Role of Prostatic Exosome miRNA-146a/TLR-4/NF- B Pathway in Chronic Inflammation in EAP Rats and Intervention with Dahuocao: Post-print

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Abstract

Background: Current treatments for chronic prostatitis (CP) have not achieved the expected efficacy, and there is an urgent need to find new therapeutic drugs. Our research group' s previous clinical studies have found that Anemone tomentosa (Maxim.) Pei has good therapeutic effects on CP, but the specific mechanism of action remains to be further investigated. Objective: To investigate the role of prostatic body-derived microRNA-146a (miRNA-146a) in regulating the Toll-like receptor 4 (TLR-4)/nuclear transcription factor B (NF- B) pathway in chronic inflammation in autoimmune prostatitis (EAP) rats and the possible mechanism of Anemone tomentosa intervention. Methods: From December 2021 to June 2022, 42 Wistar rats were divided into 7 groups using the random number table method, including a normal group, model group, Anemone tomentosa low-dose group, Anemone tomentosa medium-dose group, Anemone tomentosa high-dose group, miRNA-146a agonist (mimics) group, and miRNA-146a inhibitor group, with 6 rats in each group. After drug intervention, real-time fluorescence quantitative polymerase chain reaction (RT-PCR) was used to detect prostatic body miRNA-146a-5p mRNA, Western blotting (WB) was used to detect TLR-4, inhibitor of B (I B), phosphorylated inhibitor of B (p-I B), and tumor necrosis factor receptor-associated factor 6 (TRAF6), and enzyme-linked immunosorbent assay (ELISA) was used to detect inflammatory factors. Results: Compared with the normal group, the expression of prostatic body miRNA-146a-5p mRNA was downregulated in all groups ($P<0.01$). Compared with the model group, the expression of prostatic body miRNA-146a-5p mRNA was upregulated in the Anemone tomentosa medium- and high-dose groups and the miRNA-146a mimics group ($P<0.01$); the expression of prostatic body miRNA-146a-5p mRNA was downregulated in the miRNA-146a inhibitor

group (P<0.01). Anemone tomentosa and miRNA-146a mimics could significantly reduce the protein expression of TLR-4, p-I Bα, and TRAF6 in prostate tissue (P<0.01); promote the upregulation of I Bα protein expression in prostate tissue (P<0.01); and decrease the levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and interferon-γ (IFN-γ) in rat serum (P<0.01 or P<0.05). Anemone tomentosa and miRNA-146a mimics could alleviate inflammatory cell infiltration in prostate tissue and improve pathological damage in prostate tissue. Conclusion: Prostatic body-derived miRNA-146a can regulate TLR-4/NF- B pathway activation and participate in local pathological changes in the prostate of EAP rats. The mechanism of Anemone tomentosa against chronic inflammation in EAP rats may be related to its regulation of the prostatic body-derived miRNA-146a/TLR-4/NF- B pathway.

Full Text

Role of Prostasome-derived miRNA-146a in Regulating TLR-4/NF- B Pathway and Mechanism of Action of *Anemone tomentosa* in Treating Chronic Inflammation in a Rat Model of Experimental Autoimmune Prostatitis

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Abstract

Background: Current treatments for chronic prostatitis (CP) have not achieved the desired therapeutic efficacy, necessitating the search for novel therapeutic agents. Our previous clinical studies found that *Anemone tomentosa* (Maxim.) Pei demonstrates favorable efficacy against CP, though its specific mechanism of action requires further investigation.

Objective: To investigate the role of prostasome-derived microRNA-146a (miRNA-146a) in regulating the Toll-like receptor 4 (TLR-4)/nuclear factor- B (NF- B) pathway in chronic inflammation in experimental autoimmune prostatitis (EAP) rats and the potential mechanism of *Anemone tomentosa* intervention.

Methods: Between December 2021 and June 2022, 42 Wistar rats were randomly divided into seven groups (n=6 each): normal control, model, low-dose *A. tomentosa*, medium-dose *A. tomentosa*, high-dose *A. tomentosa*, miRNA-146a mimics, and miRNA-146a inhibitor groups. Following drug intervention, real-time polymerase chain reaction (RT-PCR) was used to detect prostasomal miRNA-146a-5p mRNA expression, western blotting (WB) was performed to measure TLR-4, inhibitor of NF- B (I B), phosphorylated I B (p-I B), and tumor necrosis factor receptor-associated factor 6 (TRAF6) protein levels, and enzyme-linked immunosorbent assay (ELISA) was used to quantify inflammatory cytokines.

Results: Compared with the normal group, all other groups showed downregulated prostasomal miRNA-146a-5p mRNA expression (P<0.01). Relative to the model group, miRNA-146a-5p expression was upregulated in the medium- and high-dose *A. tomentosa* groups and the miRNA-146a mimics group (P<0.01), while it was further downregulated in the miRNA-146a inhibitor group (P<0.01). Both *A. tomentosa* and miRNA-146a mimics significantly reduced prostate tissue expression of TLR-4, p-I Bα, and TRAF6 proteins (P<0.01), promoted I Bα protein expression (P<0.01), and decreased serum levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and interferon-γ (IFN-γ) (P<0.01 or P<0.05). Histopathological examination revealed that *A. tomentosa* and miRNA-146a mimics attenuated inflammatory cell infiltration and ameliorated prostate tissue damage.

Conclusion: Prostasome-derived miRNA-146a participates in local prostate pathology in EAP rats by modulating TLR-4/NF- B pathway activation. The anti-inflammatory mechanism of *A. tomentosa* in EAP rats may involve regulation of the prostasome-derived miRNA-146a/TLR-4/NF- B pathway.

Keywords: Chronic prostatitis; Prostasome; miRNA-146a; TLR-4; NF- B

Introduction

Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is a common urological and andrological condition, accounting for 90-95% of prostatitis cases, characterized by lower urinary tract symptoms and chronic pain without clear association with bacterial infection [1]. The pathophysiology involves complex mechanisms including occult infection, immunity, inflammation, neuroendocrine factors, intraprostatic urinary reflux, oxidative stress, and psychological factors that frequently intersect [2]. Due to its unclear and multifactorial etiology, single-modality treatments have proven largely unsuccessful, prompting a transition to multimodal approaches including pharmacological agents (antibiotics, α-blockers, anti-inflammatory drugs, phytotherapy) and non-pharmacological interventions (pelvic floor physical therapy, myofascial trigger point release, acupuncture, psychological support, extracorporeal shock wave therapy, local hyperthermia) [3]. However, a systematic review and

meta-analysis indicates that modern medicine has yet to elucidate the specific pathogenesis of CP/CPPS, and current management fails to achieve expected therapeutic outcomes [4].

In recent years, Chinese herbal medicine has gained attention as an alternative therapy for CP/CPPS, demonstrating characteristic advantages through herbal formulations or extracts [5]. Our previous research found that *Anemone tomentosa* (Maxim.) Pei significantly improves clinical symptoms in CP patients [6] and modulates inflammatory cytokine levels in CP rat models [7], exerting anti-prostatitis effects. Prostasomes, membrane vesicles secreted by human prostatic epithelial cells and the first identified reproductive tract exosomes, play important physiopathological roles in prostate diseases. Based on this, the present study employed an experimental autoimmune prostatitis (EAP) rat model to investigate the role of prostasome-derived microRNA-146a (miRNA-146a) in regulating the TLR-4/NF- B pathway in local chronic inflammation and to explore the potential mechanism of *A. tomentosa*, a single herb with "heat-clearing and dampness-removing, diuretic and strangury-relieving, qi-tonifying and kidney-nourishing" properties, in treating CP/CPPS.

Materials and Methods

Experimental Animals Five 4-month-old SPF-grade male Wistar rats weighing $(400\$\pm 20)gandforty-two8-week-oldclean-grademaleWistarratsweighing(200\pm\$15)$ g were purchased from Changsha Tianqin Biotechnology Co., Ltd. (License No. SCXK2019-0013). Animals were housed in the Guangxi Key Laboratory of Molecular Biology of Preventive Medicine of Traditional Chinese Medicine at 18-25°C with ad libitum access to standard chow and water. The study was approved by the Ethics Committee of Guangxi University of Chinese Medicine (Animal Ethics Approval No. DW20220926-202).

Experimental Instruments and Reagents Instruments: Transmission electron microscope (HITACHI, HT7800/HT7700), nucleic acid/protein quantifier (DeNovix, DS-11), electrophoresis apparatus (Beijing Liuyi, DYCZ-24DN), semi-dry transfer system (ATTO, WSE-4040, AE6675L), fluorescence quantitative PCR instrument (BIO-RAD, CFX ConnectTM), spectrophotometer (Eppendorf, BioPhotometer), optical microscope (OLYMPUS, BX43), ultracentrifuge (Hitachi, CP100MX), and microplate reader (BIO-TEK, Elx800).

Reagents: TLR4 antibody (Affinity, AF7017), TRAF6 antibody (Affinity, AF5376), p-I Bα antibody (Affinity, AF5002), I Bα antibody (Affinity, AF2002), miRcute miRNA isolation kit (Tiangen Biotech, Cat# DP501), miRcute miRNA Detection Kit (SYBR Green) (Tiangen, Cat# FP411), miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen, Cat# KR211), complete Freund' s adjuvant (Sigma-Aldrich), miRNA-146a mimics (GenePharma), miRNA-146a inhibitor (GenePharma), IL-6 (MultiSciences, EK306), IL-8 (MultiSciences, SEKR-0014), IFN-γ (MultiSciences, EK380), and TNF-α (MultiSciences, EK382).

EAP Rat Model Preparation Forty-two 8-week-old male Wistar rats were randomly divided into seven groups (n=6 each): normal control, model, miRNA-146a mimics, miRNA-146a inhibitor, low-dose *A. tomentosa*, medium-dose *A. tomentosa*, and high-dose *A. tomentosa* groups. Except for the normal group, all other groups received subcutaneous injections of a suspension containing rat prostate protein extract mixed with equal volumes of complete Freund' s adjuvant (1 ml) at multiple sites on days 0 and 30, plus intraperitoneal injection of diphtheria-tetanus-pertussis vaccine (0.5 ml). Successful modeling was confirmed by histological evidence of extensive inflammatory cell infiltration, disappearance of mucosal folds, acinar destruction, and obvious interstitial edema in prostate tissue [9].

Intervention Protocols Following successful modeling, the normal and model groups received 0.9% saline solution (4 ml/kg · d) by gavage. The low-, medium-, and high-dose *A. tomentosa* groups received *A. tomentosa* decoction at concentrations of 1.46 g/ml, 2.92 g/ml, and 5.84 g/ml respectively (4 ml/kg · d) by gavage. All treatments were administered once daily for 4 weeks. The miRNA-146a mimics group received 0.9% saline (4 $ml \cdot kg^{-1} \cdot d^{-1}$) by gavage for 4 weeks plus tail vein injection of miRNA-146a mimics (0.2 l/g) 72 hours before sacrifice. The miRNA-146a inhibitor group received 0.9% saline (4 $ml \cdot kg^{-1} \cdot d^{-1}$) by gavage for 4 weeks plus tail vein injection of miRNA-146a inhibitor (0.2 l/g) 72 hours before sacrifice [10].

Preparation of *Anemone tomentosa* Decoction Appropriate amounts of *A. tomentosa* were placed in a decoction container, soaked in 5-8 volumes of cold water for 1 hour, then boiled for 30 minutes and filtered. The residue was boiled again with 3-6 volumes of water for 30 minutes and filtered. The two filtrates were combined, centrifuged for 10 minutes (radius 10 cm, 5,000 r/min), and the supernatant was concentrated using a rotary evaporator to a final concentration of 5.84 g crude drug/ml. Before administration, the concentrate was diluted with distilled water to 1.46 g/ml, equivalent to the human-equivalent dose based on body surface area conversion.

Preparation of Rat Prostate Protein Extract Five 4-month-old SPF-grade male Wistar rats weighing (400$±$20) g were anesthetized with 1% pentobarbital sodium. After disinfection and fixation on a clean bench, the prostate was exposed via lower abdominal incision and excised. The harvested tissue was washed with cold 0.9% saline, minced, and homogenized with an equal weight of 0.9% saline containing 0.5% Triton X-100 (pre-sterilized). The homogenate was centrifuged at high speed for 30 minutes (radius 10 cm, 15,000 r/min) at 4°C. The supernatant (0.2 ml) was collected and serially diluted for concentration measurement via microplate reader. The remaining supernatant was diluted with 0.1 mol/L PBS (pH 7.2) to 60 g/L, aliquoted into cryovials, and stored at -80°C.

Prostasome Extraction and Electron Microscopy Prostasomes were extracted from prostatic fluid using ultracentrifugation. Samples were rapidly thawed at 37°C and transferred to new centrifuge tubes. Sequential centrifugation steps were performed at 4°C: 30 minutes at 4,700 r/min (radius 8.1 cm), followed by 45 minutes at 10,000 r/min (radius 8.1 cm) to remove larger vesicles. The supernatant was filtered through a 0.45 m membrane, then ultracentrifuged for 70 minutes at 112,000 r/min (radius 7.2 cm). The pellet was resuspended in 10 ml ice-cold 1×PBS and ultracentrifuged again under the same conditions. The final pellet was resuspended in 100 l ice-cold 1×PBS. For electron microscopy, 10 l samples were deposited on copper grids for 1 minute, blotted, stained with 10 l uranyl acetate for 1 minute, blotted again, air-dried for 5 minutes, and examined under transmission electron microscope.

Detection of Prostasomal miRNA-146a-5p mRNA Expression Total RNA was extracted from 200 l prostasome samples using miRcute miRNA isolation kit according to the manufacturer's protocol. Reverse transcription was performed using miRcute miRNA First-Strand cDNA Synthesis Kit (conditions: 37°C for 60 minutes). RT-PCR amplification was conducted using miRcute miRNA Detection Kit (SYBR Green) with U6 as internal reference. Relative quantification was calculated using the 2^(-ΔΔCT) method. PCR conditions: 94°C pre-denaturation for 2 minutes (1 cycle), 94°C denaturation for 20 seconds (40 cycles), and 60°C annealing for 34 seconds (40 cycles). Primer sequences are shown in Table 1 .

Western Blot Analysis of TLR-4, I B, p-I B, and TRAF6 Proteins Fifty mg of prostate tissue was minced, ground to fine powder in liquid nitrogen, and lysed in 500 l RIPA buffer. Total protein was extracted and subjected to electrophoresis and membrane transfer. Immunodetection was performed using TLR4 antibody (1:1000), TRAF6 antibody (1:1000), p-I Bα antibody (1:1000), and I Bα antibody (1:1200). Protein expression levels were quantified as the ratio of target protein gray value to internal reference gray value.

ELISA Detection of Serum TNF-α, IL-6, IL-8, and IFN-γ Serum concentrations of TNF-α, IL-6, IL-8, and IFN-γ were measured using ELISA kits according to the manufacturer' s instructions.

Statistical Analysis SPSS 22.0 software was used for statistical analysis. Normally distributed data with homogenous variance are expressed as mean ± standard deviation ($\bar{x}\pm s$) and compared using t-test between two groups or one-way ANOVA among multiple groups with LSD-t test for pairwise comparisons. Non-normally distributed data are expressed as median (interquartile range) [M(QR)] and compared using non-parametric tests. $P<0.05$ was considered statistically significant.

Results

Histopathological Examination of Prostate Tissue Under light microscopy, the model group exhibited severe prostate tissue damage compared with the normal group, including extremely irregular and deformed glandular lumens, nuclear pyknosis and necrosis of acinar epithelial cells, severe interstitial hemorrhage, and mild inflammatory cell infiltration. The miRNA-146a mimics group showed mild tissue damage with slightly disordered glandular architecture, necrotic epithelial cells, and no obvious inflammatory infiltration. The miRNA-146a inhibitor group displayed severe structural damage with disorganized glands, reduced luminal areas, epithelial cell shedding, numerous red blood cells in the stroma, and mild inflammatory infiltration. Low-dose *A. tomentosa* showed moderate damage with disorganized glands, irregular lumens, necrotic epithelial cells, obvious hemorrhage, and moderate inflammation. Medium-dose *A. tomentosa* exhibited milder damage with irregular acinar morphology and minimal inflammatory infiltration. High-dose *A. tomentosa* showed only mild damage with orderly acinar epithelium, regular lumens, minimal hyperplasia, loose connective tissue, and no inflammatory infiltration [Figure 1: see original paper].

Electron Microscopic Identification of Prostasomes Prostasomes extracted from rat prostatic fluid appeared as membrane vesicles 30-150 nm in diameter with characteristic "cup-shaped" morphology. Prostasomes were observed in all groups [Figure 2: see original paper].

Prostasomal miRNA-146a-5p Expression Compared with the normal group, all other groups showed significantly downregulated prostasomal miRNA-146a-5p mRNA expression ($P<0.01$). Relative to the model group, miRNA-146a-5p expression was upregulated in the medium- and high-dose *A. tomentosa* groups and the miRNA-146a mimics group ($P<0.01$), while it was further downregulated in the miRNA-146a inhibitor group ($P<0.01$) .

Prostate Tissue Protein Expression All groups showed upregulated TLR-4 protein expression compared with the normal group ($P<0.05$). Compared with the model group, TLR-4 expression was significantly reduced in the miRNA-146a mimics group and all *A. tomentosa* dose groups ($P<0.01$), with the high-dose group showing the most pronounced reduction, while the miRNA-146a inhibitor group showed increased expression ($P<0.05$). TRAF6 protein expression was upregulated in all groups versus the normal group ($P<0.01$) and was significantly reduced in the miRNA-146a mimics group and all *A. tomentosa* dose groups compared with the model group ($P<0.01$), with the high-dose group showing the greatest reduction. The miRNA-146a inhibitor group showed a non-significant upward trend in TRAF6 expression ($P>0.05$).

I Bα protein expression was downregulated in all groups except the miRNA-146a mimics group compared with the normal group ($P<0.05$). Compared with

the model group, I Bα expression was significantly upregulated in the miRNA-146a mimics and high-dose *A. tomentosa* groups (P<0.01). All groups showed upregulated p-I Bα expression versus the normal group (P<0.01). Compared with the model group, p-I Bα expression was significantly reduced in the miRNA-146a mimics group and all *A. tomentosa* dose groups (P<0.01), with the high-dose group showing the most marked reduction, while the miRNA-146a inhibitor group showed increased expression (P<0.01) [Figure 3: see original paper].

Serum Inflammatory Cytokine Levels All groups showed significantly elevated serum TNF-α levels compared with the normal group (P<0.01 or P<0.05). Compared with the model group, all groups except the miRNA-146a inhibitor group showed significantly reduced TNF-α levels (P<0.01 or P<0.05). Serum IL-6 levels were significantly elevated in all groups except the high-dose *A. tomentosa* group versus the normal group (P<0.01) and were significantly reduced in the miRNA-146a mimics group and all *A. tomentosa* dose groups compared with the model group (P<0.01 or P<0.05). Serum IL-8 levels were elevated in all groups except the high-dose *A. tomentosa* group compared with the normal group (P<0.01) and were significantly reduced in all groups except the miRNA-146a inhibitor group versus the model group (P<0.01 or P<0.05). Serum IFN-γ levels were significantly elevated in all groups compared with the normal group (P<0.01 or P<0.05) and were significantly reduced in the miRNA-146a mimics, medium-dose, and high-dose *A. tomentosa* groups compared with the model group (P<0.01) .

Discussion

CP/CPPS symptoms include varying degrees of chronic pelvic pain (prostate, perineum, urethra) and urinary dysfunction, significantly impacting quality of life. The estimated prevalence among Chinese men is approximately 8.4-25% [11], with recurrence rates rising to 50% with age [12]. Evidence-based systematic reviews indicate that primary therapies offering modest symptom relief include phytotherapy, α-blockers, anti-inflammatory drugs, and antibiotics, though the quality of evidence is moderate to low, suggesting unsatisfactory efficacy of current treatments [13]. The variable etiology and complex mechanisms of CP/CPPS contribute to its high incidence, refractory nature, and recurrence. Chinese herbal medicine has been widely applied as an effective alternative, attracting researcher and clinician attention due to minimal side effects and multimodal actions [5].

Traditional Chinese medicine lacks a specific disease name corresponding to CP/CPPS but recognizes its essence as a deficiency-excess complex, attributing pathogenesis to "kidney deficiency, damp-heat, and blood stasis" [14]. *Anemone tomentosa*, a perennial herb of the Ranunculaceae family primarily distributed in Guangxi and Yunnan provinces, represents authentic Zhuang medicine. With properties of acrid, slightly bitter, and neutral, it possesses "heat-clearing and dampness-removing, diuretic and strangury-relieving, qi-tonifying and kidney-

nourishing" effects [15], aligning closely with the fundamental CP/CPPS pathogenesis of "kidney deficiency with damp-heat." This study aimed to elucidate novel molecular mechanisms underlying CP/CPPS development, explain the efficacy and scientific basis of *A. tomentosa* in preventing and treating CP/CPPS, explore the advantages of ethnic medicine, and provide theoretical support for reducing recurrence and improving cure rates.

MicroRNAs are multifunctional non-coding RNAs that regulate gene expression post-transcriptionally. Toll-like receptors selectively recognize pathogen-associated molecular patterns, constituting the first line of immune defense. Excessive activation of TLR signaling pathways leads to inflammation and immune-related diseases, while miRNAs can finely regulate TLR signaling initiation, termination, and intensity, participating in immune-inflammatory pathogenesis [16]. TRAF6 enhances pro-inflammatory cytokine gene transcription to initiate inflammatory cascades and serves as a crucial adaptor protein for the TNF superfamily and TLR receptor superfamily [17]. TRAF6 activates NF- B signaling by activating I B kinase, promoting I B phosphorylation and degradation, thus acting as a key positive regulator and specific modulator of NF- B signal transduction [18]. Previous reports have confirmed that miR-146a exerts anti-inflammatory effects by targeting TRAF6. Studies demonstrate that miRNA-146a inhibits NF- B transcriptional activity and reduces downstream inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α release by suppressing TRAF6 expression in the TLR-4/NF- B pathway, ultimately inhibiting inflammatory responses [19]. Yang et al. [20] reported that exosome-derived miR-146a is upregulated in N9 microglial cells and negatively regulates TLR4 signaling by targeting TRAF6, significantly reducing IFN-β, TNF-α, and IL-1β expression.

Our study investigated the regulatory effect of prostasome-derived miRNA-146a on the TLR-4/NF- B pathway in EAP rats. The results demonstrate that miRNA-146a inhibits TLR-4/NF- B transcriptional activity and NF- B over-phosphorylation by suppressing TRAF6 expression, thereby reducing downstream inflammatory cytokines IL-6, IL-8, IFN-γ, and TNF-α release and alleviating local prostate inflammation. *A. tomentosa* intervention promoted prostasomal miRNA-146a-5p mRNA expression, suppressed TRAF6 expression, inhibited TLR-4/NF- B pathway activation, reduced downstream inflammatory factor release, and mitigated local prostate inflammation. The regulatory effect on the prostasome-derived miRNA-146a/TLR-4/NF- B pathway became more pronounced with increasing *A. tomentosa* dosage. These findings provide scientific evidence for the clinical application of *A. tomentosa* in CP/CPPS patients. Although the sample size was limited, this study preliminarily demonstrates that prostasome-derived miRNA-146a modulates TLR-4/NF- B pathway activation to participate in local prostate pathology in EAP rats, and that *A. tomentosa* exerts anti-prostatitis effects through this pathway. These results suggest that prostasomes represent a promising novel research direction in CP/CPPS pathogenesis.

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Author Contributions: LU Liangxi, WU Jinyu, and HUANG Zhimin conceived and designed the study, supervised implementation, and analyzed and interpreted the results. SHI Hong conducted feasibility analysis and experimental guidance. LU Liangxi drafted the manuscript. LU Liangxi and HUANG Zhimin prepared the animal models and performed experimental measurements. ZOU Han and ZHANG Zhiying collected and organized data. WANG Wenjie

performed statistical analysis and prepared figures. LU Liangxi and WU Jinyu revised the final manuscript and take responsibility for the paper. All authors approved the final version.

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Note: Figure translations are in progress. See original paper for figures.