Tropical Journal of Pharmaceutical Research May 2022; 21 (5): 927-932
ISSN: 1596-5996 (print); 1596-9827 (electronic)
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Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i5.3

Original Research Article

TWIST1 silencing attenuates intracranial aneurysms by inhibiting NF-κB signaling

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Sent for review: 5 January 2022 *Revised accepted: 15 April 2022*

Abstract

Purpose: *To investigate the effect of Twist family basic helix-loop-helix transcription factor 1 (TWIST1) on intracranial aneurysms.*
Methods: *A rat model of intracranial aneurysm was established by ligating the posterior branches of both the renal and left common carotid arteries. Pathological changes in the intracranial arterial wall were investigated using hematoxylin-eosin staining. TWIST1 expression was assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and western blot, while vascular smooth muscle cell apoptosis was investigated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Inflammation was evaluated using enzyme-linked immunosorbent assay (ELISA).*
Results: *Rats with intracranial aneurysms had degenerative changes in vessel wall structure. In intracranial aneurysm rats, TWIST1 was upregulated in arterial wall sections, and TWIST1 knockdown ameliorated the pathological changes to the arterial wall. TWIST1 silencing reduced serum tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) levels and suppressed vascular smooth muscle cell apoptosis in rats with intracranial aneurysms. TWIST1 knockdown increased phospho (p)-inhibitor of nuclear factor-κB (IκB) and decreased IκB and p-p65 in intracranial aneurysm rat arterial wall sections.*
Conclusion: *In intracranial aneurysms, silencing TWIST1 promoted vascular remodeling and suppresses vascular smooth muscle cell apoptosis and inflammation through inactivating NF-κB signaling, revealing TWIST1 silencing as a potential treatment strategy.*

Keywords: *TWIST1, Intracranial aneurysm, NF-κB signaling, Vascular smooth muscle, Vascular remodeling*

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INTRODUCTION

Intracranial aneurysm is a cerebrovascular disorder characterized by the expansion or dilation of blood vessels in the brain [1]. Intracranial aneurysm rupture results in serious complications such as vasospasm, subarachnoid hemorrhage, and death [2]. The mechanisms of intracranial aneurysm initiation and progression are unclear, and consequently, there are currently no effective treatments for the disease [3]. Therefore, determining the mechanisms of pathogenesis involved in intracranial aneurysm progression is urgently required.

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Pathological vascular remodeling and inflammation are implicated in intracranial aneurysm pathogenesis [4]. Vascular inflammation and other stimuli alter physiological hemodynamics in the cerebrovascular system, promote vascular smooth muscle cell apoptosis and migration, and activate vascular remodeling, which leads to intracranial aneurysm initiation and progression [4]. Therefore, inhibiting vascular inflammation and vascular smooth muscle cell apoptosis is a potential strategy for preventing intracranial aneurysms [4].

Twist family basic helix-loop-helix (BHLH) transcription factor 1 (TWIST1) participates in mesoderm specification and differentiation through binding to the nuclear distribution protein nudE homolog 1 (Nde1) E-box element of target genes [5]. Furthermore, TWIST1 is responsible for the migration, invasion, and epithelial-mesenchymal transformation of distinct cancer cells [5]. TWIST1 is an etiological gene of common vascular diseases [6]. Additionally, TWIST1 promotes the proliferation of smooth muscle cells during the progression of pulmonary hypertension [7] and is associated with the phenotypic modulation of vascular smooth muscle [8]. TWIST1 also induces endothelial cell proliferation and inflammation [9]. Therefore, TWIST1 may be involved in intracranial aneurysm progression. In this study, a rat model of intracranial aneurysm was established, and the effects of TWIST1 on vascular inflammation and vascular smooth muscle cell apoptosis were subsequently assessed.

EXPERIMENTAL

Intracranial aneurysm animal models

Male Sprague-Dawley rats (40 rats, 6 - 8 weeks old, and 200 - 300 grams in weight) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., (Shanghai, China). The rats were divided into sham (n = 10) and intracranial aneurysm (n = 30) groups. Experiments involving rats were approved by the Ethics Committee of Jiangxi Pingxiang People's Hospital (approval no. 2021022) and were conducted in accordance with the principles of the National Institutes of Health Laboratory Animal Care and Use Guidelines [10]. Rats in the model group were anesthetized by an intraperitoneal injection of 3% pentobarbital sodium, and the neck was incised to separate the subcutaneous connective tissue.

The external carotid artery was then isolated, ligated, and dissected. The right pterygopalatine artery was subsequently exposed and ligated, and the left common carotid artery was then isolated and ligated with a 5-0 surgical suture. The backs of the rats were incised, and the kidneys were pushed out. The renal capsule was separated, and the renal vein was exposed. The posterior branches of both renal arteries were ligated with a 7-0 surgical suture, and the muscularis and skin were subsequently sutured. The intracranial aneurysm rat model was confirmed using hematoxylin-eosin staining. Rats in the sham group had the right carotid bifurcation and bilateral renal arteries exposed without ligation. Rats with intracranial aneurysms were then divided into three groups: the intracranial aneurysm group (n = 10), the intracranial aneurysm with shTWIST1 group (n = 10), and the intracranial aneurysm with shNC group (n = 10). TWIST1-targeting short hairpin (sh) RNA (shTWIST1) and the negative control (shNC) were synthesized by Sangon Biotech Co. Ltd, (Shanghai, China) and were constructed into a lentiviral vector. Lentiviral vectors were then transfected into HEK-293T cells that package viral particles. The produced viral particles (1×10^8 transduction units) were injected into the rats through the caudal vein daily after the surgical induction. After 4 weeks of injections, the rats were anesthetized, and the intracranial cerebral aneurysm tissues were collected.

Hematoxylin-eosin staining

Intracranial cerebral aneurysm tissues were fixed in 4 % paraformaldehyde and embedded in paraffin. The tissues were then cut into 4 µm-thick sections. The sectioned tissues were dewaxed in xylene, dehydrated in gradient alcohol, and subsequently stained with hematoxylin (Solarbio, Beijing, China). Sections were then stained with eosin (Solarbio) and examined under an optical microscope (Shanghai Caikon Optical Instrument Co., Ltd, Shanghai, China).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Arterial wall sections were dewaxed in xylene, dehydrated in gradient alcohol, and subsequently incubated with Proteinase K (20 mg/mL; Sigma-Aldrich, St. Louis, MO, USA). The sections were then immersed in phosphate buffered saline with 0.5% H_2O_2 and incubated with 50 µL of TUNEL from the TUNEL *in situ* cell death assay kit (Roche, Basel, Switzerland). Following counterstaining with DAPI, the number of TUNEL-positive cells was counted under a microscope (Olympus, Tokyo, Japan).

Quantitative reverse transcription-PCR (qRT-PCR)

RNAs were isolated from arterial wall sections using the TRIzol kit (Invitrogen, Carlsbad, CA, USA) and were then reverse-transcribed into cDNAs. The PreTaq II kit (Takara, Dalian, Liaoning, China) was used for qRT-PCR analyses of TWIST1, IL-6, and TNF-α. The primer sequences are shown in Table 1. Expression levels of TWIST1, IL-6, and TNF-α were normalized to GAPDH.

Enzyme-linked immunosorbent assay (ELISA)

Rat blood samples were collected from the abdominal aorta and centrifuged at 1000 × g to obtain serum samples. Serum levels of TNF-α and IL-6 were measured using commercial ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot

Total proteins were extracted from intracranial cerebral aneurysm tissues using Western Blotting Lysis Buffer (Beyotime, Beijing, China) and were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electro-transferred onto PVDF membranes and blocked in 5% BSA, and the membranes were subsequently incubated overnight at 4 °C with the following primary antibodies: anti-TWIST1 and anti-β-actin (1:1500, Abcam, Cambridge, UK), anti- apoptosis regulator BAX (Bax) and anti-B-cell lymphoma 2 (Bcl-2) (1:2000, Abcam), anti-cleaved caspase-3 (1:2500, Abcam), anti-p65 and anti-p-p65 (1:3000, Abcam), anti-inhibitor of nuclear factor-κB (IκB) and anti-phospho (p)-IκB (1:3500, Abcam). Membranes were then incubated with horseradish peroxidase-labeled secondary antibody (1:4500; Abcam) and enhanced chemiluminescence (KeyGen, Nanjin, China) before analyzing the immunoreactivities.

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM) (n = 3), and were analyzed by Student's *t* test or one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software, version 11.5. A p value of < 0.05 was considered statistically significant.

RESULTS

TWIST1 knockdown ameliorates pathological changes in the intracranial arterial wall in rats with intracranial aneurysms

TWIST1 was upregulated in intracranial cerebral aneurysm tissues of rats with intracranial aneurysms compared to the sham group (Figure 1 A and B). Furthermore, the intracranial aneurysm rat model had thinner aneurysm walls than the sham group rats, suggesting that aneurysms were formed in the intracranial aneurysm rat model (Figure 1 C). Additionally, rats with intracranial aneurysms had expanded vascular walls and enlarged cerebral arteries (Figure 1 C). However, injection with shTWIST1 viral particles reduced TWIST1 expression (Figure 1 A and B), which ameliorated the previously observed degenerative changes to the vessel wall structure (Figure 1 C). Therefore, TWIST1 silencing promoted vascular remodeling in the intracranial aneurysm rat model.

Vascular smooth muscle cell apoptosis is suppressed by TWIST1 silencing

The number of TUNEL-positive vascular smooth muscle cells was higher in intracranial aneurysm model rats compared to the sham group rats (Figure 2A). Additionally, TWIST1 knockdown decreased the number of TUNEL-positive cells, indicating that cell apoptosis was suppressed (Figure 2 A). Moreover, Bcl-2 protein expression was reduced and Bax and cleaved caspase-3 protein expression were increased in the intracranial cerebral aneurysm tissues of rats with intracranial aneurysms (Figure 2 B). Furthermore, in the intracranial aneurysm rats, knockdown of TWIST1 increased Bcl-2 expression and decreased Bax and cleaved caspase-3 expression (Figure 2 B). These results suggest that TWIST1 silencing has an anti-apoptotic role in intracranial aneurysms.

Table 1: Primers for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
TWIST1	AGCAAGATTCAGACCCTCAAGC	CTCCATCCTCCAGACCGAGA
IL-6	GGATACCACTCCCAACAGACCT	CAAGTGCATCATCGTTGTTCATAC
TNF-α	TATGGCTCAGGGTCCAACTC	GGAAAGCCCATTTGAGTCCT
GAPDH	TCAACGACCACTTTGTCAAGCAGAGT	GCTGGTGGTCCAGGGGTCTTACT

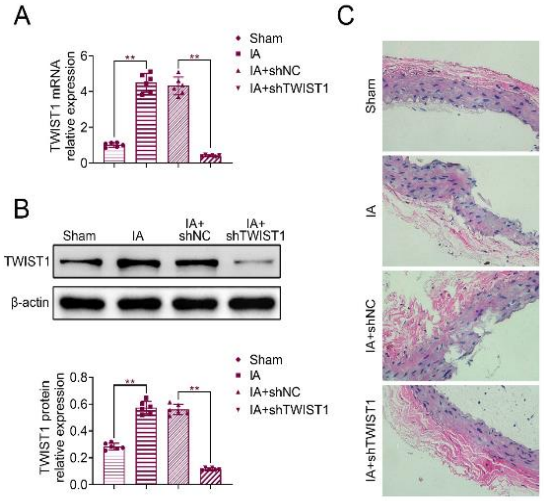

Figure 1: TWIST1 knockdown ameliorates the pathological changes to the intracranial arterial wall in rats with intracranial aneurysms. (A) TWIST1 mRNA expression was upregulated in intracranial cerebral aneurysm tissues of rats with intracranial aneurysms compared to the sham group, and injection with shTWIST1 reduced TWIST1 expression. (B) TWIST1 protein expression was upregulated in intracranial cerebral aneurysm tissues of rats with intracranial aneurysms compared to the sham group, and injection with shTWIST1 reduced TWIST1 expression. (C) Injection with shTWIST1 ameliorated the degenerative changes in vessel wall structure in rats with intracranial aneurysms. ** vs. sham or IA + shNC $p < 0.01$

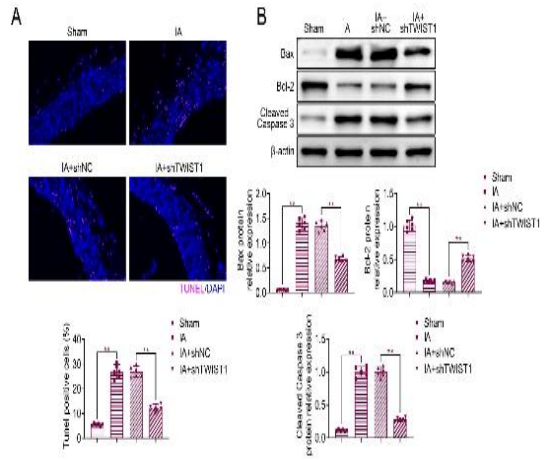

Figure 2: TWIST1 silencing suppresses vascular smooth muscle cell apoptosis. (A) Injection with shTWIST1 reduced the number of TUNEL-positive vascular smooth muscle cells in rats with intracranial aneurysms. Injection with shTWIST1 increased Bcl-2 protein expression while decreasing the expression of Bax and cleaved caspase-3 in rats with intracranial aneurysms; **$p < 0.01$ vs. sham or IA + shNC

Vascular inflammation is suppressed by silencing TWIST1

TNF-α and IL-6 mRNA expression were upregulated in rats with intracranial aneurysms (Figure 3 A), while silencing TWIST1 resulted in downregulated TWIST1 expression (Figure 3 A). Furthermore, TWIST1 silencing reduced the upregulated serum levels of TNF-α and IL-6 in rats with intracranial aneurysms (Figure 3 B), indicating that TWIST1 silencing has an anti-inflammatory effect on intracranial aneurysms.

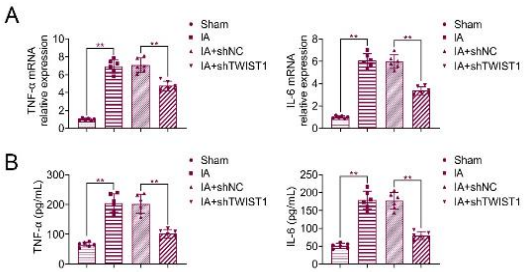

Figure 3: TWIST1 silencing suppresses vascular inflammation. (A) Injection with shTWIST1 decreased mRNA expression levels of TNF-α and IL-6 in rats with intracranial aneurysms. (B) Injection with shTWIST1 decreased serum levels of TNF-α and IL-6 in rats with intracranial aneurysms; **$p < 0.01$ vs. sham or IA + shNC

TWIST1 silencing suppresses the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling

There was no significant difference in p65 protein expression between intracranial aneurysm rats and sham-operated rats (Figure 4). Injection of shTWIST1 also had no significant effect on p65 expression (Figure 4). However, p-p65 increased in rats with intracranial aneurysms compared to the sham-operated rats (Figure 4), but p-p65 was decreased during TWIST1 knockdown (Figure 4). Furthermore, TWIST1 silencing enhanced IκB expression and reduced p-IκB in rats with intracranial aneurysms (Figure 4), revealing that TWIST1 silencing suppressed the activation of NF-κB signaling in intracranial aneurysms.

DISCUSSION

Genome-wide genotyping and transcriptomic analyses have revealed that TWIST1 expression is involved in vascular smooth muscle cell calcification and proliferation [6]. Furthermore, vascular smooth muscle cell proliferation, apoptosis, and migration influence pathological vascular remodeling and contribute to intracranial aneurysm progression [4].

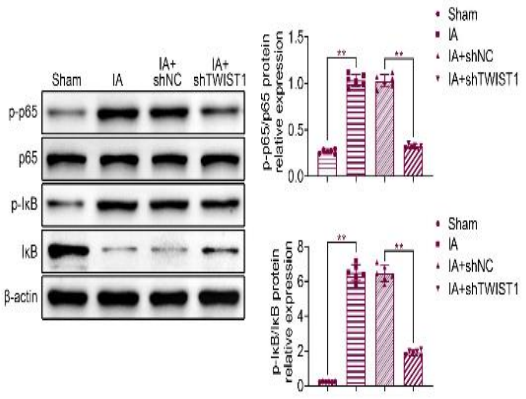

Figure 4: TWIST1 silencing suppresses the activation of NF-κB signaling. TWIST1 knockdown in rats with intracranial aneurysms enhanced IκB expression and reduced p-IκB and p-p65 compared to the shNC control rats; $^{**}p < 0.01$ vs. sham or IA + shNC

This study investigated the effects of TWIST1 on apoptosis, vascular remodeling, and inflammation in intracranial aneurysms.

It has been previously shown that TWIST1 is upregulated in carotid injury model rat vessels [8]. Results in our study coincide with this, as TWIST1 was highly expressed in the aneurysm walls of rats with intracranial aneurysms compared to the sham-operated rats. Pathological conditions induced by ligation of the posterior branches of both renal arteries and left common carotid arteries alter the cerebral vasculature blood flow, accelerate extracellular matrix degradation, and induce vascular smooth muscle cell apoptosis and migration [11].

Vascular smooth muscle apoptosis and migration results in the loss of the arterial wall smooth muscle layer, leading to the initiation of vascular remodeling during intracranial aneurysm development [12]. Furthermore, inhibiting vascular smooth muscle cell apoptosis suppresses vascular remodeling, which contributes to the intervention of intracranial aneurysms [11]. TWIST1 also regulates smooth muscle cell homeostasis [8]. In our study, TWIST1 knockdown ameliorated the integrity of the tunica intima and the structure of vascular smooth muscle cells, as well as alleviated intracranial aneurysm progression. Moreover, TWIST1 silencing increased Bcl-2 protein expression and decreased Bax and cleaved caspase-3 protein expression, inhibiting vascular smooth muscle cell apoptosis in rats with intracranial aneurysms. However, the effects of TWIST1 on vascular smooth muscle cell proliferation and migration, as well as extracellular matrix production, warrant further investigation.

Inflammatory responses stimulate vascular smooth muscle cell apoptosis and impair vessel structural integrity, leading to local arterial wall dilation during aneurysmal pathogenesis [13]. Infiltration of macrophages, monocytes, or leukocytes into the vascular wall and subsequent endothelial damage promotes the release of proinflammatory factors, such as TNF-α, IL-1β, and interferon-γ, activating apoptotic signaling in vascular smooth muscle cells [14]. Furthermore, the suppression of vascular inflammation represses cerebral aneurysm development [15].

TWIST1 induces endothelial cell inflammation and participates in vascular leakiness in atherosclerosis [9]. In this study, TWIST1 knockdown reduced TNF-α and IL-6 mRNA expression and serum levels, as well as inflammation, in rats with intracranial aneurysms.

NF-κB signaling is essential for the expression of intercellular adhesion molecules and proinflammatory molecules. Furthermore, NF-κB signaling regulates the inflammatory, migrative, and apoptotic responses of vascular smooth muscle cells. Inhibition of NF-κB signaling suppresses the inflammatory response and reduces intracranial aneurysm formation and rupture [16]. The tryptophan-arginine (Trp-Arg) domain of TWIST1 binds to the NF-κB subunit transcription factor p65 (RELA) to regulate interleukin-8 (IL-8) production [17]. TWIST1 promotes the activation of NF-κB signaling to induce the epithelial-mesenchymal transition of papillary thyroid carcinoma [17]. This study shows that injection of shTWIST1 enhanced the expression of the NF-κB inhibitor, IκB, while reducing p-p65 in rats with intracranial aneurysms, indicating that TWIST1/NF-κB is potentially involved in intracranial aneurysm progression.

CONCLUSION

The findings of this study demonstrate that TWIST1 silencing promotes vascular remodeling while suppressing vascular smooth muscle cell apoptosis and inflammation by inactivating NF-κB signaling. Therefore, TWIST1 has a potential as a novel therapeutic target to treat intracranial aneurysms.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Deng Wang designed the study and performed the experiments. Chengfu Peng supervised the data collection, analyzed the data, and interpreted the data. Dan Lai prepared the manuscript for publication and reviewed the manuscript draft. All authors read and approved the manuscript.

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REFERENCES

1. *Keedy A. An overview of intracranial aneurysms. Mcgill J Med 2006; 9(2): 141-146.*
2. *Filipce V, Caparoski A. The Effects of Vasospasm and Re-Bleeding on the Outcome of Patients with Subarachnoid Hemorrhage from Ruptured Intracranial Aneurysm. Pril 2016; 36(3): 77-82.*
3. *Hayashi K, Kataoka H, Minami M, Ikedo T, Miyata T, Shimizu K, Nagata M, Yang T, Yamamoto Y, Yokode M, et al. Association of zinc administration with growth suppression of intracranial aneurysms via induction of A20. J Neurosurg 2020;134(3):992-998.*
4. *Penn D, Witte S, Komotar R, Connolly E. The role of vascular remodeling and inflammation in the pathogenesis of intracranial aneurysms. J Clin Neurosci 2013; 21(1):28-32.*
5. *Qin Q, Xu Y, He T, Qin C, Xu J. Normal and disease-related biological functions of Twist1 and underlying molecular mechanisms. Cell Res 2012; 22(1): 90-106.*
6. *Chasman DI, Nurnberg ST, Guerraty MA, Wirka RC, Rao HS, Pjanic M, Norton S, Serrano F, Perisic L, Elwyn S, et al. Genomic profiling of human vascular cells identifies TWIST1 as a causal gene for common vascular diseases. PLoS Genet 2020; 16(1): e1008538.*
7. *Fan Y, Gu X, Zhang J, Sinn K, Klepetko W, Wu N, Foris V, Solymosi P, Kwapiszewska G, Kuebler WM. TWIST1 Drives Smooth Muscle Cell Proliferation in Pulmonary Hypertension via Loss of GATA-6 and BMPR2. Am J Respir Crit Care Med 2020; 202(9): 1283-1296.*
8. *Zhang J, Guo J-R, Wu X-L, Wang X, Zhu Z-M, Wang Y, Gu X, Fan Y. TWIST1 induces phenotypic switching of vascular smooth muscle cells by downregulating p68 and microRNA-143/145. FEBS Open Bio 2021; 11(3): 932-943.*
9. *Mahmoud MM, Kim HR, Xing R, Hsiao S, Mammoto A, Chen J, Serbanovic-Canic J, Feng S, Bowden NP, Maguire R et al. TWIST1 Integrates Endothelial Responses to Flow in Vascular Dysfunction and Atherosclerosis. Circulation Res 2016; 119(3): 450-462.*
10. *Council NR. Guide for the care and use of laboratory animals. 2010.*
11. *Li X-G, Wang Y-B. SRPK1 gene silencing promotes vascular smooth muscle cell proliferation and vascular remodeling via inhibition of the PI3K/Akt signaling pathway in a rat model of intracranial aneurysms. CNS Neurosci Ther 2019; 25(2): 233-244.*
12. *Shi Y, Li S, Song Y, Liu P, Yang Z, Liu Y, Quan K, Yu G, Fan Z, Zhu W. Nrf-2 signaling inhibits intracranial aneurysm formation and progression by modulating vascular smooth muscle cell phenotype and function. J Neuroinflam 2019; 16(1): 185-185.*
13. *Penn D, Komotar R, Connolly E. Hemodynamic mechanisms underlying cerebral aneurysm pathogenesis. J Clin Neurosci 2011; 18(11):1435-1438.*
14. *Kanematsu Y, Kanematsu M, Kurihara C, Tada Y, Tsou T-L, van Rooijen N, Lawton MT, Young WL, Liang EI, Nuki Y et al. Critical roles of macrophages in the formation of intracranial aneurysm. Stroke 2011; 42(1): 173-178.*
15. *Fukuda M, Fukuda S, Ando J, Yamamoto K, Yonemoto N, Suzuki T, Niwa Y, Inoue T, Satoh-Asahara N, Hasegawa K et al. Disruption of P2X4 purinoceptor and suppression of the inflammation associated with cerebral aneurysm formation. J Neurosurg 2021; 134(1): 102-114.*
16. *Aoki T, Frösen J, Fukuda M, Bando K, Shioi G, Tsuji K, Ollikainen E, Nozaki K, Laakkonen J, Narumiya S. Prostaglandin E2–EP2–NF-κB signaling in macrophages as a potential therapeutic target for intracranial aneurysms. Sci Signal 2017; 10(465): eaah6037.*
17. *Li S, Kendall SE, Raices R, Finlay J, Covarrubias M, Liu Z, Lowe G, Lin Y-H, Teh YH, Leigh V et al. TWIST1 associates with NF-κB subunit RELA via carboxyl-terminal WR domain to promote cell autonomous invasion through IL8 production. BMC Biol 2012; 10: 73-73.*