The conventional nonsteroidal anti-inflammatory drug sulindac sulfide arrests ovarian cancer cell growth via the expression of NAG-1/MIC-1/GDF-15

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Abstract
Although the chemopreventive and antitumorigenic activities of nonsteroidal anti-inflammatory drugs (NSAID) against colorectal cancer are well established, the molecular mechanisms responsible for these properties in ovarian cancer have not been elucidated. Therefore, there is an urgent need to develop mechanism-based approaches for the management of ovarian cancer. To this end, the effect of several NSAIDs on ovarian cancer cells was investigated as assessed by the induction of NAG-1/MIC-1/GDF-15, a proapoptotic gene belonging to the transforming growth factor-β superfamily. Sulindac sulfide was the most significant NSAID activated gene 1 (NAG-1) inducer and its expression was inversely associated with cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. This growth suppression by sulindac sulfide was recovered by transfection of NAG-1 small interfering RNA. These results indicate that NAG-1 is one of the genes responsible for growth suppression by sulindac sulfide. Furthermore, we observed down-regulation of p21 WAF1/CIP1 by introduction of NAG-1 small interfering RNA into sulindac sulfide–treated cells. In addition, to elucidate other potential molecular mechanisms involved in sulindac sulfide treatment of ovarian cancer cells, we did a membrane-based microarray experiment. We found that cyclin D1, MMP-1, PI3KR1, and uPA were down-regulated by sulindac sulfide. In conclusion, a novel molecular mechanism is proposed to explain the experimental results and provide a rationale for the chemopreventive activity of NSAIDs in ovarian cancer. [Mol Cancer Ther 2005;4(3):487–93]

Introduction
Ovarian cancer has the highest mortality rate of any of the gynecologic cancers; it is the fourth leading cause of cancer-related deaths among women in the United States and causes over 100,000 deaths annually worldwide (1, 2). Although intensive treatments for ovarian cancer, such as radical surgery, radiation therapy, and chemotherapy have improved survival rates, cure rates have stayed essentially the same over the last 20 years. Thus, early intervention with chemopreventive agents has been considered as a desirable alternative treatment for this invasive disease.

Three major chemopreventive agents, nonsteroidal anti-inflammatory drugs (NSAID), retinoids, and oral contraceptives, have been studied by many researchers for ovarian cancer (3–5). Some of the most promising pharmaceutical agents described to date for the prevention of cancer are the NSAIDs. Of all the various NSAIDs reported to inhibit the development of tumors, sulindac seems to be one of the most effective in experimental animal models (6). Sulindac itself does not inhibit cyclooxygenase (COX), but its metabolite, sulindac sulfide, is a potent nonselective inhibitor of COX. Whereas some reports suggest that some NSAIDs induce apoptosis and cell cycle arrest in human ovarian cancer cells (3, 7), the exact molecular mechanism by which NSAIDs induce antitumorigenic activity has not been investigated.

In contrast to colorectal cancer, no one has reported whether sulindac sulfide has any effect on apoptosis and cell cycle arrest in ovarian cancer cells. Epidemiologic findings regarding NSAID effects on ovarian cancer are rather conflicting. For example, an Italian study found that NSAID use was not related to ovarian cancer risk (8), whereas a hospital-based case-control study reported that long-term use of NSAIDs was associated with a reduced risk (9). In the latter case, however, there has been no reported molecular mechanism to explain the effect of NSAIDs on ovarian cancer inhibition. Therefore, it is important to know whether NSAIDs, such as sulindac sulfide, induce growth suppression in ovarian carcinoma cells, and to further investigate the molecular mechanism by which such NSAIDs may arrest ovarian cancer cell growth.

NSAID activated gene (NAG-1), also known as MIC-1, GDF-15, PTGFB, and PLAB, was identified in our laboratory as a gene regulated by NSAIDs, cyclooxygenase inhibitors based on PCR-based subtractive hybridization of indomethacin-treated human colorectal cells (10). The human NAG-1 cDNA encodes a secreted protein with
homology to members of the transforming growth factor-β superfamily and a plethora of data support the finding that NAG-1 is linked to apoptosis and that its reduced expression may enhance tumorigenesis (11–13). NAG-1 expression is up-regulated in a prostaglandin-independent manner in human colorectal cancer cells by several NSAIDs (10). Other antitumorigenic compounds, such as resveratrol (14), genistein (15), diallyl disulfide (16), catechins (17), proliferator-activated receptor-γ ligands (18), 5F-203 (19), and retinoid 6-[3-(20)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (20), also up-regulate NAG-1. Whereas some dietary factors, including resveratrol and genistein, induce NAG-1 expression through the p53 tumor suppressor protein (14, 15), NSAIDs and anoxia induce NAG-1 in a p53-independent manner (14, 21). Recently, Yamguichi et al. (22) reported that expression of NAG-1 is regulated by phosphatidylinositol 3-kinase/AKT/glycogen synthase kinase-3β pathway, the signalings of which are deregulated/inappropriately activated with high frequency in human tumors. Thus, several pathways may affect NAG-1 expression.

Although NAG-1 has been well studied in colorectal cancer as an antitumorigenic protein, the expression and regulation of NAG-1 in ovarian cancer remains to be characterized. In this report, we have found that the sulindac sulfide induces NAG-1 expression in three different ovarian cancer cell lines, which have different COX-1/COX-2 and p53 status. Therefore, we propose that NAG-1 represents a novel protein target for sulindac sulfide treatment in ovarian cancer, and that sulindac sulfide induces the expression of NAG-1 in a COX- and p53-independent manner. NAG-1 promotes growth suppression and mediates the antitumorigenic effects of NSAIDs.

Materials and Methods

Cell Lines and Reagents

Human ovarian cancer cells, SKOV3, OVCAR3, and PA-1, were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum and penicillin-streptomycin (Invitrogen, Carlsbad, CA). NSAIDs (indomethacin, NS-398, and SC-560) were purchased from Sigma (St. Louis, MO), whereas sulindac, sulindac sulfone, and sulindac sulfide were purchased from Calbiochem (San Diego, CA). Antibodies to p21 WAF1/CIP1 and actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), whereas NAG-1 antibody was described previously (10).

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, and 0.5% sodium deoxycholate]. Twenty micrograms of whole cell extracts were separated in a 4% to 7% gel (Invitrogen) and transferred onto a nitrocellulose membrane (Invitrogen). Membranes were blocked in 5% nonfat dry milk and incubated with the primary antibody. Blots were probed with primary antibody followed by labeling with horseradish peroxidase–conjugated anti-mouse, anti-rabbit (Amersham, Piscataway, NJ), or anti-goat secondary antibody (Santa Cruz Biotechnology). Bands were visualized by enhanced chemiluminescence using the ECL kit (Amersham) according to manufacturer's instruction.

Cell Viability Assay

The cell growth of the stable cell lines was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium colorimetric assay kit purchased from Promega (Madison, WI). Approximately 3 × 10^5 cells were seeded and grown in each well of 96-well plates overnight. Cell viability was measured daily for 4 days at 490 nm in an ELISA reader plate following the addition of 20 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution and incubation at 37°C for 4 hours.

NAG-1 Promoter and Luciferase Assay

SKOV3 cells were plated in six-well plates at 1 × 10^5 cells/well in DMEM supplemented with 10% fetal bovine serum. After growth for 24 hours, 1 μg NAG1 promoter containing plasmid (pNAG133/LUC) and 0.05 μg pRL-null (Promega) were transfected with FuGene6 (Roche, Indianapolis, IN) according to the manufacturer's instruction. NAG-1 promoter (pNAG133/LUC) was described previously (23). After 24 hours of transfection, cells were treated with DMSO or sulindac sulfide. After 24 hours of sulindac sulfide treatment, cells were harvested in 1× luciferase assay buffer, and luciferase activity was measured and normalized to the pRL-null luciferase activity using the Dual Luciferase Assay kit (Promega).

NAG-1 Small Interfering RNA Construct and Trypan Blue Exclusion Assay

NAG-1 small interfering RNA (siRNA) construct was kindly provided by Dr. Jim Lambert (University of Colorado, Denver, CO). Cells were transfected with pcDNA3.1 or NAG-1 siRNA before 24-hour treatment with sulindac sulfide. After 24-hour treatment with sulindac sulfide, cells were collected with trypsinization in 300 μL PBS. Cells were stained with trypan blue solution and then the viable cells were counted in a hemocytometer. Each sample was counted as three independent determinations from three independent experiments.

Reverse Transcription-PCR

Total cellular RNAs were extracted from cell lines using TRIzol reagent (Life Technologies, Gaithersburg, MD). Ten nanograms of total RNA were reverse transcribed and each set of gene-specific primers (Table 1). The thermal cycling conditions used consisted of initial denaturation at 94°C for 4 minutes, followed by 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds, and a final extension for 10 minutes at 72°C. The final PCR products were electrophoresed on a 1% agarose gel and photographed under UV light.

Microarray Experiment

The procedure for biotinylated cDNA probe synthesis was done using the Amphi-LPR labeling kit.
Table 1. Gene-specific primer sequences for reverse transcription-PCR

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequences</th>
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<tr>
<td>Cyclin D1 (NM_053056)</td>
<td>5'-TTCAAAATGTGTCGAGAAGGA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GACGAGCCGTAGTATAGGAG-3'</td>
</tr>
<tr>
<td>MMP-1 (NM_002421)</td>
<td>5'-AGATGAAAGTTGGGACAACA-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTTCTACCTCCTGTCGGCAAAT-3'</td>
</tr>
<tr>
<td>PI3KR1 (NM_181504)</td>
<td>5'-ACGTGAAGGCAATGAGAAAG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CAAAAGGAGGTGTTGTTGTA-3'</td>
</tr>
<tr>
<td>uPA (NM_002668)</td>
<td>5'-GGGGAATGAAATTTGACTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CCATTCTCCTCTCCTGTGTG-3'</td>
</tr>
<tr>
<td>GAPDH (NM_002046)</td>
<td>5'-TCAACGGATTTGCTCGATT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CTGTGTTGTCATGAGTCTCC-3'</td>
</tr>
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(Superarray, Inc., Frederick, MD). Briefly, 5 μg total RNA were used as a template for cDNA synthesis and then the cDNA was labeled with biotin-dUTP (Roche) during a PCR reaction. The reaction was stopped and denatured at 94°C for 2 minutes and the resulting DNA probe was applied to a prehybridized GEArray membrane. The hybridization was done at 60°C for 12 hours in a hybridization oven. After two-step washing at 60°C, the membrane was blocked and treated with alkaline phosphatase–conjugated streptavidin and finally exposed to CDP-Star alkaline phosphatase chemiluminescent substrate. The membrane was exposed to an X-ray film. The intensity of the spot was compared using glyceraldehyde-3-phosphate dehydrogenase as a positive control.

Results

**Sulindac Sulfide Induces NAG-1 Expression in Ovarian Cancer Cells**

Previously, we have reported that some NSAIDs induce NAG-1 expression in colorectal cancer cells (10, 24). However, there is no information regarding NAG-1 expression by NSAIDs in ovarian cancer cells. To determine whether NSAIDs increase NAG-1 expression, conventional nonselective NSAIDs (sulindac, sulindac sulfone, sulindac sulfide, and indomethacin) that inhibit COX-1 and COX-2, as well as selective COX-1 (SC-560) or COX-2 inhibitors (NS-398), were incubated with SKOV3 cells (COX-1/-2 and p53-negative cells). Cells were treated with various NSAIDs at different concentrations shown in Fig. 1A for 24 hours, and Western blot analysis was done using a NAG-1–specific antibody. Among the NSAIDs tested in this study, sulindac sulfide dramatically induced NAG-1 expression, whereas SC-560 barely induced NAG-1 expression. In agreement with previous studies with human colorectal cancer cells, sulindac and sulindac sulfone were ineffective, whereas sulindac sulfide was an excellent inducer of NAG-1 expression. Therefore, sulindac sulfide is the best NAG-1 inducer among NSAIDs tested here and, thus, was used for further studies. In addition, two other ovarian cancer cells, OVCAR3 (COX-1/-2 positive) and PA-1 (p53 positive), were examined. Interestingly, dramatic NAG-1 induction was detected in all cell lines (Fig. 1B), suggesting that sulindac sulfide induces NAG-1 expression possibly independent of COX and p53 status.

**Sulindac Sulfide Suppresses Ovarian Cancer Cell Growth and Up-Regulates NAG-1 and p21 Expression**

NAG-1 expression is linked to cell growth arrest and apoptosis (10, 11). To investigate the effects of sulindac sulfide on the growth of ovarian cancer cells in culture, SKOV3 cells were grown and treated with different concentration of sulindac sulfide (10, 20, and 30 μmol/L) for 3 days and the cell growth rate was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay at 490 nm absorbance. As shown in Fig. 2, sulindac sulfide treatment resulted in the complete inhibition of cell growth, whereas vehicle treatment did not affect any significant inhibition in SKOV3 cells, suggesting that sulindac sulfide induces cell growth arrest in SKOV3 cells in a dose-dependent manner. This data is consistent with our previous report that sulindac sulfide inhibit cell growth in HCT-116 human colorectal cancer cells (10). Based on this result, we evaluated NAG-1 expression, induced at different time and concentrations by sulindac sulfide, with Western blot analysis. Figure 3A and B shows that NAG-1 expression is dependent upon both the concentration and time of treatment. Highest expression was detected at 48 hours. In addition, a luciferase assay with the NAG-1 promoter (pNAG133/LUC) shows a similar concentration dependency for NAG-1 expression (Fig. 3C). Finally, we evaluated expression of p21 WAF1/CIP1 by Western blot analysis because Han et al. (25)
previously reported sulindac sulfide–up-regulated p21 WAF1/CIP1 in human breast cancer cells. As shown in Fig. 3D, sulindac sulfide up-regulate p21 WAF1/CIP1 at the protein level in a time-dependent manner. This result indicates that p21 WAF1/CIP1 may also be one of the genes responsible for the growth arrest induced by sulindac sulfide.

Reversal of Sulindac Sulfide–Induced Apoptosis by NAG-1 siRNA

The correlations observed between the induction of NAG-1 and cell growth arrest necessitated construction of a NAG-1 siRNA to directly assess the biological activities of sulindac sulfide and NAG-1 in ovarian cancer cells. The NAG-1 siRNA was prepared and SKOV3 cells were transiently transfected with either empty vector (pcDNA3.1) or the NAG-1 siRNA vector, followed by the treatment of sulindac sulfide. The cells transfected with the NAG-1 siRNA construct showed a repression of sulindac sulfide–induced NAG-1 expression (Fig. 4A). Next, we examined whether the suppression of NAG-1 by siRNA may attenuate sulindac sulfide–induced cell growth arrest. The empty vector and NAG-1 siRNA constructs were transiently transfected into SKOV3 cells and then the cells incubated with sulindac sulfide for 24 hours. As shown in Fig. 4B, NAG-1 siRNA transfection restored cell viability to SKOV3 cells treated with sulindac sulfide, indicating that the sulindac sulfide–induced NAG-1 expression is responsible, at least in part, for the sulindac sulfide–induced cell growth arrest observed in SKOV3 cells. These results also indicate that NAG-1 expression is directly associated with growth suppression by sulindac sulfide. To explain the molecular mechanisms of growth recovery by NAG-1 siRNA transfection, we analyzed NAG-1 and p21 expression after introduction of pcDNA3.1 or NAG-1 siRNA construct, followed by sulindac sulfide treatment. As shown in Fig. 4A, NAG-1 siRNA–transfected cells showed reduced protein expression of NAG-1, as well as p21 expression, compared with pcDNA3.1-transfected cells. These results indicate that NAG-1 is directly associated with the growth suppression caused by sulindac sulfide and p21 may be one of genes downstream of NAG-1. However, the direct molecular mechanism connecting NAG-1 and p21 remains to be elucidated.

Analysis of Gene Expression Changes by Sulindac Sulfide with Membrane-Based Microarray

To provide other potential mechanisms to explain the antitumorigenic effect of sulindac sulfide in ovarian cancer, we did a membrane-based microarray (Superarray) analysis. The Cancer Pathway Finder microarray was chosen.

Figure 2. Effect of sulindac sulfide on SKOV3 cell growth. SKOV3 cells were plated at 3,000 cells/well in a 96-well plate and incubated with vehicle (DMSO) or various concentrations of sulindac sulfide. Cell growth was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell proliferation kit (Promega). Points, mean of six replicate experiments; bars, SD.

Figure 3. NAG-1 and p21 expression by sulindac sulfide. A, SKOV3 cells were treated with three different concentrations of sulindac sulfide, and treated cells were collected for Western blot analysis using NAG-1 and actin antibodies. B, SKOV3 cells were treated with 30 μmol/L sulindac sulfide at three different time points. Cell lysates were subjected to Western blot analysis with NAG-1 and actin antibodies. C, a NAG-1 promoter construct, pNAG133/LUC (1 μg), was cotransfected with 0.1 μg pRL-null vector into SKOV3 cells using FuGene6. After 24-h transfection, cells were treated with 10, 20, or 30 μmol/L sulindac sulfide or DMSO (vehicle, VEH). The promoter activity was measured by luciferase activity after 24-h treatment. Transfection efficiency for luciferase activity was normalized to the Renilla luciferase activity. X-axis, relative luciferase unit (RLU, luciferase activity/Renilla unit). Columns, mean of three independent transfections; bars, SD. D, SKOV3 cells were treated with 20 μmol/L sulindac sulfide and cells were collected at different time points. Cell lysates were prepared and Western blot analysis was done using p21 and actin antibodies.
PI3KR1 agreed with the microarray data (Fig. 5). The characteristics evaluated by reverse transcription-PCR and the result of NAG-1 and p21 by NAG-1 siRNA points to a suitable molecular mechanism of growth recovery. These results indicate the NSAID sulindac sulfate arrests ovarian cancer cell growth via up-regulation of the NAG-1 and p21 genes. However, the exact molecular mechanism by which NAG-1 and p21 affect cell growth arrest remains to be elucidated.

Discussion

In this study, we have shown that the NSAID sulindac sulfate exerts a potential antitumorigenic effect in ovarian cancer cells through the up-regulation of the antitumorigenic protein NAG-1. Our results support the conclusion that NAG-1 has an antiproliferative activity against ovarian cancer cells. First, up-regulation of NAG-1 by sulindac sulfate treatment is inversely associated with ovarian cancer cells. Second, blocking of NAG-1 expression by NAG-1 siRNA resulted in recovery from the growth arrest caused by sulindac sulfate. Third, down-regulation of NAG-1 and p21 by NAG-1 siRNA points to a suitable molecular mechanism of growth recovery. These results indicate the NSAID sulindac sulfate arrests ovarian cancer cell growth via up-regulation of the NAG-1 and p21 genes. However, the exact molecular mechanism by which NAG-1 and p21 affect cell growth arrest remains to be elucidated.

It has been reported that 9 of 10 primary cultures of ovarian cancer cells isolated from ascite fluid remained sensitive to transforming growth factor-β growth inhibition and that these cells express all components of the transforming growth factor-β/Smad signaling pathway (26). It is notable that NAG-1 belongs to the transforming growth factor-β superfamily. Therefore, it is very interesting to examine the induction of NAG-1 by drugs including NSAIDs, retinoids, and oral contraceptives. In this report, we showed that sulindac sulfate induced NAG-1 expression. However, the other two compounds are also promising for chemoprevention against ovarian cancer. Oral contraceptives have been shown to reduce the subsequent risk of ovarian carcinoma in observation studies (27). An innovative study that supports the use of progestins as a chemopreventive agent in ovarian cancer was published (28). In addition, epidemiologic and laboratory data suggest that retinoids may have a role as preventive or therapeutic agents for ovarian cancer (29). For example, fenretinide or 4-HPR induces apoptosis in ovarian cancer cells and has few side effects compared with other vitamin A derivatives. In addition, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (AHPN/CD437), a conformationally restricted retinoid engineered to bind selectively to retinoic acid receptor-γ, also shares the apoptotic profile of 4-HPR. In ovarian cancer cells, exposure to AHPN was associated with increased expression of BAX and decreased expression of Bcl-2 (30). Indeed, we have reported that AHPN induces NAG-1 expression in lung carcinoma cells (20).

To identify other potential mechanisms, we analyzed gene expression changes in sulindac sulfate–treated ovarian cancer cells through the up-regulation of the antitumorigenic protein NAG-1. Our results support the conclusion that NAG-1 has an antiproliferative activity against ovarian cancer cells. First, up-regulation of NAG-1 by sulindac sulfate treatment is inversely associated with ovarian cancer cell growth. Second, blocking of NAG-1 expression by NAG-1 siRNA resulted in recovery from the growth arrest caused by sulindac sulfate. Third, down-regulation of NAG-1 and p21 by NAG-1 siRNA points to a suitable molecular mechanism of growth recovery. These results indicate the NSAID sulindac sulfate arrests ovarian cancer cell growth via up-regulation of the NAG-1 and p21 genes. However, the exact molecular mechanism by which NAG-1 and p21 affect cell growth arrest remains to be elucidated.
SKOV3 cells compared with vehicle-treated cells by membrane microarray. We found that four genes were dramatically down-regulated by sulindac sulfide treatment—cyclin D1, MMP-1, PI3KR1, and uPA. Increased cyclin D1 expression was detected in ovarian cancer (31) and was associated with malignancy and recurrence of ovarian cancer (32). Therefore, down-regulation of cyclin D1 by sulindac sulfide may have a significant meaning. Nishikawa et al. (33) provided evidence that MMP-1 and MMP-2 were important genes in the invasive activity of several cancer cell lines. However, in this microarray experiment, we did not detect any change in either MMP-2 or MMP-9. Therefore, MMP-1 may be one of the important targets of sulindac sulfide among the metalloproteinases. PI3KR1 is a regulatory subunit of phosphoinositide 3-kinase. Philp et al. (34) reported that PI3KR1 and serine kinase AKT were activated in SKOV3 cells and NAG-1 was identified as a downstream target of phosphoinositide 3-kinase (22). Therefore, sulindac sulfide could block the AKT pathway by down-regulation of PI3KR1. The blockage of phosphoinositide 3-kinase pathway by sulindac sulfide is under investigation. In ovarian cancer, elevated expression of uPA and PAI-1 have been reported (35, 36). Microarray data and reverse transcription-PCR data shows sulindac sulfide dramatically reduces uPA mRNA expression (Fig. 5). However, expression of those genes was not affected by NAG-1 siRNA transfection (data not shown). Taken together, the conventional NSAID sulindac sulfide has a chemopreventive effect in ovarian cancer and modulates the expression such genes as NAG-1, p21, cyclin D1, MMP-1, PI3KR1, and uPA. However, additional studies are needed to determine the relationship between the regulation of these genes and sulindac sulfide.

Acknowledgments
We thank Dr. Jeanelle Martinez and Dr. Minako Ishibashi for their helpful suggestions and comments.

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