

MiR-26 down-regulates TNF- α /NF- κ B signalling and IL-6 expression by silencing HMGA1 and MALT1

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ABSTRACT

MiR-26 has emerged as a key tumour suppressor in various cancers. Accumulating evidence supports that miR-26 regulates inflammation and tumorigenicity largely through down-regulating IL-6 production, but the underlying mechanism remains obscure. Here, combining a transcriptome-wide approach with manipulation of cellular miR-26 levels, we showed that instead of directly targeting IL-6 mRNA for gene silencing, miR-26 diminishes IL-6 transcription activated by TNF- α through silencing NF- κ B signalling related factors HMGA1 and MALT1. We demonstrated that miR-26 extensively dampens the induction of many inflammation-related cytokine, chemokine and tissue-remodelling genes that are activated via NF- κ B signalling pathway. Knocking down both HMGA1 and MALT1 by RNAi had a silencing effect on NF- κ B-responsive genes similar to that caused by miR-26. Moreover, we discovered that poor patient prognosis in human lung adenocarcinoma is associated with low miR-26 and high HMGA1 or MALT1 levels and not with levels of any of them individually. These new findings not only unravel a novel mechanism by which miR-26 dampens IL-6 production transcriptionally but also demonstrate a direct role of miR-26 in down-regulating NF- κ B signalling pathway, thereby revealing a more critical and broader role of miR-26 in inflammation and cancer than previously realized.

INTRODUCTION

MiR-26 exhibits tumour suppressor activity (reviewed in (1)) and has emerged as a key regulator in carcinogenesis and tumour progression. Ectopic expression of miR-26 inhibits proliferation, induces apoptosis and/or decreases tumorigenicity in multiple cancers, whereas down-regulation

of miR-26 was observed across multiple tumour types (2–5). An inverse relationship between levels of miR-26 and Interleukin-6 (IL-6) was observed in some tumour cells (6,7). It has been thought that miR-26 regulates inflammation and tumorigenicity largely through down-regulating IL-6.

IL-6 is a multifunctional cytokine with important roles in many chronic inflammatory diseases (8–10). IL-6 also has pro-tumourigenic activities, including promoting tumour cell proliferation and survival, stimulating angiogenesis and inducing immune tolerance (11–15). Further, IL-6 is up-regulated in many human tumours (16). All of these have focused attention on suppression of IL-6 expression as a potential anti-tumour strategy.

The mechanisms for miR-26 actions in regulating IL-6 production, inflammation and tumour proliferation remain obscure. Previously, a potential miR-26 recognition site was predicted in the 3' UTR of IL-6 mRNA (17,18). Binding of miR-26 to this site was proposed to elicit rapid degradation of IL-6 mRNA and thus silence IL-6 expression in human alveolar basal epithelial A549 cells activated by TNF- α (18). However, the region containing this site has been reported to have little effect on IL-6 mRNA levels in monkey and mouse cell models (19). Moreover, when the predicted miR-26 site in the 3' UTR of IL-6 mRNA was mutated, it had no effect on the translation of IL-6 in HeLa cells (20). These observations argue against a direct action of miR-26 on silencing the IL-6 message. Given that inflammation is a major factor contributing to malignancy and the roles of miR-26 and IL-6 in this process, it is important to understand the mechanism by which miR-26 regulates IL-6 production in the context of cellular inflammatory response.

In this study, we employed a variety of approaches to elucidate the mechanism underlying miR-26-mediated regulation of IL-6 production. Our results demonstrated that miR-26 does not directly target IL-6 transcript for rapid decay or translational repression in either human bronchial epithelial BEAS-2B or adenocarcinomic alveolar basal epithelial A549 cells. Rather, miR-26 down-regulates production of IL-6 via actions on NF- κ B signalling. Our data

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further revealed that miR-26 represses IL-6 transcription through silencing the expression of MALT1 and HMGA1, two proteins with critical functions in mediating NF- κ B signalling and tumorigenicity (21–24), in BEAS-2B cells. Moreover, we discovered an inverse relationship between levels of miR-26 and of HMGA1 or MALT1 transcripts in lung adenocarcinoma (LUAD), which is linked to LUAD patient survival. Our results not only identify a novel mechanism by which miR-26 dampens IL-6 production transcriptionally through down-regulating NF- κ B signalling pathway but also point to a direct and broader role for miR-26 in inflammation and malignancy.

MATERIALS AND METHODS

Plasmids

Renilla luciferase (RL) reporter gene driven by human GAPDH promoter (pLightSwitch-Prom-GAPDH) was purchased from SwitchGear Genomics. Firefly luciferase (FL) reporter gene driven by a minimal promoter containing an NF- κ B response element (pGL4.32[luc2P/NF- κ B-RE/Hygro]) was purchased from Promega. To construct pIL-6-FL, a 2.2-kb fragment carrying the human IL-6 promoter that contains the transcription elements described previously (25–27) was PCR-amplified using genomic DNA purified from BEAS-2B cells and inserted into pGL4.13[luc2/SV40]. The plasmids expressing FL (pGL4.13[luc2/SV40]) and RL (pGL4.74[hRLuc/TK] or pGL4.73[hRLuc/SV40]) were purchased from Promega. To create pRL-IL-6 3' UTR, pRL-IL-6 5' UTR or pRL-IL-6 ORF, the corresponding regions from a human IL-6 cDNA were inserted into the RL 3' UTR in psiCHECK2 (Promega). To construct pRL-3 \times 26(IL-6) and pRL-3 \times 26(GW182), DNA fragments containing three copies of the putative miR-26 recognition site (see Supplementary Figure S3A and B for the sequences) were synthesized (Integrated DNA Technologies) and inserted into the RL 3' UTR in psiCHECK2. The plasmid pRL-IL-6 3' UTR(Δ 26) was created by a PCR-based mutagenesis to specifically truncate the seed-binding region of the predicted miR-26 site. Plasmids carrying sequences coding for the ORF of HMGA1 or MALT1 (pIRES-HMGA1 and pcDNA-FLAG-MALT1, respectively) were purchased from Addgene.

Cell culture, transfection and dual luciferase assay

The human bronchial epithelial cell line BEAS-2B and the adenocarcinomic human alveolar epithelial A549 cell line were purchased from ATCC and cultured as instructed by the manufacturer. Cells were seeded following the first transfection of miRNA mimics and incubated for 1 day before the second transfection with both miRNA mimics and plasmid DNA (for BEAS-2B cells) or with only plasmid DNA (for A549 cells). After the second transfection, cells were incubated for 1 day before TNF- α treatment. For TNF- α stimulation, cells were cultured for 6 h (BEAS-2B) or 1 h (A549) in medium containing 50 ng/ml of TNF- α (18,28). For dual luciferase assays, BEAS-2B cells were transfected with 80 ng of plasmid(s) per 10-cm plate using X-treme GENE9 (Roche)

and A549 cells were transfected with 100 ng of plasmid(s) per 6-cm plate using Lipofectamine 2000 (Invitrogen) following the manufacturers' protocols. Transfections of miRNA mimics, miRNA antagonists (inhibitors) and siRNAs were carried out using Lipofectamine-RNAiMAX (Invitrogen) according to the manufacturer's protocol. MicroRNA and control mimics were purchased from Sigma, including miR-26a (HMI0415), miR-26b (HMI0419) and control mimics (HMC0002). The miR-26 antagonist (Cat #: 4464084) and control inhibitors (Cat #: 4464076) were purchased from ThermoFisher Scientific. Small interference RNAs (siRNAs) were purchased from Sigma and GE Dharmacon including the following: negative control siRNA (SIC001, Sigma), human HMGA1 siRNAs (#1, SASLHs01.00186717, Sigma; #2, SASLHs01.00134689, Sigma) and human MALT1 siRNAs (#1, SASLHs02.0042305, Sigma; #2, J-005936-06-07-08-09, SmartPool from GE Dharmacon). For transfection with both small RNA (i.e. miRNA mimic or siRNA) and plasmid DNA, cells were transfected with small RNAs first and then were transfected with plasmid DNA with or without small RNAs the next day. Transfected cells were harvested 42–48 h after transfection for dual luciferase assays, western blot analysis and/or RNA extractions.

For dual luciferase assays, the renilla and firefly luciferase activities in cell lysates were analysed using a Dual-Glo[®] Luciferase Assay System (Promega) according to the manufacturer's protocol, and luminescence was scanned and recorded with a Tecan Infinite[®]200 Microplate Reader (Tecan Trading AG, Switzerland).

RNA extraction and real-time PCR analysis

Total RNA was extracted using Trizol (Invitrogen) or RNeasy Mini Kit (QIAGEN) following the manufacturer's protocols. For measurements of mRNA decay in BEAS-2B cells, transcription was blocked by actinomycin D (ActD; 5 μ g/ml) and cells were harvested for RNA preparation at various subsequent time points. Real-time quantitative RT-PCR was performed using 1 μ g of total RNA in a 10 μ l reverse transcription reaction containing 50 units of MultiScribe reverse transcriptase (Applied Biosystems). The reaction was incubated at 37°C for 120 min followed by an incubation at 25°C for 10 min and then at 85°C for 5 min. After reverse transcription, 10 μ l PCR reactions containing 1X TaqMan Gene Expression Assay (Applied Biosystems), which has premixed TaqMan MGB probes and primers, 1X TaqMan Universal Master MixII (Applied Biosystems), which has DNA polymerase, dNTP, salt and buffer, and 25–50 ng of cDNA were performed using the LightCycler 384 Real-time PCR system (Roche) according to the manufacturer's protocol. Half-lives of mRNAs were determined by least squares regression of each time point data set to a one-exponential decay equation (29).

The mRNA levels of NF- κ B-responsive genes in BEAS-2B cells were analysed using the Human NF κ B Signaling Targets RT² PCR Profiler Array (Qiagen) according to the manufacturer's protocol. Data analysis was performed using Qiagen online software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

Western blot and ELISA analysis

Total cell or cytoplasmic lysates were prepared as described previously (30). Cell lysates (10 μ g) were resolved on a 7, 10 or 4–15% gradient SDS-polyacrylamide gel and analysed using an ECL Western blotting kit (Bio-Rad). The PVDF blots were probed with appropriate primary antibody at the dilutions indicated below and detected with peroxidase-conjugated secondary antibody and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Primary antibody dilutions were: anti-hMALT1 (Cell Signaling Technology), 1:3000; anti-hHMGA1 (Abcam), 1:10 000; anti- β -actin HRP-conjugated monoclonal antibody (Santa Cruz Biotechnology), 1:20 000 and anti-GAPDH monoclonal antibody (Fitzgerald), 1:20 000. Secondary antibody dilution was: goat anti-rabbit IgG HRP (ThermoScientific), 1:3000. The fluorescence signals and intensity of detected bands were captured and quantified by an imaging system (Syngene) and the software GeneTools (Syngene), respectively. Enzyme-linked immunosorbent assays (ELISA) for IL-6 in culture medium were performed according to the manufacturer's instructions (Affymetrix, eBioscience).

RNA-sequencing

We employed whole transcriptome RNA-seq to investigate changes of TNF- α responsiveness in response to alterations of miR-26 levels in a genome-wide fashion. RNA samples from BEAS-2B cells transfected with miR-26a mimic, control mimic, miR-26 antagomir or control antagomir, with or without TNF- α stimulation, were sequenced by HiSeq 2000 (Axq Technologies). The resulting 100 \times 2 paired-end RNA-seq reads were aligned to the human genome (hg19) and their abundance estimated using TopHat (v1.3.3). We verified the quality of the sequencing with FastQC. More than 12 900 human RefSeq genes can be detected through RNA-seq with expression levels more than 1 FPKM (fragments per kilobase of transcript sequence per million mapped paired-end reads) for each of the eight RNA samples. Additional details of the analysis of RNA-seq datasets can be found in the main text. Raw RNA-sequencing data have been deposited in the Gene Expression Omnibus under accession number GSE70831. All the TCGA-related analyses using Level 3 Processed RNA-seq files were downloaded from Broad Firehose database (<http://gdac.broadinstitute.org/>).

RESULTS

The 3' UTR of IL-6 mRNA does not contain a functional miR-26-responsive site for gene silencing in human BEAS-2B or A549 cells

To investigate the mechanism by which miR-26 regulates IL-6 production in the context of airway inflammation, we chose for the study a widely used human bronchial epithelial cell line BEAS-2B (e.g. see (28,31–33)). Consistent with our previous observation (28), TNF- α significantly increased IL-6 expression in BEAS-2B cells (Supplementary Figure S1A). As the miR-26 family includes miR-26a and miR-26b (34) and both are predicted to recognize target sites containing the same complementary seed region, we checked both

for their effects on IL-6 expression in BEAS-2B cells. The results showed that either miR-26a or miR-26b mimic significantly reduced IL-6 mRNA levels in TNF- α -activated BEAS-2B cells (Supplementary Figure S1B) and IL-6 protein levels in the culture medium (Supplementary Figure S1C) to similar extents.

We then tested whether the predicted miR-26 recognition site in the 3' UTR of the IL-6 mRNA is required to repress IL-6 production. Besides the predicted miR-26 recognition site, the IL-6 3' UTR contains several known or potential RNA destabilizing elements (Supplementary Figure S2), including two AU-rich elements (AREs), an upstream region that carries an endonuclease cleavage site recognized by ribonuclease ZC3H12A (Regnase I) and a potential let-7 binding site (19,35,36). As with many cytokines and chemokines, IL-6 production can be effectively down-regulated through the ARE-mediated mRNA decay pathway (19). For our test, we first introduced the entire IL-6 3' UTR into the 3' UTR of an RL reporter to create the RL-IL-6 3' UTR construct and tested the activity by dual-luciferase assay. Activity from FL mRNA served as an internal control for normalization. The results showed that the luciferase activity from the RL-IL-6 3' UTR reporter (which contains several potential RNA destabilizing elements) was dramatically lower than the RL 3' UTR control in BEAS-2B cells (Figure 1A). We then truncated either the seed region of the predicted miR-26 recognition site or an ARE in the IL-6 3' UTR to create the RL-IL-6 3' UTR(Δ 26) or RL-IL-6 3' UTR(Δ AREI) construct. The dual-luciferase assay showed that the activity derived from the RL-IL-6 3' UTR(Δ AREI) reporter is appreciably higher than that from the reporter carrying IL-6 3' UTR (Figure 1A), consistent with a gene silencing function of the ARE (19). On the other hand, the activity derived from the RL-IL-6 3' UTR(Δ 26) reporter is similar to that from the reporter carrying IL-6 3' UTR (Figure 1A). Thus, the predicted miR-26 recognition site does not contribute to the activity of IL-6 3' UTR to down-regulate IL-6 level.

We then tested whether the predicted miR-26 recognition site in IL-6 3' UTR, albeit is dispensable, might be responsive to miR-26 for gene silencing function. We introduced three consecutive copies of the site (Supplementary Figure S3A) into the RL reporter to create the RL-3 \times 26(IL-6) construct and transfected it into BEAS-2B cells with miR-26 mimics or a negative control mimic. For a positive control, we introduced three consecutive copies of a miR-26 recognition site found in human TNRC6A (37), also known as GW182 (Supplementary Figure S3B), into the RL 3' UTR to create RL-3 \times 26(GW182). The dual-luciferase assay showed significantly lower activity from the RL-3 \times 26(GW182) transcript in the presence of either miR-26a or miR-26b mimic than in the presence of the control mimic (Figure 1B, C, and Supplementary Figure S4A). In contrast, the respective luciferase activities derived from the RL 3' UTR, RL-IL-6 3' UTR, RL-IL-6 3' UTR(Δ 26) and RL-3 \times 26(IL-6) transcripts in the presence of miR-26 mimics were similar to the corresponding activities in the presence of a control mimic (Figure 1B, C, and Supplementary Figure S4A). Taken together, these results indicate that the IL-6 3' UTR lacks a miR-26-responsive site in BEAS-2B cells.

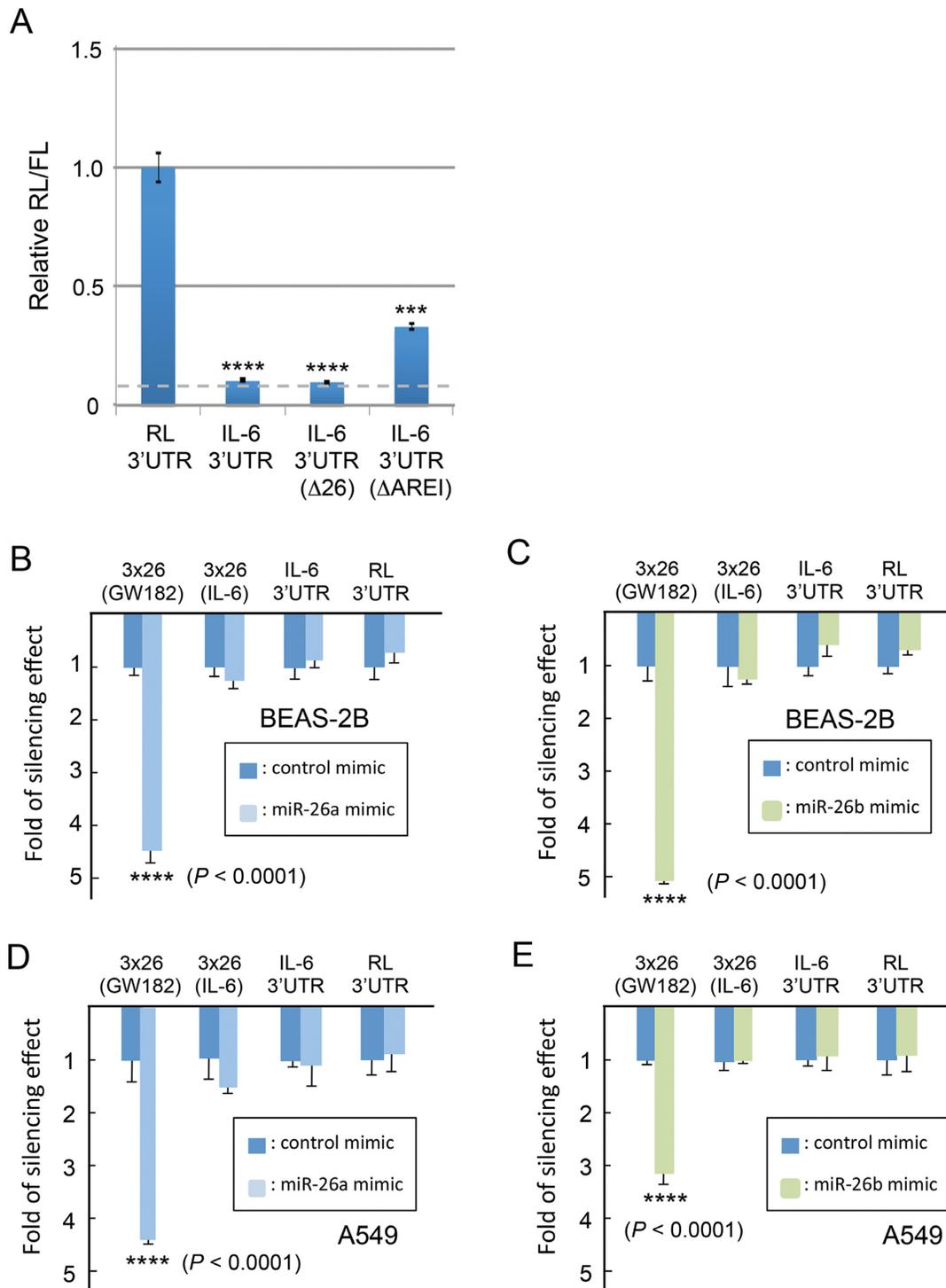


Figure 1. The 3' UTR of IL-6 mRNA does not contain a functional miR-26-responsive site for gene silencing in human BEAS-2B or A549 cells. (A) Histogram showing that the predicted miR-26 recognition site in the IL-6 3' UTR is dispensable for silencing function. RL activities in BEAS-2B cells expressing RL reporter mRNA carrying IL-6 3' UTR, IL-6 3' UTR with miR-26 seed region truncated (IL-6 3' UTR(Δ26)) or IL-6 3' UTR with deletion of an ARE (IL-6 3' UTR(ΔAREI)) were detected by dual-luciferase assay. FL activity derived from the same plasmid carrying the RL gene served as a control for normalization. The RL/FL activity detected in cells expressing the RL 3' UTR control mRNA was set as 1. All data represent the mean \pm standard error ($n = 3$). (B–E) Histograms showing relative changes of RL activity derived from the indicated reporter mRNAs in TNF- α -stimulated BEAS-2B (B and C) or A549 (D and E) cells in the presence of miR-26a (light blue bars), miR-26b (light green bars), or a control miRNA mimic (dark blue bars). The RL reporter mRNA carrying three copies of the predicted miR-26 recognition site from human GW182 (3 \times 26(GW182)) served as a positive control. FL activity, derived from the same plasmid carrying the RL reporter gene, was used for normalization. The relative silencing effects were measured by comparing the RL/FL activity detected in cells expressing each reporter mRNA as indicated in the presence of miR-26 mimics with that detected in cells expressing the corresponding reporter mRNA in the presence of the control miRNA mimic (set as 1, representing no silencing effect). All data represent the mean \pm standard errors ($n = 3$). T-test was done to assess statistical significance. *** $P < 0.001$; **** $P < 0.0001$.

Since a previous study suggested that miR-26 directly targets the predicted miR-26 recognition site in IL-6 3' UTR in A549 cells (18), we also looked for silencing function of the IL-6 3' UTR, IL-6 3' UTR(Δ 26) or 3 \times 26(IL-6) in response to miR-26 mimics or a control mimic in A549 cells. The dual-luciferase assay (Figure 1D, E, and Supplementary Figure S4B) showed that the activity derived from the positive control RL-3 \times 26(GW182) transcript was effectively repressed by miR-26 mimics, whereas the activities derived from the reporters carrying the IL-6 3' UTR, IL-6 3' UTR(Δ 26) or 3 \times 26(IL-6) were similar with miR-26 mimics and control mimic in A549 cells. Thus, the predicted miR-26 recognition site in IL-6 3' UTR does not respond to miR-26 mimics in either BEAS-2B or A549 cells. We conclude that the 3' UTR of the IL-6 mRNA does not contain a functional miR-26-responsive site.

MiR-26 does not directly target IL-6 mRNA per se for gene silencing

We then considered whether miR-26 might exert gene silencing effects by targeting an unexpected recognition site in other regions of the IL-6 transcript. We introduced the 5' UTR or the open-reading frame (ORF) of IL-6 into the 3' UTR of RL mRNA and evaluated the corresponding luciferase activities in BEAS-2B cells co-transfected with the miR-26a mimic or a control mimic. The dual-luciferase assay (Figure 2A) showed that while miR-26a mimic appreciably repressed the luciferase activity derived from the control RL-3 \times 26(GW182) transcript, it had little effect on the activities from the transcripts containing either IL-6-5' UTR or IL-6-ORF. These results indicate that the entire IL-6 transcript lacks a sequence suitable for direct interaction with miR-26 to exert a gene silencing function.

As mammalian miRNAs silence their direct targets largely through eliciting rapid degradation of their mRNAs (38–40), to further corroborate the above findings, we checked whether miR-26 can promote IL-6 mRNA degradation. We performed time-course experiments using actinomycin D to block transcription in BEAS-2B cells activated by TNF- α . The results showed that while the miR-26a and miR-26b mimics significantly reduced the stability of the positive control mRNA, MAP kinase 6 (MAPK6) transcript (Figure 2B), neither of them had a destabilizing effect on the IL-6 mRNA (Figure 2C). Collectively, we conclude that miR-26 does not directly target the IL-6 transcript for gene silencing.

MiR-26 down-regulates IL-6 production through dampening IL-6 transcription activated by TNF- α /NF- κ B signalling

The steady-state level of a cytoplasmic mRNA represents a balance between its biogenesis in the nucleus and its degradation in the cytoplasm (29). In light of the finding that miR-26 does not directly target the IL-6 transcript for mRNA degradation (Figures 1 and 2), we tested whether miR-26 down-regulates the IL-6 level by repressing IL-6 transcription activated by TNF- α . We constructed a human IL-6 promoter-driven FL reporter gene (IL-6-FL) containing the transcription elements described previously (25–27). Results from dual luciferase assays with this reporter (Figure 3A) showed that the human IL-6 promoter responds

strongly to TNF- α treatment, giving a \sim 15x induction of luciferase activity in the presence of the control miRNA mimic (Figure 3A), which directly parallels the level of TNF- α induction seen from the endogenous IL-6 gene in BEAS-2B cells (Supplementary Figure S1A). In the presence of the miR-26a mimic, the induction of luciferase activity derived from IL-6-FL was only \sim 5x, one-third of the induction in the presence of the control mimic. Moreover, the steady-state level of endogenous IL-6 mRNA in the presence of the miR-26a mimic was \sim 35% (i.e. also about one-third) of the level in the presence of the control mimic (Figure 3B). In contrast, there was little induction of luciferase activity derived from a negative control RL reporter gene driven by human GAPDH promoter (GAPDH-RL) (Figure 3C), and the GAPDH promoter activity was the same with the miR-26a mimic as with the control mimic (Figure 3C). These results indicate that miR-26 down-regulates the TNF- α -induced IL-6 promoter activity.

Since TNF- α can activate NF- κ B, a transcription factor that plays an important role in TNF- α -mediated activation of many genes encoding cytokines and chemokines, including IL-6 (25,41), we tested whether miR-26 can compromise TNF- α -mediated activation of NF- κ B signalling. We used the dual luciferase assay to measure the effect of the miR-26a mimic on the activity of an FL reporter gene driven by a minimal promoter containing a copy of the NF- κ B response element. With the control mimic, TNF- α stimulation gave a \sim 250x induction of luciferase activity from this reporter (Figure 3D). In the presence of miR-26a mimic, the TNF- α induction of activity driven by this NF- κ B-responsive promoter was $<$ 100x, which is \sim 38% of the induction in the presence of the control mimic (Figure 3D). Collectively, the results (Figure 3A–D) demonstrated that the miR-26a mimic blunts TNF- α activation of both the IL-6 and NF- κ B promoters, but not the GAPDH promoter. We conclude that miR-26 decreases IL-6 production through silencing the transcription of IL-6 promoter that is activated by TNF- α /NF- κ B signalling.

Investigating the effect of miR-26 on TNF- α /NF- κ B-responsive genes at the transcriptome level

As NF- κ B signalling plays a key role in the activation of many cytokine and chemokine genes, our findings (Figure 3A–D) suggest a previously unknown and broad effect of miR-26 on NF- κ B-responsive genes. Thus, we tested whether miR-26 also down-regulates expression of other genes besides IL-6. We first carried out qRT-PCR analysis using a NF- κ B signalling target gene array containing 84 NF- κ B-responsive genes. We readily detected more than 58 genes expressed in BEAS-2B cells in two replicates. At least 31 of these expressed genes (Supplementary Table S1) had a $>$ 2-fold induction by TNF- α treatment in the presence of a control miRNA mimic. Analysis using the data from both PCR-array experiments and a high-depth RNA sequencing experiment (see below) showed that the induction levels of 17 of the 31 TNF- α /NF- κ B-responsive genes in the presence of the miR-26a mimic were appreciably reduced compared with the control values (Figure 3E). We conclude that miR-26 can down-regulate the activation of many NF- κ B-responsive genes in BEAS-2B cells. Many of these genes,

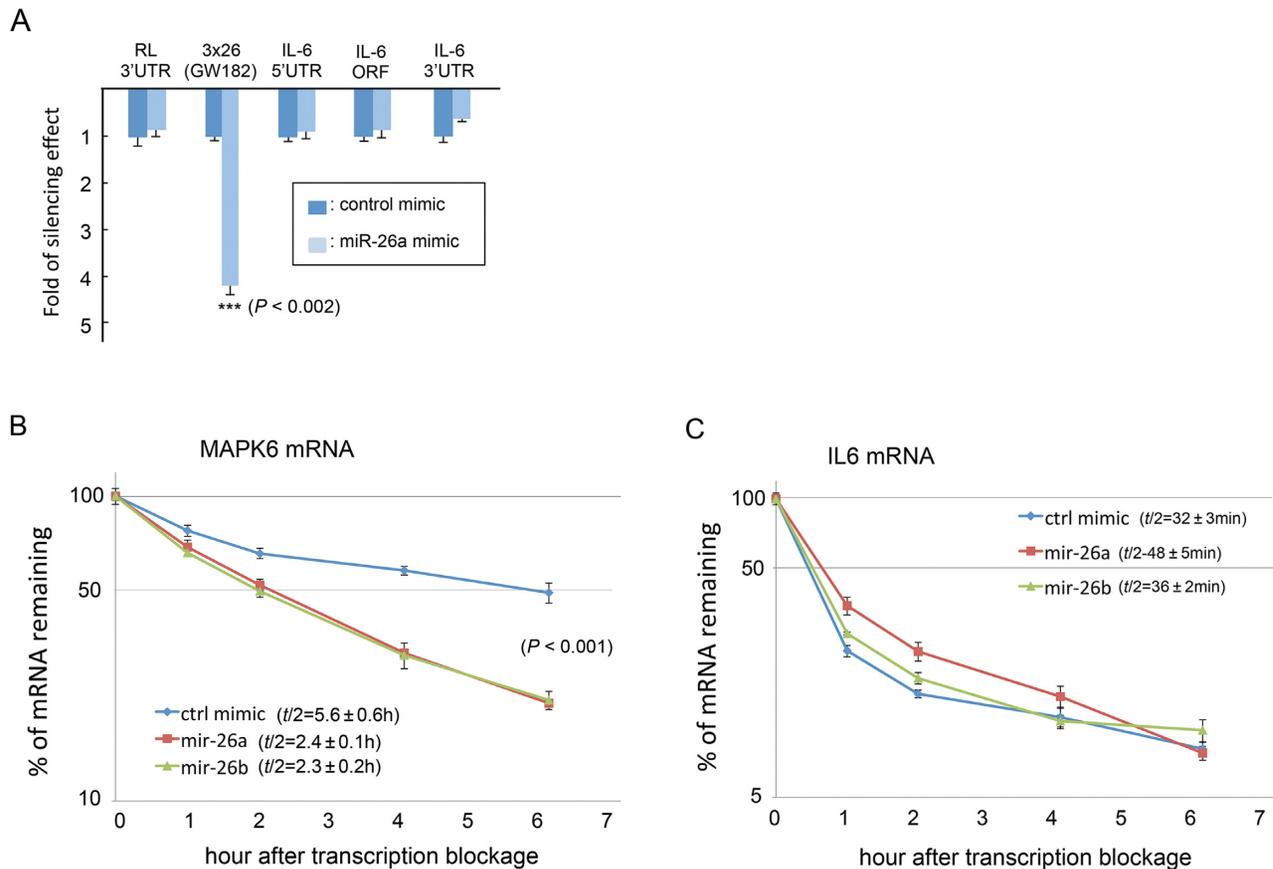


Figure 2. The IL-6 mRNA is not a direct target of miR-26 for gene silencing. (A) Histogram showing that the IL-6 5' UTR, IL-6 ORF and IL-6 3' UTR do not exhibit any silencing effect in cells treated with miR-26a mimic. The RL reporter mRNA carrying three copies of the predicted miR-26 recognition site from human GW182 (3×26(GW182)) served as a positive control. RL activities derived from the indicated RL reporter mRNAs in BEAS-2B cells in the presence of miR-26a (light blue bars) or a control miRNA mimic (dark blue bars) were detected by dual-luciferase assay. FL activity, derived from the same plasmid carrying the RL reporter gene, was used for normalization. The relative silencing effects were measured by comparing the RL/FL activity detected in cells expressing each reporter mRNA as indicated in the presence of miR-26a mimic with that detected in cells expressing the corresponding reporter mRNA in the presence of the control miRNA mimic (set as 1, represents no silencing effect). (B and C) miR-26a and miR-26b mimics destabilize the MAPK6 mRNA (B), but neither destabilizes IL-6 mRNA (C). BEAS-2B cells transfected with miR-26a (red line), miR-26b (green line) or a control miRNA mimic (blue line) were stimulated with TNF- α for 6 h and then treated with Actinomycin D (ActD; 5 μ g/ml) to block transcription. Cells were harvested immediately (time 0) and after 1, 2, 4 or 6 h of ActD treatment. The levels of endogenous MAPK6 mRNA (B) or IL-6 mRNA (C) were quantified by real-time RT-PCR and normalized to the amount of an internal control, GAPDH mRNA. Half-lives shown in the semi-log plots were obtained by least squares analysis of the percentage of mRNA remaining as a function of time. All data represent the mean \pm standard errors ($n = 3$). T-test was done to assess statistical significance.

such as C3, CXCL1, CXCL2, IL-6, CXCL8 (IL-8), SOD2 and MMP9, are directly related to inflammation and tissue remodelling (42,43).

To better characterize the wider effects of miR-26 on TNF- α /NF- κ B signalling, we performed high-depth ($5-7 \times 10^7$ reads) RNA sequencing (RNA-seq) using RNA samples from TNF- α -stimulated or non-stimulated BEAS-2B cells that had been transfected with either miR-26a or negative control mimics. We used this transcriptome-based approach to identify additional TNF- α /NF- κ B-responsive genes besides IL-6 and the other 16 genes described above (Figure 3E) whose transcription activation by TNF- α is affected either directly or indirectly by changes in miR-26 level. We also performed parallel high-depth RNA-seq using RNA samples from cells treated with a miR-26 inhibitor (antagomir) to deplete endogenous miR-26 or with a control inhibitor. We looked for genes whose activation by TNF- α is not only repressed by the miR-26a mimic but

also enhanced (de-repressed) by the miR-26a inhibitor. We used as a baseline the response to TNF- α treatment (calculated as the ratio of signals with and without TNF- α) in the presence of a control mimic, compared to the response with a miR-26 mimic (Supplementary Figure S5, left), or in the presence of a control inhibitor, compared to the response with a miR-26 inhibitor (Supplementary Figure S5, right). The genes exhibiting a differential response to the two conditions (i.e. induction by TNF- α is repressed by a miR-26 mimic or induction by TNF- α is enhanced by a miR-26 inhibitor) are predicted to be miR-26-affected. We modelled the 2-fold changes as a linear relationship and calculated the distance from each gene to the identity line. Using the distribution of the distances for all expressed genes, we calculated z scores to quantify the responses to the miR-26a mimic (Z_{mimic}) or inhibitor ($Z_{\text{inhibitor}}$). We then looked for genes whose induction decreased in the presence of a miR-26a mimic ($Z_{\text{mimic}} < 0$) and increased in the presence of a

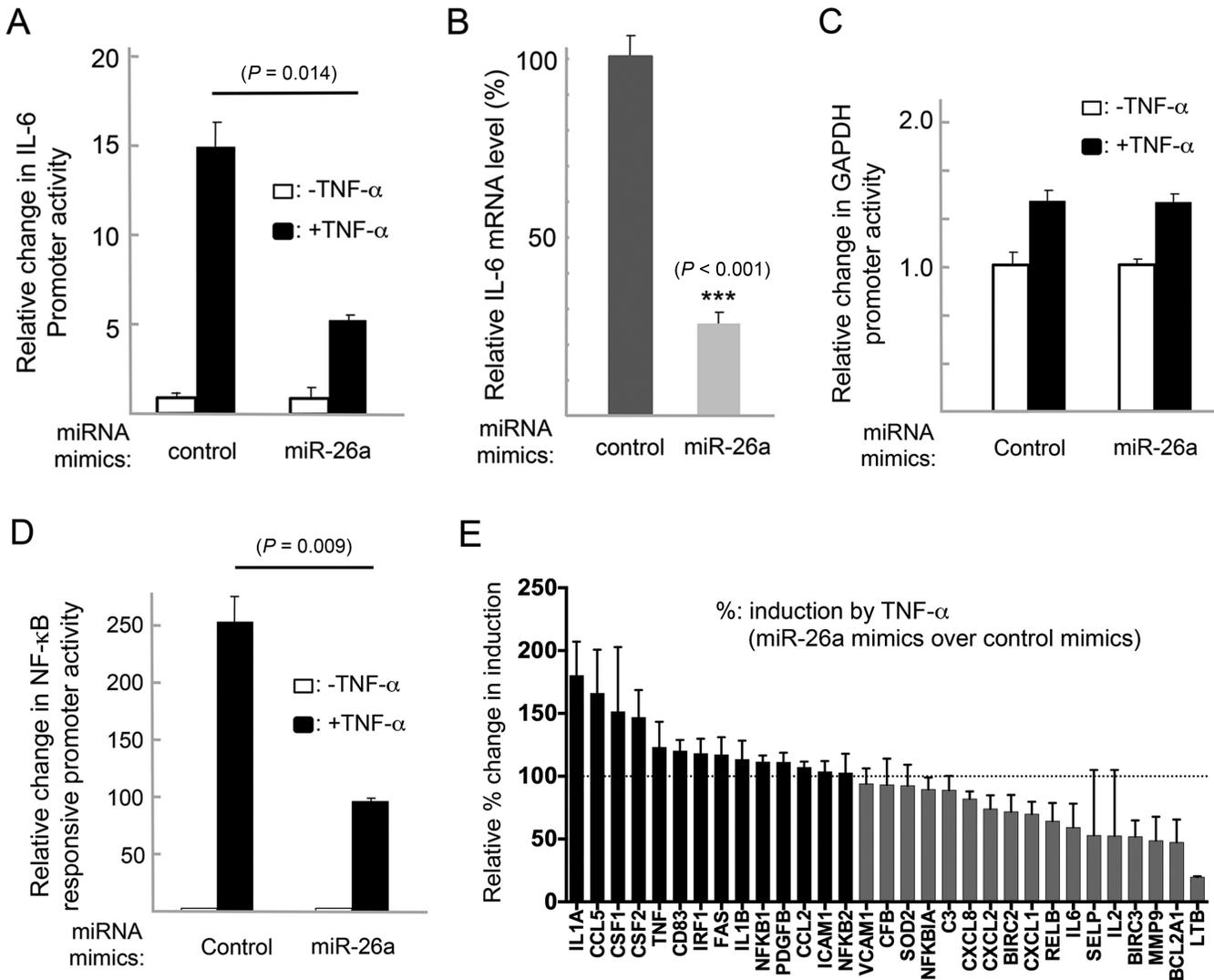


Figure 3. MiR-26 dampens IL-6 production through silencing IL-6 transcription activated by TNF- α /NF- κ B signalling axis. (A) Histogram showing relative changes in luciferase activity following TNF- α treatment of BEAS-2B cells expressing an FL reporter mRNA driven by the human IL-6 promoter in the presence of the miR-26a or a control miRNA mimic. Cells were co-transfected with a plasmid expressing RL as an internal control for luciferase activity quantification. (B) Histogram showing relative levels of endogenous IL-6 mRNA in the TNF- α -stimulated BEAS-2B cells transfected with the miR-26a or a control miRNA mimic. Endogenous IL-6 mRNA was quantified by real-time RT-PCR and normalized to the amount of an internal control, GAPDH mRNA. The IL-6 mRNA level in cells transfected with the control miRNA mimic was set as 100%. (C and D) Histogram showing relative changes in luciferase activity following TNF- α treatment of BEAS-2B cells expressing a luciferase reporter mRNA driven by human GAPDH promoter (C) or by a minimal promoter containing the NF- κ B responsive element (D) in the presence of the miR-26a or a control miRNA mimic. Cells were co-transfected with a plasmid expressing a different luciferase as an internal control for luciferase activity quantification. All data represent the mean \pm standard errors ($n = 3$). T-test was done to assess statistical significance. (E) MiR-26 down-regulates many NF- κ B-responsive genes in BEAS-2B cells. Bar graph showing relative mRNA levels (miR-26a mimic/control mimic) for 31 NF- κ B-responsive genes in TNF- α -stimulated BEAS-2B cells. The mRNA expression of these genes was readily detected by RT-qPCR based assay in two replicates using Qiagen human NF- κ B-responsive gene PCR array, and each gene was induced at least 2-fold by TNF- α treatment. These changes were further confirmed in an RNA-Seq experiment (Supplementary Table S1), except for 6 that lacked sufficient read depth. The bars represent the mean induction of the three experiments (except for the ones undetectable by RNA-Seq), and the error bars show the SEM. Gray bars: TNF- α -induced expression is decreased by miR-26 mimic; black bars: TNF- α -induced expression is either unaffected or enhanced by miR-26 mimic.

miR-26a inhibitor ($Z_{\text{inhibitor}} > 0$) (Figure 4A). Using this approach, we identified 103 genes (Figure 4A and Supplementary Table S2) whose induction by TNF- α was affected by changes in the level or activity of cellular miR-26 (Figure 4A and B).

To attribute functional categories to these 103 miR-26-affected genes, we looked for enriched Gene Ontology terms using GATHER (44). The functional categories most as-

sociated with these genes were immune response (FDR = 2×10^{-9}) and inflammatory response (FDR = 1×10^{-7}) (Supplementary Table S3). These functions are consistent with cellular inflammatory response and gene activation involving TNF- α /NF- κ B signalling (45). Indeed, the top hit from Ingenuity analysis of the 103 genes was a network that contains the NF- κ B complex as a major hub (Supplementary Figure S6).

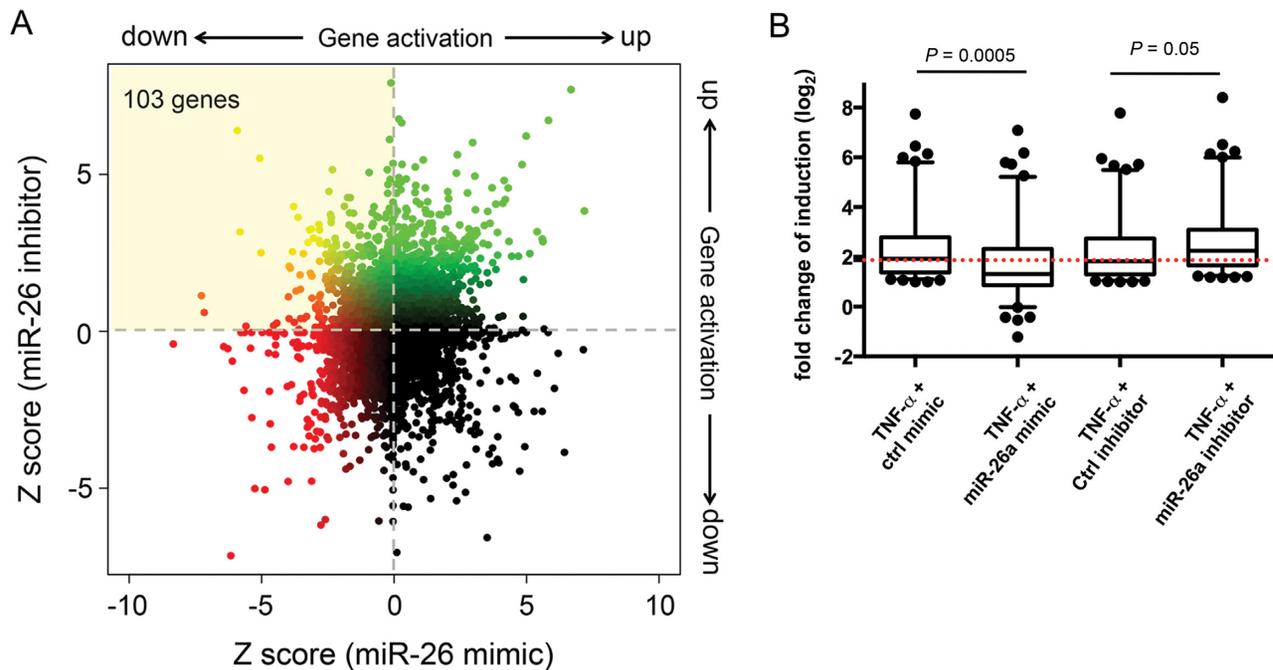


Figure 4. Transcriptome-wide screening in BEAS-2B cells for genes whose induction by TNF- α is sensitive to changes in miR-26a level. (A) Scatter plot showing identification of genes that are sensitive to changes in cellular miR-26 level. Each gene's Z-score for miR-26 inhibitor is plotted as a function of that gene's Z-score for a miR-26 mimic (see the main text and the legend to Supplementary Figure S5 for more details). A total of 103 genes (see Supplementary Table S2) in the upper left area with a Z-score (miR-26 mimic treated) < 0 and a Z-score (miR-26 inhibitor treated) > 0 were considered affected and subjected to further bioinformatics analysis. Green dots: up-regulated genes with a Z-score > 0 upon miR-26 inhibitor treatment; red dots: down-regulated genes with a Z-score < 0 upon miR-26 mimic treatment; yellow and orange dots: genes that satisfy both conditions. (B) Boxplot showing relative changes in TNF- α -mediated induction of the 103 genes identified in panel A when miR-26 levels were increased by miR-26 mimic or reduced by miR-26 inhibitor. The red dotted line marks a 4-fold induction by TNF- α treatment. T-test was done to assess statistical significance.

To further address the involvement of NF- κ B signalling in controlling the activation of these 103 genes by TNF- α , we also used the GATHER application to identify the transcription factor binding sites that are the most common among the 103 genes. Remarkably, the three most significant hits were all matrices for NF- κ B complex (FDR = 0.03) (Supplementary Table S4). In addition, we used the Ingenuity Pathway Analysis algorithm to look for upstream signalling pathways that might lead to the TNF- α activation of the 103 genes. The top three hits were TNF (P -value of overlap = 7.04×10^{-31}), NF- κ B complex (P -value of overlap = 4.12×10^{-24}) and RelA (P -value of overlap = 5.28×10^{-17}) (Supplementary Table S5). Taken together, the results of these unbiased analyses of the 103 miR-26-affected genes are all consistent with their being activated through NF- κ B signalling pathway.

Identification of NF- κ B signalling factors targeted by miR-26

The results described above suggest that miR-26 might act through silencing one or more factors required for proper activation of the NF- κ B signalling pathway by TNF- α . Therefore, we analysed the RNA-seq datasets to identify NF- κ B signalling related factors whose mRNA levels were lowered by miR-26 mimics in both TNF- α -stimulated and non-stimulated cells. We first calculated Z-scores for genes from the differences in their RNA levels in non-stimulated BEAS-2B cells treated with either miR-26a mimic or a con-

trol mimic. We identified 949 genes whose expression decreased upon miR-26a mimic treatment (Z-score < -1) (Supplementary Figure S7A). To filter out potential indirect targets, we focused on the transcripts that were also enriched in Ago-CLIP-Seq tags with miR-26 using the 'starBase' platform (46,47). This analysis yielded a group of 396 potential miR-26a direct targets. To identify miR-26a targets likely to be involved in NF- κ B signalling, we separately compiled a list of 334 reported or potential TNF- α /NF- κ B signalling related factors (23,48–53) (Supplementary Table S6) and then selected genes appearing in both this list and the group of potential miR-26a direct targets. This analysis yielded four candidates for NF- κ B signalling related miR-26 targets: BAG4, HMGA1, MALT1 and MAP3K1.

We performed a similar analysis using RNA-seq data obtained from BEAS-2B cells stimulated with TNF- α , producing a group of 804 transcripts whose mRNA levels are lowered by miR-26a mimics in TNF- α -activated cells (Z-score < -1) (Supplementary Figure S7B). Of these transcripts, 122 were either marginally or significantly enriched in Ago-CLIP-Seq tags with miR-26. Four of these also appeared on the list of reported or potential TNF- α /NF- κ B signalling related factors (Supplementary Table S6) and were thus candidates for NF- κ B signalling related miR-26 targets: HMGA1, MALT1, PPP2R5E and ZNF462. Collectively, the above analyses of TNF- α -stimulated and non-stimulated conditions yielded two common genes, HMGA1 and MALT1; both genes have an established role in mediat-

ing NF- κ B signalling (22–24,54). Therefore, we focused on these two genes in subsequent studies.

MiR-26 down-regulates IL-6 and many other NF- κ B-responsive genes through silencing HMGA1 and MALT1

To verify that miR-26 can silence HMGA1 and MALT1 expression, we first performed western blot analysis (Figure 5A) and showed that the miR-26a mimic, but not a control mimic, greatly reduces the level of HMGA1 and moderately diminishes the level of MALT1 in BEAS-2B cells. As mammalian miRNAs silence their direct targets largely through eliciting rapid degradation of their mRNAs (38–40), we also checked the destabilizing effects of miR-26 on the HMGA1 and MALT1 transcripts. The results (Figure 5B) showed that the miR-26a mimic reduces the half-life of the HMGA1 mRNA from >12 to ~6.8 h and of the MALT1 transcript from ~6.9 to ~3.7 h. In contrast, little effect on IL-6 mRNA stability was observed (Figures 2C and 5B). These results not only demonstrate the destabilizing effects of miR-26 on the HMGA1 and MALT1 transcripts but also further substantiate that IL-6 transcript is not a direct target of miR-26. Moreover, knocking down either HMGA1 or MALT1 dramatically reduces the IL-6 mRNA levels in TNF- α -activated BEAS-2B cells (Figure 5C and D), indicating that both HMGA1 and MALT1 are required for a full TNF- α mediated induction of IL-6 expression in BEAS-2B cells.

We then tested whether HMGA1 and MALT1 are involved in the miR-26-mediated repression of the 17 NF- κ B-responsive genes described in Figure 3E (gray bars). We knocked down both HMGA1 and MALT1 simultaneously to an extent similar to that seen with miR-26a mimics in TNF- α -activated cells (Figure 6A), and the levels of IL-6 transcript were reduced to ~20% of the control levels (Figure 6B). We then evaluated the impact of this knockdown on the 17 miR-26-affected NF- κ B-responsive genes using NF- κ B target gene PCR array (Figure 6C). Fourteen of the 17 NF- κ B-responsive genes (including IL-6) that were down-regulated by miR-26a were also down-regulated to a comparable extent by the knockdown of HMGA1 and MALT1 (Figure 6C). Taken together, our results indicate that the suppressive effect of miR-26a on many NF- κ B-responsive genes, including IL-6, is largely attributable to the ability of miR-26 to silence HMGA1 and MALT1.

Inverse relationship between levels of miR-26 and of HMGA1 or MALT1 transcripts in lung adenocarcinoma

The observation that miR-26 down-regulates NF- κ B signalling is consistent with the notion that miR-26 levels may be altered in human cancers (2–5). We thus used the starBase Pan-Cancer Networks platform to analyze clinical mRNA and miRNA expression profiles of 12 cancer types from The Cancer Genome Atlas (TCGA) data portal (46,47). We found that 9 of the 12 cancers exhibit decreased levels of miR-26a (Supplementary Table S7). We then used the starBase platform to perform a Pearson correlation analysis of the miR-26a levels and the mRNA levels of HMGA1 or MALT1 in different cancers. The results (Supplementary Table S8) showed that four of the nine cancer types, including breast cancer, head and neck squamous

cell carcinoma, lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), display a significant inverse relationship between miR-26a and HMGA1 mRNA levels. On the other hand, colon and rectal adenocarcinoma and glioblastoma multiform exhibit a marginal inverse relationship between miR-26a and MALT1 mRNA levels (Supplementary Table S9). As our study uses a human bronchial epithelial cell line model, it is worth noting that both lung-related cancers (LUAD and LUSC) display a significant inverse relationship between miR-26a and HMGA1 mRNA levels (Figure 7A).

We then focused on LUAD and LUSC cancers for patient survival analysis. We first stratified the TCGA datasets of LUAD and LUSC patients according to differential miR-26 expression and performed Kaplan–Meier analysis, focusing on patient groups with high levels of miR-26a relative to those with low levels to measure survival time for each of the two cancers. This analysis did not lead to any significant correlation between miR-26a expression levels and mortality due to LUAD or LUSC (data not shown). However, when we looked for the combined change of miR-26a levels and HMGA1 levels in a reverse manner (Figure 7B), we discovered that the survival rate of LUAD patients with high miR-26a levels and low HMGA1 mRNA levels (blue line, 50% survival at 53 months) is higher than that of the LUAD patients with low miR-26a levels and high HMGA1 mRNA levels (red line, 50% survival at 20 months). At 120 months post prognosis, the difference between the two groups is even larger, with only 5% survival for tumours with low miR-26a expression and high HMGA1 expression versus 20% survival in the group with high miR-26a and low HMGA1 (Figure 7B). The overall survival for LUAD patients analysed using miR-26a/MALT1 datasets also showed a similar trend, albeit less significant than that using the miR-26a/HMGA1 datasets (Figure 7C). It is worth noting that at 120 months post prognosis, the difference became quite marked, with no survival for patients with tumours expressing low miR-26a and high MALT1 expression versus 20% patient survival with tumours expressing high miR-26a and low MALT1 expression (Figure 7C). Similar analyses of patient survival using the LUSC dataset did not yield significant correlations (data not shown).

We also analysed the IL-6 mRNA levels in LUAD patients. IL-6 mRNA levels were slightly higher in the low miR-26a LUAD patients than in the high miR-26a patients (data not shown). However, when we compared IL-6 mRNA levels between patient groups by factoring in the inverse relationship between miR-26a and HMGA1 transcript levels, we observed a significant difference in IL-6 mRNA levels ($P = 0.001$, Student's *t* test) (Figure 7D, left). Likewise, we also observed a difference of IL-6 mRNA levels in the case of miR-26a versus MALT1 patient groups (Figure 7D, right), albeit somewhat less significant. Collectively, these analyses reveal associations between miR-26a-regulated changes in HMGA1, MALT1 and IL-6 mRNA levels and LUAD patient survival. In particular, miR-26a and HMGA1 transcript levels may have prognostic value in cancer patients with lung adenocarcinoma.

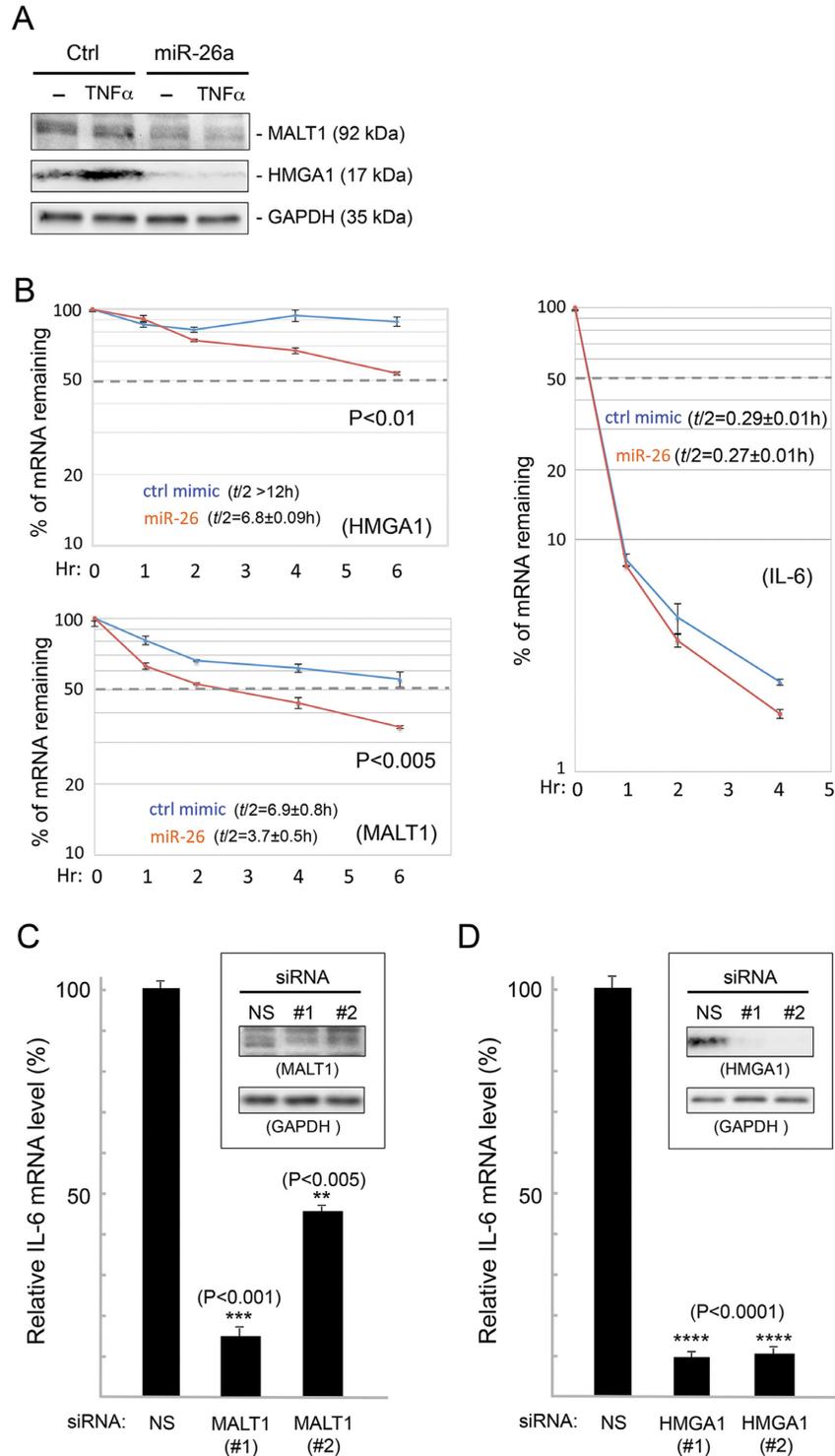


Figure 5. MiR-26 down-regulates IL-6 production through silencing HMGA1 and MALT1. (A) Western blot showing silencing effects of miR-26a at the protein levels of HMGA1 and MALT1 in both non-activated and TNF- α -activated BEAS-2B cells. (B) Effects of miR-26a mimics on decay kinetics of HMGA1, MALT1 and IL-6 mRNAs. BEAS-2B cells were activated by TNF- α and then treated with actinomycin D (ActD; 5 μ g/ml) to block transcription. Cells were harvested immediately (time 0) and after 1, 2, 4 or 6 h of ActD treatment. Transcript levels were quantified by real-time RT-PCR and normalized to the amount of an internal control, GAPDH mRNA. Half-lives shown in the semilog plots were obtained by least squares analysis of the percentage of mRNA remaining as a function of time. (C and D) Effects of knocking down HMGA1 (C) or MALT1 (D) on IL-6 mRNA levels. BEAS-2B cells were activated with TNF- α for 6 h. For HMGA1 knockdown, two different individual siRNAs (#1 and #2) were used. For knocking down MALT1, one individual siRNA (#1) and a pool of four different siRNAs (GE Dharmacon SmartPool; none of these four siRNAs shares the same sequence as #1 siRNA) (#2) were used. IL-6 mRNA levels were quantified by real-time RT-PCR and normalized to the amount of GAPDH mRNA. The IL-6 mRNA level detected in the cells transfected with the control (NS) siRNA (after normalization) was set as 100%. Insets in panels C and D are western blots showing the knockdown efficiency. All data represent the mean \pm standard error ($n = 3$). T-test was done to assess statistical significance.

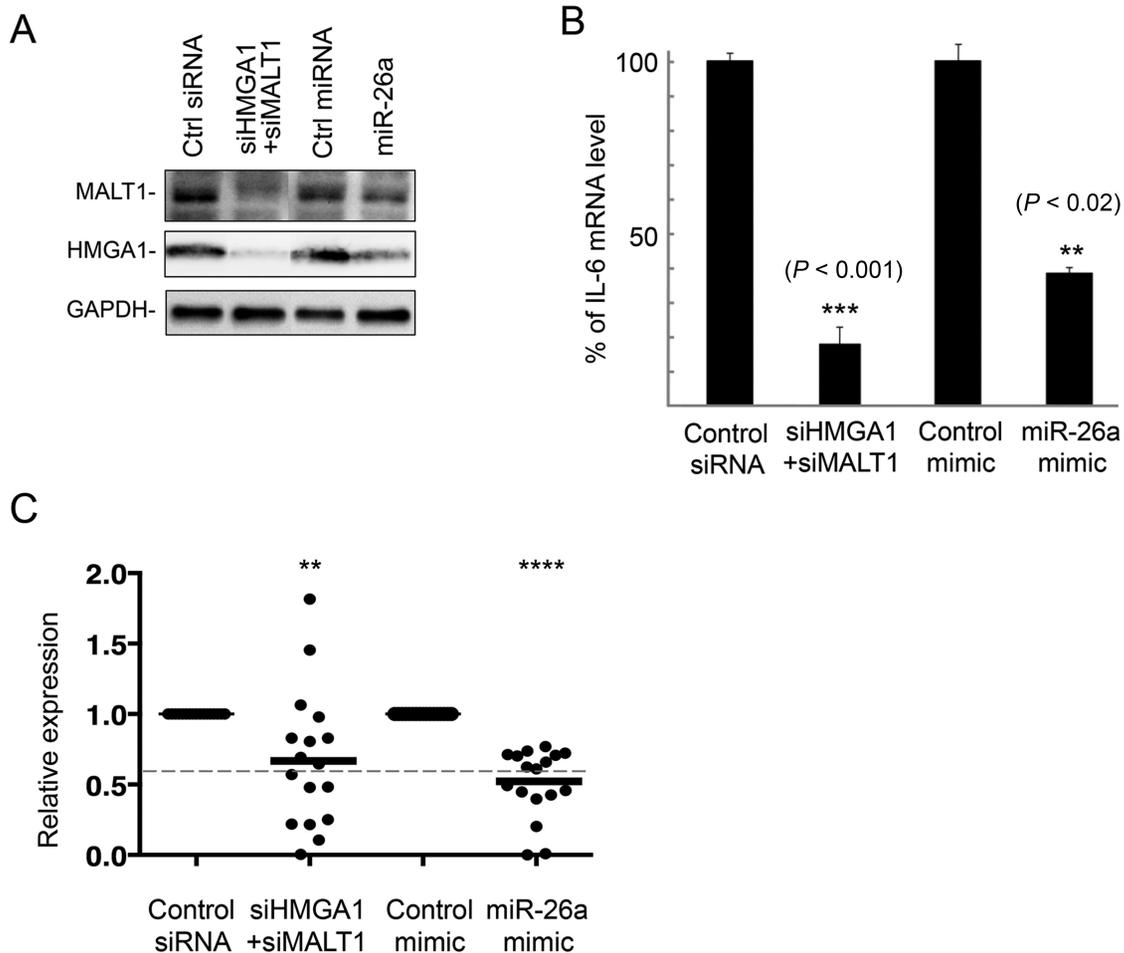


Figure 6. Silencing MALT1 and HMGA1 largely accounts for the suppressive effect of miR-26a on the induction of IL-6 and many other NF- κ B-responsive genes. (A and B) Effects of RNAi knockdown of MALT1 and HMGA1 and miR-26a mimic treatment on IL-6 mRNA levels in BEAS-2B cells. (A) Western blot showing the efficiency of silencing HMGA1 and MALT1. (B) Histogram showing IL-6 mRNA levels quantified by real-time RT-PCR and normalized to GAPDH mRNA. The IL-6 mRNA level detected in cells transfected with control siRNA or control miRNA mimic (after normalization) was set as 100%. All data represent the mean \pm standard error ($n = 3$). (C) Effects of knocking down both MALT1 and HMGA1 or effects caused by the miR-26 mimic on the TNF- α -induced expression of NF- κ B-responsive genes. The relative mRNA level of each of the 17 NF- κ B-responsive genes, described in Figure 3E (gray), in cells transfected with miR-26a mimic or with HMGA1 and MALT1 siRNAs was normalized to mRNA level of the same gene in cells transfected with control miRNA mimic or control siRNA (set as 1). The scattering and average of the mRNA levels of the 17 genes under these different conditions were plotted as a combined bee swarm representation. A dashed line was drawn to assist comparisons of average fold change in each treatment. T-test was done to assess statistical significance. ** $P < 0.01$; **** $P < 0.0001$.

DISCUSSION

In this study, we found that miR-26 dampens IL-6 expression by down-regulating the TNF- α /NF- κ B signalling axis through silencing two NF- κ B signalling factors, HMGA1 and MALT1, and not by directly targeting IL-6 mRNA. These findings are at odds with a previous study by another group which, based on some circumstantial observations, suggested that miR-26 silences IL-6 expression by directly targeting a predicted recognition site in IL6 3' UTR for rapid mRNA decay in A549 cells (18). Moreover, our data showed that through down-regulating NF- κ B signalling, miR-26 dampens the expression of not only IL-6 but also many other NF- κ B-responsive genes. We further discovered that poor patient prognoses in human lung adenocarcinoma are associated with the combination of low miR-26 levels and high HMGA1, MALT1 or IL-6 levels but not with any of them individually.

Several lines of direct evidence indicate that miR-26 does not directly target IL-6 mRNA for gene silencing. First, truncating the seed region of the predicted miR-26 recognition site does not affect the silencing function of IL-6 3' UTR (Figure 1A; compare IL-6 3' UTR(Δ 26) and IL-6 3' UTR). This finding is consistent with an earlier study which showed that mutating the predicted miR-26 site does not change translation of IL-6 mRNA in HeLa cells (20). Moreover, a sub-region of IL-6 3' UTR containing the predicted miR-26 was shown to have little destabilization effect on the mRNA in either monkey COS-7 or mouse NIH3T3 cells (19). Together, these observations indicate that the predicted miR-26 recognition site in IL-6 3' UTR is not only dispensable but also nonfunctional for gene silencing. Second, even when miR-26 mimics were introduced into either BEAS-2B or A549 cells, the expression levels of a reporter bearing either the entire IL-6 3' UTR or three copies of

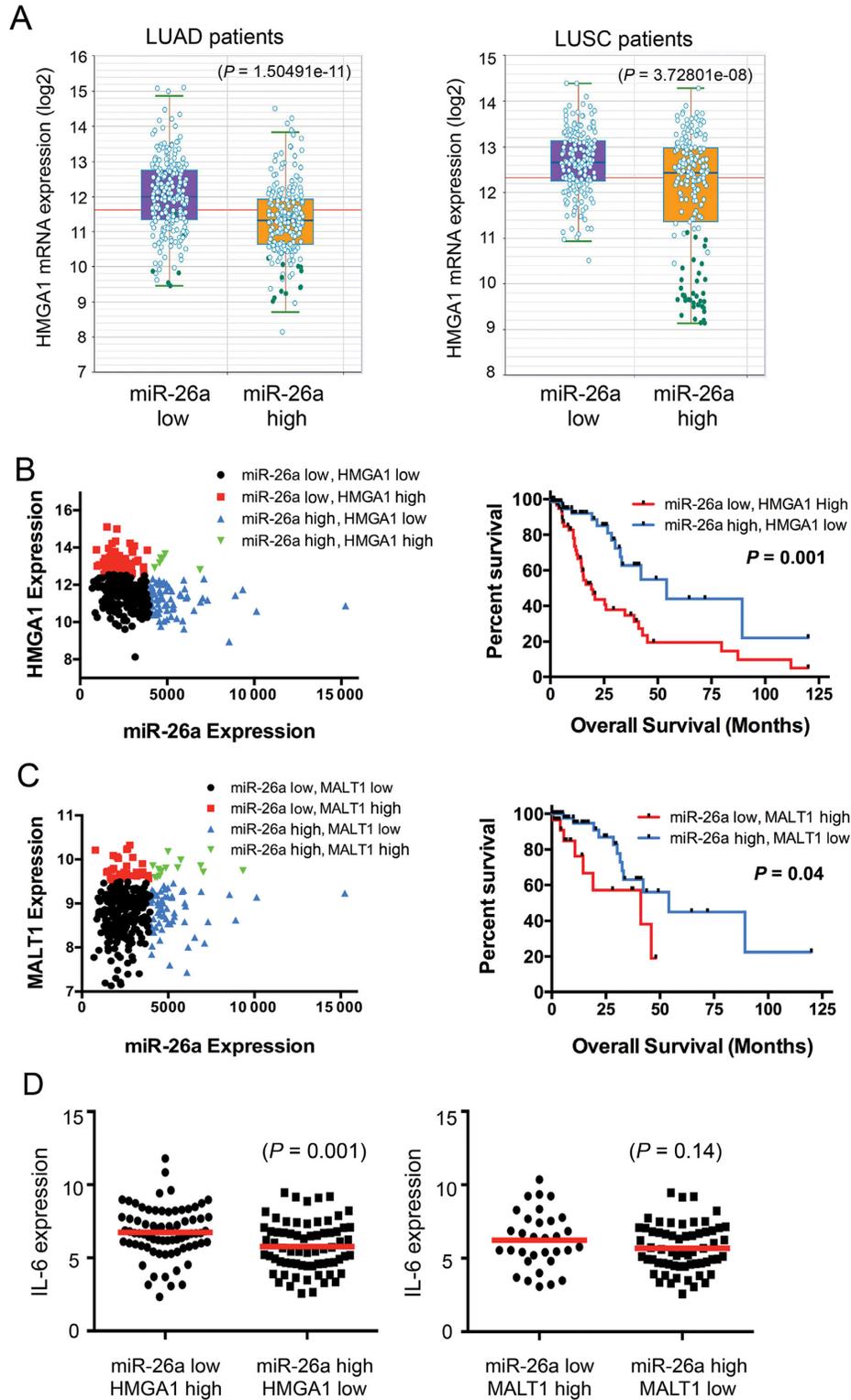


Figure 7. Relative levels of miR-26a versus HMGA1 or MALT1 and lung adenocarcinoma patient survival. (A) Inverse relationship between expression levels of miR-26a and HMGA1 mRNA levels in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) tumours. Patients were first stratified into high or low miR-26a expression and then plotted for the mRNA levels of HMGA1. T-test analysis was done using two independent datasets retrieved from TCGA, using the ‘starBase V2’ platform. Blue open circles: cancer patients; green solid circles: healthy controls. (B and C) The prognostic significance of miR-26a and HMGA1 (B) or miR-26a and MALT1 (C) for LUAD patients was assessed using Kaplan–Meier analysis. LUAD patients were first stratified into high or low miR-26a expression and then plotted for mRNA levels of HMGA1 (B, left) or MALT1 (C, left). To plot patient survival rate (right), we focused on the patients with high miR-26a and low HMGA1 (B) or with high miR-26a and low MALT1 (C) levels (blue lines), and on patients with low miR-26a and high HMGA1 (B) or with low miR-26a and high MALT1 (C) levels (red lines). (D) Bee swarm plots showing the relative IL-6 mRNA levels of LUAD patients as grouped and plotted in panels B and C (red and blue). T-test was done to assess statistical significance.

the predicted IL-6 miR-26 recognition site exhibited only a marginal change (Figure 1B–D and Supplementary Figure S4). These findings indicate that the 3' UTR of the IL-6 mRNA does not contain a functional miR-26-responsive site in either BEAS-2B or A549 cells. Third, the 5' UTR and ORF of IL-6 mRNA do not respond to miR-26 mimics either (Figure 2A), indicating that the entire IL-6 transcript does not contain any functional miR-26 recognition site for gene silencing. Fourth, miR-26 mimics can destabilize MAPK6 (Figure 2B), HMGA1 and MALT1 transcripts (Figure 5B) but cannot destabilize the IL-6 mRNA (Figures 2C and 5B). Clearly, miR-26 down-regulates IL-6 production through an indirect route rather than by directly targeting the IL-6 transcript for rapid decay or translation repression.

Our finding that miR-26 mimics repress the IL-6 promoter activity enough to account for the decrease in the steady-state level of IL-6 mRNA by miR-26 mimics in TNF- α -activated BEAS-2B cells (Figure 3A and B) not only further substantiates that miR-26 does not directly target IL-6 mRNA for rapid decay but also unravels the real mechanism underlying miR-26 mediated down-regulation of IL-6 production. Moreover, we demonstrated that miR-26 mimics can decrease TNF- α -mediated activation of a minimal promoter containing a DNA element responsive to NF- κ B (Figure 3D), an essential modulator of transcription of many genes involved in cytokine and chemokine production (including IL-6) and cell survival and proliferation (e.g. (45,48,53,55)). Combining a transcriptome-wide approach (high-depth RNA sequencing and bioinformatics analyses) with manipulation of cellular miR-26 levels (Supplementary Tables S1–S6; Figures 4–6, Supplementary Figures S5 and S6), our study demonstrates a novel role of miR-26 in dampening the TNF- α -activated expression of many NF- κ B-responsive genes related to inflammation, proliferation and stress via silencing NF- κ B related factors HMGA1 and MALT1 in human bronchial epithelial BEAS-2B cells.

The identification of HMGA1 mRNA as a direct target of miR-26 (Figure 5A, B, and Supplementary Figure S7) is consistent with a previous observation through dual-luciferase analysis, which showed that deletion of a predicted miR-26 recognition site in the 3' UTR of HMGA1 mRNA abolished miR-26-mediated gene silencing of HMGA1 (56). Furthermore, using Ago-CLIP-seq databases, we found that the single miR-26 recognition site in HMGA1 mRNA is highly enriched in Ago-CLIP sequence tags (Supplementary Figure S8). Moreover, our time-course experiment showed that miR-26 mimic significantly destabilizes HMGA1 mRNA (Figure 5B). All these findings further substantiate that HMGA1 is a direct target of miR-26 and also validate the approach we used to identify NF- κ B factors targeted by miR-26. Our data also show that miR-26 mimics can down-regulate MALT1 expression by promoting its mRNA decay (Figure 5B), a prominent mechanism of miRNA-mediated gene silencing (38–40). Thus far, no predicted miR-26 recognition site has been identified in the 3' UTR of MALT1, and we did not find any site of the MALT1 3' UTR with a significant enrichment of Ago-CLIP sequence tags that could be potentially recognized by miR-26 (data not shown). One possibility is that MALT1 mRNA carries an unconventional miR-

26 recognition site that evades the prediction by a known algorithm commonly used. While the present data do not rule out the possibility that MALT1 may be indirectly silenced by miR-26, the expression of both HMGA1 and MALT1 is appreciably dampened by miR-26 (Figure 5A and B). Moreover, we also show that both HMGA1 and MALT1 are required for a full TNF- α mediated induction of IL-6 expression (Figure 5C and D). Knocking down both HMGA1 and MALT1 had a silencing effect on many NF- κ B-responsive genes, including IL-6, similar to that caused by miR-26 (Figure 6). Collectively, our data support the notion that the suppressive effect of miR-26 on many NF- κ B-responsive genes is largely attributable to the ability of miR-26 to silence HMGA1 and MALT1.

The important issue regarding whether and how miR-26 might be linked to the control of NF- κ B signalling was never addressed previously, although miR-26a was shown to inhibit cell proliferation and cell motility in bladder cancer through silencing of HMGA1 (56). Our present finding that miR-26a can repress the activation of NF- κ B-responsive genes via the ability to silence MALT1 and HMGA1 (Figures 5 and 6) not only reveals a novel role of miR-26 in down-regulating inflammatory mediator production in bronchial epithelia cells but also provides further mechanistic insight into how miR-26 may accomplish its role by silencing NF- κ B signalling. HMGA1 is a non-histone, chromatin-binding protein that is highly expressed during embryogenesis and in some poorly differentiated cancers. Along with NF- κ B and other promoter-binding transcription factors in the nucleus, HMGA1 is thought to activate inflammation and proliferation related genes (23,24). On the other hand, MALT1 has been shown to regulate NF- κ B activation in lymphocytes through recruiting and activating the cytoplasmic I κ B kinase (IKK) complex (22,54), which is involved in propagating the lymphocyte response to inflammation. MALT1 can also activate NF- κ B in an IKK-independent manner in lymphocyte by cleaving a NF- κ B inhibitor, RelB, to facilitate DNA-binding by RelA or c-Rel-containing NF- κ B complexes for transcriptional activation. Thus, our findings (Figures 5 and 6) support a model (Figure 8) illustrating how miR-26 can dampen activation of NF- κ B signalling pathway quite effectively. In this model, up-regulating miR-26 has a two-pronged action, decreasing levels of HMGA1 in the nucleus and MALT1 in the cytoplasm (Figure 8).

Another critical point revealed by our study is that evaluation of miR-26 as a silencer for a given cytokine needs to consider the possibility of indirect actions on signalling pathways regulating transcription, as well as direct actions on the transcript for the cytokine. Our finding that poor patient prognoses in human lung adenocarcinoma are associated with the combination of low miR-26 levels and high HMGA1, MALT1 or IL-6 levels but not with any of them individually (Figure 7) also highlights the importance of the interplay among these factors in regulating inflammation and tumourigenicity. Along this line, it is worth noting that IL-6 up-regulation is found to reduce miR-26a expression in hepatocellular carcinoma cells (7), suggesting that a negative feedback loop may exist between IL-6 and miR-26 expression. As inflammation is a major factor contributing to malignancy and IL-6 is a prominent pro-inflammatory

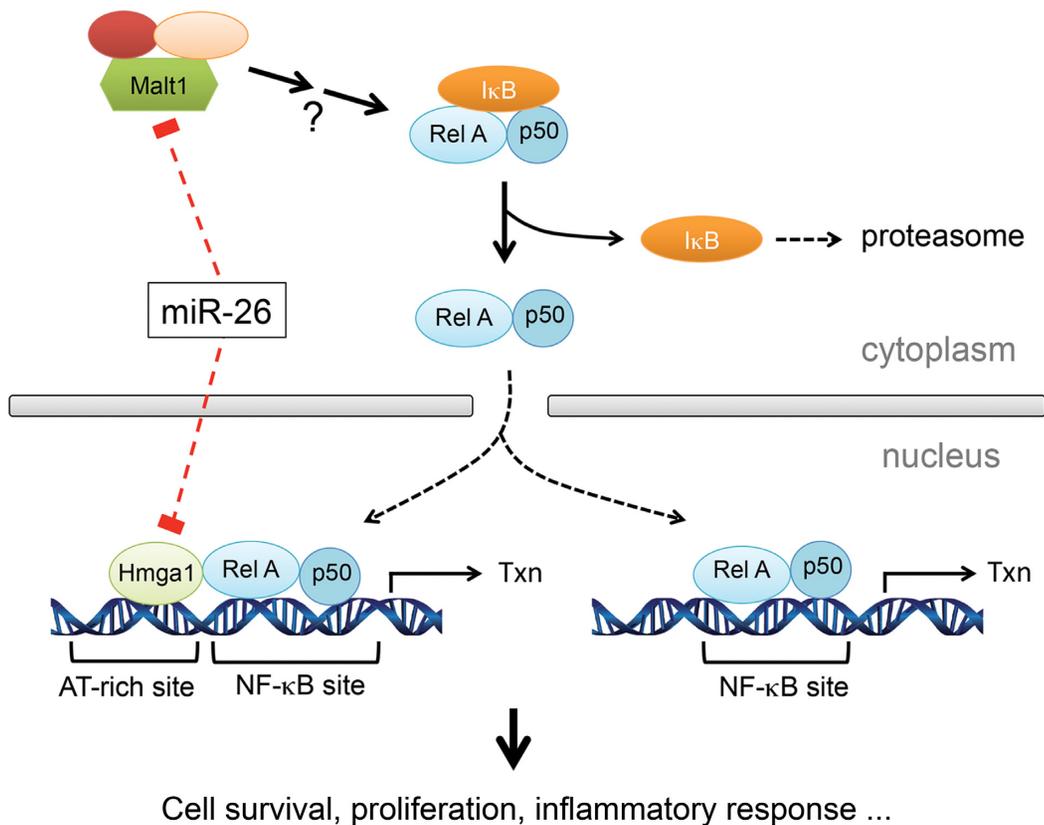


Figure 8. Schematic illustrating proposed regulation of NF- κ B signalling by miR-26 via silencing HMGA1 and MALT1. For simplicity, the action of MALT1 to decrease RelB levels and enhance NF- κ B signalling is not depicted. Note that HMGA1 acts in concert with NF- κ B to mediate transcription of some NF- κ B-responsive genes by binding to an AT-region near the NF- κ B binding site. See the Discussion for more details.

mediator (14,57,58), one important implication from our present findings is that miR-26 may have a role in limiting chronic airway inflammatory disease through silencing NF- κ B signalling. This notion is supported by mouse model studies showing the functional significance of NF- κ B-driven processes in orchestrating events pertinent to human asthma (59–62). Also, a recent study (63) reported that broadly induced overexpression of miR-26a in a transgenic mouse model is well tolerated by the animals without any obvious side-effects and, importantly, to not be oncogenic. Our results raise the possibility that miR-26 mimics may be useful in dampening activation of the NF- κ B pathway in epithelial cells, as part of therapeutic modification of pathological airway inflammation. The present findings also point to the possibility of exploiting the actions of miR-26 to manage chronic inflammation and related malignancies in general.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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