Decreased Level of PDCD4 (Programmed Cell Death 4) Protein Activated Cell Proliferation in the Lung of A/J Mouse

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Abstract

Background: Programmed cell death 4 (PDCD4), a protein that binds to eukaryotic initiation factor 4A (eIF4A), inhibits the initiation of translation. Although a number of tumor suppressors target transcription, Pdc4 is the first suppressor targeting protein translation, and has also been suggested to function as a tumor suppressor gene in human cancer. The majority of tumor suppressors are mutationally inactivated, but the expression of Pdc4 is downregulated with progression in a number of human cancer sites, including the lung.

Methods: An aerosol of lentivirus-shRNA *Pdc4* was delivered into A/J mice, through a nose-only inhalation system twice a week for 1 month.

Results and Conclusions: Downregulated *Pdc4* resulted in increase levels of antiapoptotic and uPA-regulated proteins. We also found that downregulated *Pdc4* induced the mTOR/p70S6K pathway and cell-cycle proteins. Our results suggest that Pdc4 may perform a critical function in the regulation of lung cancer cell proliferation.

Key words: aerosol gene delivery, shRNA *Pdc4*, lung cell proliferation

Introduction

PROGRAMMED CELL DEATH 4 (Pdc4) was originally identified in a screen for genes activated during apoptosis.⁽¹⁾ Pdc4 has been linked to the process of apoptosis in response to different inducers, and has been shown to be regulated by Akt, an important antiapoptotic regulator.⁽²⁾ Pdc4 has been described as a novel tumor suppressor gene that inhibits oncogenesis by suppressing gene transcription and translation.⁽³⁾

The mammalian target of rapamycin (mTOR) plays an important role in regulating protein translation, cell proliferation, and survival. Activation of mTOR and its effectors is induced by various cytokines, hormones, and receptor tyrosine kinases, and such activation is important for the

generation of mitogenic and cell proliferative response.⁽⁴⁾ Ribosomal p70S6Kinase 1 (p70S6K1) is a major downstream target of mTOR; the p70S6 kinase1 (p70S6K1) exerts its function in regulating protein synthesis, cell proliferation, cell cycle progression, and apoptosis in response to growth factors and other cellular signals.⁽⁵⁾

The serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) are involved in the control of extracellular matrix turnover, cell migration, invasion, and cell signaling, leading to a variety of different responses, under both physiological and pathological conditions.⁽⁶⁾ Recent studies show a close relationship of the uPA system and cell proliferation/apoptosis. uPA/uPAR is responsible for the activation and release of different growth factors and modulates

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the cell proliferation/apoptosis ratio through the dynamic control of cell-matrix interactions.⁽⁷⁾ Recent studies also suggest that expression levels of uPAR, are downregulated in several cancers and regulated by *Pdcd4*.⁽⁸⁾

To better understand the mechanism underlying the effects of suppressed *Pdcd4* in lung cell proliferation, we induced a downregulation of *Pdcd4* expression *in vivo* via the aerosol delivery of shRNA. An investigation of the molecular consequences of downregulated *Pdcd4* revealed the regulation of the cell cycle progression proteins uPA and uPAR levels and apoptosis regulatory proteins. These *in vivo* results provide additional insight into the role of *Pdcd4* in controlling cell growth and proliferation.

Materials and Methods

Materials

The monoclonal antibody against PDCD4, Akt1, and phospho-Akt1 (Ser473) was generated via a general method described elsewhere.^(9,10) Antibodies against phospho-Akt1 (Thr308), p21, p27, CDK4, cyclinD1, PCNA, BclxL, BCL2, uPA, uPAR, PAI-1, p53, p70S6K, phospho-p70S6K (Thr 389), and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-p53 (Ser20), phospho-mTOR (Ser 2448), eIF4E and 4E-BP1, and mTOR antibodies were obtained from Cell Signaling (Beverly, MA, USA). pENTR/U6TM entry vector, pLenti6/BLOCK-iTTM-DEST vector kits and lentiviral expression plasmids were purchased from Invitrogen (Carlsbad, CA, USA).

Lentivirus-shRNA *Pdcd4* cloning

To generate shRNA expression viral vectors, double-stranded oligonucleotides coding for shRNA targeting the mouse *Pdcd4* (Gene bank number: NM_011050) (*Pdcd4* shRNA: 5'-AAAGAGTATTTACTCTCTGGA-3') were cloned into the pENTR/U6TM entry vector (Invitrogen). The shRNA expressing the lentivirus vector (pLenti6/BLOCK-iTTM-DEST vector) was constructed in accordance with the manufacturer's protocol (BLOCK-iTTM Lentiviral RNAi Expression System, Invitrogen).

Production of lentivirus-shRNA *Pdcd4*

A total of 5×10^6 293FT cells were seeded in 10-cm diameter dishes 24 h prior to transfection in DMEM (WelGENE, Daegu, Korea) with 10% fetal bovine serum and penicillin (100 IU/mL), in a 5% CO₂ incubator. A total of 12 μ g of plasmid DNA was used for the transfection of one dish: 3 μ g of expression plasmid, and 9 μ g of transfer vector plasmid viral power packaging plasmids (pLP1, pLP2, pLP/VSV-G). An equal volume of lipofectamine reagent was added to this DNA mixture and the complexes were incubated at room temperature for 20 min prior to transfection. After 24 h, the medium (10 mL) was replaced with DMEM. The viral supernatant was collected after 48 h and the viral concentration was determined using an HIV-1 p24 ELISA kit (Perkin-Elmer, Boston, MA, USA).

Aerosol delivery of lentivirus-shRNA *Pdcd4*

Experiments were conducted on 6-week male A/J mice (three mice/group), purchased from Joongang Laboratory

Animals (Seoul, Korea). The animals were maintained in a laboratory animal facility with temperature and relative humidity maintained at $23 \pm 2^\circ\text{C}$ and $50 \pm 20\%$ of relative humidity under a 12-h light/dark cycle. All methods used this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-080313-2). Mice were placed in a nose-only exposure chamber (NOEC) and exposed to the aerosol, which was generated using a patented nebulizer (Korea Patent #20304964) designed to minimize sample loss as well as shearing force. For aerosol delivery, the A/J mice were exposed to an aerosol of containing lentivirus-shRNA *Pdcd4* solution (50 mL) containing 40 ng/mL of lentivirus-shRNA *Pdcd4* for 1 month.⁽¹⁰⁾ The A/J mice were allowed to inhale the aerosolized lentivirus-shRNA *Pdcd4* from the nebulizer for 30 min twice a week for 1 month, after which the mice were sacrificed, and lung samples were collected for further analysis. The untreated mice and mice treated with scramble shRNA (scrambled) were used as controls.

Western blot analysis

The protein concentration of the homogenized lysates was assessed using a Bradford kit (Bio-Rad, Hercules, CA, USA) and equal amounts (50 μ g) of protein were separated on SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia, Cambridge, UK). After blocking the membranes for 1 h in TTBS containing 5% skim milk for 1 h, immunoblotting was conducted by overnight incubation at 4°C with corresponding primary antibodies in 5% skim milk and then with secondary antibodies conjugated to horseradish peroxidase (HRP) for 3 h at room temperature or overnight at 4°C . After washing, the bands of interest were pictured using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The results were quantified with the LAS-3000 measurement program.

Reverse transcriptase-polymerase chain reaction experiments

Total RNA was isolated from the lung tissue with Trizol reagent (Invitrogen). Primers used for the polymerase chain reaction (PCR) were designed to be isoform specific. The sequences were as follows: for *Pdcd4*, sense 5'-GGT TGC TAG ATA GGC GGT CC-3' and antisense 5'-GTG ATT GAC AGG CTG TTG CC-3'; for GAPDH (glyceraldehyde phosphate dehydrogenase), sense 5'-GAA GGA CTC ATG ACC ACAG-3' and antisense 5'-CTTCAC CAC CTT CTT GATG-3'. The amplification conditions were as follows: 94°C for 5 min; 30 cycles of 30 sec each of denaturation at 94°C , annealing at 54°C for *Pdcd4* and 50°C for GAPDH, and extension at 72°C ; and a final extension for 5 min at 72°C .

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were cut at 5 μ m and transferred to plus slides (Fisher Scientific, Pittsburgh, PA, USA). The tissues were deparaffinized in xylene and rehydrated through a gradient of alcohol. The tissue sections were incubated in 150 μ L proteinase K, washed, and incubated in 0.3% hydrogen peroxidase (AppliChem, Darmstadt, Germany) for 30 min to quench endogenous peroxidase activity. After washing in PBS, the tissue sections were incu-

bated in 3% BSA in PBS for 1 h at room temperature to block unspecific binding sites. Primary antibodies were applied on tissue sections overnight at 4°C. The following day, the tissue sections were washed and incubated with secondary HRP-conjugated antibodies for 2 h at room temperature. After washing, the tissue sections were counterstained with Mayer's Hematoxylin (DAKO, Carpinteria, CA, USA), and the slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY, USA). The staining intensity of PDCD4, uPA, CDK4, p70S6K, and PCNA for the quantification of IHC analysis was conducted using In Studio version 3.01 programs (Pixera, San Jose, CA, USA).

Data analysis

Quantification of Western blot analysis was conducted using Multi Gauge version 2.02 programs (Fujifilm). All results are expressed as the means $\pm$ SE. The results were analyzed via Student's *t*-test (Graph Pad Software, San Diego, CA, USA). **p* < 0.05 was considered significant and ***p* < 0.01 highly significant compared with the corresponding control values.

Results

Activated expression of antiapoptotic protein by *Pdcd4* shRNA

Lentiviral shRNA targeting *Pdcd4* was delivered to the lungs of 6-week old A/J mice via aerosol as described in Materials and Methods. Lungs from the mice receiving aerosol *Pdcd4* shRNA, scrambled shRNA (control) or untreated mice were harvested for analyses. To determine the effects of aerosol-delivered shRNA *Pdcd4* on the expression level of PDCD4 reverse transcriptase-PCR and Western blot were carried out in the lungs of A/J mice. As shown in Figure 1, lentivirus-shRNA *Pdcd4* suppressed the mRNA and protein expression of PDCD4 (Fig. 1A and B). The results of immunohistochemical analysis also clearly showed that lentiviral delivery of shRNA *Pdcd4* could significantly suppressed the protein expression of PDCD4 (Fig. 1C). The suppressed level of PDCD4 protein was confirmed after quantifying the staining of PDCD4 (Fig. 1D). We also investigated the effects of aerosol-delivered shRNA *Pdcd4* on the expression levels of apoptosis-related proteins. The results revealed that lentivirus-shRNA *Pdcd4* significantly increased the levels of antiapoptotic proteins, including BCL-2 and BCL-XL in the lungs (Fig. 1E). Densitometric analysis of bands of interest clearly verified the results of our Western blot analyses (Fig. 1F).

Knockdown of *Pdcd4* increased levels of uPA and uPAR proteins

Signals mediated by uPA and its receptor uPAR have been implicated in tumor cell invasion, survival, and metastasis in a variety of cancers, including lung cancer.⁽¹¹⁾ Interestingly, *Pdcd4* was reported to downregulate uPAR via its promoter region,⁽⁸⁾ and therefore, a decrease in *Pdcd4* would be predicted to increase uPA/uPAR levels. Toward this end, we assessed the effects of aerosol-delivered shRNA *Pdcd4* on the uPA/uPAR system in the lungs of A/J mice by Western blotting. Our results demonstrated that aerosol delivery of shRNA *Pdcd4* significantly increased the protein expressions of uPA and uPAR, whereas the level of the uPA inhibitor

PAI-1 protein remained unchanged (Fig. 2A). The results of Western blot analysis were verified via densitometric analysis, as is shown in Fig. 2B. The increased uPA expression in the lungs was further confirmed by immunohistochemical analysis (Fig. 2C). The relative intensity of staining for uPA was clearly consistent with the increase observed in Figure 2D.

shRNA *Pdcd4* increased Akt/mTOR pathway in the lungs of A/J mice

The Akt/mTOR pathway controls cellular protein translation through regulation of 70-kDa ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein1 (4E-BP1) phosphorylation. Protein translation is closely related with cancer cell growth.⁽¹²⁾ In this study, we investigated the changes in protein expression of Akt1, phospho-Akt1(Ser473), phospho-Akt1(Thr308), mTOR, [phospho-mTOR (Ser2448)], p70S6K, and phospho-p70S6K (Thr389). Our results showed that shRNA *Pdcd4* significantly increased Akt1 and phospho-Akt1 (Ser473) (Fig. 3A and B). Lentiviral delivery of shRNA *Pdcd4* increased p70S6K, mTOR, and eIF4E protein levels. However, shRNA *Pdcd4* did not affect the protein expression of total 4E-BP1 (Fig. 3C and D). In addition, immunohistochemistry analysis showed that aerosol delivered lentivirus-shRNA *Pdcd4* increased the expression of p70S6K in the lungs of A/J mice (Fig. 3E). Quantification of the relative intensity of staining verified the increased expression of p70S6K (Fig. 3F).

shRNA *Pdcd4* induced cell cycle progression in the lungs of A/J mice

The potential ability of *Pdcd4* to regulate cell growth was evaluated by Western blot analysis of cell cycle regulated proteins in the lung after *Pdcd4* knockdown. The repeated aerosol delivery of lentivirus-shRNA *Pdcd4* significantly reduced the levels of p21 and phospho-p53 (Ser 20), whereas p27 and cyclin D1 protein levels were unchanged (Fig. 4A and B). Also, aerosol delivery of lentivirus-shRNA *Pdcd4* induced a significant increase in CDK4 and PCNA expression (Fig. 4A) as shown by densitometric analysis (Fig. 4B). In addition, immunohistochemistry analysis showed that aerosol delivered lentivirus-shRNA *Pdcd4* increased the expression of PCNA and CDK4 in the lungs of A/J mice (Fig. 4C). Quantification of the relative intensity of staining verified the increased expression of PCNA and CDK4 (Fig. 4D). These results clearly indicated that the downregulation of *Pdcd4* facilitated the cell cycle and cell proliferation in the murine lung.

Discussion

Pdcd4 was originally identified as a transcript upregulated in apoptotic cells. Further studies revealed that *Pdcd4* mRNA is frequently downregulated in several types of tumors, thus making *Pdcd4* a candidate tumor suppressor gene.⁽¹³⁾ It was also reported that the tumor suppressor property of *Pdcd4* is a consequence of its downstream effects on numerous cellular processes important for tumor progression, including AP-1 activation and invasion, metastasis and cell cycle control.^(3,14) The significance of *Pdcd4* suppression for tumor progression was also demonstrated for human nonsmall cell lung carcinoma (NSCLC), where *Pdcd4* loss was correlated with poor

FIG. 1. Downregulation of PDCD4 protein expression. (A) Lysates from the lungs of A/J mice treated with aerosol-delivered shRNA *Pdc4* was analyzed for protein and mRNA levels of PDCD4 by Western blot and reverse transcriptase-polymerase chain reaction analysis. (B) Bands of interest were analyzed further by densitometer. (C) Immunohistochemical analysis of PDCD4 (original magnification, $\times 200$; bar = $50\ \mu\text{m}$). (D) PDCD4 positive staining was determined by counting four randomly chosen fields per section, thereby determining the percentage of DAB-positive cells per 100 cells at $\times 200$ magnification. (E) Western blot analysis of BCL-xL and BCL2 proteins. (F) Bands of interest were further analyzed by densitometry. Each bar represents the mean $\pm$ SE ($n = 3$). * $p < 0.05$ was considered significant and ** $p < 0.01$ highly significant compared with corresponding control. CON, control; Scram, Scramble control; shPdc4, shRNA *Pdc4*-delivered lungs.

FIG. 2. Western blot and IHC analysis of uPA-related proteins in the lungs of A/J mice. **(A)** Lysates from the lungs of A/J mice treated with aerosol-delivered shRNA *Pdcd4* were analyzed for levels of uPA, uPAR, and PAI-1 proteins by Western blot analysis. **(B)** Bands of interest were further analyzed by a densitometer. **(C)** Immunohistochemical analysis of uPA (original magnification, $\times 200$; bar = 50 μm). **(D)** uPA positive staining was determined by counting four randomly selected fields per section, determining the percentage of DAB-positive cells per 100 cells at $\times 200$ original magnification. Each bar represents the mean $\pm$ SE ($n = 3$). * $p < 0.05$ was considered significant and ** $p < 0.01$ highly significant compared with the corresponding control. CON, control; Scram, Scramble control; shPdcd4, shRNA *Pdcd4*-delivered lungs.

prognosis and survival.⁽³⁾ The current study was performed to elucidate the effects of repeated aerosol shRNA *Pdcd4* delivery on lung cell proliferation. Only a few reports document the interaction between the uPA/uPAR-system and the Bcl-2 protein family. It is reported that the level of Bcl-2 in malignant glioma cell lines positively correlates with the expression of metalloproteinase and cell surface-uPA.⁽¹⁵⁾ uPA, a serine protease, is able to bind to cell membrane receptor, uPAR, and thereby initiates signal transduction, cell migration, invasion, and proliferation.⁽¹⁶⁾ Binding of uPA to uPAR mediates cell proliferation in several cell types including nonmalignant lung epithelial cells, lung carcinoma-derived cells.⁽¹⁷⁾ Stimulation of the lung epithelium by uPA elicits proliferative responses via signaling mechanism that are in-

completely characterized at present.⁽¹⁸⁾ We have shown that shRNA-mediated downregulation of *Pdcd4* increased expression of antiapoptotic proteins (Bcl-XL and Bcl-2) and uPA-related proteins (uPA and uPAR), while a decrease in expression level of PAI-1 as an inhibitor of uPA was observed in the lungs of A/J mice (Figs. 1 and 2). These findings demonstrated that downregulated *Pdcd4* could affect the Bcl-2 family protein and regulate the uPAR/uPA system.

The phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is involved in many different cellular processes, including proliferation, differentiation, and apoptosis.⁽¹⁹⁾ Our result showed that shRNA *Pdcd4* increased Akt1 and phosphor-Akt1 (Ser473). One of the downstream targets of Akt is represented by the mammalian target of rapamycin (mTOR), a highly

FIG. 3. Western blot analysis of Akt1-related proteins in the lungs of A/J mice. (A) Lysates from the lungs of A/J mice treated with aerosol-delivered shRNA *Pdc4* were analyzed for protein levels of Akt1, phospho-Akt1 (Ser 473) and phospho-Akt1 (Thr308) by Western blot. (B) Bands of interest were further analyzed by a densitometer. (C) Western blot analysis of mTOR, phospho-mTOR at Ser 2448, p70S6K, phospho-p70S6K at Thr389, eIF4E, and 4E-BP1 proteins. (D) Bands of interest were further analyzed by using a densitometer. (E) Immunohistochemical analysis of p70S6K (original magnification, $\times 400$; bar = 20 μm). (F) p70S6K positive staining was determined by counting four randomly selected fields per section, determining the percentage of DAB-positive cells per 100 cells at $\times 400$ original magnification. Each bar represents the mean $\pm$ SE ($n = 3$). * $p < 0.05$ was considered significant and ** $p < 0.01$ highly significant compared with corresponding control. CON, control; Scram, Scramble control; shPdc4, aerosol *Pdc4*-delivered lungs.

conserved serine/threonine protein kinase that is essential for the regulation of cell growth and proliferation, by controlling these processes at the translational level. In mammals, Akt can phosphorylate mTOR on Ser2448 to activate this kinase,⁽²⁰⁾ and subsequently activated mTOR leads to translation initiation

through phosphorylation of p70S6K and 4E-BP1 protein.⁽²¹⁾ Recent studies have shown that an important downstream target for the kinase activity of p70 S6K is PDCD4,⁽²²⁾ a tumor suppressor protein that blocks cap-dependent translation by inhibiting the RNA helicase eIF4A.⁽²³⁾ p70S6 kinase is a ubiq-

FIG. 4. Analysis of signal proteins for cell-cycle regulation. (A) Lysates from the lungs of A/J mice treated with aerosol-delivered shRNA *Pdcd4* were analyzed for protein levels of p53, phospho-p53 (Ser 20), p21, p27, CDK4, cyclinD1, and PCNA. (B) Bands of interest were analyzed further by densitometry. (C) Immunohistochemical analysis of CDK4 and PCNA in the lungs (original magnification, $\times 200$; bar = $50 \mu\text{m}$). (D) CDK4 and PCNA positive staining were determined by counting four randomly selected fields per section, determining the percentage of DAB positive cells per 100 cells at $\times 200$ original magnification. Each bar represents the mean $\pm$ SE ($n=3$). * $p < 0.05$ was considered significant and ** $p < 0.01$ highly significant compared with the corresponding control. CON, control; Scram, Scramble control; sh*Pdcd4*, shRNA *Pdcd4*-delivered lungs.

uitously expressed serine/threonine protein kinase that phosphorylates the 40S ribosomal protein S6 in response to mitogen stimulation. Inactivation of p70S6 kinase is known to suppress cell growth and survival.⁽²⁴⁾

Our result indicated that mTOR, phospho-mTOR (Ser2448) and phospho-p70S6K (Thr389) protein levels were significantly increased by aerosol delivery of shRNA *Pdcd4* in the

lung of A/J mice, suggesting that shRNA *Pdcd4* can regulate cell proliferation (Fig. 3).

mTOR can promote the phosphorylation of p70S6K and 4E-BP1, thus stimulating the translation of the proteins required for the cell cycle progression from the G1 to S phase.⁽²⁵⁾ Cyclin D1 and its catalytic partner CDK4 are known to play important roles in the G1/S checkpoint of the cell cycle.^(26,27)

The complex formed by CDK4 and Cyclin D1 has been strongly implicated in the control of cell proliferation.⁽²⁸⁾ The result revealed that aerosol delivery of shRNA *Pdcd4* significantly increased CDK4 protein level (Fig. 4A and B). In addition, cell proliferation marker protein such as PCNA expression was increased (Fig. 4C and D).

Previous work had identified p21 (Waf1/Cip1) as a potential target for PDCD4.⁽¹⁴⁾ In addition, our previous data showed that aerosol-delivered *Pdcd4* regulated lung cancer growth through increasing the cell cycle inhibitors, such as p21 and p27, while suppressing cell proliferation proteins, such as cyclin-dependent kinase 4 and proliferating cell nuclear antigen.⁽²⁹⁾ p21(Waf1/Cip1) has originally been identified as an inhibitor of G1 cyclin-dependent kinases that mediates the p53-induced growth arrest following DNA damage.⁽³⁰⁾ In addition, there are several reports showing that p21 (Waf1/Cip1) may also influence cell proliferation in a positive manner. It has long been known that p21 (Waf1/Cip1) functions as an assembly factor in the activation of cyclin D1/Cdk complexes.⁽³¹⁾ Our results clearly demonstrated that shRNA *Pdcd4* decreased phosphorylation of the Ser-20 residue of p53 expression while total p53 protein expression remained unchanged (Fig. 3A and B). Also, down-regulation of the *Pdcd4* leads to reduction of p21 expression while p27 expression remained unchanged (Fig. 4A and B). These data indicate that expression of shRNA *Pdcd4* can promote cell cycle progression.

In summary, the aerosol delivery of shRNA *Pdcd4* successfully decreased PDCD4 protein levels in the lungs after 1 month of treatment. The decreased PDCD4 protein correlated with cell proliferation as defined by induction of the mTOR/p70S6K pathway and cell cycle regulated proteins such as p21, CDK4, and PCNA in the lungs of A/J mice. Decreased *Pdcd4* also resulted in an increase in apoptotic proteins BCL2 and BCL-xL, and suggest that decreased *Pdcd4* levels might influence tumorigenesis by both increased proliferation and decreased apoptosis. The reciprocal would then suggest that in healthy cells, elevated *Pdcd4* levels suppress tumorigenesis by negatively regulating cell proliferation and promoting apoptosis of target cells.

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Author Disclosure Statement

The authors declare that there are no conflicts of interest.

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