Slug promotes p53 and p21 protein degradation by inducing Mdm2 expression in HCT116 colon cancer cells

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Abstract. Our previous study revealed that the tumor suppressor/transcription factor p53 directly binds to its transcriptional target, p21, and that the p53/p21 complex binds to zinc finger protein SNAI2 (Slug), a tumor promoter/transcription factor; thereby promoting the degradation of Slug by Mdm2, an E3 ligase. The present study demonstrated that Slug reduced the cellular expression levels of p53 and p21 in HCT116 colon cancer by decreasing their protein stability. In parallel, Slug increased the mRNA and protein expression levels of Mdm2 in these cells. Moreover, knockdown of Mdm2 using specific small interfering RNAs abolished the ability of Slug to induce the degradation of p53 and p21. Considering the well-known function of Mdm2 in facilitating p53 and p21 degradation, these data suggested that Slug promoted p53 and p21 degradation by inducing Mdm2 expression. Moreover, Slug increased ubiquitination levels of p53 in HCT116 cells. This is consistent with the fact that Mdm2 induces p53 degradation by ubiquitinating p53, and further confirmed that Mdm2 acted downstream of Slug. Comparative studies using HCT116 cells and their p53- or p21-knockout variants have revealed that Slug requires p21 to induce p53 degradation. This result is consistent with our previous study, which revealed that Mdm2 acts more efficiently on p53 in the p53/p21 complex compared with on p53 alone. By contrast, Slug did not require p53 to induce p21 degradation, suggesting that p53 was dispensable in Mdm2-mediated p21 degradation. Notably, the ability of Slug to increase/decrease Mdm2/p53 and p21 levels, respectively, was not confined to HCT116 cells alone, but was also confirmed in A549 and H460 lung cancer cells. Collectively, the results of the present study suggested that Slug could counter p53 and p21. The balance between these two opposing groups (Slug vs. p53/p21) may depend on environmental stresses and the internal physiology of cells.

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Introduction

Tumor suppressors and tumor promoters regulate cell functions in an opposite manner. For example, while tumor promoters generally support cell growth, survival, and invasion, tumor suppressors inhibit these cell functions. Accordingly, networks of the two opposing groups have evolved for their coordinated regulation, which is essential for cells to adapt to changes in the external environment and internal physiology. An imbalance in such networks can cause tumor initiation and progression (1), and therefore, elucidating the functional interaction between tumor suppressors and tumor promoters is required for an improved understanding of not only cell function regulation, but also the process leading to tumor initiation and progression.

Slug, a member of the Snail family of transcription factors (2), is upregulated in various types of cancers (3-7). Since Slug upregulation facilitates epithelial-mesenchymal transition and the subsequent invasion and metastasis of cancer cells (2,3), it is considered as a tumor promoter. Slug is downregulated by Mdm2, a ring-finger-containing E3 ligase that ubiquitinates Slug, leading to its proteasome-dependent degradation. It has been reported that the action of Mdm2 on Slug is facilitated by the binding of Slug to p53, a tumor suppressor/transcription factor (8). Previous studies from our group have shown that p53 can form a complex with its transcriptional target p21^{WAF1/CIP1}, and the p53/p21 complex rather than p53 alone is the functional unit that binds to Slug to promote Mdm2-mediated degradation of Slug (9). The p53/p21 complex consistently suppresses cancer cell invasion (9), suggesting that the complex acts as a tumor suppressor that can degrade a tumor promoter, such as Slug, and inhibit the spread of cancer cells. In this regard, it is interesting to speculate that Slug might not be unilaterally attacked by p53 and p21, but instead might have a tool to counter p53 and p21 to maintain the balance between itself and the p53/p21 complex. To date, however, this question has not been addressed.

Mdm2 can also directly bind to p53 and p21, inducing proteasomal degradation (10-14). While Mdm2 ubiquitinates p53 for p53 degradation (10-12), Mdm2 facilitates the binding of p21 to the proteasome without ubiquitinating p21 (13,14). Our recent studies revealed that Mdm2 binds to p53 in the p53/p21 complex more efficiently than p53 alone (15). Accordingly, the binding of p21 to p53 promoted Mdm2-mediated p53 degradation. However, it remains unclear whether p53 also influences p21 stability. Considering that the regulation of p53 and p21 protein stability is important for controlling their activities in cells (10-14), Slug may influence the protein stability of p53 and p21. Here, we investigated this possibility using HCT116 colon cancer cells, because in contrast to numerous other cancer cells in which p53 is mutated, HCT116 cells express p53 wild-type. Moreover, p53- and p21-knockout variants of HCT116 cells are also available (16). Therefore, HCT116 cells and their variants have been widely used as models to investigate the functions of p53 and p21. Our data demonstrate that Slug facilitates the degradation of p53 and p21 proteins by inducing Mdm2 expression, which acts as a mediator of such actions of Slug. While Slug requires p21 to induce p53 degradation, we found that p53 does not influence Slug-induced p21 degradation. Our data support not only the existence of mutual interactions between Slug and p53/p21 to balance their tumor-promoting and -suppressing functions, respectively, but also provide new insights into the mechanism wherein the stability of p53 and p21 proteins is regulated.

Materials and methods

Antibodies, small-interfering RNAs, and chemicals. The following antibodies were used in the study: Anti-Slug (Abnova), anti-p53 and anti-Mdm2 (Santa Cruz Biotechnology, Inc.), anti-p21 (Cell Signaling Technology), and anti-β-actin (Sigma-Aldrich; Merck KGaA). Small-interfering RNAs (siRNAs) were purchased from Ambion. When two sets of siRNAs were used, they were numbered as siRNA-1 and -2. Their catalog numbers are as follows: Slug siRNA-1 and -2, S13128 and S224653; Mdm2 siRNA, S224037. Cycloheximide was purchased from Sigma-Aldrich; Merck KGaA.

Cell culture and treatments. Sources and authentication of HCT116 colon cancer cells, their p53- or p21-knockout variants, H460 and A549 lung cancer cells have been described previously (17,18). Cells were regularly screened for mycoplasma contamination using MycoAlert Mycoplasma Detection Kits (Lonza, Switzerland). Cells were cultured in McCoy's 5A (HCT116 cells and their variants) or RPMI-1640 medium (H460 and A549 cells) supplemented with 10% heat-inactivated FBS. For gene knockdown, siRNAs targeting the specified genes were introduced into cells using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.). The treated cells were used for experiments after 24 h of recovery.

Western blot analysis. Cell lysates, prepared via a previously described method (19), were subjected to SDS-PAGE. The resolved proteins were electrotransferred onto nitrocellulose membranes (Millipore) and analyzed using the indicated antibodies and an ECL detection system (Bio-Rad) (19).
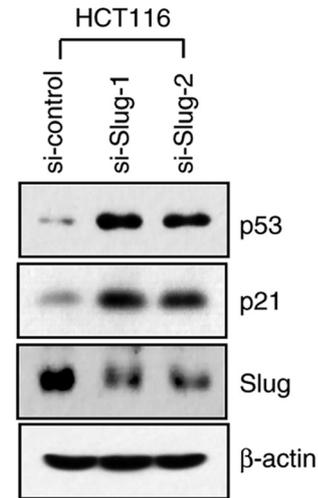
Figure 1. Slug decreases cellular levels of p53 and p21. HCT116 cells were treated either with a control or two sets of Slug-targeting siRNAs. After 24 h of incubation, cellular expression levels of p53, p21 and Slug were compared using western blotting. β-actin was used as the loading control. siRNA, small interfering RNA; Slug, zinc finger protein SNAI2.

Quantitative real-time PCR. Total RNA was extracted using the Hybrid-R RNA preparation kit (GeneAll Biotechnology, Korea) and reverse transcribed into cDNA using an RT-for-PCR kit (GeneAll Biotechnology). Quantitative real-time PCR was carried out using the SYBR-Green Real-time PCR Premix (Enzynomics, Korea) using the following primers: Mdm2, 5'-TAGTATTTCCCTTTCCTTTGATGA-3' and 5'-CAC TCTCCCCTGCCTGATAC-3'; Slug, 5'-CATGCCTGTCAT ACCACAAC-3 and 5'-GGTGTCAGATGGAGGAGGG-3'; GAPDH, 5'-CATCTCTGCCCCCTCTGCTGA-3' and 5'-GAA TGACCTTGCCCAGAGCCT-3'. The results of real-time PCR amplifications were analyzed using an IQ-5 Real-Time System (Bio-Rad). GAPDH was used as an internal control for normalization.

Data presentation and statistical analysis. The results shown in this study are representative of at least three independent experiments. Statistical significance ($P < 0.05$) of the graphic data was determined using Student's t-test or two-way ANOVA with Sidak's multiple comparisons test (GraphPad Software).

Results

Slug decreases the cellular levels of p53 and p21. Considering that p53 and p21 cooperate to promote Slug protein degradation (9), cells may have established a system for Slug to counter such actions of p53 and p21. To investigate this possibility, we knocked down Slug expression in HCT116 colon cancer cells using two sets of specific siRNAs. Slug knockdown using either siRNA resulted in an increase in the cellular levels of p53 and p21, as analyzed by western blotting (Fig. 1), suggesting that Slug reduces the levels of p53 and p21 proteins in the cells. Hereafter, only si-Slug-1 was used and is referred to as Slug siRNA.

Slug reduces p53 protein stability in a p21-dependent manner. Given that the cellular levels of p53 are controlled mainly via the regulation of its protein stability (10-12), the reduction in
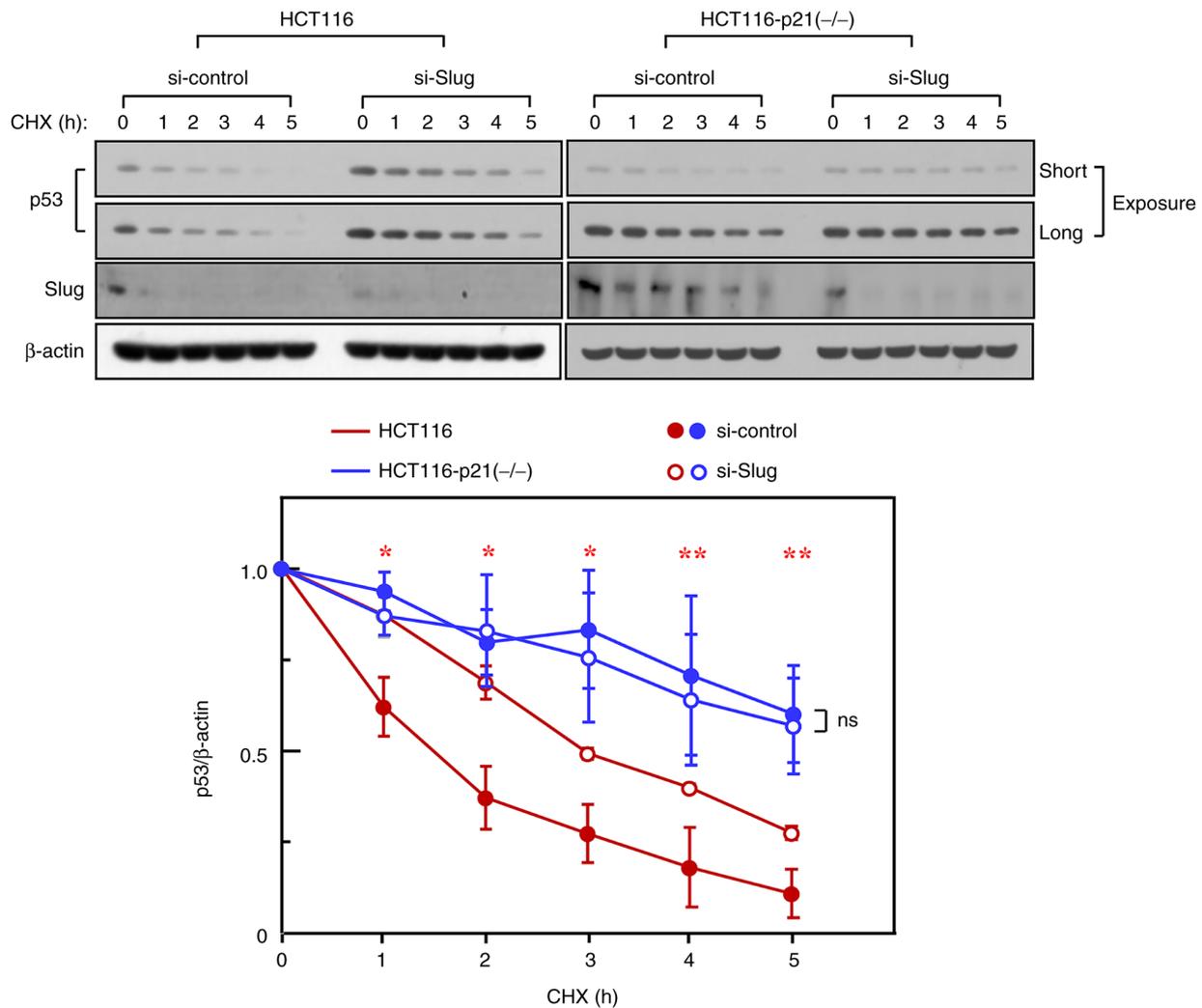
Figure 2. Slug promotes p53 protein degradation in a p21-dependent manner. HCT116 cells and their p21-knockout variants were treated with control or Slug siRNA. After 24 h of recovery, the cells were additionally treated with CHX (10 μM) and further incubated for the indicated periods. p53 levels were analyzed by western blotting and normalized to those of β-actin. Western blotting results are representative of both short and long exposures for more precise comparison. Their mean values and standard deviations are shown in the plots. *P<0.005, **P<0.05 si-control vs. si-Slug in HCT116 cells. ns, non-significant; siRNA, small interfering RNA; Slug, zinc finger protein SNAI2; CHX, cycloheximide.

p53 levels by Slug might reflect the ability of Slug to reduce p53 protein stability. To investigate this possibility, HCT116 cells were incubated in the presence of cycloheximide, an inhibitor of protein synthesis. The results showed the subsequent reduction of p53 protein, thereby indicating its degradation. The degradation of p53 was effectively reduced upon Slug knockdown (Fig. 2), suggesting that Slug promotes p53 protein degradation.

We have previously reported that p53 and p21 can form a complex via their direct interaction (9,20). In this regard, it was interesting to speculate whether p21 influences Slug-induced p53 degradation. To address this question, we used HCT116 p21-knockout variant cells. The stability of p53 protein in these variants was much higher than that in HCT116 parental cells, as reported recently (15). Notably, in contrast to HCT116 parental cells, Slug knockdown in p21-knockout cells did not further increase p53 stability (Fig. 2), indicating that Slug requires p21 to promote p53 degradation.

Slug reduces p21 protein stability in a p53-independent manner. We next investigated the possible influence of Slug

on p21 protein stability and the probable requirement of p53 for such an action of Slug. HCT116 parental cells and their p53-knockout variants were used for this purpose. Slug knockdown increased p21 protein stability in the parental cells, indicating that Slug also promotes p21 degradation. However, in contrast to the effect of p21 knockout on p53 stability, p53 knockout did not significantly influence the basal stability of p21 as well as the Slug knockdown-induced increase in p21 stability (Fig. 3). Therefore, Slug does not require p53 to promote p21 degradation.

Slug requires Mdm2 to reduce p53 and p21 levels. Since Mdm2 is involved in the degradation of both p53 and p21 (10-14), we investigated whether Mdm2 plays a role in Slug-induced degradation of p53 and p21. Towards this, we knocked down Mdm2 in HCT116 cells. As expected, Mdm2 knockdown enhanced the levels of p53 and p21 (Fig. 4A, lanes 1 and 3). However, the levels of both proteins were not further increased upon the additional knockdown of Slug (Fig. 4A, lanes 3 and 4). This observation is in contrast with the ability of Slug knockdown
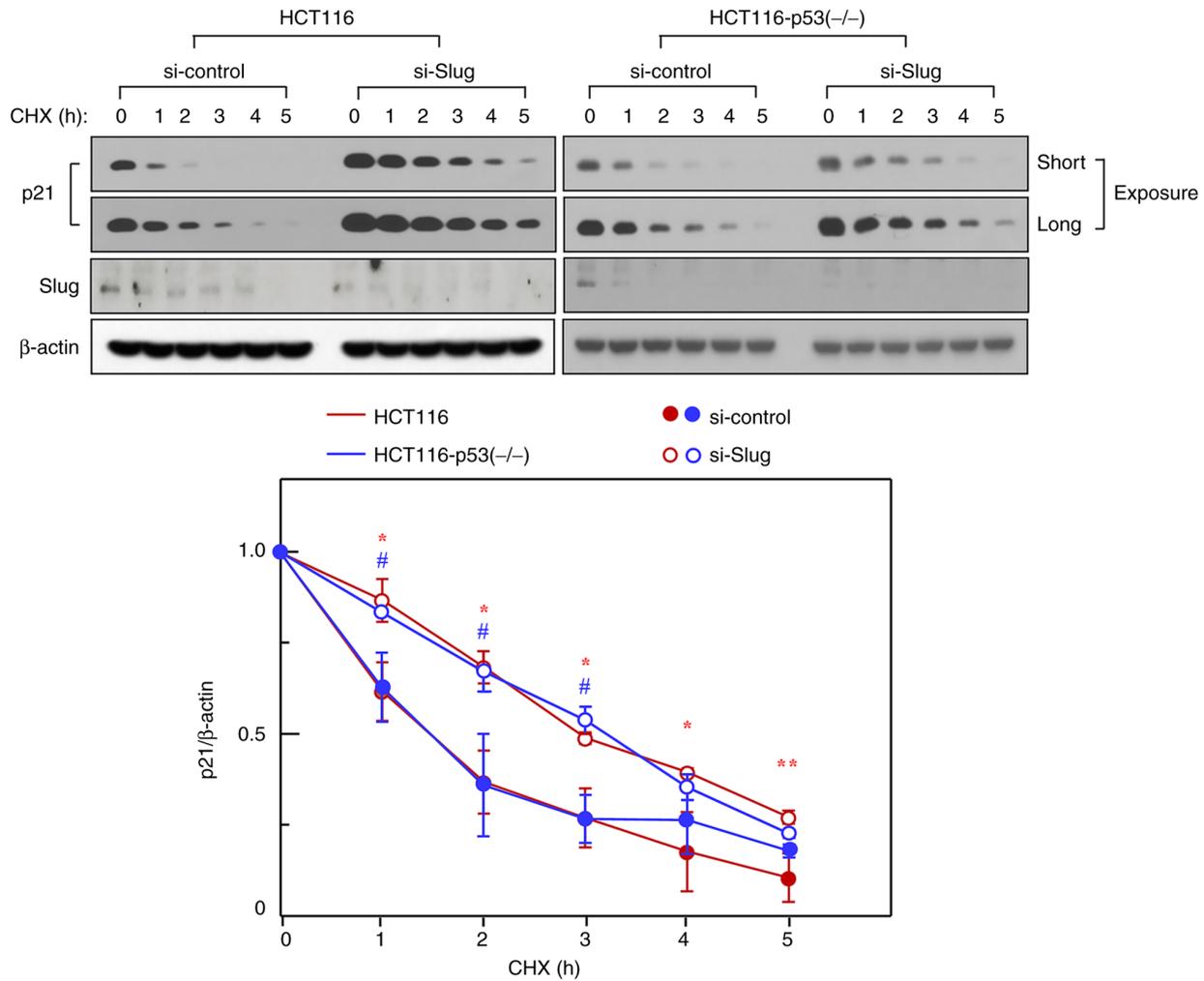
Figure 3. Slug promotes p21 protein degradation in a p53-independent manner. HCT116 cells and their p53-knockout variants were treated with control or Slug siRNA for 24 h, and then incubated in the presence of CHX (10 μM) for the indicated periods. p21 and Slug levels were analyzed. *P<0.005, **P<0.05 si-control vs. si-Slug in HCT116 cells. #P<0.005 si-control vs. si-Slug in p53-knockout variants. siRNA, small interfering RNA; Slug, zinc finger protein SNAI2; CHX, cycloheximide.

to increase p53 and p21 levels in control cells (Fig. 4A, lanes 1 and 2), suggesting that Slug requires Mdm2 to promote p53 and p21 degradation.

Slug induces Mdm2 expression. While analyzing Mdm2 expression, we unexpectedly observed that Slug knockdown reduced Mdm2 protein levels (Fig. 4A). This led us to investigate whether Slug also influences Mdm2 mRNA levels. Analysis by quantitative real-time PCR revealed that Mdm2 mRNA levels in HCT116 cells were significantly reduced upon Slug knockdown (Fig. 4B), suggesting that Slug induces Mdm2 expression. These findings also suggest that Slug promotes p53 and p21 degradation by inducing Mdm2.

Slug increases p53 ubiquitination. While Mdm2 promotes p21 degradation without ubiquitinating p21 (13,14), Mdm2-mediated ubiquitination of p53 is required for p53 degradation (10-12). Therefore, to further confirm the involvement of Mdm2 in the actions of Slug, we investigated whether Slug also influences p53 ubiquitination. As expected, levels of p53 ubiquitination were greatly reduced upon Mdm2 knockdown (Fig. 4C), consistent with the fact that Mdm2 is a major

E3 ligase of p53. Notably, Slug knockdown also effectively reduced p53 ubiquitination, indicating the ability of Slug to promote p53 ubiquitination. These data support the notion that Mdm2 can act downstream of Slug.

Slug influences Mdm2, p53, and p21 levels in multiple cell types. To investigate whether Slug regulates Mdm2, p53, and p21 levels in other cell types, we knocked down Slug in A549 and H460 lung cancer cells. As in the case of HCT116 cells, Slug knockdown decreased Mdm2 levels and increased p53 and p21 levels (Fig. 4D). Therefore, the ability of Slug to induce Mdm2 expression and thereby promote p53 and p21 degradation may not be confined to a particular cell type.

Discussion

Our studies using HCT116 colon cancer cells have shown that Slug reduces the cellular levels of p53 and p21 by decreasing their protein stability. This phenomenon appears to be mediated by Mdm2, since Slug increases Mdm2 expression and the prevention of this event by Mdm2 knockdown abolishes the ability of Slug to downregulate p53 and p21. Considering
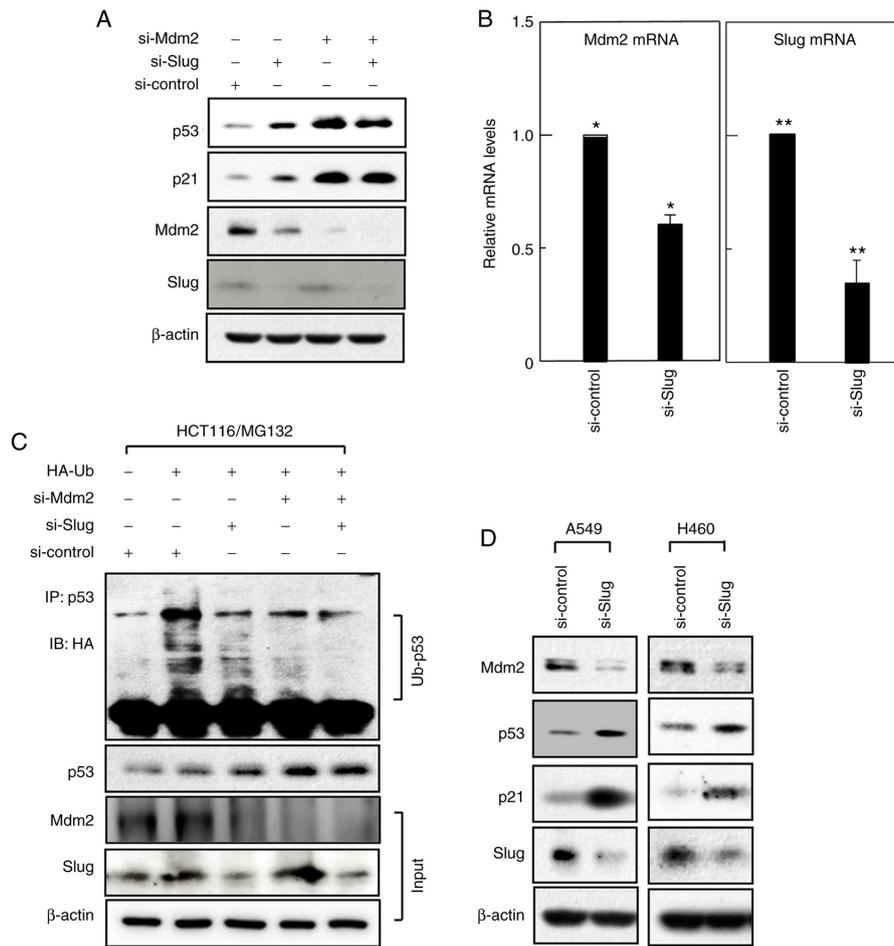
Figure 4. Slug increases Mdm2 expression. (A) HCT116 cells were treated with siRNAs targeting Slug or Mdm2 in the combination indicated. After 24 h of incubation, expression levels of the specified proteins were compared using western blotting. (B) Mdm2 and Slug mRNA levels in HCT1116 cells, treated with control or Slug siRNA, were compared by reverse transcription-quantitative PCR. (C) HCT116 cells were transfected with the expression vector for HA-tagged ubiquitin along with siRNAs targeting Slug or Mdm2 in the indicated combinations. The transfectants were treated with MG132 (10 μM) for 2 h. The precipitated proteins were analyzed by western blotting using anti-HA and anti-p53 antibodies. Effects of siRNAs were confirmed by analyzing cell lysates using anti-Mdm2 and anti-Slug antibodies (input). (D) A549 and H460 cells were treated with control or Slug siRNA for 24 h. Levels of the indicated proteins were compared. [*]P<0.005, si-control vs. si-Slug for Mdm2 mRNA. [**]P<0.05, si-control vs. si-Slug for Slug mRNA. siRNA, small interfering RNA; Slug, zinc finger protein SNAI2; HA-Ub, HA-ubiquitin; IP, immunoprecipitation; IB, immuonoblotting.

these results, we propose that Slug induces Mdm2, which in turn, acts on p53 and p21, promoting their degradation, at least in HCT116 cells. However, the application of this model to other cell types may be possible because we further verified the ability of Slug to increase and decrease Mdm2 and p53/p21 levels, respectively, using A549 and H460 lung cancer cells.

We have recently reported that Mdm2 acts more efficiently on p53 in the p53/p21 complex than p53 alone (15). In this regard, our finding that Slug requires p21 for inducing p53 degradation is consistent with the view that Mdm2 mediates Slug-induced p53 degradation. This view was further supported by the finding that Slug promotes p53 ubiquitination. In sharp contrast, we found that p53 is dispensable for Slug-induced p21 degradation, suggesting that Mdm2 can act on p21 in the absence of p53. The ability of Mdm2 to induce p21 degradation in p53-null cells has also been reported by other investigators (14). Notably, our direct comparative analysis of p21 stability in control and p53-knockout cells revealed that the presence of p53 does not further facilitate the ability of Slug (i.e., Mdm2 in this case)
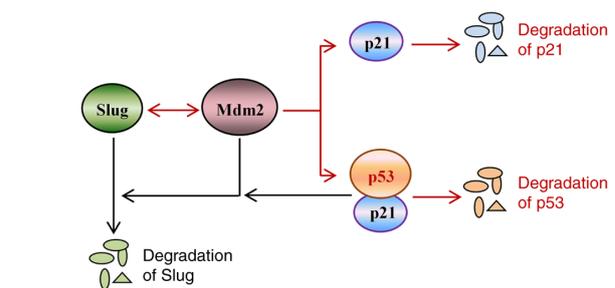
Figure 5. Schematic model representing the functional relationship between Slug, Mdm2, p53 and p21. Slug increased cellular levels of Mdm2 mRNA and protein. As Mdm2 could stabilize Slug mRNA by binding to it, an amplification loop between Slug and Mdm2 expression appeared to exist (indicated by two-headed arrow). Mdm2 could act on p53 and p21, inducing their degradation. While Mdm2 was not required p53 for p21 degradation, its action on p53 required p21 since the p53/p21 interaction promoted the p53/Mdm2 interaction. This oncogenic situation is indicated by red arrows. Mdm2 could also induce proteolytic cleavage of Slug, which was facilitated by the p53/p21 complex. This tumor-suppressing pathway is indicated by black arrows. This model suggested that Mdm2 could exert dual functions by mediating either the tumor-promoting function of Slug or tumor-suppressing function of the p53/p21 complex; the balance between these two opposing pathways may change depending on the external environment and internal physiology of cells. Slug, zinc finger protein SNAI2.

to induce p21 degradation. Our model for the role of Mdm2 in Slug-induced p53 and p21 degradation is schematically depicted in Fig. 5.

It should be noted that we verified the ability of Slug to induce Mdm2 expression by knocking down Slug. To further confirm this, we overexpressed Slug in HCT116 cells. However, this treatment did not noticeably influence the levels of Mdm2 and p53, as analyzed by western blotting (data not shown). Although the reason for these unexpected results is not clear, it may reflect that Slug is constitutively expressed in the cells to functionally saturated levels. Slug has been consistently reported to be upregulated in numerous cancer cells (3-7). The constitutive upregulation of Slug may be the reason that in sharp contrast to Slug knockdown, further elevation of Slug levels does not markedly influence its downstream target molecules.

Mdm2 is generally considered as an oncogenic protein since it degrades tumor suppressors such as p53 and p21 (21). However, the targets of Mdm2 are not confined to cellular proteins, and Mdm2 can also bind to mRNAs. For example, we showed that Mdm2 can stabilize Slug mRNA by binding to it (22). This observation, along with the ability of Slug to increase Mdm2 mRNA levels suggests the existence of an amplification loop between Slug and Mdm2 at least at the mRNA level to activate the process leading to p53 and p21 degradation (Fig. 5).

It is well known that Mdm2 is a transcriptional target of p53 (23). This raises the question as to why a tumor suppressor, such as p53, induces the expression of an oncogenic protein, such as Mdm2. Notably, Mdm2 does not always act as an oncogenic component and often performs contrasting functions by contributing to tumor suppression (24). For example, as stated earlier, Mdm2 can induce the proteolytic degradation of Slug, which is promoted by the action of the p53/p21 complex (8,9) (Fig. 5). Moreover, Mdm2 can facilitate the translocation of p53 from the nucleus to the cytosol (25,26), where p53 binds to Bcl-2 family proteins, suppressing and promoting cancer cell invasion and apoptosis, respectively, depending on the stress context (17,27-29). The dual role of Mdm2 is consistent with the induction of its expression by components with contrasting functions, such as p53 and Slug.

Given that Slug can act as a transcriptional repressor (2), Slug may reduce levels of p53 and p21 proteins not only by decreasing their stability but also by downregulating their mRNA levels. Although this latter possibility was beyond the scope of this study, future investigation of this possibility may provide a global view of the regulation of p53 and p21 levels by Slug.

In conclusion, we have demonstrated the ability of Slug to promote p53 and p21 degradation. Given that p53 and p21 can also promote Slug degradation (9), the balance between these two opposing components (p53/p21 vs. Slug) may be critical for cells to adapt to changes in the external environment and internal physiology, and the failure to do so may contribute to tumor initiation and progression.

Acknowledgements

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

JK, JL, UK, JKP and HDU performed the experiments and interpreted the results. HDU conceived and designed the current study and wrote the manuscript. All authors have read and approved the final manuscript. JK and HDU confirm the authenticity of all raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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