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Skinner, Heath D.; Zheng, Jenny Z.; Fang, Jing; Agani, Faton; and Jiang, Bing-Hua, "Vascular Endothelial Growth Factor Transcriptional Activation Is Mediated By Hypoxia-Inducible Factor 1A, Hdm2, And P70S6K1 In Response To Phosphatidylinositol 3-Kinase/Akt Signaling" (2004). *Faculty Scholarship*. 726.

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# Vascular Endothelial Growth Factor Transcriptional Activation Is Mediated by Hypoxia-inducible Factor 1 $\alpha$ , HDM2, and p70S6K1 in Response to Phosphatidylinositol 3-Kinase/AKT Signaling\*

Received for publication, April 13, 2004, and in revised form, July 26, 2004  
Published, JBC Papers in Press, August 26, 2004, DOI 10.1074/jbc.M404097200

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**Vascular endothelial growth factor (VEGF) expression is elevated in ovarian and other cancer cells. However, the mechanism that causes the increase in VEGF expression still remains to be elucidated. In this study, we demonstrated that activation of PI3K signaling mediated VEGF protein expression at the transcriptional level through hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) expression in human ovarian cancer cells. We found that inhibition of PI3K activity by LY294002 decreased VEGF transcriptional activation and that forced expression of AKT completely reversed the inhibitory effect. HDM2 and p70S6K1 are two downstream targets of AKT that mediate growth factor-induced VEGF transcriptional activation and HIF-1 $\alpha$  expression. The inhibition of PI3K by LY294002 inhibited p70S6K1 and HDM2 activity in the cells. Forced expression of p70S6K1 or HDM2 reversed LY294002-inhibited VEGF transcriptional activation and HIF-1 $\alpha$  expression. This study identifies a potential novel mechanism responsible for increased VEGF expression in ovarian cancer cells. It also indicates the important role of VEGF and HIF-1 in ovarian tumorigenesis and angiogenesis, which is mediated by the PI3K/AKT/HDM2 and AKT/p70S6K1 pathways in ovarian cancer cells.**

Vascular endothelial growth factor (VEGF)<sup>1</sup> is essential for both physiological and pathological angiogenesis and has been shown to play a critical role in ovarian cancer. Many studies have shown that increased VEGF expression correlates with poor prognosis in ovarian cancer patients (1–3). VEGF is expressed by ovarian cancer cells (4) and is found at high levels in the malignant ascites of humans as well as in animal models (5–7). Flt-1 and KDR, the two VEGF receptors, are expressed in both ovarian cancer cells and the ovarian tumor vasculature (8, 9). VEGF production correlates with ovarian cancer cell proliferation (10), whereas inhibition of VEGF, whether pharmacologically or by an inhibitory antibody, decreases ascite formation and mortality in ovarian cancer models (11–14).

\* This work was supported by National Institutes of Health Grant RR16440 (to B.-H. J.) and NS41309 (to F. A.) and by American Cancer Society Research Scholar Grant 04-076-01-TBE (to B.-H. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>1</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; PI3K, phosphatidylinositol 3-kinase; IGF, insulin-like growth factor; ELISA, enzyme-linked immunosorbent assay; Luc, luciferase; CMV, cytomegalovirus.

VEGF is a primary transcriptional target of hypoxia-inducible factor 1 (HIF-1). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits (15, 16). HIF-1 $\beta$  is constitutively expressed in cells, whereas HIF-1 $\alpha$  expression is up-regulated by hypoxia, as well as by a variety of growth factors and oncogenes (16–23). HIF-1 has previously been shown to play a crucial role in both angiogenesis and tumor growth (24–27). HIF-1 activity is primarily regulated by the levels of HIF-1 $\alpha$  in the cells. Inhibition of HIF-1 $\alpha$  expression leads to decreased tumor size *in vivo*, whereas increased HIF-1 $\alpha$  expression has the reverse effect (25–27). In some cancers, HIF-1 $\alpha$  expression is associated with tumor aggressiveness and patient mortality (28–31). Under hypoxic conditions, HIF-1 $\alpha$  expression is controlled primarily at the post-transcriptional level, due to an inability to bind the E3 ubiquitin ligase, von Hippel Lindau protein. However, the mechanism of HIF-1 $\alpha$  expression induced by growth factor stimulation has not been completely elucidated. PI3K signaling was shown to regulate HIF-1 $\alpha$  expression in some cell systems in response to growth factors and hypoxia (20–23, 32, 33), whereas it was shown not to be involved in HIF-1 $\alpha$  regulation in other cell lines (34).

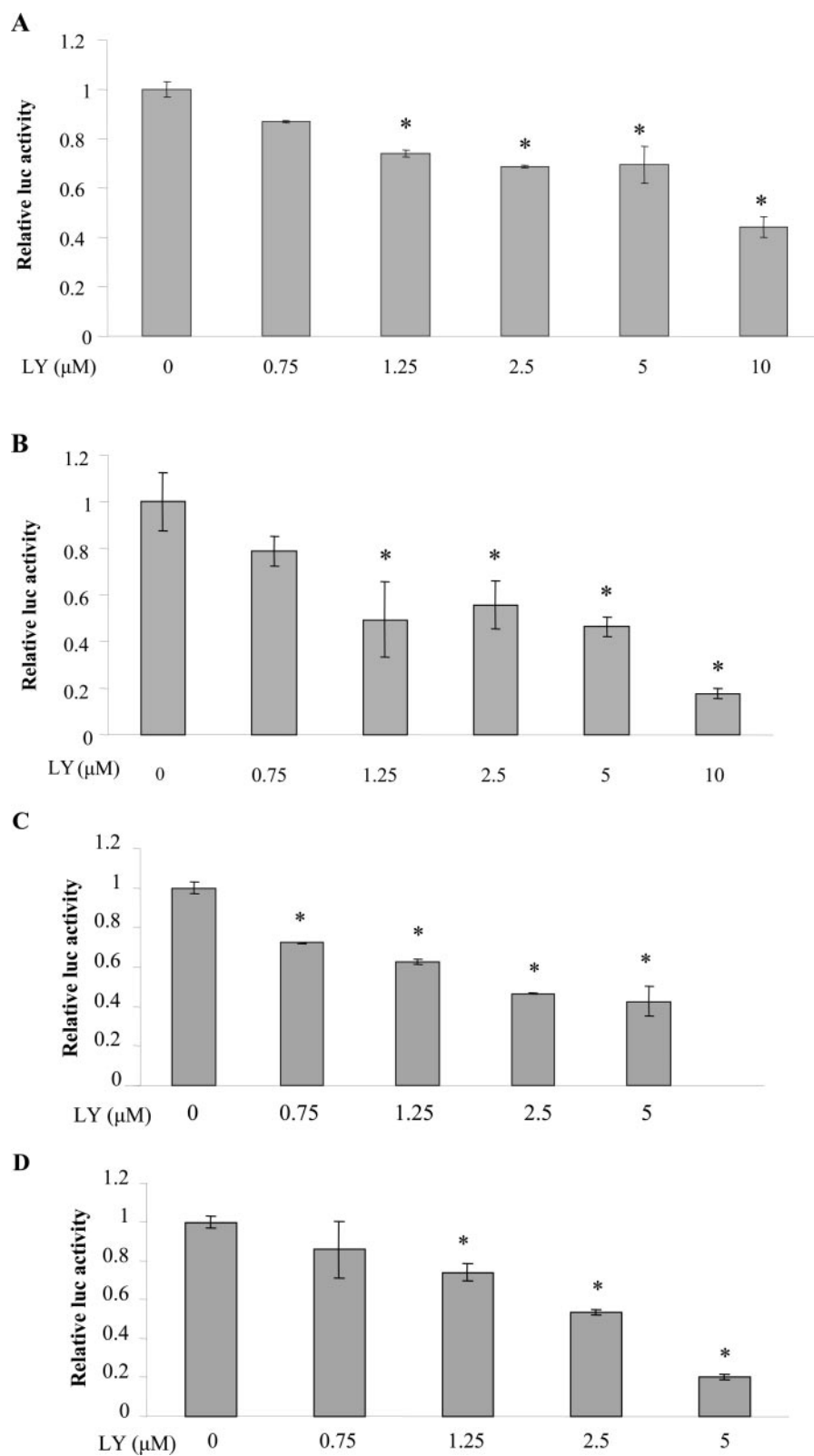
PI3K signaling is frequently up-regulated in ovarian cancer cells. *PI3CA*, the gene that encodes the p110 catalytic subunit of PI3K, is increased in copy number in 40% of ovarian cancer occurrences (35). The p85 $\alpha$  regulatory subunit of PI3K is also frequently mutated in ovarian cancer (36). AKT1 and AKT2 are both activated in a large number of ovarian carcinomas, with activation being associated with a higher tumor stage (37, 38). Pharmacologic inhibition of PI3K decreased ovarian cancer cell proliferation and tumor growth *in vivo* and rendered ovarian cancer cells more sensitive to chemotherapy agents (35, 39, 40).

In this study, we wanted to determine whether PI3K signaling regulates VEGF expression and transcriptional activation and to determine whether PI3K-mediated VEGF expression is regulated by HIF-1 $\alpha$  expression in ovarian cancer cells. We further investigated the possible mechanism by which PI3K signaling mediates VEGF and HIF-1 $\alpha$  expression and identified potential signaling molecules for regulating VEGF and HIF-1 $\alpha$  expression.

## MATERIALS AND METHODS

**Reagents and Cell Culture**—The human ovarian cancer cell lines A2780, A2780/CP70, OVCAR-3, and SKOV-3 were maintained in RPMI medium supplemented with 10% fetal bovine serum (Intergen, Purchase, NY), 0.2 units/ml human insulin (Sigma), 2 mM glutamine, 50 units/ml penicillin, and 50  $\mu$ g/ml streptomycin (Invitrogen). Human umbilical vein endothelial cells were maintained in EGM endothelial cell medium (Cambrex, East Rutherford, NJ). All cells were cultured at 37 °C in a 5% CO<sub>2</sub> incubator, and trypsin (0.25%) was used to detach adherent cells for subculture. LY294002 and wortmannin were

**FIG. 1. The effects of PI3K inhibitor LY294002 (LY) on VEGF transcriptional activation and VEGF protein level.** A and B, OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well on a 6-well plate the day before the transfection. To test VEGF transcriptional activation, the cells were co-transfected with pCMV- $\beta$ -galactosidase plasmid and a VEGF reporter containing a 2.6-kb human VEGF promoter fragment in the pGL2 vector, pVEGF-Luc. After the transfection, the cells were cultured for 12 h followed by incubation in the absence or presence of LY294002 at the indicated concentrations for 24 (A) and 36 h (B). The relative luciferase activity was determined by the ratio of luciferase: $\beta$ -galactosidase activity and normalized to the control in the cells. C and D, cells were co-transfected with pCMV- $\beta$ -galactosidase plasmid and a VEGF reporter containing a 46-bp functional human VEGF promoter with the HIF-1 binding site, pMAP11wt. The cells were cultured and treated with LY294002 for 24 h (C) and 36 h (D) as described above. E, the cells were co-transfected with pCMV- $\beta$ -galactosidase plasmid and the pMAP11mut, which contained 3-bp substitutions at the HIF-1 binding site of pMAP11wt. The cells were treated with LY294002 as described above for 36 h. F and G, OVCAR-3 (F) and A2780/CP70 (G) cells were treated with Me<sub>2</sub>SO or LY294002 for 24 and 36 h. VEGF protein levels were analyzed by ELISA using 100  $\mu$ l of media as described under "Materials and Methods," and the values were normalized to the control. \* indicates a significant difference from the control ( $p < 0.05$ ).



obtained from Calbiochem, and rapamycin was obtained from Cell Signaling Technology (Beverly, MA).

**Immunoblotting Analysis**—Cells were washed in cold  $1 \times$  phosphate-buffered saline and lysed with radioimmune precipitation buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 2 mM aprotinin, and 2 mM pepstatin on ice for 30 min. Cellular debris was removed by centrifugation at 13,000 rpm

for 15 min at 4  $^{\circ}$ C. Total cellular protein concentration was assayed using Bio-Rad $\text{\textcircled{R}}$  protein assay reagent. Aliquots (40  $\mu$ g) of protein were loaded onto a SDS/polyacrylamide gel and resolved by gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane in 20 mM Tris-HCl (pH 8.0) with 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with either 5% nonfat dry milk in  $1 \times$  Tris-buffered saline or 5% bovine serum albumin in  $1 \times$  Tris-buffered saline and incubated with protein specific antibodies. Proteins were detected via horseradish peroxidase-conjugated antibodies (PerkinElmer Life

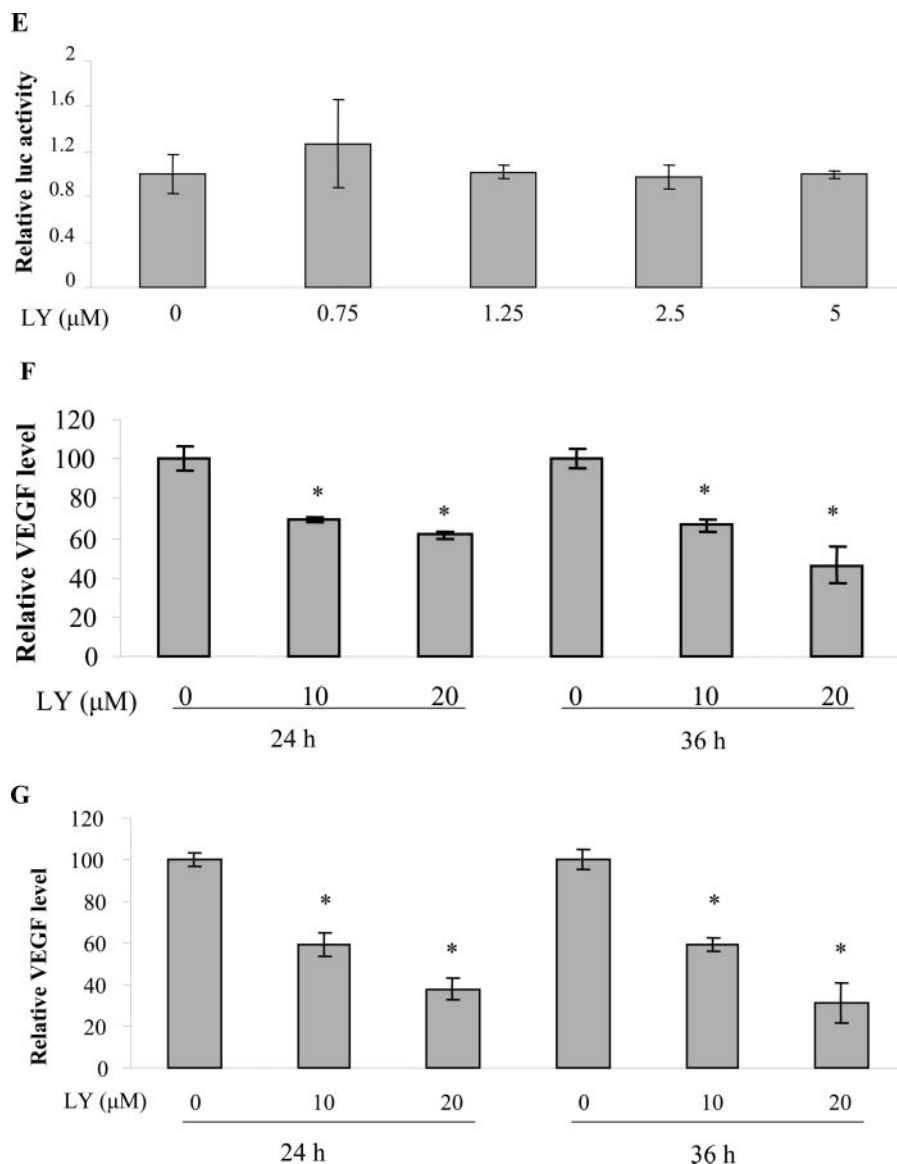


FIG. 1—continued

Sciences) and visualized through enhanced chemiluminescence reagent (PerkinElmer Life Sciences).

**cDNA Constructs**—HIF-1 $\alpha$  wild type and HIF-1 $\alpha$  dominant negative were cloned into the pCEP4 vector (Invitrogen) as we described previously (1). The human VEGF reporter was constructed by inserting a 2.65-kb KpnI-BssHIII fragment of human VEGF gene promoter into the pGL2-basic vector (Promega, Madison, WI) (pVEGF-Luc). The pMAP11wt VEGF reporter was constructed by PCR amplification of a fragment of the VEGF promoter from -985 to -939. This fragment regulates VEGF transcription in response to hypoxia and contains the HIF-1 binding site. The pMAP11mut was constructed by substituting 3 bp in the HIF-1 binding sequence of the pMAP11wt VEGF reporter. This 3-bp substitution abolishes HIF-1 binding to the region. The  $\beta$ -galactosidase gene driven by the CMV promoter was used as a control plasmid for transfection efficiency.

**Transient Transfection and Luciferase Assays**—OVCAR-3 cells were seeded in a 6-well plate at a density of  $0.3 \times 10^6$  cells/well the day before the transfection. The cells were washed twice with warm Hank's buffered salt solution (Invitrogen) and then transfected with LipofectAMINE (Sigma) per the manufacturer's instructions. Briefly, the DNA was incubated with 5  $\mu$ l/well LipofectAMINE in serum-free Opti-MEM medium (Invitrogen) for 30 min. This solution was then added to the cells and allowed to incubate at 37  $^{\circ}$ C for 4.5 h. The LipofectAMINE was then removed, and cells were cultured as described above. For the luciferase assays, cells were transfected with VEGF promoter reporter and pCMV- $\beta$ -galactosidase (control). The cells were cultured for 12 h after transfection followed by incubation in the absence or presence of LY294002 or rapamycin. At the end of incubation, the cells were col-

lected in luciferase lysis buffer (Promega, Madison, WI) per the manufacturer's instructions. Briefly, 250  $\mu$ l of luciferase lysis buffer was added to each well and placed at -70  $^{\circ}$ C until frozen. Cells and lysis buffer were allowed to thaw and then collected and stored at -70  $^{\circ}$ C until use. Aliquots of protein samples were used for luciferase assay using luciferase substrate (Promega, Madison, WI) and measured by a monolight luminometer.  $\beta$ -Galactosidase activity was determined using 40  $\mu$ g of cellular protein extracts by the hydrolysis of *o*-nitrophenyl-b-D-galactopyranoside at 37  $^{\circ}$ C for 1 h.

**Stable Transfection**—A2780/CP70 cells were transfected using pcDNA3 vector alone or pcDNA3-HDM2, which contains a neomycin resistance cassette. The cells were cultured overnight after transfection followed by the addition of G418. The G418 resistant cells were pooled after 2 weeks and cultured as described above in media supplemented with G418.

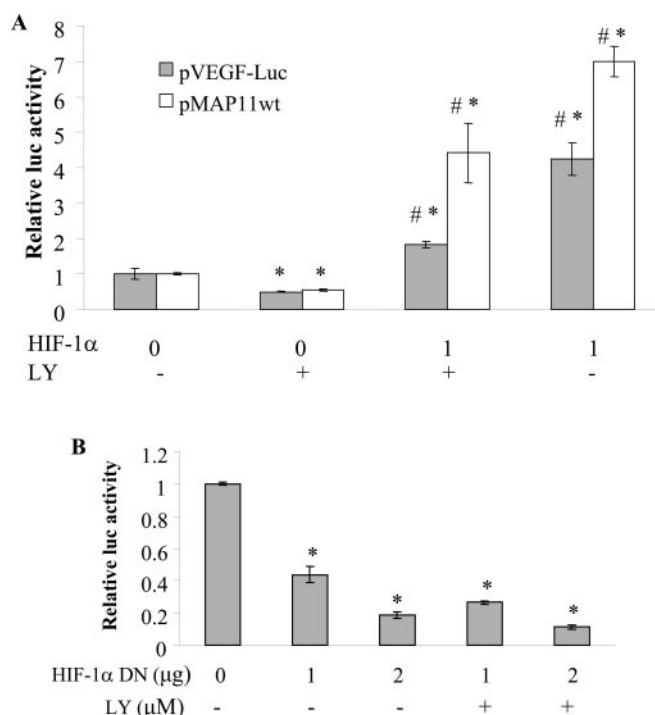
**VEGF ELISA**—Media were collected from cells and centrifuged at 800 rpm for 4 min at room temperature to remove any cellular debris and then stored at -70  $^{\circ}$ C. Wells of a 96-well plate were coated with VEGF polyclonal capture antibodies (R&D Systems, Minneapolis, MN) overnight at 4  $^{\circ}$ C. Aliquots of media were then added to each well and allowed to incubate at room temperature. VEGF monoclonal detection antibody coupled to horseradish peroxidase (R&D Systems) was added to the wells and incubated. The wells were washed, the levels of VEGF were detected using 2,2'-azino-bis(3-ethylbenzothione-6-sulfonic acid as substrate, and 1%  $H_2O_2$  was then added. The color change was then measured in a 96-well micro plate reader and compared with the VEGF standard in the same assay.

## RESULTS

**The PI3K Inhibitor LY294002 Inhibited VEGF Transcriptional Activation and Protein Expression in Ovarian Cancer Cells**—Increased VEGF expression is an important factor for inducing ovarian tumorigenesis; however, the mechanism of its elevation still remains to be elucidated. To determine whether PI3K activity plays a role in VEGF transcriptional activation, OVCAR-3 cells were transfected with a VEGF promoter reporter containing a 2.6-kb human VEGF promoter. Inhibition of PI3K activity by LY294002 inhibited the VEGF reporter activity (Fig. 1, A and B). This result indicates that PI3K activity is required for VEGF transcriptional activation. It is known that VEGF transcription is mainly regulated by HIF-1 in response to hypoxia. To test whether the HIF-1 binding site at the VEGF promoter is important for PI3K-mediated VEGF transcriptional activation, we constructed a VEGF reporter containing a functional promoter fragment with the HIF-1 binding site. Inhibition of PI3K by LY294002 also inhibited the VEGF reporter in a time- and dose-dependent manner (Fig. 1, C and D). Mutation of the HIF-1 binding site abolished the inhibitory effect of LY294002 on VEGF transcriptional activity (Fig. 1E). Thus, the inhibitory effect of LY294002 on VEGF transcriptional activation requires the HIF-1 binding site at the VEGF promoter. To determine whether LY294002 treatment affects VEGF protein levels, OVCAR-3 and A2780/CP70 cells were treated with LY294002. A2780/CP70 cells contain the lost function of p53 protein, which would test the effect of PI3K inhibition without p53 activity in the cells. The VEGF protein levels in the medium were measured by ELISA. As shown in Fig. 1, F and G, LY294002 treatment significantly decreased VEGF protein levels in both cell lines.

**LY294002-inhibited VEGF transcriptional Activation Was Reversed by HIF-1 $\alpha$** —To further study whether the inhibitory effect of LY294002 on VEGF transcriptional activation depends on HIF-1 $\alpha$  expression, the cells were co-transfected with VEGF promoter reporters and HIF-1 $\alpha$  plasmids. Transfection with HIF-1 $\alpha$  alone significantly increased the VEGF reporter activity, completely reversed LY294002-inhibited VEGF transcriptional activation, and resulted in even higher levels of activity (Fig. 2A). This result shows that HIF-1 $\alpha$  is sufficient to induce VEGF transcriptional activation in the cells. To test whether HIF-1 activity is required for the VEGF expression, the cells were transfected with HIF-1 $\alpha$  dominant negative construct, which inhibited the VEGF promoter activity in a dose-dependent manner (Fig. 2B). The inhibition was not affected significantly by combined treatment with LY294002 and the HIF-1 dominant negative construct (Fig. 2B). These results suggest that LY294002-inhibited VEGF transcriptional activation depends on HIF-1 $\alpha$  expression.

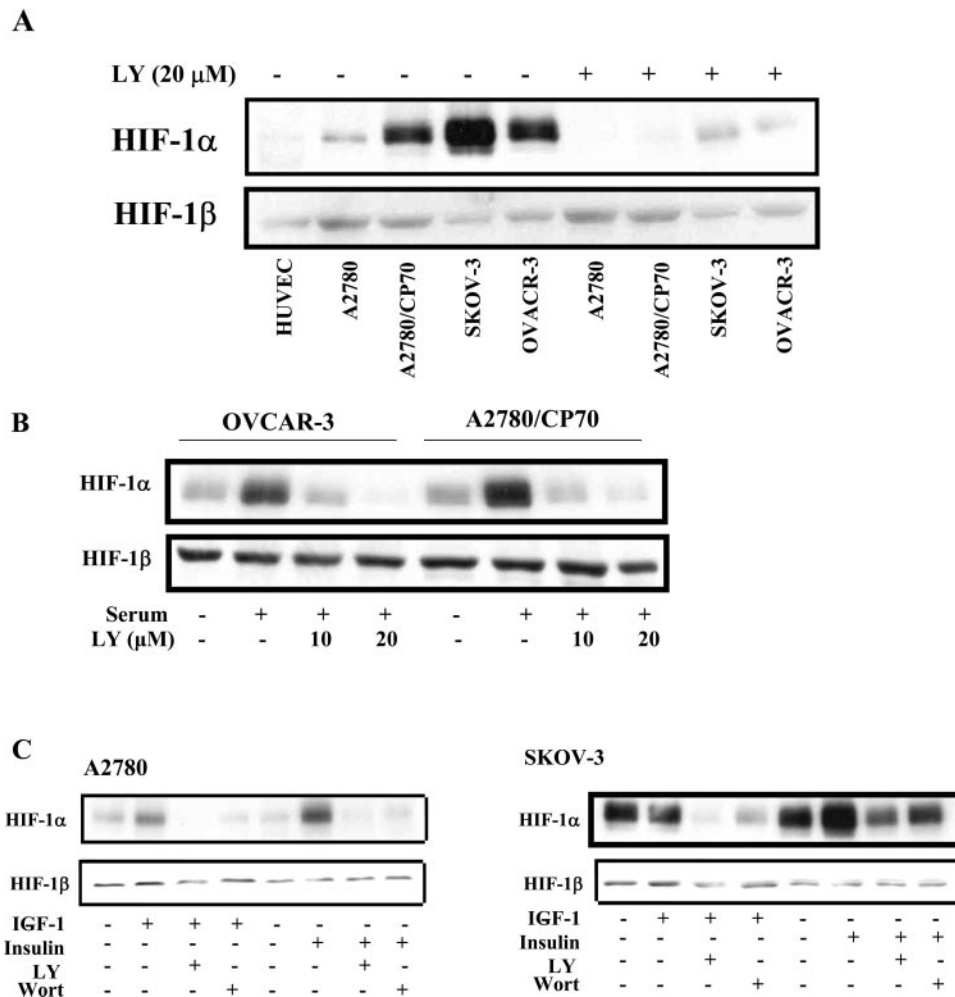
**HIF-1 $\alpha$  Protein Expression in Ovarian Cancer Cells Required PI3K Activity**—To determine whether HIF-1 $\alpha$  expression is mediated by PI3K in ovarian cancer cells, HIF-1 $\alpha$  protein levels were measured in several ovarian cancer cells in the absence or presence of LY294002. HIF-1 $\alpha$  protein levels in human endothelial cells (human umbilical vein endothelial cells) were used as a negative control. HIF-1 $\alpha$  expression levels in all ovarian cancer cell lines were much higher than those in human umbilical vein endothelial cells (Fig. 3A). LY294002 treatment specifically inhibited HIF-1 $\alpha$ , but not HIF-1 $\beta$  expression, indicating that HIF-1 $\alpha$  expression in these cells required PI3K activation. To determine whether PI3K is required for growth factor-induced HIF-1 $\alpha$  expression, the cells were cultured in serum-free medium for 24 h followed by the addition of 10% serum, insulin, or IGF-1. Treatment with serum, insulin, or IGF-1 greatly increased HIF-1 $\alpha$  expression in OVCAR-3, A2780/CP70, and A2780 cell lines, and the induced HIF-1 $\alpha$



**FIG. 2. HIF-1 $\alpha$  reversed LY294002 (LY)-inhibited VEGF transcriptional activation.** A, OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well on a 6-well plate the day before the transfection. The cells were co-transfected with pCMV- $\beta$ -galactosidase, pCEP4-HIF-1 $\alpha$ , and pVEGF-Luc or pMAP11wt plasmids. The parental pCEP4 vector was used to adjust the equal amount of plasmid used for each transfection. The cells were incubated for 12 h after transfection and then treated with Me<sub>2</sub>SO alone or 10  $\mu$ M LY294002 for 24 h. The relative luciferase activity was determined by the ratio of luciferase: $\beta$ -galactosidase activity and normalized to the control. B, the cells were co-transfected with pCMV- $\beta$ -galactosidase, pVEGF-Luc, and pCEP4 vector or HIF-1 $\alpha$  dominant negative construct. The cells were incubated for 12 h after transfection and then treated with Me<sub>2</sub>SO alone or 10  $\mu$ M LY294002 for 24 h. The relative luciferase activity was determined as described above. \* indicates a significant difference from the control ( $p < 0.05$ ). # indicates a significant difference from the LY294002-treated group ( $p < 0.05$ ).

expression was inhibited by LY294002 (Fig. 3, B and C). Due to the high basal level in SKOV-3 cells, HIF-1 $\alpha$  expression was not induced by IGF-1 but induced by insulin (Fig. 3C). Both the basal level and induced HIF-1 $\alpha$  expression were inhibited by LY294002 treatment. These data further confirm that PI3K mediates VEGF transcriptional activation through HIF-1 $\alpha$  but not HIF-1 $\beta$  levels in the ovarian cancer cells with different properties.

**AKT Is Essential for PI3K-mediated VEGF Transcriptional Activation**—AKT is a known target of PI3K. To confirm that AKT was activated by serum and inhibited by LY294002 in the ovarian cancer cells, the cells were cultured in serum-free medium for 24 h, pretreated with LY294002 for 30 min, and then stimulated by serum for 1.5 h. AKT activation, indicated by its protein phosphorylation, was increased by serum stimulation and inhibited by LY294002 (Fig. 4A). To determine whether expression of active form of AKT is sufficient to restore LY294002-inhibited VEGF transcriptional activation, OVCAR-3 cells were transfected with VEGF reporter and myristylated AKT. Transfection of AKT significantly increased VEGF reporter activity and completely reversed LY294002-inhibited VEGF reporter activity in a dose-dependent manner (Fig. 4, B and C). Because the full activation of AKT still requires PI3K activity, the reporter activity is much lower in the presence of LY294002 than that in the absence of LY294002 (Fig. 4). These data indicate that AKT is a sufficient target of PI3K for mediating VEGF expression.



**FIG. 3. HIF-1 $\alpha$  expression is inhibited by PI3K inhibitor LY294002 (LY) in ovarian cancer cells.** *A*, four human ovarian cancer cell lines (A2780, A2780/CP70, SKOV-3, and OVCAR-3) were cultured to confluence in 60-mm dishes in RPMI supplemented with 10% fetal bovine serum, 5% L-glutamine, 5% penicillin-streptomycin, and 0.2% insulin at 37 °C in 5% CO<sub>2</sub>. The cells were treated with Me<sub>2</sub>SO or 20  $\mu$ M LY294002 for 6 h. The HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels were analyzed by immunoblotting using antibodies specific for HIF-1 $\alpha$  and HIF-1 $\beta$ . *HUVEC*, human umbilical vein endothelial cells. *B*, the cells were cultured to 90% confluence and then switched to serum-free medium for 24 h. The cells were incubated in the absence or presence of 10% serum and LY294002 (10 or 20  $\mu$ M) for 6 h. Aliquots (40  $\mu$ g) of total cellular proteins were analyzed by immunoblotting using antibodies specific for HIF-1 $\alpha$  and HIF-1 $\beta$ . *C*, the serum-starved cells were pretreated with Me<sub>2</sub>SO, 20  $\mu$ M LY294002, or 100 nM wortmannin (*Wort*) for 30 min followed by the addition of 200 nM insulin or 4 nM IGF-1 as indicated for 6 h. HIF-1 $\alpha$  and HIF-1 $\beta$  proteins were analyzed as described above.

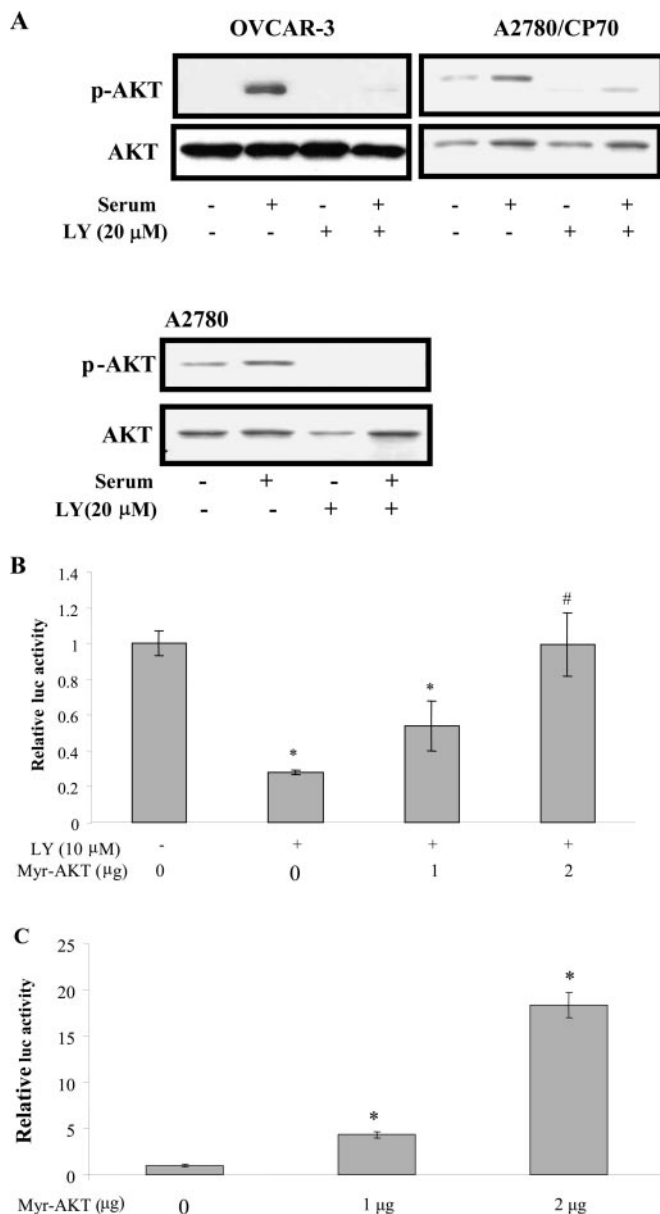
**Rapamycin Decreased VEGF Transcriptional Activation and VEGF Protein Production**—Rapamycin is a specific inhibitor for mammalian target of rapamycin and p70S6K1. To investigate whether rapamycin inhibited VEGF transcriptional activation, OVCAR-3 cells were transfected with the VEGF reporter followed by the treatment with solvent alone or rapamycin. VEGF reporter activity was decreased by rapamycin treatment in a dose-dependent manner (Fig. 5, *A* and *B*), suggesting that activation of mammalian target of rapamycin and p70S6K1 is involved in VEGF transcriptional activation. To determine whether rapamycin treatment also inhibited VEGF protein levels in ovarian cancer cells, the cells were treated with rapamycin, and VEGF levels were analyzed by ELISA. Rapamycin treatment decreased VEGF protein levels in both cell lines in a dose-dependent manner (Fig. 5, *C* and *D*).

**P70S6K1 Was Sufficient to Reverse LY294002-Inhibited VEGF Transcriptional Activation**—To test whether expression of an active form of p70S6K1 is sufficient to reverse LY294002-inhibited VEGF transcriptional activation, OVCAR-3 cells were transfected with a VEGF reporter and a constitutively active form of p70S6K1. As shown in Fig. 6, forced expression of p70S6K1 greatly increased VEGF reporter activity and re-

versed LY294002-inhibited VEGF transcriptional activation in a dose-dependent manner. These data indicate that p70S6K1 is an important downstream target of PI3K and AKT for inducing VEGF transcriptional activation in ovarian cancer cells.

**Rapamycin Inhibited HIF-1 $\alpha$ , but Not HIF-1 $\beta$ , Expression in OVCAR-3 and A2780/CP70 Cells**—To determine whether rapamycin inhibits HIF-1 $\alpha$  and HIF-1 $\beta$  expression in response to growth factor stimulation, OVCAR-3 and A2780/CP70 cells were cultured in serum-free medium for 24 h followed by the addition of serum, IGF-1, or insulin. Rapamycin specifically inhibited HIF-1 $\alpha$  but not HIF-1 $\beta$  expression in response to serum, IGF-1, and insulin (Fig. 7). This result indicates that rapamycin may inhibit VEGF transcriptional activation through a decrease of HIF-1 $\alpha$  expression in the cells.

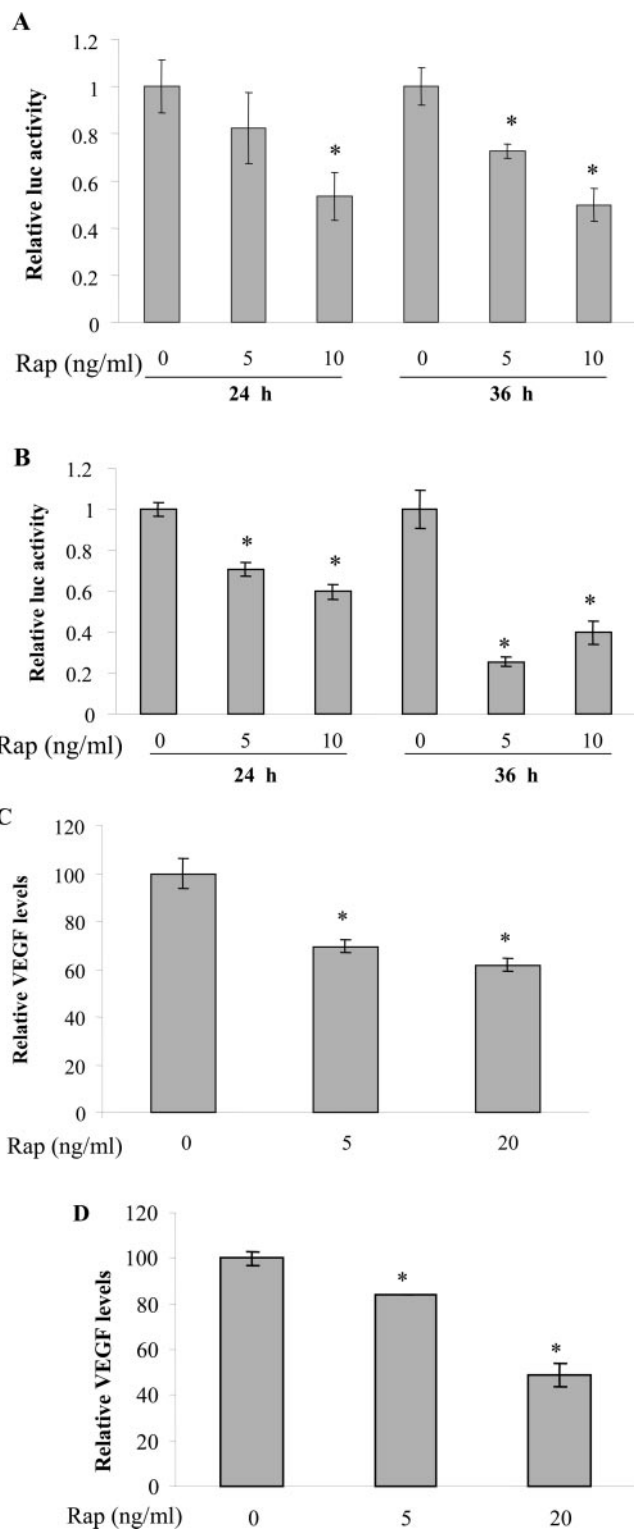
**Inhibition of PI3K Decreased HDM2 Phosphorylation and Expression in Ovarian Cancer Cells**—Previous studies have indicated that AKT may up-regulate the function of HDM2 via phosphorylation (41, 42). To determine whether inhibition of PI3K/AKT by LY294002 affects HDM2 phosphorylation and protein levels, OVCAR-3 cells were treated with LY294002 and analyzed by immunoblotting for HDM2 expression. Both



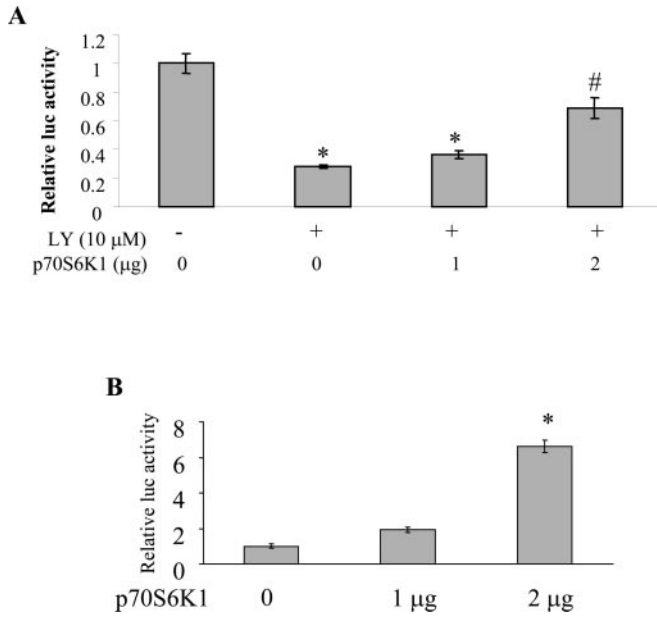
**FIG. 4. AKT is essential for VEGF transcriptional activation.** A, AKT activation was inhibited by LY294002 (LY) in ovarian cancer cells. A2780, A2780/CP70, and OVCAR-3 cells were cultured to 90% confluence and switched to serum-free medium for 24 h. The cells were pretreated with LY294002 for 30 min followed by treatment with 10% fetal bovine serum for 1.5 h. The phospho-AKT (p-AKT) and total AKT protein levels were analyzed by immunoblotting. B and C, AKT reversed LY294002-inhibited VEGF transcriptional activation. OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well on a 6-well plate the day before the transfection. The cells were co-transfected with pCMV- $\beta$ -galactosidase control, the pVEGF-Luc reporter, and a constitutively active AKT (Myr-AKT) plasmid or the vector. The cells were cultured for 12 h after transfection and then treated with Me<sub>2</sub>SO and 10  $\mu$ M LY294002 (B) or with Me<sub>2</sub>SO alone (C) for 24 h. The relative luciferase activity was determined by the ratio of luciferase: $\beta$ -galactosidase activity and normalized to the control. \* indicates a significant difference from the control ( $p < 0.05$ ). # indicates a significant difference from the LY294002 treatment ( $p < 0.05$ ).

HDM2 phosphorylation and protein expression were inhibited by LY294002 (Fig. 8).

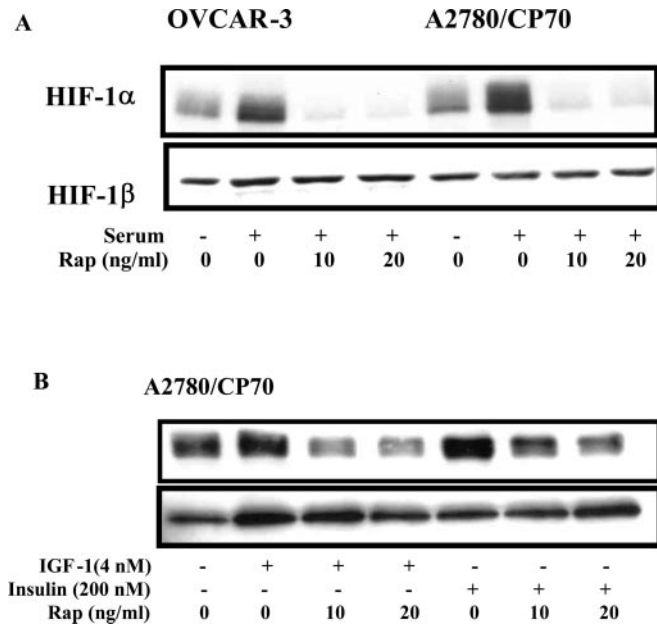
**Forced Expression of HDM2 Reversed LY294002-inhibited VEGF Transcriptional Activation, VEGF Expression, and HIF-1 $\alpha$  Expression**—To determine whether HDM2 acts downstream of PI3K to affect VEGF transcriptional activation, OVCAR-3 cells were transfected with VEGF reporter and



**FIG. 5. Rapamycin (Rap) inhibited VEGF transcriptional activation and VEGF protein levels in ovarian cancer cells.** A and B, to test the effect of rapamycin on VEGF transcriptional activation, OVCAR-3 cells were co-transfected with pCMV- $\beta$ -galactosidase plasmid and pVEGF-Luc (A) or pMAP11wt plasmid (B). The cells were incubated for 12 h after transfection followed by the treatment with Me<sub>2</sub>SO, or rapamycin (5 or 10 ng/ml) for 24 and 36 h. The relative luciferase activity was determined by the ratio of luciferase: $\beta$ -galactosidase activity and normalized to the control. C and D, to study the VEGF protein levels in the cells, OVCAR-3 (C) and A2780/CP70 (D) cells were treated for 24 h with rapamycin. The VEGF protein levels in the cultured medium were analyzed by sandwich ELISA as described under "Materials and Methods," and the VEGF protein levels were normalized to the control as the relative VEGF protein levels. \* indicates a significant difference from the control ( $p < 0.05$ ).

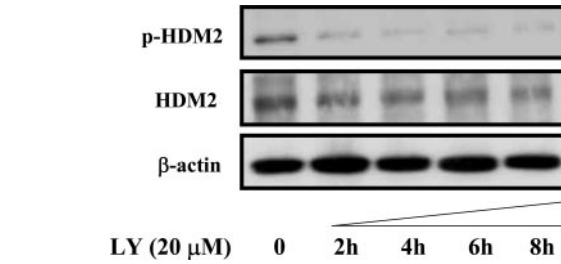


**FIG. 6. Expression of p70S6K1 reversed LY294002 (LY)-inhibited VEGF transcriptional activation.** OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well on a 6-well plate the day before the transfection. The cells were transfected with pCMV- $\beta$ -galactosidase, pVEGF-Luc, and p70S6K1 or vector plasmid. The cells were cultured and treated with  $\text{Me}_2\text{SO}$  and LY294002 (A) or  $\text{Me}_2\text{SO}$  alone (B) for 24 h as described under "Materials and Methods." The relative luciferase activity was determined in the cells as described above. \* indicates a significant difference from the control ( $p < 0.05$ ). # indicates a significant difference from the LY294002-treated group ( $p < 0.05$ ).

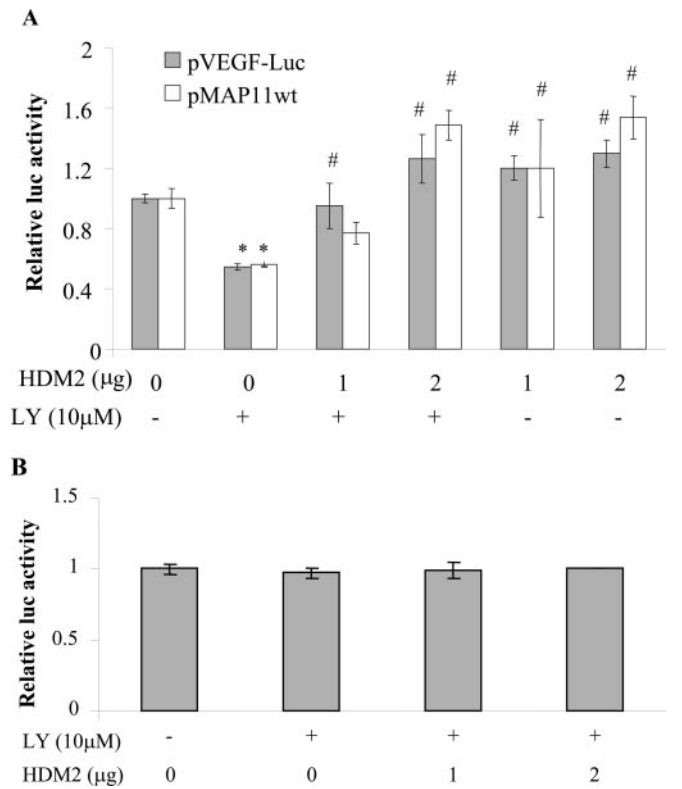


**FIG. 7. Rapamycin (Rap) inhibited HIF-1 $\alpha$  expression in ovarian cancer cells.** A, OVCAR-3 and A2780/CP70 cells were cultured to 90% confluence and switched to serum-free medium for 24 h. The cells were pretreated with rapamycin at the indicated concentrations for 30 min and then incubated with or without 10% fetal bovine serum for 6 h. HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels were analyzed by immunoblotting using antibodies against HIF-1 $\alpha$  and HIF-1 $\beta$ . B, A2780/CP70 cells were cultured as described above and then pretreated with rapamycin as indicated for 30 min followed by the incubation with or without 4 nM IGF-1 or 200 nM insulin for 6 h. HIF-1 $\alpha$  and HIF-1 $\beta$  were analyzed as described above.

HDM2 plasmids. After the transfection, the cells were incubated in the absence or presence of LY294002. Forced expression of HDM2 reversed LY294002-inhibited VEGF reporter



**FIG. 8. LY294002 (LY) treatment inhibited HDM2 phosphorylation and protein levels.** OVCAR-3 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum to 80% confluence and then treated with 20  $\mu$ M LY294002 for different times as indicated. Aliquots (40  $\mu$ g) of cellular protein extracts were analyzed using antibodies specific for phospho-HDM2 (p-HDM2), total HDM2, and  $\beta$ -actin by immunoblotting.



**FIG. 9. HDM2 reversed LY294002 (LY)-inhibited VEGF transcriptional activation.** OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well. A, to test the effect of HDM2 on VEGF transcriptional activation, the cells were co-transfected with pCMV- $\beta$ -galactosidase, HDM2, and pVEGF-Luc or pMAP11wt plasmids as indicated. The cells were cultured overnight and then treated with  $\text{Me}_2\text{SO}$  alone or 10  $\mu$ M LY294002 for 24 h. The relative luciferase activity was determined as described in the legend for Fig. 1. B, to test whether the HIF-1 binding in the VEGF promoter is essential for mediating VEGF transcriptional activation, cells were co-transfected with pCMV- $\beta$ -galactosidase and pMAP11mut plasmid with the empty vector or pCMV-HDM2. The cells were treated as described above, and the relative luciferase activity was determined in the cells. \* indicates a significant difference from the control ( $p < 0.05$ ). # indicates a significant difference from the LY294002-treated group ( $p < 0.05$ ).

activity in a dose-dependent manner (9A), indicating that HDM2 was sufficient to restore LY294002-inhibited VEGF transcriptional activation. To test whether the HIF-1 binding site is required for HDM2-mediated VEGF reporter activity, we used the mutant VEGF reporter at the HIF-1 DNA binding site. Neither LY294002 nor HDM2 affected the mutant VEGF reporter activity (Fig. 9B), indicating that LY294002 and HDM2 regulate the reporter activity through the HIF-1 binding site.



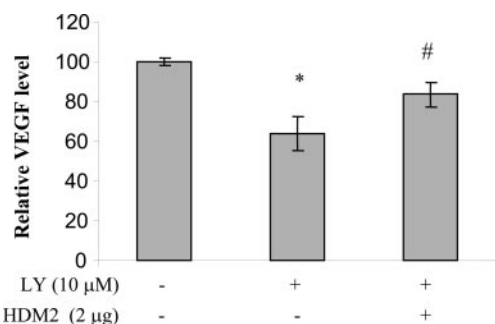


FIG. 10. **HDM2 transfection increased VEGF protein production in the presence of LY294002 (LY).** OVCAR-3 cells were seeded at  $0.5 \times 10^6$  cells/well on a 6-well plate the day before the transfection. The cells were transfected with 2  $\mu$ g of empty vector or pCMV-HDM2 plasmid. The cells were cultured for 12 h after transfection and then treated with Me<sub>2</sub>SO or 10  $\mu$ M LY294002 for 24 h. The VEGF protein levels in the medium were analyzed by ELISA and normalized to the control. \* indicates a significant difference in VEGF levels from the control. # indicates a significant difference in VEGF levels from the LY294002-treated group ( $p < 0.05$ ).

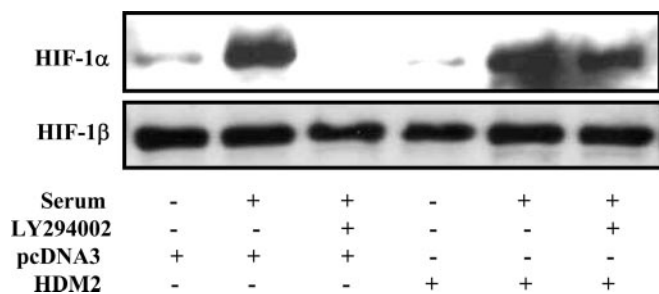


FIG. 11. **HDM2 reverses LY294002 (LY)-inhibited HIF-1 $\alpha$  expression.** A2780/CP70 cells were transfected with either pcDNA3 vector or pcDNA3-HDM2 as described previously. After the transfection, the cells were selected with G418 for 2 weeks. Resistant colonies were pooled and cultured in the presence of low levels of G418 to maintain selection pressure. The cells were cultured in serum-free medium for 24 h followed by incubation in the absence or presence of 10% serum and 20  $\mu$ M LY294002 for 6 h. Aliquots of protein extracts were analyzed using antibodies specific for HIF-1 $\alpha$  and HIF-1 $\beta$  by immunoblotting.

**HDM2 Restored LY294002-inhibited VEGF Protein Production**—To study whether HDM2 reversed LY294002-inhibited VEGF protein expression, the cells were transiently transfected with vector alone or HDM2 followed by treatment with LY294002. HDM2 partially reversed the inhibitory effect of LY294002 on VEGF protein levels (Fig. 10). These data indicate that HDM2 plays an important role in PI3K-mediated VEGF transcriptional activation and VEGF protein expression.

To determine whether HDM2 restores LY294002-inhibited HIF-1 $\alpha$  expression, A2780/CP70 cells stably expressing HDM2 or vector alone were cultured in serum-free medium and then treated with 10% serum in the presence or absence of LY294002. Expression of HDM2 in the cells reversed LY294002-inhibited HIF-1 $\alpha$  expression (Fig. 11). Taken together, these results suggest that PI3K and AKT mediate VEGF transcriptional activation through HIF-1 $\alpha$  expression and that HDM2 and p70S6K1 signaling pathways are two parallel pathways that mediate this process.

#### DISCUSSION

It is known that VEGF plays an important role in ovarian tumorigenesis and angiogenesis. However, the mechanism by which VEGF expression is elevated is not completely understood. Genes encoding PI3K are frequently amplified in copy number in ovarian cancer cells, leading to activation of PI3K signaling (35, 36). In this study, we showed that VEGF protein expression and transcriptional activation were induced by

PI3K activation in ovarian cancer. To understand the mechanism of the increased VEGF expression, we found that PI3K signaling up-regulated VEGF expression through HIF-1 $\alpha$ . VEGF and HIF-1 are known to increase tumor growth and angiogenesis. Thus, PI3K may increase ovarian tumor growth and angiogenesis through VEGF and HIF-1 expression.

To identify the downstream mediators of PI3K necessary for regulating HIF-1 $\alpha$  and VEGF expression in ovarian cancer cells, we investigated potential downstream targets of PI3K in response to growth factor stimulation. We found that AKT was essential for VEGF transcriptional activation in the cells. In this study, we were particularly interested in the downstream targets of AKT that mediate VEGF transcriptional activation in ovarian cancer cells. We found that p70S6K1 and HDM2 are two parallel pathways that mediate growth factor-induced VEGF transcriptional activation and HIF-1 $\alpha$  expression. These results are consistent with recent studies demonstrating that AKT activation increased HDM2 phosphorylation and its activity (41, 42). These results may provide useful information to understand VEGF and HIF-1 regulation in other human cancer cells.

Overall, these results identify a novel mechanism by which the observed PI3K activation and other oncogenic signals in ovarian cancer cells may increase VEGF expression, which in turn induces angiogenesis and tumor growth. We have identified several members of the signaling molecules including AKT, HDM2, and p70S6K1 that are necessary for VEGF transcriptional activation and HIF-1 $\alpha$  expression. This study may also provide useful information and potential targets for anti-ovarian cancer therapy in the future.

**Acknowledgments**—We thank Dr. John Blenis and Dr. Zhi-Min Yuan (Harvard Medical School, MA) for kindly providing p70S6K1 and HDM2 constructs, respectively.

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