Regulation of Angiogenic Factors by HDM2 in Renal Cell Carcinoma

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Abstract

The oncogene HDM2 has been implicated in the regulation of the transcription factor, hypoxia inducible factor (HIF). We show in von Hippel-Lindau (VHL)-defective renal carcinoma cells that express constitutively high levels of HIF-1α and HIF-2α that down-regulation of HDM2 by siRNA leads to decreased levels of both HIF-1α and HIF-2α protein levels. However, we show a differential regulation of HDM2 on the HIF angiogenic targets, vascular endothelial growth factor (VEGF), plasminogen activator inhibitor-1 (PAI-1), and endothelin-1 (ET-1): siRNA to HDM2 leads to increased expression of VEGF and PAI-1 proteins but decreased levels of ET-1. We show that HDM2-mediated regulation of these proteins is independent of VHL and p53 but dependent on a novel action of HDM2. Ablation of HDM2 leads to phosphorylation of the transcription factor, hypoxia inducible factor (HIF). We show that regulation of these angiogenic factors is dependent on ERK1/2 phosphorylation, which can be reversed by addition of the MAP/ERK1/2 kinase inhibitors PD98059 and PD184352. This study identifies a novel role for the HDM2 oncoprotein in the regulation of angiogenic factors in renal cell carcinoma. [Cancer Res 2008;68(2):545–52]

Introduction

The oncoprotein HDM2 is an E3 ubiquitin ligase that regulates the stability of the tumor suppressor p53 (1, 2). HDM2, by action of its E3 ligase activity, ubiquitinates p53, targeting it for degradation by the proteasome (3). In addition to its function as a negative regulator of p53, HDM2 also has a number of p53-independent activities. HDM2 interacts with a variety of factors affecting RNA biosynthesis, DNA synthesis, cell cycle control, transcription, and cell surface receptor regulation (for review, see ref. 4). Of interest is the regulation of HDM2 on the transcription factor, hypoxia inducible factor (HIF). HIFs are heterodimeric complexes comprising two subunits — an oxygen regulated subunit, HIF-1α or HIF-2α, and a constitutively expressed subunit, HIF-1β — that bind to hypoxia response elements in the promoters of target genes involved in a variety of cellular functions such as erythropoiesis, angiogenesis, and glucose metabolism. Ravi et al. (5) first suggested that HDM2 might function as an E3 ligase for HIF-1α using HDM2 mutants deficient in E3 ligase activity. A second group (6) proposed that the orphan nuclear receptor, Nur77, stabilizes HIF-1α by inhibition of HDM2. Although it has been shown that HDM2 and HIF-1α directly interact with each other (7), no evidence yet exists that HDM2 directly targets HIF-1α for degradation. In fact, we (8) and other groups (9, 10) have shown that HDM2 can positively regulate HIF-1α expression. Both HIF-1α (8, 11–14) and HDM2 (8, 11, 15) are downstream targets of the phosphatidylinositol 3-kinase/Akt/PKB pathway, and we have recently found that in cells treated with insulin-like growth factor-1 (IGF-1), increased HIF-1α was dependent, in part, upon the presence and phosphorylation of HDM2 (8). Subsequently, other groups have shown that pharmacologic inhibition of the PI3K/Akt/PKB pathway leads to ablation of HDM2, HIF-1α, and vascular endothelial growth factor (VEGF; refs. 13, 16).

Von Hippel-Lindau (VHL) disease is a rare inherited cancer syndrome where patients have an increased predisposition to hemangioblastomas of the central nervous system and retina, pheochromocytoma, and clear cell renal carcinoma (17). VHL disease arises from biallelic loss or inactivation of the VHL tumor suppressor gene that also accounts for the majority of sporadic clear cell renal carcinomas (18). The VHL gene encodes pVHL, a protein of ~30 kDa that functions as an E3 ligase component of a multiprotein ubiquitin ligase complex that targets substrate proteins for degradation by the proteasome. The molecular mechanisms by which loss of VHL leads to an increased propensity for an angiogenic phenotype have recently been elucidated. HIF-1α and HIF-2α are two of the best described substrates of pVHL (19). At normal oxygen tension, HIF-α subunits are hydroxylated on conserved proline residues by a family of prolyl hydroxylases (PHD; refs. 20–22). Hydroxylated HIF-α subunits are subsequently recognized and targeted for degradation by pVHL. Under hypoxic conditions, the PHDs are inactive due to a requirement of molecular oxygen for hydroxylation. This allows HIF-α to escape VHL-mediated proteosomal degradation leading to stabilization and accumulation of HIF-α. Accumulation of HIF-α subunits and up-regulation of HIF-dependent target genes, such as VEGF, occur in cells deficient for pVHL, leading to the angiogenic phenotype typically seen in VHL-associated renal carcinoma.

In this study, we show for the first time that HDM2 regulates constitutively expressed HIF-1α and HIF-2α in VHL-defective renal carcinoma cells. However, we show that loss of HDM2 leads to a differential regulation of HIF angiogenic targets [VEGF, plasminogen activator inhibitor-1 (PAI-1), and endothelin-1 (ET-1)] at the protein level. We show that these effects seem to be independent of both p53 and pVHL but dependent on activation of extracellular-regulated kinase 1/2 (ERK1/2) signaling. Finally, we show that HDM2-mediated differential regulation of angiogenic factors in renal carcinoma cells can be reversed by pharmacologic inhibition using the MAP/ERK kinase (MEK)1/2 inhibitors PD98059 and PD184352. Our studies highlight the importance of the HDM2 oncoprotein in the differential regulation of secreted factors involved in angiogenesis in renal cell carcinoma.

Materials and Methods

Materials. Mouse anti-HDM2 and mouse anti-p53 (DO1) antibodies were purchased from Oncogene Science. Mouse anti-p21WAF1 (EA10) antibody

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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was obtained from Calbiochem (Merck Biosciences). Rabbit antiphospho-
p44/42 (pERK1/2) antibody and the MEK inhibitor PD98059 were purchased from Cell Signaling Technology. Rabbit anti-ERK antibody (tERK) was obtained from Santa Cruz Biotechnology. Mouse anti–HIF-1α antibody was obtained from BD Biosciences. Rabbit anti–HIF-2α antibody (ab199) and mouse anti–α-tubulin were purchased from Abcam. Mouse anti-cyclin D1 (DCS-6) was obtained from NeoMarkers (Lab Vision). Camptothecin and mouse anti–α-tubulin antibody were purchased from Sigma. The MEK

Figure 1. Effect of HDM2 siRNA on secreted angiogenic proteins in RCC4 and 786-O cells. A, RCC4 cells were transfected with siRNA to HDM2 or a control inverse duplex (Inv) for 24 h. Panels, whole-cell lysates were assayed by Western blot for HDM2. Tubulin was used as a load control. Graphs, conditioned medium was harvested 24 h after transfection, and secreted protein levels of VEGF, PAI-1, and ET-1 were analyzed by ELISA. B, 786-O cells were transfected with siRNA to HDM2 or a control inverse duplex for 24 h. Panels, whole-cell lysates were assayed by Western blot for HDM2. Tubulin was used as a load control. Graphs, conditioned medium was harvested 24 h after transfection, and secreted protein levels of VEGF, PAI-1, and ET-1 were analyzed by ELISA. Three independent experiments were performed. Columns, mean; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001 as determined by t test.

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Figure 2. Effect of HDM2 siRNA on HIF-α protein expression. RCC4 (A and B) and 786-O cells (C) were transfected with siRNA to HDM2 or a control inverse duplex. A, mRNA expression of HDM2 was determined by real-time quantitative PCR relative to GAPDH. B and C, whole-cell lysates were assayed by Western blot for HDM2, HIF-1α, and HIF-2α proteins. Tubulin was used as a load control.
inhibitor, PD184352, was a kind gift from Dr Simon Cook (The Babraham Institute, Cambridge, United Kingdom).

Cell culture. All tumor cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% FCS purchased from Harlan, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glutamine (all purchased from Life Technologies). The RCC4 renal carcinoma cells were gifts from Professor Patrick Maxwell (Imperial College, London, United Kingdom; ref. 19). Renal carcinoma cells (786-O) were gifts from Professor William G. Kaelin, Jr. (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) and have been described previously (23).

siRNA duplexes and transient transfections. A total of five siRNA duplexes were designed to HDM2 (Genbank accession number NM_002392) and evaluated. The most efficient duplex for silencing HDM2 targeted nucleotides 627 to 647 (5'-AATCAGCAGGAATCATCGGAC) was obtained as a gel-purified annealed duplex from Dharmacon and used in all experiments (designated oligo1 in Supplementary Data). Two other sequences from the five tested were used to independently confirm the results obtained with oligo1. These were designated oligo2 and oligo3 and were purchased from QIAGEN. An HDM2 control inverse sequence (5'-CTTCGTGAGAATTGGCTTCC) and reverse (5'-CAAAGGCCCTCTT-CAGCCTTG) primers for HDM2 were purchased from Invitrogen. Expression of target gene mRNA relative to reference gene mRNA (GAPDH) was calculated using the Relative Expression Software Tool, and statistical significance was determined using the Pair Wise Fixed Reallocation Randomization Test1 (26, 27).

Results

HDM2 differentially regulates angiogenic factors. Recent reports have identified a role for HDM2 in the regulation of HIF-1α and VEGF (8, 10). However, little is known about the role of HDM2 in the regulation of HIF-α subunits and HIF target genes in the

1 http://www.gene-quantification.com
levels of HIF-1α and 786-O renal carcinoma cells that constitutively express high levels of VEGF, PAI-1 and ET-1 was investigated in RCC4 context of renal cell carcinoma. To address this, the effect of HDM2 on multiple HIF-transcriptional targets was investigated in RCC4 and 786-O renal carcinoma cells that constitutively express high levels of HIF-1α and/or HIF-2α due to loss of VHL function (19, 23). Inhibition of HDM2 by siRNA significantly increased VEGF and PAI-1 protein levels, whereas ET-1 was significantly down-regulated in both RCC4 and 786-O cells (Fig. 1A–B; Supplementary Fig. S1). To address whether HDM2 also affected constitutive basal expression of HIF-α subunits, RCC4 and 786-O cells were treated with siRNA to HDM2. Significant knockdown of HDM2 mRNA and protein levels were achieved (Fig. 2A–C). Ablation of HDM2 led to decreased protein levels of both HIF-1α and HIF-2α in RCC4 cells (Fig. 2B; Supplementary Figs. 1A–B) and decreased HIF-2α protein levels in 786-O cells that only express this HIF-α subunit (Fig. 2C). In addition, reverse transcription-PCR experiments revealed that both HIF-1α and HIF-2α mRNA were decreased after HDM2 siRNA (Supplementary Fig. S2). These data identify a role for HDM2 in the regulation of HIF-1α and HIF-2α in normoxia and are consistent with previous studies that show a positive regulation of HDM2 on growth factor–induced HIF-1α (8, 9).

To determine whether HDM2-mediated regulation of angiogenic factors was dependent on the expression of the HIF-α subunits due to loss of VHL function, similar experiments were performed in RCC4 cells that express pVHL (19). A similar pattern of regulation of VEGF, PAI-1, and ET-1 was observed in VHL-competent RCC4 cells after siRNA to HDM2 (Fig. 3A), indicating that the effects observed were not dependent on the expression of VHL and were therefore unlikely to be HIF-α dependent. In fact, recent reports have shown that HIF-1α and HIF-2α display a distinct specificity in their activation of HIF target genes with a predominant role for HIF-2α in regulation of HIF transcriptional targets in renal cell carcinoma (25, 28, 29). Raval et al. (29) showed that HIF-2α primarily regulates VEGF, cyclin D1, and transforming growth factor-α, whereas HIF-1α regulates BNIP3. We recently confirmed that VEGF is predominantly regulated by HIF-2α rather than HIF-1α in RCC4 cells and, in addition, showed that both PAI-1 and urokinase-type plasminogen activator receptor are also regulated by HIF-2α in these cells (25). Therefore, to further investigate the extent to which HIF-α regulates protein levels of VEGF, PAI-1, and ET-1, siRNA techniques to HIF-1α and HIF-2α were used. Ablation of HIF-2α, but not HIF-1α, reduced VEGF and PAI-1 protein (data not shown), consistent with our previous results (25), and partially reduced ET-1 protein levels (Fig. 3B). Thus, loss of HIF-1α had no significant effect, and loss of HIF-2α only partially inhibited the basal expression of VEGF, PAI-1, and ET-1 in these cells (Fig. 3B; ref. 25), suggesting that other mechanisms are also involved in their regulation. Indeed, our data indicate that although HDM2 can clearly regulate HIF-α expression in VHL-defective renal carcinoma cells, HDM2 also differentially regulates angiogenic factors in these cells independently of HIF-α status.

HDM2-mediated differential regulation of angiogenic factors is not dependent on p53. We have previously shown that the effects of HDM2 on IGF-1–induced HIF-1α expression is independent of p53 (8). However, because HDM2 regulates the degradation of p53 (1, 2), we were interested in determining whether a potential induction of p53 by treatment of cells with HDM2 siRNA occurred, and whether this contributed to the increase in secretion of angiogenic proteins. Of particular interest was PAI-1 because this is also known to be a p53 target gene (30). Treatment of RCC4 cells with siRNA to HDM2 led to a modest but reproducible increase in p53 protein levels and an induction of the p53 target, p21 (Fig. 4A–B). However, the increased PAI-1 protein levels observed after HDM2 siRNA treatment were not reversed by cotransfection with siRNA to p53 (Fig. 4C).
addition, p53 siRNA treatment alone had no significant effect on basal PAI-1 levels (Fig. 4C). In contrast, however, p21 expression was blocked by both p53 siRNA alone or in combination with HDM2 siRNA (Fig. 4B). These data indicate that the increase in PAI-1 (Fig. 4C) and VEGF proteins (Fig. 4D) observed after HDM2 siRNA treatment was not due to activated p53 but suggest that other mechanisms are involved.

HDM2 regulates ERK1/2 signaling in renal cell carcinoma. Both the Ras-MEK-ERK and the PI3K-Akt/PKB signaling pathways have been shown to regulate HDM2 levels (8, 11, 31), VEGF (12, 32), and PAI-1 (33, 34). To determine whether signaling via either of these pathways was affected by siRNA to HDM2, levels of phospho-ERK1/2 and phospho-Akt/PKB were investigated. No changes were observed in phosphorylated Akt/PKB signaling upon addition of HDM2 siRNA (data not shown). However, a significant up-regulation of phosphorylated ERK1/2 was observed upon treatment of RCC4 and 786-O cells with siRNA to HDM2 (Fig. 5A; Supplementary Fig. S1B). Increased ERK1/2 phosphorylation was observed in both RCC4 and 786-O cells expressing pVHL (Fig. 5A).

To determine whether other inhibitors of HDM2 could elicit similar effects on ERK1/2 phosphorylation, RCC4 cells were treated with the DNA-damaging agent, camptothecin, which has previously been shown to transiently down-regulate HDM2, leading to stabilization of p53 (35). Camptothecin treatment led to down-regulation of HDM2 in RCC4 cells (Fig. 5B) as has been shown previously in other cell types (35). Activation of p53 and induction of p53 targets p21 and cyclin D1 were observed after camptothecin treatment (Fig. 5B). Importantly, a significant increase in phospho-ERK1/2 was observed at 24 h (Fig. 5B) in concert with a significant increase in VEGF protein levels (Fig. 5C). Taken together these results implicate a role for ERK1/2 in the HDM2-mediated effects observed in renal carcinoma cells.

Regulation of angiogenic proteins is dependent on ERK1/2 activity. To further explore the role of ERK1/2 signaling in the regulation of VEGF, PAI-1, and ET-1, RCC4 cells were treated with the MEK1/2 inhibitors PD98059 and PD184352. Both PD98059 and PD184352 inhibited phosphorylated ERK1/2, had no effect on HIF-1α and HIF-2α proteins, but reduced both basal VEGF and PAI-1 levels (Fig. 6A), indicating that phosphorylated ERK1/2 was important for the positive regulation of VEGF and PAI-1. Conversely, basal ET-1 levels were increased after MEK1/2 inhibition, indicating that ET-1 expression was negatively regulated or repressed by phosphorylated ERK1/2 (Fig. 6A). To investigate the effects of MEK1/2 inhibition on the increase in VEGF mediated by siRNA to HDM2, RCC4 cells were treated with PD98059 or PD184352 in the presence of HDM2 siRNA (Fig. 6B). Importantly, PD98059 and PD184352 also inhibited VEGF in the presence of HDM2 siRNA, further supporting a role for MEK1/2-ERK1/2

Figure 5. Induction of ERK1/2 phosphorylation after inhibition of HDM2. A, the cell lines as indicated were treated with siRNA to HDM2 or a control inverse duplex. Twenty-four hours after transfection, whole-cell lysates were assayed by Western blot for HDM2, pERK1/2, and total ERK1/2 (tERK1/2). Tubulin was used as a load control. B, RCC4 cells were treated with 2 μmol/L camptothecin (CPT) or vehicle alone (DMSO) for 24 h. Whole-cell lysates were assayed for HDM2, p53, p21, cyclin D1, pERK1/2, and tERK1/2. Tubulin was used as a load control. C, conditioned medium was harvested from the experiment described in B and analyzed for secreted protein levels of VEGF. Three independent experiments were performed. Columns, mean; bars, SE. *, P < 0.05 as determined by t test.
signaling in the regulation of secreted angiogenic factors in renal cell carcinoma cells.

Taken together, these data suggest that HDM2 is clearly important for HIF-α expression in normoxia in renal carcinoma cells that have lost VHL function. Interestingly, HDM2 also regulates the differential expression of several angiogenic factors analyzed herein. This novel function of HDM2 seems to be independent of HIF-α and p53 status but dependent on MEK1/2-ERK1/2 activity.

Discussion

In this study, we investigated the role of HDM2 in the regulation of HIF-α protein levels and secreted angiogenic factors known to be HIF targets in renal carcinoma cells. We have shown a requirement for HDM2 in the normoxic expression of HIF-1α and HIF-2α in these cells. Down-regulation of HDM2 led to a decrease in protein levels of both HIF-α subunits. Surprisingly, however, only one of three HIF angiogenic targets selected for analysis, ET-1, was down-regulated by loss of HDM2 as might be expected due to the decreased protein levels of both HIF-α subunits. Instead, VEGF and PAI-1 were significantly induced by down-regulation of HDM2. It seemed unlikely that the effects of HDM2 on VEGF and PAI-1 were exerted through increased HIF transcriptional activity as both HIF-1α and HIF-2α mRNA and protein (Fig. 2; Supplementary Figs. S1–2) were reduced by loss of HDM2, and VEGF mRNA was not affected (Supplementary Fig. S3). Moreover, no significant differences were observed between VHL-defective cells that express HIF-1α and VHL-competent cells that do not. The finding that HIF-1α does not contribute at all to the regulation of VEGF, PAI-1, and ET-1 (Fig. 3B; ref. 25) nor to the HDM2-mediated differential regulation of these factors and that HIF-2α only partially contributes to these effects further supports the notion that HDM2 can regulate these angiogenic factors independently of HIF-α.

Another potential explanation for these results could be due to the stabilization and activation of p53 by loss of HDM2. However, the significance of p53 in renal cell carcinoma has been the subject of conflicting reports as it is not clear whether p53 is functional in these
cells. One study has shown in several wild-type p53 renal carcinoma cell lines that p53 was not transcriptionally active (36). A second study using predominantly different renal carcinoma cell lines showed that p53 is functional and regulated by HDM2 (37). Although the renal carcinoma cell lines used in this study do not overlap with those used in either of these other two reports (36, 37), our data suggest that p53 is functional in renal carcinoma. We showed that siRNA to HDM2 led to a modest but reproducible increase in p53 protein levels and activation of p21, a p53 target gene (Fig. 4). Down-regulation of p53 by siRNA also decreased p21. As P1 is also a p53 target gene, we wanted to assess whether HDM2 siRNA-induced activation of p53 was responsible for the increase in P1-AI protein levels observed. However, cotransfection of both HDM2 and p53 siRNA did not reduce secreted P1-AI protein, indicating that p53 was not responsible for the increased P1-AI protein levels observed after HDM2 siRNA. In addition, our observations indicated that p53 status did not affect the ability for HDM2 siRNA to induce VEGF, suggesting that other mechanisms were involved.

We (32) and others have shown that ERK1/2 signaling regulates HIF activity and VEGF induction in response to growth factor signaling. In this report, we show that levels of VEGF, P1-AI, and ET-1 are all dependent on ERK1/2 activity. Pharmacologic inhibition of ERK1/2 signaling reduced both VEGF and P1-AI and, interestingly, induced ET-1 protein (Fig. 6f). It seemed likely, therefore, that an increase in ERK1/2 phosphorylation would lead to a corresponding increase in VEGF and P1-AI and decreased ET-1. Interestingly, our data show that this is a possibility as increased ERK1/2 activity mediated by HDM2 siRNA corresponded to increased VEGF and P1-AI and to decreased ET-1. Therefore, the ability of HDM2 to differentially regulate the expression of angiogenic factors is likely to be dependent on its capacity to regulate MEK1/2/ERK1/2 activity in renal carcinoma cells. It has previously been shown that activation of the Ras-Raf-MEK/ERK1/2 pathway can induce HDM2 expression (31), which can be attenuated with the MEK1/2 inhibitors PD98059 and U0126 (38). Our data indicate that down-regulation of HDM2 can also activate ERK1/2 signaling, suggesting the possibility of a feedback loop between HDM2 and ERK1/2 pathways. How loss of HDM2 mediates an increase in ERK1/2 phosphorylation in renal carcinoma cells is an interesting question. It is tempting to speculate that HDM2 may regulate the stability of a kinase or phosphatase that affects MEK1/2/ERK1/2 signaling.

Of interest is the potential significance of HDM2 regulation of ERK1/2 signaling in renal cell carcinoma. Our experiments with VH1-competent cells revealed that this was not dependent on expression of pVHL. A more comprehensive analysis of cell lines however should determine whether this is a unique pathway in renal carcinoma. Inhibition of HDM2 is currently considered an attractive strategy for anticancer therapy both in its role as a negative regulator of p53 and in its p53-independent activities (4, 39). The use of small molecule inhibitors of HDM2 as potential anticancer agents has also recently gained momentum (16, 40–42). Interestingly, two recent studies (10, 42) have highlighted the antiangiogenic effects of Nutlin-3, which prevents the association of HDM2 with p53, resulting in strong stabilization of both p53 and HDM2 proteins. These reported effects of Nutlin-3 are not in discordance with our results shown here, as LaRusch et al. (10) have shown that Nutlin-3 most likely exerts its antiangiogenic effects via a HIF-dependent mechanism. In our study, we have revealed a more complex role of HDM2 in renal cell carcinoma. Using siRNA techniques, ablation of HDM2 can lead to activation of the Ras-Raf-MEK/ERK1/2 pathway and to differential regulation of angiogenic proteins that is independent of HIF-α. Other studies have also shown that sustained induction of phosphorylated ERK1/2 can also abrogate apoptosis (43). These data, therefore, have implications for the potential use of HDM2 inhibitors in renal carcinoma. In conclusion, our studies show that HDM2 affects distinct cellular pathways that may independently govern HIF-α protein abundance and levels of angiogenic proteins that may have important consequences for the development of renal cell carcinoma.

Acknowledgments

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