Induction of Differentiation-dependent Apoptosis in Human Esophageal Squamous Cell Carcinoma by Adenovirus-mediated \(p21^{sd1}\) Gene Transfer

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ABSTRACT

When keratinocytes withdraw from the cell cycle, they migrate from the basal to the superficial layers of the epidermis and undergo morphological and biochemical changes during the process of terminal differentiation. These differentiation features of keratinocytes are known to be altered or reduced in esophageal cancer cells. Therefore, we examined the effects of transferring the cyclin-dependent kinase inhibitor \(p21^{sd1}\) gene into human esophageal cancer cell lines as well as normal keratinocytes using an adenovirus vector system. Ectopic expression of \(p21^{sd1}\) protein resulted in cell cycle arrest at the \(G_1\) phase and produced morphological changes, such as enlarged nuclei and a flattened cellular shape, changes specific to the differentiated phenotype. The human involucrin protein is a specific product of keratinocyte differentiation, which is selectively expressed in the suprabasal epidermal layers. Western blot analysis and immunohistochemical staining demonstrated that involucrin expression was 3- to 5-fold enhanced by the forced expression of \(p21^{sd1}\) in esophageal cancer cells, whereas only a mild up-regulation up to 1.2-fold occurred in normal keratinocytes. We also found that exogenous introduction of the \(p21^{sd1}\) gene transcriptionally activated the upstream promoter function of the involucrin gene. These stimulatory effects on involucrin expression were not observed when another cyclin-dependent kinase inhibitor gene, \(p16^{INK4a}\), was transduced. Moreover, \(p21^{sd1}\) expression in esophageal cancer cells transduced with \(p21^{sd1}\) led to a rapid apoptotic cell death after a transient dormant phase, although keratinocytes transduced with \(p21^{sd1}\) survived longer by terminally withdrawing from the cell cycle. These results may have an important implication for understanding the biology of differentiation-dependent apoptosis in human esophageal squamous cell carcinoma.

INTRODUCTION

Squamous cell differentiation is a multistep process that is followed by the expression of many squamous cell-specific genes for proteins such as keratin 1, involucrin, and cornifin. Of these, the involucrin protein was first discovered as the most abundant envelope component in human keratinocytes and subsequently shown to be synthesized as a cytoplasmic soluble precursor during squamous maturation (1, 2). Normally, the cross-linked envelope of the keratinocyte is formed in the last stage of its terminal differentiation. In the normal epidermis, the basal layer located adjacent to the dermis consists of relatively undifferentiated, proliferative cells, whereas the maturing terminal cells of the upper spinous layers show differentiation-specific morphological changes and express involucrin (1, 3). Moreover, in psoriasis and other benign epidermal hyperplasias, involucrin can be detected in most of the suprabasal layers of involved skin (4). These observations suggest that the onset of involucrin expression is an early marker of terminal differentiation (5–7), although the mechanism responsible is not well understood.

A wide variety of biological events and mechanisms appear to have roles in the development and progression of esophageal squamous cell carcinoma. These abnormalities include mutations in the tumor suppressor \(p53\) gene; allelic loss at various chromosomal loci; altered expression of genes involved in the regulation of cell proliferation, such as cyclin D1, MDM2, and \(c-erbB\); and infection with human papilloma virus (8–10). In addition, recent studies have demonstrated that the disruption of normal squamous cell differentiation may be one of the mechanisms for esophageal cancer development. Of special interest is the fact that involucrin expression is likely to disappear in severe dysplasias, premalignant lesions, and squamous cell carcinoma (11). Indeed, immunohistochemical analysis showed...
that the expression of involucrin was diminished in the immature tumor cells in proportion to the malignant potential, suggesting that the up-regulation of involucrin expression may directly lead a poorly differentiated squamous cell carcinoma into a terminal differentiation pathway. These findings prompted us to study the effect of the Cdk3 inhibitor p21 sdi1, which is associated with differentiation in some circumstances, on involucrin expression in esophageal squamous cell carcinoma cell lines as well as normal human keratinocytes.

p21, also known as sdi1 (12), Cip1 (13), WAF1 (14), or mda-6 (15), was originally identified as a molecule that regulates the transition from the G1 phase to the S phase of the cell cycle. During skeletal muscle differentiation, the muscle-specific transcriptional regulator MyoD increases p21 sdi1 expression, thereby inducing terminal cell-cycle arrest (16). Moreover, it has been reported that p21 protein expression is more frequently detected in well-differentiated non-small cell lung carcinoma than in poorly differentiated tumors (17). Overexpression of p21 sdi1 has been shown to induce differentiation in monoblastic cell lines (18). Together, these observations suggest that p21 sdi1 is involved in a terminal differentiation program in normal as well as cancer cells; the effect on esophageal cancer cells, however, has not been elucidated. Nevertheless, the defect in the pathway of terminal differentiation is clearly one of the most important abnormalities in esophageal carcinogenesis.

In the present study, we demonstrated that ectopic p21 sdi1 gene transfer augments involucrin expression and induces differentiation followed by a rapid cell death with apoptosis in human esophageal cancer cell lines but has no significant effects

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3 The abbreviations used are: Cdk, cyclin-dependent kinase; NHEK, normal human epidermal keratinocyte; CMV, cytomegalovirus; MOI, multiplicity of infection.
on p21<sup>sdi1</sup>-expressing normal keratinocytes. Our data may thus have important implications for understanding a novel function of p21<sup>sdi1</sup> on differentiation-dependent apoptosis.

**MATERIALS AND METHODS**

**Cells and Culture Conditions.** The human esophageal squamous cell carcinoma cell lines TE-1, which carries a homozygous p53 gene mutation, and TE-3, which contains wild-type p53, were routinely cultured in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. The NHEK line NHEK4031 (Clonetics, Walkersville, MD) was grown in monolayer culture in KBM medium (Clonetics) supplemented with 0.003% bovine pituitary extract, 50 mg/ml gentamicin sulfate, 0.05 mg/ml of amphotericin B, 0.1 ng/ml of human epidermal growth factor, 5 mg/ml of insulin, and 0.5 mg/ml of hydrocortisone. The transfected embryonic kidney cell line 293 was grown in DMEM with high glucose (4.5 g/l), supplemented with 10% FCS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin.

**Recombinant Adenoviruses.** The recombinant adenovirus vectors expressing human p21<sup>sdi1</sup> or p16<sup>INK4a</sup> cDNA we used were previously constructed and characterized (19, 20). The resultant viruses were named Ad5CMVp21 or Ad5CMVp16. Adenoviral vectors containing luciferase cDNA (Ad5RSVLuc) or no expression cassette (dl312) were used as control vectors. The viral stocks were quantified by a plaque-forming assay using 293 cells and then stored at −80°C.

**Construction of Reporter Vector.** The reporter vector we used was constructed as previously reported (21). Briefly, a HindIII-Xhol fragment of the involucrin 5′-untranslated region encompassing a region sufficient for dictating tissue-specific and differentiation-dependent expression was subcloned into the 5′ and used in transient transfection analyses.

**Western Blot Analysis.** Cells were collected by trypsinization and washed twice in cold PBS. Cells were then lysed in SDS solubilization buffer [62.5 mM Tris-HCl (pH 6.8) containing 10% glycerol, 5% β-mercaptoethanol, and 2% SDS (Sigma, St. Louis, MO)]. Protein concentrations were determined using the Bio-Rad protein determination method (Bio-Rad, Hercules, CA). Equal amounts of proteins (15 μg) were boiled for 5 min and electrophoresed under reducing conditions on 12.5% (w/v) polyacrylamide gels. Proteins were then electrophoretically transferred to Hybond polyvinylidene difluoride transfer membranes (Amersham, Arlington Heights, IL) and incubated first with primary antibodies against p21 (EA10; Oncogene Science, Manhasset, NY) or involucrin (SY5; NovoCASTRA, United Kingdom) and then with a peroxidase-linked secondary antibody. An enhanced chemiluminescence Western blotting system (Amersham Japan) was used to detect secondary probes.

**Immunohistochemistry.** Immunohistochemical staining was performed using a HISTOFINE kit (Nichirei, Tokyo, Japan). Cells were grown on chamber slides (Nunc, Naperville, IL) and infected with adenoviruses. Following fixation with 10% formaldehyde, the slides were blocked with 1% BSA for 30 min and incubated with the primary antibody overnight at 4°C in a high-humidity chamber. After one rinsing, the slides were incubated with biotinylated rabbit antimouse IgG for 10 min and then with avidin-biotin-peroxidase complex for 10 min. Peroxidase activity was detected using a 3, 3′-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. Slides were counterstained with methyl green. The primary antibodies used were anti-p21 antibody (EA10; Oncogene Science) and anti-involucrin antibody (SY5; NovoCASTRA). Negative control slides were processed with mouse preimmune immunoglobulin.

**Flow Cytometric Analysis.** Trypsinized adherent and floating cells were collected, washed twice with cold PBS, and resuspended in PBS containing 0.1% Triton X-100 and 0.1% RNase for 5 min at room temperature. The samples were then stained with propidium iodide (0.1 mg/ml), filtered through a 40-μm-pore-size nylon mesh, and analyzed in a cell sorter (FACScan; Becton Dickinson, Mountain View, CA) for DNA content. Cell debris and fixation artifacts were gated out, and G1, S, and G2-M populations were quantified using the ModFit LT program for Macintosh (version 1.01; Verity Software House, Inc.).

**Transfection Procedure and Analysis of Reporter Gene Activity.** Cells (10<sup>5</sup>/well) were seeded on 6-well tissue culture plates, incubated until 50% confluent, and then washed twice with OPTI-MEM I Reduced Medium (Life Technologies, Inc.), followed by the addition of 2.0 ml of OPTI-MEM I Reduced Medium containing 5 μg of reporter plasmid, 0.25 μg of control luciferase plasmid, and 15 μl of LIPOFECT ACE Reagent (Life Technologies, Inc.). Cells were incubated for 24 h, infected with Ad5CMVp21 or control dl312 vector, and then grown for an additional 24 h before harvest. For harvest, cells were washed twice in PBS and scraped into cell-culture lysis reagent (Promega, Madison, WI). The cell extracts were immediately assayed for luciferase activity using the Promega Dual-Luciferase Reporter Assay System and a Berthold luminometer.

**RESULTS**

**Ectopic p21<sup>sdi1</sup> Expression in Human Esophageal Cancer Cells and NHEKs.** We used a replication-deficient adenovirus vector carrying a p21<sup>sdi1</sup> gene under the control of the CMV promoter (Ad5CMVp21) to achieve efficient gene transfer into human cancer cells as well as normal cells. Monolayer cultures of TE-1 and TE-3 human esophageal cancer cells and NHEKs were infected with either Ad5RSVLuc or Ad5CMVp21 at an MOI of 100. Western blot analysis demonstrated that readily detectable p21<sup>sdi1</sup> protein expression was seen in all cells as early as 24 h after infection but not in parental and Ad5RSVLuc-infected cells (Fig. 1A).

To further confirm the p21<sup>sdi1</sup> expression in individual cells, immunohistochemical staining was performed using antibody against p21<sup>sdi1</sup> after 48 h of infection. As shown in Fig. 1B, positive nuclear staining for p21<sup>sdi1</sup> was detected in 80–90% of TE-1 cancer cells, whereas most Ad5CMVp21-infected NHEKs expressed p21<sup>sdi1</sup> protein primarily in the cytoplasm. These findings suggest that the adenovirus-mediated gene transfer and expression were highly efficient in human esophageal cancer cells as well as in normal keratinocytes. There was no
Differentiation-dependent Apoptosis by p21 Gene Transfer

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apparent toxicity in response to adenovirus infection up to 200 MOI (data not shown).

Effect of \textit{p21}\textsuperscript{sdi1} Gene Transfer on Cell-cycle Progression and Morphology of TE-1, TE-3, and NHEKs. To investigate the function of ectopically transferred \textit{p21}\textsuperscript{sdi1}, we next performed cell-cycle analysis on TE-1 and TE-3 cells as well as NHEKs. Cells were infected with either Ad5CMVp21 or Ad5RSVLuc at an MOI of 100, harvested at 48 h postinfection, and then assayed for DNA content by flow cytometry. Ad5CMVp21-infected cells had a lower fraction of S-phase cells (mock: 21.8%, Ad5RSVLuc: 23.4%, Ad5CMVp21: 8.9% for TE-1; mock: 25.4%, Ad5RSVLuc: 34.6%, Ad5CMVp21: 8.9% for TE-1; mock: 25.4%, Ad5RSVLuc: 34.6%, Ad5CMVp21: 8.9%

Fig. 2  A, cell cycle distribution of TE-1, TE-3, and NHEK cells transduced with Ad5CMVp21 or Ad5RSVLuc. Cells were infected with either Ad5CMVp21 or Ad5RSVLuc at 100 MOI for 48 h, trypsinized, and then analyzed for DNA content by propidium iodide staining and flow cytometry. A normal cell-cycle curve was maintained in Ad5RSVLuc-infected cells, whereas a marked S-phase reduction was observed in cells transduced with Ad5CMVp21. \textit{B}, phase-contrast photomicrographs (×200) of mock-infected, Ad5RSVLuc-infected, and Ad5CMVp21-infected cells 72 h after infection. Most of the Ad5CMVp21-infected cells became greatly enlarged in average diameter when compared with mock-infected or Ad5RSVLuc-infected cells. \textit{C}, photomicrograph of TE-1 cells cultured in serum-depleted medium. No morphological changes were observed.

Fig. 3  Increased expression of involucrin protein. \textit{A}, total cell extracts were prepared from parental TE-1, TE-3, and NHEK cells mock-infected or infected with either Ad5RSVLuc or Ad5CMVp21 at an MOI of 100 and then analyzed on Western blots using antibodies against human involucrin. \textit{B}, a comparison of involucrin protein expression after quantification by densitometric scanning using NIH Image software and normalization by dividing the actin signal (shown in Fig. 1A) for each sample. \textit{C}, immunohistochemical analysis for involucrin expression. Mock-infected, Ad5RSVLuc-infected, and Ad5CMVp21-infected TE-1 cells were stained with anti-involucrin antibody 72 h after infection.
Differentiation-dependent Apoptosis by p21 Gene Transfer.

p21 was evident (Fig. 3C). Cytoplasmic, and in most cells, heterogeneous staining was observed immunohistochemically. Staining with anti-involucrin antibody 72 h after infection showed involucrin protein to be expressed in a 5-fold, 3-fold, and 1.2-fold increase of involucrin levels in NHEKs, suggesting that overexpression of p21sdi1 predominately arrested the cell cycle at the G1 checkpoint (Fig. 2A).

Arrested TE-1 and TE-3 cells and NHEKs showed morphological changes, including an enlarged, flattened shape, an increased cytoplasmic-to-nuclear ratio, and a decreased cell density, all of which are consistent with a differentiation phenotype. 72 h after Ad5CMVp21 infection (Fig. 2B). We further examined whether withdrawal of cells from the cell cycle induced by serum deprivation could cause alterations in cell morphology. Although the growth of cells cultured in the absence of serum was significantly reduced as evidenced by a decreased cell density, cells exhibited neither a flattened morphology nor an enlarged nuclei (Fig. 2C). Thus, the morphologically altered differentiated phenotype appeared not to be due to the withdrawal from the cell cycle.

Enhanced Involucrin Protein Expression by Ectopic p21sdi1 Gene Transfer. To further study the relationship between the exogenous p21sdi1 expression and differentiation, we examined the levels of endogenous involucrin protein in Ad5CMVp21-infected cells. Involucrin is a major envelope protein expressed in the early stage of keratinocyte differentiation and thus, is an appropriate marker for differentiation. Western blot analysis showed that infection with Ad5CMVp21 at an MOI of 100 resulted in up-regulation of involucrin protein expression (Fig. 3A). A modest expression of involucrin was detected in parental TE-1 cells; involucrin levels, however, were highly elevated upon infection with Ad5CMVp21. In contrast, enhancement of involucrin expression in NHEKs was less pronounced than in esophageal cancer cell lines. An up-regulation of involucrin was not produced in any cell lines by control Ad5RSVLuc infection.

Densitometric analysis of the sample band normalized to actin and shown in Fig. 1A revealed that Ad5CMVp21 resulted in a 5-fold, 3-fold, and 1.2-fold increase of involucrin levels in TE-1, TE-3, and NHEK cells, respectively, over the levels in mock-infected cells at 24 h after infection (Fig. 3B).

TE-1 cells, which underwent remarkable morphological changes following infection with Ad5CMVp21, were also analyzed immunohistochemically. Staining with anti-involucrin antibody 72 h after infection showed involucrin protein to be cytoplasmic, and in most cells, heterogeneous staining was evident (Fig. 3C).

Transcriptional Activation of Involucrin by Ectopic p21sdi1 Gene Transfer. To establish that induction of involucrin protein expression was dependent on transcriptional regulation by p21sdi1, we transiently transfected TE-1 and TE-3 cells and NHEKs with an upstream promoter sequence of the involucrin gene linked to a luciferase reporter gene. Transient transfection analysis demonstrated that treatment of transfected cells with Ad5CMVp21 at an MOI of 100 led to a ~1.5- to 2-fold induction of the involucrin promoter activity in all types of cells by 24 h after infection (Fig. 4). In contrast, the promoter activity in TE-1 and TE-3 cells was unaffected by infection with an adenovirus carrying the p16INK4a cDNA (Ad5CMVp16).

Effect of p21sdi1 Gene Transfer on the Long-term Growth of TE-1 Cells and NHEKs. We finally investigated the effects of ectopic p21sdi1 expression on the long-term growth curve of TE-1 cells as well as normal keratinocytes. TE-1 cells and NHEKs that remained attached as a monolayer were assessed for viability by measuring trypan blue uptake for 18 days following infection of Ad5CMVp21 at 100 MOI for 24 h. As illustrated in Fig. 5A, Ad5CMVp21 infection suppressed the growth of NHEKs and then caused a gradual decrease in cell number up to 18 days, whereas it induced a rapid cell death in TE-1 cells after a remarkable growth arrest for ~8 days.

To obtain further details of the rapid cell death in TE-1 cells after a transient growth arrest, dying TE-1 cells were stained with the DNA-intercalating dye Hoechst 33342 and then examined by fluorescence microscopy. The analysis showed that Ad5CMVp21-infected TE-1 cells underwent apoptosis, which was characterized by highly condensed and partially fragmented nuclei, ~8 days after infection (Fig. 5B), whereas only a few NHEKs did so over 18 days.

DISCUSSION

Human esophageal squamous cell carcinoma exhibits a reduced degree of differentiation, as evidenced by the decreased capacity to form cross-linked envelopes, thus suggesting that these tumor cells frequently display a defect in the terminal differentiation pathway (3). Involucrin is a major component of the cornified envelope and is considered to be an appropriate marker for terminal differentiation. The results described above demonstrate that adenovirus-mediated transfer of the p21sdi1 gene up-regulates involucrin protein expression and induces...
terminal differentiation in human esophageal squamous cell carcinoma cells, thereby leading to sequential apoptotic cell death.

Adenovirus-mediated gene transfer is a highly efficient gene delivery system. We used a recombinant adenovirus to overexpress p21\textsuperscript{sd1} in normal and neoplastic squamous cells. Preliminary titration studies showed that transient transgene expression in most populations of infected cells can be expected at an MOI of 100. In fact, strong induction of exogenous p21\textsuperscript{sd1} expression was achieved in both human esophageal cancer cells and NHEKs by Ad5CMVp21 infection; the localization of transduced p21\textsuperscript{sd1} protein, however, was completely divergent: nuclear in the esophageal cancer cells \textit{versus} cytoplasmic in the NHEKs (Fig. 1). p21\textsuperscript{sd1} protein has an intrinsic nuclear local-
izing signal that could facilitate nuclear entry of associated cyclin E-Cdk2 complexes as well as cyclin D-Cdk4 complexes (22). In normal keratinocytes, exogenous p21sdi1 may be dispensable for survival because the cells already have sufficient endogenous p21sdi1. Thus, the overexpression of p21sdi1 protein in the cytoplasm of the normal cells may be explained by their machinery for defending themselves.

Despite this difference in cellular localization, transduced p21sdi1 blocked cell cycle progression and caused differentiation-specific morphological changes in normal and neoplastic cells (Fig. 2). Simply arresting cells was apparently not sufficient to activate the differentiation pathway because withdrawal from the cell cycle by serum deprivation could not cause alterations in cell morphology (Fig. 2C). These results suggest that overexpression of exogenous p21sdi1 can lead directly into a differentiation program.

Our data demonstrate that ectopic p21sdi1 expression increases cytoplasmic levels of involucrin protein in monolayer cultures of NHEKs as well as esophageal squamous carcinoma cell lines (Fig. 3). This stimulatory effect of p21sdi1 gene transfer on expression of involucrin occurred at the level of transcriptional regulation (Fig. 4). Ad5CMVp21 infection activated transcription of the involucrin promoter similarly in normal and neoplastic cells, the magnitude of protein induction, however, was higher in the cancer cells than in the normal cells because the basal level of involucrin expression was relatively high in the normal keratinocytes. Harvat et al. (23) has reported that NHEK expressing Cdk inhibitors (e.g., p27kip1, p21WAF1/Cip1, p16INK4a) induced expression of differentiation-specific genes, including involucrin and cornifin, only when placed in suspension. Cunto et al. (24) have reported that adenovirus-mediated transfer of the p21sdi1 gene but not the p16INK4a gene transcriptionally inhibited the expression of differentiation markers in primary mouse keratinocytes. One possible explanation for the discrepancy between our data and these results may be the differences in cell source and/or experimental systems (e.g., vector constructs). Our data, at least, provide evidence that p21sdi1 gene transfer can positively regulate endogenous involucrin expression, leading to a terminal differentiation, in human esophageal squamous cell carcinoma cell lines.

The signals that induce differentiation-specific involucrin expression have been intensively studied. It has been reported that Fos-related antigen, junB, and junD activate the involucrin promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP1 binding sites (25). The AP2-like transcriptional factor that binds to keratinocyte differentiation factor promoter through the AP2-like transcriptional factor that binds to Cdk as well as proliferating cell nuclear antigen, have been identified (26, 27). p21sdi1 itself is not likely to be a transcriptional factor. Therefore, transcriptional activation of the involucrin promoter by p21sdi1 gene transfer may be indirect, presumably through other transcriptional factors, although the precise mechanism of this stimulatory effect remains to be identified.

Our own flow cytometric analysis of cell cycle distribution revealed that Ad5CMVp21-infected normal keratinocytes as well as esophageal cancer cells exited the S phase after the accumulation in the G1 phase (Fig. 2A); in the long-term, however, tumor cells expressing the p21sdi1 gene underwent apoptosis after a transient dormant period, whereas normal keratinocytes expressing p21sdi1 gradually declined in number and survived longer (Fig. 5). The fate of p21sdi1-expressing keratinocytes seems to be physiological because NHEKs are not immortalized cells. In contrast, the rapid cell death observed in tumor cells is not physiological. These results suggest that the long-term effects of ectopic p21sdi1 gene transfer diverge between normal keratinocytes and esophageal squamous cell carcinoma cells, although the immediate growth-inhibitory effects are almost the same.

In summary, our results demonstrate that introduction of the p21sdi1 gene into human esophageal squamous cell carcinoma cells can cause involucrin expression and induce the differentiated phenotype, leading to terminal apoptotic cell death. The efficacy of the differentiation therapy for hematopoietic diseases has been proven; the antitumor effects against solid tumors, however, are not clear. Our present study showing the potential of p21sdi1 to induce differentiation may therefore provide a clue as to a successful differentiation-directed cancer therapy.

ACKNOWLEDGMENTS

We thank Drs. Hirotoshi Takashima and Masafumi Kataoka for helpful discussion and Ms. Monica Contreras for her expert assistance.

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