Induction of amphiregulin by p53 promotes apoptosis via control of microRNA biogenesis in response to DNA damage

Naoe Taira, Tomoko Yamaguchi, Junko Kimura, Zheng-Guang Lu, Shinji Fukuda, Shigeki Higashiyama, Masaya Ono, and Kiyotsugu Yoshida

Department of Biochemistry, The Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan; Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan; Department of Chemistry and Molecular Genetics, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan; and Chemotherapy Division and Cancer Proteomics Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

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Upon DNA damage, tumor suppressor p53 determines cell fate by repairing DNA lesions to survive or by inducing apoptosis to eliminate damaged cells. The decision is based on its posttranslational modifications. Especially, p53 phosphorylation at Ser46 exerts apoptotic cell death. However, little is known about the precise mechanism of p53 phosphorylation on the induction of apoptosis. Here, we show that amphiregulin (AREG) is identified for a direct target of Ser46 phosphorylation via the comprehensive expression analyses. Ser46-phosphorylated p53 selectively binds to the promoter region of AREG gene, indicating that the p53 modification changes target genes by altering its binding affinity to the promoter. Although AREG belongs to a family of the epidermal growth factor, it also emerges in the nucleus under DNA damage. To clarify nuclear function of AREG, we analyze AREG-binding proteins by mass spectrometry. AREG interacts with DEAD-box RNA helicase p68 (DDX5). Intriguingly, AREG regulates precursor microRNA processing (i.e., miR-15a) with DDX5 to reduce the expression of antiapoptotic protein Bcl-2. These findings collectively support a mechanism in which the induction of AREG by Ser46-phosphorylated p53 is required for the microRNA biogenesis in the apoptotic response to DNA damage.

H omestasis is maintained by a balance between cell proliferation and cell death. Activation of oncogenic transcription factor (e.g., c-Myc) and growth factor [e.g., epidermal growth factor (EGF) family proteins] destroys the balance, leading to tumorigenesis. EGF family contains transforming growth factor α (TGF-α), heparin binding-EGF, and amphiregulin (AREG). These growth factors are catalyzed by matrix metalloproteases (MMPs) at the plasma membrane, which enables them to act as a ligand for EGF receptor (EGFR) (1). MMPs are frequently overexpressed in tumors, suggesting that EGF family proteins, besides AREG, mainly function as tumor facilitators. In this context, AREG is exerted as a bifunctional growth modulator (2). However, a mechanism for AREG-mediated growth suppression remains unclear. AREG is initially synthesized as a pro-AREG that encodes 252 aa. Pro-AREG is translocated to the cell surface and then processed by one of MMPs, ADAM17. Soluble AREG directly binds to EGFR as a ligand to transmit growth signal (3). However, AREG is not able to activate EGFR signaling effectively compared with other EGF ligands (4). It is well established that AREG translocates to the plasma membrane; however, few reports showed that AREG localizes in the nucleus (5, 6). Recent study has demonstrated that AREG translocates to the inner nuclear membrane by a retrograde trafficking and attenuates global transcription (7). Taken together, AREG has multiple functions not only as a ligand for EGFR.

Tumor suppressor p53 transactivates numerous target genes in response to DNA damage to prevent tumorigenesis. The target genes exhibit diverse functions including cell cycle arrest, DNA damage repair, or apoptosis induction (8). The intracellular functions and localization of p53 are regulated by its posttranslational modifications. Especially, Ser46 phosphorylation is a key modification to eliminate cancer cells by inducing apoptosis (9). Previously, we have demonstrated that DYRK2 phosphorylates p53 at Ser46 under severe DNA damage (10, 11). We also found that DYRK2 is activated by ataxia-telangiectasia mutated (ATM), which senses DNA damage, and triggers apoptotic cell death (12). If Ser46 phosphorylation could be induced by chemical compounds, the compounds might enable tumor cells to induce cell death. Therefore, Ser46 phosphorylation could be a molecular target for cancer therapy. Recent study demonstrated that Ser46 phosphorylation-dependent apoptosis is also induced by mutant huntingtin (mHtt) (13). Because the mHtt elicits ROS reproduction and mitochondria dysfunction, DNA damage response is constitutively activated in the mHtt-expressing cells. Thus, Ser46 phosphorylation is widely linked in human disease development. However, it is unclear how Ser46-phosphorylated p53 induces cell death.

Here, we report that AREG is identified as a Ser46 phosphorylation responsible gene by the microarray analysis. AREG interacts with DEAD-box RNA helicase p68 (DDX5) to regulate precursor microRNA processing. In this regard, Ser46-phosphorylated p53

Significance

The tumor suppressive function of p53 is tightly regulated by its posttranslational modifications. Although Ser46 phosphorylation is a critical modification for apoptosis induction, a molecular mechanism by which Ser46-phosphorylated p53 induces apoptosis remains unclear. Here, we clarify that amphiregulin (AREG) is specifically induced in a Ser46 phosphorylation-specific manner. Notably, AREG colocalizes with DEAD-box RNA helicase p68 (DDX5) in the nucleus and regulates tumor suppressive microRNA biogenesis in response to DNA damage. These findings support a model in which Ser46-phosphorylated p53 orchestrates tumor suppressive microRNA expression in the apoptotic response to DNA damage.

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Data deposition: The complete expression datasets have been deposited in the Array Express database, www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-2556).

To whom correspondence should be addressed. E-mail: kyo@ikey.jp.

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facilitates tumor suppressive miRNA maturation via AREG, leading to apoptotic cell death.

**Results**

**Identification of AREG as a Direct Target of Phosphorylated p53 at Ser46.** In an attempt to isolate target genes of p53 that are especially phosphorylated at Ser46, p53-deficient H1299 lung adenocarcinoma cells or SaOS-2 osteosarcoma cells were transfected with Flag vector, Flag-p53 wild type (WT), or the Flag-p53 mutant in which Ser46 was replaced by Ala (S46A). As shown previously, ectopic expression of p53-WT in p53-deficient cells was sufficient for induction of Ser46 phosphorylation (14). We obtained similar convincing evidence for Ser46 phosphorylation with p53-WT, but not p53-S46A (Fig. S1A). To explore target genes that depend on Ser46 phosphorylation, we purified mRNA from these transfected cells to apply the microarray analysis. Whereas ~54,000 probe sets were analyzed, to isolate p53 target genes, we compared gene expression profiles in p53-WT–transfected cells with those in the empty vector-transfected cells. As targets for p53, 793 or 1,804 probes, which were more than 1.5-fold increase, were chosen in H1299 or SaOS-2 cells, respectively. To further screen out target genes induced by phosphorylated p53 at Ser46, the expression profiles in p53-WT–transfected cells were compared with those in the p53-S46A transfectant. As targets for phosphorylated p53 at Ser46, 11 or 28 probes, which were more than a 1.5-fold increase, were chosen in H1299 or SaOS-2 cells, respectively. Eventually, one probe has been selected, which conforms the criteria for the transcriptional elevation, in a Ser46 phosphorylation-dependent manner in both cell lines (Fig. 1A). Surprisingly, the probe was coded for AREG that is known as a member of EGF. Analysis of microarray data indicated that induction of AREG mRNA in p53-WT is significantly higher than that in p53-S46A (Fig. 1B). To verify this finding, we performed real-time RT-PCR and semiquantitative RT-PCR. In concert with the microarray data, expression of p53-WT markedly increased AREG mRNA in H1299 cells (Fig. 1B and Fig. S1B). Moreover, in comparison with transfection with p53-WT, induction of AREG expression was diminished in cells transfected with p53-S46A (Fig. 1B and Fig. S1B). Taken together, these findings demonstrated that the transcription of AREG is up-regulated by Ser46-phosphorylated p53. To further examine AREG expression in protein levels, H1299 cells were transfected with p53, then cell lysates were analyzed by Western blotting. In accordance with mRNA levels, AREG protein was highly inducible in cells transfected with p53-WT (Fig. 1C). By contrast, introduction of p53-S46A was little, if any, effect on inducible AREG expression (Fig. 1C), suggesting its dependency on Ser46 phosphorylation in protein levels.

p53 Binds to and Transactivates the AREG Promoter. To define the potential p53-responsive elements in the AREG promoter, we searched the consensus p53-binding site (15) and found two matches for this sequence within 500 bp of the AREG initiation codon (Fig. 2A). To confirm that p53 binds to these elements in vivo, we performed chromatin immunoprecipitation (ChIP) assays. Chromatin was isolated from H1299 cells transfected with Flag vector, Flag-p53 WT, or Flag-p53 S46A and was immunoprecipitated with an anti-Flag antibody. Immunoprecipitated DNA was then analyzed by PCR with primers amplifying the AREG promoter region encompassing the putative p53-binding consensus elements (p53-CE; −561 to −361). The elements were specifically immunoprecipitated with anti-Flag in p53-WT–transduced cells, suggesting that p53 actually binds to AREG promoter in vivo (Fig. 2B). Moreover, there was no remarkable occupancy of p53-S46A on the p53-CE of AREG promoter (Fig. 2B), indicating that Ser46 phosphorylation is prerequisite for recruitment of p53 to the AREG promoter. To extend this finding in the physiological condition, human osteosarcoma U2OS cells were left untreated or treated with adriamycin (ADR). The analysis of ChIP showed that there was little immunoprecipitation of DNA fragments containing p53-CE in unstimulated cells (Fig. 2C). By contrast, occupancy of the AREG promoter by p53 was markedly increased after ADR stimulation (Fig. 2C). As a control, there was no detectable p53 occupancy of a control region (CR; −853 to −695) in the AREG promoter upstream to the p53-CE (Fig. 2C). Importantly, there was little, if any, p53 occupancy in cells pretreated with pofitherin-α, a specific p53 inhibitor (Fig. 2C). Previous study showed that DYRK2 is responsible for Ser46 phosphorylation in response to DNA damage (10). In this context, the demonstration that there was also little, if any, immunoprecipitates of p53-CE by silencing DYRK2 supports a model in which Ser46 phosphorylation triggers the p53 occupancy of AREG promoter (Fig. 2C).

To further prove the role of Ser46 phosphorylation for the promoter binding, we performed ChIP analysis with the anti-phospho-p53 (Ser46) antibody. Initially, we validated whether this antibody is useful for the immunoprecipitation. The results demonstrated that endogenous phosphorylated p53 is successfully immunoprecipitated with the antibody in nonsilencing siRNA (control siRNA)-transfected cells after DNA damage (Fig. S2A). Notably, in the absence of DYRK2, there was little, if any, immunoprecipitation of p53 (Fig. S2A), indicating that the phospho-Ser46–specific antibody is applicable for immunoprecipitation with high specificity. To verify the result, we monitored the endogenous phosphorylation levels of p53 (Fig. S2B). As shown previously, phosphorylation of p53 at Ser46 was abrogated...
In DYRK2 knockdown cells. Based on these findings, we performed ChIP analysis with this antibody. The results showed that phosphorylated p53 is recruited to the AREG promoter under genotoxic stress. By contrast, the recruitment was diminished by knocking down DYRK2 (Fig. S2C). These results strengthen the evidence that phosphorylated p53 at Ser46 is specifically recruited to the AREG promoter in response to DNA damage.

To establish whether p53 binding is functionally relevant, we investigated the p53-CE in the AREG promoter for the ability to drive a luciferase reporter gene (AREG-Luc) in response to cotransfection of p53 into H1299 cells (Fig. 2D). In concert with findings obtained from ChIP analysis, upon exposure to ADR, the luciferase activity was significantly up-regulated in cells transfected with p53 WT (Fig. 2D). By contrast, ectopic expression of p53-S46A attenuated ADR-induced luciferase activity in comparison with that of p53-WT (Fig. 2D). Deletion of p53-CE (Δp53CE-AREG-Luc) completely abrogated the luciferase activity in p53-WT–transfected cells after DNA damage (Fig. 2D). Identical effects were obtained in p53-S46A–transfected cells (Fig. 2D). To confirm that endogenous p53 plays a role in the induction of AREG by DNA damage, U2OS cells were transfected with AREG-Luc or Δp53CE-AREG-Luc followed by treatment with etoposide (ETO). As found in transfected H1299 cells, DNA damage enhanced the luciferase activity, whereas the absence of p53-CE completely impaired its induction (Fig. 2E). Significantly, silencing p53 conferred resistance to the augment of ETO-induced luciferase activity regardless of the presence or absence of p53-CE (Fig. 2E). Taken together, these findings indicated that p53 transactivates AREG via p53-CE on the AREG promoter in response to DNA damage.

**p53 Induces AREG in Response to DNA Damage.** To determine whether p53 physiologically induces AREG expression after genotoxic stress, U2OS cells were transfected with a control siRNA or a siRNA targeting for p53 (p53 siRNA) followed by treatment with ADR. Analysis of real-time RT-PCR revealed that upon exposure of cells to ADR, AREG mRNA was increased at relatively later periods (Fig. 3A), which specifically coincided with the level of Ser46 phosphorylation (14). Importantly, abrogation of p53 expression completely suppressed increment of AREG mRNA after ADR treatment (Fig. 3A), indicating that DNA damage-induced augment of AREG mRNA requires p53. To extend these findings in protein levels, U2OS cells were transfected with the control siRNA, or the p53 siRNA followed by ADR exposure. As shown for the mRNA levels, the expression of AREG was elevated at 24 h in mock-transfected U2OS cells (Fig. S3), indicating relatively later-phase induction similar to Ser46 phosphorylation. In sharp contrast, there was no detectable AREG expression in cells silenced for p53 (Fig. S3). These results demonstrated that AREG is a target of p53 in response to DNA damage. Given that Ser46-phosphorylated p53 is recruited to the AREG promoter (Fig. 2 B and D), we assessed whether AREG expression coincides with the Ser46 phosphorylation level. AREG was detectable at 24 h after ADR exposure in accordance with Ser46 phosphorylation (Fig. 3B). However, in DYRK2 knockdown cells, AREG expression was not up-regulated by DNA damage (Fig. 3B and Fig. S2B). Importantly, DNA damage-induced Ser46 phosphorylation was markedly attenuated in DYRK2 knockdown cells. Taken together, AREG is induced by p53 in a Ser46 phosphorylation–specific manner.

Accumulating lines of evidence show that AREG is transported to the plasma membrane and is secreted as a ligand for EGFR (16). However, recent study demonstrated that intracellular AREG translocates to the inner nuclear envelope and then regulates chromatin remodeling (7). These reports thus suggest that AREG function is diverse, which is determined by its cellular localization. Given that AREG is shedded by ADAM17 on the cell surface and secreted from cells, we measured the AREG levels in the cell culture media. Of note, AREG was initially discovered in MCF-7 cells (17). As reported previously, AREG was released to extracellular fluid in response to TPA stimulation in MCF-7 cells (Fig. 3C). In contrast, AREG production was attenuated by cotreatment with ADAM17 inhibitor, TAPI. Importantly, there was little, if any, increase AREG production in response to various DNA damage stimuli. Furthermore, in U2OS cells, AREG production levels were

**Fig. 2.** AREG is a direct target gene of p53. (A) Schematic diagram of AREG promoter and p53 consensus motif. Capital letters indicate nucleotides that match the p53 consensus sequence. (B) H1299 cells were transfected with Flag-vector, Flag-p53 WT, or the S46A mutant. For ChIP assay, chromatin–protein complexes were immunoprecipitated with anti-Flag. Precipitated chromatin fragments were amplified by PCR with specific primers for p53-CE in the AREG promoter. (C) U2OS cells were transfected with nonsilencing siRNA (control siRNA) or DYRK2-specific siRNA, and then left untreated or treated with ADR and/or p53 inhibitor. Chromatin–protein complexes were immunoprecipitated with normal mouse IgG or anti-p53. Real-time PCR amplification was performed to analyze the chromatin-immunoprecipitated fragments by using primers that are designed for AREG promoter (AREG), or the outside of AREG promoter control region (CR). The data were normalized to the level of input control. (D) H1299 cells were cotransfected with pGL3 vector, AREG-Luc, or Δp53CE-AREG-Luc and Flag-p53 WT or the S46A mutant, and then treated with 0.5 μg/mL ADR for 24 h. The relative increase in luciferase activity was compared with cells transfected with pGL3 vector. **P < 0.01. (E) U2OS cells were transfected with the reporter vectors and the control siRNA or p53-specific siRNA, and then treated with 10 μM ETO for 24 h. Luciferase assays were performed as described above.
dominantly localized in the nucleus upon ETO treatment (Fig. 3).
AREG was not expressed in the plasma membrane, but pre-
expression was increased after DNA damage. Notably,
p53 and is targeted to the nucleus in response to DNA damage. 
The results demonstrated that endogenous DDX5 interacts with 
AREG in the presence of ETO (Fig. 4, Center). These results 
support our model in which AREG modulates the microRNA 
processing with DDX5–Drosha complex. To clarify subcellular 
localization of DDX5 following DNA damage, U2OS cells were 
immunostained with anti-DDX5 and anti-AREG antibodies. As 
shown previously, AREG localized in the nucleus under the 
DNA-damaged condition (Fig. 3D). In this circumstance, DDX5 
colocalized with AREG in the nucleus (Fig. 4B). Taken together, 
these findings demonstrate that nuclear AREG interacts with 
DDX5 upon genotoxic stress.

Fig. 3. AREG is induced by p53 in response to DNA damage. (A) U2OS cells were transfected with control siRNA or p53 siRNA to reduce endogenous p53 expression. At 48 h after transfection, cells were left untreated or treated with ADR for the indicated times. Total RNA was analyzed by semi-quantitative real-time PCR. (B) U2OS cells were transfected with control siRNA or DYRK2 siRNA and then treated with ADR for the indicated times. Cell lysates were subjected to immunoblot analysis with the indicated antibodies. Cell lysates were also analyzed by Western blotting. (C) MCF-7 and U2OS cells were treated with 100 nM TPA, 10 μM TAPI, 2 μg/mL ADR, or 20 μM ETO for 24 h. The amount of secreted AREG in the culture medium was measured by ELISA. The data were normalized to values from each untreated cells. The results are represented as mean ± SD obtained from triplicate ELISA values. (D) U2OS cells were treated with 10 μM ETO for 24 h. Cells were immunostained with anti-AREG (FITC). The nuclei were detected with DAPI.

remaining unchanged even after treatment with TPA or geno-
To examine where AREG localizes under DNA damage condi-
tion, U2OS cells were immunostained with anti-AREG antibody (Fig. 3D). As similarly shown for Western blotting (Fig. 3B), 
AREG expression was increased after DNA damage. Notably, 
AREG was not expressed in the plasma membrane, but pre-
Drosha complex. To clarify subcellular 
localization of DDX5 following DNA damage, U2OS cells were 
immunostained with anti-DDX5 and anti-AREG antibodies. As 
shown previously, AREG localized in the nucleus under the 
DNA-damaged condition (Fig. 3D). In this circumstance, DDX5 
colocalized with AREG in the nucleus (Fig. 4B). Taken together, 
these findings demonstrate that nuclear AREG interacts with 
DDX5 upon genotoxic stress.

AREG Interacts with DDX5 and Drosha. To characterize nuclear 
function of AREG, we intended to identify AREG-associated 
protein by mass spectrometry. U2OS cells were transfected with 
YFP-vector or YFP-tagged AREG, and cell lysates were immu-
noprecipitated with anti-GFP antibody. Immune complexes were 
prepared by ImmunoPrecipitation with Anti-AREG (IP) or AREG. 
We determined whether Drosha also interacts with AREG. 
Given that DDX5 is one of the components of Drosha complex, 
we determined whether Drosha also interacts with AREG. 
Endogenous Drosha was immunoprecipitated with anti-Drosha 
bound protein (Fig. S4A and B). The MS peaks of immunoprecipitated AREG was detected by these analyses (Fig. S4 C and D). To confirm this 

Fig. 4. AREG associates with DDX5 with Drosha. (A) U2OS cells were treated with 10 μM ETO for 24 h. Lysates were immunoprecipitated with normal mouse IgG, anti-DDX5, or anti-Drosha followed by immunoblot analysis with indicated antibodies. Cell lysates were also analyzed by Western blotting. (B) U2OS cells were treated with 10 μM ETO for 24 h. Cells were immunostained with anti-AREG (FITC) and anti-DDX5 (rhodamine). The nuclei were detected with DAPI.

AREG Regulates microRNA Processing To Induce Apoptosis. Previous studies have shown that DDX5 engages microRNA processing, especially among the converting step from primary microRNA (miRNAs) to precursor miRNA (19). DDX5 regulates miRNAs (i.e., miR-15a) biogenesis in response to DNA damage. In ad-
dition, because Ser46 phosphorylation is an indispensable mod-
ification for p53-dependent apoptosis, it is plausible that AREG 
exerts induction of apoptosis. Based on these findings, we hy-
thesized that AREG regulates tumor suppressive miRNAs 
processing via DDX5 interaction. To address this possibility,
precursor miRNA was isolated from U2OS cells and then analyzed by real-time PCR. As shown by Suzuki et al. (19), pre-miR-15a was increased by DNA damage, whereas pri-miR-15a levels remained unchanged (Fig. 5A and B). This data thus confirmed that miR-15a expression is posttranscriptionally regulated. In AREG knockdown cells, pre-miR-15a levels were significantly reduced, indicating that AREG modulates miR-15a processing (Fig. 5D). Because a major target of the miR-15a is antiapoptotic protein Bcl-2 (20), we examined Bcl-2 expression under the same conditions (Fig. 5C). Bcl-2 expression was declined upon severe DNA damage. By contrast, the decrease was little, if any, observed in AREG knockdown cells. This finding indicates that AREG induces Bcl-2 expression through processing of miR-15a. To assess whether endogenous AREG is involved in apoptosis induction, U2OS cells were transfected with the control siRNA or the AREG siRNA followed by treatment with ADR. Silencing AREG significantly attenuated apoptosis elicited by ADR (Fig. 5D). To further investigate whether AREG regulates apoptosis induction via the miRNA biogenesis, miR-15a inhibitor was cotransfected with control siRNA or AREG siRNA. In the control siRNA-transfected U2OS cells, DNA damage-induced apoptosis was remarkably suppressed in the presence of miR-15a inhibitor (Fig. 5D). This result supported that miR-15a is a prerequisite miRNA for apoptosis induction. In contrast, apoptosis induction was not attenuated by AREG depletion regardless of miR-15a inhibitor transfection, suggesting that AREG induces apoptosis via miR-15 induction (Fig. 5D).

To examine whether AREG modulates the microRNA biogenesis besides miR-15a, the expression levels of other precursor miRNAs were monitored by real-time PCR. Given that miR-34 is one target for p53 in response to DNA damage (21), we analyzed pri-miR-34 expression in U2OS cells. As shown previously, pri-miR-34 expression increased following genotoxic stimuli. In AREG-silencing cells, pri-miR-34 expression was induced at a comparable level to that in control siRNA-transfected cells. This finding demonstrated that miR-34 transcription is induced by DNA damage, which is independent of AREG (Fig. S6A). Pri-miR-143 expression remained unchanged after ADR treatment in both transfectants (Fig. S6B). These results suggest that AREG is not involved in microRNA transcription. We further examined expression levels of pre-miR-34 and pre-miR-143 under the same experimental conditions. They were increased by DNA damage. By contrast, in AREG knockdown cells, they were markedly attenuated, suggesting that AREG regulates the processing of miR-34 and miR-143 (Fig. S6C and D). Taken together, these findings indicate that AREG contributes to the microRNA biogenesis, not only in miR-15, but also other miRNAs.

We also examined whether DDX5 or DDX17 modulates AREG-mediated apoptotic cell death. U2OS cells were cotransfected with AREG siRNA and DDX5 siRNA or DDX17 siRNA. Apoptosis induction was monitored by TUNEL assay. As previously shown (Fig. 5D), AREG knockdown was associated with the attenuation of apoptotic cell death compared with the control siRNA transfection (Fig. S7). Importantly, additional knockdown of DDX5 or DDX17 had no further effect on apoptosis induction (Fig. S7). These results suggest that AREG is essential for the process of precursor microRNAs involved in apoptosis regulation.

Taken together, these findings demonstrated that AREG induces apoptosis in response to genotoxic insult by modulating the microRNA processing within the Drosha complex (Fig. 6).

Discussion

Tumor suppressor p53 has a great variety of function to prevent tumor progression. Especially, apoptotic induction is an effective...
mechanism for tumor elimination. Despite phosphorylation of p53 at Ser46 that dramatically induces apoptosis, how phosphorylated p53 induces apoptosis remains unclear. To identify Ser46 phosphorylation-responsive genes, gene expression profiles from p53 WT cells were compared with those from S46A mutant. We successfully identified AREG as a phospho-Ser46 responsive gene. Notably, Ser46 phosphorylation allows p53 to target AREG promoter. Given that Ser46-phosphorylated p53 induces the expression of p53A1PI that promotes apoptosis (14), Ser46 phosphorylation changes promoter affinity of p53 to the promoters with proapoptotic genes.

Recent studies demonstrated the function of p53 in the microRNA biogenesis. miR-34 was originally identified as a microRNA that is directly transactivated by p53 (21, 22). Suzuki et al. showed the promoters with proapoptotic genes. miR-34 was originally identified as a microRNA and posttranscriptional levels. The present study shows that p53 engages microRNA production via AREG, which is a target for p53, indicating that p53 indirectly modulates the microRNA biogenesis. In this context, p53 modiﬁed the microRNA biogenesis in the various steps. In response to DNA double-strand break, ATM phosphorylates downstream effectors to transduce the damage signal. ATM regulates the microRNA biogenesis via KSRP phosphorylation (25). Taken together, these tumor suppressor mechanisms increase the microRNA biogenesis, suggesting that dysregulation of microRNA metabolism is observed in tumors. Accumulating evidence has demonstrated that microRNA levels are decreased in tumors (24), and that defects in microRNA processing are observed in tumor (25). In these regards, posttranscriptional regulation for the microRNA biogenesis is fundamental for tumor inhibition. We reported that DYRK2-depleted cells contribute to tumorigenesis in vivo (26, 27). In the current study, AREG expression remained little, if any, detectable in the DYRK2 knockdown cells. Loss of AREG could not suppress Bcl-2 expression under severe DNA damage mainly due to the failure of pri-miR-15a processing. In this context, the defect in the microRNA biogenesis might accelerate tumorigenesis.

AREG was originally identified in MCF7 cells treated with TPA. Furthermore, AREG is well known as a bifunctional protein for cell growth (17). Initially, AREG is synthesized as a precursor protein, and the precursor is transported to the plasma membrane and proteolytically cleaved by ADAM17 (28). Cleaved AREG binds to EGFR to facilitate receptor phosphorylation. Thus, secreted AREG functions as a ligand to promote proliferation. In contrast, other reports suggested that AREG has a tumor inhibitory function (2, 17). Intriguingly, AREG translocated to the inner nuclear membrane to induce heterochromatinization, thereby suppressing transcription (7). In ovarian cells, AREG localizes in the nucleus (5, 6); however, nuclear function of AREG has remained to be elucidated. In the present study, we clarified that nuclear AREG modulates tumor suppressive miRNA expression via microRNA processing to inhibit tumor growth. AREG possesses two nuclear localization sequences; however, machinery of nuclear trafficking of AREG is not fully understood. Elucidation of the regulation for intranuclear localization of AREG is requisite for characteristics of its function.

Materials and Methods
Experimental details are described in SI Materials and Methods. All results are expressed as mean values ± SD of at least three independent experiments. Unpaired Student t test was used to generate statistical analysis. All microarray data (29) have been deposited in the ArrayExpress database under accession no. E-MEXP-2556.

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Supporting Information

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SI Materials and Methods

Cell Culture. U2OS cells were cultured in RPMI medium 1640 supplemented with 10% (vol/vol) heat-inactivated FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. H1299 cells, SaOS-2 cells, and MCF-7 cells were grown in DMEM containing 10% FBS and supplements. Cells were treated with adriamycin (ADR; Sigma-Aldrich) or etoposide (ETO; Sigma-Aldrich).

Microarray Analysis. H1299 cells and SaOS-2 cells were transfected with Flag-tagged p53 wild type or S46A mutant. Total RNA were purified and used for microarray analysis by using the Human Genome U133 plus 2.0 array (Affymetrix) as described (1). The results from Genechip analysis are available at www.ebi.ac.uk/arrayexpress, under accession no. E-MEXP-2556.

Plasmid and siRNA. Human p53 was cloned into pcDNA3-FLAG vector (2). Alanine-substituted mutant of p53 was constructed by site-directed mutagenesis. Promoter region of amphiregulin (AREG) was cloned into the pGL3 basic vector. Human AREG was subcloned into the pEYFP vector (3). The control siRNA and DYRK2-, p53-, and AREG-specific siRNA were purchased from Qiagen and Invitrogen. Plasmids or siRNA were transfected by using FuGENE9 (Roche) or Lipofectamine RNAi MAX (Invitrogen), respectively. The miR-15a inhibitor was obtained from Ambion. The inhibitor was transfected by using Lipofectamine 2000 (Invitrogen).

Immunoblot, Immunoprecipitation, and Immunofluorescence Analyses. Cells lysates were prepared as described (4). Lysates were separated by SDS/PAGE and transferred to nitrocellulose membrane. The membranes were incubated with anti-phospho-p53 (Ser46) (Bio-academia), anti-p53 (Santa Cruz Biotechnology), anti-Flag (Sigma-Aldrich), anti-GFP (Nacalai tesque), anti-Bcl-2 (Zymed), anti-DYRK2 (Human Protein Atlas), anti-DEAD-box RNA helicase p68 (DDX5) (Millipore), anti-DDX5X (abcam), anti-Drosha (Santa Cruz Biotechnology), or anti-tubulin (Sigma-Aldrich). Polyclonal antibody to amino acids 233–250 of the human AREG was generated (5). Immune complexes were incubated with secondary antibodies and visualized by chemiluminescence (Perkin-Elmer). For immunoprecipitation, lysates were incubated with anti-GFP (MBL), anti-DDX5, or anti-Drosha for 2 h at 4 °C. Immune complexes were precipitated by protein G. For immunofluorescence, cells were left untreated or treated with ETO for 24 h. Cells were fixed in methanol for 5 min and permeabilized with 1% Triton X-100 for 15 min. After washing once with PBS, cells were blocked with goat serum in PBS for 1 h. After washing with PBS-T (0.05% Tween-20) three times, cells were incubated with anti-AREG and/or anti-DDX5. Immune complexes were then stained with a goat anti-rabbit secondary antibody-conjugated FITC or a goat anti-mouse secondary antibody-conjugated rhodamine. Nuclei were stained with DAPI (Vector Laboratories).

Densitometric Analysis. The band intensity was calculated by the densitometric analysis with the ImageJ program. The score was normalized by the signal intensity of nondamaged cells.

Semiquantitative Real-Time PCR. Precursor microRNA expression was analyzed by real-time PCR as described (6).

Reporter Assay. Luciferase activities were measured at 48 h after transfection by using the Bright-Glo Luciferase assay system (Promega) according to the manufacturer’s protocol. The relative increase in activity compared with cells transfected with pGL3 vector was determined as described (7).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assay was performed as described (8). Immunoprecipitation was carried out with normal mouse IgG, anti-Flag, anti-p53, or anti-phospho-p53 (Ser46). Immunoprecipitated DNA fragments were amplified and subjected to semiquantitative RT-PCR. The data were normalized for the level of input control. The following primers were used for ChIP assays. For AREG promoter, 5‘-gtaccttcatacataaataggca-3’ and 5‘-ggtcagataggtgtagagagaa-3’; Control region (CR), 5‘-catatccacctggttaacat-3’ and 5‘-ggcggaaatttaaactccctc-3’.

AREG Production. Cells were plated onto 96-well culture plate and treated with 12-O-tetradecanoylphorbol-13-acetate (TPA), TNF-alpha protease inhibitor (TAPI), ADR, or ETO for 24 h. The amount of secreted AERG was measured by using human amphiregulin DuoSet (R&D Systems) according to the manufacturer’s protocol.

Mass Spectrometry Analysis. U2OS cells were transfected with YFP vector or YFP-tagged AREG. After 48 h, cells were harvested and cell lysates were immunoprecipitated with GFP antibody, and then binding proteins were eluted with 10% (vol/vol) sodium deoxycholate (SDC) solution. Samples were digested with sequencing grade modified trypsin (Promega) overnight. For LC-MS analysis, peptides were purified and resuspended with 0.1% formic acid. Acquired data from LC-MS were analyzed and quantified by using 2DICAL software (9).

Apoptosis Assay. Transiently transfected U2OS cells were cultured with poly-d-lysine–coated 4-well chamber slides. Apoptosis was detected by TUNEL assays by using DeadEnd Fluorometric TUNEL System (Promega). Statistical comparisons within the treatment groups were made by the Student t test. A P value of <0.05 was considered to be statistically significant.

Fig. S1. AREG is induced by phosphorylated p53 at Ser46. (A) SaOS-2 cells were transfected with Flag-vector, Flag-p53 wild type, or the S46A mutant. Cell lysates were subjected to immunoblot analysis with indicated antibodies. (B) H1299 cells were transfected with Flag-vector, Flag-p53 WT, or the S46A mutant. Total RNA was analyzed by RT-PCR.
Fig. S2. Phosphorylated p53 selectively binds to AREG promoter. U2OS cells were transfected with nonsilencing siRNA (control siRNA) or DYRK2 siRNA, and then treated with ADR. (A) Lysates were immunoprecipitated with normal mouse IgG or anti–phospho-p53 (Ser46) [anti–p-p53 (Ser46)]. Immune complexes were subjected to immunoblot analysis with anti-p53. (B) Cell lysates were analyzed by Western blotting with indicated antibodies. (C) Chromatin–protein complexes were immunoprecipitated with anti–p-p53 (Ser46). Precipitated chromatin fragments were amplified by real-time PCR with specific primers for the AREG promoter. *P < 0.05.
AREG is a physiological target gene for p53. U2OS cells were transfected with control siRNA or p53 siRNA and then treated with ADR for the indicated hours. Cell lysates were subjected to immunoblot with anti-AREG (Top), anti-p53 (Middle), or anti-tubulin (Bottom).

Mass spectrometric analysis for AREG binding protein. The MS data were obtained from three independent experiments. The results from 2DICAL analysis were shown in A and C. The MS peaks were developed in various combinations of axes; the m/z (x axis) and intensity axes (y axis) (Upper Left), the signal intensity (Left; y axis) and a box-and-whisker diagram (Upper Right), a grayscale intensity pattern of the RT (Lower Left; x axis), the m/z (x axis), and RT (y axis) (Lower Right). The results from Mascot search were shown in B and D. The results from A and B showed the MS peak of 493 m/z matched DDX5. The MS peak of 602 m/z matched AREG (C and D).
**Fig. S5.** DDX5 interacts with AREG. U2OS cells were transfected with YFP-vector or YFP-tagged AREG. Lysates were immunoprecipitated with anti-GFP. Immune complexes were subjected to immunoblot analysis with indicated antibodies.

**Fig. S6.** AREG regulates biogenesis of microRNAs. U2OS cells were transfected with control siRNA or AREG siRNA and then treated with ADR for the indicated times. Total RNA was amplified with specific primers for pri-miR-34 (A), pri-miR-143 (B), pre-miR-34 (C), or pre-miR-143 (D). Data represent mean ± SD of relative induction.
Fig. S7. AREG provokes apoptotic cell death via the microRNA processing. U2OS cells were cotransfected with AREG siRNA and DDX5 siRNA or DDX17 siRNA. Apoptosis induction was monitored by TUNEL assay. Knockdown efficiency of DDX5 or DDX17 was determined by immunoblot analysis. n.s., not significant.

Table S1. List of peptides from YFP-AREG immunocomplex

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Relative ratio (compared with YFP-vector)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AREG</td>
<td>5.57</td>
<td>THSMIDSSLSK</td>
</tr>
<tr>
<td>DDX5</td>
<td>1.47</td>
<td>APILIATDVASR</td>
</tr>
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<td>LLQLVEDR</td>
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