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Lenti-ShRNA-Mediated *FAM54A* Knockdown Suppresses Proliferation and Induces Apoptosis in Human Burkitt Lymphoma Cells

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Abstract

Objectives: Burkitt lymphoma is a kind of non-Hodgkin B-cell-derived malignancy originating from germinal centre B cells. *FAM54A* has been proven to be involved in various physiological and pathological processes of cancers, but the biological function of *FAM54A* in Burkitt lymphoma remains unclear. Thus, the aim of our research was to elucidate the roles of *FAM54A* in the proliferation, apoptosis and cell cycle of Burkitt lymphoma.

Methods: A Burkitt lymphoma cell line (Namalwa) was chosen to perform the following experiments. *FAM54A*-shRNA and negative control-shRNA lentiviruses that were synthesized by Qiagen were used to transfect targeted cells to knockdown *FAM54A* or as a negative control. Then, cell proliferation, cell cycle and cell apoptosis were detected by using MTS assay, propidium iodide staining and Annexin V-APC staining, respectively.

Results: Our results showed that high expression of *FAM54A* protein was found in the Namalwa cell line. Furthermore, MTS analysis revealed that knockdown of *FAM54A* obviously inhibited cell proliferation in Namalwa cells. Moreover, cell cycle analysis showed that knockdown of *FAM54A* induced Namalwa cell apoptosis and arrested the cell cycle in G2/M phase.

Conclusion: These findings suggest that *FAM54A* is essential for Namalwa cell proliferation and may be a potential therapeutic target for the treatment of Burkitt lymphoma.

Keywords: Burkitt lymphoma; *FAM54A*; shRNA; Proliferation; Apoptosis

Introduction

Burkitt lymphoma (BL) is a kind of non-Hodgkin B-cell-derived malignancy originating from germinal centre (GC) B cells [1-3] with highly invasive and proliferative ability. Clinically, it is divided into three subtypes: endemic, sporadic and immunodeficiency-related [4]. Many studies have reported that the occurrence of the disease is related to Epstein-Barr (EB) virus infection, human immunodeficiency virus (HIV) infection and chromosomal translocation. Among the factors that cause BL, the presence of *MYC* translocation caused by the fusion of the *MYC* gene and immunoglobulin gene is a key event, resulting in abnormal expression of the gene, so further promoting the proliferation of BL cells [5,6].

FAM54A is a highly conserved gene family that controls embryonic development and cell differentiation and is associated with cell metabolism [7]. Aberrant regulation of the gene is associated with the occurrence of malignant tumours [8]. However, the effects of *FAM54A* on BL are still undetermined, so the aim of this research was to explore the biological function and potential mechanism of *FAM54A* in human BL. To our knowledge, this study is the first to deeply characterize the role of *FAM54A* in BL pathogenesis and confirm the markedly high expression level of *FAM54A* in BL tissues by western blotting (WB). Knockdown of *FAM54A* with the lenti-shRNA system [9] significantly inhibited cell proliferation. Furthermore, we found that knockdown of *FAM54A* could induce cell apoptosis and cause cell cycle arrest in G2/M phases. These outcomes indicate that *FAM54A* may play an essential role in cell proliferation and progression of BL.

Methods

Cell lines and cell culture

A human BL cell line (Namalwa) and human renal epithelial cells (293T) were purchased from Biological Science (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with penicillin (100 U/ml)-streptomycin (100 μg/ml) and 10% foetal bovine serum (FBS) in 5% CO_2 at 37°C.

Western blot analysis

The cells were harvested and lysed using ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS, 1 mM EDTA, 1% NP-40) supplemented with 1 mM protein inhibitor and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 minutes on ice. The pyrolysis products were centrifuged at 10,000 × g at 4°C for 10 minutes, and the supernatant was collected. The protein concentration was detected by a bicinchoninic acid (BCA) detection kit (HyClone-

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Pierce, USA). Total proteins were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to a previously reported method [10] and then transferred to polyvinylidene fluoride (PVDF) membranes. Mouse anti-GAPDH and *FAM54A* antibodies were used to detect targeted proteins (Santa Cruz Biotechnology, USA). Finally, the proteins were visualized using enhanced chemiluminescence (ECL) regent (Piscataway, NJ, USA).

Recombinant lentiviral vector production

According to the principle of RNA interference, multiple RNA interference target sequences were designed based on the *FAM54A* sequence (Accession no. NM_001099286). After evaluation and determination by design software, the target sequence 5'-AATTGTGGAAATGCAGGAA-3' in the full-length sequence of *FAM54A* was selected as the most suitable interference target by GeneChem company (Shanghai, China). After detecting the knockdown efficiency, stem-loop oligonucleotides were synthesized and inserted into the lentivirus-based GV115-GFP vectors with AgeI/EcoRI sites (GeneChem, Shanghai, China). Lentivirus particles were prepared as described in a previous report [11].

Cells transfection

For lentivirus transfection, the target cells were cultured in a 6-well microplate, and then *FAM54A*-shRNA lentivirus and negative control (NC)-shRNA lentivirus were added according to the appropriate multiplicity of infection (MOI). After 72 hours of transfection, the cells were observed by a fluorescence microscope (NIB900, Olympus, Japan). After 120 hours of transfection, the cells were collected to detect the knockdown efficiency by WB.

Cell proliferation assay

Cell growth was measured by the MTS (3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, USA) [12]. In brief, cells transfected with *FAM54A*-shRNA or NC-shRNA lentivirus were placed into 96-well plates at a density of 2×10^3 cells/well, One, 2, 3, 4 and 5 days after incubation, the medium was replaced with 100 µl of fresh serum-free RPMI-1640 medium with 20 µl CellTiter 96® AQueous One Solution reagent in each well. After incubation for 4 hours at 37°C, the 96-well plate was placed on the oscillator and vibrated for 2-5 minutes. Then, the optical density (OD) value in each well was read by detecting the absorbance at 490 nm using a microplate spectrophotometer.

Cell cycle analysis

Cell cycle distribution was analysed using Propidium Iodide (PI) following the manufacturer's instructions as previously described [13]. Briefly, Namalwa cells that were transfected with *FAM54A*-shRNA or NC-shRNA lentivirus were collected, washed twice with ice-cold phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol at 4°C for 1 hour, and stained with PI (GeneChem, Shanghai, China). Finally, cell cycle analysis was performed by flow cytometry. Each experiment was performed in triplicate.

Cell apoptosis detection

The apoptosis rate of cells was determined by Annexin V-APC staining according to the kit protocols (Santa Cruz, USA). Namalwa cells (6×10^5 cells/well) were plated onto 6-well plates after infection with *FAM54A*-shRNA or NC-shRNA lentivirus. After 5 days of culture, the cells were washed, collected, and resuspended in PBS buffer, and the cell concentration was adjusted to 1×10^6/ml. Then, 5 µl Annexin V (0.1 µg/µl) was added to 100 µl cell suspension and incubated at room temperature in the dark for 30 minutes on ice. The cells exposed to different treatments were subjected to fluorescent-activated cell sorting (FACS) analysis. All experiments were performed in triplicate.

Statistical analysis

The statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test was used for raw data analysis. For all statistical tests, p-values less than or equal to 0.05 were considered to be statistically significant.

Results

FAM54A protein was highly expressed in a human BL cell line

The expression level of *FAM54A* protein in a human BL cell line (Namalwa) and human renal epithelial cells (293T) was examined by WB. As depicted in Figure 1, the expression of *FAM54A* at the protein level was significantly higher in Namalwa cells.

Determination of knockdown efficiency by WB

Knockdown efficiency was determined by detecting *FAM54A* protein expression levels in 293T cells infected with *FAM54A*-shRNA or NC-shRNA lentivirus by WB. Compared to that in NC-shRNA lentivirus-transfected cells, the expression level of the protein in 293T cells transfected with *FAM54A*-shRNA lentivirus was greatly decreased, which indicated efficient knockdown of the target gene (Figure 2).

Lentivirus-mediated knockdown of *FAM54A* in the human BL cell line Namalwa

To explore the function of *FAM54A*, we knocked down *FAM54A* in the Namalwa cell line. After 3 days of transfection, fluorescence imaging technology was applied to determine the transfection efficiency in cells. The results showed that the percentage of infected cells was >50% for both the *FAM54A*-shRNA and NC-shRNA lentiviruses (Figure 3). *FAM54A* protein expression levels were assessed by WB at day 5 post infection in cells infected with either the *FAM54A*-shRNA or NC-shRNA lentivirus. The results in the Namalwa cell line were similar to those in 293T cells. *FAM54A*-shRNA cultures had obviously lower protein expression compared to NC-shRNA cultures (Figure 4).

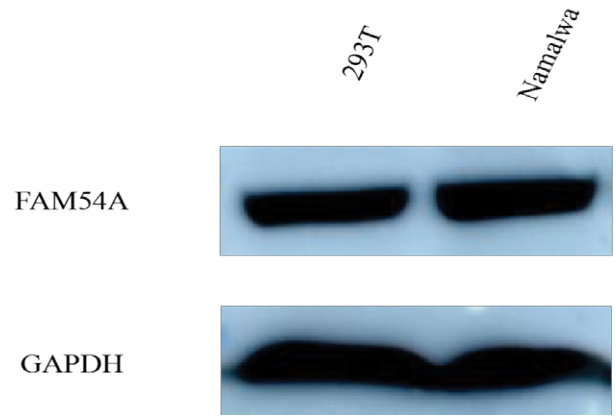

Figure 1: The expression level of FAM54A protein in 293T cells and Namalwa cells. WB was used to analyse FAM54A protein expression levels in both cell lines. GAPDH was used as a loading control.

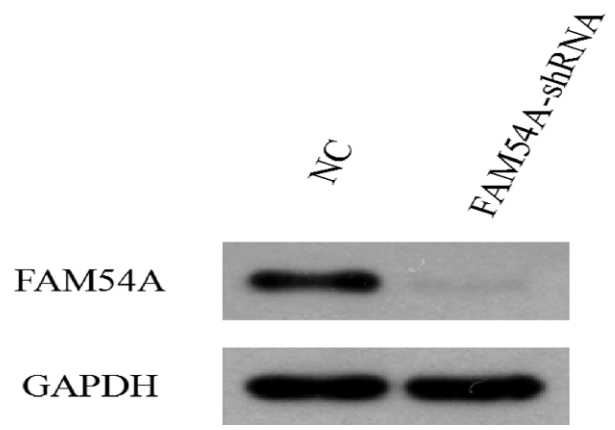

Figure 2: Knockdown of FAM54A protein expression in 293T cells. WB was used to detect the expression of FAM54A protein in *FAM54A*-shRNA or NC-shRNA lentivirus-transfected 293T cells. GAPDH was used as a loading control.

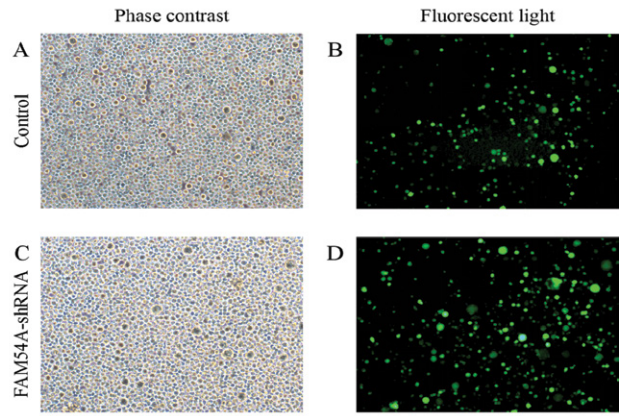

Figure 3: Evaluating the infection efficiency of *FAM54A*-shRNA and NC-shRNA lentiviruses in Namalwa cells. Namalwa cells were transfected with *FAM54A*-shRNA or NC-shRNA lentivirus and observed by fluorescence microscopy on the third day after infection. Approximately 50% of the cells expressed green fluorescent protein (GFP). Magnification, 100x. Fluorescent and white light images of the cells are shown.

Knockdown of *FAM54A* inhibits human BL cell proliferation *in vitro*

To study the effect of *FAM54A* on cell growth, *FAM54A*-shRNA lentivirus was transfected into Namalwa cells to silence the gene. Then, cells expressing *FAM54A*-shRNA or NC-shRNA lentivirus were plated into 96-well plates and analysed by MTS for 5 days. The effect of *FAM54A* downregulation on the proliferation of Namalwa cells was detected by MTS assay. Compared to that of NC cells, the absorbance of *FAM54A*-shRNA-transfected Namalwa cells at 490 nm was significantly lower (Figure 5), indicating that the proliferation rate of *FAM54A*-shRNA lentivirus-transfected cells was lower than that of NC cells ($p<0.05$). Thus, the experimental data showed that the downregulation of *FAM54A* noticeably suppressed cell proliferation.

Knockdown of *FAM54A* in Namalwa cells increases cell apoptosis

To determine whether the expression of *FAM54A* protein affects the apoptosis of BL cells, we knocked out *FAM54A* in Namalwa cells. Annexin V staining and flow cytometry were applied to detect the apoptosis rate (Figure 6A). As shown in Figure 6B, detailed data demonstrated that the apoptosis rate of *FAM54A*-shRNA lentivirus-transfected cells was significantly higher than that of NC cells (NC 2.97 ± 0.02% vs. *FAM54A*-shRNA 4.93 ± 0.07%, $p<0.001$). These results implied that the lack of *FAM54A* protein expression is a critical factor in cell apoptosis in Namalwa cells.

Knockdown of *FAM54A* causes cell cycle arrest in human BL cells in vitro

To determine the detailed mechanism by which *FAM54A* knockdown led to cell proliferation inhibition, the cell cycle distribution of *FAM54A*-shRNA or NC-shRNA lentivirus-transfected cells was analysed by flow cytometry (Figure 7A). The cell cycle distribution of NC-shRNA lentivirus-transfected cells was as follows: G0/G1 phase, 39.91 ± 0.75%; S phase, 36.19 ± 0.99%; and G2/M phase, 23.91 ± 0.28%, and the cell cycle distribution of the *FAM54A*-shRNA lentivirus-transfected group was as follows: G0/G1 phase, 36.46 ± 0.36%; S phase, 33.16 ± 0.69%; and G2/M phase, 30.38 ± 0.59%. As shown in Figure 7B, compared with that in the NC group, the cell proportion of the *FAM54A*-shRNA lentivirus-transfected group showed a significant decrease in cell in G0/G1 phase ($p<0.05$) but showed a significant increase in G2/M phase cells ($p<0.05$). In summary, the data indicate that *FAM54A* regulates cell proliferation and that downregulation of *FAM54A* can render cell cycle arrest in G2/M phase.

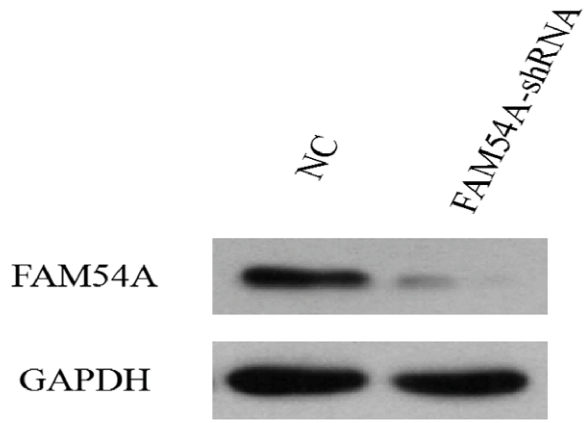

Figure 4: Knockdown of FAM54A protein expression in the cell line Namalwa. FAM54A protein expression was analysed by WB in *FAM54A*-shRNA and NC-shRNA lentivirus-transfected Namalwa cells. GAPDH was used as a loading control.

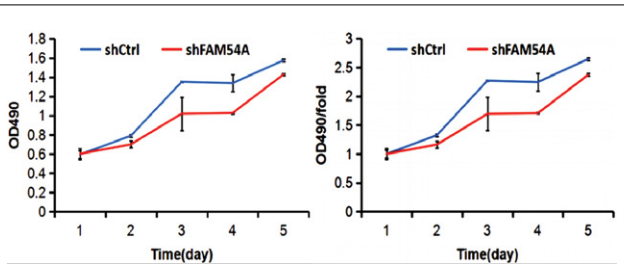

Figure 5: Effect of *FAM54A* knockdown on Namalwa cell growth. Cells were plated into 96-well plates and infected with *FAM54A*-shRNA or NC-shRNA lentivirus and cell growth was evaluated every day for 5 days (NC vs. *FAM54A*-shRNA, $p<0.05$).

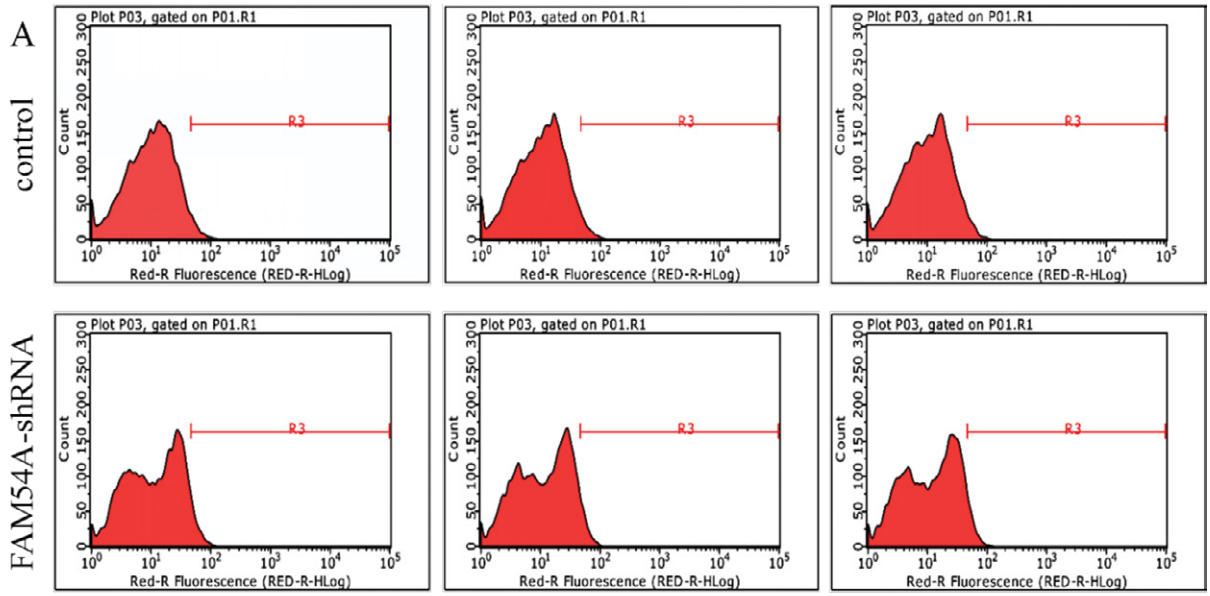

Figure 6: *FAM54A* knockdown increases apoptosis in Namalwa cells. (A) Cell apoptosis was determined by Annexin V staining and flow cytometry. The experiment was repeated in triplicate. (B) The quantitative data are shown. Note that compared with that in the NC group, apoptosis in the *FAM54A*-shRNA lentivirus-transfected group was significantly increased (***p<0.001).

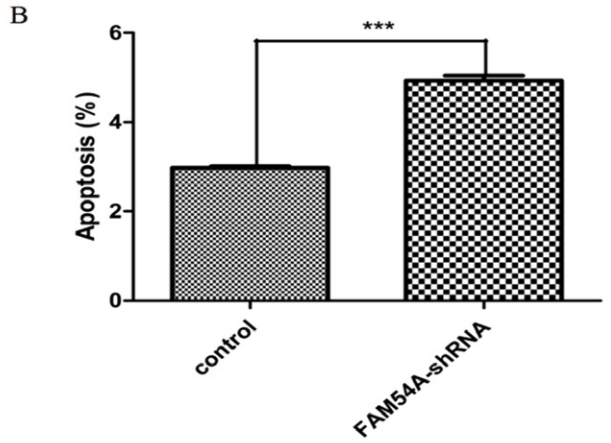

Figure 7: Knockdown of *FAM54A* results in cell cycle arrest. (A) Cell cycle distribution was analysed by flow cytometry. Each experiment was repeated in triplicate. (B) Compared with that in the NC group, the cell proportion of the *FAM54A*-shRNA lentivirus-transfected group showed a significant decrease in G0/G1 phase cells (*p<0.05); however, it showed a significant increase in G2/M phase cells (*p<0.05).

Discussion

BL is one of the most common causes of cancer-related death worldwide because it is difficult to detect in the early stage and the disease has progressed to late stage by the time it is diagnosed accurately, thus missing the optimal treatment window [14]. Therefore, early diagnosis and targeted therapy are key to a better prognosis of patients with BL. Additionally, *FAM54A*, also known as mitochondrial fission regulator 2 (*MTFR2*) or DUF729 domain-containing protein 1 (*DUFD1*), plays an important role in regulating the structure and function of mitochondria. *FAM54A* protein is also related to membrane-enriched subcellular fractions, including mitochondria. It has been proven that inhibition of Dufd1 expression in testicular germ cell lines can seriously impair oxygen consumption, suggesting that the gene is necessary for mitochondrial respiration [15]. Therefore, the downregulation of *FAM54A* expression in tumour cells will be beneficial to inhibit the consumption and utilization of oxygen and the synthesis of adenosine triphosphate (ATP) by tumour cells, further leading to the death of tumour cells due to hypoxia. This mechanism may provide a novel therapeutic strategy against tumours. At the same time, this protein is also an important nuclear protein and is considered a key molecular target in cancers, infectious diseases and other diseases. In 2009, Persson et al. [8] reported that *FAM54A* is positively correlated with inflammation and tumour progression. To determine whether this critical molecular marker may provide a novel strategy and insight into the early diagnosis and targeted treatment of BL, we explored the role of *FAM54A* in tumourigenesis and progression of BL.

First, we measured the expression level of *FAM54A* protein in a human BL cell line (Namalwa), and the results showed that the expression level of *FAM54A* protein was very high in the cancer cell line. Therefore, we speculated that it probably played a role in promoting cell growth in BL and acted as an oncogene.

Next, we transfected Namalwa cells using *FAM54A*-shRNA lentivirus, which significantly downregulated the endogenous expression level of *FAM54A* protein. Then, cell proliferation viability was detected by MTS assay. Our results showed that knockdown of *FAM54A* obviously led to an obvious inhibition of cell proliferation in BL. Furthermore, the results of cell cycle and apoptosis analyses revealed that downregulation of *FAM54A* negatively regulated BL progression by inducing apoptosis and blocking the cell cycle in Namalwa cells.

Conclusion

Although detailed reference literature on *FAM54A* is very limited because few studies have been carried out to explore it carefully, our present study demonstrates that *FAM54A* protein is highly expressed

in a human BL cell line. Knockdown of *FAM54A* suppresses cell proliferation by causing cell cycle arrest and inducing cell apoptosis. These findings not only elucidate the effects of *FAM54A* on BL but also provide a novel strategy and insight into improving the prognosis of BL and promoting the development of early diagnosis and targeted therapy for human BL. However, the detailed molecular mechanism involved in the effects of *FAM54A* on BL still needs further investigation.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding

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Availability of Data and Materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' Contributions

YG, WXH, YB and XCY analyzed and interpreted the all experimental data. ZRY, CMX, LRF and ZHD performed the relevant experiments, respectively. YG and JZS were major contributors in writing the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

I would like to declare on behalf of my co-authors that this research is not involved in informed consent, because it does not involve in human participants and/or animals research.

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