggggATATCCATgATCTTCCTTCCCTATTC. Levels of U6 small nuclear RNA were used for normalization using the forward and reverse primers. U6-F 5′-cgcttcggcagcacatatacta-3′ and U6-R 5′-cgcttcacgaatttgcgtgtca-3′, respectively. GenEX software (TATAA Biocenter, Munich, Germany) was used to analyze real-time PCR data. A paired Student's *t*-test was performed to determine the difference in the level of miRNA expression between noncancerous and cancer tissue.

2.5. Bioinformatics

Identification of potential mRNA targets for the differentially expressed miRNAs was performed using TargetScan [15], miRANDA, and PicTar databases. The most recent TargetScan predictions were used to identify the putative miRNA targets. These included the 3′ UTR targets reported by Lewis et al. [16].

2.6. Construction of adenoviral vector

To address whether miR-29b could exert effects in myeloma cells, modified adenoviral vectors carrying the miR-29b gene were constructed. A genomic fragment of human miRNA-29b was cloned from the pcDNA3.0-miR-29b precursor and amplified by PCR, using the primer pair 5′-gggggTACCCTTCAggAAgCTggTTTC-3′ and 5′-ggggATATCTACATg TgAggCA ggTTCTCAC-3′. New restriction sites were introduced to blunt-end one or both ends with T4 DNA. The sequence of the amplified segments was verified by DNA sequencing.

The shuttle vectors were linearized with *Pme*I and transformed into electrocompetent Ad5f11p cells, generating recombinant adenovirus plasmid Ad5f11p-CMV-miR-29b. Replication-deficient adenoviral vectors pAdEasy-1/F11P containing the human miR-29b (Ad5f11p-miR-29b) were constructed using the pAdEasy-1 system (Stratagene), according to the manufacturer's protocol. The construction of replication-deficient adenoviruses containing no transgene (Ad5f11p-null) or green fluorescent protein (Ad5f11p-GFP) was carried out in a similar way to that of Ad5f11p-miR-29b. Ad5f11p-CMV-GFP was used as mock product to evaluate infection efficiency of Ad5f11p-miR-29b analysis. The null adenovirus was used as a control sample.

The recombinant plasmid, Ad5f11p-miR-29b, was purified by cesium chloride gradient ultracentrifugation and transfected into human fetal HEK-293 kidney cells. The final plaque-forming units (pfu) were determined by titration on HEK293 cells under an agarose overlay. Titers were determined by absorbance measurement (number of viral particles per ml). Transgene expression was assessed by PCR using miR-29b primers [17]. Cells were transduced with recombinant Ad5f11p-GFP at increasing multiplicities of infection (MOIs; number of viral particles per cell) for 48 h after infection at 150 MOI, and transduction efficiency was determined by visualization of GFP-expressing cells. To examine the biological

effects of miR-29b in MM, miR-29b was overexpressed in two human myeloma cell lines (HMCLs; SKO-007 and XG-7).

2.7. Western blot

Cells were washed twice with 1× phosphate buffered saline (PBS) and suspended in lysis buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride membranes. After probing with anti-Mcl-1 (R&D International) and anti-GAPDH (Zhongshan Goldenbridge Biotechnology Co. Ltd., China), the antigen–antibody complex was visualized by the enhanced chemiluminescence reagent, Supersignal (Pierce, Rockford, IL) and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.8. Apoptosis assays

Cells were stained with annexin V conjugated to fluorescein isothiocyanate (Caltag Laboratories, Burlingame, CA, USA) and PE active caspase-3 (BD), according to the manufacturer's protocol. The stained cells were analyzed on a flow cytometer using the CellQuest program.

2.9. Pan-caspase inhibition by Z-VAD-FMK

After transduced with miR-29b, RPMI SKO-007 cells were incubated with 0.05 mM pan-caspase inhibitor, Z-VAD-FMK (R&D) for 60 min. The extent of cell survival (FITC/PI cells) was determined by flow cytometry at time intervals of 12, 24, and 48 h post-infection. Untreated and null infected cells were considered as control samples.

2.10. Cell proliferation assays

For cell proliferation assays, SKO-007 and XG7 cell lines were infected with Ad5f11p-miR-29b. After 48 h, infected cells were collected and resuspended. Cells were plated at a density of 4000 cells per well in a total of 100 μl of medium (96-well plates) and examined after 12, 24, 36 and 48 h. Each well received 20 μl CCK-8 solution and incubated for a further 4 h. Optical density (OD) values were determined at a wavelength of 450 nm using a microplate reader (Varoskan Flash, Thermo Scientific). Analysis of cell proliferation was carried out using CCK8 detection.

XG7 cells were starved overnight in RPMI 0.5% FBS and then cultured both with and without 10 ng/ml IL-6 for 6 h. Cells were then infected with Ad5f11p-miR-29b or Ad-null and examined after 12, 24 and 48 h intervals. The effect of apoptosis in XG7 cells was stained with both annexin V and PI and determined using flow cytometry, Western blot analysis was performed to examine the effects of IL-6 on the regulation of anti-apoptotic proteins.

2.11. Statistical analysis

All reported values are given as the mean ± standard deviation, unless otherwise stated. Differences were assessed by two-tailed Student's *t*-test using Excel software. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1. Downregualtion of miR-29b in human myeloma cells

Several miRNAs showed differential expression in the microarray hybridization results, all with the potential to target genes involved with hematopoiesis and/or the immune response. The most notable case was that of the miR-29 family (miR-29a, miR-29b and miR-29c). The results of PCR analysis of the expression of the mature miR-29 family in three myeloma cell lines (SKO-007, U266 and XG7) showed that miR-29b was significantly downregulated in all three cell lines, compared with miR-29a and miR-29c (Fig. 1A). These observations indicate that miR-29b could be an important regulator of myeloma cells and may be involved the procession of multiple myeloma pathology.

3.2. Construction and preparation of Ad5f11p-miR-29b

Both transfected HEK293 cells and un-transfected HEK293 cells expressed hsa-miR-29b (Fig. 1B), but the level of miR-29b expression in the former cells was dramatically increased compared to the latter. The results indicate that the Ad5f11p-CMV-miR-29b express vector was successfully constructed. The observed endogenous expression of miR-29b in HEK293 cells is consistent with reported studies from the literature [18].

With increasing values for MOI, the percentage of GFP-positive cells reached 90.81% and 92.12% for SKO-007 and XG7 cells, respectively, having been infected by Ad5f11p-CMV-GFP at 150 MOI (Fig. 1C).

PCR analysis of the miR-29b gene transduction efficiency showed that SKO-007, U266 and XG7 expressed endogenous miR-29b and served as positive controls (Fig. 1D). Quantitative real-time PCR analysis of myeloma cells 24 h after infection with Ad-miR-29b or Ad-null showed miR-29b overexpression in all.

3.2.1. Ad-miR-29b-transduced cells

Fiber modification by Ad5f11p-miR-29b significantly increased the gene transduction ability of miR-29b in all tested cells. These results satisfy the requirements for the infection and function of myeloma cells in vitro.

3.3. miR-29b targets Mcl-1 in multiple myeloma cells

Analysis of candidate target genes for miR-29b using TargetScan revealed perfect complementarity between miR-29b and the 3'UTR of Mcl-1 over the first nine nucleotides (including the 2–7), consistent with a known miR-29 binding site (Fig. 2A). The analysis revealed that the Mcl-1 gene 3′-UTR harbors one putative binding site for miR-29b and, importantly, that the site is conserved across various species (Fig. 2A).

Western blot analysis of protein extracts obtained from the myeloma cells (at baseline and at 12, 24 and 48 h following Ad5f11p-miR29b infection), demonstrated that the overexpression of miR-29b dramatically downregulated Mcl-1 protein levels in a time-dependent manner (Fig. 2B), and that this downregulation was most prominent after 48 h. In contrast, Ad-null infected cells showed no reduction in Mcl-1 expression levels (Fig. 2C). In the context of previous studies, which have indicated that the inhibition of target gene expression by miRNAs may be mediated by mRNA degradation [19], our findings showed that Mcl-1 mRNA expression is not altered by the overexpression of miR-29b (Fig. 2D).

3.4. MiR-29b overexpression inhibits growth and induces apoptosis of multiple myeloma cells

Overexpression of miRNA-29b in the SKO-007 and XG7 HMCLs resulted in inhibition of cell growth in both cell lines by CCK-8 test (Fig. 3A and B, respectively) compared to their respective controls. Apoptosis of SKO-007 and XG7 cells showed a time and concentration-dependent increase upon exposure to overexpression of miR-29b, (Figs. 3C and D, respectively). XG7 cells were more susceptible to miR29b-induced apoptosis, demonstrated by a total increase by

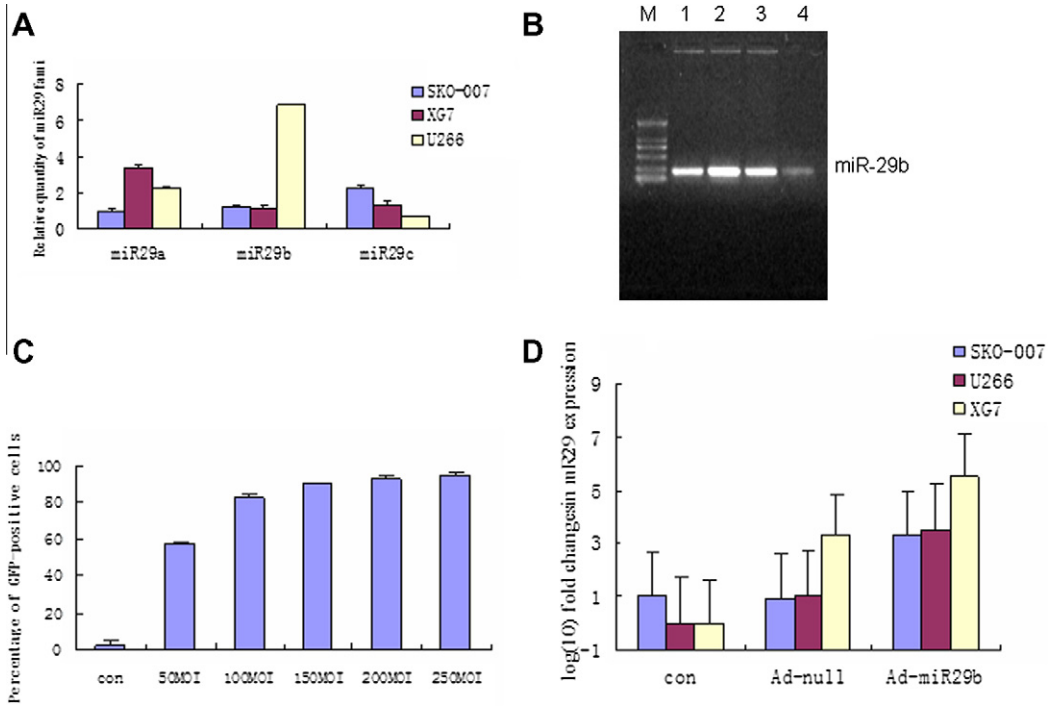

Fig. 1. miR-29b is downexpressed in human myeloma cells. (A) Expression of hsa-miR-29a, hsa-miR-29b and hsa-miR-29c in SKO-007, U266, and XG7 cells, confirmed by quantitative real-time PCR. The data shown represent mean ± SD from two independent experiments in triplicates and have been normalized to U6. (B) Identification of recombinant adenoviral vectors carrying miR-29b with polymerase chain reaction (PCR) methods. Lane M showed the DNA marker composed of 2000, 1000, 750, 500, 250 and 100 bp bands. 1–4 are the PCR products of vector and virus transduced cells. (C) Gene transducer efficiency of Ad5f11p-GFP at variable MOIs. (D) Changes in the expression of miR-29b after infection with Ad5f11p-miR-29b in three myeloma cell lines. Bars represent standard error from three separate experiments; one asterisk indicates $p \leqslant 0.05$, and two asterisks indicate $P \leqslant 0.01$.

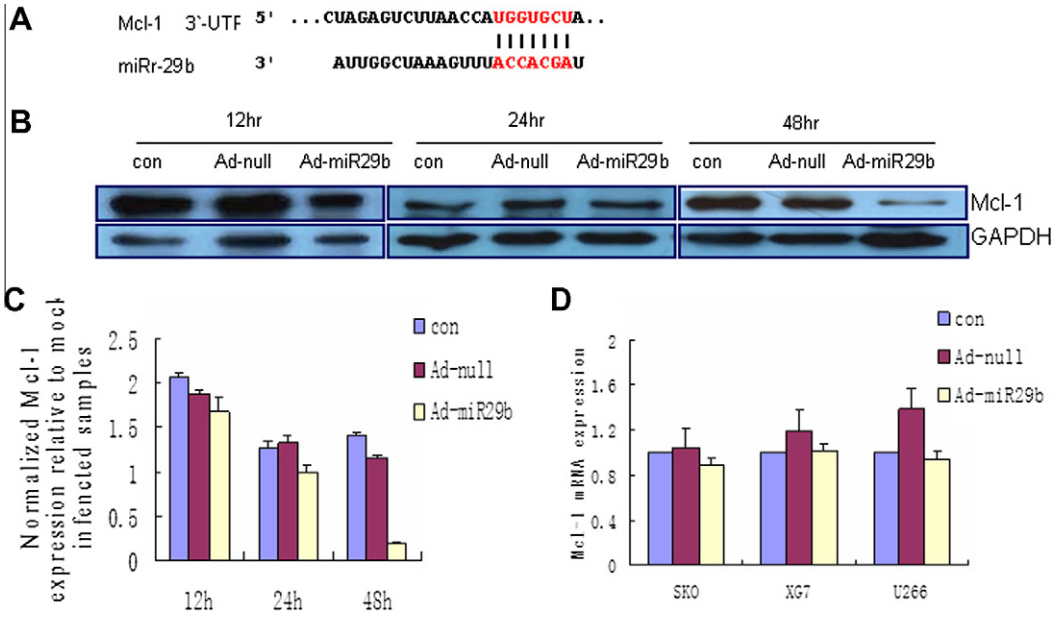

Fig. 2. Identification of Mcl-1 as a potential target of miR-29b. (A) Predicted interactions between miR-29b with the 3'-untranslated region (3′-UTR) of Mcl-1(TargetScan). Seed regions are highlighted in bold. (B) Mcl-1 protein expression in SKO-007 and XG7 cells after 12, 24 and 48 h of infection with Ad5f11p-miR-29b. (C) Summary of changes in Mcl-1 protein level 12, 24, and 48 h after infection with Ad5f11p-miR-29b in SKO-007 and XG7 cells. Error bars indicate standard deviation. (D) Quantitative RT-PCR of Mcl-1 mRNA after 24 h of infection of three myeloma cell lines with Ad5f11p-miR-29b. The results are shown as average mRNA expression after normalization with GAPDH mRNA. Data represent the average of three independent experiments ± SD.

a factor of 3.4 at 12, 24 and 48 h compared to a factor of 2 for SKO-007 cells, as measured by the annexin-V assay ($p < 0.01$). Additionally, the percentage of apoptotic cells in the Ad5f11p-miR-29b infected XG7 cells was found to be higher than that in Ad5f11p-null infected SKO-007 cells ($p < 0.05$; Fig. 3D).

3.5. Caspase-3 activation is involved in miR-29b induced apoptosis of multiple myeloma cells

Apoptosis induction by miR-29b was found to be associated with caspase-3 activation (Fig. 3E and F). At 12, 24 and 48 h after

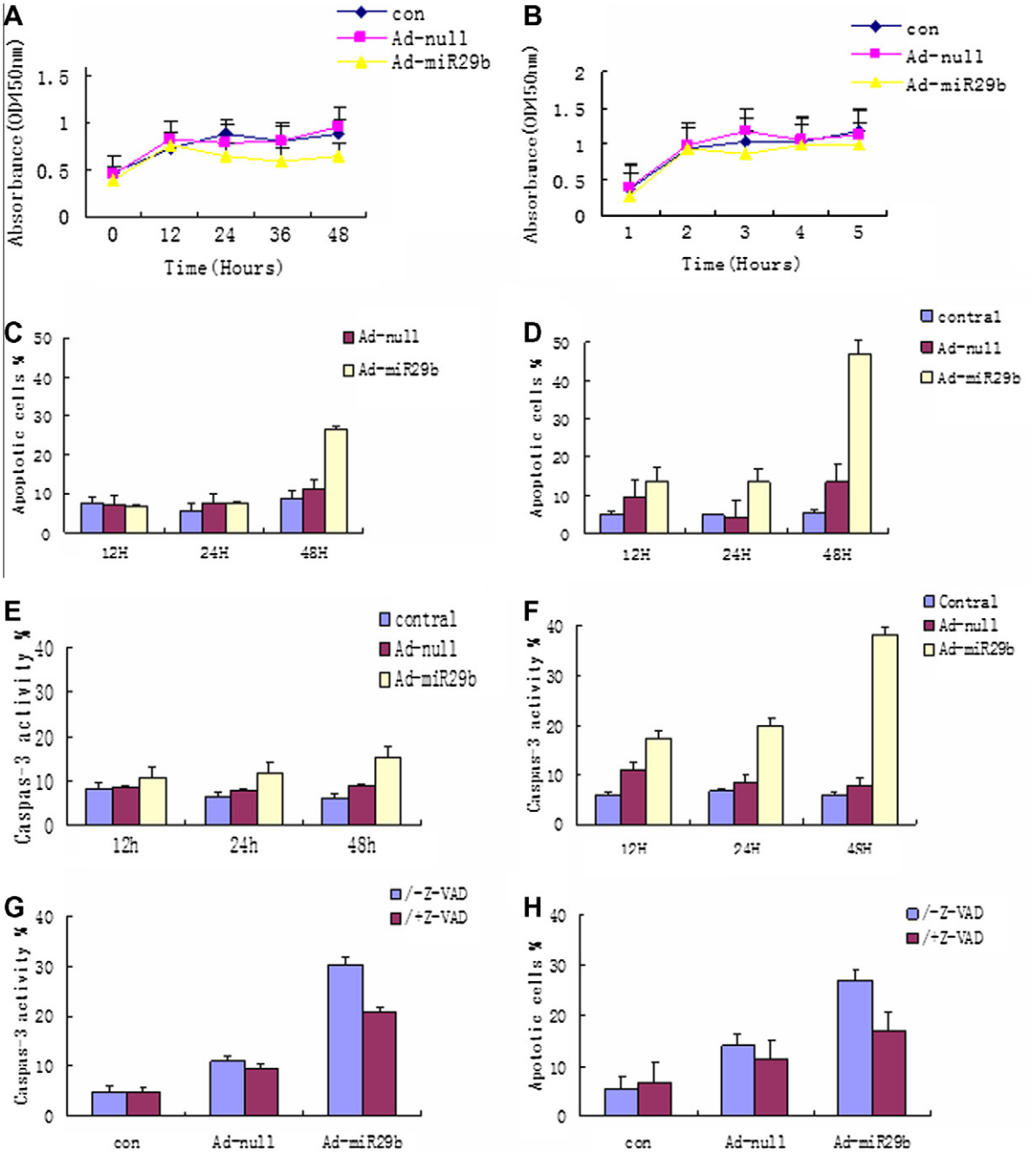

Fig. 3. MiR-29b induces apoptosis and inhibits proliferation. (A and B) MiR-29b reduces cell growth and induces apoptosis in three cell lines. Cell growth curves of SKO-007 cells (A, left panel) or XG7 cells (B, right panel) infected in vitro with adenovirus miR-29b and their respective controls (without, or empty vector). (C and D) Annexin V/PI assays in SKO-007 (C, left panel) and XG7 (D, right panel) cells after12, 24 and 48 h of infection with adenovirus miR-29b or controls (without infection or empty vector). The results are shown as percentage of apoptotic cells. Data are the average of three independent experiments ± SD. *p*-values were obtained using *t*-tests. $^{*}p < 0.05$. (E and F) The regulation of apoptosis by miR-29b was confirmed by measuring caspase-3 activities. SKO-007 (E) and XG7 (F) cells were infected with adenovirus miR-29b or controls (without infection or empty vector) and. After 48 h, luminescence was measured using the caspase-3 Glo Assay (Promega). *p*-Values were obtained using *t*-tests. Values are mean ± SD; $n = 3$. (G and H) Overexpression miR-29b induces apoptosis and trigger activation of caspase-3 which partially protected by Z-VAD-FMK in HMCLs. SKO-007 cells were treated with Ad5f11p-miR-29b or null vector alone or in the presence of Z-VAD-FMK (200 μM) for different times. (G) Caspase activity was checked for statistical differences. (H) Viability, expressed as the percentage of annexin V and PI negative cells, is shown for each time at the bottom of the figure.

miR-29b overexpression in SKO-007 and XG7 cells, caspase-3 activation levels were increased compared with the Ad-null control cells and coincident with increased apoptosis. The annexin-V expression in Ad-miR-29b infected cells in the presence or absence of Z-VAD-FMK are shown in Fig. 3G. The results indicate that the increase in apoptosis is partially restricted by Z-VAD-FMK in SKO-007 cells (Fig. 3H). The efficiency of Z-VAD-FMK is evident since caspase-3 activation was largely blocked in the presence of this inhibitor.

3.6. Overexpression of miR-29b inhibits IL-6-induced upregulation of Mcl-1

Results indicate that the percentage of apoptotic XG7 cells was not reduced following the culturing of Ad-miR-29b-infected cells with IL-6 (Fig. 4A). As shown in Fig. 4B, following infection of Ad5f11p-miR-29b, the level of IL-6-induced Mcl-1 expression decreased in XG7 cells in a time-dependent manner, compared to that of Ad5f11p-null infected cells and uninfected cells. Notably,

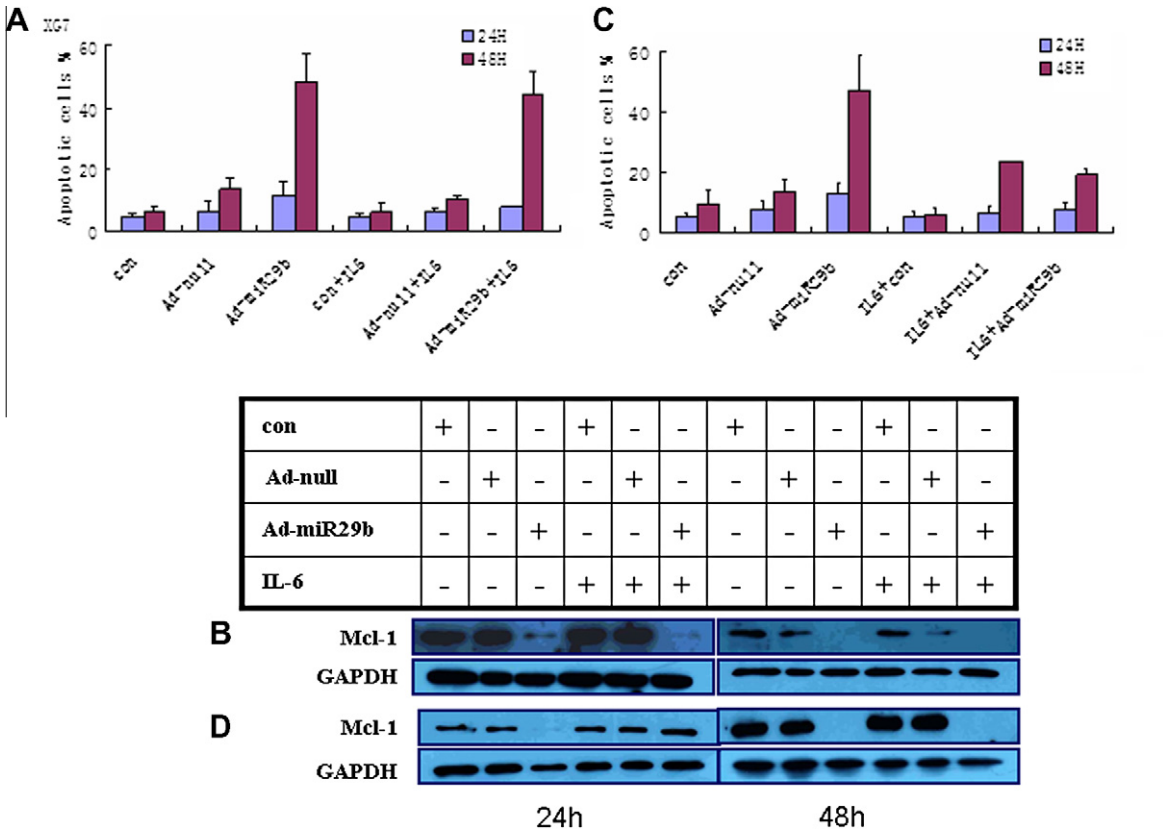

Fig. 4. Mir-29b overexpression inhibit Mc1-1 expression and block inhibitory effects of IL-6 on XG-7 cell apoptosis. (A and B) MiR-29b interrupts IL-6-induced Mcl-1 upregulation in XG7 cells. (A) Annexin V/PI assays apoptosis of XG7 cells with or without IL-6 stimulation XG7 cells after12, 24 and 48 h of infection with adenovirus miR-29b or controls (without infected, or empty vector). (B) Mcl-1 protein expression of with or without IL-6-stimulating in XG7 cells after 12, 24 and 48 h of infection with Ad5f11p-miR-29b or controls (without infected cells and empty vector (EV) for cells). The protein loading control was performed using GAPDH. (C and D) MiR-29b-induced cell death is partially rescued by IL-6. (C) Apoptosis was assessed IL-6-triggered XG7 cells were infected with adenovirus miR-29b or controls (without infected, or empty vector) after 12, 24 and 48 h. (D) Western blotting of Mcl-1 protein expression in IL-6-stimulated XG7 cells that were infected with adenovirus miR-29b or controls (without infected, or empty vector infected) after 12, 24 and 48 h.

the downregulation of Mcl-1 was already marked 12 h after Ad5f11p-miR-29b infection, indicative of the rapid kinetics of this process. These results demonstrate that miR-29b at least partially interrupts the process of IL-6-induced upregulation of Mcl-1 and suppresses the protective effect of IL-6 in myeloma cells.

The results of flow cytometry analysis on the level of miR-29b-induced apoptosis in the presence of IL-6 is shown in Fig. 4C. Gated analysis on XG7 cells showed that IL-6 reduced miR-29b-induced apoptosis from approximately 21% to 15% (24 h after Ad-miR-29b infection) and from 47% to 23% (48 h after Ad-miR-29b infection). The results of Mcl-1 expression determined by Western blot analysis are shown in Fig. 4D. This shows that IL-6 significantly upregulated the Mcl-1 protein level at both the 24 and 48 h time intervals, which had been depressed by miR-29b in XG7 cells. These results demonstrate that IL-6 partially protects myeloma cells against miR-29-induced apoptosis.

4. Discussion

As a result of the similar chemical structures of miRNA to DNA, we created a novel and efficient adenovirus Ad5f11p-CMV-miR-29b, to conduct this series of molecular biology experiments. The data presented here illustrate the expression characteristics of miR-29b in HMCL cell lines and primary MM cells. Findings show that enforced expression of miR-29b downregulates Mcl-1 expression, inhibits cell growth and induces apoptosis in HMCLs. These results indicate that inhibitory effects of miR-29b are not the result of apoptosis and are likely to contribute to the tumor suppressor activity of miRNAs. The tumor suppressor effect of miR-29b in MM was further demonstrated in vivo.

Although deregulation of miR-29b in a certain type of cancer suggests a potential involvement in the malignancy, experimental evidence has previously been lacking. Our results indicate that there is a significant increase of miR-29b expression among three myeloma cell lines following infection with Ad5f11p-miR-29b. This is potentially very useful for scientists that specialize in the research of miRNA function using adenoviral vectors. Indeed, it has been shown here that this is an effective strategy to determine target genes and to identify pathways regulated by miRNAs.

The implication of quantitative RT-PCR analysis in HMCLs, which showed no change in the level of Mcl-1 mRNA after infection of miR-29b relative to the control, is that reduction of Mcl-1 protein levels by miR-29b occurs at a translational suppression stage rather than at mRNA degradation. The downregulation of Mcl-1 protein during apoptosis is consistent with previous studies [20–24] and suggests that Mcl-1 could be important for survival of myeloma cells.

The pan-caspase inhibitor Z-VAD-FMK largely inhibited the expression of caspase-3. However, the fact that ZVAD-FMK only partially protected against apoptosis increase after miR-29b treatment indicates that apoptosis is not solely due to caspase-3 activation. Recently, it has been reported that Mcl-1 is a substrate for caspases during Fas-mediated apoptosis [25].

In the present study, we also analyzed the relationship between miR-29b expression and IL-6 activity during Mcl-1 expression in

MM. Our results demonstrate for the first time that miR-29b-induced cell death is partially prevented by IL-6. However, with increasing time after miR-29b infection, the magnitude of this effect gradually decreases, which indicates that miR-29b could completely depress the expression of Mcl-1.

In conclusion, the results of this in vitro study demonstrate that miR-29b could be a tumor suppressor that operates by targeting critical oncogenic pathways, thereby suggesting that miRNA-based therapy could enable a novel pro-apoptotic approach to improving treatment response and disease progression rates in patients with multiple myeloma.

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