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**Genistein inhibits oxidative stress and alleviates vascular remodeling in hypertension through the GSK3 β /FYN/Nrf2 pathway**

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Original article

# Genistein inhibits oxidative stress and alleviates vascular remodeling in hypertension through the GSK3β/FYN/Nrf2 pathway

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ABSTRACT

Hypertension raises cardiovascular risk *via* vascular remodeling, worsened by oxidative stress-linked VSMC dysfunction. Genistein (Gen), an antioxidant isoflavone, protects against cardiovascular diseases, but its role in hypertensive remodeling is unclear. This study explores if Gen eases remodeling by inhibiting oxidative stress *via* the GSK3β/FYN/Nrf2 pathway. *In vivo*, spontaneously hypertensive rats (SHR) and normotensive WKY rats got Gen (10–20 mg·kg$^{-1}$·d$^{-1}$) or valsartan (Val) for 10 weeks; aortic tissues were tested for morphology (HE/Masson), proliferation (PCNA), migration (MMP2/9), oxidative stress (NOX4), and VSMC phenotype (*α*-SMA/OPN). *In vitro*, SHR/WKY VSMCs were treated with Gen (10–40 μmol·L$^{-1}$) to check proliferation (EdU), migration (scratch/Transwell), ROS, and Nrf2 markers. Network pharmacology predicts Gen inhibits vascular remodeling *via* reducing hypertension-related oxidative stress, verified by EdU, scratch, Transwell, and Western blot. Gen reduced SHR blood pressure, vascular wall thickness, and fibrosis, reversing PCNA, MMP2/9, NOX4, OPN overexpression and *α*-SMA underexpression. In VSMCs, it dose-dependently inhibited proliferation/migration, reduced ROS, and restored Nrf2 pathway activity. Network analysis found 52 shared targets, with GSK3β, FYN, Nrf2 as key nodes. Gen and pGSK3β inhibitor SNP reversed GSK3β/FYN overexpression and Nrf2 underexpression; Nrf2 inhibitor ML385 barely affected pGSK3β/FYN. Thus, Nrf2 may lie downstream of GSK3β and FYN. Gen inhibits oxidative stress injury in the aortic tissue of SHR rats through the GSK3β/FYN/Nrf2 pathway, thereby reducing vascular remodeling in hypertension, lowering blood pressure, and exerting a protective effect on vascular lesions in hypertension. These findings provide new experimental evidence for Gen as a potential therapeutic agent against hypertensive vascular remodeling.

## 1. Introduction

Hypertension is the leading independent risk factor for global disease burden and mortality, closely related to major diseases such as heart failure [1], myocardial infarction [2], stroke [3], vascular dementia [4], and chronic kidney disease [5]. It has affected over one billion young people worldwide [6] and is a key pathological basis for multi-system organ damage. Its occurrence and development are accompanied by vascular remodeling (VR) [7], which is characterized by pathological phenomena such as thickening of the vascular media, abnormal accumulation of extracellular matrix, phenotypic transformation of vascular smooth muscle cells (VSMCs), and dysregulation of vascular tone [8], seriously impairing the functions of target organs such as the heart, brain, and kidneys. VR is driven by multiple factors in a coordinated manner, with oxidative stress being one of the core mechanisms [9]. Under normal physiological conditions, the vascular oxidation and antioxidant systems maintain a balance; in hypertension, continuous high pressure activates cellular signaling pathways, upregulates the expression of NADPH oxidase, and promotes the generation of reactive oxygen species (ROS), disrupting the redox balance and triggering oxidative stress [10, 11]. ROS, as signaling molecules, further activate pathways such as NF-κB [12] and MAPK [13], promoting VSMC proliferation, migration, and extracellular matrix synthesis, and accelerating the process of VR. In the endogenous antioxidant defense system, nuclear factor erythroid 2-related factor 2 (Nrf2) forms a protective network for ROS clearance by activating antioxidant genes such as heme oxygenase-1 (*HO-1*) and quinone oxidoreductase 1 (*NQO1*), alleviating oxidative stress-mediated vascular damage and inhibiting hypertension-related VR. However, under continuous high pressure, the Nrf2 defense system may become decompensated due to excessive oxidative stress pressure, exacerbating vascular damage [14, 15]. Therefore, finding reliable targets to activate Nrf2 and inhibit oxidative stress has become a key direction for improving hypertension-re-

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lated vascular remodeling.

Genistein (Gen) is a common isoflavone compound found in leguminous plants. Due to its chemical structure being similar to that of estradiol, it can specifically bind to estrogen receptors ERα and ERβ, exhibiting estrogenic activity and is classified as a phytoestrogen [16]. Although its health promotion mechanism is not yet fully understood, it has been listed by the US Food and Drug Administration as a dietary supplement and is widely consumed globally through legume foods and supplements [17]. Studies have shown that Gen can regulate lipid metabolism and improve bone remodeling balance, potentially preventing chronic diseases such as cardiovascular diseases [18], obesity metabolic syndrome [19], and postmenopausal osteoporosis [20], and its health benefits and molecular mechanisms are receiving increasing attention.

GSK3β plays a critical regulatory role in vascular remodeling [21]. As an upstream hub of the pathway, activated GSK3β promotes nuclear translocation of the downstream kinase FYN [22-24], which in turn suppresses the activity of the transcription factor Nrf2. This leads to Nrf2 nuclear export, degradation, and downregulation of downstream antioxidant gene expression, ultimately exacerbating oxidative stress and vascular injury. Conversely, inhibition of GSK3β attenuates the negative regulation of Nrf2 by FYN, allowing Nrf2 to accumulate in the nucleus and activate antioxidant defenses, thereby alleviating vascular remodeling [25, 26]. Notably, plant-derived bioactive components, such as flavonoids and polyphenols, often act through the Nrf2 antioxidant signaling axis in the prevention and treatment of various metabolic and cardiovascular diseases [27]. For instance, in a hyperglycemic environment, resveratrol enhances the antioxidant capacity of osteoblasts by promoting Nrf2 nuclear translocation *via* AKT signaling while inhibiting its GSK3β/FYN-mediated nuclear export [28]. Ginseng and its active components exert protective effects across diabetes and its complications by activating the Nrf2 antioxidant pathway [29]. As one of the primary active constituents of ginseng, ginsenoside Rb1 can activate the intracellular Nrf2 antioxidant signaling pathway, thereby playing a central protective role in various cardiovascular and cerebrovascular diseases [30]. These findings collectively suggest that targeting Nrf2 and its upstream regulatory network may represent a common mechanism through which plant-derived compounds exert broad cardiovascular and metabolic protective effects. Based on the above regulatory relationship and existing research, we propose the hypothesis that Gen may inhibit oxidative stress damage by intervening in the GSK3β/FYN/Nrf2 pathway, thereby ameliorating hypertension-associated vascular remodeling.

Network pharmacology integrates multiple disciplines to construct a "multi-component–multi-target–multi-pathway" framework, breaking through the traditional single-target research model [31], providing new strategies for the modernization of traditional Chinese medicine, and revealing the clinical effect mechanisms of multi-component networked synergy in medicinal plants. In this study, the spontaneously hypertensive rat (SHR) was used as the model in the *in vivo* experiment, with the Kyoto Wistar rat (WKY) as the control; in the *in vitro* experiment, primary vascular smooth muscle cells from both were used to construct a hypertension-related vascular remodeling model. By leveraging network pharmacology and molecular docking, the mechanism by which Gen improves oxidative stress, inhibits abnormal activities of smooth muscle cells, and thereby inhibits vascular remodeling was explored, providing a theoretical basis for the clinical treatment of hypertension.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Genistein (≥ 98%, B21039) was purchased from Shanghai Yuanye (Shanghai, China). MMP9 (1∶1000, BM4089), MMP2 (1∶1000, BM4075), PCNA (1∶5000, 10205-2-AP), α-SMA (1∶1000, BM3902), OPN (1∶1000, Proteintech, 22952-1-AP), Nrf2 (1∶2000, Proteintech, 16396-1-AP), HO-1 (1∶1000, Proteintech, 10701-1-AP), NQO1 (1∶1000, Proteintech, 11451-1-AP), GSK3β (1∶5000, Proteintech, 82061-1-RR), pGSK3β (1∶2000, Proteintech, 67558-1-Ig), FYN (1∶5000, Proteintech, 60330-1-AP), GAPDH (1∶50 000, Proteintech, 60004-1-Ig), β-actin (1∶20 000, Proteintech, 66009-1-Ig) and EdU Imaging Kits, all purchased from APE × Bio (Houston, Texas, USA). Dulbecco's modified Eagle's medium (DMEM), trypsin, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA).

### 2.2. Animal experiments

Fourteen male WKY rats and twenty-eight male SHR rats were used for the experiment. They were acclimatized in a standard laboratory with a temperature of 26 ± 1 °C, a relative humidity of 55%, and a 12-h light/dark cycle for one week, with free access to food and water. Subsequently, they were randomly divided into six groups of six rats each: the WKY group, the WKY + Gen-H group, the SHR group, the SHR + Gen-L group, the SHR + Gen-H group, and the SHR + valsartan (Val) group. The low dose of Gen was 10 $mg \cdot kg^{-1} \cdot d^{-1}$, and the high dose was 20 $mg \cdot kg^{-1} \cdot d^{-1}$. The Val group was 10 $mg \cdot kg^{-1} \cdot d^{-1}$. Gen and Val were prepared as 20 $mg \cdot mL^{-1}$ stock solutions and dissolved in normal saline for daily gavage intervention for 10 weeks. Blood pressure was monitored every 14 days (± 6 h) using non-invasive tail artery pressure measurement (Model BP-2010A, Softron Biotechnology, Beijing, China). After the intervention, the rats were anesthetized with sodium pentobarbital, blood was collected for serological analysis, and the thoracic aorta was removed. Half of it was used for pathological sections, and the remaining tissue was stored at −80 °C. All experimental procedures strictly followed the ARRIVE Guidelines 2.0 and were approved by the Bioethics Committee of Shihezi University (Approval No. A2025-622).

### 2.3. Measurement of blood pressure

During the experiment, blood pressure was monitored every 14 days (± 6 h) at 10∶00 ± 30 min daily using non-invasive tail artery pressure measurement, and body weight was recorded simultaneously. Before measurement, the rats were pre-adapted on a 37 °C operating table, fixed, and the tail artery was located. The automatic pressure measurement was conducted three times (with an interval of ≥ 5 min), and the abnormal values were excluded to obtain the average value. After the intervention, the thoracic aorta was removed, fixed in 4% paraformaldehyde, and embedded in paraffin.

### 2.4. Cell culture

Select 6–8 week-old male WKY and SHR rats, anesthetize them, and remove the aorta. Store it in double-antibody buffer solution, rinse it with PBS, then separate the outer membrane and inner membrane while retaining the middle layer and cutting it into pieces. Digest the tissue with collagenase I, terminate the reaction with complete medium, centrifuge, resuspend the cells, and inoculate for cultivation. Change the medium for the first time after 72 h, and do so daily thereafter. When the cell confluence reaches 80%–90%, perform subculture. During subculture, clean, digest the cells, centrifuge and resuspend them, and inoculate them into new dishes. For the P1–P3 generations, gradually reduce the serum concentration in the DMEM medium. Observe under a microscope and identify using cell immunofluorescence method. VSMCs were isolated from SHR and WKY rats as shown in Figs. S1A and S1B. Cells from passages 3 to 6 were used in subsequent experiments.

### 2.5. Hematoxylin-eosin staining

First, the aortic smooth muscle was fixed in 4% paraformaldehyde. Then, before staining with hematoxylin and eosin (H&E), the vascular tissue was dehydrated, embedded, sectioned and dewaxed. Finally, the sections were stained and mounted for microscopic observation.

### 2.6. Masson staining

Vascular tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Subsequently, the vascular sections were stained for collagen according to the Masson's trichrome method to determine the degree of fibrosis. Finally, the sections were stained and mounted for microscopic observation of the fibrotic areas.

### 2.7. Immunohistochemistry (IHC)

The continuous sections of vascular tissue were incubated with specific antibodies against PCNA, MMP2 and MMP9. Subsequently, the corresponding secondary antibodies were applied to the sections. Then, the sections were stained with 3,3′-diaminobenzidine to obtain positive results, which appeared as a brown color. Finally, microscopic observation was conducted.

### 2.8. Western blot

Extract protein from vascular tissues or aortic smooth muscle cells. Perform Western blotting as described in previous work [32]. The antibodies include: PCNA, MMP2/9, $\alpha$-SMA, OPN, NOX4, Nrf2, HO-1, NQO1, pGSK3β, GSK3β, FYN, GAPDH and β-actin. To normalize the data, we use GAPDH and β-actin as references.

### 2.9. EdU assay for cell proliferation

Vascular smooth muscle cells were seeded in 12-well plates at a density of $8 \times 10^3$ cells per well. After adhering to the plates, the cells were treated with 10, 20, and 40 μmol·L$^{-1}$ Gen for 24 h. EdU was added according to the manufacturer's instructions, and the nuclei were stained with Hoechst 33342. After incubation, images were captured under a fluorescence microscope at appropriate wavelengths. Five random areas in each group were selected to assess EdU incorporation, and the proportion of EdU-positive cells was calculated using ImageJ software.

### 2.10. Scratching experiment

Uniformly inoculate VSMCs into the medium-sized dish. When the cell confluence reaches 90%, use the tip of a 1 mL pipette to draw vertical lines at the bottom of the culture plate. After 24 h of line drawing, take pictures of the migration area in the selected region and compare them with the images at 0 h. Take at least five pictures for each well, and then use ImageJ software to quantitatively analyze the migration area of each group.

### 2.11. Transwell assay

VSMCs were uniformly inoculated in Transwell chambers. 200 μL of cell suspension was added to the upper chamber, and 700 μL of complete medium containing 10% FBS was injected into the lower chamber. After the cells adhered, the medium in the lower chamber was replaced with medium containing 10, 20, and 40 μmol·L$^{-1}$ Gen, and the cells were further cultured for 24 h. The chambers were then removed, rinsed with PBS, fixed with 4% paraformaldehyde for 15 min, stained with 0.1% crystal violet at room temperature for 15 min, washed with PBS three times, and the non-migrated cells in the upper chamber were removed. Five 100 × fields were randomly selected under an inverted microscope, and the number of migrated cells in the lower chamber was counted to calculate the migration rate for analysis.

### 2.12. Network pharmacology

The potential targets and pathways of Gen in oxidative stress and hypertension-induced vascular remodeling were predicted through network pharmacology. The targets of Gen were queried using three databases: SwissTargetPrediction (http://www.swisstargetprediction.ch/), HERB (http://herb.ac.cn/), and TargetNet (http://targetnet.scbdd.com/). The targets related to hypertension-induced vascular remodeling and oxidative stress were obtained from five databases: GeneCards (https://www.genecards.org/), OMIM (http://omim.org/), PharmGKB (https://www.pharmgkb.org/), CTD (https://ctdbase.org/), and TTD (https://db.idrblab.net/ttd/). All targets were converted into UniProt ID format through UniProt (https://www.uniprot.org/), and a Venn diagram was drawn using the Omicshare (https://www.omicshare.com/) database to screen for common targets. Then, a PPI was constructed using String (https://string-db.org/), and finally, Cytoscape 3.9.1 software was used to visualize the PPI and predict KEGG and GO enrichment pathways.

### 2.13. Molecular docking

Verification of key targets by molecular docking technology: the 3D structure of Gen was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/), and the appropriate protein-ligand complex structures were downloaded from the PDB database (https://www.rcsb.org/). Autodock software was used to preprocess Gen and the targets respectively, and then molecular docking was performed using PyMOL software. The binding degree of the protein and the small molecule was evaluated by measuring the binding energy.

### 2.14. Statistical analysis

All the test data are based on the results of 3 to 7 replicate samples. Statistical analysis was performed using one-way ANOVA with GraphPad Prism software (version 9.0, GraphPad Software Inc., San Diego, CA, USA) for comparisons between multiple groups. All results are presented as mean ± SEM.

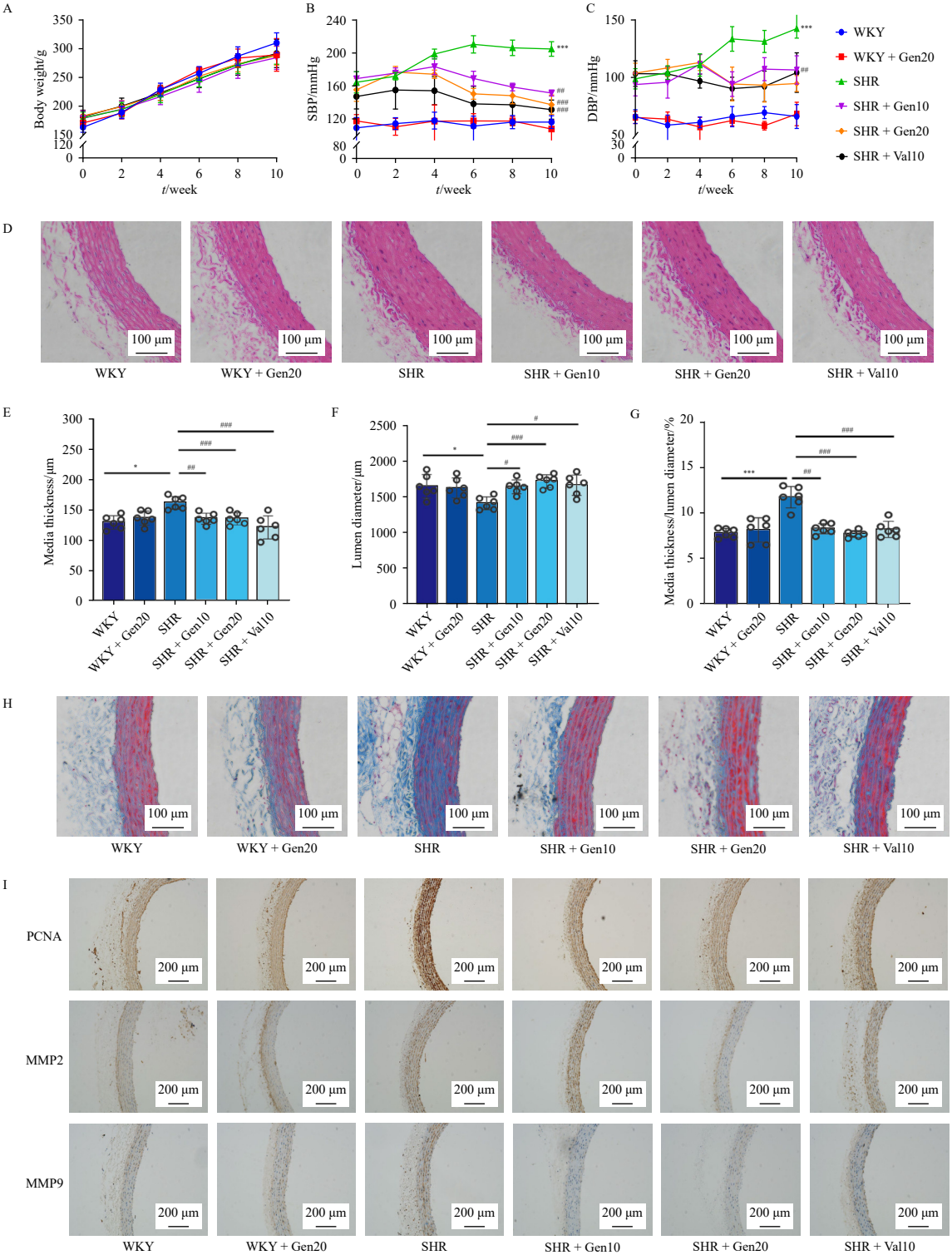
## 3. Results

### 3.1. Gen lowers the blood pressure of SHR rats and improve their vascular proliferative remodeling

After one week of adaptive feeding, baseline blood pressure was measured and physiological indicators were monitored in WKY and SHR rats. After 10 weeks of drug intervention, there was no difference in body weight among the groups and the growth curves were normal as shown in Fig. 1A. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the SHR + Gen low-dose (10 mg·kg$^{-1}$) and high-dose (20 mg·kg$^{-1}$) groups decreased after 4 weeks of intervention and was significantly lower than that of the SHR group at week 10, with the high-dose group approaching the positive drug Val group (Figs. 1B and 1C). Meanwhile, the concentration of angiotensin II (AngII) in the serum was detected using ELISA as shown in Fig. S2A. The content of AngII in SHR rats was higher than that in WKY rats. After administration of Gen, the content of AngII decreased.

Histological examination showed that in HE staining, the vascular structure of the WKY group was normal, the media was

thickened and the lumen was narrowed in the SHR group, and the structure improved after Gen and Val intervention (Figs. 1D–1G). Masson trichrome staining as shown in Fig. 1H indicated that the collagen fibers in the WKY group were orderly, the collagen deposition was disordered in the SHR group, and the deposition decreased after Gen and Val intervention. IHC as shown in Fig. 1I indicated that the expression of PCNA (proliferation marker), MMP2/9 (migration-promoting proteins) was enhanced in the SHR group, and weakened after Gen and Val intervention. There was no difference between the WKY + Gen-H (20 mg·kg$^{-1}$) group and the control group. Immunofluorescence double staining showed that the expression of OPN and NOX4 was upregulated and the expression of $\alpha$-SMA was downregulated in the SHR group, and the opposite was observed after Gen and Val treatment (Figs. 1J and 1K). Moreover, there was no difference between the WKY + Gen-H (20 mg·kg$^{-1}$) group and the WKY group in the above indicators. Concurrently, we detected the level of NOX4 in the serum of hypertensive rats, the results show that the content of NOX4 in the serum of SHR rats is higher than that in normotensive WKY rats; after Gen and Val intervention, the content of NOX4 in the serum of SHR rats decreased. Meanwhile, the content of NOX4 in the serum of WKY rats treated with Gen alone showed no significant difference compared with that of normal WKY rats (Fig. S2B). The above results indicate that Gen can inhibit the proliferation and remodeling of aortic vascular tissue in SHR rats and alleviate oxidative stress damage.



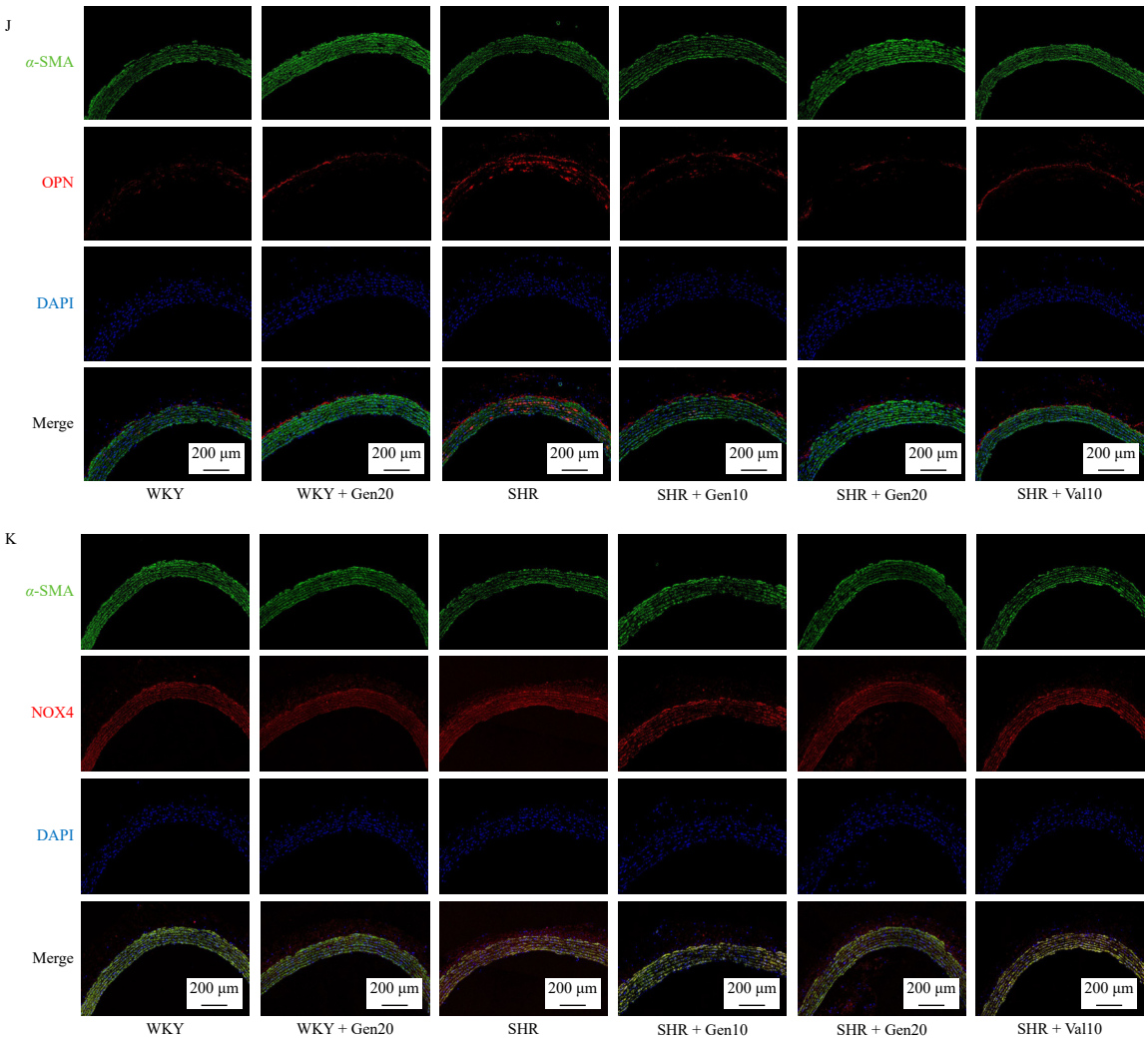



**Fig. 1** The protective effect of Gen on the blood vessels and blood pressure of SHR rats. (A–C) Gen reduces blood pressure in SHR rats. (D–G) Media thickness, lumen diameter, and their ratio were quantified by H&E staining for each group (H&E stain, 100 ×). (H) Masson staining of SHR rats. (I) IHC for the detection of PCNA, MMP2/9 expression in the aorta (200 ×). (J and K) Immunofluorescence staining for the detection of *α*-SMA, OPN and NOX4 expression in the aorta (200 ×). All data are presented as mean ± SEM ($n$ = 6). $P$-values were determined by ANOVA followed by a post hoc Tukey test, $^{*}P < 0.05$, $^{***}P < 0.001$, SHR *vs* WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR *vs* SHR + Gen/Val.

### *3.2. Gen inhibits the proliferation and phenotypic transformation of vascular smooth muscle cells in SHR rats*

To determine the concentration of Gen that inhibits the proliferation of vascular smooth muscle cells, CCK-8 assay was performed at concentrations of 10, 20, and 40 μmol·L$^{-1}$ (Fig. S1D). Additionally, CCK-8 assay indicated that Gen at concentrations below 90 μmol·L$^{-1}$ had no significant toxicity to vascular smooth muscle cells (Fig. S1C). EdU incorporation experiments (Figs. 2A and 2B) demonstrated that the proliferative activity of VSMCs from SHR was significantly higher than that from normotensive rats (WKY). After intervention with different concentrations of Gen, the proportion of EdU-positive cells in VSMCs from SHR decreased in a dose-dependent manner, while high-dose Gen treatment had no significant effect on the proliferation of normal VSMCs from WKY. Western blot analysis (Figs. 2C and 2F) further confirmed that the expression level of proliferating cell nuclear antigen (PCNA) in VSMCs from the SHR group was significantly upregulated, and Gen intervention could effectively inhibit its expression. High-dose Gen treatment had no significant effect on PCNA expression in the normal WKY group. These results suggest that Gen can selectively inhibit the abnormal proliferation of VSMCs under hypertensive conditions without significantly interfering with the physiological functions of normal VSMCs.

The expression levels of *α*-SMA and OPN in VSMCs were detected by Western blotting (Figs. 2D–2F). The results showed that compared with the WKY group, the expression of OPN protein in VSMCs of SHR rats was upregulated, while the expression of *α*-SMA protein was downregulated. After treatment with low, medium and high doses of Gen, the expression of OPN protein gradually weakened, while the expression of *α*-SMA protein gradually increased. This indicates that Gen can inhibit the transformation of VSMCs from the contractile type to the synthetic type in SHR rats.

### *3.3. Gen inhibits the migration of vascular smooth muscle cells in SHR rats*

The horizontal migration ability of VSMCs was analyzed through scratch assays, and their transmembrane longitudinal migration ability was detected by Transwell assays. Meanwhile, the expression levels of migration-related proteins MMP2 and MMP9 were examined by Western blotting to explore the effect of Gen on the migration of VSMCs in SHR rats. The results of the scratch test are shown in Figs. 3A and 3C. The lateral migration area of VSMCs in the SHR group increased after scratching compared with the WKY group. However, after administration of low, medium and high doses of Gen, the lateral migration ability of cells gradually decreased compared with the SHR group, indicating that Gen can inhibit the lateral migration ability of VSMCs in

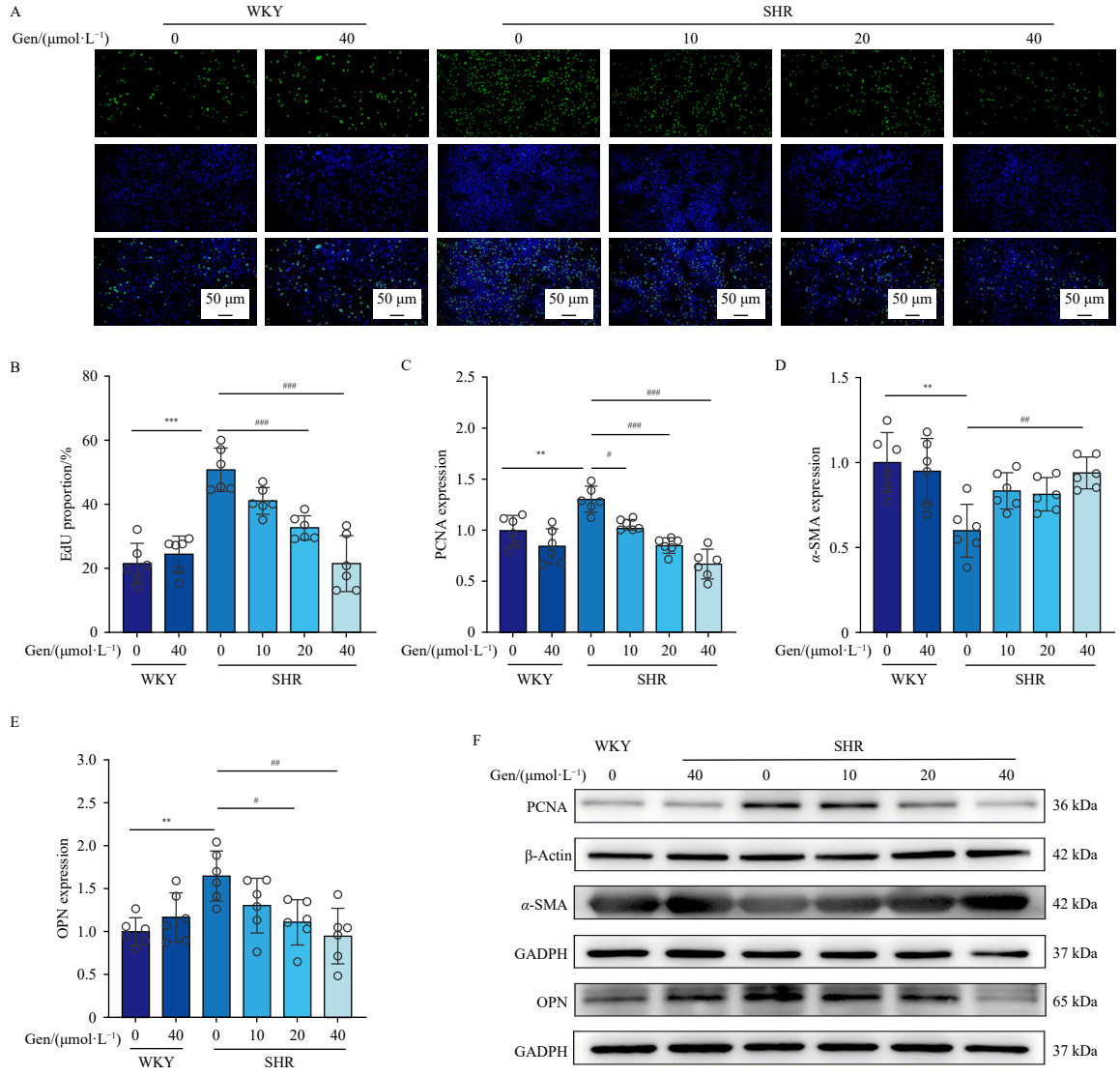



**Fig. 2** Gen can inhibit the proliferation and phenotypic transformation of vascular smooth muscle cells in SHR rats. (A and B) EdU staining test was used to detect the cell proliferation (50 ×). (C–F) The expression of PCNA, α-SMA, OPN in VSMCs was detected by Western blot experiment. All data are presented as mean ± SEM ($n = 6$). *P*-values were determined by ANOVA followed by a post hoc Tukey test. $^{**}P < 0.01$, SHR *vs* WKY; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, SHR + Gen *vs* SHR.

SHR rats. After high-dose Gen intervention in the WKY group, the lateral migration ability of cells showed no significant difference from the normal group, suggesting that Gen has no significant effect on the lateral migration ability of normal cells.

The results of the Transwell migration assay (Figs. 3A and 3D) indicated that the number of cells that crossed the Transwell chamber in the SHR group was significantly higher than that in the WKY group after 24 h, suggesting that the longitudinal migration ability of VSMCs in the SHR group was significantly enhanced. The number of cells that crossed the membrane in the Gen low-, medium-, and high-dose intervention groups decreased in a dose-dependent manner. Moreover, there was no significant difference in the longitudinal migration ability between the WKY + Gen-L group and the WKY group, indicating that Gen could inhibit the longitudinal migration ability of VSMCs in SHR rats without significantly affecting normal cells.

The Western blot results (Figs. 3B, 3E and 3F) indicated that after 24-h culture, the protein expression levels of MMP2 and MMP9 in VSMCs of SHR rats were significantly higher than those in the WKY group. After treatment with low, medium and high doses of Gen, the expression of MMP2 and MMP9 was dose-dependently downregulated. After treatment with high-dose Gen in normal cells, the expression levels of MMP2 and MMP9 were not significantly different from those in the WKY group. Based on the above experimental data, it can be concluded that Gen has an inhibitory effect on the migration ability of VSMCs in SHR rats, and has no significant effect on normal cells.

### *3.4. Gen inhibits oxidative stress injury of vascular smooth muscle cells in SHR rats*

To explore the protective effect of Gen on oxidative stress in VSMCs of SHR rats, the intracellular ROS level was detected by DCFH-DA fluorescent probe, and the expression of key proteins HO-1 and NQO1 in the Nrf2 pathway was detected by Western blotting. As shown in Figs. 4A and 4C, the ROS fluorescence intensity in the SHR group was significantly higher than that in the WKY group, suggesting that the oxidative stress injury was aggravated. After intervention with low, medium and high doses of Gen, the intracellular ROS content was decreased, and the fluorescence intensity was dose-dependently weakened. Meanwhile, in the normal group, the intracellular ROS content and probe fluorescence intensity of VSMCs did not change significantly after high-dose Gen intervention.

The Western blotting results (Figs. 4B, 4D–4F) showed that the expression of NQO1, HO-1 and nuclear Nrf2 proteins in VSMCs of SHR rats was significantly lower than that in the WKY group. However, after intervention with low, medium and high

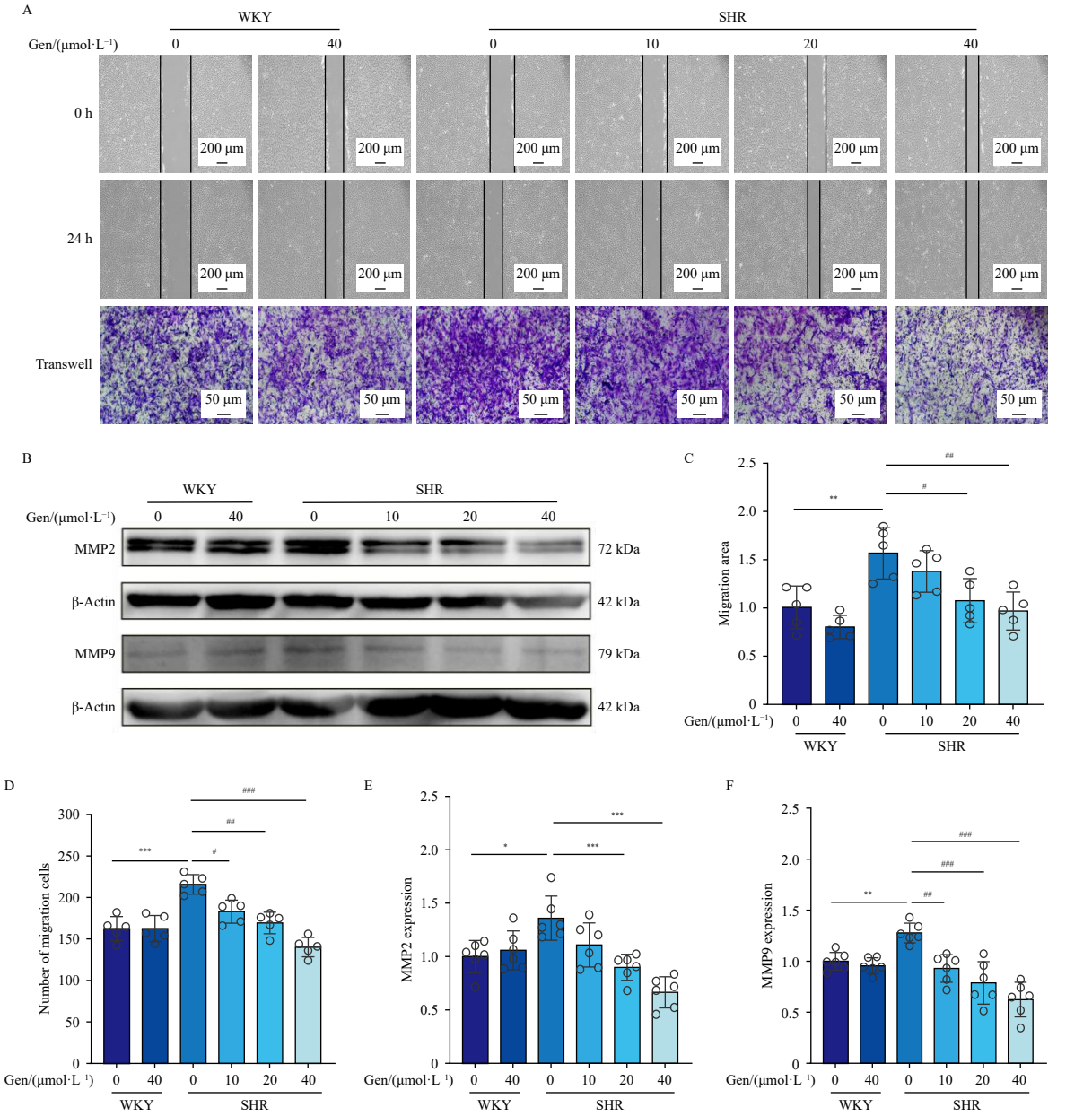



**Fig. 3** Gen can inhibit the migration of vascular smooth muscle cells in SHR rats. (A, C and D) Scratch and Transwell assays for detecting the migration of VSMCs (200 ×, 50 ×). (B, E and F) Detection of MMP2 and MMP9 protein expressions in VSMCs by Western blot. All data are presented as mean ± SEM ($n$ = 5–6). $P$-values were determined by ANOVA followed by a post hoc Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, SHR *vs* WKY; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR + Gen *vs* SHR.

doses of Gen, the expression of NQO1, HO-1 and nuclear Nrf2 proteins in VSMCs gradually increased with the increase of the dosage, suggesting that Gen can improve the imbalance of the antioxidant system in VSMCs of SHR rats and restore their antioxidant capacity.

### *3.5. Network pharmacology predicts that Gen inhibits oxidative stress and improves vascular remodeling in hypertension by targeting core proteins GSK3β, FYN, and Nrf2, which is validated through in vitro and in vivo experiments*

To investigate Gen protective mechanisms in hypertension, we integrated computational target prediction and network analysis. Potential targets of Gen were identified from SwissTargetPrediction, TargetNet, and HERB databases. Disease-related targets for hypertension vascular remodeling and oxidative stress were collected from GeneCards, CTD, OMIM, TTD, and PharmGKB. The intersection revealed 52 common targets (Fig. 5A), which were used to construct a protein-protein interaction network *via* STRING (Fig. 5B) and analyzed in Cytoscape (Fig. 5C). Analysis of the protein-protein interaction network revealed that GSK3β, FYN, and Nrf2 (corresponding to NFE2L2 in the network diagram) have degree values ranking among the top 20 out of 52 targets, indicating their role as core hubs in regulating vascular function. In contrast, targets such as ALPL and CFTR exhibited low degree values, positioning them as peripheral nodes in the network with relatively limited global regulatory influence. Furthermore, GO functional enrichment and KEGG pathway (Figs. S3A and S3B) analyses demonstrated that GSK3β, FYN, and Nrf2 were significantly enriched in pathways directly related to vascular protection, including “oxidative stress regulation”, “inflammatory response”, and “vascular endothelial function”. Other targets, such as ESR1, TTR, and ACHE, were primarily associated with mammary, thyroid, or neural functions and showed weaker direct relevance to vascular protection. Additionally, the mechanisms of some highly connected targets like EGFR and PPARG are

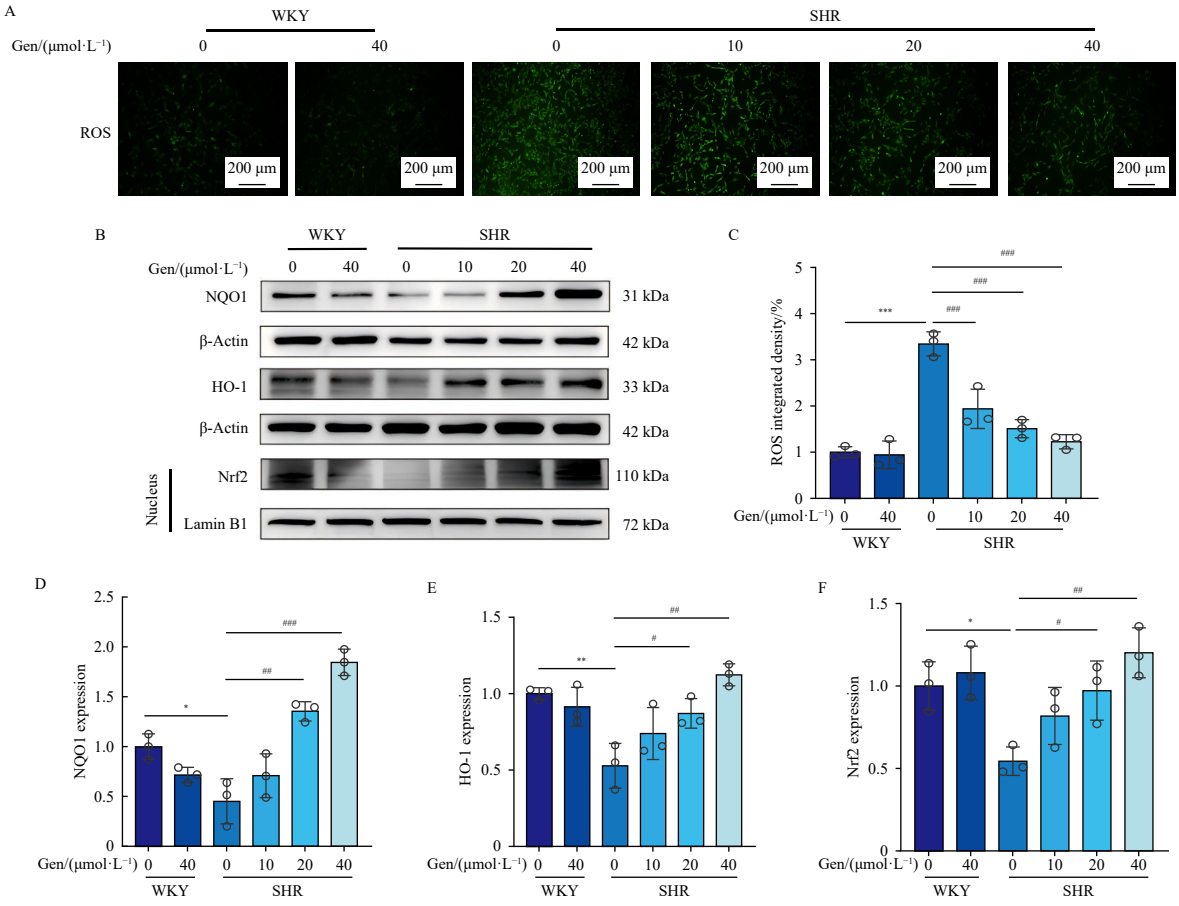
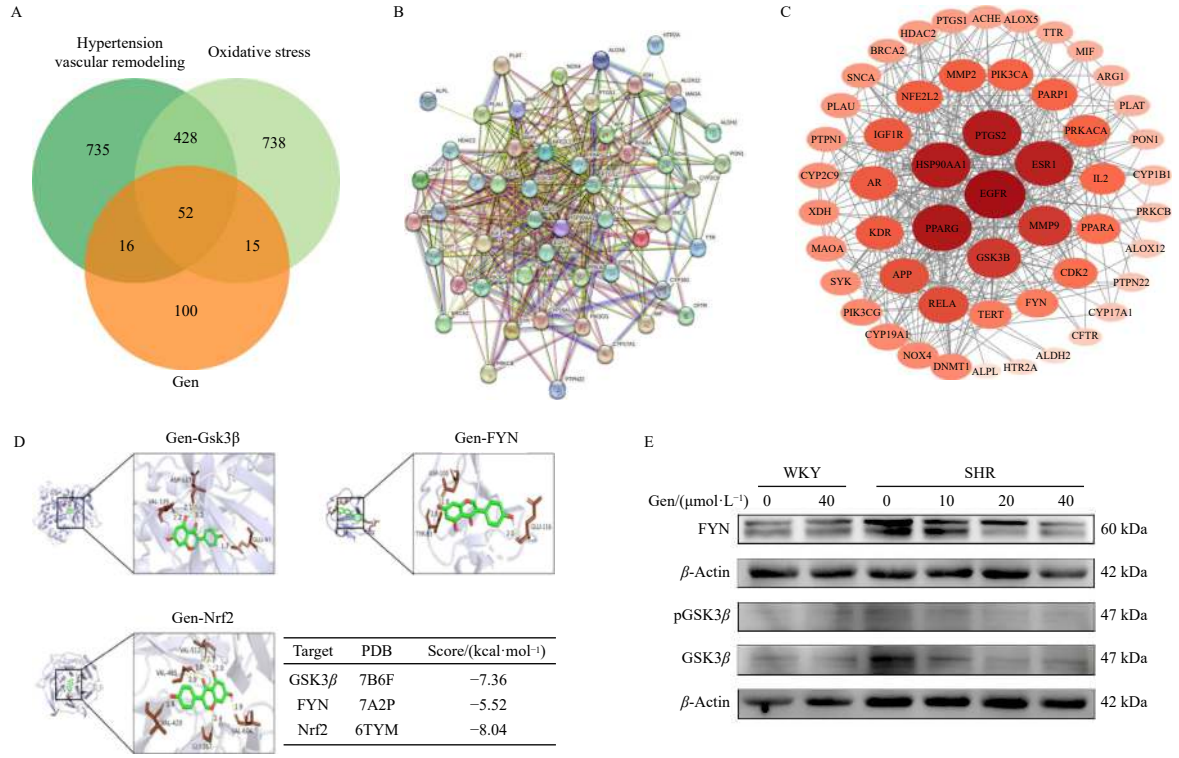



**Fig. 4** Gen can inhibit oxidative stress injury of vascular smooth muscle cells in SHR rats. (A and C) Levels of reactive oxygen species (200 ×). (B, D–F) Detection of NQO1, HO-1 and Nrf2 protein expressions in VSMCs by Western blot. All data are presented as mean ± SEM ($n$ = 3). $P$-values were determined by ANOVA followed by a post hoc Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, SHR *vs* WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR + Gen *vs* SHR.

already well-established [33-36]. In addition, literature reports indicate that [23, 37] GSK3β activates FYN, which subsequently regulates Nrf2, indicating FYN may bridge this pathway. Based on their network prominence, literature support, and functional relevance, we identified GSK3β, FYN, and Nrf2 as the core targets and selected them for further investigation.




| Target | PDB | Score/(kcal·mol⁻¹) |
|---|---|---|
| GSK3$\beta$ | 7B6F | −7.36 |
| FYN | 7A2P | −5.52 |
| Nrf2 | 6TYM | −8.04 |

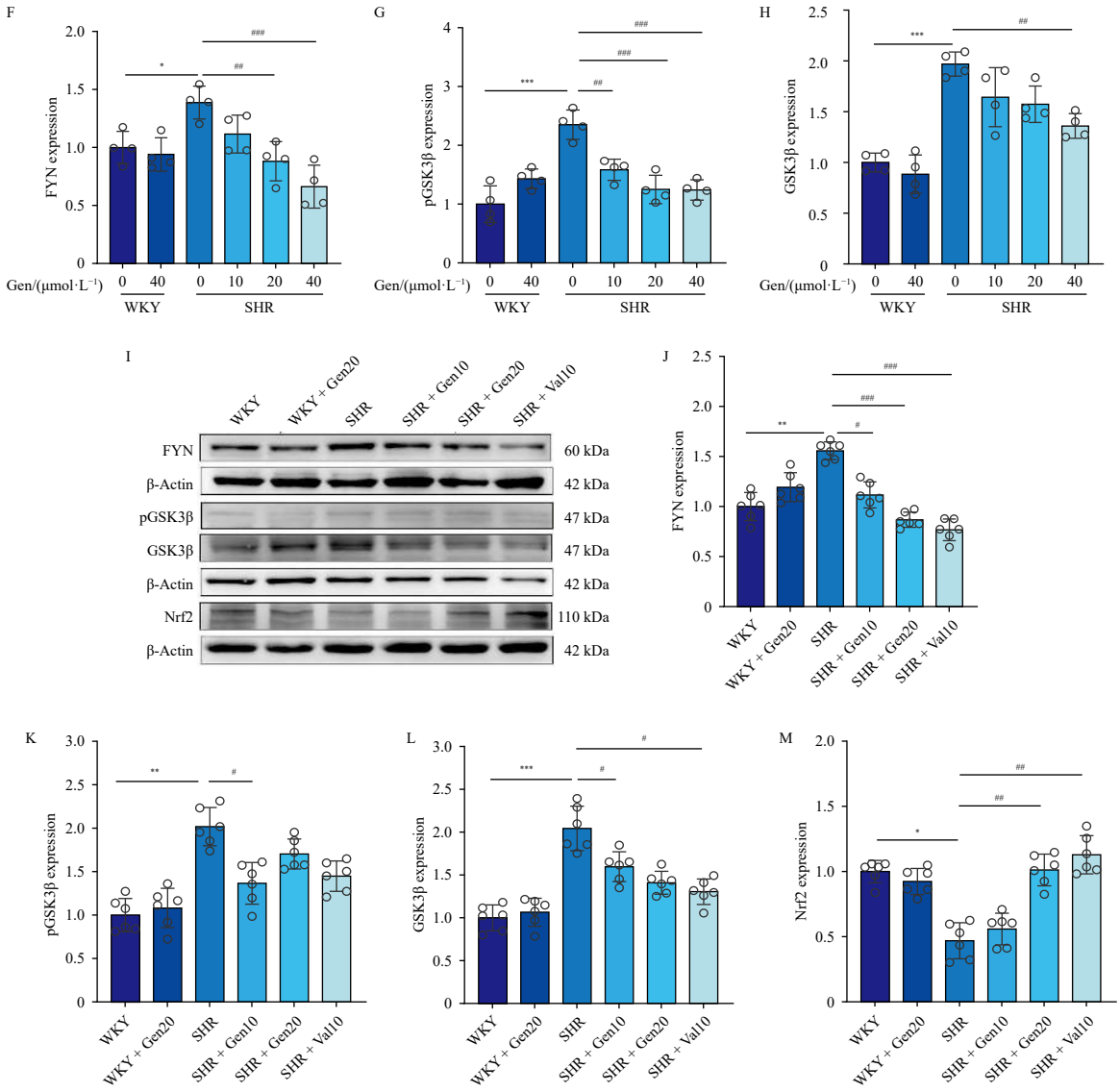



**Fig. 5** Network pharmacology predicts the mechanism by which Gen inhibits oxidative stress injury and improves vascular remodeling in hypertension, and this has been validated through *in vitro* and *in vivo* experiments. (A)Venn diagram of Gen–oxidative stress–hypertensive vascular remodeling targets. (B and C) Visualization network diagram of the common target points of Gen–oxidative stress–hypertensive vascular remodeling, and sorted according to the degree values. (D) Molecular docking model of Gen with GSK3β, FYN and Nrf2. (E–M) The expression of FYN, GSK3β and Nrf2 proteins in vascular smooth muscle cells and SHR rats were detected by Western blotting. All data are presented as mean ± SEM ($n$ = 4–6). $P$-values were determined by ANOVA followed by a post hoc Tukey test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, SHR *vs* WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR + Gen/Val *vs* SHR.

Molecular docking was performed to evaluate Gen binding to GSK3β, FYN, and Nrf2. Gen's structure was sourced from TCMSP, while protein structures (GSK3β: 7B6F; FYN: 7A2P; Nrf2: 6TYM) were obtained from PDB. The calculated binding energies (−7.36, −5.52, and −8.04 kcal·mol$^{-1}$, respectively) all fell below the −5 kcal·mol$^{-1}$ threshold, indicating strong binding. Molecular interactions were visualized using PyMOL (Fig. 5D). These results confirm Gen's high affinity for all three targets, supporting their selection for further mechanistic study.

Based on the results of network pharmacology, molecular docking predictions, and literature hints, Western blotting was used to detect the protein expression levels of GSK3β, pGSK3β, and FYN in the primary extracted aortic VSMCs. The results are shown in Figs. 5E–5H. *In vitro* experiments, compared with the WKY group, the expression of FYN, pGSK3β, and GSK3β in the SHR group increased. After Gen intervention, the expression of FYN, pGSK3β, and GSK3β in SHR rats aortic smooth muscle decreased, the protein expression of these targets *in vivo* was also detected, and the results were consistent with those *in vitro*. The results are shown in Figs. 5I–5M.

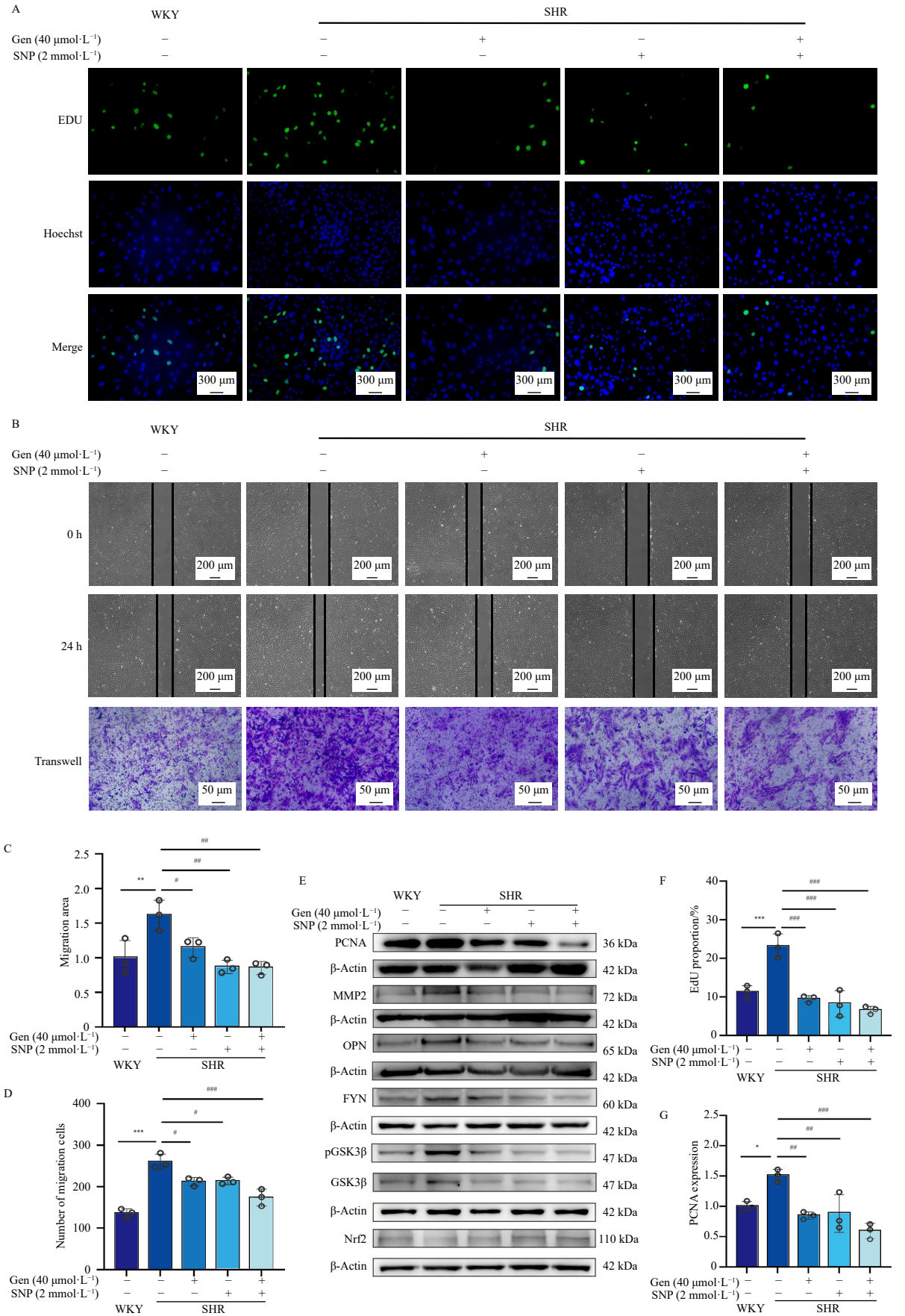
### *3.6. PGSK3β inhibitor SNP inhibits the proliferation, migration, and phenotypic transformation of aortic VSMCs in SHR rats and oxidative stress damage, reversing the expression of FYN and Nrf2*

To further verify this mechanism, the study employed the pGSK3β inhibitor SNP (2 mmol·L$^{-1}$) [23] *in vitro* intervention. A total of 5 groups were set up, namely: WKY group, SHR group, SHR + Gen group, SHR + SNP group, SHR + Gen + SNP group. During the experiment, the proliferation ability, migration ability and phenotypic transformation ability of cells in each group were detected using the EdU assay, scratch assays and immunoblotting quantitative detection method.

The experimental results are shown in Figs. 6A–6D and 6F. After 24 h of intervention with Gen, SNP, and Gen + SNP, compared with the WKY group, the proportion of EdU-stained positive VSMCs in SHR group increased, as well as enhanced lateral and longitudinal migration abilities. When Gen and SNP were given, the proportion of EdU-stained cells and the migratory ability in SHR rat VSMCs decreased, indicating that Gen and pGSK3β inhibitor SNP had inhibitory effects on the proliferation and migra-

tion of VSMCs in SHR rats.

At the same time, Western blot experiments were conducted to detect the expression levels of proliferation indicators PCNA, migration indicator MMP2, phenotypic transformation indicators OPN and target protein FYN, pGSK3β and Nrf2. The results are shown in Figs. 6E, 6G–6M. Compared with the WKY group, the



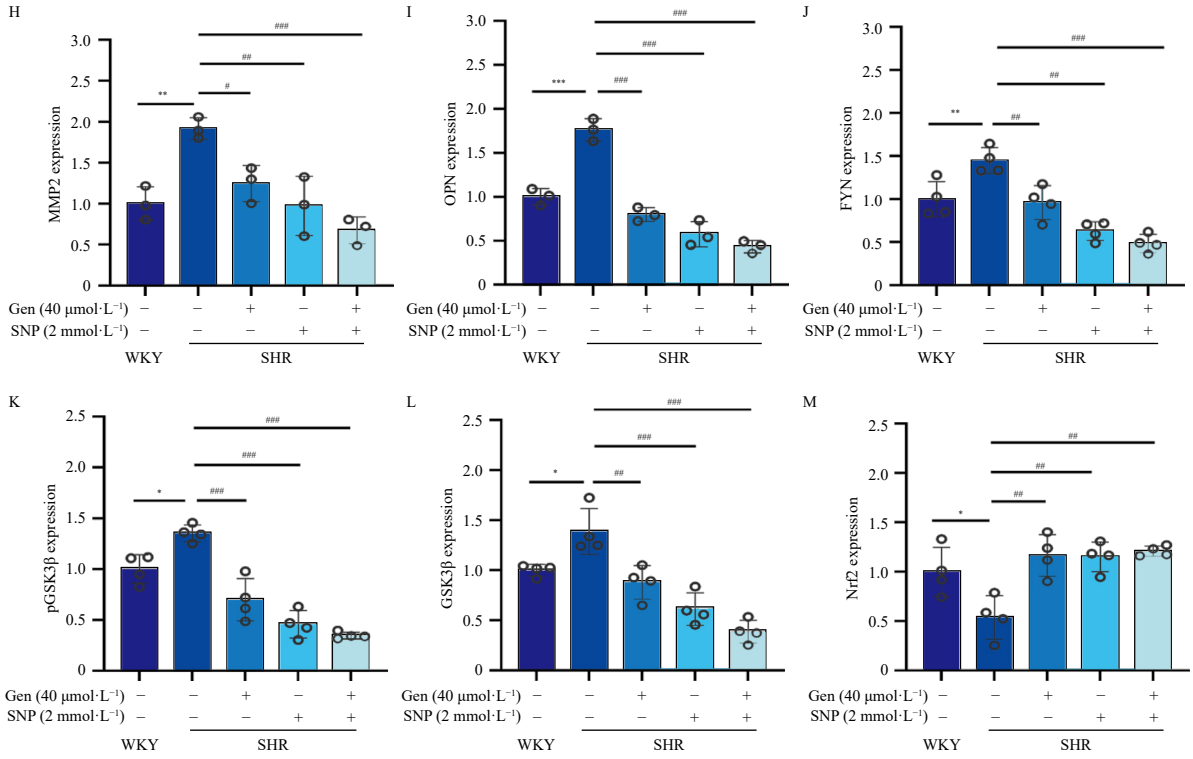



**Fig. 6** The effect of Gen on VSMCs of SHR rats after SNP treatment. (A, F) EdU staining test was used to detect the cell proliferation. (B–D) Scratch and Transwell assays for detecting the migration of VSMCs. (E, G–M) Detection of PCNA, MMP2, OPN, FYN, pGSK3β, GSK3β and Nrf2 protein expressions in VSMCs by Western blot. All data are presented as mean ± SEM ($n$ = 3–4). $P$-values were determined by ANOVA followed by a post hoc Tukey test. $^{**}P < 0.01$, $^{***}P < 0.001$, SHR *vs* WKY; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR + Gen/SNP/Gen + SNP *vs* SHR.

expression levels of PCNA, MMP2, OPN, FYN and pGSK3β proteins in SHR group were increased, while the protein expression level of Nrf2 decreased. After administration of Gen and SNP, the expression showed a reversed trend, and the reversal in the SHR + Gen + SNP group was more obvious to the naked eye. Overall, the results of the EdU assay and Western blot experiment show that Gen has the same inhibitory effect as the pGSK3β inhibitor SNP.

### *3.7. Gen alleviates oxidative stress damage and inhibits the process of hypertension-induced the proliferation and migration of vascular smooth muscle cells by regulating the GSK3β/FYN/Nrf2 signaling pathway*

To conduct a more in-depth verification, the Nrf2 inhibitor ML385 (2 μmol·L$^{-1}$) [38] was added, and the groups were divided into five: WKY, SHR, SHR + Gen, SHR + ML385, and SHR + Gen + ML385. EdU and scratch assays were used to detect the proliferation and migration abilities of cells in each group. The experimental results are shown in Figs. 7A, 7B, 7D and 7E. The results indicated that the Nrf2 inhibitor ML385 had no effect on the proliferation and migration of smooth muscle cells in SHR rats, indicating that Gen and pGSK3β inhibitor SNP could effectively inhibit the proliferation and remodeling of aortic VSMCs in SHR rats and oxidative stress damage, and this inhibitory effect was not affected by Nrf2 inhibitor ML385.

Based on the above verification, In the experiment, a new group named SHR + Gen + SNP + ML385 was added. The expression of various target proteins was detected through immunoblotting experiments. The results are shown in Figs. 7C, 7F–7I. There was no significant difference in protein expression between the SHR + Gen + SNP group and the SHR + Gen + SNP + ML385 group. There was a significant difference in protein expression between the SHR + Gen + ML385 group and the SHR + Gen + SNP + ML385 group, indicating that the expression of GSK3β and FYN is may not be affected by Nrf2.

Based on the above experimental results, it can be inferred that GSK3β is upstream of Nrf2, and Gen alleviate oxidative stress damage and inhibit the process of hypertension-induced vascular remodeling by regulating the GSK3β/FYN/Nrf2 signaling pathway.

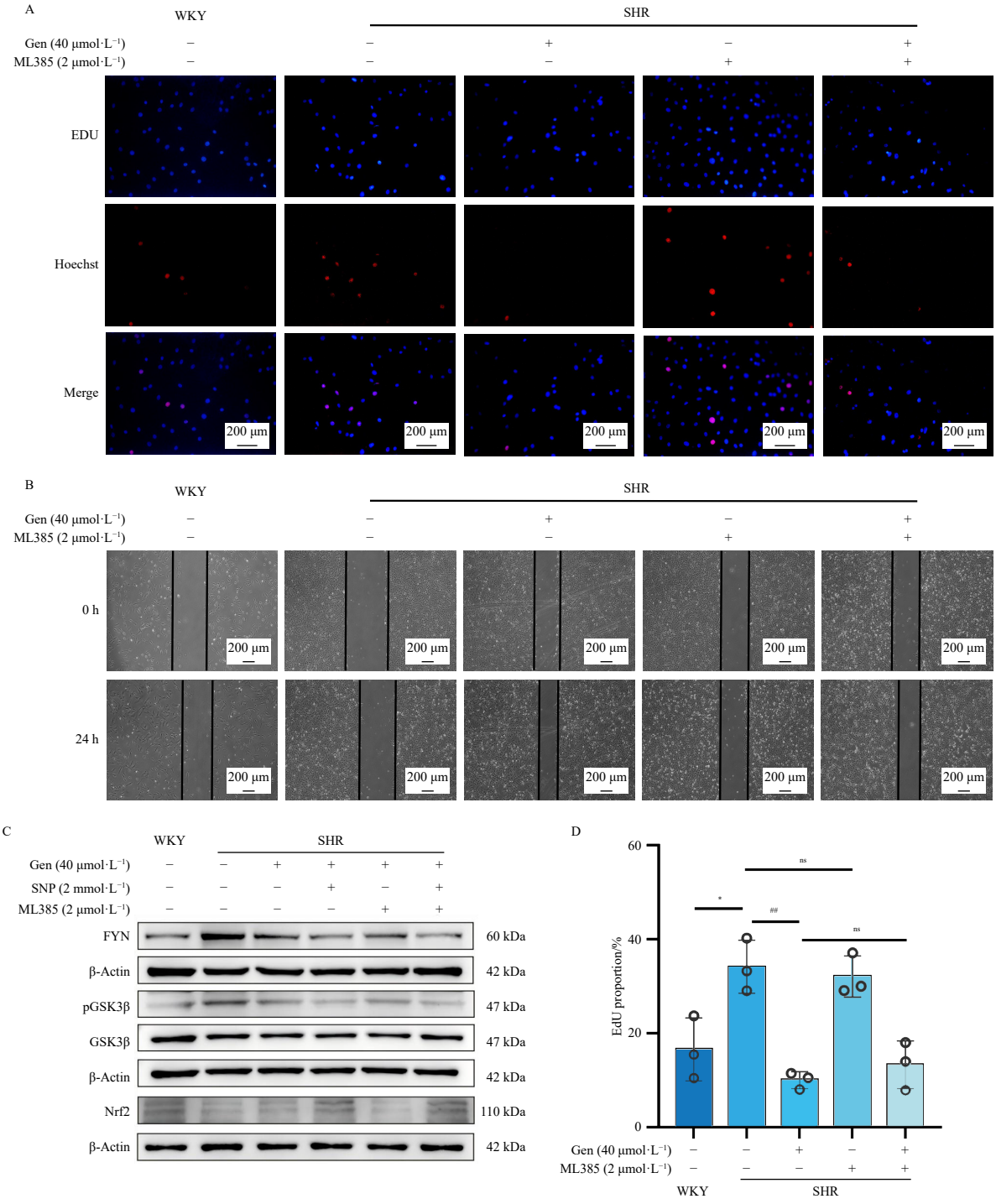
## 4. Discussion

Hypertension, as a major chronic disease threatening human health, still faces a severe prevention and control situation. According to data from the World Health Organization, the number of adult hypertensive patients worldwide has reached 1.28 billion [39]. In China, over 50% of cardiovascular disease deaths are directly related to poor blood pressure control [40]. Vascular remodeling is an important initiating factor and indicator in the course of hypertension. The structural remodeling of the arterial wall may induce hypertension, and it itself is also a pathological result caused by the continuous increase in blood pressure [41]. The vicious cycle formed by this is specifically manifested as thickening of vascular media, increase of extracellular matrix, and transformation of VSMCs' phenotype [8], and studies have confirmed that intervening in vascular remodeling can effectively lower blood pressure [42-44]. In addition, oxidative stress also plays a key role in the process of hypertension, where abnormal accumulation of ROS is an important initiating factor for triggering a series of pathological changes [45]. Under the state of hypertension, various pathways can promote the generation of ROS in large quantities, promoting the proliferation and migration of VSMCs, damaging vascular contraction and relaxation functions, and ultimately triggering pathological vascular remodeling [46], accelerating the course of hypertension. Gen, a natural isoflavone compound, has been proven to have the effects of inhibiting angiogenesis [47], antioxidation [48], and protecting the cardiovascular system [49]. Studies have found that it can reduce intracellular ROS levels and alleviate oxidative damage [50, 51], but the mechanism of its effect on vascular remodeling in hypertension is still unclear.

Based on its antioxidant properties, conducting research is expected to provide new ideas for the prevention and treatment of vascular remodeling in hypertension.

This study selected spontaneously hypertensive rats (SHR) as the classic animal model to simulate hypertension and its secondary hypertension-related cardiovascular diseases. Homologous WKY rats were used as the normal control group to minimize the interference of genetic background differences. The study found that Gen could effectively lower the blood pressure of SHR rats and improve their abnormal vascular morphology. The data showed that the systolic and diastolic blood pressures of SHR rats were significantly higher than those of WKY rats before the experiment, and continued to rise. At the 4th week of treatment, the blood pressure of SHR rats receiving the combined treatment of Gen and Val began to decrease, and by the 10th week, it had approached the level of WKY rats, which was similar to the results of previous studies [52]. This indicates that the drug has a significant blood pressure-lowering effect. Through HE staining and Masson staining, it was observed that SHR rats had abnormal morphological changes such as thickened blood vessels. After Gen treatment, these abnormal phenomena were significantly improved, indicating that the drug can reduce the thickness of the aortic wall and delay the process of vascular fibrosis.

Furthermore, the research also revealed that Gen inhibited the abnormal proliferation migration, phenotypic transformation, and oxidative stress damage of the aortic vessels and VSMCs of SHR rats. It is reported that in cardiovascular proliferative diseases, PCNA and MMP2/9 are involved in vascular remodeling [53], and VSMC phenotypic transformation aggravates remodeling [54], and abnormal accumulation of ROS triggers pathological changes



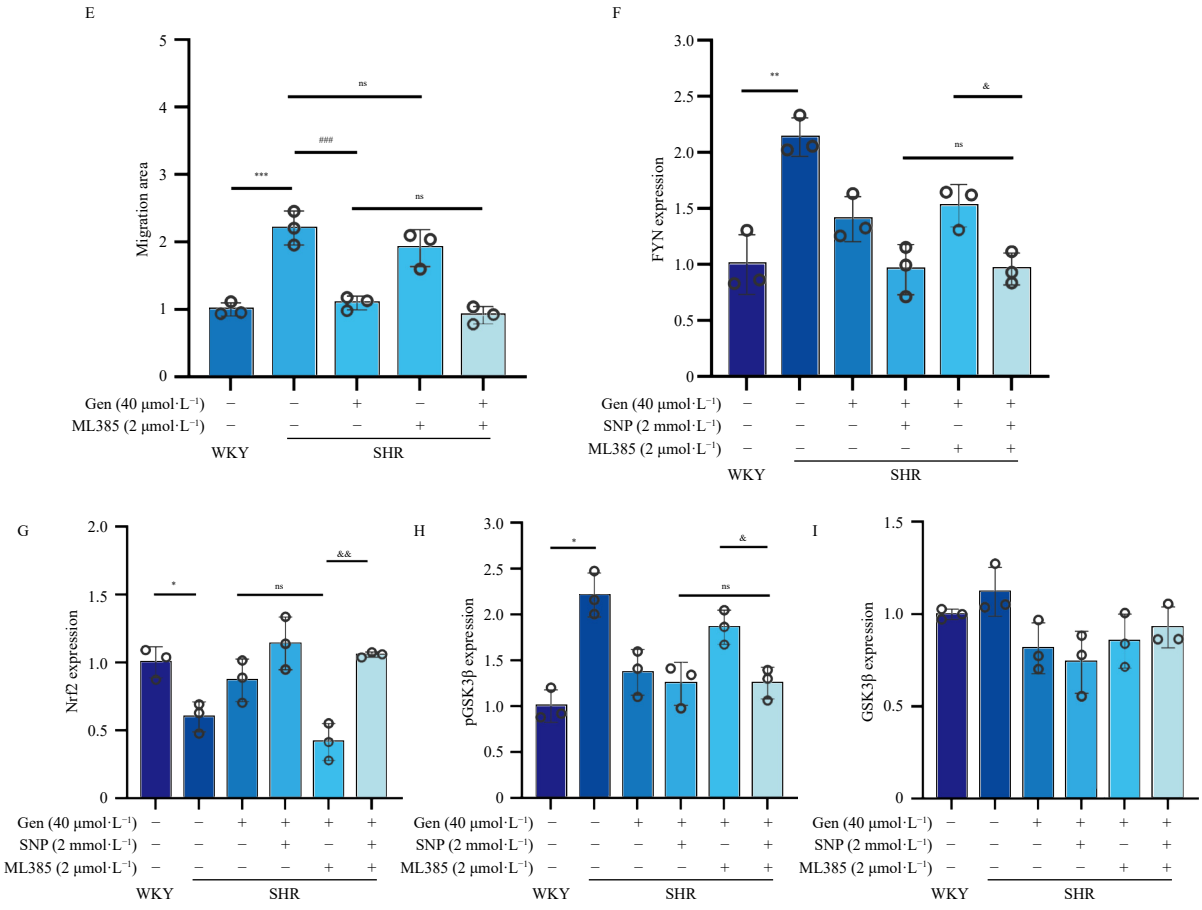



**Fig. 7** The effect of Gen on VSMCs of SHR rats after SNP and ML385 treatment. (A and D) EdU staining test was used to detect the cell proliferation. (B and E) Scratch assays for detecting the migration of VSMCs. (C, F–I) Detection of FYN, pGSK3β, GSK3β and Nrf2 protein expressions in VSMCs by Western blot. All data are presented as mean ± SEM ($n$ = 3). $P$-values were determined by ANOVA followed by a post hoc Tukey test. $^{**}P < 0.01$, $^{***}P < 0.001$, SHR *vs* WKY; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, SHR + Gen *vs* SHR; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, Gen + ML385 *vs* Gen + ML385 + SNP.

through related pathways. Oxidative stress, characterized by increased ROS production or decreased antioxidant capacity, is a key mechanism in the pathogenesis of hypertension. It can reduce the bioavailability of nitric oxide (NO) and trigger endothelial dysfunction. NADPH oxidase (Nox) and xanthine oxidase (XO) in the vascular wall are the main sources of ROS. The uncoupling of eNOS function and damage to the mitochondrial respiratory chain can also promote ROS production, thereby activating RAAS, enhancing sympathetic nerve activity, etc., and exacerbating hypertension [55]. In damaged astrocytes of Alzheimer's patients and APP/PS1 double transgenic mice, the level of NOX4 (the main source of ROS) increases, leading to increased mtROS production, impaired mitochondrial metabolism, and fragmentation; at the same time, it inhibits the cellular antioxidant system, further intensifying oxidative stress [56]. This study confirmed that Gen can down-regulate the expression of NOX4, and this effect is related to its antioxidant and anti-vascular remodeling effects. However, the current conclusion mainly relies on protein expression data. Considering the complex feedback regulation relationship between the reactive oxygen species generation system and the antioxidant system, the change in expression level alone is not sufficient to clarify whether the down-regulation of NOX4 is an upstream driving factor of oxidative stress or a downstream adaptive response taken by the body to alleviate oxidative stress [57]. To clarify the specific functional role and causal relationship of NOX4 in the mechanism of action of Gen, future studies will detect the enzymatic activity of NOX4 [58], and conduct experiments with NOX4-specific agonists or inhibitors [59]. This will help verify whether NOX4 is a key target of Gen and systematically depict its position in the multi-target antioxidant network.

In addition, the effect of the high-dose Gen group was similar to that of the antihypertensive drug Val. Val can reduce oxidative stress and alleviate vascular remodeling [60, 61], suggesting that Gen has the same effect as Val, and the administration alone does not cause damage to the aorta. However, the specific molecular mechanisms by which Gen exerts these combined effects, especially how it precisely regulates the relevant signaling pathways, still require further in-depth investigation.

In order to explore the mechanism by which Gen inhibits oxidative stress-induced damage and alleviates vascular remodeling in hypertension, this study, through network pharmacology and molecular docking methods, discovered that the role of Gen in reducing oxidative stress damage in hypertension and improving vascular remodeling may be closely related to three targets, GSK3β, FYN, and Nrf2. GSK3β, as a key regulatory factor in vascular remodeling [21], has been shown that Akt phosphorylation can activate GSK3β, which then upregulates cyclin D1, promoting cell cycle progression and leading to excessive proliferation of pulmonary artery smooth muscle cells and inhibition of cell apoptosis, thereby exacerbating pulmonary vascular remodeling [22]. FYN is an important member of the Src family of tyrosine kinases. Studies have found that sulforaphane can stimulate the Akt/GSK3β/FYN pathway, reduce the amount of FYN entering the nucleus and its transport effect on Nrf2, allowing Nrf2 to accumulate in the nucleus and activate, thereby promoting the expression of downstream antioxidant genes and enhancing cellular antioxidant capacity [23]. Other studies have shown that GSK3β has a regulatory effect on the activity and localization of FYN [24-26]. When GSK3β is activated, it promotes FYN to transport Nrf2 from the nucleus to the cytoplasm and its degradation, resulting in re-

duced expression of Nrf2 downstream antioxidant genes and decreased cellular antioxidant capacity, thereby affecting vascular remodeling.

In this study, pGSK3β inhibitor SNP and Nrf2 inhibitor ML385 were added and the groups were reclassified. Through EdU, scratch, Transwell, and Western blot experimental results, Gen and pGSK3β inhibitor SNP could effectively inhibit the proliferation and remodeling of aortic VSMCs in SHR rats and oxidative stress damage, and this inhibitory effect was not affected by Nrf2 inhibitor ML385. In the simulated atherosclerosis model, Dong et al. [62] discovered that alpinetin activated Nrf2 by regulating GSK3β/FYN, improving the redox homeostasis mediated by Nrf2, thereby inhibiting macrophage infiltration and atherosclerosis. Tong et al. [63] found that in the myocardial infarction model, bFGF activated Nrf2-mediated antioxidant defense through the Akt/GSK3β/FYN pathway, thereby alleviating myocardial infarction injury and cardiomyocyte apoptosis. Thus, it can be inferred that GSK3β is upstream of Nrf2, and Gen may regulate the GSK3β/FYN/Nrf2 signaling pathway to alleviate oxidative stress damage and thereby inhibit the process of hypertension-induced vascular remodeling. Furthermore, previous studies have indicated that Gen can reduce the production and signaling of AngII by inhibiting angiotensin-converting enzyme (ACE) activity [64] and downregulating AT1 receptor expression [65]. AngII can induce ROS generation *via* NADPH oxidase [66] and activate the GSK3β/FYN pathway, thereby suppressing Nrf2-mediated antioxidant defense [67]. The present study demonstrates that Gen can inhibit the negative regulation of Nrf2 by GSK3β/FYN, enhance antioxidant capacity, and alleviate oxidative stress. This effect not only directly mitigates AngII-induced vascular injury but may also, through negative feedback regulation, reduce the activity of the renin-angiotensin system (RAS), ultimately contributing to lowered AngII levels and improved vascular remodeling, which is consistent with the results of this study that Gen alleviates vascular remodeling in hypertension.

## 5. Conclusion

In conclusion, this study provides sufficient evidence to demonstrate that Gen can effectively alleviate oxidative stress damage and inhibit hypertensive vascular remodeling. This is mainly due to the fact that Gen regulates the GSK3β/FYN/Nrf2 pathway, inhibits the phosphorylation of GSK3β, blocks the activation of FYN, thereby preventing the downregulation of Nrf2 by FYN, restoring the intracellular antioxidant defense system, effectively inhibiting oxidative stress damage, ultimately improving hypertensive vascular remodeling and controlling the course of hypertension. Growing evidence suggests that various plant-derived bioactive components, ranging from ginsenosides to tea polyphenols, exert protective effects in different disease models by modulating the Nrf2 antioxidant signaling axis. This study, for the first time, integrates Gen into this framework. Not only does it clarify the mechanistic positioning of Gen within the relevant research field, but it also provides novel insights and directions for the prevention and treatment of hypertensive vascular lesions.

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## Supporting information

Supporting information of this paper can be requested by sending E-mail to the corresponding author.

## Conflict of interest

The authors declare no potential competing interests, including employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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