MicroRNAs (miRNAs) constitute a class of small noncoding RNAs that play important roles in a variety of biological processes including development, apoptosis, proliferation, and differentiation. Here we show that the expression of miR-199a and miR-199a* (miR-199a/a*), which are processed from the same precursor, is confined to fibroblast cells among cultured cell lines. The fibroblast-specific expression pattern correlated well with methylation patterns: gene loci on chromosome 1 and 19 were fully methylated in all examined cell lines but unmethylated in fibroblasts. Transfection of miR-199a and/or -199a* mimetics into several cancer cell lines caused prominent apoptosis with miR-199a* being more pro-apoptotic. The mechanism underlying apoptosis induced by miR-199a was caspase-dependent, whereas a caspase-independent pathway was involved in apoptosis induced by miR-199a* in A549 cells. By employing microarray and immunoblotting analyses, we identified the MET proto-oncogene as a target of miR-199a*. Studies with a luciferase reporter fused to the 3’-untranslated region of the MET gene demonstrated miR-199a*-mediated down-regulation of luciferase activity through a binding site of miR-199a*. Interestingly, extracellular signal-regulated kinase 2 (ERK2) was also down-regulated by miR-199a*. Coordinated down-regulation of both MET and its downstream effector ERK2 by miR-199a* may be effective in inhibiting not only cell proliferation but also motility and invasive capabilities of tumor cells.
substantial apoptosis (33–37). Accordingly, *MET* is considered an important target for anti-cancer therapy, and inhibitors of *MET* signaling such as ligand antagonists (38–40), kinase inhibitors (41), and receptor competitors (42, 43) have been developed. In addition, the inhibition of *MET* expression by siRNA technology may be a useful approach.

The present study demonstrates that *MET* proto-oncogene is negatively regulated by *miR-199a**. The expression of *miR-199a* was silent in proliferating cells except fibroblasts and in accordance with its silent expression, the *miR-199a* locus was heavily methylated in non-expressing cell lines. When introduced into tumor cells, *miR-199a* induced pronounced apoptosis, suggesting that *miR-199a* is a putative tumor suppressor. Intriguingly, mitogen-activated protein kinase (MAPK) ERK2, which is one of the downstream effectors of *MET*, was also down-regulated by *miR-199a*. Thus, promoting apoptosis through the inhibition of the *MET* signaling pathway by *miR-199a* may hold great promise as a potential therapy for a variety of primary and metastatic tumors.

**EXPERIMENTAL PROCEDURES**

RNA and DNA Oligonucleotides—RNA and DNA oligonucleotides were synthesized by Bioneer (Daejeon, Republic of Korea). The 19-mer target sequence of 3 siRNAs targeting *H11032*/*H11003* was: 5’-CUGGUUAUCAUCUGGGAAGA-3’ (siMet-1), 5’-GUGAAGAUCCCAUGUGUCUCA-3’ (siMet-2), 5’-CAGGGUGGUGGUUUCUGAU-3’ (siMet-3), and 5’-CCUACGCCACAAUUCUGU-3’ (NC), respectively. NC siRNA was used as a non-silencing control siRNA. A 2-nt overhang, dTdT, was added to 3’ of all siRNAs. miRNA mimetics were purchased from Dharmacon (Lafayette, CO). All siRNAs and miRNA mimetics were resuspended in diethyl pyrocarbonate-treated water to a final concentration of 30 μM.

**Cell Lines and Culture Conditions**—Human cell lines A549 (lung cancer cell line), HeLa (cervix adenocarcinoma), CDD-986sk (normal skin fibroblasts), MCF-7 (breast carcinoma), PC-3 (prostate cancer cell line), KB (oral epidermoid carcinoma), K562 (myeloid leukemia), Jurkat (T cell leukemia), Raji (Burkitt lymphoma), PWR-1E (normal prostate epithelial cell), MCF10A (normal breast epithelial cell line), JEG3 (choriocarcinoma), SiHa (cervical carcinoma), and SK-OV-3 (ovarian carcinoma) were obtained from the American Type Culture Collection (Manassas, VA) and Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI 1640 (A549, HeLa, KB, PC-3, MCF-7, JAR, and SK-OV-3), Dulbecco’s modified Eagle’s medium (CDD-986sk, K562, JEG3, and SiHa), keratinocyte serum-free medium (PWR-1E), or a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 (MCF10A) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified 5% CO2 incubator. Synovial fibroblasts isolated from the knee synovium of two individuals with no known joint disease were obtained from Asterand, Inc. (Detroit, MI). In addition, synovial fibroblasts were isolated from two patients diagnosed with rheumatoid arthritis and prepared for cell culture as described before (44). Synovial fibroblasts were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum and antibiotics (100 units of penicillin/ml and 100 μg of streptomycin/ml). The medium was replaced every 2–3 days and synovial fibroblasts between passages 3 and 6 were used in experiments as indicated. All culture media and fetal bovine serum were purchased from Invitrogen.

**Quantitative Real-time PCR Analysis of MicroRNAs**—Total RNAs were isolated from cultured cells using the mirVana miRNA isolation kit (Ambion, Austin, TX). Total RNAs from human tissues were obtained from Ambion. For quantitative analysis of miRNAs, two-step TaqMan real-time PCR analysis was performed using primers and probes obtained from Applied Biosystems (Foster City, CA). Briefly, cDNA was made from total RNA in 15-μl reactions using murine leukemia virus reverse transcriptase and specific primers for each miRNA contained in the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). The reverse transcriptase reaction was performed by sequentially incubating at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. Each PCR mixture (20 μl) contained 1.3 μl of reverse transcriptase product, 10 μl of TaqMan 2× Universal PCR Master Mix, and 1 μl of the appropriate TaqMan MicroRNA Assay (20×) containing primers and probes for the miRNA of interest. The mixture was initially incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. PCR were performed in triplicate using a DNA Engine Opticon system (MJ Research Inc.). All RNA samples were normalized relative to human 18 S rRNA.

**DNA Methylation Analysis Using Combined Bisulfite Restriction Analysis (COBRA) and Bisulfite Sequencing**—Genomic DNA was extracted from cultured cells using the DNA Wizard Genomic DNA Purification Kit (Promega). DNA from human normal and tumor tissues were obtained from Biochain (Hayward, CA). Prior to treatment with bisulfite, DNA was digested with Ncol (New England Biolabs, Ipswich, MA). The digested DNA was purified with phenol/chloroform extraction, precipitated with ethanol, and resuspended in Tris-EDTA buffer. A bisulfite reaction was performed with 0.8 μg of Ncol-digested DNA using EZ DNA Methylation Kit (Zymo Research, Orange, CA). After bisulfite reaction and purification, 250 ng of bisulfite-converted DNA was used as a template for each PCR analysis. PCR was carried out in a 20-μl reaction mixture containing specific primers (10 pmol each) using AccuPower PCR premix (Bioneer, Korea). The amplification cycle was 94 °C for 40 s, 57 °C for 50 s, and 72 °C for 60 s for 35 cycles. Primers used to amplify the DNA fragment (19-F1) containing the *miR-199a* locus on chromosome 19 were 5’-GGTTGGTGGAA-AATTGATATTTTTG-3’ and 5’-AAATTTCTCAAACCCACAAACTTTT-3’. Primers used to amplify the DNA fragment (19-F2) upstream of the *miR-199a* locus on chromosome 19 were 5’-TTTTGTTTTAGGAAATTTTAAAGG-3’ and 5’-AATACAAACCATTCTAAAATAC-3’. Primers used to amplify the DNA fragment (1-F2) upstream of the *miR-199a* locus on chromosome 1 were 5’-AGTGAAGGTATGATTTGATTTG-3’ and 5’-CATATATAAACTCTCCACCCACC-3’. Primers used to amplify the DNA fragment (1-F3) upstream of the *miR-199a* locus on chromosome 1 were 5’-GGTTGGTGGTTAGGATTATATATT-3’ and 5’-ACTTTCTCAAACCACCCACCCCTC-3’. A DNA fragment (1-F1) containing the *miR-199a* locus on
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chromosome 1 was amplified using nested PCR. First, DNA fragment was amplified using a forward primer (|

PCR was then used as a template for a second PCR to amplify DNA fragment 1-F1, using a forward primer (|

A luciferase reporter carrying the 3'-UTR of MET was constructed as follows. An 1850-bp fragment containing the majority of the 3'-UTR fragment of the MET gene, including the predicted target site for miR-199a*, was amplified by PCR from a full-length MET cDNA clone (NM_000245, obtained from RZPD, Imagenes GmbH, Berlin, Germany) using F1 and R1 primers that create XhoI and NotI sites, respectively (see supplemental Table S1 for primer sequences). The XhoI-NotI-digested product was cloned into the 3'-UTR of the luciferase gene in the pSICHECK-2 vector (Promega). To delete the predicted miR-199a* target site from the 3'-UTR fragment of MET, DNA fragments containing 1035 bp upstream and 795 bp downstream of the target site were separately amplified using F1/R2 and F2/R1 primers, respectively. After digestion with XbaI, ligation of upstream and downstream fragments, and digestion with XhoI and NotI, the 3'-UTR fragment with a deletion in the miR-199a* binding site was cloned into XhoI/NotI-digested pSICHECK-2. The cells were cotransfected in 6-well plates using Lipofectamine 2000 (Invitrogen) with 400 ng of the 3'-UTR-luciferase report vector and 10 nm miRNA mimetics or negative control mimetic (Dharmacon). Forty-eight hours after transfection, firefly and Renilla luciferase activities were measured consecutively by using dual-luciferase assays (Promega) according to the manufacturer’s protocol.

RESULTS

Expression of miR-199a/a* Is Confined to Fibroblasts among Proliferating Cell Lines—We recently constructed a library of small RNAs from synovial tissue isolated from rheumatoid arthritis (RA) patients. Sequencing of 2000 clones revealed that miR-199a/a* is one of several highly expressed miRNAs in RA
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As shown in Fig. 2, CpG density is high in the miR-199a/a* locus on chromosome 19, and a predicted CpG island (CpG island searcher) is present between ~130 and 540 bp upstream of the mature miR-199a sequence (another program, the CpG plot program at EBI predicted a 134-bp CpG-dense region between 269 and 402 bp upstream of mature miR-199a). In contrast, the miR-199a/a* locus on chromosome 1 is relatively CpG poor. Sequencing analysis of 20 clones obtained from the genomic DNA of A549 and HeLa cells following bisulfite conversion and PCR amplification revealed that 94.7 and 94.6% of analyzed CpG sites around the miR-199a/a* locus on chromosome 19 were methylated in A549 and HeLa cells, respectively (Fig. 2). On chromosome 1, a similar level of DNA methylation was detected: 94.6 and 97.6% in A549 and HeLa cells, respectively. In contrast, the same CpG sites were unmethylated in synovial fibroblasts and CCD-986sk skin fibroblast cells. Therefore, there is a good correlation between DNA hypomethylation and miR-199a/a* gene expression.

Next, we extended our methylation analysis to additional normal and cancer cell lines using bisulfite PCR followed by COBRA. The COBRA technique allows the quantification of the methylation level in CpG sites within CpG islands by assessing the availability of CpG-containing restriction sites for enzymatic cleavage following bisulfite treatment. In this case, GCGC sites (HhaI restriction site) were assessed. GCGC sites can only be cleaved by HhaI if the internal CpG is methylated, which protects the cytosine from conversion to uracil during bisulfite treatment. The ratio of restricted (methylated) to unrestricted (unmethylated) PCR products is a quantitative measure of methylation (see top map in Fig. 2 for the location of 5 CpG sites analyzed by COBRA). As shown in Fig. 3A, the analyzed CpG sites on chromosome 19 were almost completely methylated in all examined normal and tumor cell lines (except fibroblasts) as indicated by complete digestion of the PCR fragment by HhaI. Due to lack of enzyme sites suitable for COBRA analysis, the methylation patterns of 5 CpG sites upstream of the miR-199a/a* locus on chromosome 1 were examined by bisulfite sequencing. As shown in Fig. 3E, the locus on chromosome 1 was heavily methylated in all examined cell lines except K562 erythroleukemia cells. The hypomethylation of the miR-199a/a* locus on chromosome 1 appears to be specific to K562 cells because the same locus was heavily methylated in Jurkat (T cell lymphoma) and Raji (B lymphocytes from Burkitt’s lymphoma) cells as well as peripheral blood leukocytes (supplemental Fig. S2). These results suggest a critical role of DNA methylation in suppressing the expression of the miR-199a/a* gene in most proliferating cells except fibroblast cells.

To examine the functional significance of DNA methylation in transcriptional repression of the miR-199a/a* gene, A549 cells were treated with the DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-azaC), and/or the histone deacetylase inhibitor, trichostatin A, and the level of miR-199a/a* transcripts was measured by quantitative real-time PCR. As shown
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To examine the molecular mechanism(s) by which miR-199a and -199a* induce apoptosis in cancer cells, Western blot analysis was performed on samples isolated from A549 cells transfected with miR-199a and/or -199a* mimetics at 72 h after transfection. First, the activation of caspase enzymes via a proteolytic cascade was examined by measuring cleavage of caspases and their substrate, poly(ADP-ribose) polymerase (PARP). Cleavage of PARP by caspases is recognized as a hallmark of apoptosis (49). As can be seen in Fig. 4B, PARP was cleaved when cells were transfected with miR-199a but not when they were transfected with miR-199a*. Consistent with PARP cleavage, all examined caspases including initiator (CASPASE 9 and CASPASE 8) and effector (CASPASE 3 and CASPASE 7) were cleaved in cells transfected with miR-199a. These results indicate that the apoptosis pathway induced by miR-199a is caspase-dependent, whereas miR-199a* causes a caspase-independent cell death pathway in A549 cells.

To further clarify the mechanisms underlying miR-199a- or -199a*-mediated apoptosis, we next examined the relative levels of pro-apoptotic and anti-apoptotic proteins. As depicted in Fig. 4B, the level of the pro-apoptotic BAX protein was slightly increased in cells transfected with miR-199a, whereas levels of BID and BAD, other pro-apoptotic proteins, were increased when cells were transfected with miR-199a*. The apoptosis inducing factor, which plays an important role in caspase-independent apoptosis, was decreased in cells transfected with miR-199a*. Notably, the amount of anti-apoptotic BCL-XL protein was significantly reduced in cells transfected with miR-199a, suggesting that decrease in the level of BCL-XL may be responsible for the miR-199a*-induced apoptosis. Although treatment with Z-VAD-fmk, a pan-caspase inhibitor, inhibited miR-199a*-mediated apoptosis in A549 cells, the same treatment did not block miR-199a*-mediated apoptosis, as expected, given the lack of caspase activation in cells treated with miR-199a* (data not shown).

MET Is a Target of miR-199a*—To identify gene targets regulated by miR-199a/a*, we used a microarray approach to characterize changes in mRNA levels after transfection with miR-199a and -199a* mimetics. Although miRNAs regulate gene expression mostly at the translational level, it is becoming clear that miRNAs can also negatively regulate gene expression through effects on mRNA degradation (3, 50–52). After transfection with miR-199a and -199a* mimetics, we identified hundreds of genes with altered mRNA expression profiles (73 and 24 genes were up-regulated, and 318 and 183 genes were down-

in supplemental Fig. S3, the miR-199a/a* genes were significantly reactivated by treatment with 5-azaC alone or 5-azaC followed by trichostatin A. These results indicate that DNA methylation is one mechanism that transcriptionally silences miR-199a/a* genes in A549 cells.

In adult tissues, the miR-199a/a* loci on chromosomes 1 and 19 were heavily methylated in most tissues with breast, colon, and testes being less methylated (Fig. 3, B and F). Consistent with a low level of expression in brain, the miR-199a/a* gene was heavily methylated in that tissue. In fetal tissues, the miR-199a/a* gene was heavily methylated in brain, and less methylated in lung (Fig. 3C). Notably, the miR-199a/a* gene was hypomethylated in fetal skin, which is in good agreement with the lack of methylation in fibroblast cells (supplemental Fig. S4). In breast tumor tissues, the miR-199a/a* gene was slightly more methylated in 4 of 5 examined tissues compared with matched normal breast tissues (Fig. 3D).

miR-199a/a* Induce Apoptosis in A549 Cells—The silencing of miR-199a/a* in most proliferating cell lines prompted us to investigate whether miR-199a/a* functions as a tumor suppressor. As shown in Fig. 4A, the transient introduction of miR-199a and/or -199a* induced apoptosis in A549 cells compared with cells transfected with negative control mimetic, as assessed by cell morphology and Annexin V staining. miR-199a* caused more pronounced apoptosis than miR-199a in several cancer cell lines such as A549, PC3, KB, and MCF7 cells (Fig. 4A and supplemental Fig. S5) and its pro-apoptotic activity was concentration dependent (data not shown).
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regulated in cells transfected with miR-199a* and -199a mimetics, respectively). By comparing these genes with miR-199a* gene targets predicted by a web-based computer program at the Sanger Institute (miRanda algorithm), we narrowed down the number of putative target genes to 38 genes for miR-199a* and 23 genes for miR-199a (see supplemental Table S2 for a list of putative target genes). Subsequent target validation by Western blot analysis revealed that the MET proto-oncogene is downregulated in A549 and HeLa cells transfected with miR-199a* (Fig. 5A).

To demonstrate that MET is a target of miR-199a*, effects of miR-199a* mimics on expression of the luciferase gene were determined using a MET 3′-UTR-luciferase reporter construct. The reporter plasmid bearing the 3′-UTR of the MET gene was cotransfected with miR-199a*, miR-199a, or negative control mimetics and luciferase activity was measured using a dual luciferase assay at 48 h after transfection. When cotransfected with miR-199a*, luciferase activity was decreased to 19% of the level in cells transfected with a negative control (Fig. 5B). More importantly, luciferase activity was not reduced when the predicted binding site for miR-199a* was deleted from the 3′-UTR. Very similar results were obtained using a shorter MET 3′-UTR fragment (data not shown). Taken together, immunoblotting and reporter assay data indicate that the MET proto-oncogene is a target of miR-199a*.

Although the expression of miR-199a*/a* was silent in many proliferating cells, miR-199a/a* was significantly expressed in fibroblasts. To determine whether inhibition of miR-199a* in fibroblasts can lead to an increase in the level of MET protein, normal synovial fibroblasts were treated with anti-miR-199a* inhibitor for 24 h and the MET protein was detected using Western blot analysis. Consistent with the role of miR-199a* in down-regulating MET, treatment of fibroblasts with the anti-miR-199a* inhibitor resulted in up-regulation of MET, indicating a relief in miR-199a*-mediated translational inhibition by anti-miR-199a* inhibitor (supplemental Fig. S6).

ERK2 Is Down-regulated by miR-199a*—To examine the effects of MET down-regulation on signaling pathways regulated by MET, we performed Western analysis to determine changes in the amount and phosphorylation of AKT and ERK1/2, which are two major effector molecules downstream of MET. As depicted in Fig. 6A, the Akt level was slightly decreased in cells transfected with miR-199a. Interestingly, the level of ERK2 was significantly decreased in cells transfected with miR-199a*, but ERK1 levels were unaffected, and levels of MAPKs SAPK/INK and p38 were also unaffected.

Because MET is down-regulated by miR-199a*, it may be that the decrease in signaling from MET may result in the decrease in the level of ERK2. To test this possibility, we transfected 3 siRNAs targeting MET mRNA into A549 cells and assayed their effect on ERK2 levels. As shown in Fig. 6C, MET protein was down-regulated by each of the 3 siRNAs, but ERK2 protein was unaffected. These data suggest that the down-regulation of MET is not a cause of the decrease in ERK2 protein.
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As expected from the reduced level of ERK2, the amount of phosphorylated ERK2 was also reduced in cells transfected with miR-199a* (Fig. 6B). Thus, miR-199a* controls MET signaling at multiple points including affecting MET receptor levels and affecting levels of the downstream effector ERK2.

**DISCUSSION**

In this study, we show that MET proto-oncogene is a target of miR-199a*. Several lines of evidence indicate that miR-199a* is a putative tumor suppressor. 1) The expression of miR-199a/a* is silenced in all proliferating cell lines tested except fibroblasts. 2) Introduction of miR-199a/a* caused apoptosis in cancer cells. 3) miR-199a* down-regulates MET proto-oncogene and also down-regulates ERK2, an effector downstream of MET.

In support of our finding that miR-199a/a* is a putative tumor suppressor, it was recently reported that the expression of miR-199a/a* was significantly reduced in ovarian cancer and hepatocellular carcinoma when compared with normal tissues (53, 54). Another interesting role of miR-199a* was recently reported. The miR-199a* homolog in mouse, mnu-mir-199a*, regulates COX-2 expression in mouse uterus during implantation (55).

miR-199 genes are located within the intron of DYNAMIN genes that are large GTPases involved in vesicle formation during receptor-mediated endocytosis (56). There are three DYNAMIN genes in mammals and each human DYNAMIN gene contains an miR-199 gene (57). In human, more than 25% of miRNAs are located in introns of coding genes and in many cases, miRNAs are coexpressed with their host transcripts (58). However, the miR-199a/a* gene is located in the opposite direction to the DYNAMIN genes suggesting it is transcribed from its own promoter. The miR-199a/a* gene on chromosome 1 is relatively CpG poor and not associated with a CpG island. Interestingly, the miR-214 gene is located ~5.7 kb downstream of miR-199a/a* gene in the same direction of transcription, suggesting cotranscription by the same promoter. This hypothesis is supported by a similar pattern of expression of miR-199a/a* and miR-214 across a variety of tissues and cell lines (59, 60). Analysis of transcripts after knockdown of Drosha by siRNA or transfection of a large genomic DNA fragment containing upstream of the miR-199a/a* gene may be necessary for promoter analysis.

MET tyrosine kinase receptor is frequently amplified or mutated in a variety of solid tumors and the inhibition of its amplified signaling leads to the inhibition of cell proliferation and metastasis. Hence, it is an attractive target for anti-cancer therapy and more than 5 MET inhibitors including monoclonal antibodies and small molecule inhibitors are currently being tested in clinical trials. Recently, MET amplification has been implicated in drug resistance to the epidermal growth factor receptor kinase inhibitors gefitinib and erlotinib in lung cancers (61). ERBB3 (HER3)-dependent activation of phosphatidylinositol 3-kinase, which is triggered by amplified MET receptor, appeared to confer resistance to gefitinib and concurrent inhi-
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bition of both epidermal growth factor receptor and MET suppressed the growth of resistant cell lines. Similar coactivation of multiple receptor tyrosine kinases including MET was recently reported in brain tumor glioblastoma multiforme, suggesting the importance of combination therapy to treat tumors resistant to single agents (62). In this regard, it is important to note that miR-199a* down-regulated both MET receptor and the downstream signaling component ERK2. Because down-regulation of MET by siRNA did not result in the decrease in ERK2, it is likely that miR-199a* down-regulates ERK2 directly by targeting ERK2 mRNA or indirectly by targeting another protein whose reduction in turn affects the level of ERK2 protein. All mimetics were purchased from Dharmacon. For detection of phosphorylated proteins, cells transfected with mimetics were transferred to serum-free media at 32 h post-transfection and cultured 16 h. Following an overnight starvation, cells were treated with HGF (40 ng/ml, Sigma) for 10 min before harvesting to prepare cell lysates. It is notable that the down-regulation of ERK2 by the miR-199a* mimetic was less pronounced when cells were serum starved overnight. When cells were grown in complete media, ERK2 was significantly down-regulated with miR-199a* mimetic treatment, irrespective of HGF treatment, suggesting that serum starvation may affect the level of ERK2 down-regulation. A549 cells were transfected with siRNAs targeting MET at 100 nM concentration. Total cell lysates were prepared at 72 h post-transfection and subjected to Western blot analysis. NC, negative control siRNA; 1, siMet-1; 2, siMet-2; 3, siMet-3.

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