Activation of p53 by MEG3 Non-coding RNA*

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MEG3 is a maternally expressed imprinted gene suggested to function as a non-coding RNA. Our previous studies suggest that MEG3 has a function of tumor suppression. The tumor suppressor p53 plays a central role in tumor suppression and mediates the functions of many other tumor suppressors. Therefore, we hypothesized that MEG3 functions through activation of p53. We found that transfection of expression constructs for MEG3 and its isoforms results in a significant increase in p53 protein levels and dramatically stimulates p53-dependent transcription from a p53-responsive promoter. Using this as the functional assay, we demonstrated that the open reading frames encoded by MEG3 transcripts are not required for MEG3 function, and the folding of MEG3 RNA is critical to its function, supporting the concept that MEG3 functions as a non-coding RNA. We further found that MEG3 stimulates expression of the growth differentiation factor 15 (GDF15) by enhancing p53 binding to the GDF15 gene promoter. Interestingly, MEG3 does not stimulate p21CIP1 expression, suggesting that MEG3 can regulate the specificity of p53 transcriptional activation. p53 degradation is mainly mediated by the mouse double minute 2 homolog (MDM2). We found that MDM2 levels were down-regulated in cells transfected with MEG3, suggesting that MDM2 suppression contributes at least in part to p53 accumulation induced by MEG3. Finally, we found that MEG3 is able to inhibit cell proliferation in the absence of p53. These data suggest that MEG3 non-coding RNA may function as a tumor suppressor, whose action is mediated by both p53-dependent and p53-independent pathways.

RNAs that do not encode any proteins and function at the RNA level are non-coding RNAs (ncRNAs). In recent years, emerging evidence indicates that they play important roles in regulating cellular and biological functions (1, 2). Besides the well-known transfer RNAs and ribosomal RNAs, ncRNAs can be categorized into small nuclear RNAs, small nucleolar RNAs, microRNAs, small interfering RNAs, and medium/large ncRNAs that do not belong to the aforementioned RNAs (1, 3). Small nuclear RNAs and small nucleolar RNAs are usually 60–300 nucleotides long and are mainly involved in ribosome biogenesis and RNA splicing (4). MicroRNAs and small interfering RNAs are mostly 22 nucleotides long and function to negatively regulate gene expression at the posttranscriptional level (3). The medium/large ncRNAs are usually 1–2 kb in length and may carry polyadenyl tail, which are mRNA-like except that they do not encode any proteins. ncRNAs in this class are particularly interesting because they appear to have distinctive functions based on published reports (2) and participate in a variety of cellular functions. For example, H19 was reported to suppress growth of embryonal tumors and is believed to be an ncRNA tumor suppressor (5). The B2 transcript of non-small cell lung cancer line has been shown to inhibit growth of lung cancer cells (6). The steroid receptor RNA activator functions as a co-activator in regulating transcription by nuclear receptors (7). However, the cellular functions of many such medium/large ncRNAs remain elusive.

Human maternally expressed gene 3 (MEG3) is an mRNA-like RNA with a length of ~1.6 kb nucleotides (GenBankTM NR_002766) (8). Its mouse homologue is Gt12 (8). MEG3/Gt12 is a single copy gene and is not embedded within another cellular gene. In addition, there is no evidence suggesting that MEG3/Gt12 is antisense to any gene transcripts. MEG3/Gt12 with the paternally imprinted gene Dlk1 defines the Dlk1-Gt12 imprinted locus, which lies on human chromosome 14q and mouse chromosome 12q (9, 10). The imprinting of this gene is controlled by an intergenic germ line-derived differentially methylated region (11). Genomic structure analysis reveals that the MEG3/Gt12 gene consists of 10 exons (8). It generates multiple transcripts, attributed to alternative splicing (12, 13). MEG3 gene transcripts consist of several small open reading frames (ORFs). No consensus Kozak sequences are found in their initial ATG region. In addition, the putative proteins encoded by those ORFs do not resemble any known functional proteins or peptide. Therefore, it has been suggested that MEG3/Gt12 is an ncRNA (8, 12). However, because the function of MEG3 has remained largely unknown, a functional assay did not exist to confirm its ncRNA identity.

MEG3 is expressed in many normal human tissues, with the highest expression found in the brain and pituitary gland (14). In the normal pituitary, it is co-localized to gonadotroph-producing cells (14). However, we found that human pituitary tumors of a gonadotroph cell lineage do not express MEG3 (14).
In addition, MEG3 is not expressed in many human cancer cell lines, including brain cancer derived lines (14, 15). The loss of MEG3 expression in tumors has been found to be, at least in part, the result of hypermethylation in the MEG3 gene promoter region as well as the intergenic germ line-derived differentially methylated region (15, 16). Furthermore, we found that ectopic expression of one MEG3 isoform, MEG3a, suppresses growth of several human cancer cell lines in culture (14). These studies suggest that the MEG3 gene may play a role in tumor suppression.

Tumor suppression is a cellular defense mechanism preventing the neoplastic transformation of normal cells, and the tumor suppressor p53 plays a central role in tumor suppression (17). As a transcription factor, p53 functions by regulating expression of its target genes (18, 19). p53 is activated by many stress signals, such as sustained mitogenic stimulation and DNA damage (19). Its activation leads to cell cycle arrest, replicative senescence, or apoptosis depending on the strength of the signal and the cellular context (20). p53 also suppresses tumor development through activities unrelated to cell cycle regulation, such as inhibition of tumor angiogenesis (21). Therefore, functional inactivation of p53 has been found in most human cancers (17). The most common cause of p53 inactivation is a mutation in the p53 gene. Other causes that functionally inactivate p53 include interaction with viral oncoproteins and genetic alterations in genes whose products affect the function of p53, such as those that interact with, or transmit information, to and from p53 (17).

p53 also plays a critical role in mediating tumor suppression functions of other tumor suppressors, such as ARF (22), BRCA1 (23), NF2 (24), PTEN (25), and VHL (26). Therefore, we hypothesized that p53 may also mediate MEG3 function. We found that ectopic expression of MEG3 activates p53 and stimulates its transcription activation activity. Using this as the functional assay, we demonstrate that MEG3 functions as a non-coding RNA. In addition, we show that MEG3 stimulates expression of GDF15 through activation of p53. Finally, we demonstrate that MEG3 is able to inhibit cell proliferation in the absence of p53. Our data suggest that MEG3 ncRNA has a tumor suppression function, which is mediated by both p53-dependent and p53-independent pathways.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—MEG3 cDNA and its isoforms, MEG3a, MEG3b, MEG3c, and MEG3d were subcloned into the expression vector pCI under the control of the cytomegalovirus (CMV) promoter. The resultant constructs were designated as pCI-MEG3, pCI-MEG3a, pCI-MEG3b, pCI-MEG3c, and pCI-MEG3d, respectively. A fragment of approximate 400 bp was deleted from the 5′-end of MEG3 cDNA in pCI-MEG3 to generate pCI-MEG3-del5. pCI-MEG3b-del5 was similarly constructed. DNA fragments of MEG3 containing ORF1, ORF2, ORF3, ORF1 + 2, ORF1 + 3, or ORF2 + 3 were obtained from MEG3 cDNA and inserted into pCI to generate pCI-MEG3-ORF1, pCI-MEG3-ORF2, pCI-MEG3-ORF3, pCI-MEG3-ORF12, pCI-MEG3-ORF13, and pCI-MEG3-ORF23, respectively. MEG3 expression constructs carrying point mutations that cause a frameshift as well as create a premature in-frame stop codon in each ORF were generated using a PCR-based site-directed mutagenesis method with pCI-MEG3 as the template as described previously (27). ORF1, ORF2, and ORF3 were destroyed in pCI-MEG3–1T23, pCI-MEG3–12A3, and pCI-MEG3–123C, respectively. Both ORF1 and -3 were destroyed in pCI-MEG3–1T23C, whereas all three ORFs were destroyed in pCI-MEG3–1T23A3. A fragment between SalI and TfiI in MEG3 cdNA was deleted to generate pCI-MEG3-dST. A fragment between SphI and HindIII was deleted to generate pCI-MEG3-dSH. The CMV promoter was removed from pCI-MEG3b by restrictive enzyme digestion to generate pCI-MEG3b-P. The p53-responsive reporter construct p53-Luc was obtained from Stratagene (La Jolla, CA). The control plasmid pCMVβ expressing β-galactosidase was obtained from BD Clontech Laboratories (Palo Alto, CA). pCMS-d2EGFP is an expression vector containing an expression cassette of destabilized green fluorescent protein (d2EGFP) under the control of an SV40 early gene promoter. MEG3 cDNA was cloned into this vector under the control of a CMV promoter to generate pCMS-d2EGFP-MEG3. Expression vectors for p14ARF, and MEG3-del5 were similarly constructed. A DNA fragment containing sequences of the GDF15 promoter from nucleotide position −920 to +85 was amplified by PCR using a human genomic clone as template (clone CTC-251H24, from Invitrogen, Carlsbad, CA), and subsequently cloned into pGL3-basic (Promega, Madison WI) to generate pGDF15–920-Luc. A fragment of 220 bp between −920 and −701 containing the distal p53 binding site was removed from pGDF15–920-Luc to generate pGDF15–700-Luc. To generate pGDF15–920mt-Luc, the proximal p53 binding site in the GDF15 promoter (between nucleotides +19 and +39) was deleted in pGDF15–920-Luc by the PCR based site-directed mutagenesis method as describe previously (27). The pGDF15–700mt-Luc was similarly generated. All GDF15 promoter constructs were confirmed by DNA sequencing.

**Cell Culture, Transfection, and Luciferase Assay**—HCT116 and U2OS cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to the vendor’s instructions. HCT116p53+/− and HCT116p53−/− cells were kindly provided by Dr. Bert Vogelstein and maintained in McCoy 5A medium conditioned with 10% fetal bovine serum. Cells were transfected with Mirus TransIT-LT1 reagent (Mirus Bio, Madison, WI) overnight as previously described (27). For p53 reporter assays, cells in 12-well plates were transfected with plasmid DNAs containing 50 ng of p53-Luc, 0.2 μg of pCMVβ, and others as indicated. For GDF15 promoter assays, cells were transfected with 50 ng of reporter constructs and 0.1 μg of pCMVβ, plus 0.2 μg of pCI-p14ARF, pCI-p53, pCI-MEG3, or pCI-MEG3-del5 as indicated. Twenty-four hours after incubated with fresh culture medium, cells were lysed and luciferase activities were measured as previously described (27). The luciferase activity was finally normalized against the β-galactosidase activity from the same well.

**Northern and Western Blotting**—Cells were lysed with TRIzol reagent (Invitrogen) to isolate total RNA according to the manufacturer’s instruction, or lysed with radioimmune precipitation assay buffer to obtain total protein as previously
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MEG3 Reverse Transcription-PCR—Total RNA from normal colon mucosa and HCT116 cells was isolated using TRizol reagent following the manufacturer’s instructions. MEG3 transcripts were detected using OneStep reverse transcription-PCR kit from Qiagen. The primers used for reverse transcription-PCR were 5′-CCACTCCGGTACTAATCAGCTC-3′ (forward) and 5′-TAGTGCCCTGTGGAGTGTAG-3′ (reverse). As a control, the transcript of β-actin was also detected. The primers were 5′-CATGTACGTTGCTATCCAGGC-3′ (forward) and 5′-CTCCCTAATGTCACGCAG-3′ (reverse). The PCR products were resolved by 2% agarose gel and visualized with ethidium bromide staining.

BrdUrd Incorporation Assay—Cells were grown on coverslips in 6-well plates. After transfection, cells were labeled with 10 μM BrdUrd for 20 min and fixed with 3% paraformaldehyde in phosphate-buffered saline for 10 min. The fixed cells were treated with 0.2% Triton X-100 in phosphate-buffered saline for 10 min. The fixed cells were incubated with anti-BrdUrd antibody in phosphate-buffered saline at least three times between each treatment. The coverslips were mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA). The number of BrdUrd labeled cells per 100 GFP-expressing cells was counted under a fluorescence microscope and designated as the BrdUrd-labeling index (BrdUrd-LI). BrdUrd-LI in cells transfected with pCMS-d2-MEG3 was designated as (BrdUrd-LI)MEG3 and BrdUrd-LI in cells transfected with pCMS-d2EGFP only was (BrdUrd-LI)GFP. The relative BrdUrd-LI for MEG3-transfected cells was calculated as follows: [(BrdUrd-LI)MEG3/(BrdUrd-LI)GFP] × 100. The relative BrdUrd-LI for cells transfected with other constructs was similarly calculated.

ChIP Assay—The chromatin immunoprecipitation assay was performed with ChIP-IT kit from Active Motif following the manufacturer’s instructions (Active Motif, Carlsbad, CA). HCT116p53+/+ cells in P100 culture dishes were transfected overnight with pCI, pCI-p14ARF and pCI-MEG3, respectively. Twenty-four hours after transfection, cells were fixed with 1% formaldehyde. Cells were lysed and subsequently sonicated with a Branson 250 sonifier. Nine 15-s bursts of sonication (output was set at 3 and duty cycle at 30) were applied with 30-s pauses to avoid overheating. A portion of the sonicated lysates was used to purify chromatin DNA and used as input DNA. Lysates equal to 45 μg of chromatin DNA from each sample were used in immunoprecipitation with 3 μg of either normal rabbit IgG or an anti-p53 antibody (FL-393, Santa Cruz Biotechnology). The precipitated chromatin DNA was purified and subjected to PCR analysis. Primers used to detect GDF15 promoter and p21CIP1 promoter are as follows. For GDF15 promoter DNA containing the distal p53 binding site, 5′-AGGATGCCCTCTCCAGTGCA-3′ (forward) and 5′-GATCTCGACCCCCAACAGCT-3′ (reverse); for GDF15 promoter containing the proximal p53 binding site, 5′-CATGTGCAGCGACCAGCAGC-3′ (forward) and 5′-GACCACTCCCGGCTCAAACT-3′ (reverse); for p21CIP1 promoter containing distal p53 binding site, 5′-GATCTCGACCCCCAACAGCT-3′ (forward) and 5′-TTGCGCTCAAACT-3′ (reverse) and 5′-GACCACTCCCGGCTCAAACT-3′ (reverse).

RESULTS

MEG3 Activates p53 in Human Cancer Cells—The MEG3 contains 10 exons and is known to express multiple alternatively spliced transcripts (8, 12, 13). We isolated five of them from a human fetal liver cDNA library as described previously (14). Sequence analysis revealed that one of the cDNAs was reported previously as the human MEG3 transcript by Miyoshi et al. (GenBankTM NR_002766) (8). It contains sequences from all exons but 5, 6, and 7 (Fig. 1A). MEG3a, which we have previously reported (14), contains an additional exon 6 compared with MEG3. The rest of the isolated cDNAs are new MEG3 isoforms, designated as MEG3b, MEG3c, and MEG3d, respectively (Fig. 1A).

p53 has been shown to mediate functions of many tumor suppressors, such as ARF (22), BRCA1 (23), PTEN (25), and VHL (26). We hypothesized that p53 may also play a role in the anti-proliferative function of MEG3. To investigate this possibility, we transfected expression constructs for MEG3 and its isoforms into, HCT116, a human colon cancer cell line which contains wild-type (wt) p53 and does not express MEG3 (Fig. 1B). We found that cells transfected with MEG3 or any of its isoforms had significant increases in p53 protein levels (Fig. 1C).

To investigate whether the accumulated p53 in MEG3-transfected cells is functional, we used a p53-responsive reporter construct (p53-Luc) to measure p53 activity after co-transfection with expression constructs of MEG3 and its isoforms into HCT116p53+/+ cells. p53-Luc contains multiple p53 binding sites at its promoter region controlling the luciferase cDNA and has been widely used in quantifying p53 activity. As shown in Fig. 1D, luciferase activities were significantly higher in HCT116p53+/+ cells transfected with any of the MEG3 expression constructs than in cells transfected with blank vectors (Fig. 1D). To exclude the possibility that transcription stimulation
from p53-Luc by MEG3 and its isoforms is cell line-specific, we did similar p53 reporter assays in the osteosarcoma cell line U2OS, which expresses functional p53 (Fig. 1E). As shown in Fig. 1E, transfection of MEG3 and its isoforms significantly stimulates luciferase expression from p53-Luc (Fig. 1E). These data indicate that the p53 protein accumulated by transfection of MEG3 and its isoforms is functionally active.

To ensure that the activation of luciferase expression from p53-Luc by MEG3 and its isoforms is p53-dependent, we did similar experiments in HCT116p53−/− cells, which are isogenic to HCT116p53+/+ cells and do not express functional p53. As a positive control, we co-transfected 50 ng of p53-Luc with 50 pg of a wt p53 expression construct and found that the induction of luciferase expression was ~40-fold compared with that in cells transfected with vector only (Fig. 1F). In contrast, MEG3 and its isoforms failed to stimulate any luciferase expression even at 400 ng per transfection in these cells (Fig. 1F), indicating that stimulation of expression from p53-Luc by MEG3 and its isoforms is p53-dependent.

**p53 Activation Requires MEG3 Transcription**—To investigate whether MEG3 and its isoforms activate p53, we introduced their expression into MEG3-deficient cells by transfection. One possibility was that p53 activation might be the result of a cellular stress response to the introduction of certain foreign DNAs, such as MEG3 and its isoforms. To exclude this possibility, we transfected HCT116p53+/+ cells with pCI-MEG3b-P, which was created by deletion of the CMV promoter from pCI-MEG3b. As expected, no MEG3b transcript was detected in cells transfected with pCI-MEG3b-P by northern blotting (Fig. 2A). Importantly, transfection of this plasmid also failed to cause p53 accumulation in HCT116p53+/+ cells (Fig. 2A). Consistent with this observation, transfection of pCI-MEG3b-P did not stimulate luciferase expression from p53-Luc in HCT116p53+/+ cells (Fig. 2B). These data indicate that transfection of MEG3 DNA does not cause p53 activation. Instead, transcription of MEG3 is required for p53 activation.

Because p53 is a stress-response protein, another possibility was that its activation could be provoked by the high level of cellular MEG3 transcripts in the transfected cells, leading to p53 accumulation. We reasoned that if p53 activation is a non-specific response to high levels of MEG3 transcripts, expression of MEG3 mutants with a relatively small deletion at comparable levels should also be able to elicit a similar response, leading to p53 activation. However, if p53 activation by transfection of MEG3 constructs is a MEG3 sequence-specific event, small deletions in MEG3 may abolish its ability to activate p53, regardless of the mechanisms by which MEG3 activates p53. To exclude this possibility, we deleted a DNA fragment of ~400 bp from the 5′-end of MEG3 and MEG3b. We then transfected their expression constructs into HCT116p53+/+ cells. We found that truncated MEG3 and MEG3b (MEG3-del5 and MEG3b-del5, respectively) failed to induce any significant increase in p53 levels, regardless of the fact that expression of both deletion mutants was comparable to that of their wt counterparts as demonstrated by northern blotting (Fig. 2C). Similarly, these deletion mutants failed to stimulate luciferase expression from p53-Luc in HCT116p53+/+ cells, whereas the wt MEG3 and MEG3b did (Fig. 2D). To demonstrate that this is not an event specific to HCT116, we did similar experiments in U2OS cells. We found that expression of MEG3 and MEG3b significantly induced p53 accumulation, whereas expression of MEG3-del5 and MEG3b-del5 failed to do so (Fig. 2E). In addition, MEG3 and MEG3b significantly stimulated luciferase expression from the p53 reporter construct in U2OS cells, whereas their respective mutants did not (Fig. 2F). These data indicate that p53 activation by MEG3 and MEG3b is a specific event, which requires full-length MEG3 transcripts, and is not due to accumulation of random RNA transcripts.
p53 Activation Does Not Require MEG3 Translation—The MEG3 transcripts encode multiple small open reading frames. Three of them encode hypothetical peptides with greater than fifty amino acid residues in MEG3. We assigned them in MEG3 as MEG3-ORF1, -ORF2, and -ORF3, respectively (Fig. 3A). To functionally test whether these hypothetical peptides or proteins are responsible for p53 activation, we created a series of mutant MEG3 cDNAs. As illustrated in Fig. 3A, DNA fragments containing one or two ORFs were cloned into pCI to generate constructs expressing each ORF alone or two ORFs together. In addition, a single nucleotide was inserted or deleted after the initial ATG in each ORF to cause a frameshift as well as to create a premature in-frame stop codon in the ORF, making the ORF untranslatable (Fig. 3A). Expression constructs carrying these cDNAs are able to make full-length MEG3 transcripts but are unable to express one or more hypothetical proteins encoded by these ORFs. As expected, transfection of constructs containing wt MEG3 caused a significant increase in expression of luciferase from p53-Luc as well as in p53 protein in HCT116p53H11001H11001 cells compared with that in cells transfected with the blank vector (Fig. 3, A and B), whereas transfection of constructs carrying individual ORFs (ORF1, ORF2, and ORF3) failed to do so (Fig. 3, A and B). Interestingly, transfection of constructs containing the full-length MEG3 cDNA with one or more untranslatable ORFs (ORF1T23, ORF12A3, ORF123C, ORF1T23C, and ORF1T2A3C) dramatically stimulated expression of luciferase and resulted in significant increases in p53 levels, both of which were comparable to those in cells transfected with the wt MEG3 construct (Fig. 3, A and B). Similar results were also observed with a set of MEG3b constructs carrying similar mutations as described in MEG3 (data not shown). These data suggest that proteins or peptides encoded by those ORFs are not involved in activation of p53, even if synthesized.
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Therefore, translation of these ORFs is not required for p53 activation by MEG3.

MEG3 Function Depends on the Secondary Structure of the MEG3 Transcript—Results from experiments described above suggest that the full-length transcript is important for MEG3 to function. To further investigate how critical the full-length transcript is to MEG3 function, we made two mutant MEG3 cDNAs with small deletions. One deletion of 151 bp, designated as an ST deletion, removes half of the ORF2 region (Fig. 4A, ST). The other deletion of 163 bp, designated as a SH deletion, removes a fragment between ORF2 and ORF3 (Fig. 4A, SH). We tested their ability to activate luciferase from p53-Luc in HCT116p53 transfected with p53-Luc, pCMV/Luc, ST and SH deletions (28, 29). MEG3 RNA folding software Mfold to predict the secondary structure of MEG3 function. To investigate this further, we used the nucleic acid folding software Mfold to predict the secondary structure of the full-length MEG3 transcript is to MEG3 function, we made two mutant MEG3 cDNAs with small deletions. One deletion of 151 bp, designated as an ST deletion, and with SH deletion (SH), b1, b2, and b3 represent three major RNA folding branches in Group 2.

The functional loss in MEG3-dSH. These data indicate that specific RNA folding is critical to MEG3 activation of p53.

MEG3 Down-regulates MDM2 Expression—p53 degradation is mainly mediated by MDM2 (30). To determine whether p53 accumulation is attributed to inhibition of MDM2, we examined MDM2 protein levels in HCT116 cells after transfection with MEG3 expression constructs. As expected, we found that the MDM2 protein level was significantly elevated in cells transfected with the p14ARF construct compared with that in cells transfected with blank vector or with MEG3-del5 (Fig. 5A). In contrast, the MDM2 level in cells transfected with expression constructs for MEG3 was clearly lower than that in cells transfected with the blank vector or with MEG3-del5 (Fig. 5A). Similarly, the MDM2 level was lower in cells transfected with MEG3b compared with that in cells transfected with blank vector or with MEG3b-del5 (Fig. 5A). These data suggest that MEG3 and MEG3b suppress expression of MDM2.

We noticed that the MDM2 protein level is very low in HCT116 cells. To further investigate whether MEG3 suppresses MDM2 expression, we stimulated MDM2 expression by treating cells with the DNA-damaging agent actinomycin D (Act D). Treatment with Act D (1.5 nM) for 4 h significantly elevated MDM2 levels in HCT116 cells (Fig. 5B, lane N/T). Transfection of blank vector did not affect MDM2 induction by Act D (Fig. 5B, lane Vector). However, MDM2 induction by Act D was much less significant in cells transfected with MEG3 and

FIGURE 4. RNA folding is critical to MEG3 function. In A: left panel, schematic representation of expression constructs for the wt and mutant MEG3 cDNAs (open box, open reading frame) right panel, HCT116p53 cells were transfected with p53-Luc, pCMV/Luc, and MEG3 constructs as indicated in the left panel. Luciferase activities were measured as described under “Experimental Procedures.” The data were plotted as described in Fig. 1. * p < 0.001 compared with the value from cells transfected with wt MEG3 construct. B, RNA folding predicted by Mfold (28, 29). The stems and loops of folding are categorized into three groups as indicated. Group 2 is framed and enlarged for comparison among the wt MEG3 (WT), MEG3 with ST deletion (ST), and with SH deletion (SH). b1, b2, and b3 represent three major RNA folding branches in Group 2.

FIGURE 5. MEG3 down-regulates MDM2. A, HCT116p53 cells were transfected with expression constructs as indicated. MDM2 protein was detected by Western blotting. B, non-transfected HCT116p53 cells (N/T) and cells transfected with expression constructs as indicated were treated with (+) or without (−) Act D (1.5 nM) for 4 h. Cell lysates were collected as described under “Experimental Procedures.” MDM2 proteins were detected by Western blotting. β-Actin was probed as an equal loading control.
MEG3 up-regulates GDF15 by enhancing p53 binding to the GDF15 gene promoter—The GDF15 gene promoter contains two p53 binding sites (33, 35, 36). One overlaps nucleotides from −841 to −819 from the transcription initiation site, designated as the distal p53 binding site; the other contains nucleotides from +19 through +39, designated as the proximal p53 binding site. We hypothesized that MEG3 stimulates GDF15 expression by activating p53, which binds to its recognition sites in the GDF15 gene promoter, whereby stimulating transcriptional expression of the gene. To explore this possibility, we examined whether these p53 binding sites are required for MEG3 to activate transcription from the promoter. We made several mutant GDF15 promoter reporter constructs in which one or both p53 binding sites are deleted as shown in Fig. 7A. We first tested whether p14ARF is able to activate their transcription in HCT116p53−/− cells. As shown in Fig. 7B, p14ARF, as expected, stimulates luciferase expression from pGDF15−920-Luc. It also significantly stimulates luciferase activities from pGDF15−700-Luc, which lacks the distal p53 binding site. Interestingly, the transcription activation from pGDF15−920mt-Luc, which contains the distal p53 binding site but does not have the proximal site, by p14ARF was significantly reduced compared with that from pGDF15−920-Luc. Furthermore, p14ARF failed to stimulate any transcription from pGDF15−920mt-Luc, suggesting that MEG3 stimulation of GDF15 expression requires the presence of functional p53. These data demonstrate that p53 activated by MEG3 is indeed biologically functional and is able to stimulate expression of the endogenous target genes.

To investigate whether MEG3 stimulates GDF15 expression by increasing transcription of the GDF15 gene, we cloned a human genomic DNA fragment containing a GDF15 gene promoter consisting of nucleotides from −920 through +85 and inserted this DNA fragment into pGL3-basic to generate a GDF15 gene promoter reporter construct, designated as pGDF15−920-Luc. We co-transfected this construct with expression constructs for p14ARF, MEG3, or MEG3-del5, respectively, into HCT116p53−/− cells. We found that both p14ARF and MEG3 significantly stimulated luciferase expression from pGDF15−920-Luc, whereas MEG3-del5 failed to do so (Fig. 6C). In addition, we found that co-transfected with pGDF15−920-Luc into p53-null HCT116p53−/− cells p14ARF and MEG3 failed to activate transcription from pGDF15−920-Luc, although p53 significantly stimulated luciferase expression from pGDF15−920-Luc in these cells (Fig. 6D). These data indicate that MEG3 stimulates transcription activities from the GDF15 gene promoter, and this stimulation requires the presence of functional p53; consistent with the finding that MEG3 increased GDF15 protein levels in cells containing functional p53, but not in cells without p53 (Fig. 6, A and B).

MEG3 up-regulates GDF15 expression by enhancing p53 binding to the GDF15 gene promoter. As shown in Fig. 7A, p14ARF is able to activate their transcription in HCT116p53−/− cells. As shown in Fig. 7B, p14ARF, as expected, stimulates luciferase expression from pGDF15−920-Luc. It also significantly stimulates luciferase activities from pGDF15−700-Luc, which lacks the distal p53 binding site. Interestingly, the transcription activation from pGDF15−920mt-Luc, which contains the distal p53 binding site but does not have the proximal site, by p14ARF was significantly reduced compared with that from pGDF15−920-Luc. Furthermore, p14ARF failed to stimulate any transcription from pGDF15−920mt-Luc, suggesting that MEG3 stimulation of GDF15 expression requires the presence of functional p53. These data demonstrate that p53 activated by MEG3 is indeed biologically functional and is able to stimulate expression of the endogenous target genes.

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FIGURE 7. p53 binding sites are required for MEG3 to stimulate transcription from the GDF15 promoter. A, schematic representation of reporter constructs for GDF15 promoter. Open ovals, p53 binding sites; D, distal p53 binding site; P, proximal p53 binding site; oval with a cross, p53 binding site deleted. B, HCT116p53+/− cells were transfected GDF15 promoter constructs as indicated plus pCI (open bar) or pCI-p14ARF (solid bar); C, HCT116p53−/− cells were transfected with GDF15 promoter constructs as indicated plus pCI (open bar) or pCI-p53 (solid bar); D, HCT116p53+/− cells were transfected with GDF15 promoter constructs as indicated plus pCI (open bar) or pCI-MEG3 (solid bar). The luciferase assay was performed as described under “Experimental Procedures.” The data were plotted as arbitrary units, where the activity from the cells transfected with pCI vector was set as 1. The value is represented as mean ± S.D. for results from at least three independent experiments. *, p < 0.001 compared with values from cells transfected with blank vectors.

700mt-Luc, which had both p53 binding sites deleted (Fig. 7B). Similar data were obtained in the p53-null HCT116p53−/− cells with a construct expressing wt p53 (Fig. 7C). These data suggest that the proximal p53 binding site is the major p53 binding location through which p53 regulates GDF15 expression. We then examined how these mutations affect MEG3 function in activating the GDF15 promoter. As shown in Fig. 7D, a similar pattern was observed compared with those found in Fig. 7 (B and C). Transcription activation by MEG3 was significantly compromised in the promoter with the proximal p53 binding site deleted, and deletion of both p53 binding sites completely diminished the transcription activation from the GDF15 promoter by MEG3 (Fig. 7D). These data indicate that activation of GDF15 promoter by MEG3 is mediated by these p53 binding sites.

To further investigate how MEG3 increases expression of the GDF15 gene, we examined the interaction of p53 protein with the endogenous GDF15 gene promoter using ChIP assays. HCT116p53+/− cells were transfected with blank vector or expression constructs of MEG3 and p14ARF. The p53-bound chromatin DNAs were immunoprecipitated by anti-p53 antibodies. The amount of DNAs precipitated that contain the GDF15 gene promoter sequences were evaluated by PCR. The p53-bound DNAs carrying the distal or proximal p53 binding sites of the GDF15 gene promoter were identified by PCR using the site-specific primers. As shown in Fig. 8A, we found that amounts of the p53-bound DNAs containing the distal p53 binding site detected in cells transfected with either p14ARF or MEG3 are similar to those detected in cells transfected the blank vector (Fig. 8A). In contrast, amounts of the p53-bound DNAs containing the proximal p53 binding site detected in cells transfected with p14ARF or MEG3 were significantly more than those detected in cells transfected with the blank vector (Fig. 8B). These data indicate that p14ARF and MEG3 both increase p53 binding to the GDF15 promoter and the proximal p53 binding site is the primary location through with p14ARF and MEG3 regulate transcription of the GDF15 gene.

We also investigated p53 binding to the p21CIP1 promoter after transfection of p14ARF and MEG3. The p21CIP1 gene promoter consists of two p53 binding sites; distal and proximal p53 sites (30). Using ChIP assays, we found that transfection of p14ARF significantly increased p53 binding to both p53 sites in the p21CIP1 promoter (Fig. 8, C and D). However, transfection of a MEG3 expression construct failed to increase p53 binding to either site in the p21CIP1 promoter (Fig. 8, C and D). These data demonstrate that p53 activated by MEG3 does not stimulate transcription of the p21CIP1 gene, indicating that p21CIP1 is not a target gene of MEG3.
MEG3 Inhibits Cell Proliferation in the Absence of p53—p53 activation has been shown to induce growth arrest in HCT116 cells (37). We therefore examined whether MEG3 suppresses growth in HCT116 cells. We cloned MEG3 cDNA into the expression vector pCMS-d2EGFP, which contains an expression cassette of destabilized green fluorescent protein (d2EGFP). The resultant construct designated as pCMS-d2EGFP-MEG3 is illustrated in Fig. 9A. After transfection, cells expressing MEG3 are identified by expression of d2EGFP under a fluorescence microscope. p14ARF is known to activate p53 by blocking MDM2 function, which leads to growth arrest (17). Therefore, we constructed pCMS-d2EGFP-p14ARF expressing p14ARF as a positive control construct. We transfected these constructs into HCT116p53+/+ cells. Cell proliferation was determined by examination of BrdUrd incorporation in cells expressing d2EGFP. As expected, expression of p14ARF dramatically reduced BrdUrd incorporation in HCT116p53+/+ cells compared with expression of d2EGFP only (Fig. 9B). We also observed that BrdUrd incorporation was significantly lower in cells expressing MEG3 than in control cells (Fig. 9B), indicating that MEG3 inhibits proliferation in human tumor cells as does its isoform MEG3a (14). To exclude the possibility that growth suppression by transfection of pCMS-d2EGFP-MEG3 was due to introduction of foreign DNA, the CMV promoter was removed from pCMS-d2EGFP-MEG3 to generate pd2-MEG3-P, which expresses d2EGFP but not MEG3. We found that transfection of this construct does not inhibit BrdUrd incorporation in HCT116 cells (Fig. 9B), eliminating the possibility that MEG3 induced growth suppression is a nonspecific cellular response to the transfection of foreign DNA. These results show that transcriptional expression of MEG3 is necessary for MEG3 inhibition of cell proliferation.

To investigate whether growth suppression by MEG3 is mediated by p53, we performed BrdUrd incorporation assays in HCT116p53−/− cells after transfection with pCMS-d2-MEG3 and control constructs. HCT116p53−/− is an isogenic cell line of HCT116 in which the p53 gene is knocked out by targeted deletion (38). Lack of p53 expression in HCT116p53−/− cells was confirmed by Western blotting (Fig. 9C). As expected, expression of p14ARF does not inhibit DNA synthesis in HCT116p53−/− cells (Fig. 9D). However, MEG3 expression results in a significant decrease in BrdUrd incorporation in the p53 null cells, which is comparable to that observed in HCT116p53+/+ cells transfected with the MEG3 construct (Fig. 9D). This indicates that MEG3 inhibits cell proliferation in the absence of p53.

**DISCUSSION**

**MEG3 Acts as a Non-coding RNA**—The MEG3/Gtl2 gene is known to generate multiple transcripts by alternative splicing (12, 13). We have isolated five MEG3 cDNA species from a human fetal liver cDNA library (14). MEG3 and all its isoforms contain multiple open reading frames. However, none of them contain a consensus Kozak sequence in their initial ATG region. Because of this characteristic, MEG3/Gtl2 has been suggested to function as an RNA (8, 12). However, appropriate systems to test this hypothesis have not been previously available. We found that re-expression of MEG3 induces p53 accumulation and stimulates transcription from a p53-responsive promoter in MEG3-deficient cells (Fig. 1). Using this as a functional assay, we found that transcription of MEG3 is required to activate p53 (Fig. 2), whereas translation of ORFs encoded by MEG3 transcripts is not required for activation (Fig. 3). It is interesting to point out that the ability to activate p53 varies significantly among MEG3 isoforms (Fig. 1), although the majority of their sequences are the same and differ only in a small stretch of sequences in the middle of cDNA resulting from the inclusion of different exons during RNA splicing (Fig. 1A). This small sequence variation may cause changes in RNA secondary structures, which may account for the significant differences seen in their ability to activate p53.

That the RNA secondary structure is critical to MEG3 function is further demonstrated by experiments with MEG3-dST and MEG3-dSH. The secondary structure of MEG3 RNA predicted by nucleic acid folding software Mfold consists of many stems and loops. They are mainly classified into three groups (Fig. 4B). Group 2 contains three main branches, b1, b2, and b3 (Fig. 4B). Branch b2 is formed by folding of sequences between nucleotides 460 and 720, whereas Branch b3 is folded by sequences between nucleotides 720 and 870. The ST deletion shortened Branch b2 but did not affect Branches b1 and b3.
Interestingly, MEG3 carrying this deletion still functions as wt MEG3 (Fig. 4). The SH deletion removed most sequences in Branch b3. However, this deletion altered all three branches in Group 2 (Fig. 4B). The structural change is much more dramatic than that caused by a ST deletion. In addition, the ability of MEG3 carrying the SH deletion to activate p53 is significantly diminished compared with that of wt MEG3, although we observed that MEG3 with the SH deletion is still able to activate transcription from p53-Luc by ~5-fold compared with control (Fig. 4A). These data demonstrate the importance of RNA folding to the function of MEG3 and further indicate that MEG3 acts as an ncRNA.

**MEG3 Activates p53**—Normally, p53 protein is short-lived attributed to its rapid degradation mediated by MDM2 and other E3 ligases (39). Activation of p53 mostly results from inhibition of MDM2 function through various mechanisms, including phosphorylation, acetylation, and sumoylation of p53 and MDM2, inhibition of p53 ubiquitination, and blockage of phosphorylation, acetylation, and sumoylation of p53 and other E3 ligases (39). Activation of p53 mostly results from inhibition of MDM2 and/or p53 to block MDM2-mediated p53 degradation. It has been reported that the complex of 5L-MDM2-p53 contains 5 S sion of proteins that modify p53 and/or MDM2. It has been shown that ncRNAs can act as co-activators, such as SRA in steroid receptor-mediated transcription activation (7). Thus, one possibility is that MEG3 functions as a transcriptional co-activator to stimulate expression of proteins that modify p53 and/or MDM2. It has been reported that the complex of 5L-MDM2-p53 contains 5 S rRNA (41), suggesting that MDM2 and p53 are able to interact with RNA directly or indirectly, although whether 5 S rRNA affects p53 function is not clear. Therefore, another possible way for MEG3 RNA to activate p53 is to form complexes with MDM2 and/or p53 to block MDM2-mediated p53 degradation.

**GDF15 Is a Target Gene of MEG3**—The tumor suppressor p53 is a transcription factor, which exerts its tumor suppression function by regulating expression of its target genes. We showed that expression of MEG3 ncRNA activates p53. To demonstrate that the p53 activated by MEG3 was biologically active in regulating endogenous genes, we examined two p53 target genes, p21CIP1 and GDF15, and found that only GDF15 was up-regulated by MEG3, and this transcription activation required the presence of functional p53 (Fig. 6), suggesting that GDF15 is a MEG3 target gene. GDF15 is a member of the transforming growth factor-β superfamily of proteins (42). It is also known as macrophage inhibitory cytokine-1 (43), prostate-derived factor (44), placental transforming growth factor-β (45), placental bone morphogenetic protein (46), and non-steroidal anti-inflammatory drug-activated gene (34). GDF15 has been shown to inhibit proliferation of several cancer cell lines in culture, including the colon cancer line HCT116 (34, 47) and breast cancer line MCF7, and prostate cancer line DU145 (35, 48), inhibit colony formation of the osteosarcoma line U2OS and HCT116 in soft agar (33, 34) and suppress tumor formation of HCT116 and the glioblastoma line LN-Z308 in nude mice (34, 49). In addition, overexpression of GDF15 suppresses development of intestinal neoplasia in mice (50). These results indicate that GDF15 plays an important role in tumor suppression. In this study, we demonstrated that GDF15 is a target gene of MEG3 and that introduction of MEG3 expression suppresses proliferation of HCT116 cells (Fig. 9). Because p21CIP1 is not activated by MEG3 in this cell line, it is likely that inhibition of cell proliferation by MEG3 is mediated by GDF15, which would be consistent with the previous reports by others (34, 47). Previously, we have shown that expression of MEG3 is lost in non-functioning human pituitary tumors and many other human cancer cell lines (14, 16). Furthermore, re-expression of MEG3a inhibits proliferation of several cancer cell lines in culture (14). Taken together, these results indicate that MEG3 has a tumor-suppressive function, which is, at least in part, mediated by GDF15.

Many anti-tumor drugs have been shown to up-regulate GDF15 expression (51). It has been demonstrated that drugs, such as etoposide, genistein, and resveratrol, stimulate GDF15 expression by activation of p53 (32, 33, 35). The GDF15 gene promoter contains two p53 binding sites. We performed in vitro reporter assays using the GDF15 promoter controlling expression of luciferase. We found that deletion of the distal p53 binding site did not significantly affect promoter activation by MEG3 (Fig. 7D). In contrast, deletion of the proximal p53 binding site significantly reduced promoter activation by MEG3 (Fig. 7D). Furthermore, we found that MEG3 enhanced p53 binding only to the proximal and not the distal site of the endogenous GDF15 promoter as demonstrated by ChIP assays (Fig. 8). These data indicate that the distal p53 binding site is not important for MEG3 activation of the GDF15 gene. Rather, it is the proximal p53 site that plays a primary role in mediating transcriptional activation of the GDF15 gene by MEG3. It is noteworthy that the promoter with a deletion at the proximal p53 site was still somewhat responsive to MEG3 in reporter assays (~920mt-Luc, Fig. 7D), although p53 binding to this site was not increased in the endogenous promoter by ChIP assays (Fig. 8A). This discrepancy is likely caused by the characteristics of the in vitro reporter assay, in which a reporter construct carrying a piece of promoter DNA is introduced into cells by transient transfection. Because the promoter DNA introduced exogenously does not have a chromatin structure, unlike the endogenous gene promoter, transcription factors are able to scan freely along the promoter DNA to find their interaction sites. Thus, transcription from the promoter is likely to be mediated by the most favorable sites. However, the less favorable sites in the promoter can be used by transcription factors to activate transcription in the absence of the primary interaction sites. These less favorable sites may not be accessible in the endogenous environment due to the presence of histones. Therefore, in reporter assays, the transcription activation by MEG3 in the promoter with the proximal p53 site deleted is most likely mediated by the distal p53 binding site. Consistent with this, when both sites were deleted, MEG3 activation of the promoter was abolished completely (Fig. 7D).
MEG3 Modulates p53 Specificity in Activation of Gene Expression—It is well documented that p21^{CIP1} is a p53 target gene (31, 37). As expected, transfection of p14^{ARF} resulted in a significant increase in p53 levels, leading to a dramatic rise in p21^{CIP1} expression in HCT116p53^{+/+} cells (Fig. 6A). In addition, p14^{ARF} also induced expression of GDF15 (Fig. 6A). However, it is intriguing that transfection of MEG3 resulted only in elevated expression of GDF15, but not p21^{CIP1}, although p53 levels were significantly increased in the transfected cells (Fig. 6A). These results suggest that p53 proteins activated by p14^{ARF} are different from those activated by MEG3. Indeed, ChIP assays demonstrated that p53 activated by p14^{ARF} bound to the p21^{CIP1} promoter as well as the GDF15 promoter (Fig. 8), and p53 activated by MEG3 bound to the GDF15 promoter only (Fig. 8A). This may explain why p14^{ARF} was able to stimulate transcription of both p21^{CIP1} and GDF15, whereas MEG3 could only activate transcription of GDF15. These data also indicate that MEG3 can modulate the specificity of p53 in activating its target genes. As shown in Fig. 6A, it appeared that p53 activated by MEG3 was quantitatively less than that activated by p14^{ARF}. However, the GDF15 levels were comparable in cells expressing p14^{ARF} and MEG3 (Fig. 6A). This could suggest that the GDF15 promoter is more sensitive to lower levels of p53 compared with the p21^{CIP1} promoter. Therefore, it is possible that MEG3 selectively regulates its target genes by modulating p53 levels. It has been demonstrated that the apoptotic-stimulating proteins of p53 activate p53 by interacting with the p53 DNA binding domain and this interaction makes p53 prefer binding to the promoters of bax and pig3 but not to those of p21^{CIP1} and MDM2 (52). In contrast, c-Abl interacts with p53 and enhances p53 binding to the p21^{CIP1} promoter but not to the bax promoter (53). Therefore, by analogy, it is possible that MEG3 RNA stabilizes p53 by direct interaction with the protein and renders the p53 preferential binding to the GDF15 promoter over the p21^{CIP1} promoter.

*p53-independent Function of MEG3*—It has been well documented that p53 plays a critical role in regulating cell proliferation (17). In addition, GDF15 has been shown to inhibit growth in several human tumor cell lines (33–35, 47, 48, 54). Because MEG3 activates p53 and induces expression of GDF15 (Figs. 1 and 6), we expected that MEG3 would inhibit proliferation in HCT116 cells. As expected, we found that MEG3 significantly inhibits BrdUrd incorporation in HCT116p53^{+/+} cells (Fig. 9B). Surprisingly, MEG3 also inhibited proliferation in HCT116p53^{−/−} cells (Fig. 9D), indicating that MEG3 can inhibit proliferation without activating p53 and GDF15. However, it is important to point out that the data showing MEG3 inhibition of cell proliferation in the absence of p53 is not in any way an indication that p53 is not involved in MEG3 inhibition of cell proliferation in the presence of functional p53. Because it has been demonstrated that GDF15 inhibits proliferation of HCT116 cells (34, 47), it is more likely that GDF15 mediates MEG3 inhibition of cell proliferation in the presence of p53. These data suggest that MEG3 can inhibit cell proliferation through two pathways, which are p53-dependent and p53-independent. The former is mediated by GDF15 and the latter is GDF15-independent. This is not surprising considering that many tumor-suppressive genes are multifunctional and can suppress tumor growth through activating various pathways. For example, ARF (p14^{ARF} for human and p19^{ARF} for mice) is well known to activate p53 by inhibition of MDM2 function (22). It can also induce G_{1} cell cycle arrest as well as apoptosis in the absence of p53 in certain cells (55, 56). PTEN and BRCA1 have been shown to regulate transcription activity of p53 (23, 57). They have also been shown to suppress cell growth in the absence of p53 (58–60).

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