miR-142-5p regulates CD4+ T cells in human non-small cell lung cancer through PD-L1 expression via the PTEN pathway

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Abstract. The present study aimed to evaluate the function of microRNA (miR)-142-5p on cancer immunity to induce apoptosis in human non-small cell lung cancer (NSCLC) and its mechanism. miR-142-5p expression was upregulated, and CD4+ T cell levels were reduced in patients with NSCLC. Overexpression of miR-142-5p expression inhibited the cancer effects of CD4+ T cells on NSCLC cell lines, and downregulation of miR-142-5p increased the cancer effects of CD4+ T cells on NSCLC cell lines, compared with the control group. In addition, we found that overexpression of miR-142-5p suppressed PTEN protein expression and induced PI3K, p-Akt and PD-L1 protein expression in an in vitro model of NSCLC. Downregulation of miR-142-5p induced PTEN and PD-L1 protein expression and suppressed PI3K and p-Akt and protein expression in an in vitro model of NSCLC. The suppression of PD-L1 reduced the cancer effects of CD4+ T cells on NSCLC cell lines following miR-142-5p downregulation. The inhibition of PTEN also reduced the cancer effects of CD4+ T cells on NSCLC cell lines following miR-142-5p downregulation. Therefore, our study demonstrated that miR-142-5p regulated CD4+ T cells in human NSCLC through PD-L1 expression via the PTEN pathway.

Introduction

Lung cancer is a malignant tumor of the highest morbidity and mortality worldwide. It is also a major disease threatening human life and health. The global cancer burden is increasingly aggravated, as is suggested in the World Cancer Report released by the World Health Organization (WHO) International Agency for Research on Cancer in 2014. Lung cancer was ranked at the top among new common cancers in 2012 (1). There were approximately 1.8 million lung cancer cases, accounting for 13% of the total common cancer cases (1). In addition, lung cancer was also ranked at the top among the common causes of cancer-related deaths. There were approximately 1.6 million death cases, accounting for 19.4% of the total cases. Of these, Chinese cases accounted for over 1/3 (2).

Primary lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for 85% of all lung cancer cases (2). It mainly includes 3 types, namely, lung adenocarcinoma, lung squamous cell carcinoma and large cell carcinoma (2). To date, surgical treatment is the most effective method for NSCLC. In comparison, SCLC is generally more sensitive to chemotherapy and radiotherapy (2).

Circulating miRNAs can serve as disease markers. Such a discovery has aroused extensive interest from numerous scientists in recent years (1). Particularly, miRNAs as tumor diagnostic markers have attracted wide attention (1). A miRNA is an endogenous non-coding small molecular RNA. It is highly conserved, with a length of approximately 18-23 bases. Notably, detecting changes in substances contained in the blood can indicate the health condition of patients. This is a minimally invasive method that can alleviate patient suffering (3). Therefore, detecting blood miRNA content is a good screening method or auxiliary diagnosis (3).

CD4+ T cells can secrete cytokines. Thus, they can activate monocytes, macrophages and NK cells. In addition, they can induce immune response in monocytes, macrophages and NK cells. Therefore, they can exert an antitumor function (4). CD4+ T cells can also secrete IL-2 as a second signal. Subsequently, it can activate CD8+ T cells and participate in the immune response against tumors (4,5). CD8+ T cells are inhibitory T cells that exert an immunosuppressive function. The body can only exert normal immune function under the balanced status of the two. Research has found that lung cancer patients are under immunosuppressive status (5). This can be attributed to the joint action of multiple immunosuppressive factors (4). These inhibitory factors can suppress the maturation and differentiation of CD4+ T cells. This can thereby decrease the number of CD4+ T cells and weaken the immuno-monitoring function on tumor cells (4).

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PD-L1 and PD-L2 are newly discovered B7 family costimulatory molecule ligands. They share the common receptor PD-1 (6). In addition, they can bind with receptor PD-1 to inhibit T-cell proliferation and excessive activation (6). In this way, it plays a negative regulatory role in cellular immune response (7). Concurrently, it also exerts a regulatory role in humoral immunity by affecting cytokine secretion (7). The PD-L1 and PD-L2/PD-1 pathways play important roles in autoimmune tolerance and the immune escape mechanism of tumor cells (7). At present, numerous tumors are found to express PD-L1. PD-L1 expression in tumor cells can weaken the immunogenicity of tumors. Furthermore, it can influence tumor cells to produce specific T-cell response (7). Moreover, it can suppress the production of tumor immune response. PD-L1 expressed in tumor cells can induce specific CTL apoptosis. Thus, it allows the tumor cells to develop immune escape (7). Talebi et al revealed that miR-142 regulates T-cell differentiation in an animal model of multiple sclerosis (8). The present study aimed to evaluate the function of miR-142-5p on cancer immunity to induce apoptosis in human non-small cell lung cancer (NSCLC) and its mechanism.

Materials and methods

Patients and flow cytometry. A total of 20 patients with NSCLC and a total of 20 normal specimens were collected from the Department of Thoracic Surgery of Shenzhen People's Hospital. The patients were aged from 55 to 65 years. Peripheral blood was collected and rapidly frozen in liquid nitrogen and stored at -80°C. Ethical approval was obtained from the Shenzhen People's Hospital.

Serum was collected after centrifugation at 1000 x g for 10 min at 4°C and used to assess CD4+ T cells. Immune cell suspensions were prepared and stained with anti-CD4+CD25hi+Foxp3+ T cell-APC (anti-mouse antibody; eBioscience; Thermo Fisher Scientific, Inc.) and calculated using the SYBR-Green qPCR SuperMix-UDG reagents (Invitrogen; Ribobio, Co., Ltd., Guangzhou, China) with Platinum Bulge-Loop™ miRNA qRT-PCR Primer Set (Guangzhou Ribobio, Co., Ltd., Guangzhou, China) for 15 min at room temperature. Flow cytometry was performed using BD AccuriC6 (BD Biosciences, Franklin Lakes, NJ, USA) and data was analyzed using FlowJo software (FlowJo, LLC, Ashland OR, USA).

Quantitative real-time PCR (qRT-PCR). Total RNA from serum and cultured cells samples was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcribe reactions were performed to compound cDNA using M-MLV reverse transcriptase (Promega Corp., Madison, WI, USA). miR-142-5p expression was detected using a Bulge-Loop™ miRNA qRT-PCR Primer Set (Guangzhou Ribobio, Co., Ltd., Guangzhou, China) with Platinum SYBR-Green qPCR SuperMix-UDG reagents (Invitrogen; Thermo Fisher Scientific, Inc.) and calculated using the 2−ΔΔCt method. PCR primers of miR-142-5p were as follows: forward, 5'-AACGCTTCACGAATTTGCGT-3' and reverse, 5'-TCTTGAACCCTCATCCTGT-3'; and PCR primers of U6 were: forward, 5'-TCTGCTTCGGCAGCGAC-3' and reverse, 5'-AAAGCTTACGGATATTGCGT. The qRT-PCR thermostability conditions were as follows: initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 25 sec, 60°C for 30 sec and 72°C for 30 sec.

Cell culture and reagents. NSCLC cell line A549 was cultured with Dulbecco's modified Eagle's medium (DMEM; Whittaker BioProducts, Walkersville, MD, USA) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin in humidified air at 37°C with 5% CO₂. miR-142-5p, anti-miR-142-5p and negative mimics were transfected into A549 cells using Lipofectamine™ 2000 (Invitrogen, Thermo Fisher Scientific, Inc.). PBMCs were acquired from the same donor for preparation of non-adherent responder T-cells (NAC) and monocytes (MN) and incubated in complete RPMI-1640 (Whittaker BioProducts) supplemented with 5% PHS in 25 cm² tissue culture flasks (2.5x10⁵ cells/flask) in the presence of MTB H37RvL (1 µg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) for 5 days. PBMCs (5x10⁵) were seeded onto the cultured A549 cells by transfection for 24 h (1:5; A549:PBMCs) in 10 µg/ml of PHA (Sigma-Aldrich, St. Louis, MO, USA).

MTT assay, LDH activity level and flow cytometric analysis of apoptosis. Cells were assessed using an MTT assay. MTT solution (20 µl) was added to the cells after transfection at 24, 48 and 72 h. Following incubation for 4 h, the previous medium was removed and 150 ml dimethyl sulfoxide (DMSO) was added to the cells for 20 min at 4°C. The optical density (OD) was read at 570 nm using Bio-Rad Microplate Reader Model 680 (Bio-Rad Laboratories, Hercules, CA, USA).

To assess the LDH activity level after transfection at 24 h, the cells were harvested using an LDH level kit (Beyotime Institute of Biotechnology, Nanjing, China). The OD was read at 450 nm using Bio-Rad Microplate Reader Model 680 (Bio-Rad Laboratories).

To assess apoptosis using flow cytometry, after transfection at 24 h, the cells were harvested and stained with FITC-Annexin V and 7-AAD. The cells were analyzed with BD AccuriC6 (BD Biosciences) and data was analyzed using FlowJo software (FlowJo, LLC).

Determination of the concentration of cytokines using ELISA. Cellular supernatant was collected after centrifugation at 1000 x g for 10 min at 4°C. CCL11, CCL22 and IFN-γ levels were assessed using ELISA kits. The OD was read at 450 nm using Bio-Rad Microplate Reader Model 680 (Bio-Rad Laboratories).

Western blotting. Cells were harvested and washed with PBS. Briefly, total proteins were extracted by disrupting cells in RIPA lysis buffer and assessed using a BCA assay (both from Beyotime Institute of Biotechnology). Total protein (50 µg) was separated on 10% polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked at room temperature for 1 h with 5% non-fat milk in TBST and the membranes were incubated at 4°C overnight with the following antibodies: PD-L1 (1:500; cat. no. sc-293425), PTEN (1:500; cat. no. sc-6817-R), PI3K (1:500; cat. no. sc-7175), p-Akt (1:200; cat. no. sc-7985-R), Akt (1:500; cat. no. sc-8312) and GAPDH (1:5,000; cat. no. sc-25778; all from Santa Cruz Biotechnology, Inc., Dallas TX, USA). After being washed with TBST, the membranes were incubated with goat anti-rabbit peroxidase-conjugated secondary antibodies (1:5,000; 273
cat. no. sc-2005 or cat. no. sc-2004; Santa Cruz Biotechnology) at room temperature for 1 h. Protein expression was detected using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology).

**Determination of caspase-3/9 activity.** Cells were harvested and washed with PBS. Briefly, total proteins were extracted by disrupting cells in RIPA lysis buffer and assessed using a BCA assay (both from Beyotime Institute of Biotechnology). Total protein (10 µg) was used to assess caspase-3/9 activity levels using Caspase-3/9 activity kits (Beyotime Institute of Biotechnology). The OD was read at 405 nm using Bio-Rad Microplate Reader Model 680 (Bio-Rad Laboratories).

**Statistical analyses.** All data are represented as the mean ± SD of three independent experiments. All data were evaluated using Student's t-test or one-way analysis of variance (ANOVA) and Tukey's post test. Values were considered significant when P<0.05.

**Results**

**miRNA-142-5p expression and CD4+ T cells in NSCLC patients.** To determine miRNAs in NSCLC patients, we examined the expression levels of CC chemokines which interact with cell surface chemokine receptor CCR4, in 136 NSCLC patients by gene chip or qPCR analyses. As revealed in Fig. 1A and B, miRNA-142-5p expression was upregulated in NSCLC patients, compared with the control group. Then, we also found that the CD4+CD25hi+Foxp3+ T cell expression level was upregulated in NSCLC patients, compared with the control group (Fig. 1C). The levels of IFN-γ, CCL17 and CCL22 were reduced in NSCLC patients, compared with the control group (Fig. 1D-F).

**Overexpression of miR-142-5p expression inhibits the cancer effects of CD4+ T cells in NSCLC cell line A549.** To detect the effect of miR-142-5p on the cancer effects of CD4+ T cells on NSCLC cell line A549, we overexpressed miR-142-5p expression using miR-142-5p mimics (Fig. 2A). Following a significant increase of miR-142-5p expression in A549 cells, these cells were co-cultured with CD4+ T cells. Following co-culture for 24, 48 and 72 h, the overexpression of miR-142-5p reduced the cancer effects of CD4+ T cells. miR-142-5p overexpression promoted cell growth, and reduced the level of LDH activity, the apoptosis rate and caspase-3/9 activities in A549 cells compared with the control group (Fig. 2B-G).

**Downregulation of miR-142-5p increases the cancer effects of CD4+ T cells in NSCLC cell line A549.** Next, we also used anti-miR-142-5p mimics to decrease the level of miR-142-5p...
expression in A549 cells and compared this level with the control group (Fig. 3A). Following the decrease of miR-142-5p expression, the cells were co-cultured with CD4+ T cells. Then, at 24, 48 and 72 h of co-culture, it was observed that downregulation of miR-142-5p increased the cancer effects of CD4+ T cells. Downregulation of miR-142-5p reduced cell growth, and increased the activity level of LDH, the apoptosis rate and caspase-3/9 activities compared with the control group (Fig. 3B-G). Therefore, the anti-effects of miR-142-5p in NSCLC may involve CD4+ T cells, however, the mechanism warrants further investigation.

The effects of miR-142-5p on CD4+ T cells in NSCLC cell line A549 via the PTEN pathway. We investigated the effects of
miR-142-5p on the PTEN pathway, to validate the bioinformatics analysis. As revealed in Fig. 4A, PD-L1 and PTEN are the potential target genes of miR-142-5p. Then, in the co-culture model, overexpression of miR-142-5p suppressed PTEN and PD-L1 protein expression in A549 cells compared with the control group (Fig. 4B-D). Furthermore, we also found that overexpression of miR-142-5p induced PI3K and p-Akt protein expression in the co-culture model, compared with the control group (Fig. 4E-G). In the co-culture model, downregulation of miR-142-5p induced PTEN and PD-L1 protein expression in A549 cells compared with the control group (Fig. 5A-C). Moreover, downregulation of miR-142-5p suppressed PI3K and p-Akt protein expression in the co-culture model compared with the control group (Fig. 5D-F). Collectively, miR-142-5p promoted antitumor immunity in NSCLC by blocking the PD-L1/PD-1 pathway via the PTEN pathway.

**Effects of miR-142-5p on the concentration of CCL17, CCL22 and IFN-γ levels.** Next, we analyzed CCL17, CCL22 and IFN-γ levels in the medium of the co-culture model using ELISA kits. Downregulation of miR-142-5p increased CCL17, CCL22 and IFN-γ levels in the medium of the co-culture model compared with the control group (Fig. 6A-C). Overexpression of miR-142-5p reduced CCL17, CCL22 and IFN-γ levels in the medium of the co-culture model compared with the control group (Fig. 6D-F). These results revealed that miR-142-5p regulates the PD-L1/PD-1 pathway to secrete CCL17, CCL22 and IFN-γ into the tumor microenvironment for antitumor immunity in NSCLC cells.

**Inhibition of PTEN also reduces the cancer effects of CD4+ T cells in NSCLC cell line A549 following miR-142-5p downregulation.** Considering the potential role of the PTEN/PI3K/Akt signaling pathway in miR-142-5p-promoted antitumor immunity in NSCLC cells, we wanted to further elucidate the involvement of PTEN in the stability regulation of PD-L1. The PTEN inhibitor (VO-Ohpic trihydrate) suppressed PTEN and PD-L1 protein expression in A549 cells in the co-culture model compared with the miR-142-5p downregulation group (Fig. 7A-C). In addition, the inhibition of PTEN induced PI3K and p-Akt protein expression in the co-culture model compared with the miR-142-5p downregulation group (Fig. 7D-F). Moreover, the inhibition of PTEN inhibited the levels of CCL17, CCL22 and IFN-γ in the medium of the co-culture model compared with the miR-142-5p downregulation group (Fig. 7G-I). Finally, inhibition of PTEN also reduced the cancer effects of CD4+ T cells in A549 cells following miR-142-5p downregulation compared with the miR-142-5p downregulation group (Fig. 8).

**Suppression of PD-L1 reduces the cancer effects of CD4+ T cells in NSCLC cell line A549 following miR-142-5p downregulation.** Investigation of the function of PD-L1 in the cancer effects of CD4+ T cells in NSCLC cell line A549 following miR-142-5p downregulation revealed that PD-L1
inhibitor (PD1-PDL1 inhibitor 1) suppressed the PD-L1 protein expression in the co-culture model of A549 cells as well as the levels of CCL17, CCL22 and IFN-γ in the medium of the co-culture model compared with the miR-142-5p downregulation group (Fig. 9). The suppression of PD-L1 reduced the cancer effects of CD4+ T cells on cell growth, the LDH...
activity levels, the apoptosis rate and caspase-3/9 activities in A549 cells in the co-culture model, compared with miR-142-5p downregulation group (Fig. 10).

Discussion

Lung cancer is one of the high-risk factors of malignancy-induced deaths at present (9). No detection means with strong specificity are presently available due to the lack of obvious early clinical features and symptoms (10). As a result, most lung cancer patients are at the advanced stage at the time of diagnosis. This has severely affected the therapeutic effect and prognosis of patients. Cellular immunity is the major mechanism against tumors. Moreover, T cells are important effector cells in antitumor immunity. It can complete immune regulatory function through lymphocyte subsets with various functions (10). Therefore, we hypothesized that miRNA-142-5p expression was upregulated in NSCLC patients. Talebi et al revealed that miR-142 regulates T-cell differentiation in an animal model of multiple sclerosis (8). Hou et al revealed that the levels of CD4+CD25+FOXP3+/CD4+ T cells and FOXP3 mRNA were significantly higher in lung cancer patients than in healthy controls (11), which is in keeping with Fig. 1. Kotsakis et al revealed that particular CD4+ Treg subtypes are elevated in NSCLC patients (12), which is in keeping with Fig. 1.

Tumor genesis, development, infiltration and metastasis are the collective action of multiple factors (9). However disordered autoimmune monitoring and immune tolerance are the basic factors of tumor formation (10). In recent years, the discovery of CD4+CD25+ regulatory T cells (Tregs) has added to the understanding of immune tolerance and immune regulation. CD4+CD25+ Treg cells are derived from the thymus. It is an important component of the immune system which helps to maintain immune homeostasis (10). It is characterized by immune nullipotence and immune suppression. It can directly contact effector cells (9). Thus, it can inhibit the activation and proliferation of potential autoimmune T cells in the body (9). Notably, it plays an important role in regulating tumor immunity and autoimmunity (10). Therefore, we hypothesized that miRNA-142-5p expression was upregulated in NSCLC patients. Talebi et al revealed that miR-142 regulates T-cell differentiation in an animal model of multiple sclerosis (8).
CCL17 can be secreted by dendritic cells and endothelial cells. CCL22 is mainly derived from macrophages and dendritic cells differentiated from monocytes. CCL17 shares the common receptor molecule CCR4 with CCL22 (13, 14). The chemokine receptor plays a key role in guiding cell migration in tissue along with the chemokine gradient. CCR4 is expressed in T cells, NK cells, monocytes, and the eosinophil surface (14, 15). Among them, CCR4+ T cells are the important effector cells of CCL17 and CCL22. Both CCL17 and CCL22 act on CCR4+ T cells. However, they play distinct roles in tumor immunity (14). CCL17 may play a role against tumor cell immunity (15). However, CCL22 may promote the formation of tumor immunity tolerance of Treg cells. IFN-γ is the potent activator of monocytes/macrophages. It can extensively enhance the expression of the MHC-6-type antigen by all types of cells (16). Therefore, it can amplify the recognition stage of immune response. IFN-γ can directly stimulate T- and B-cell differentiation as well as CTL maturation. In addition,
it can stimulate B cells to secrete antibodies (6). IFN-γ can suppress lymphocytes under certain circumstances, especially Th2 cells (6). We found that the downregulation of miR-142-5p increased CCL17, CCL22 and IFN-γ levels in the medium of the co-culture model. Karu et al revealed that miR-142-5p regulates tumor cell PD-L1 expression and enhances antitumor immunity via increase of IFN-γ and TNF-α (17).

Malignant tumor patients are mostly associated with immunological dysfunction. T lymphocytes are the most important components in cellular immunity (10). They play an immune regulatory role through T lymphocyte subsets with various functions (10). Peripheral T cell subsets can also be divided into CD4+ helper/inducer T cells and CD8+ inhibitory/cytotoxic T cells (9). CD4+ cells can assist B cells in secreting antibodies and regulating the immune responses of other T cells (18). CD8+ cells mostly manifest cytotoxicity. They are the major cytotoxic effector cells (9). In this study, we found that overexpression of miR-142-5p expression inhibited the cancer effects of CD4+ T cells in NSCLC cell line A549. Ding et al indicated that the inhibition of miR-142-3p/5p causes CD4+ T-cell activation in systemic lupus erythematosus (19).

PD-L1 can transfer immune inhibitory signals to T cells after binding with receptor PD-1. Thus, it can inhibit T-cell immunity (20). Moreover, it plays a negative regulatory role in the immune response. The PD-1/PD-L1 interaction can suppress proliferation and activation of CD4+ and CD8+ T cells. In addition, it can downregulate the expression and secretion of IL-2 and IFN-γ. Furthermore, it induces cell cycle arrest at the G0/G1 stage (20). The outcome of their interaction depends on T-cell antigen receptor, which accounts for the major biological effect of the PD-1/PD-L1 pathway (21). In addition, this pathway exhibits more evident inhibitory effects on CD8+ T cells than on CD4+ T cells (21). Our study revealed that the effects of miR-142-5p on CD4+ T cells in NSCLC cell line A549 were achieved via the PTEN pathway. Jia et al revealed that miR-142-5p enhances antitumor immunity via tumor cell PD-L1 expression (22).
The PD-L1 signaling pathway can regulate T-cell activation. One of its mechanisms of action is the direct inhibition of T cells (23). It manifests as T-cell proliferation inhibition, cytokine secretion suppression and cytotoxic reaction (24). PD-L1 protein can induce massive transformation of CD4+ T cells into Treg cells. This molecular mechanism includes blocking PI3K to initiate new signals (25). In addition, it is accompanied with the upregulation of PTEN expression (25). PTEN is the first tumor suppressor gene discovered with dual specific phosphatase activity of lipid phosphatase and protein phosphatase. Abrupt PTEN changes can be observed in most tumors (25). Loss of PTEN function can activate the PI3K/Akt signaling pathway, thus participating in cancerogenesis (25,26). In addition, in the present study we revealed that the suppression of the PD-L1 or PTEN inhibitor reduced the cancer effects of CD4+ T cells in NSCLC cell line A549 (25,26). In this study, we found that NSCLC cells.

Collectively, we revealed that miR-142-5p expression was upregulated in NSCLC patients. In this study, we found that miR-142-5p regulated CD4+ T cells in human non-small cell lung cancer through PD-L1 expression via the PTEN pathway (Fig. 11). Our findings revealed that miR-142-5p may be a novel therapeutic target for NSCLC.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JW designed the experiment; XL, BP and GD performed the experiment; JW and XL analyzed the data; JW wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Ethical approval was obtained from the Shenzhen People's Hospital.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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JW designed the experiment; XL, BP and GD performed the experiment; JW and XL analyzed the data; JW wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Consent for publication

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