

The expression and regulation of DFNA5 in human hepatocellular carcinoma DFNA5 in hepatocellular carcinoma

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Abstract Hepatocellular carcinoma is a primary malignancy of hepatocytes which accounts for 80 % of all primary liver cancers. DFNA5 has been identified as a tumor suppressor gene with an important role in several frequent forms of cancers, while little is known about its role in hepatocellular carcinoma. Through comparison of the DFNA5 protein expression in hepatocellular carcinoma cells (HepG2) with human fetal lung fibroblast cells (MRC5), we found that the DFNA5 protein expression in hepatocellular carcinoma cells was significantly lower than that in normal cells. The transfection of DFNA5 gene into HepG2 cells could increase DFNA5 protein expression, which subsequently led to inhibition of cell proliferation. Underlying mechanism study revealed that decreased proliferation was due to increased apoptosis and cell cycle arrest. In view of the important role of DFNA5 gene in carcinogenesis, these findings are expected to provide new understanding on development and treatment of human hepatocellular carcinoma.

Keywords DFNA5 · Hepatocellular carcinoma · Apoptosis · Cell cycle arrest

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Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of hepatocytes which accounts for 80 % of all primary liver cancers and ranks globally as the fourth leading cause of cancer-related death [1]. Epidemiological studies have provided overwhelming evidence that cirrhosis is the strongest and the most common risk factor for HCC, particularly cirrhosis related to hepatitis C virus and hepatitis B virus infections [2, 3]. Epigenetic changes such as DNA methylation and histone modification have recently been shown to play important roles in tumorigenesis [4]. Genomic hypomethylation increases chromosome instability [5] while localized hypermethylation decreases tumor suppressor gene expression [6], thus increasing the risk of HCC development. A number of tumor suppressor genes, including *p16^{INK4A}* [7], suppressor of cytokine signaling-1 [8], adenomatous polyposis coli [9], glutathione-S-transferase pi class gene [10] and E-cadherin [11], are silenced by DNA methylation in HCC, and this process often starts at the preneoplastic stage [12]. Moreover, genes involved in cell cycle checkpoints [13], apoptosis [14], and gene transcription [15] all have been shown to play important roles in HCC.

The human deafness, autosomal dominant 5 gene (DFNA5) was first discovered in Dutch family with autosomal dominant hearing loss [16]. Since its identification, several published papers have shown that DFNA5 is a tumor suppressor gene and is epigenetically inactivated in several types of cancer, including gastric [17], colorectal [18] and breast cancer [19]. Forced expression of DFNA5 in colorectal carcinoma cell lines can decrease cell growth and colony-forming ability [18]. In breast carcinoma, the methylation status of DFNA5 is correlated with lymph node metastasis [19]. However, whether DFNA5 gene is involved in the carcinogenesis of HCC has never been explored before. In this study,

we aimed to investigate the DFNA5 expression in HCC and explore its underlying molecular mechanism.

Materials and methods

Cell lines

Human cell line HepG2 (hepatoblastoma) and human fetal lung fibroblast cell (MRC5 cell line) were obtained from the American Tissue Culture Collection (ATCC, Rockville, USA). HepG2 and MRC5 were respectively cultured in PRMI1640 medium and high glucose dulbecco's modified eagle's medium (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum, 100 µ/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator with 5 % CO₂.

Transfection experiments

Eukaryotic cell expression plasmid pEGFP-N1-DFNA5 was purchased from Yingrui Biotech (Changsha, China). The HepG2 cells at logarithmic phase were seeded in 6-well plates at a density of 2×10^5 cells/well and grown for 24 h. Cells were transfected with pEGFP-N1-DFNA5 (with 1 µg plasmid) using Lipofectamine 2000 reagent according to the protocols provided by the manufacturer (Invitrogen, Carlsbad, CA).

Western blotting

At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry (BD Biosciences, Heidelberg, Germany). Both of the HepG2 and MRC5 cells were washed three times and lysed in ice-cold lysis buffer for 30 min, followed by centrifuging at 12,000 rpm for 10 min at 4 °C. The sediment was discarded and the protein concentration was measured by bicinchoninic acid method. Equivalent amounts of denatured cellular lysates (20 µg) from each cell line were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene membrane using electro-transfer system. The membranes were blocked with 5 % skimmed milk solution for 2 h at room temperature. Next, the membranes were incubated with primary antibodies (1:1,000 dilution), including anti-DFNA5 antibody (sigma), anti-FAS antibody, anti-FasL antibody, and anti-cleaved caspase 8 antibody (Cell Signaling, USA) at 4 °C overnight, followed by a secondary antibody (Anti-rabbit IgG, HRP-linked Antibody, 1:5,000 dilution, Cell Signaling, USA) for 1 h at room temperature. Immunoblots were developed using the enhanced chemiluminescence western blotting kit (Santa Cruz Biotechnology). β-actin protein level was measured as reference.

Tetrazolium-based colorimetric assay (MTT test)

At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted using flow cytometry (BD Biosciences, Heidelberg, Germany) and prepared into single cell suspension. The cells were seeded in 96-well plate in triplicate at a density of 2×10^3 /well and grown for two days, followed by the addition of 50 µl MTT (1 mg/ml) in each well. After a 4 h culture, the supernatant was discarded, 150 µl dimethyl sulfoxide was added to each well, and the plate was shaken for 10 min to dissolve the MTT. Absorbance of each well was measured by 96-well microplate reader at 570 nm wavelength and recorded.

Measurements of cell apoptosis

At 24 or 48 h after transfection, the HepG2 cells were collected by centrifugation at 1,000 rpm for 5 min and washed twice by PBS. The precipitate was resuspended in 500 µl binding buffer and stained with Annexin V-FITC/propidium iodide (PI) (Kaiji Biotechnology, Nanjing, China) for 5–15 min in the dark at room temperature. Apoptotic cells were analyzed by flow cytometry within 1 h and apoptosis index (AI) was calculated using the following formula: $AI = \text{apoptosis cells}/\text{total cells} \times 100 \%$.

Measurement of cell cycle

At 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry and centrifuged at 1,000 rpm for 5 min, followed by washing twice with PBS. After fixation in pre-cooled 70 % ethanol at 4 °C for 2 h, the fixation buffer was discarded, the cells were resuspended in PBS and passed through 400 mesh. After that, the cells were centrifuged at 1,000 rpm for 5 min and the PBS was discarded. The cells were then stained with PI for 30 min in the dark at 4 °C. The cell cycle was analyzed by flow cytometry.

Statistical analysis

All statistical analyses were performed using *t* test. *P*-values below 0.05 were considered statistically significant. Statistics were calculated using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

DFNA5 expression in HepG2 and MRC5 cell lines

Western blotting analysis using anti-DFNA5 antibody was done to evaluate the DFNA5 expression in HepG2 and

MRC5 cells. We found that the DFNA5 expression level in MRC5 cells was significantly higher than that in HepG2 cells. The quantitative analysis of the expression level through scanning the image density revealed that the DFNA5 expression level in HepG2 cells was only equal to around 20 % of that in MRC5 cells (Fig. 1).

The expression level increases after transfection of DFNA5 into HepG2 cells

Since the DFNA5 expression in HepG2 cell cline had been demonstrated to be much lower than that in normal cells, whether transfection of DFNA5 into HepG2 cells could increase the DFNA5 expression became a subject of interesting investigation. The transfection experiment was performed on HepG2 cells using lipofectamine 2000. At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry and the DFNA5 expression level was detected by western blotting. The results indicated that the DFNA5 expression level in DFNA5 transfected HepG2 cells was significantly increased at 24 h after transfection and 48 h after transfection ($p < 0.05$, Fig. 2).

Effect of transfection of DFNA5 into HepG2 cells on cell proliferation and apoptosis

To investigate how the transfection of DFNA5 into HepG2 cells affects the cell proliferation, we performed the

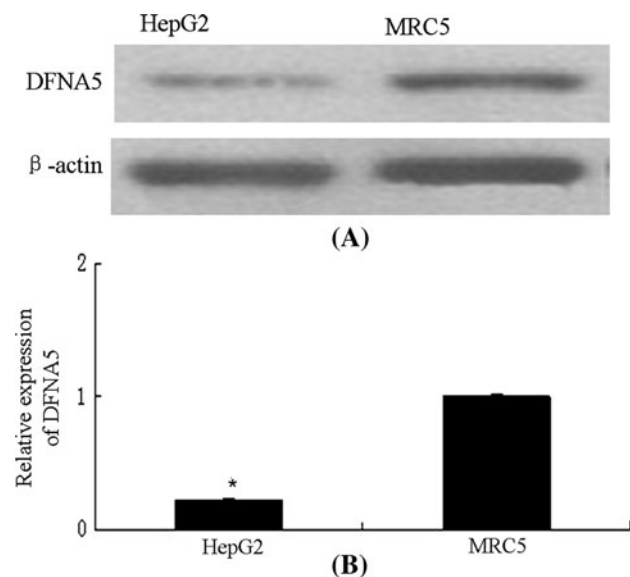


Fig. 1 DFNA5 protein expression in HepG2 cells and MRC5 cells. HepG2 cells and MRC5 cells at logarithmic phase were collected and subjected to western blotting analysis for DFNA5 protein expression using anti-DFNA5 antibody. The DFNA5 expression in HepG2 cells was significantly lower than that in MRC5 cells. β -actin expression served as internal control. **a** electrophoretogram; **b** quantitative analysis. *compared with MRC5 cells, $P < 0.05$

transfection experiment as mentioned above and compared the viability of DFNA5 transfected HepG2 with the viability of non-transfected HepG2 cells through MTT assay at 24 or 48 h after incubation. As expected, the viability of non-transfected HepG2 cells didn't show much difference between 24 and 48 h (90–88 %), while DFNA5 transfected HepG2 cells showed a decreasing viability after transfection in a time-dependent manner. The viability of transfected cells decreased from 86 to 60 %, which was significantly lower than that of non-transfected cells ($p < 0.05$, Fig. 3).

Simultaneously, apoptosis experiments was also conducted on HepG2 cells at 48 h after transfection, and the results showed that the apoptotic rate in the DFNA5 transfected HepG2 cells was around 30 %, which was significantly higher than that in non-transfected cells with 5 % apoptotic rate (Fig. 4).

Changes in apoptosis-related proteins after transfection of DFNA5 into HepG2 cells

We have demonstrated that the transfection of DFNA5 into HepG2 cells could significantly increase apoptosis rate, while we are not clear how the apoptosis related proteins mediate the apoptosis process. Here, the expressions of Fas, Fas ligand (FasL), and caspase 8, were analyzed by western blotting in the DFNA5 transfected HepG2 cells. We found that the expressions of Fas and caspase 8 were significantly increased after transfection in a time-dependent manner, while the expression of FasL was reduced after transfection in a time-dependent manner (Fig. 5).

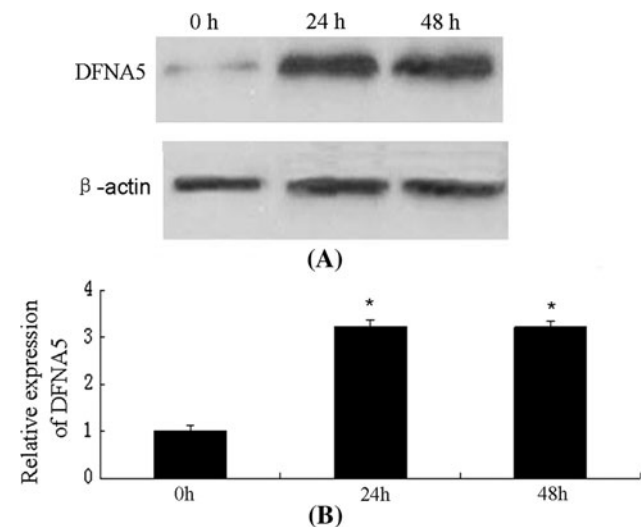


Fig. 2 DFNA5 protein expression in DFNA5 transfected HepG2 cells. At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry. HepG2 cells were collected and subjected to western blotting analysis for DFNA5 protein expression using anti-DFNA5 antibody. The DFNA5 expression level in HepG2 cells significantly increased after transfection. **a** electrophoretogram; **b** quantitative analysis. *compared with 0 h, $P < 0.05$

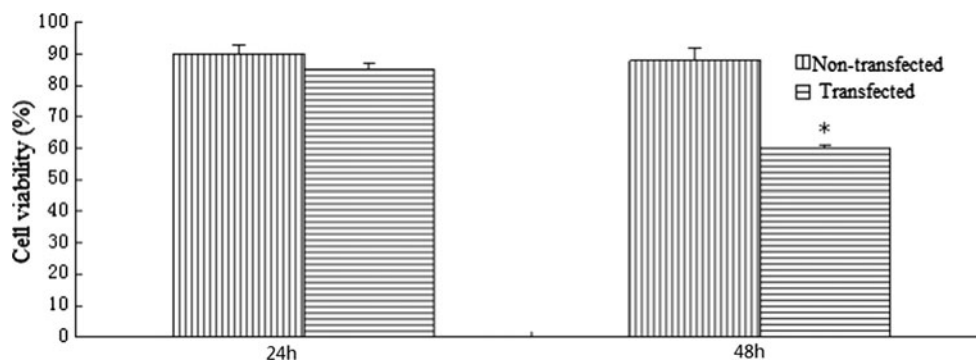


Fig. 3 Cell viability decreased 48 h after transfection of DFNA5 gene into HepG2 cells. At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry. MTT assay was performed to analyze cell viability. The viability of

non-transfected HepG2 cells didn't show much difference between 24 h and 48 h (90 %–88 %), while the viability of DFNA5 transfected cells decreased from 86 % to 60 %, which was significantly lower than that of non-transfected cells (* $p < 0.05$)

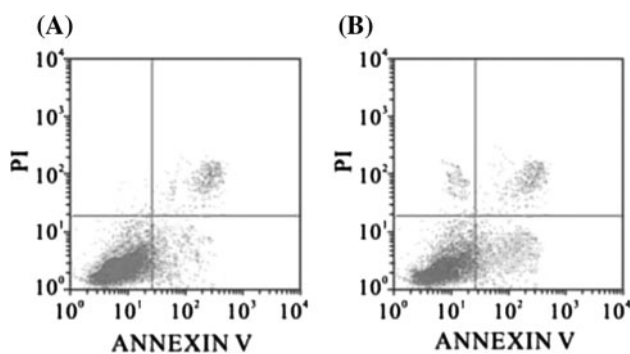


Fig. 4 Effect of transfection of DFNA5 gene into HepG2 cells on cell apoptosis. At 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry and stained with Annexin V-FITC/propidium iodide to detect apoptosis. The cell apoptotic rate in the DFNA5 transfected HepG2 cells (b) was strikingly higher than in non-transfected cells (a)

Effect of transfection of DFNA5 into HepG2 cells on cell cycle

To determine the nature of decreased cell proliferation in DFNA5 transfected HepG2 cells, we performed cell cycle analysis through flow cytometry. As shown in Fig. 6, transfection of DFNA5 into HepG2 cells enhanced G2/M arrest with 31.3 % of population arrested in the G2/M, compared to 6.2 % of population arrested in the G2/M in the non-transfected HepG2 cells. So the increased G2/M arrest in transfected HepG2 cells might account for the decreased cell proliferation.

Discussion

Aberrant methylation of DFNA5 has been recently detected in gastric, colorectal and breast cancer [17–20], which leads to the down-regulation of DFNA5 compared with matched normal tissues or cells. Our study was the first to

investigate the role of DFNA5 in HCC. As expected, the DFNA5 expression level was also significantly lower in HCC cell than that in normal human MRC5 cell. In order to explore the underlying molecular mechanism of DFNA5 in HCC cell, we transfected DFNA5 into HepG2 cell and found that the DFNA5 expression level was significantly increased after transfection. Subsequent studies indicated that DFNA5 overexpression could inhibit HCC cell proliferation through induction of cell apoptosis and G2/M arrest. These results are in accordance with a recent study described by de Beeck et al. [21]. They report that DFNA5 is composed of two domains: the first region induces apoptosis when transfected in HEK293T cells and the second region masks and probably regulates this apoptosis inducing capability [21].

Apoptosis is the most common forms of eukaryotic cell death, acting as a physiological suicide mechanism to preserve homeostasis, and occurs naturally during tissue turnover [22]. Any decrease in apoptotic rate may disturb this homeostasis and result in cancer formation and progression [23]. Fas is a member of a subgroup of the tumor necrosis factor receptor superfamily that contain an intracellular death domain and can initiate apoptosis signaling [24]. It has been reported that HCC tissues express Fas less frequently and more weakly than noncancerous tissues [25]. FasL is normally expressed by T lymphocytes when they are activated [26]. FasL has been reported to be high expressed in many human tumor cells and promotes apoptosis of T Lymphocytes [27], ultimately leading to immune evasion of tumor cells [28]. Low expression of Fas and high expression of FasL cause tumor metastasis [29]. Caspase-8 is an aspartate-specific cysteine protease and present in healthy cells as an inactive proenzyme. Through complex proximity-driven conformational changes, pro-caspase-8 molecules within the death-inducing signaling complex (DISC) gain full enzymatic activity [30]. This active caspase-8 can leave the DISC to target its specific

Fig. 5 Effect of transfection of DFNA5 gene into HepG2 cells on Fas, Fas L and caspase 8 protein expression. At 24 or 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry and subjected to western blotting analysis for DFNA5 protein expression using anti-Fas antibody, anti-FasL antibody, and anti-cleaved caspase 8 antibody. The expression of Fas and caspase 8 significantly increased after transfection in a time-dependent manner, while the expression of FasL decreased after transfection in a time-dependent manner. **a** electrophoretogram; **b** quantitative analysis. *compared with 0 h, $P < 0.05$

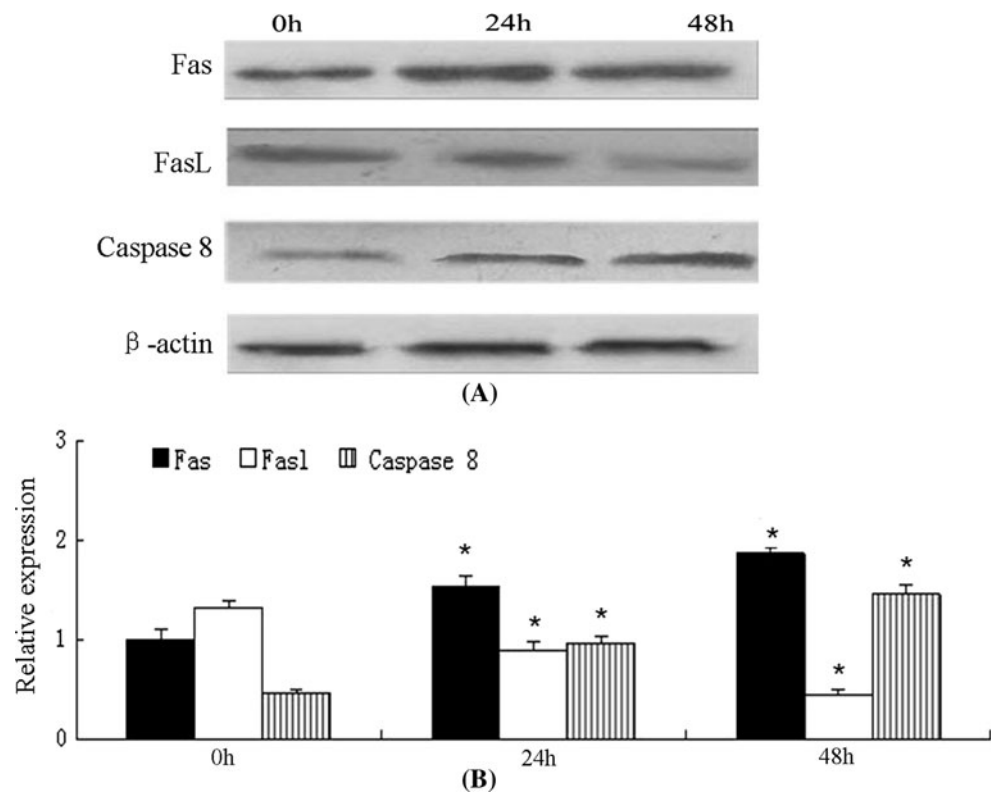
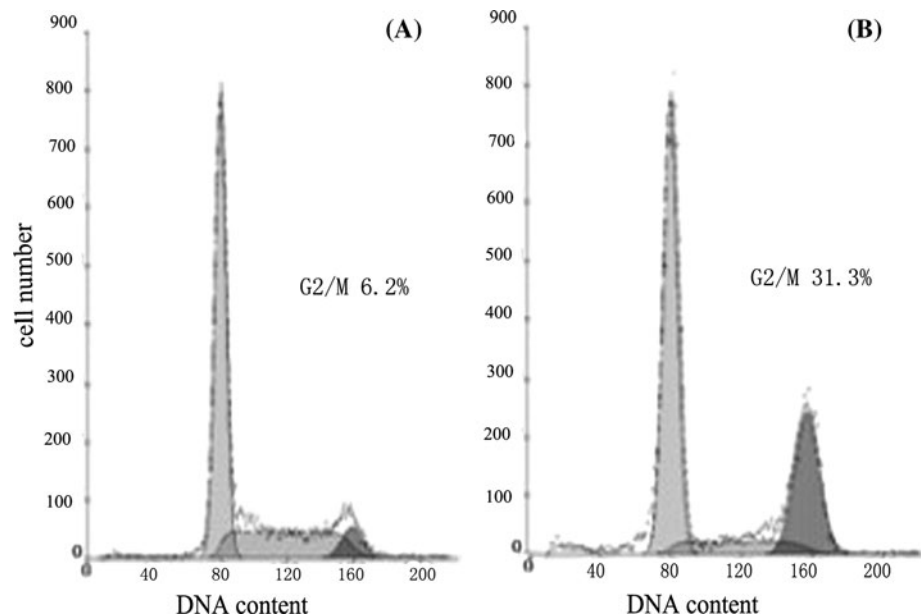


Fig. 6 Effect of transfection of DFNA5 gene into HepG2 cells on cell cycle. At 48 h after transfection, the fluorescence-labeled HepG2 cells were sorted by flow cytometry and subjected to cell cycle analysis. Transfection of DFNA5 into HepG2 cells enhanced G2/M arrest with 31.3 % of population arrested in the G2/M (**b**), compared to 6.2 % of population arrested in the G2/M in the non-transfected HepG2 cells (**a**)



substrates within the cytosol, such as effector caspases (caspase-3), which is critical for Fas-mediated apoptosis [31]. A characteristic feature of human cancer is their inability to undergo apoptosis in response to apoptotic stimuli, and the inactivation of caspase-8 by genetic or epigenetic mechanisms is one mechanism to evade apoptosis [32, 33]. In this study, we transfected DFNA5 into

HepG2 cells and increased the Fas and caspase-8 protein expression, while decreased FasL protein expression, which finally contribute to the induction of apoptosis and prevention of tumor counterattack.

It is known that cell growth is controlled by cell cycle progression, a highly regulated process. The standard cell cycle is divided into four non-overlapping phases, namely

G1, S, G2 and M phases in sequence. Each phase has checkpoints that cause cell cycle arrest and activation of repair mechanisms. Unlike normal cells that rely on the G1 checkpoint to protect against DNA damage, cancer cells are more dependent on G2 checkpoint for DNA damage repair [34]. Our study demonstrated that transfection of DFNA5 into HepG2 cells induced G2/M arrest, which leads to inhibition of cell proliferation [35]. While the underlying mechanism on how the transfection of DFNA5 into HepG2 cells to induce G2/M arrest requires further investigations.

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