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RESEARCH

Ginkgo biloba extract mediates HT22 cell proliferation and migration after oxygen–glucose deprivation/reoxygenation via regulating RhoA-ROCK2 signalling pathway

Dexiu Wang[1,2] · Xin Zhao[3] · Jinghan Li[2] · Yang Song[2] · Weida Chen[3] · Xin Cai[2] · Ruofan Liu[3] · Zetao Chen[3,4]

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Abstract

Vascular dementia (VD) is a neurocognitive disorder resulting from cerebral vascular disorders, leading to the demise of neurons and cognitive deficits, posing significant health concerns globally. Derived from *Ginkgo biloba* leaves, EGb761 is a potent bioactive compound widely recognized for its benefits in treating cerebrovascular diseases. Previous studies have demonstrated that the administration of EGb761 to VD rats enhances the proliferation, differentiation, and migration of neurons, effectively alleviating cognitive dysfunction. However, the specific mechanisms by which EGb761 exerts its remedial influence on VD persist in ambiguity. This investigation utilized an integrated approach incorporating network pharmacology with experimental procedures on HT-22 mouse hippocampal neuronal cells amidst oxygen-glucose deprivation and reoxygenation (OGD/R) to delve into certain repercussions of EGb761 on cell proliferation and migration. Results revealed that ras homolog family member A (RHOA) and B-cell lymphoma 2 (BCL-2) are potential targets of *Ginkgo biloba* leaves. Target genes are mainly enriched in pathways including those involved in growth hormone synthesis, secretion and action and the neurotrophin signalling pathway. Cellular experiments further demonstrated that the application of EGb761 notably enhanced the viability, proliferation, and migration of HT22 cells subjected to OGD/R through RhoA-ROCK2 pathway. In conclusion, our findings indicated that EGb761 significantly enhances neuronal proliferation and migration following OGD/R injury by targeting the RhoA-ROCK2 signalling pathway, thus offering valuable insights into its potential as a treatment for VD.

✉ Ruofan Liu
71002179@sdutcm.edu.cn

✉ Zetao Chen
71000808@sdutcm.edu.cn

Dexiu Wang
2022140033@sdutcm.edu.cn

Xin Zhao
71001594@sdutcm.edu.cn

Jinghan Li
L15978330825@163.com

Yang Song
yangs337@stu.sdsmu.edu.cn

Weida Chen
2021111092@sdutcm.edu.cn

Xin Cai
caixin@sdsmu.edu.cn

1 First Clinical Medical College, Shandong University of Traditional Chinese Medicine, Jinan 250355, P.R. China

2 School of Basic Medical Sciences, Shandong Second Medical University, Weifang 261053, P.R. China

3 Department of Geriatric Medicine, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, No.42 Wenhua West Road, Jinan 250011, P.R. China

4 Subject of Integrated Chinese and Western Medicine, Shandong University of Traditional Chinese Medicine, Jinan 250355, P.R. China

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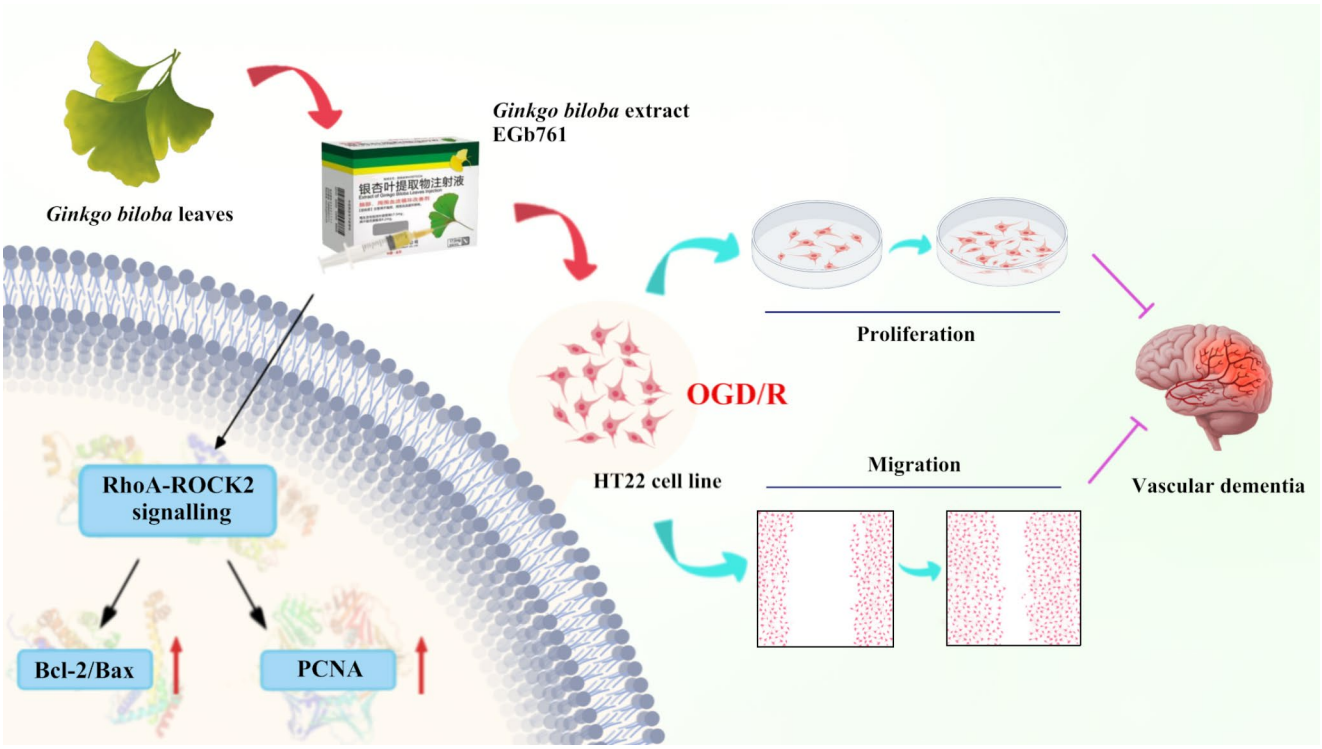
Graphical abstract



EGb761 mediates HT22 cell proliferation and migration after OGD/R via regulating RhoA-ROCK2 signalling pathway

Keywords EGb761 · Vascular dementia · Proliferation · Migration · RhoA-ROCK2 pathway

Introduction

Vascular dementia (VD) is a neurocognitive disorder characterised by cognitive decline resulting from vascular damage or small vessel disease within the brain (Bir et al. 2021). Culminating in the cessation of cellular vitality, these pathophysiological processes activate mechanisms linked to excitotoxicity, mitochondrial discordance, inflammatory reaction and oxidative stress (Li et al. 2021; Tian et al. 2022). Such a condition is prominently characterised by symptoms of memory loss and cognitive deficits, thereby exerting a severe and distressing impact on affected individuals (Bir et al. 2021). By constituting about 15% of cognitive decline cases worldwide, VD has attracted considerable attention as a noteworthy health issue (Torres-Simon et al. 2022).

The inability of mature neurons in the mammalian central nervous system to fully regenerate after trauma is broadly acknowledged. However, recent studies have shown that humans continue to generate new neurons from adulthood to advanced age (Tobin et al. 2019). These findings firmly conclude that the capacity for neurogenesis remains consistent across different ages of the brain. Another study indicates that the number of proliferating and developing neurons is markedly reduced within the brains of patients with cognitive impairment compared to healthy individuals. The observed decrease in developing neurons is believed to exacerbate cognitive deficits among affected patients. It has been further demonstrated that elderly individuals without cognitive impairment or psychiatric disorders have a greater number of proliferating neural cells in the brain. Following neurogenesis, these neurons continuously mature, differentiate, and migrate toward damaged areas of the brain, facilitating the timely formation of neural circuits, which shapes the brain structure and function, and enhances cognitive abilities (Boldrini et al. 2018). Furthermore, this reaffirms that the decline in hippocampal neurogenesis would be associated with impaired cognitive and emotional abilities. Consequently, the promotion of neurogenesis, proliferation and migration of neurons toward damaged areas may be a novel therapeutic approach for neurological disorders such as VD.

Rho family GTPases are indispensable in governing the cytoskeleton and are pivotal in cell proliferation, differentiation, and migration (Hall and Lalli 2010; Billuart et al. 2001). Serving as molecular toggles, they would typically function by oscillating between a dormant

GDP-bound state and an active GTP-bound state. Upon activation, these GTPases interact with specific effectors (Yoon et al. 2006). Among them, GTP-bound RhoA has the capacity to stir Rho-associated kinase (ROCK), which includes ROCK1 and ROCK2. (Fujita and Yamashita 2014) Although similar, ROCK1 and ROCK2 are not always identical (Hartmann et al. 2015). In the domain of various neuronal activities like proliferation, migration, dendritic growth, and axonal expansion, the signalling cascade of RhoA/ROCK serves as a pivotal function (Fujita and Yamashita 2014; Rico et al. 2004). Research indicates that the signalling pathway of RhoA/ROCK, under the influence of IL-1β or Wnt5a, would promote neurite outgrowth and upregulate neuronal factors such as NT3 and Ngn1 (Park et al. 2018). Yet to be resolved is whether the RhoA-ROCK2 pathway promotes neuronal proliferation and migration, thereby serving as a crucial therapeutic target for VD.

The utilisation of *Ginkgo biloba L.* leaves for medicinal purposes can be dated back to as early as 1509 (Mostafa et al. 2016). Extracts of *Ginkgo biloba* leaves have demonstrated therapeutic efficacy in managing disorders including cognitive impairment, Alzheimer's disease as well as cardiovascular disease (Chan et al. 2007). EGb761, a standardised extract, is derived from the foliage of *Ginkgo biloba*, formulated to contain 24% flavonol glycosides and 6% terpene lactones (National Toxicol Program 2013). These components were considered to have potential medical applications owing to their bioactive properties, particularly their antioxidant and anti-apoptotic effects, as well as their ability to enhance cognitive abilities, contributing to the treatment of cerebrovascular ailments (DeFeudis and Drieu 2000). For example, EGb761 can alleviate memory impairment by attenuating synaptic damage elicited by the scopolamine in mice (Zhang et al. 2022). Additionally, EGb761 has emerged in other studies as being capable of significantly stimulate hippocampal neuron cell proliferation while manifesting dose-dependent nuances (Müller et al. 2012). In young (8-week-old) mice, a 28-day treatment regimen involving EGb761 (40 mg/kg or 100 mg/kg) yielded a progressive increase specifically within the proliferative and differentiative abilities of the dentate gyrus, correlating with dosage (Yoo et al. 2011). In light of this, the central purpose was to ascertain whether EGb761 can exert neuroprotective effects by increasing neuronal proliferation and migration through the RhoA-ROCK2 pathway in VD.

Materials and methods

Materials and reagents

The standardized liquid formulation of EGb761 was procured from Yue Kang Pharmaceutical Group (Beijing, China). DMEM along with FBS were brought in from subsidiary of Thermo Fisher Scientific, Gibco (Waltham, MA, USA). Cell Counting Kit-8 (CCK-8) solution and 5-Ethynyl-2'-deoxyuridine (EdU) was sourced from Beyotime Biotechnology (Nanjing, China). The antibodies including rabbit anti-RhoA, rabbit anti-ROCK2 and rabbit anti-PCNA were procured by way through the company of Cell Signalling Technology (USA). Proteintech (Rosemont, IL, USA) provided rabbit anti-Bcl-2 and mouse anti-Bax antibodies. Fasudil hydrochloride (Fasu) was purchased from MedChemExpress (Princeton, NJ, USA).

Identification of bioactive components and potential targets of Ginkgo biloba leaves

The assay of high-performance liquid chromatography (HPLC) was employed with the aim of analysing the EGb761 sample. The outcomes are presented as Fig. S1 in the supplementary materials.

Bioactive components of *Ginkgo biloba* leaves were sourced from the Database of Traditional Chinese Systems Pharmacology (TCMSP) (https://tcmsp-e.com/tcmsp.php) by integrating the threshold of oral bioavailability (OB) ≥ 30% along with drug likeness (DL) ≥ 0.18%. Predicted biological counterparts corresponding to these active ingredients were anticipated and acquired using the data repository of Swiss Target Prediction (http://www.swisstargetprediction.ch), the data repository of Super Pred (https://prediction.charite.de) and by directly searching "*BAI GUO* YE" (Chinese term for *Ginkgo biloba* leaves) as keywords in the HERB (http://herb.ac.cn) Database, along with pertinent literature (Xuerui et al. 2016; DeFeudis et al. 2003; Peng et al. 2013; Chao and Chu 2004).

Prediction of therapeutic targets for VD

By utilizing 'vascular dementia' as a keyword, therapeutic targets related to VD were acquired by querying the data repository of GeneCards (https://www.genecards.org), PharmGKB (https://www.pharmgkb.org), as well as the data repository of OMIM (https://www.omim.org), Drugbank (https://go.drugbank.com) and TTD (http://db.idrblab.net/ttd), along with relevant literature (Lu et al. 2023; Zhang et al. 2021; Shi et al. 2016).

Construction of protein protein interaction (PPI) network and subsequent analysis

To visually illustrate the intersection between genes related to VD disease targets and those associated with *Ginkgo biloba* leaves, a Venn diagram was generated using the R tools. Subsequently, the intersected genes were employed, leveraging the STRING Database (https://string-db.org), to forge protein-protein (PPI) interaction network, with the search term 'Homo sapiens' specified. Cytoscape 3.9.1 was used to facilitate further explorations of protein interactions in the acquired network. Topological intricacies of the acquired network, specifically the betweenness centrality (BC), closeness centrality (CC), and degree centrality (DC) of each target, were computed through CytoNCA, and the core targets within the acquired PPI network were selected by values exceedingly twice the median of DC and surpassing the respective medians of BC and CC.

Analysis of functional enrichment

To further investigate the underlying mechanisms of *Ginkgo biloba* leaves on treating VD, gene ontology (GO) analysis and enrichment analysis of Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways were conducted by using the integrated data repository of DAVID (version 6.8) and subsequently visualised through the R tools. A network diagram of "compound-target-pathway" interactions was assembled utilizing the tool of Cytoscape 3.9.1 in order to visualise the intricate relationships.

Molecular docking

In order to assess binding affinity between bioactive compounds and target proteins, thereby creating an insight into their potential therapeutic efficacy and mechanisms of action, molecular docking was performed. Chemical architectures of the bioactive compounds of *Ginkgo biloba* leaves were retrieved from PubChem Database, and protein configurations of targets were obtained from the PDB Database. Conducting Schrödinger's Ligand docking calculations through AutoDockTools 1.5.7 software to assess the docking score (affinity). A heatmap depicting binding energies resulting from molecular docking of main component compounds with 5 core targets is generated, higher absolute docking scores indicate greater binding affinity.

Induction of OGD/R injury and EGb761 administration in HT22 cells

We allocated HT22 cells into control, OGD/R, and EGb761 (at concentrations of 25, 50, and 100 μg/mL) groups. The control group was submerged in a 10% FBS fortified DMEM medium, as well as penicillin at a concentration of 100 units/mL accompanied with the preparation of 100 μg/mL streptomycin under optimized and standardized cultivation milieu within the incubator (37 °C, 5% CO_2). In order to mimic ischaemia/reperfusion (I/R) injury, cells in the group of OGD/R and EGb761 group were exposed to OGD for the optimal duration, according to our previous experiment (Yin et al. 2024). Following OGD, the nutrient solution immediately substituted with DMEM including penicillin-streptomycin antibiotic and FBS, with EGb761 stood applied at the beginning of reoxygenation, and subsequently the cells underwent a 24 h-incubation to facilitate reoxygenation.

Cell counting kit-8 assay

Within the 96-well plates, cells from each group were seeded. Thereafter, CCK-8 solution was added following incubation for 2 h. Utilizing a microplate reader, the absorbance at 450 nm for each well would be accurately quantified.

EdU assay

Within 96-well plates, inoculations of cells in each group were conducted, where cells were cultured for 2 h with EdU medium, immersed for 30 min within cell fixative reagent, subsequently cultured with glycine (2 mg/mL) for 5 min, and incubated for 10 min with 100 μL penetrant. Cells were then subjected to staining at room temperature for 30 min, followed by 3 times of decolourisations and washes with 100 μL of penetrant in a shaker. Subsequent to this, a 30-min Hoechst staining protocol was applied to the cells and after which they were inspected under a fluorescent microscope.

Wound healing assay

The medium was drained and were given two PBS washes upon reaching approximately 80% cell density. A straight line was evenly marked in the centre of each well with pipette tips. The medium was rinsed twice with PBS in order to purge the unattached cells, followed by incubation in DMEM. Inspections on the cell migration was performed at the time period of 0, 6, 12 and 24 h, and photographed for statistical analysis.

Western blot analysis

Lysis buffer was applied to HT22 cells, followed by their lysis on ice. Collected lysates were transferred into EP tubes and centrifuged with the whirring speed at 12,000 rpm for

a quarter-hour at the setup of 4 °C. Following centrifugation, supernatant was gathered, followed by the determination of protein concentrations by the application of the BCA method. Amalgamated with SDS-PAGE protein loading buffer, the remaining protein samples were subjected to denaturation by heating for 10 min, followed by packaging. Subsequently, the proteins in each group were subjected to electrophoresis, transferred to membranes, sealed with non-fat milk and subjected to 4 °C overnight incubation with primary antibodies, predominantly rabbit anti-RhoA (1:1000), rabbit anti-ROCK2 (1:3000), rabbit anti-PCNA (1:2000), rabbit anti-Bcl-2(1:800), mouse anti-Bax (1:1000), and mouse anti-β-actin (1:1000). The following day, the membranes were subjected to a further incubation phase with secondary antibodies for 1 h, which were comprised by goat anti-rabbit polyclonal antibody (1:2000) coupled with goat anti-mouse monoclonal antibody (1:10000) conjugated with HRP. Subsequently, membranes have been subjected to ECL chemiluminescence reagent for imaging using an ultra-sensitive chemiluminescence imager, facilitating quantitative analysis of proteins.

Table 1 Twenty-seven bioactive compounds identified from *Ginkgo biloba* leaves using the TCMSP database

No.	Molecule Name	OB (%)	DL
1	(-)-catechin	49.68	0.24
2	(+)-catechin	54.83	0.24
3	beta-sitosterol	36.91	0.75
4	Bilobalide	84.42	0.36
5	bis[(2 S)−2-ethylhexyl] benzene-1,2-dicarboxylate	43.59	0.35
6	campest-5-en-3beta-ol	37.58	0.71
7	Chryseriol	35.85	0.27
8	Diosmetin	31.14	0.27
9	Ethyl oleate (NF)	32.4	0.19
10	Flavoxanthin	60.41	0.56
11	Genkwanin	37.13	0.24
12	ginkgolide B	44.38	0.73
13	ginkgolide C	48.33	0.73
14	ginkgolide J	44.84	0.74
15	Ginkgolide M	49.09	0.75
16	Isogoycyrol	40.36	0.83
17	isorhamnetin	49.6	0.31
18	kaempferol	41.88	0.24
19	Laricitrin	35.38	0.34
20	Linolenic acid ethyl ester	46.1	0.2
21	luteolin	36.16	0.25
22	Luteolin-4′-glucoside	41.97	0.79
23	Mandenol	42	0.19
24	quercetin	46.43	0.28
25	sesamin	56.55	0.83
26	Stigmasterol	43.83	0.76
27	Syringetin	36.82	0.37

Statistical analysis

Statistical one-way ANOVA has been employed to specifically compare amidst multiple groups. Statistical evaluations were executed through the utilization of GraphPad Prism software 7.0. A significance level of $P < 0.05$ was deemed significant in statistical analysis. Mean ± standard deviation (SD) statistics were used for the showcase of data. A minimum of three trials were performed for each experiment to ensure reliability.

Results

Acquisition of therapeutic targets for VD and PPI network analysis

A total of 27 bioactive compounds of *Ginkgo biloba* leaves were retrieved from the database of TCMSP (Table 1). Subsequently, an assembly of 782 potential targets associated with bioactive compounds were predicted. Further explorations yielded a cumulative count of 1166 targets related to VD. The overlapping zone of predicted targets and anti-VD targets was eventually determined and visually represented, revealing 235 potential targets for therapeutic intervention using *Ginkgo biloba* leaves against VD (Fig. 1A), including RHOA, BCL2, ROCK2, and PCNA. A PPI network was coherently formed (Fig. S2). By the utilization of Cytoscape, topological relationships were calculated, leading to the identification of 35 core targets with strong interconnections, including RHOA and BCL2 (Fig. 1B).

Enrichment analysis of functions and pathways

Analyses through GO and KEGG were conducted revealing significant involvement of the intersected targets such as positive regulation of cell growth, positive regulation of cell migration alongside with negative regulation of neuronal apoptotic process; as well as targeting the cellular nucleus, mitochondrion and cell surface (Fig. 1C). Additionally, notably enriched pathways correlating with network targets were mapped, including growth hormone synthesis, secretion and action and the neurotrophin signalling pathway. (Fig. 1D). Subsequently, a network diagram of "compound-target-pathway" interactions was constructed (Fig. 1E).

Molecular docking

For the purpose of evaluating the binding affinity bridging the active compounds of *Ginkgo biloba* leaves and the corresponding target proteins, molecular docking trials were implemented, and a heat map was generated to visualise

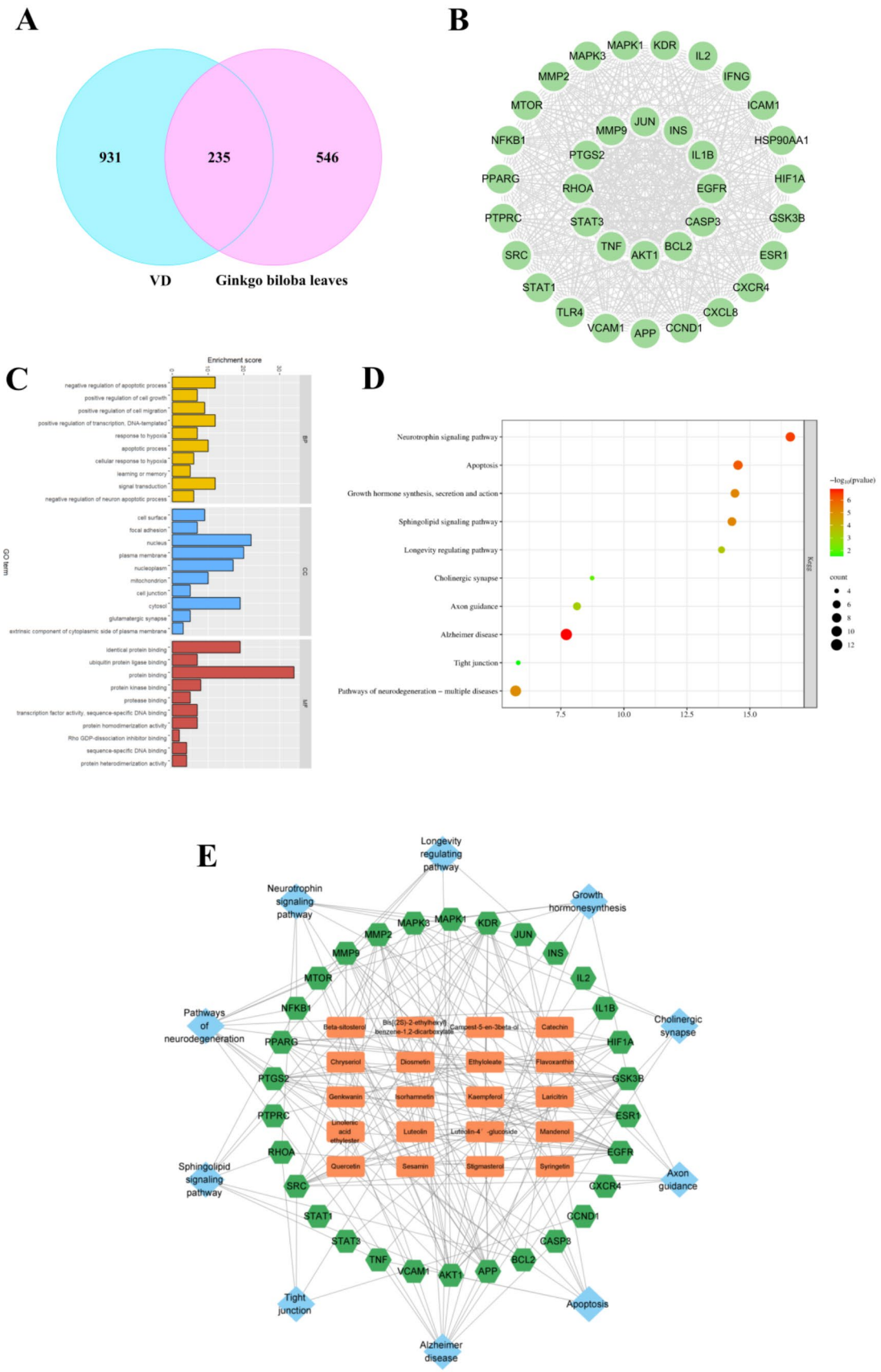

A
931
235
546
VD
Ginkgo biloba leaves
B
MAPK1
KDR
IL2
MAPK3
IFNG
MMP2
ICAM1
MTOR
JUN
INS
MMP9
HSP90AA1
NFKB1
PTGS2
IL1B
PPARG
HIF1A
RHOA
EGFR
PTPRC
STAT3
CASP3
GSK3B
TNF
BCL2
SRC
AKT1
ESR1
STAT1
CXCR4
TLR4
CXCL8
VCAM1
APP
CCND1
C
Enrichment score
negative regulation of apoptotic process
positive regulation of cell growth
positive regulation of cell migration
positive regulation of transcription, DNA-templated
response to hypoxia
apoptotic process
cellular response to hypoxia
learning or memory
signal transduction
negative regulation of neuron apoptotic process
cell surface
focal adhesion
nucleus
plasma membrane
nucleoplasm
mitochondrion
cell junction
cytosol
glutamatergic synapse
extrinsic component of cytoplasmic side of plasma membrane
identical protein binding
ubiquitin protein ligase binding
protein binding
protein kinase binding
protease binding
transcription factor activity, sequence-specific DNA binding
protein homodimerization activity
Rho GDP-dissociation inhibitor binding
sequence-specific DNA binding
protein heterodimerization activity
GO term
BP
CC
MF
D
Neurotrophin signaling pathway
Apoptosis
Growth hormone synthesis, secretion and action
Sphingolipid signaling pathway
Longevity regulating pathway
Cholinergic synapse
Axon guidance
Alzheimer disease
Tight junction
Pathways of neurodegeneration – multiple diseases
7.5
10.0
12.5
15.0
KEGG
−log10(pvalue)
count
E
Longevity regulating pathway
Neurotrophin signaling pathway
Growth hormonesynthesis
Pathways of neurodegeneration
Cholinergic synapse
Sphingolipid signaling pathway
Axon guidance
Tight junction
Apoptosis
Alzheimer disease
Beta-sitosterol
Bis[(2S)-2-ethylhexyl] benzene-1,2-dicarboxylate
Campest-5-en-3beta-ol
Catechin
Chryseriol
Diosmetin
Ethyloleate
Flavoxanthin
Genkwanin
Isorhamnetin
Kaempferol
Laricitrin
Linoleric acid ethylester
Luteolin
Luteolin-4'-glucoside
Mandenol
Quercetin
Sesamin
Stigmasterol
Syringetin

Fig. 1 Identification of core targets of *Ginkgo biloba* for the alleviation of VD, and GO and KEGG inspections. **A** Venn diagram illustrating 235 intersected targets between the bioactive constituents of EGb761 and their relevant VD associated targets. **B** PPI network interlaced by the relatively pivotal targets. **C** Bar chart of GO analysis, the bar height represents enrichment score of each section. **D** Enrichment chart of KEGG analysis. Magnitudes of dots indicate target gene counts of each pathway, and dot colour depicts the related P adjusted values. **E** The "compound-target-pathway" network

the specific results. Results indicated that the main active ingredients in *Ginkgo biloba* leaves exhibited strong binding capabilities to core targets of VD and their associated pathway targets, including BAX, BCL2, RHOA, ROCK2, PCNA. Surprisingly, all assessed binding affinity values were below −5, indicating that the primary components of *Ginkgo biloba* leaves exhibit significant binding efficiency with the aforementioned targets. Specifically, isorhamnetin demonstrated notable binding affinity with BCL2 (affinity score = −7.7), while quercetin exhibited strong affinity with ROCK2 (affinity score = −8.5) and Ginkgolide B was found to bind effectively with ROCK2 (affinity score = −9) (Fig. 2).

EGb761 increased the viability of HT22 cells exposed to OGD/R

With the intent of simulating the pathological state of cerebral I/R injury, OGD/R was applied to HT22 cells, functioning as a representative in vitro model. By utilizing the CCK-8 assay, the vitality of cells was probed. Following OGD/R, the viability of HT22 cells markedly declined while in contradistinction to the control group. HT22 cells subjected to different durations of OGD were examined under a microscope. For groups with OGD durations of 0, 1, and 2 h, the cell morphology was spindle, the cell membrane was smooth, and the adherent growth was observed. However, following 4 h of OGD, the cells began to shrink and adopt a round shape, accompanied by reduced cytoplasmic volume and a flattened appearance, as the duration of OGD time increased, the cells became progressively rounder, and the number of adherent cells decreased. Results of the CCK-8 experiment demonstrated a gradual decline in cell viability with prolonged OGD duration (Fig. 3A-B). Based on the previous experiment (Yin et al. 2024), an OGD duration resulting in 50% ~ 60% cell viability was deemed optimal, with 4 h identified as the most suitable OGD duration. To explore the impact of assorted EGb761 concentration levels (25, 50, 100, and 200 μg/mL) on HT22 cellular vitality under the stimulated milieu of OGD/R, we utilized the CCK-8 assay. Research outcomes has disclosed a pronounced diminution of cellular vitality within the OGD/R group in opposition to the control group, while the EGb761 group demonstrated a notable elevation in cell viability in comparison with the group subjected to OGD/R. Maximum cellular vitality was notably recorded at an EGb761 concentration level reaching 50 μg/mL, and subsequent cell experiments were conducted at this concentration (Fig. 3C).

EGb761 increased OGD/ R-induced proliferation of HT22 cells

To delve deeper into the specifics about whether EGb761 amplified the proliferation of HT22 cells under OGD/R-induced circumstances, assessment of the expression of PCNA and Bcl-2/Bax was conducted. Results revealed lower expression levels of PCNA and Bcl-2/Bax in OGD/R group while evaluated against the control group, and the expression levels in EGb761 group was higher measured against the group underwent OGD/R solely (Fig. 4A-C), indicating a significant enhancement in post-OGD/R HT22 cell proliferation due to EGb761 treatment. In order to further validate our findings, EdU staining was conducted. Findings revealed that compared to control group, the OGD/R group had a substantially reduced number of cells with proliferative activity, indicating a weakened proliferative capacity of cells after treated with OGD/R. Conversely, the EGb761-treated group exhibited a significantly higher number of proliferated cells in opposition to the OGD/R group (Fig. 4D-G), which highlighted a substantial elevation in the proliferative ability of the cells underwent EGb761treatments.

EGb761 increased OGD/ R-induced migration of HT22 cells

HT22 cells underwent allocations among following groups: control, OGD/R and EGb761 groups to investigate the capability of EGb761 on migration, and wound healing assays were employed, wherein cells from all groups migrated towards the bare zone of the scratch as the experiment progressed (Fig. 5A). In comparison with the control group, the migration rate was notably slower in the group of OGD/R, whereas the EGb761 group demonstrated increased migration velocity surpassing the findings of the OGD/R group (Fig. 5B). These findings suggest that EGb761 may promote the migration of HT22 cells amidst the OGD/R circumstances.

RhoA-ROCK2 signalling pathway involved in the effects of EGb761 on OGD/R-induced proliferation and migration of HT22 cells

Pursuing an investigation of the involvement of RhoA-ROCK2 signalling pathway in the modulatory effects of EGb761 on cell proliferation and migration prompted by

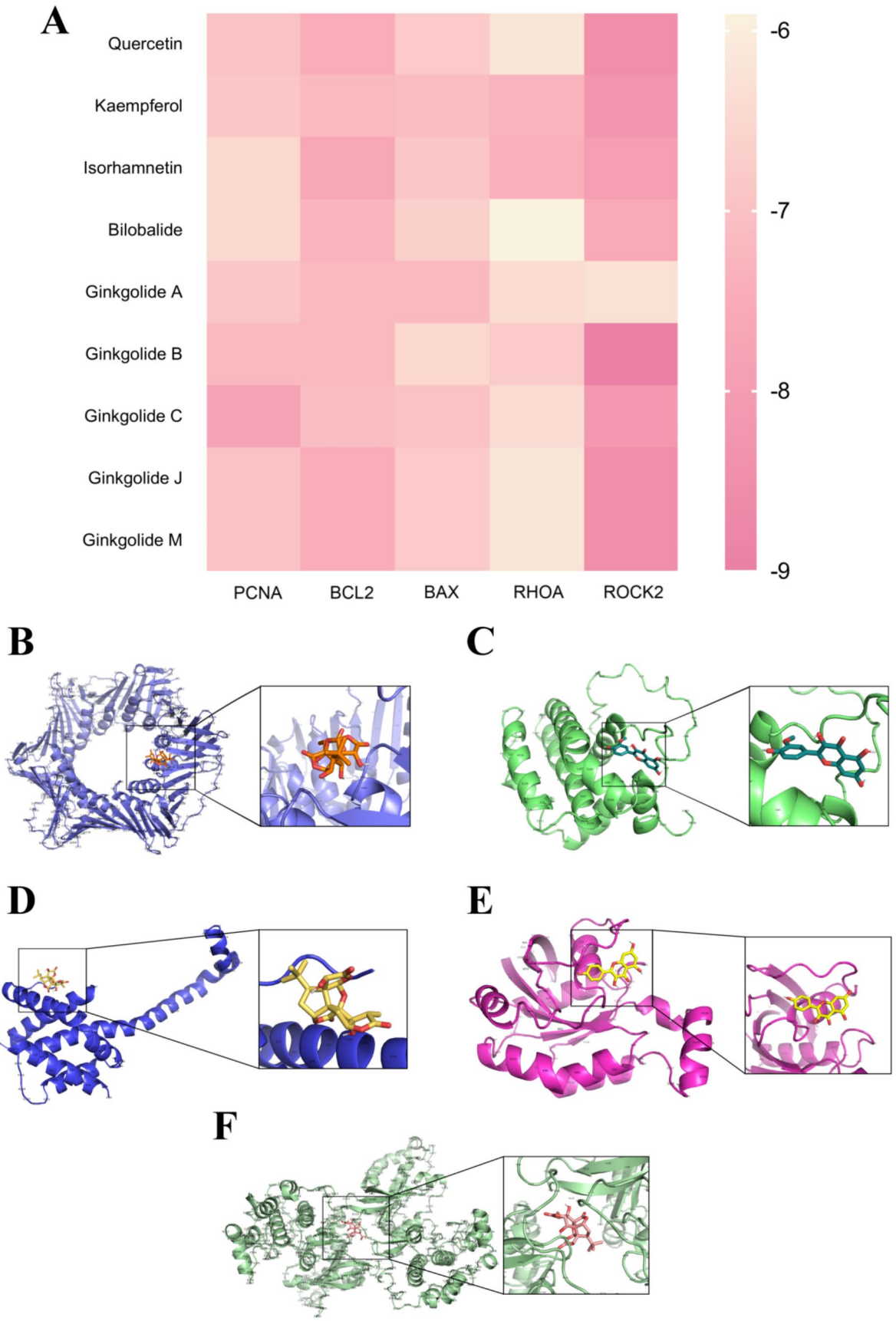

A
Quercetin
Kaempferol
Isorhamnetin
Bilobalide
Ginkgolide A
Ginkgolide B
Ginkgolide C
Ginkgolide J
Ginkgolide M
PCNA
BCL2
BAX
RHOA
ROCK2
-6
-7
-8
-9
B
C
D
E
F

Fig. 2 Molecular docking results. **A** Heatmap of molecular docking results (kcal/mol), indicating the magnitude of the binding energy, with lower values signifying heightened estimated binding stability between ligands and targets. **B–F** Visualization of docking complexes formed by the ligand and receptor protein at the lowest binding energy, presented utilizing PYMOL software: Ginkgolide C and PCNA; Isorhamnetin and BCL2; Ginkgolide A and BAX; Kaempferol and RHOA; Ginkgolide B and ROCK2

OGD/R, Fasu, the inhibitor of RhoA-ROCK2 signalling pathway, was used. A total of four groups of cells were allocated: control, OGD/R, EGb761 and EGb761 + Fasu groups, and various assays including western blot, wound healing and EdU were employed. Through western blot examination, a reduction in the observed quantities of RhoA and ROCK2 within the group of EGb761 + Fasu has been revealed, while matched against the EGb761 assembly (Fig. 6). Contrasting with the EGb761 group, findings pointed to subdued lower expression of PCNA and Bcl-2/Bax ratio in the EGb761 + Fasu group. (Figs. 7A-C) Similarly, EdU results showed significantly fewer proliferating cells within the assembly of EGb761 + Fasu while juxtaposed against the EGb761 group, indicating diminished proliferation ability. (Fig. 7D-E) The wound healing indicated a reduced migration capability of cells in the group of EGb761 + Fasu as opposed to the EGb761 group at 6, 12, and 24 h (Fig. 7F-G). Overall, it has been conclusively proposed and inferred that the inhibition of RhoA-ROCK2 signalling pathway reduces cell proliferation and migration, and that EGb761 boosts these through the RhoA-ROCK2 signalling pathway.

Discussion

Neuronal proliferation and migration are essential for the formation of neural circuits and are critical for cognitive functions, including memory acquisition and retention. Enhancing neurogenesis, along with promoting neuronal proliferation and migration, emerges as a vital therapeutic approach for alleviating cognitive impairments. In response to neurological insults such as cerebral I/R injury, neurons located within the CA1 region of the hippocampus, one of the most sensitive areas, undergo apoptosis (Wang et al. 2011). Endogenous neural stem cells differentiate into neurons that subsequently proliferate rapidly and migrate towards damaged regions. (Tang et al. 2022) These new neurons integrate into existing neural networks, thereby markedly aiding in the rejuvenation of cognitive functions (Toda et al. 2018). Investigations has discovered that suppression of miR-155 has demonstrated a capacity to offer protective effects by enhancing the survival capacity and migration regarding SH-SY5Y cell that experienced OGD/R conditions, while also mitigating apoptosis and reducing the necrotic zone in middle cerebral artery occlusion (MCAO) mouse (Zhang et al. 2020). Therefore, this study aims to explore strategies for enhancing neurogenesis, as well as promoting neuronal proliferation and migration in the context of cerebral I/R injuries, which are hypothesized to be critical therapeutic approaches for ameliorating cognitive disorders associated with VD.

EGb761 is a standardised extract extensively utilized for the amelioration and deterrence of neurodegenerative dementias. Its multifaceted neuroprotective effects are attributed to its ability to modulate several biological processes, including potent anti-inflammatory and antioxidant activities, the inhibition of apoptosis, and other critical cellular protective mechanisms. Researches had discovered that EGb 761 conspicuously intensified the proliferation of cells within the hippocampal region of 6 months and 22 months TgAPP/PS1 mice (Tchantchou et al. 2007). In addition to its effects on neurogenesis, EGb761 induced prominent reductions within the infarct volume in I/R rats, as in comparison to the vehicle-treated group. This reduction is accompanied by a marked inhibition of neuronal apoptosis, as well as substantial increases in hippocampal cellular proliferation and migration (Sun et al. 2013). Furthermore, our previous experiments on rat models revealed that EGb761 not only delays the progression of cognitive decline but also improves learning and memory capacities (Yin et al. 2024). Despite these promising findings, whether EGb761 can promote neuronal proliferation and migration to exert its neuroprotective effects in VD is yet not clear and remains to be elucidated.

Network pharmacology is a systems biology approach that addresses multiple targets, drug-drug interactions and the overall effect of a drug within an organism. Core targets of Ginkgo biloba for the treatment of VD were initially identified, enabling a deeper understanding of the intricate molecular mechanisms underlying its therapeutic potential. Results of the analysis revealed 235 potential targets, with a PPI network highlighting 35 crucial targets, including RHOA and BCL2. To further explore the biological pathways involved in the treatment of VD, GO enrichment and KEGG pathway analyses were also performed in order to provide valuable insights into the contributions of the identified targets and their interconnected pathways. Molecular docking confirmed strong binding between the main active compounds of *Ginkgo biloba* and the identified core targets, including BAX, BCL2, RHOA, ROCK2, PCNA. These findings provide compelling evidence that the identified core targets focus on proliferation and migration, and play a pivotal role in the therapeutic mechanisms of EGb761.One of the core targets identified in this study is proliferating cell nuclear antigen (PCNA), a fundamental protein which would be crucial for DNA synthesis and repair processes

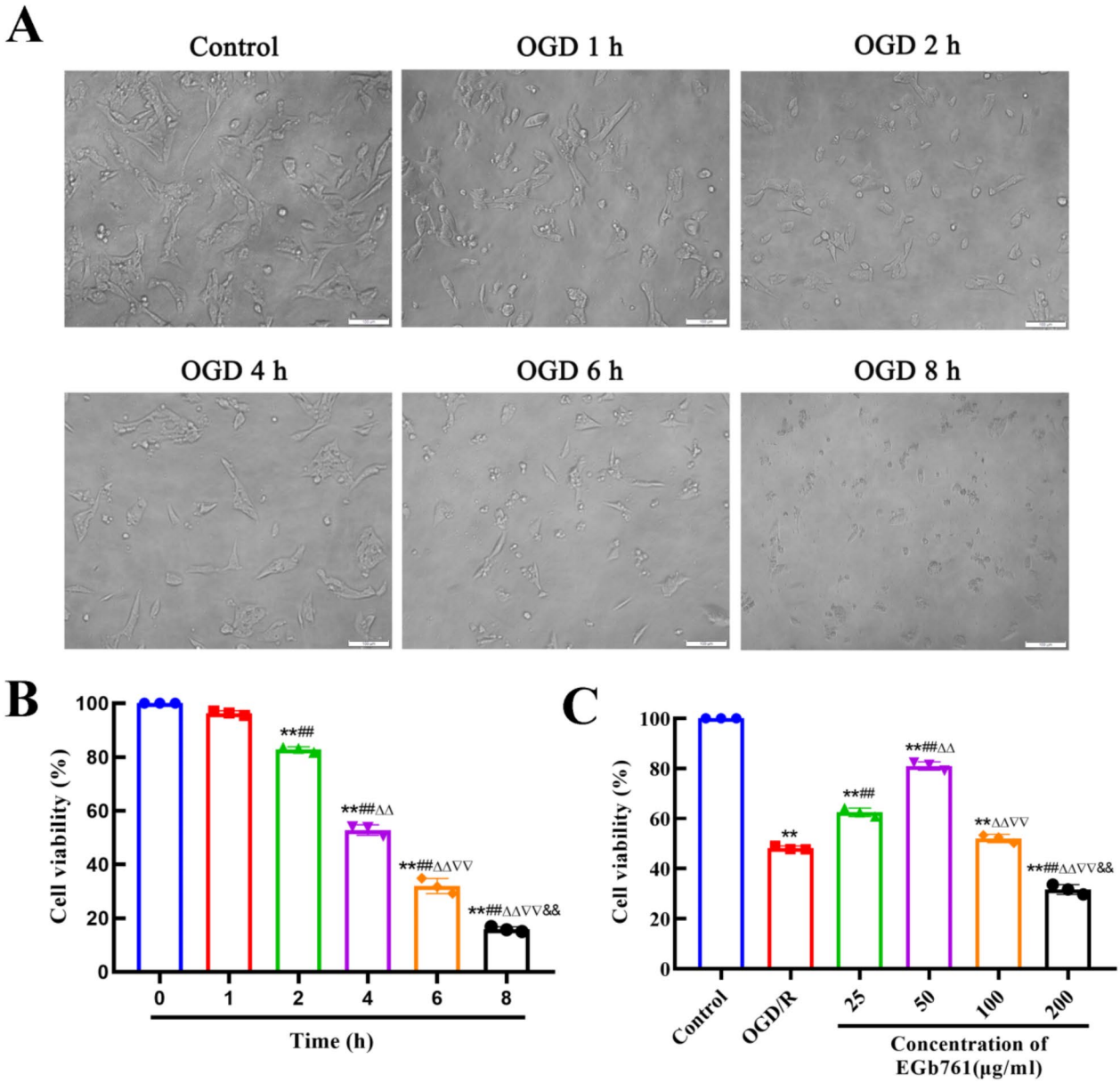


Fig. 3 Microscopic morphology and viability assessment of HT22 cells. **A** Morphology of HT22 cells at different time of OGD. Bar = 100 μm. **B** Assessment of HT22 cell viability at different time of OGD conducting CCK-8 assay. ($n = 3$) $^{**}P < 0.01$ vs.0 h group, $^{\#\#}P < 0.01$ vs.1 h group, $^{\Delta\Delta}P < 0.01$ vs. 2 h group,$^{\nabla\nabla}P < 0.01$ vs. 4 h group, $^{\&\&}P < 0.01$ vs. 6 h group. **C** Viability of HT22 cell subjected by OGD/R after EGb761 treatment, assessed using CCK-8 asay. ($n = 3$) $^{**}P < 0.01$ vs. Control group, $^{\#\#}P < 0.01$ vs. OGD/R group, $^{\Delta\Delta}P < 0.01$ vs. 25 μg/mL group,$^{\nabla\nabla}P < 0.01$ vs. 50 μg/mL group, $^{\&\&}P < 0.01$ vs. 100 μg/mL group

within proliferating cells, signifies active cell proliferation and serves as a vital role in preserving cellular equilibrium (Witko-Sarsat et al. 2010). In neurological diseases, elevated PCNA expression has been linked to enhanced DNA repair, facilitating the recovery from cerebral I/R injury by promoting cell proliferation, and ultimately improving cognitive function (Wang et al. 2022). Furthermore, studies by Matthew K. Tobin have disclosed an interrelation in the plenitude of DCX^+ $PCNA^+$ cells and interactive functionalities among presynaptic SNARE proteins, suggesting that an elevated count of DCX^+ $PCNA^+$ cells are associated with improved cognitive performance and mental acuity (Tobin et al. 2019). BAX and BCL2 are also closely linked to cellular proliferation and apoptosis, making their regulation as a potential therapeutic strategy for VD. In addition, RHOA and ROCK2, key components of the RhoA-ROCK2

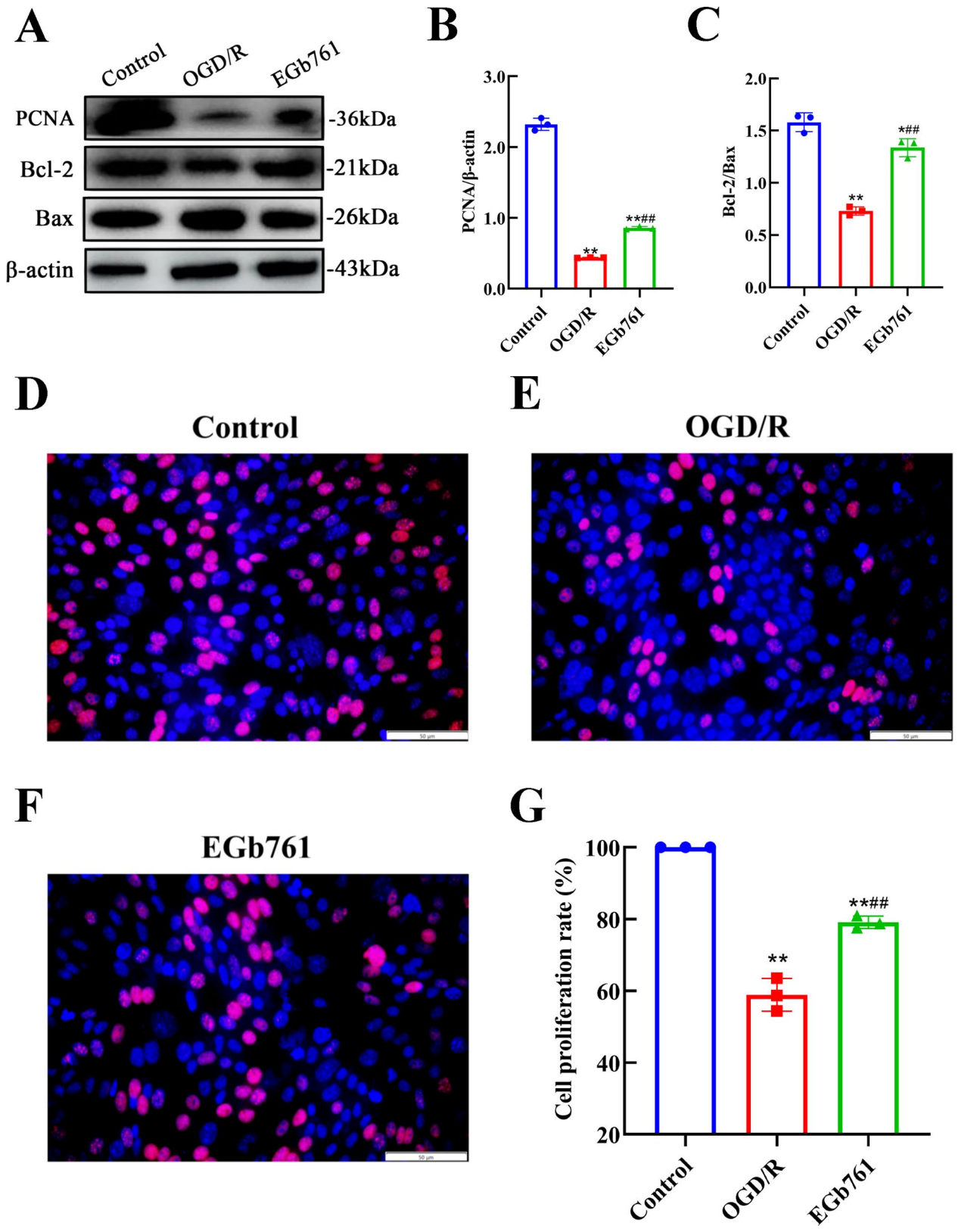


Fig. 4 EGb761 amplified HT22 cells' proliferation provoked by OGD/R. **A–C** EGb761's influence on PCNA level and Bcl-2/Bax in OGD/R-intervened HT22 cells. **D–G** The proliferative influence attributed by EGb761 on OGD/R-induced HT22 cells, assessed by EdU assay. Bar = 50 μm. ($n = 3$) $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group, $^{\#\#}P < 0.01$ vs. OGD/R group

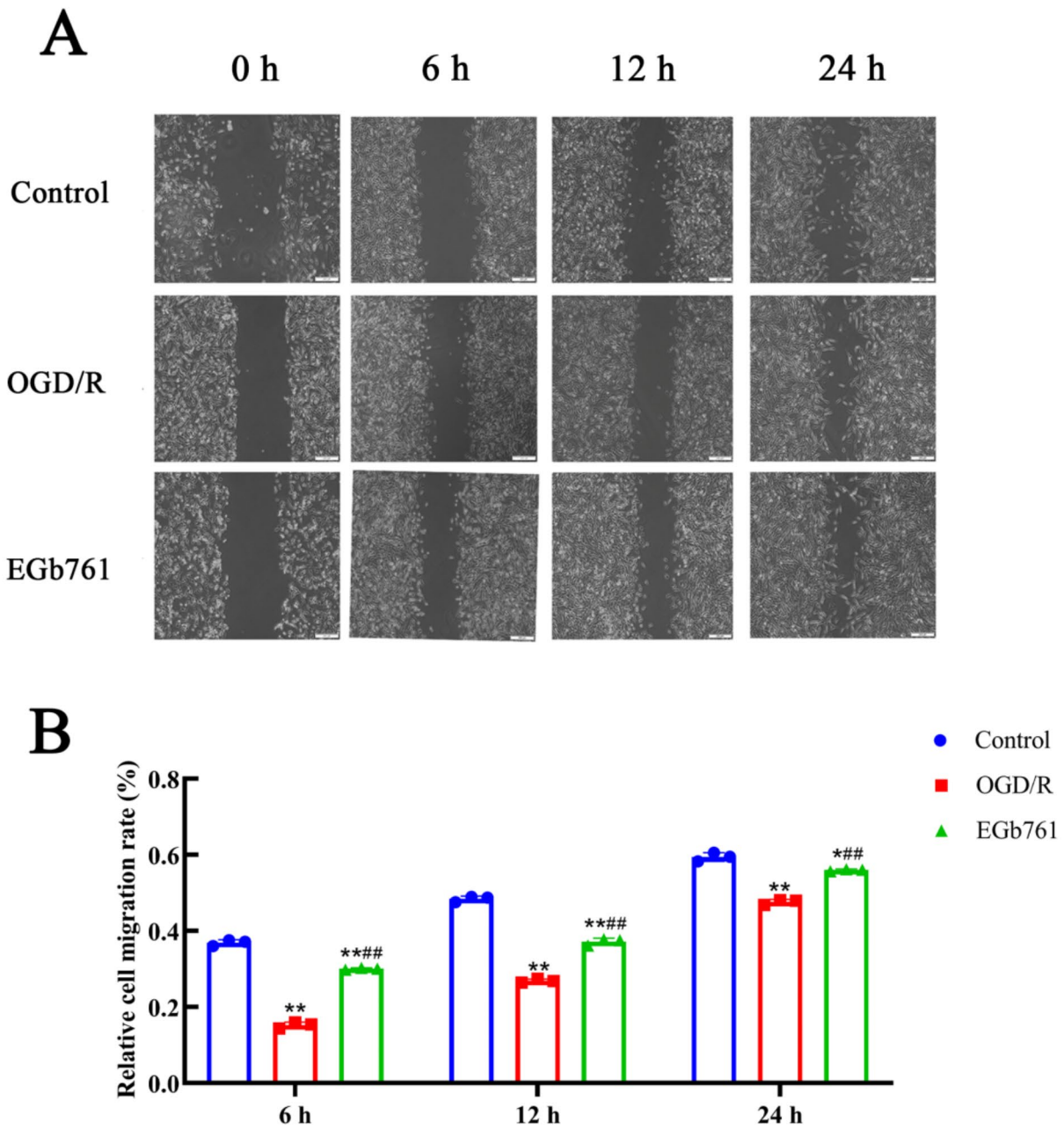


Fig. 5 Migration effect of EGb761 on OGD/R-induced HT22 cells detected by the wound healing assay. **A** The migration of post-OGD/R HT22 cells treated with EGb761 observed under the microscope at 6, 12, and 24 h. Bar = 200 μm. **B** Statistical analysis of migration in OGD/R-induced HT22 cells receiving EGb761 treatment at 6, 12, and 24 h. ($n = 3$) $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control group, $^{\#\#}P < 0.01$ vs. OGD/R group

signaling pathway, regulate cellular growth, differentiation, migration, and development, with its activation promoting cellular proliferation (Ma et al. 2018). This pathway enhances neurite extension in hippocampal neurons, while suppression of RhoA diminishes axon arborization in embryonic mouse neurons (Chan et al. 2007; Ahnert-Hilger et al. 2004). By modulating these core targets and the signalling pathways involved in neuronal proliferation and migration, it remains uncertain whether these targets indeed contribute to the therapeutic effects of EGb761 in VD.

While our findings suggest the potential role of BAX, BCL2, PCNA and RhoA-ROCK2 signalling pathway as

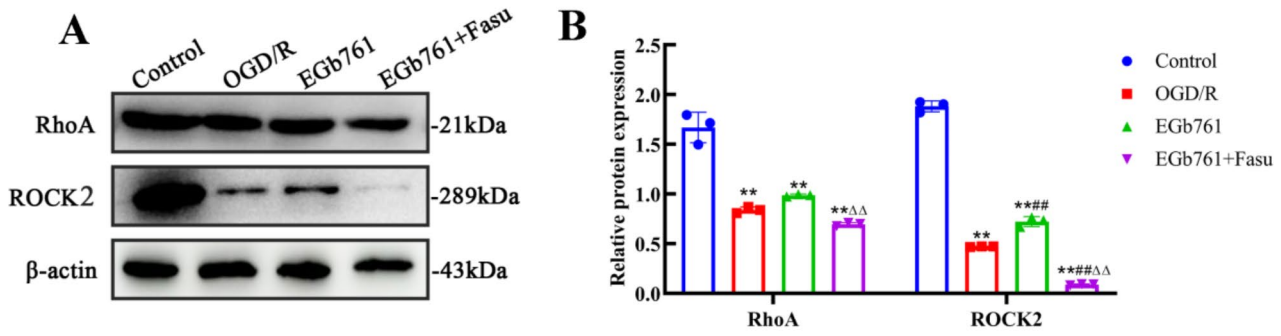


Fig. 6 Effect of the inhibitor Fasu on the RhoA-ROCK2 signalling pathway in OGD/R-intervened HT22 cells. **A** Band articulation of RhoA and ROCK2 levels in the experimental context of HT22 cells in the control, OGD/R, EGb761, and EGb761 + Fasu groups. **B** Statistical analysis of RhoA and ROCK2 expression.($n = 3$) $^{**}P < 0.01$ vs. Control group, $^{\#\#}P < 0.01$ vs. OGD/R group. $^{\Delta\Delta}P < 0.01$ vs. EGb761 group

therapeutic targets in these processes, their precise involvement remain unclear and warrant further investigation to validate their relevance for drug intervention. Building upon the previous findings, cellular experiments were conducted to further elucidate the neuroprotective mechanisms of EGb761 and its effects on these targets. The experimental results demonstrated that EGb761 significantly increased PCNA expression and upregulated the Bcl-2/Bax ratio, thereby enhancing the proliferation and migration abilities of OGD/R-exposed HT22 cells, confirming the neuroprotective effects of EGb761. Moreover, detailed cellular assays demonstrated that EGb761 treatment led to pronounced upregulation of RhoA and ROCK2 expression levels compared to the untreated model group. Conversely, the group treated with EGb761 in combination with the Fasu exhibited downregulation amidst the levels of RhoA, ROCK2, PCNA, and the Bcl-2/Bax ratio, accompanied by diminished cellular proliferation and migration compared to treatment with EGb761 alone. These findings underscore the vital influence of RhoA-ROCK2 signalling pathway in stimulating the proliferation and migration of neurons, suggesting that EGb761 can effectively promote neuronal proliferation and migration in OGD/R-induced HT22 cells through the RhoA-ROCK2 signalling pathway.

During this study, core targets were identified as pivotal regulators in these processes and are considered potential therapeutic candidates for the treatment of VD. Experimental findings demonstrated that EGb761 effectively enhances neuronal proliferation and migration in HT22 cells subjected to OGD/R, primarily through the activation of the RhoA-ROCK2 signalling pathway, underscoring the significance of this pathway in the neuroprotective effects of EGb761. However, in vitro experiments may not be able to fully replicate the in vivo environment of VD. While cell culture models provide valuable insights into the molecular and cellular effects of EGb761, they lack the complexity of whole organisms, such as interactions between different cell types, the influence of systemic factors like hormones and cytokines, as well as the impact of the blood-brain barrier. To comprehensively elucidate the therapeutic effects and mechanisms of EGb761, our future research will incorporate in vivo studies to simulate the complicated biological environment of living organisms, integrating bioinformatics and metabolomics to explore EGb761's impact from molecular interactions to systemic outcomes, offering deeper insights into its potential for treating VD. In summary, EGb761 has shown potential in enhancing neuronal proliferation and migration through the pathway of RhoA-ROCK2, with neuroprotective effects evident in cellular models, highlighting the remedial promise of aiming at the signalling pathway of RhoA-ROCK2 in mitigating VD.

Conclusion

This study employed an integrated approach merging network pharmacology alongside with the vitro experimental validation in order to delve into the curative potential of EGb761 for VD. Several key targets including RHOA, ROCK2, and PCNA were initially identified as potential therapeutic targets for VD. Subsequent in vitro experiments demonstrated that EGb761 effectively promotes neuronal proliferation and migration through the pathway of RhoA-ROCK2 amidst OGD/R-exposed HT22 cells, underscoring its therapy effect in brain tissue injury repair in VD and provide further preclinical evidence supporting its clinical application.

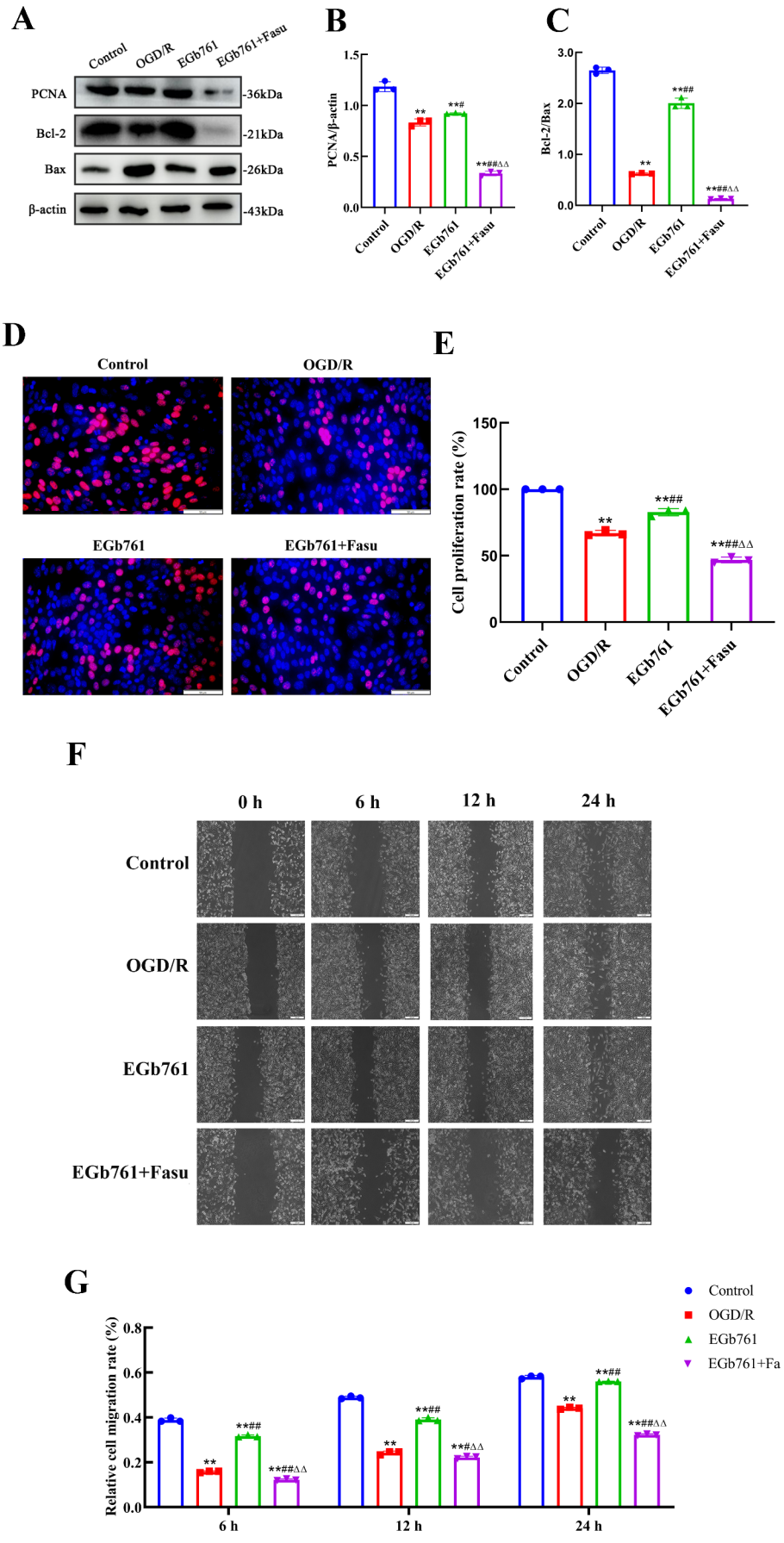


Fig. 7 Involvement of the signalling pathway of RhoA-ROCK2 in the EGb761 mediated proliferative and migratory responses of OGD/R-induced HT22 cells. **A–C** The expression of PCNA and Bcl-2/Bax in the control, OGD/R, EGb761, and EGb761 + Fasu groups. **D–E** Cell proliferation of OGD/R-induced HT22 cells in the control, OGD/R, EGb761, and EGb761 + Fasu groups, assessed by EdU assay. Bar = 50 μm. **F–G** Cell migration of OGD/R-induced HT22 cells in the control, OGD/R, EGb761, and EGb761 + Fasu groups, evaluated by the wound healing assay. Bar = 200 μm. ($n = 3$) $^{**}P < 0.01$ vs. Control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. OGD/R group. $^{\Delta\Delta}P < 0.01$ vs. EGb761 group

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Author contributions Dexiu Wang: Writing– original draft, Conceptualization, Methodology, Investigation, Funding acquisition. Xin Zhao: Methodology, Investigation, Formal analysis. Jinghan Li: Methodology, Investigation, Data curation. Yang Song: Investigation, Formal analysis, Data curation. Weida Chen: Methodology, Investigation, Funding acquisition, Data curation. Xin Cai: Methodology, Investigation, Formal analysis, Resources. Ruofan Liu: Writing– review & editing, Project administration, Conceptualization. Zetao Chen: Writing– review & editing, Project administration, Conceptualization.

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Data availability Data is provided within the manuscript or supplementary information files.

Declarations

Consent for publication All authors agreed with the content and that all gave explicit consent to submit and publish.

Competing interests The authors declare no competing interests.

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