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·Original Article·

MiR-206 decreases brain-derived neurotrophic factor levels in a transgenic mouse model of Alzheimer's disease

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ABSTRACT

MicroRNA alterations have been reported in patients with Alzheimer's disease (AD) and AD mouse models. We now report that miR-206 is upregulated in the hippocampal tissue, cerebrospinal fluid, and plasma of embryonic APP/PS1 transgenic mice. The increased miR-206 downregulates the expression of brain-derived neurotrophic factor (BDNF). BDNF is neuroprotective against cell death after various insults, but in embryonic and newborn APP/PS1 mice it is decreased. Thus, a specific microRNA alteration may contribute to AD pathology by downregulating BDNF.

Keywords: miR-206; brain-derived neurotrophic factor; APP/PS1; cell death; Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in the population >65 years old[1]. It is associated with impairments in memory, language, behavior, and cognition[1]. The brain of AD patients is characterized by the presence of senile plaques and neurofibrillary tangles together with synaptic and neuronal cell loss[1]. Many microRNAs (miRNAs) have been implicated in AD[2-4]. miRNAs are a group of short (~21–23 nucleotides) non-coding RNAs[5] with conserved roles in *Drosophila*, rodents, monkeys, and humans[6-9]. miRNA precursors are first processed into single-stranded miRNAs that bind to the 3' untranslated region (UTR) of the complementary target mRNA sequences[10], leading to translational repression or target degradation[11, 12]. A specific miRNA can have several hundred target gene sequences and regulate many pathways[13, 14].

Neurotrophins have been reported to play important roles in the physiopathology of AD[15]. Brain-derived neurotrophic factor (BDNF) is the most widely expressed neurotrophin in the central nervous system[16], and plays critical roles in neurite outgrowth, directional guidance, the induction of long-term potentiation, and neurotransmitter release[17]. It also regulates gene transcription, such as nuclear factor kappa B and c-Jun N-terminal kinase-p53-Bax, and activates intracellular signaling pathways, including Ras/mitogen-activated protein kinase, phosphatidylinositol 3/Akt, and phospholipase C pathways[18]. Moreover, BDNF has been associated with AD pathology. In cultured neurons, BDNF protects against amyloid β $(Aβ)_{1-42}$ toxicity[19]. It also rescues the damage induced by amyloid in rodent and primate models of AD[20]. On the other hand, $Aβ_{1-42}$ decreases BDNF expression in cortical neurons and affects its intracellular trafficking[21]. In AD transgenic mouse models, BDNF is negatively correlated with the cerebral amyloid burden[22]. In addition, genetic association studies have linked BDNF polymorphisms to an increased risk of AD in humans[23-26]. Taken together, BDNF is implicated in AD pathogenesis.

In the present study, we set out to determine the mechanism of how BDNF is altered and whether some miRNA regulation is involved in such alteration.

MATERIALS AND METHODS

Cell Culture

Primary neurons were cultured from embryonic day 14 (E14) APP/PS1 (APPswe/PS1ΔE9) mouse hippocampus, following the regulations of Peking University Animal Care and Use Committee as previously described[27]. SH-Sy5y cells were cultured with minimum essential medium (Invitrogen, Carlsbad, CA).

Reagents and Sequences of miRNAs and 3'UTR and Real-time PCR

Mouse miR-206, scrambled microRNA control (5'-GTGTAACACGTCTATACGCCCA-3'), mimic miR-206 (5'-UGGAAUGUAAGGAAGUGUGUGG-3'), an miR-206 inhibitor (5'-ACAUGCUUCUUUAUAUCCUCAUA-3'), mutant miR-206 (5'-UGGAAUGUAAGG**CC**GUGUGUGG-3'), and complementary sequence (5'-ACATTCC-3') were all purchased from Qiagen (Hilden, Germany). The altered mouse BDNF mRNA 3'UTR sequences used were as follows: mutant BDNF mRNA 3'UTR: 5'-ACAAACC-3' and MAP2 3'UTR: 5'-CATATTCATTCTTTACAAACCATAG-3'. All WT and modified 3'UTR sequences were cloned into a pLenti-Luc-UTR or pLenti-EGFP-UTR vector backbone (Abm, Richmond, Canada). miRNAs were transfected into SH-Sy5y cells with HiPerFect transfection agent (Qiagen). BDNF (Sigma, St. Louis, MO), $A\beta_{1-42}$ (Bachem, Torrance, CA), staurosporine (STS, Sigma), etoposide (Etop, Sigma) and glutamate (Sigma) were stored in 100× stock solution and diluted with culture medium before use. Real-time PCR was done following the described procedure[28]. miR-206 levels in tissue, serum, and cerebrospinal fluid (CSF) were measured by the TaqMan MicroRNA assay kit for Mmu-miR-206 according to the manufacturer's instructions (Life Technologies Corp., Carlsbad, CA).

Western Blots and Measurement of Neuronal Cell Death

Western blots were performed following the protocols described previously[27]. Anti-BDNF (Abcam, Cambridge, UK) and actin (Sigma) antibodies were diluted at 1:1 000 for Western blots as primary antibodies. The relative density was calculated as the total absolute density of BDNF/actin control. Secreted BDNF was calculated by equal loading of the same volume of culture medium. Cell death was measured by terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) using *in situ* cell death detection kit I according to the manufacturer's description (Roche, Quebec, Canada)[27].

Luciferase Reporter Assays

The effect of miR-206 on BDNF expression was investigated using the Dual-Luciferase Reporter Assay System kit (E1910; Promega, Madison, WI). The constructs with the firefly (*Photinus pyralis*) luciferase gene downstream of the BDNF 3'UTR were co-transfected with WT, mutant, scrambled, or mimic miR-206, or with miR-206 inhibitor, into SH-Sy5y cells. Firefly luminescence was detected as indicated by the manufacturer. For the control of transfection efficiency, *Renilla* (*R. reniformis*) luciferase construct was co-transfected into the cells. The activity was corrected as (firefly/*Renilla* luciferase) ×10 000. The luminescence was expressed as relative light units[29].

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA kits were used to determine the concentrations of BDNF (R&D Systems, Minneapolis, MN) in the hippocampus, CSF, and plasma, as well as TrkA (R&D Systems), TrkB (R&D Systems) and $p75^{NTR}$ (EIAAB Inc.) levels in the hippocampus of WT and APP/PS1 mice. The relative optical intensity of each well was read by a microplate reader (BioRad 480; BioRad, Hercules, CA).

Statistical Analysis

Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Sheffé's test. $P < 0.05$ was taken as statistically significant.

RESULTS

BDNF mRNA 3'UTR Was a Target of miR-206

In APP/PS1 mice at E14, the miR-206 level in the hippocampus was about 6-fold that in WT mice (Fig. 1A). As previous studies showed that miRNAs can be secreted into the CSF and plasma[2], we also assessed their miR-206 levels, and found that both were upregulated in APP/PS1 mice compared with the WT mice at E14 (Fig. 1A).

The 3'UTR of human BDNF mRNA contains 3 complementary sequences for miR-206 (Fig. 1B). In SH-Sy5y cells, luciferase reporter assays were performed

in which the expression of firefly was regulated by the BDNF mRNA 3'UTR region complementary to miR-206, and *Renilla* was an internal control for transfection efficiency. We found that miR-206 induced a dose-dependent inhibition of luciferase activity, whereas mutant miR-206 and scrambled control miRNA had no such effect (Fig. 1C, left panel). The mimic of miR-206 markedly inhibited the luciferase activity, while the inhibitor of miR-206 increased it (Fig. 1C, left panel). In SH-Sy5y cells, the EGFP reporter assay confirmed the inhibitory effect of miR-206 on the BDNF mRNA 3'UTR (Fig. 1C, right panel).

To determine the specificity of miR-206 targeting BDNF mRNA, we tested other miRNAs with proposed roles in AD[3] against luciferase reporter activity regulated by the BDNF mRNA 3'UTR. Only miR-206 effectively inhibited luciferase activity (Fig. 1D). We then mutated the BDNF mRNA 3'UTR complementary sequence and performed a luciferase assay with either scrambled control miRNA or miR-206. Our results showed that miR-206 only suppressed the luciferase activity with the WT BDNF mRNA 3'UTR, but not with the mutant 3'UTR (Fig. 2A), suggesting the specificity of the interaction between miR-206 and the BDNF mRNA 3'UTR. To further validate that the BDNF mRNA 3'UTR was one of the targets for miR-206, we made mutant a BDNF mRNA 3'UTR with two repeats of the putative complementary binding domain (BD) sequence (2BD) and three repeats of the BD sequence (3BD) for each putative binding site. The luciferase reporter system showed that miR-206 did not suppress the luciferase activity regulated by the unrelated MAP2 mRNA 3'UTR (Fig. 2B). With the BDNF mRNA 3'UTR, luciferase activity was greatly reduced in a BD dose-dependent manner (Fig. 2B). A time-course study showed that miR-206 began to inhibit luciferase activity from 12 h of treatment (Fig. 2C). Western blotting further confirmed that miR-206 decreased the BDNF level (Fig. 2D). miR-206 downregulated the levels of both BDNF in cellular extracts and secreted BDNF, whereas the miR-206 inhibitor increased both cellular and secreted BDNF (Fig. 2D).

BDNF Was Decreased in AD Transgenic Mice and Protected Against Cell Death

ELISA showed that the BDNF level in hippocampal tissue was decreased in APP/PS1 mice compared with WT mice (Fig. 3A) at the E14 and newborn (P0) stages. The levels of BDNF in the CSF and plasma were also significantly lower

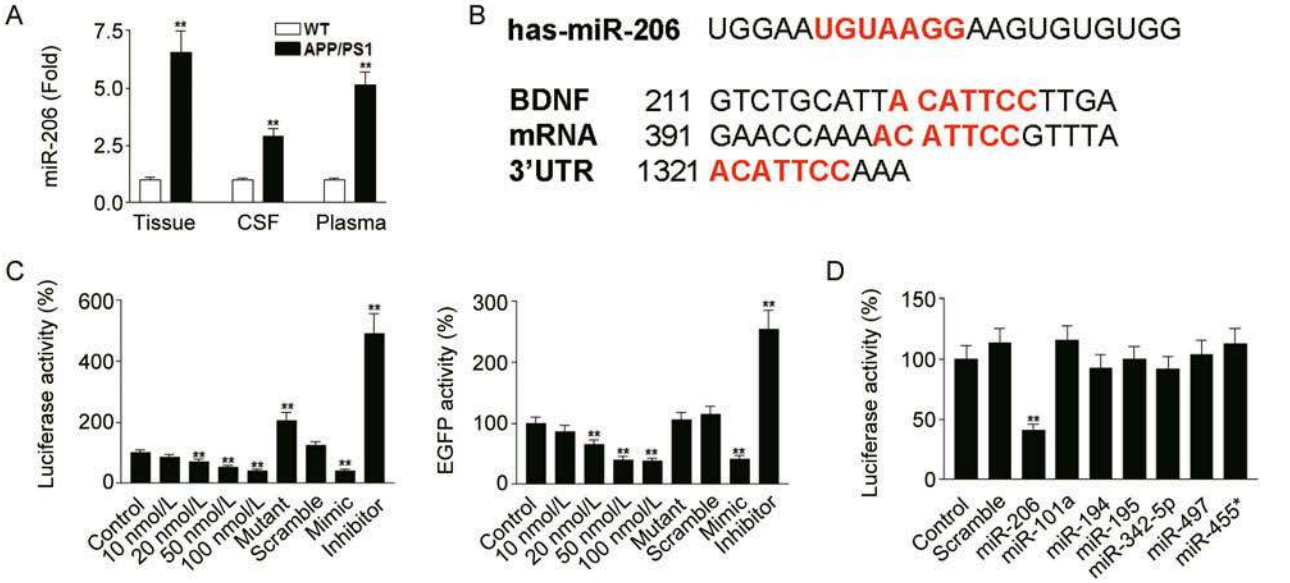

Fig. 1. miR-206 regulated the BDNF mRNA 3'UTR. A: miR-206 was increased in hippocampal tissue, CSF, and plasma of APP/PS1 mice compared with WT mice at E14. B: Sequence alignment of human miR-206 and the BDNF mRNA 3'UTR region. C: Dose responses of miR-206 in the luciferase reporter assay (left) and EGFP reporter assay (right) in SH-Sy5y cells. D: Other microRNAs were tested against luciferase activity driven by the BDNF mRNA 3'UTR. Only miR-206 specifically decreased the luciferase activity.

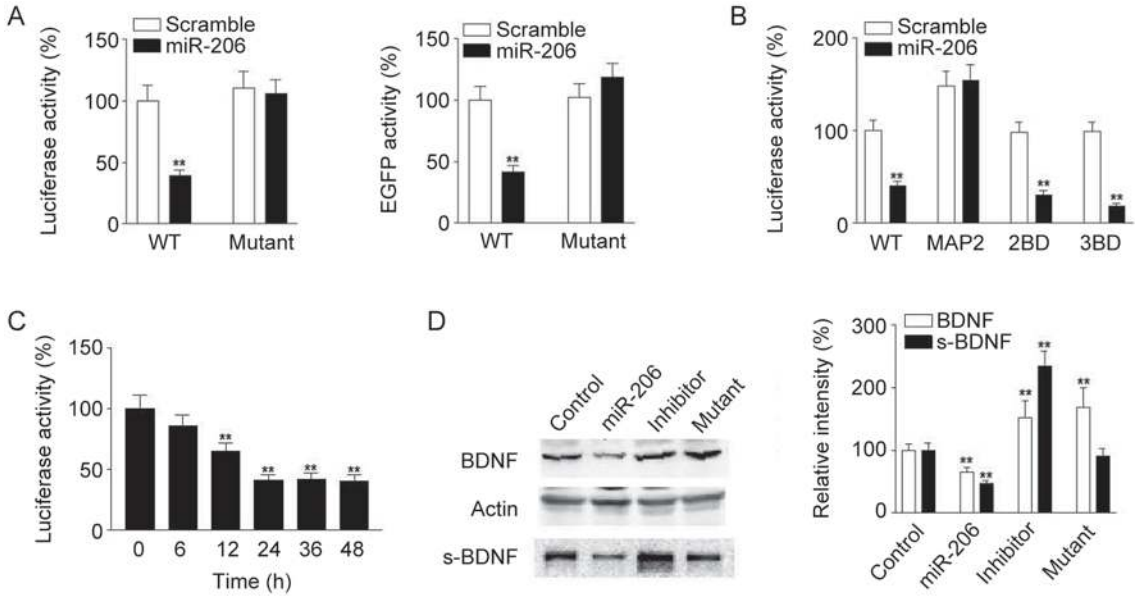

Fig. 2. miR-206 decreased BDNF expression. A: Left panel, miR-206 reduced the luciferase activity with the WT BDNF mRNA 3'UTR, but not with the mutant BDNF mRNA 3'UTR. Right panel, miR-206 reduced the EGFP reporter activity with the WT BDNF mRNA 3'UTR, but not with the mutant BDNF mRNA 3'UTR. B: miR-206 did not alter the expression with an unrelated MAP2 mRNA 3'UTR. With BDNF mRNA 3'UTRs constructed with 2 or 3 duplicates of the putative binding domain (2BD, 3BD) of each binding site, miR-206 decreased the luciferase activity remarkably in a dose-dependent manner. C: Time-course of miR-206 inhibition of the BDNF mRNA 3'UTR. D: Western blots of BDNF showed that a mimic of miR-206 (lane 2) reduced, whereas an inhibitor of miR-206 (lane 3) increased the BDNF level, including BDNF from cell extracts and secreted BDNF (s-BDNF) from the medium. Mutant miR-206 (lane 4) did not alter BDNF levels. Data are mean ± SE (*n* = 3 for each group). ***P* <0.01 compared with control.

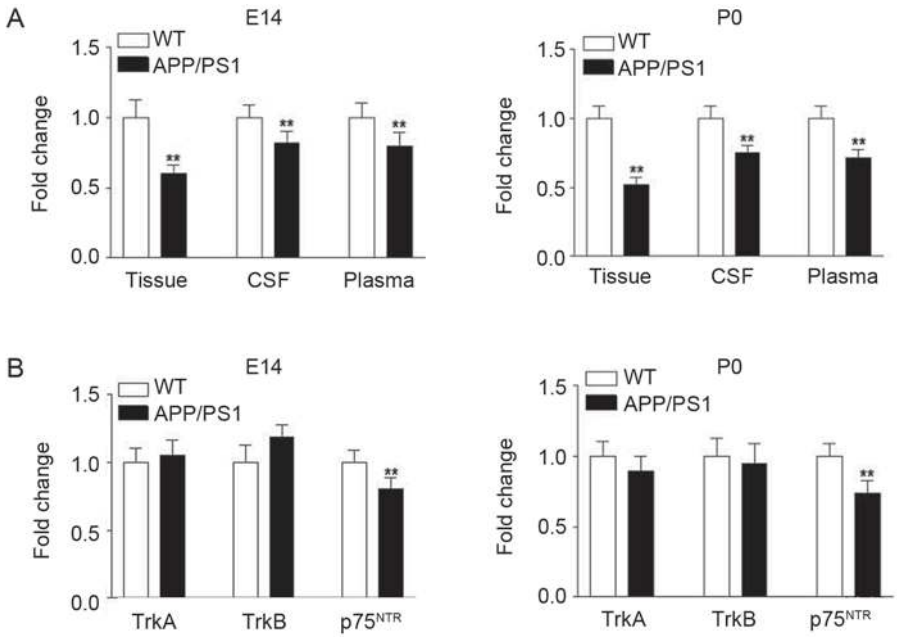

Fig. 3. BDNF was downregulated in APP/PS1 mice. A. At E14 and P0, BDNF decreased in the hippocampal tissue, CSF, and plasma in APP/PS1 mice compared with WT mice. B. At E14, TrkA and TrkB did not change significantly in APP/PS1 mouse hippocampal tissue. p75NTR decreased in APP/PS1 compared with WT mice.

in APP/PS1 mice than in WT mice at both stages (Fig. 3A). We also assessed the levels of TrkA, TrkB, and $p75^{NTR}$, receptors for BDNF[18], in hippocampal tissues at E14 and P0, and found no significant changes of TrkA and TrkB levels at either stage (Fig. 3B). The $p75^{NTR}$ level was lower in the APP/PS1 than in the WT mouse tissue (Fig. 3B).

As neuronal loss has been demonstrated in AD pathology[1], we then assessed the vulnerability of cultured WT and APP/PS1 neurons to various insults. Our data showed that extracellular $A\beta_{1-42}$[27, 29-32], serum deprivation[31, 33], staurosporine[31, 33], Etop[31] and glutamate[34] induced more severe cell death in cultured APP/PS1 neurons than in WT neurons (Fig. 4). Exogenous administration of BDNF at 1 μmol/L[35, 36] decreased the vulnerability of APP/PS1 neurons to all the above insults (Fig. 4), suggesting that BDNF plays an important role in neuronal vulnerability and viability/death.

DISCUSSION

In the present study, we found that miR-206 was upregulated in transgenic APP/PS1 mice. A similar increase

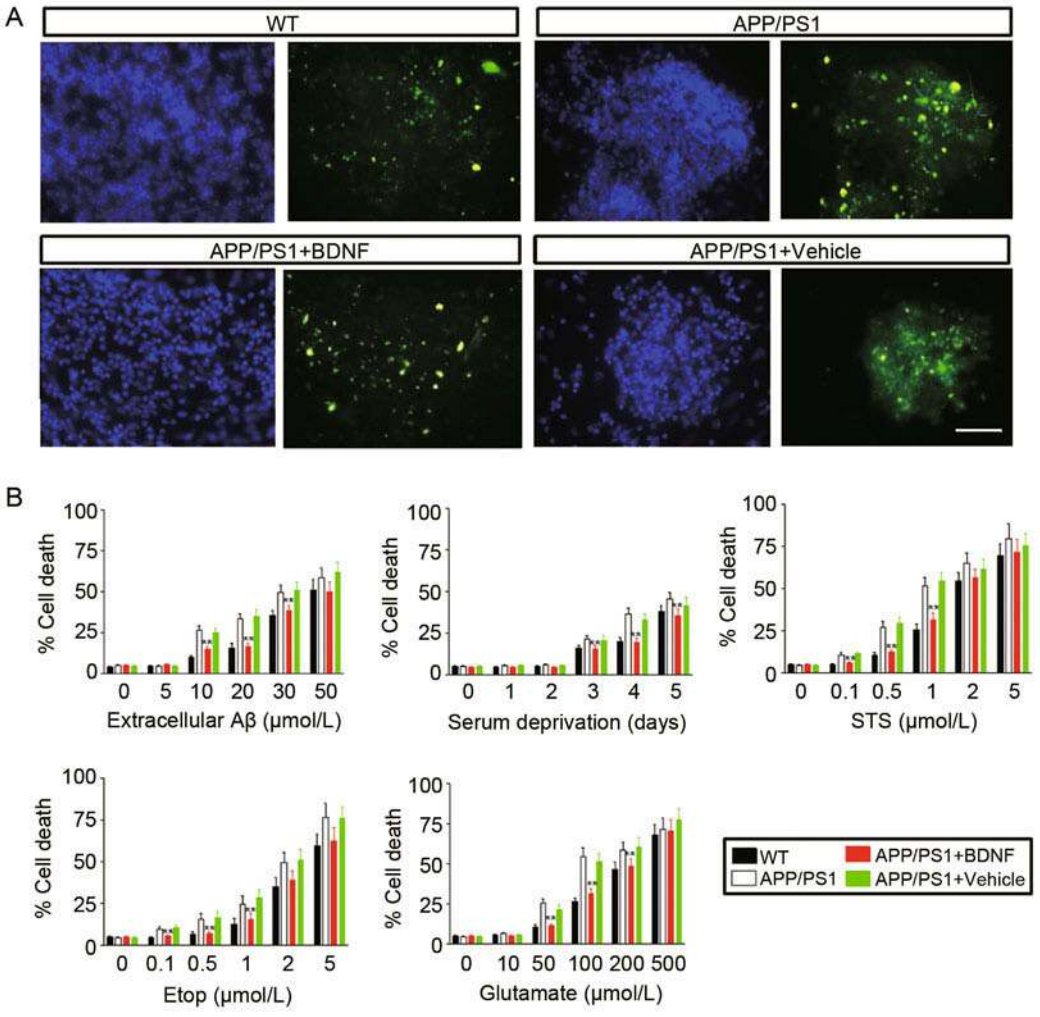

Fig. 4. BDNF was protective against cell death. A: Representative images of cultured WT and APP/PS1 neurons treated with BDNF or vehicle in the presence of 10 μmol/L extracellular $A\beta_{1-42}$. Blue, Hoechst staining indicating nuclei; green, TUNEL staining indicating apoptotic cells. Scale bar, 50 μm. B: In cultured WT and APP/PS1 neurons, toxicity was induced by extracellular $A\beta_{1-42}$, serum deprivation, STS, Etop, and glutamate. The neurons were treated with BDNF at 1 μmol/L in the culture medium. Cell death was measured 24 h after treatment. Data are mean ± SE (n = 3 for each group). **P <0.01 compared with APP/PS1 neurons.

of miR-206 has been reported in Tg2576 AD transgenic mice and in the temporal cortex of human AD brains[37]. Furthermore, miR-206 targeted the 3'UTR of BDNF mRNA and decreased the BDNF level in AD transgenic mouse neurons, which is also consistent with the report that miR-206 targets BDNF transcripts[37]. Further, BDNF was protective against cell death induced by extracellular $A\beta_{1-42}$, STS, Etop, serum deprivation, and glutamate. The mechanism of BDNF protection is yet to be fully understood. It has been reported that $A\beta_{1-42}$ inhibits the BDNF-induced expression of Arc, an immediate-early gene responding to the BDNF signal, in cultured neurons[38-40]. Arc is associated with synaptic plasticity and the induction of long-term potentiation[41] and may be linked to AD pathology. A reduced level of BDNF in AD has also been associated with increased pro-inflammatory activity[42-44], alterations of oxidative stress and mitochondrial dysfunction[45], downregulation of hippocampal neurogenesis[46, 47], and GSK3β hyperactivity[48-50]. Further studies are needed to discover the mechanisms of miR-206 regulation in AD animal models and BDNF protection. Finally, AD has been considered to be an aging-related disorder. Our data showed that developmental defects, including microRNA regulation and BDNF expression and secretion may be involved in AD pathogenesis.

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