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Research Article

Inhibitor Targeting METTL3 Partially Reverses Damage Associated with Pulmonary Arterial Hypertension in Mouse and Cell Models.

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Abstract

Background: Pulmonary arterial hypertension is a potentially fatal condition with no effective cure. Recent studies have shown that progressively increasing pulmonary vascular resistance and irreversible pulmonary vascular remodeling are key factors contributing to pulmonary hypertension. Methyltransferase like 3 (METTL3), a key N6-Methyladenosine (m6A) methyltransferase, it has been shown that elevated levels of METTL3 expression is a risk factor for pulmonary hypertension, Elevated levels of METTL3 contribute to vascular remodeling in the lungs, resulting in increased pulmonary artery pressure and right ventricular hypertrophy.

Methods: To obtain a model of pulmonary hypertension, we placed C57BL/6 male mice in a 500-liter ventilated room with a 10% oxygen concentration for four weeks. After mice were placed in a hypoxic incubator for two weeks, they were started on intraperitoneal injections of STM2457 once a day for two weeks, after which right ventricular pressure was measured using a Miller catheter, right ventricular remodeling is determined using hematoxylin-eosin staining, right ventricular hypertrophy was assessed using the right ventricular/left ventricular + septum (RV/LV+S) ratio and relative expression of TNF-α, IL-1β, IL-6 protein by western blot. The impact of STM2457 on Human Pulmonary Artery Smooth Muscle Cells (HPASMCs) under hypoxia was examined by evaluating their viability, proliferation, migration and the expression of IL-1β, IL-6, and TNF-α proteins.

Results: In mouse models of hypoxic PAH, METTL3 expression was significantly increased and pulmonary vascular remodeling. Treatment with STM2457 significantly decreased right ventricular pressure and hypertrophy, and inhibited molecules associated with the inflammation signaling pathway. Additionally, the inhibition of METTL3 by STM2457 attenuated hypoxia-induced proliferation and migration of HPASMCs, while further inhibiting molecules associated with the inflammation signaling pathway.

Conclusions: Based on the results obtained above, we are confident that treatment with STM2457 targeting METTL3 significantly reduces damage in PAH model mice and cells.

Keywords: METTL3, STM2457; IL-6; Pulmonary Arterial Hypertension (PAH); HPASMCs, Human pulmonary artery smooth muscle cells.

Introduction

Pulmonary Arterial Hypertension (PAH) is a deadly condition with a high death rate. It denotes a group of diseases characterized by heightened pulmonary vascular resistance caused by remodeling in the pre-capillary resistance arterioles [1-5]. Recent studies have found that genetic mutations, immune dysfunction and inflammation are closely related to the pathogenesis of PAH. NF-κB exerts a regulatory role in the inflammatory response of PAH, which can eventually lead to the activation of downstream cytokines [6-10]. Inflammatory cytokines exacerbate vascular remodeling pathology by promoting apoptosis and proliferation of vascular smooth muscle cells [11-13]. In PAH, the hyperproliferation and reduced apoptosis of Pulmonary Artery Smooth Muscle Cells (PASMC) worsen vascular remodeling [14]. Hence, reducing inflammation will be crucial for mitigating the pathogenic process of PAH.

N6-Methyladenosine (m6A), which has garnered growing attention in recent years, is the most abundant RNA methylation in eukaryotes [15,16].

Previous research has identified notably higher m6A levels in both patients with Idiopathic Pulmonary Arterial Hypertension (IPAH) and experimental Pulmonary Hypertension (PH) models, in comparison to control lung sections [17]. A methyltransferase complex multimer composed of methyltransferase-like enzymes 3 and 14 (METTL3, METTL14) catalyzes the formation of m6A [18]. The m6A modification of Phosphatase and Tensin homolog (PTEN) is regulated by METTL3 and leads to a decrease in the expression of PTEN protein levels, which in turn promotes the activation of the PI3K/Akt axis and leads to hypoxia-induced proliferation of PASMCs [19,20]. Under hypoxic conditions,

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lowering or inhibiting METTL3 protein expression can inhibit PASMC proliferation, thereby attenuating pulmonary vascular remodeling and ultimately alleviating pulmonary hypertension [19]. Previous studies have shown that in urologic cancers, high expression of METTL3 positively promotes the activation of NF-κB and SHH-GL1 pathways, while negatively inhibiting the PTEN signaling pathway [21]. Taken together, it is reasonable to hypothesize that inhibition of METTL3 may attenuate PAH by inhibiting the NF-κB signaling pathway. Therefore, this study aimed to determine if inhibiting METTL3 could mitigate the effects of hypoxia on pulmonary hemodynamics, inflammatory responses, and NF-κB signaling in PAH mouse and cellular models.

Materials and Methods

Drug and Antibodies

The METTL3 inhibitor STM2457 (SJ-MX0125) was obtained from Shandong Sparkjade Biotechnology Co., Ltd. Antibodies against TNF-α (26162-1-AP), IL-6 (21865-1-AP), GAPDH (23660-1-AP) and IL-1b (16806-1-AP) and were from Proteintech Group.

Cell Culture and Treatment

According to the manufacturer's instructions, HPASMCs (CP-H243, Procell) were cultured using the complete medium specifically designed for HPASMCs (CM-H243, Procell). Cells were cultured in an humidified incubator set at 37°C, 5% CO_2 concentration. PASMCs were exposed to the METTL3 inhibitor STM2457 (Shandong Sparkjade Biotechnology Co., Ltd.) for 24 hours following the manufacturer's protocol. To simulate the hypoxic pathological process of pulmonary hypertension in vitro,

Cells were cultured in a hypoxic incubator (HF100 incubator, Harmonic Bio-pharmaceuticals Holding Co., Ltd., Shanghai, China) with a gas composition of 92% nitrogen, 5% carbon dioxide, and 3% oxygen. PASMCs at passages 4-7 were used, and after inoculation into 6-well plates, they were maintained in the anoxic environment of the incubator for 24 hours.

Animals

Male C57BL/6 mice aged 8-12 weeks were used for all animal experiments in this study. The Ethics Committee for Laboratory Animal Welfare, Institute of Biology, Shandong Academy of Sciences approved the study. Six male C57BL/6 mice were used in each group to complete the experiment.

Mouse Models for PAH

The methodology employed to develop the pulmonary arterial hypertension mouse model is based on prior research findings [22,23]. We randomly divided C57BL/6 mice into two groups for modeling pulmonary hypertension. Mice were maintained in a hypoxic 500-liter ventilated chamber containing 10% oxygen concentration for 4 weeks [24]. After acclimating for two weeks in a hypoxic environment, the treatment group received daily intraperitoneal injections of STM2457 (50mg/kg) for two consecutive weeks. Control mice were administered an equivalent volume of solvent, in which the drug was dissolved, via intraperitoneal injection. The animal facility maintained a constant temperature of 22°C under a 12-hour light-dark cycle. All mice were housed in this controlled environment and provided with standard mouse chow and autoclaved water ad libitum.

2.5 Phenotyping of mice with pulmonary arterial hypertension

Following established protocols, we exposed the right jugular vein by incising the neck skin of the mice. Subsequently, a 1.4F Millar Mikro-tip catheter converter was inserted into the right ventricle through the exposed jugular vein to measure Right Ventricular Pressure (RVSP) in both the experimental and control groups of mice. Using scissors, we isolated the Right Ventricle (RV) from the heart of each mouse, leaving the Left Ventricle plus septum (LV + Septum). Each part was weighed using a precision scale and the measurements were recorded. The degree of right ventricular hypertrophy was quantified using the ratio RV/(LV + Septum) [25]. Hematoxylin and eosin-stained lung images of mice were analyzed using case viewer software. Outer and inner diameters of pulmonary vessels were measured, and the medial wall thickness was calculated using the formula: vascular media wall thickness = (outer diameter - inner diameter) / (2 × outer diameter) [26].

Cell Proliferation Assays

5 x 10^3 PASMCs were inoculated in each well of a 96-well plate, and then the different concentrations of STM2457 inhibitor (5 wells were set up for each concentration) were treated for 24 hours. Subsequently, 10 μL of CCK-8 reagent (Beyotime Biotechnology) was added to each well, and the mixture was incubated for 1-2 hours. and finally, the 96-well plate was placed into a Microplate Reader, and the values of the proliferation levels of the cells were measured at 450 nm [27,28].

Cell Migration

Each well in a 6-well plate was inoculated with 5 x 10^5 PASMCs cells and then incubated for 24 hours at 37□°C with 5% CO_2 concentration. When the monolayer cells grew to about 90% of the area of each well, we used 200 μL pipette tips to make a scratch in each well, at the same time, STM2457 inhibitor and solvent were added to incubate for 18 hours, and finally, the migration distance was recorded by microscope and calculated by Image J Software.

Western Blot Analysis

Mouse lung tissues obtained from mice isolated after cervical dislocation [29, 30]. The lung tissue and collected PASMCs samples were lysed on ice at □4°C by adding Cell lysis buffer for Western and IP buffer (P0013, Beyotime) containing PMSF (ST506, Beyotime). Cell lysates were centrifuged at 4°C, 12000 rpm for 30 minutes, the supernatant was collected, the following precipitate was discarded, and then 5× SDS loading buffer was added to the supernatant and analyzed by western blotting as in previous studies □[30]. Detailed information and sources of antibodies to the proteins of interest in this study can be accessed in the Drugs and Antibodies section.

Statistical Analysis

We designed the experiment and completed the statistical analysis of the data in accordance with previous studies □[30]. Mean±SE represents all quantitative data. Statistical analysis between two groups utilized a two-tailed Student's t-test, while comparisons among multiple groups employed one-way ANOVA followed by Tukey's post hoc test for data analysis [50]. We considered p-value less than 0.05 as statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001.

Results

As previous studies showed that METTL3 increased in mouse PAH models, we wondered whether the pulmonary hypertension phenotype could be reversed with STM2457, an inhibitor of METTL3. To investigate the therapeutic effect of STM2457 on experimental PAH, we maintained mice in a hypoxic environment for four weeks., with STM2457 (50mg/kg) administrated daily for the latter 2 weeks. The control group mice were treated with the same hypoxia environment, and with the same volume of solvent as in the treatment group for the latter 2 weeks. Hemodynamic data demonstrated that STM2457 treatment attenuated the hypoxia-induced elevation of RVSP (Figure 1A). RVSP was significantly higher in mice exposed to hypoxia

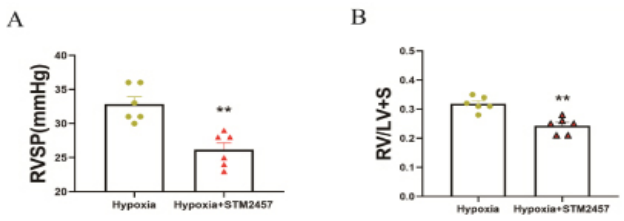

Figure 1: STM2457 partially inhibited the right ventricular pressure and right ventricular hypertrophy in hypoxia-induced PAH. (A) STM2457 partially alleviated the elevated RVSP caused by hypoxia stimulation. (B) STM2457 partially alleviated the right ventricular hypertrophy (RV/LV+S) caused by hypoxia stimulation. ** p <0.01 vs controls.

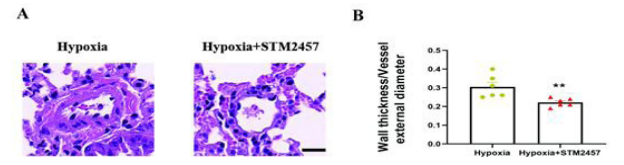

Figure 2: STM2457 partially reversed vascular remodeling in hypoxia-induced PAH. (A) Shows representative H&E staining images of lung tissues. (B) Presents quantified data on pulmonary artery medial wall thickness index. STM2457 significantly attenuated the hypoxia-induced thickening of medial walls in muscular pulmonary arteries (** p < 0.01 vs. controls).

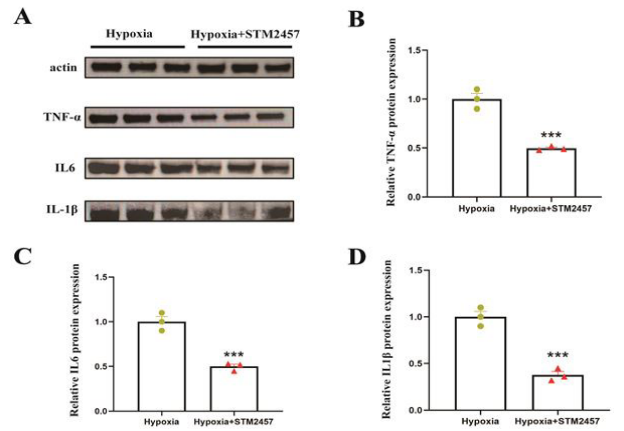

Figure 3: Effects of STM2457 on TNF-α, IL-6, IL-1β in the therapeutic treatment of hypoxia-induced PAH. (A) Western blotting analysis showed that the TNF-α, IL-6, IL-1β induced by hypoxia could be reversed by STM2457 in lungs. (B) Quantifications of TNF-α expression in lungs. (C) Quantifications of IL-6 expression in lungs. (D) Quantifications of IL-1β expression in lungs. ** p <0.01 vs controls.

compared to those treated with STM2457. Analysis of hypertrophy indicated a notable increase in the RV/LV+S ratio in control mice (Figure 1B), which was absent in mice treated with STM2457. The impact of STM2457 on pulmonary vascular remodeling was initially assessed using H&E staining. Figures 2A and 2B illustrate significantly thicker pulmonary artery medial walls in hypoxia-exposed PAH mice, a condition mitigated by STM2457 treatment. Previous studies have found that elevated levels of TNF-α, IL-6 and IL-1β expression enhance distal arterial vascular muscularization and promote right ventricular hypertrophy. These mechanisms are likely to influence the pathogenesis of pulmonary hypertension. As depicted in (Figure 3), STM2457 markedly suppressed the hypoxia-induced expression of IL-6, IL-1β, and TNF-α in lung tissue. Furthermore, we employed the CCK8 assay to assess PASMC viability and investigate the potential impact of STM2457 on cell proliferation. However, as shown in Figure 4, 50 μM to 200μM STM2457 significantly attenuated hypoxia-challenged PASMCs viability elevation. We selected 150μM of STM2457 for the next experiments. We used the scratch wound assay to detect the effect of STM2457 on the hypoxia-induced migration capacity of PASMCs. Interestingly, the scratch-wound assay (Figure 5) demonstrated that STM2457 dose-dependently inhibited PASMC migration induced by hypoxia. Further western blot analysis confirmed that treatment with 150μM STM2457 reversed the up-regulation of IL-6, IL-1β, and TNF-α (Figure 6).

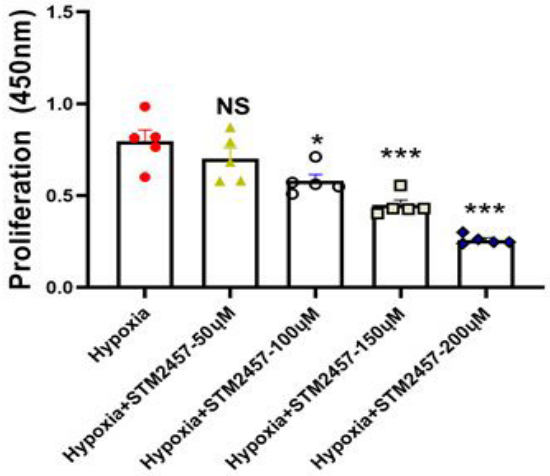

Figure 4: The impact of STM2457 on hypoxia-induced HPASMC proliferation was assessed using the CCK-8 assay. The results indicated that STM2457 dose-dependently inhibited cell proliferation under hypoxic conditions. Statistical analysis revealed significance levels as follows: NS (Not Significant), * p<0.05 vs controls, ** p <0.01 vs controls, *** p <0.001 vs controls.

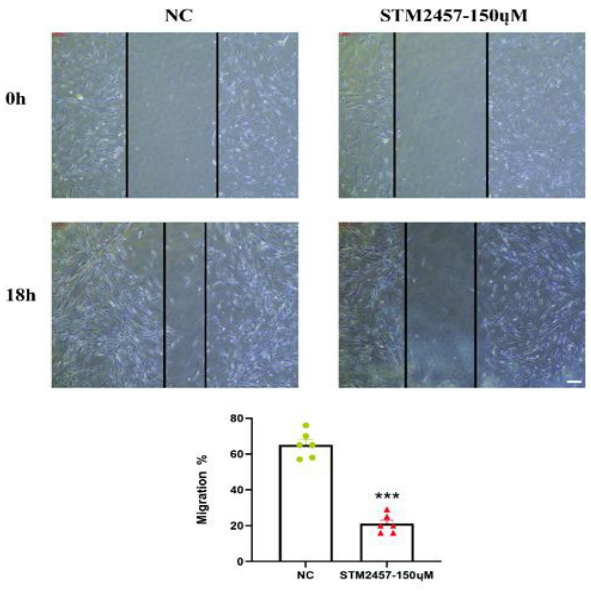

Figure 5: The effect of STM2457 on hypoxia-induced HPASMC migration was evaluated using a scratch assay, demonstrating significant inhibition by STM2457. Statistical analysis indicated. *** p <0.001 vs controls.

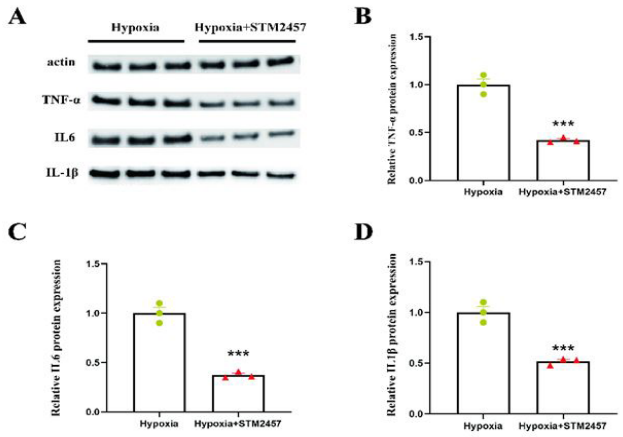

Figure 6: Effects of STM2457 on TNF-α, IL-6, IL-1β. (A) Western blotting analysis showed that the TNF-α, IL-6, and IL-1β induced by hypoxia could be reversed by STM2457 in PASMCs. (B) Quantifications of TNF-α expression in PASMCs. (C) Quantifications of IL-6 expression in PASMCs. (D) Quantifications of IL-1β expression in PASMCs. ***p <0.001 vs controls.

Discussion

Pulmonary Arterial Hypertension (PAH) is marked by structural changes in the pulmonary vasculature and sustained elevation of pulmonary arterial pressure. These conditions contribute to the rapid progression of the disease, leading to right heart failure (RHF), and ultimately, mortality [31,32]. In this work, we evaluated the effects and molecular mechanisms of STM2457 directed against METTL3 in PAH models induced by hypoxia. Our findings suggest that hypoxia-induced PAH mice have typical features of pulmonary hypertension such as pulmonary artery remodeling, right ventricular hypertrophy, and dysfunction, and STM2457 significantly inhibits the above progression. A significant inhibitory effect of STM2457 was found in hypoxia-induced PASMCs proliferation in vivo and vitro. Furthermore, it was demonstrated that STM2457, restrains the NF-κB signaling pathway and inhibits PASMCs proliferation. In conclusion, these findings illustrate that STM2457 may alleviate vascular remodeling in Pulmonary Arterial Hypertension (PAH) via a novel mechanism. Additionally, this study identifies STM2457 as a promising innovative target for treating hypoxic PAH.

As the most abundant RNA methylation in eukaryotes, m6A has attracted increasing attention and research in recent years [33]. The modification of m6A is involved in important physiological responses by affecting the processes of mRNA splicing, export, translation and decay. The modifications of m6A are not static but dynamically regulated over time and in response to environmental changes. Methyltransferases (writers), demethylases (erasers), and binding proteins (readers) are responsible for catalyzing, removing, and recognizing the modifications of m6A, respectively [18,34]. The “writers” are primarily composed of METTL3 and METTL14, while the “erasers” include FTO and ALKBH5. The “readers” comprise YTHDF1, YTHDF2, and YTHDF3, among others. These enzymes work in concert to modulate the m6A modification, thereby affecting mRNA metabolism and protein expression [15,34]. METTL3, as an m6A methylase has been confirmed to participate in the regulation of pulmonary hypertension [19,35,36]. In this study, we reported that the METTL3 inhibitor STM2457 partially reversed pulmonary vascular remodeling to ameliorate the hypoxia-induced pulmonary hypertension phenotype in mice.

Prior research has highlighted multiple factors influencing pulmonary vascular remodeling in Pulmonary Arterial Hypertension (PAH), with a crucial emphasis on the excessive proliferation of pulmonary smooth muscle cells [37]. Many studies have shown that the pathogenesis of various heart diseases is closely related to the NF-κB signaling pathway. Inflammation is known to be one of the essential features of PAH, and NF-κB and (IL□)-1β have been shown to promote an inflammatory response that affects the course of pulmonary hypertension in both human PAH and animal models of PAH [38-40]. Mitani Y et al. demonstrated that pyrrolidine dithiocarbamate attenuates the progression of pulmonary hypertension in rats with monocrotaline-induced PAH by suppressing the NF-κB signaling pathway [40]. Studies have demonstrated that zinc finger protein A20 mitigates hypoxia-induced pulmonary hypertension by suppressing NF-κB signaling pathway activation and the excessive proliferation of pulmonary artery smooth muscle cells [41]. NF-kB/TNF-α pathways were upregulated after hypoxia induction in important organs of rats, such as heart, liver, spleen, lung, kidney, brain and muscle [42]. With the prolongation of hypoxia induction in lung tissues, expression levels of these relative genes were gradually increased [43,44]. Previous experimental investigations [45,46] have indicated the involvement of the NF-κB pathway in Reactive Oxygen Species (ROS) production and polymorphonuclear neutrophil infiltration during PAH. NF-κB also plays a regulatory role in the inflammatory response of PAH, potentially leading to the activation of downstream cytokines [47,48]. Cancer-Associated Fibroblast (CAF)-produced METTL3-derived IL-18 counteracts Non-Small Cell Lung Cancer (NSCLC) immunosuppression by driving the NF-κB pathway [49]. Elevated expression of METTL3 was confirmed in lung tissue of pulmonary hypertension, and depletion of METTL3 can inhibit the proliferation, invasion, and migration of PASMCs [19,50]. Therefore, novel approaches to inhibit NF-κB signaling pathway activation and inflammation may hold new promise for the treatment of PAH. Interestingly, STM2457 effectively reduces the proliferation and migration of PASMCs by inhibiting the NF-κB signaling pathway, thereby mitigating vascular remodeling.

Conclusion

In summary, the administration of STM2457 directed at METTL3 reduced right ventricular pressure, and hypertrophy, remodeling and the molecules associated with the inflammation signaling pathway associated with PAH. Additionally, our findings demonstrate that blocking METTL3 reversed hypoxia-induced proliferation and migration of HPASMCs, and suppressed molecules involved in inflammatory signaling pathways. This identifies a novel potential target for treating pulmonary arterial hypertension.

Authors’ Contributions

Hui Li conceived and designed the study, drafted the manuscript, critically revised the manuscript, and supervised the study; Jiajian Gu and Zhixin Niu performed experiments; Zhixin Niu and Yuanhao Sun analyzed the data; All authors read and approved the final manuscript.

Ethics Approval and Consent To Participate

Animal care and experimental procedures were approved by □Ethics Committee for Laboratory Animal Welfare, Institute of Biology, Shandong Academy of Sciences, China (No:SWS20240108). Animal experiments conformed to the guidelines of the Care and Use of Animals for Research by the Ministry of Science and Technology of the P. R. China (2006–398). Euthanasia was performed by anesthesia with injection of Tribromoethyl alcohol (30μL/g) followed by cervical dislocation. The HPASMCs (CP-H243) was obtained from Wuhan Pricella Biotechnology Co., Ltd.

Human and Animal Rights

No humans were used as experimental subjects for this study. All animal experiments were conducted in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Consent for Publication

Not applicable.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (Hui Li] upon request.

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Conflict of Interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of the article.

Acknowledgements

Declared none.

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