Tetramethylpyrazine downregulates transcription of the CXC receptor 4 (CXCR4) via nuclear respiratory factor-1 (Nrf-1) in WERI-Rb1 retinoblastoma cells

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Abstract. Tetramethylpyrazine (TMP; an extract of the Chinese herbal medicine, Chuanxiong) has been shown to exert remarkable antiretinoblastoma (RB) effects. Based on our previous study, the target gene was found to be C-X-C chemokine receptor type 4 (CXCR4). CXCR4 is a prognostic marker in various types of cancer, but the exact mechanisms underlying the regulation of CXCR4 expression by TMP in WERI-Rb1 cells have yet to be fully elucidated. In the present study, it was revealed that TMP significantly downregulated CXCR4 expression and inhibited CXCR4 promoter activity in WERI-Rb1 cells, indicating that TMP inhibits CXCR4 expression in WERI-Rb1 cells through transcriptional regulatory mechanisms. Among the numerous transcription factors involved in CXCR4 function, including Yin Yang 1 (YY1), nuclear respiratory factor-1 (Nrf-1), Krüppel-like Factor 2 (KLF2), specificity protein 1 (SP1), and nuclear factor-κB subunit 1 (NF-κB1), only TMP led to a significant downregulation of Nrf-1 expression. Chromatin immunoprecipitation assays further indicated that Nrf-1 directly binds to the promoter region of CXCR4, and silencing Nrf-1 via siRNA transfection notably inhibited CXCR4 expression in WERI-Rb1 cells. In addition, the expression levels of both Nrf-1 and CXCR4 increased concomitantly with WERI-Rb1 cell density. Furthermore, the downregulation of Nrf-1 and CXCR4 expression in RB by TMP was confirmed *in vivo*. Taken together, the results of the present study have uncovered a novel mechanism in which CXCR4 expression is regulated by Nrf-1 in WERI-Rb1 cells, thereby identifying novel potential targets for the treatment of RB, and providing evidence for the clinical application of TMP in adjuvant retinoblastoma therapy.

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Introduction

Retinoblastoma (RB) is a pediatric retinal tumor and the most common primary intraocular childhood malignancy (1-3), with an incidence of ~1 in 15,000 live births (4). The occurrence and development of tumors seriously damages the function of the retina. In addition, the current effective treatment methods for RB, including local chemotherapy and radiotherapy, can lead to further retinal damage (5). Recent progress in RB diagnosis and treatment has pushed the goal of RB management from life-saving to eye and vision preservation. Numerous types of neuron exist in the retina, including ganglion cells, bipolar cells, horizontal cells, and photoreceptor cells. Light is collected by these neurons and transformed to electric signals that are transmitted to the brain, leading to the phenomenon of vision. Thus, ideal therapeutic agents for the adjuvant therapy of RB should possess the following properties: Inhibition of tumor cells, proliferation and protection of neurons against damage from excitotoxicity induced by tumor cells, and radiotherapy.

Chuanxiong has been used in Chinese traditional medicine for more than 2,000 years. Its bioactive component, tetramethylpyrazine (TMP), was extracted from Chuanxiong in 1973 (6). According to data from the China Food and Drug Administration (CFDA), there are 132 pharmaceutical factories that produce TMP injections or tablets in China (7). TMP has been widely used in the clinic to treat ischemia, cerebral infarction, degenerative diseases, and so on, albeit there are mild side effects (8-11). Furthermore, a paper published by our research group identified that TMP exerts neuroprotective effects, and revealed a mechanism underpinning TMP-mediated treatment that involved the inhibition of C-X-C chemokine receptor type 4 (CXCR4) expression in cerebral neurocytes and glioma cells (12). Accumulating evidence has confirmed that TMP can

significantly attenuate chemotherapeutic multidrug resistance, and is able to inhibit the proliferation and metastasis of various types of cancer cells, including melanoma cells, lung cancer and gastric carcinoma cells (13-18). More importantly, in our previous study, it was shown that TMP can significantly inhibit RB cell growth, and that CXCR4 is the target gene (19).

The chemokine receptor CXCR4 belongs to a large superfamily of G protein-coupled receptors with a 7-transmembrane spanning structure. CXCR4 is widely expressed in numerous types of cancerous tissues, including lung, kidney, breast, and retinal tumors, and is considered to serve a pivotal role in a number of biological processes that promote cancer growth and spreading, including angiogenesis, invasion, locomotion, extravasation, directional migration, homing, and cell survival (20-26). Therefore, CXCR4 has been shown to be a prognostic marker in various types of cancer, including leukemia, breast cancer, and prostate cancer (27-29). In addition, certain eye diseases have been shown to be associated with abnormal activation of CXCR4, such as primary open-angle glaucoma, angiogenesis, and eye inflammation (30,31). In our previous study, it was shown that CXCR4 is overexpressed in RB cells, and is a target gene of TMP to inhibit the growth of RB cells (19). Nevertheless, the mechanism underlying the regulation of CXCR4 expression by TMP in RB cells is not well defined.

The regulation of transcription is a vital process in all living organisms, and has a strong impact on gene expression. CXCR4 transcription is controlled by various mechanisms, depending on the cell type. In oral cancer cells, Krüppel-like factor 2 (KLF2) was shown to reduce CXCR4 promoter activity as a negative regulator of CXCR4 expression (32). In breast cancer cells, nuclear factor-κB (NF-κB) could directly bind to the CXCR4 promoter region (from -66 to +7 bp) and positively regulate CXCR4 expression, thus promoting tumor migration and metastasis (33). Our previous study also demonstrated that NF-κB and Nrf-1 transcriptionally co-regulate CXCR4 in corneal neovascularization (34). In addition, the transcription factors c-myc, Yin Yang 1 (YY1), specificity protein 1 (SP1), and hypoxia-inducible factor-1α (HIF-1α) have also been reported to be involved in the transcriptional regulation of CXCR4 (35-37). However, the transcription factors regulating CXCR4 expression by TMP in RB cells remain unidentified.

Therefore, the aim of the present study was to investigate the possible transcriptional mechanism by which TMP mediates the downregulation of CXCR4 in RB cells *in vitro* and *in vivo*. The results obtained will lead to the identification of novel potential targets for the treatment of RB, and provide evidence for the clinical application of TMP in adjuvant therapy of RB.

Materials and methods

Ethics statement. Thirty-two female nude mice (4-5 weeks old), weighing between 16 and 20 g, were obtained from the Laboratory Animal Center, Sun Yat-sen University (Guangzhou, China). All experimental procedures were performed in accordance with the ARVO Statements for the Use of Animals in Ophthalmic and Vision Research, and were approved through the Institutional Animal Ethical Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (permit no. 2014-007). All animals were maintained in a room (temperature: 20-26˚C, atmosphere: 40-70%) with a light schedule of alternating 12 h periods of light and dark, and received adequate amounts of food and water freely.

Cell culture. Cells of the human WERI-Rb1 RB cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Invitrogen® RPMI-1640 medium (Thermo Fisher Scientific, Inc, Waltham, MA, USA) supplemented with 10% FBS containing 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. Primary mouse retinal neurocytes were cultured as described previously (38). For co-culture assay, Transwell 12-well plates (0.4 μm pore size; BD Biosciences, Bedford, MA, USA) were used. Approximately $5x10^4$ primary mouse retinal neurocytes were cultured for 6 days in the lower chamber filled with 1 ml medium, and subsequently 200 μl WERI-Rb1 cell suspension ($7.5x10^5$ cells/ml) was seeded in the upper chamber. Cells were maintained in RPMI-1640 and DMEM medium (1:1) supplemented with 10% FBS. For the cell density assay, the WERI-Rb1 cells were seeded in 6-well plates and maintained in 2 ml Complete™ RPMI-1640 medium at different densities ($1x10^5$, $2.5x10^5$, $5.0x10^5$, $7.5x10^5$, and 10^6 cells/ml) for 24 h; subsequently, cell proteins were extracted for performing western blot assays. TMP was purchased from Sigma (now a brand of Merck KGaA; Darmstadt, Germany) and dissolved in component solvent (DMSO: Saline=1:1) to an appropriate concentration. The component solvent was applied as a control in all experiments.

Immunohistofluorescence assay. Cultured primary retinal neurocytes or WERI-Rb1 cells were fixed with ice-cold 4% paraformaldehyde for 15 min. For mono-staining (of Map-2 or CXCR4), the fixed cells were blocked with 10% normal goat serum for 30 min. For double-staining (CXCR4 and Nrf-1), the fixed cells were incubated with 0.1% Triton X-100 for 10 min, and then blocked with 10% normal goat serum for 30 min. Subsequently, cells were incubated overnight at 4˚C with primary antibodies against Map-2 (1:100; cat. no. BM1243, Boster Biological Technology, Ltd., Wuhan, China), CXCR4 (1:100; cat. no. ab2074, Abcam, Cambridge, UK) and Nrf-1 (1:100; cat. no. sc-23624, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Alexa Fluor 555 anti-rabbit lgG (1:500; cat. no. 4413, Cell Signaling Technology, Inc., Dallas, TX, USA) and Alexa Fluor 488 anti-goat lgG (1:500; cat. no. A-11055, Thermo Fisher Scientific, Inc.) were used as secondary antibodies, and nuclei were stained with DAPI. Images were captured using fluorescence microscopy (Leica Microsystems, Wetzlar, Germany; original magnification, x100).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA from WERI-Rb1 cells following treatment with TMP was isolated with Invitrogen® TRIzol® reagent (Thermo Fisher Scientific, Inc.). The DNA contaminants in total RNA isolates were eliminated by treatment with DNAse I for 30 min at 37˚C. One microgram of total RNA was subjected to reverse transcription using a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) following the manufacturer's protocol. Expression levels of CXCR4 and Nrf-1 were measured by RT-qPCR with the Roche

LightCycler® 480 System (Roche, Indianapolis, IN, USA). The PCR program was as follows: 94˚C for 5 min, followed by 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec. The following primer pairs were used: CXCR4, 5'-CTT ATCCTGCCTGGTATTGTC-3' (forward) and 5'-CAATGT AGTAAGGCAGCCAAC-3' (reverse); Nrf-1, 5'-GGAATTCCC ATGGAGGAACACGGAGTGAC-3' (forward) and 5'-CGG GATCCCGTTATTTCCTTTTCAGTTGCTG-3'; (reverse); and β-actin, 5'-TCACCCACACTGTGCCCAT-3' (forward) and 5'-TCTTTAATGTCACGCACGATT-3' (reverse). Relative target gene expression levels (measured against β-actin) were calculated using the $2^{\Delta\Delta Cq}$ method (39).

Western blot assay. Western blotting assays were performed according to a standard protocol. Briefly, whole proteins were extracted by using a radioimmunoprecipitation (RIPA) lysate kit (Beyotime Institute of Biotechnology, Jiangsu, China). The protein concentration was determined using the bicinchoninic acid (BCA) method. Equal amounts of protein (30 μg/well) were separated on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis, which were subsequently electrophoretically transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, then incubated with primary antibodies. The following primary antibodies were used: CXCR4 (1:500; cat. no. ab2074, Abcam), Nrf-1 (1:1,000; cat. no. ab175932, Abcam), YY1 (1:300; cat. no. sc-1703x, Santa Cruz Biotechnology, Inc.), KLF2 (1:300; cat. no. ab203591, Abcam), SP1 (1:300; cat. no. BA1402, Boster Biological Technology, Ltd.), NF-kB1 (1:500; cat. no. BA1297, Boster Biological Technology, Ltd.) and GAPDH (1:1,000; cat. no. 10494-1-AP, ProteinTech Group, Inc., Chicago, IL, USA). GAPDH served as the loading control. Protein bands were detected using an enhanced chemiluminescence detection system (EMD Millipore, Billerica, MA, USA).

Cell Counting Kit-8 (CCK-8) cell viability assay. The effect of TMP on the cell viability of retinal neurocytes and WERI-Rb1 cells in a co-culture system was measured using a CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Following treatment with 200 μM TMP or vehicle control for the relevant time periods (24, 48 or 72 h), WERI-Rb1 cells were transferred to new wells. Subsequently, CCK-8 reagent was added to each well, followed by incubation at 37˚C for an additional 1 h. The absorbance of the product was measured at 450 nm using a fluorescence plate reader (Power Wave XS; BioTek China, Beijing, China). Cell viability was calculated by determining the optical density ratio of a treated culture over an untreated control.

RNA interference. The siRNA sequences used for targeted silencing of Nrf-1 and control sequences were as follows: Human Nrf-1 siRNA: 5'-CGTTAGATGAATATACTAC-3' and the control, GGUUUGGCUGGGGUGUUAUdTdT. The oligos were purchased from Guangzhou RiboBio (Guangzhou, China). WERI-Rb1 cells ($2x10^6$ cells in 60 mm dishes) in good condition underwent lipidmediated transfection using Invitrogen® Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, Inc.), as recommended by the manufacturer. The mRNA and protein expression levels of Nrf1 and CXCR4 were measured by RT-qPCR or western blotting assays, respectively, at 24 or 48 h after transfection.

Reporter and plasmid construction. A fragment spanning from -1,981 to +80 bp that included the CXCR4 promoter sequence was produced by PCR with the forward primer, 5'-GGGGTACCCCACACAATTCTGAATCCTGCCT-3', and the common reverse primer, 5'-CCGCTCGAGCGGTCC AGATGCGGTGGCTACTG-3'. This fragment was fused to the promoter-less firefly luciferase gene of pGL3-Basic vector (Promega Corporation, Madison, WI, USA) to generate the constructed plasmid, named 'PGL3-hCXCR4-pro'. PGL3-hCXCR4-pro was derived from the pGL3-Basic vector (Promega Corporation), in which human CXCR4 cDNA was inserted into pGL3-Basic vector using the restrictive digestion sites, *Kpn*I and *Xho*I.

CXCR4 promoter-reporter assay. WERI-Rb1 cells ($2x10^6$ cells in 60 mm dishes) were transfected using Invitrogen® Lipofectamine™ 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfected plasmids contained 2 μg expression plasmid PGL3-hCXCR4-pro or pGL3-Basic vectors, and 100 ng *Renilla* luciferase reporter plasmid, pCMV-RL (Promega Corporation). The pCMV-RL plasmid encoding *Renilla* luciferase was included in all the samples to monitor transfection efficiency. At 24 h post-transfection, the levels of firefly and *Renilla* luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase Assay system (Promega Corporation). The levels of firefly luciferase activity were normalized against Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using the ChIP assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY, USA), according to the manufacturer's protocol. Approximately $5x10^5$ cells/ml were used for each assay. WERI-Rb1 cells were cross-linked by the addition of 1% formaldehyde for 10 min at room temperature, and the reaction was terminated upon the addition of glycine (final concentration, 0.125 M). Subsequently, cells were collected and incubated in 600 μl SDS lysis buffer containing protease inhibitors (2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 mM PMSF) for 10 min on ice. The samples were sonicated to yield fragments of chromatin of ~0.5 kb in length on ice (the duration of the sonication process was 8 min (6 sec ON-12 sec OFF) and the power was set at 400 W). After sonication, the lysate was centrifuged at 16,800 g for 10 min at 4˚C. The supernatant was diluted in ChIP dilution buffer that included the protease inhibitors. A total 5% of the supernatant was saved as input DNA, and primary rabbit antibody Nrf-1 (Santa Cruz Biotechnology, Inc.) or rabbit normal IgG (Merck KGaA) was added to the supernatant and incubated overnight at 4˚C with rotation. Following incubation with protein A-agarose, the immune complexes were eluted with elution buffer. A part of the captured immune-complex was subjected to western blotting analysis to detect whether the captured chromatins contained Nrf-1. Cross-linking was reversed by heating at 65˚C overnight. RNA was subsequently degraded with RNase A for 30 min, whereas protein was degraded with proteinase K for 2 h. DNA was purified using an Ez-ChIP™ polypropylene spin

column (Merck Millipore), and subjected to PCR amplification using the following primers for H-CXCR4-CHIP: 5'-GAC CACCCGCAAACAGCAGG-3 (sense) and 5'-GCAGCCAAC AAACTGAAGTTTC-3' (antisense).

Murine xenograft model of retinoblastoma. All experimental procedures were approved by the Ethical Committee of Zhongshan Ophthalmic Center, Sun Yat-sen University (permit no. 2014-007). Female athymic nude mice aged 4-5 weeks old were obtained from the Laboratory Animal Center, Sun Yat-sen University. They were maintained in a standard environment with filter tops. After the WERI-Rb1 cells had been resuspended in PBS, the mice were anesthetized with 3% isoflurane delivered by mask, and $1x10^5$ cells were injected into the vitreous cavity of the right eye using a Hamilton needle. All surgical procedures were performed under sterile conditions with a dissecting microscope; the left eyes were used as untreated controls. The eyes were observed on a weekly basis for tumor development. At 2 weeks after the mice had been xenotransplanted intravitreally with WERI-Rb1 cells, fundus photographs (Phoenix MicronIV™; Phoenix Research Labs, Pleasanton, CA, USA) were taken for all animals. All animals were subsequently randomly divided into two groups (n=16 mice in each group). Mice were anesthetized with 3% isoflurane delivered by mask, and each treatment group then received vitreous injections with TMP at a final dose of 200 μM, whereas the control group received vitreous injections with balanced salt solution. At 48 h following vitreous injection, the mice were euthanized by carbon dioxide inhalation (flow rate: 2-3 l/min) in a 10 l sealed box, and death of the mice was verified through several indicators, including absence of a heartbeat, respiratory arrest, and rigor mortis. The globes (n=4 for each group) were enucleated and fixed in 4% paraformaldehyde for 12 h. The eyeballs were subsequently processed into paraffin-embedded sections, and sequential meridian sections (6 μm thick) were created and stained with hematoxylin and eosin (H&E). To detect the mRNA and protein levels of Nrf-1 and CXCR4 following TPM treatment, tumor tissue was collected and analyzed by RT-qPCR and western blotting, respectively, after vitreous injection with TPM for 48 h (n=6 for each group).

Statistical analyses. All experiments were performed at least three times. Data are expressed as the mean ± SD. Differences between mean values were evaluated using two-tailed Student's t-test (for 2 groups) or analysis of variance (ANOVA; >2 groups). SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. P<0.05 was considered to indicate a statistically significant value.

Results

TMP significantly promotes retinal neurocyte survival and suppresses WERI-Rb1 cell growth in co-culture systems. To assay the bioactivity of TMP on primary retinal neurocytes and WERI-Rb1 cells, a co-culture system was used to mimic RB physiological conditions. First, the primary rat retinal neurocytes were cultured 1 day after birth, and Map-2 staining was performed to verify the identity of these cells. As denoted by the green coloration in Fig. 1A, the majority of the cells were Map-2-positive cells. Subsequently, the primary retinal neurocytes were co-cultured with WERI-Rb1 cells, and treated with TMP or vehicle. At different time points following treatment, the cell viability of neurocytes and tumor cells were measured by CCK-8 assay. Our data showed that the viability of WERI-Rb1 cells was significantly inhibited following TMP treatment compared with the controls (at 24 h: Control, 1; and TMP, 0.82±0.04; at 48 h: Control, 1; TMP, 0.77±0.04; and at 72 h: Control, 1; TMP, 0.70±0.08) (P<0.05; Fig. 1B), results which were consistent with our previous study (19). Simultaneously, the viability of retinal neurocytes was significantly enhanced by TMP compared with controls (at 24 h: Control, 1; TMP, 1.10±0.04; at 48 h: Control, 1; TMP, 1.23±0.10; and at 72 h: Control, 1; TMP, 1.35±0.04) (P<0.05; Fig. 1C). These results indicated that TMP may serve as an ideal therapeutic agent for the adjuvant treatment of RB.

TMP downregulates CXCR4 expression in WERIRb1 cells. CXCR4 fulfills a critical role in fundamental aspects of cancer, including proliferation, migration, invasion, and metastases (20-26). CXCR4 was found to be strongly expressed on the nuclear membrane of WERI-Rb1 cells (Fig. 2A). At 24 h after treatment with 200 μM TMP, total RNA and whole-cell lysates were extracted for RT-qPCR and western blot analyses, respectively. As shown in Fig. 2B, the level of CXCR4 mRNA in WERI-Rb1 cells following TMP treatment was significantly decreased compared with the control (control, 1; TMP, 0.63±0.14; P<0.05). The western blot assay also revealed that TMP treatment led to a notable attenuation of the CXCR4 protein level in WERI-Rb1 cells compared with the control (Fig. 2C). The relative quantification of CXCR4 expression is shown as a histogram in Fig. 2D (control, 1; TMP, 0.64±0.06; P<0.05).

To examine whether TMP regulated CXCR4 expression in WERI-Rb1 cells at the transcriptional level, a firefly luciferase reporter was constructed using the predicted CXCR4 promoter region (-1,981 to +80 bp) (Fig. 2E). Luciferase assay revealed that TMP significantly inhibited CXCR4 reporter activity (Control, 1; CXCR4Pro, 62.90±7.79; and CXCR4-Pro + TMP, 49.67±11.96) (P<0.05; P<0.001; Fig. 2F). These results indicated that TMP downregulates CXCR4 expression in WERI-Rb1 cells via inhibition of its transcription.

The transcription factor Nrf-1 is downregulated by TMP in WERI-Rb1 cells. To identify the transcription factors regulated by TMP in WERI-Rb1 cells, several reported transcription factors (i.e., KLF2, SP1, Nrf-1, YY1, and NF-κB1) that bind to the CXCR4 promoter were analyzed. After treatment with TMP for 24 or 48 h, whole-cell lysates of WERI-Rb1 were analyzed by western blotting. As shown in Fig. 3A, Nrf-1 expression was markedly downregulated in accordance with the expression of CXCR4 in WERI-Rb1 cells; however, the expression levels of KLF2, SP1, YY1, and NF-κB1 in WERI-Rb1 cells were not significantly altered following TMP treatment. The relative expression levels were then quantified by densitometry and normalized against GAPDH levels. As shown in Fig. 3B, Nrf-1 expression was significantly decreased by 27.62±7.62 and 33.65±9.52%, respectively, at 24 and 48 h following TMP treatment. Similarly, CXCR4 expression was decreased by 35.87±8.89 and 45.40±11.75%, respectively. For

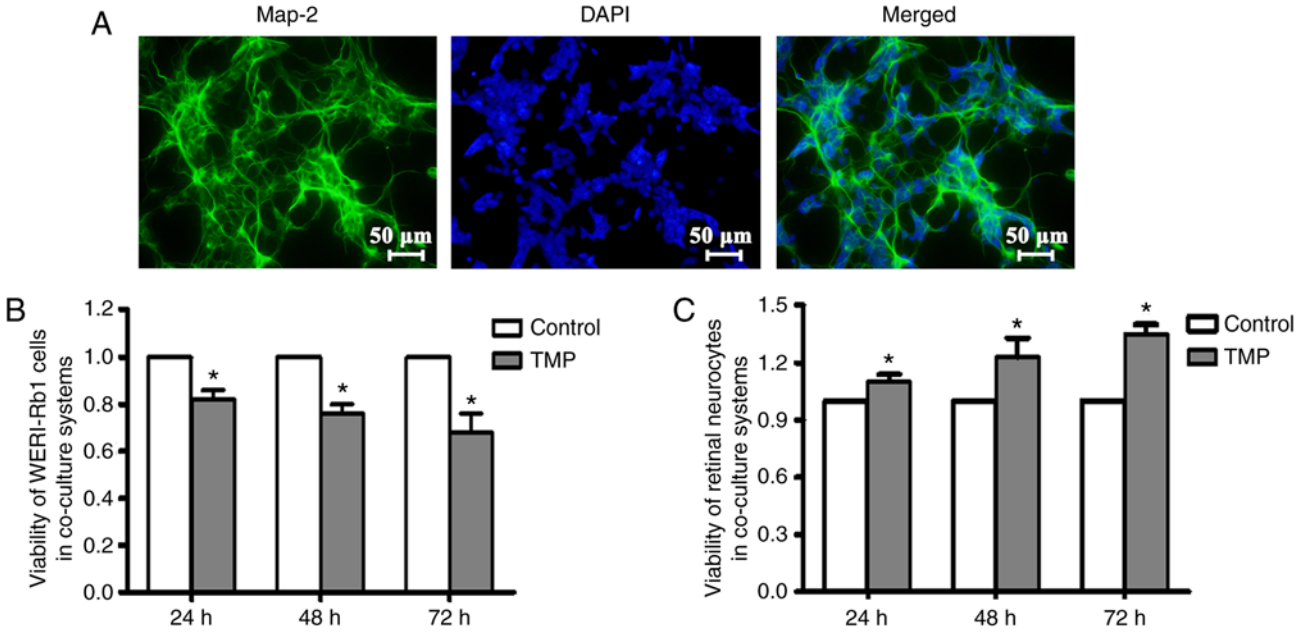

Figure 1. TMP significantly promotes retinal neurocyte survival and suppresses WERI-Rb1 cell growth in co-culture systems. (A) Immunohistofluorescence experiments revealed that Map-2 stained positively in the majority of primary retinal neurocytes. Scale bars, 50 μm. (B) The viability of WERI-Rb1 cells was significantly inhibited at 24 h after TMP treatment, compared with controls at 48 or 72 h in the co-culture system. (C) The viability of primary retinal neurocytes was significantly promoted by TMP compared with controls at 24, 48 or 72 h in the co-culture system. All these results were confirmed by three independent experiments. *P<0.05 comparing TMP treatment with the control. TMP, tetramethylpyrazine.

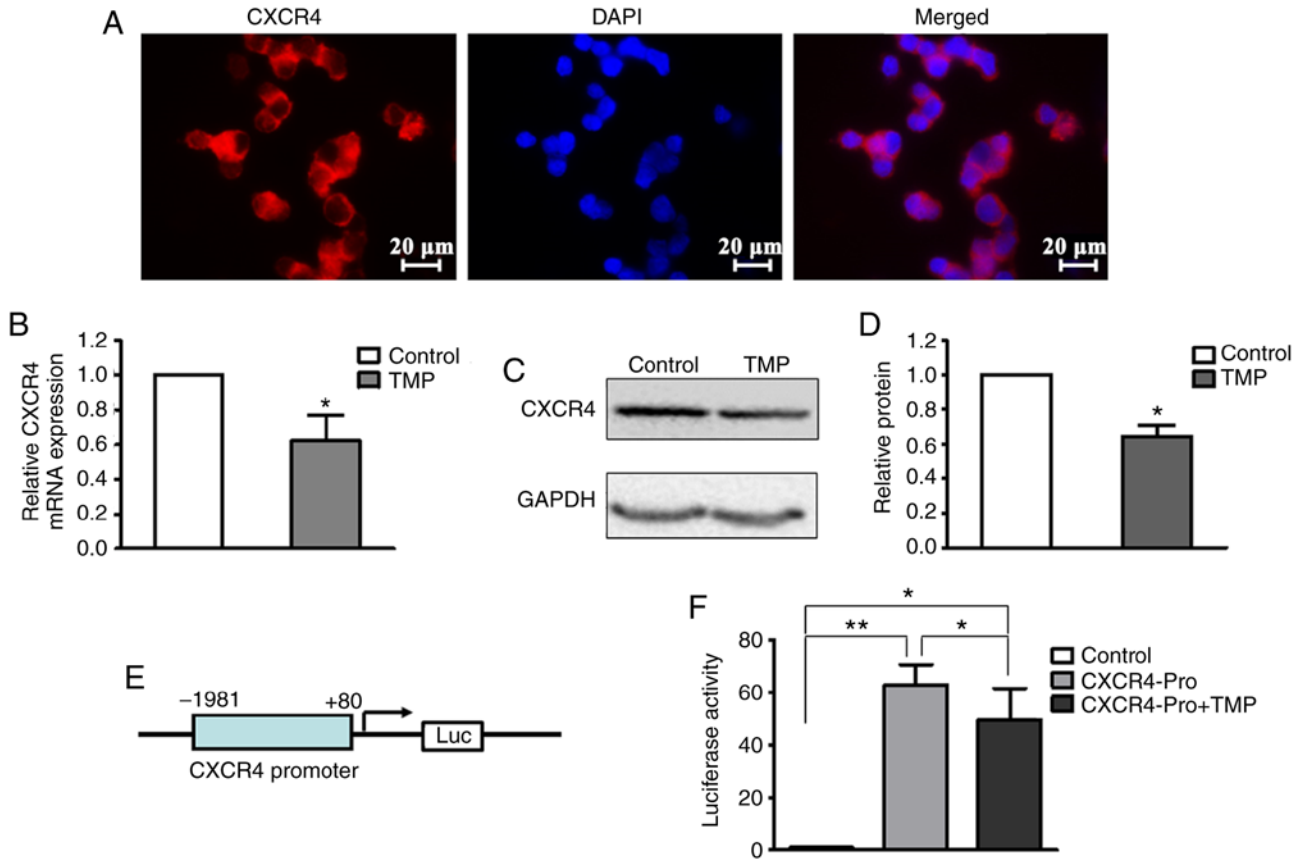

Figure 2. TMP downregulates CXCR4 expression in WERI-Rb1 cells. (A) Immunohistofluorescence assays revealed that CXCR4 (red) was expressed on the nuclear membrane of WERI-Rb1 cells. Scale bars, 20 μm. (B) RT-qPCR assays revealed that the level of mRNA expression of CXCR4 is downregulated following TMP (200 μM) treatment for 24 h. *P<0.05 comparing TMP treatment with the control. (C and D) The protein expression of CXCR4 in WERI-Rb1 cells was notably decreased by TMP, and the relative quantification of CXCR4 protein expression is presented as a histogram. *P<0.05 comparing TMP treatment with the control. (E) Schematic diagram of the PGL3-hCXCR4-Pro-luc reporter. (F) Luciferase activity analysis revealed that TMP significantly inhibits CXCR4 promoter activity. Statistically significant differences between the CXCR4-Pro and control, CXCR4-Pro and CXCR4-Pro + TMP, and CXCR4-Pro + TMP and the control are indicated (*P<0.05; **P<0.001). TMP, tetramethylpyrazine; CXCR4, C-X-C chemokine receptor type 4.

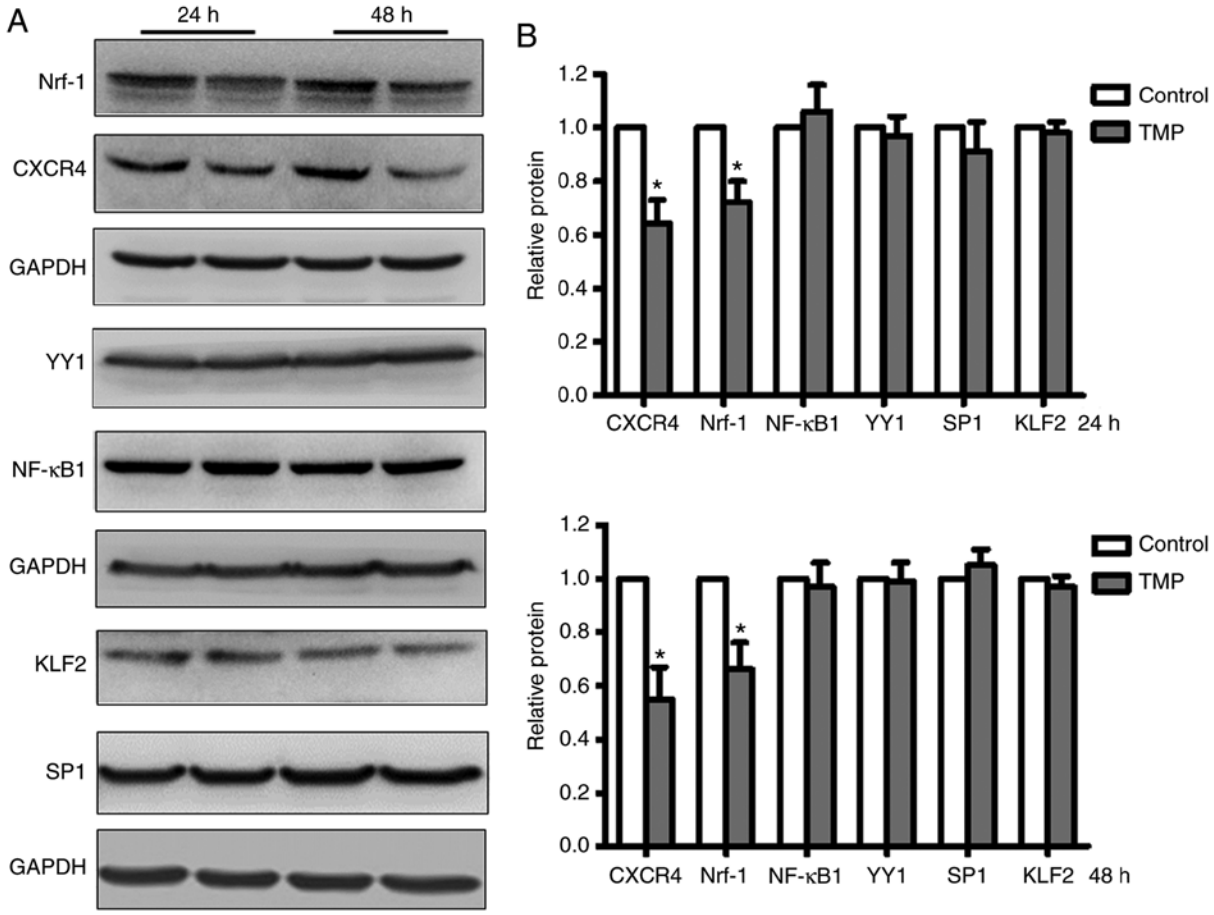

Figure 3. The transcription factor Nrf-1 is downregulated in WERI-Rb1 cells following TMP treatment. (A) Several CXCR4-associated transcription factors (KLF2, SP1, Nrf-1, YY1, NF-κB1) were analyzed by western blotting in WERI-Rb1 cells after TMP treatment for 24 or 48 h. (B) Relative quantification of protein expression levels revealed that the expression levels of Nrf-1 and CXCR4 were significantly decreased; however, the expression levels of KLF2, SP1, YY1, and NF-κB1 were not significantly altered in WERI-Rb1 cells following treatment with TMP (200 μM) for 24 or 48 h. *P<0.05 comparing TMP treatment with the control. TMP, tetramethylpyrazine; CXCR4, C-X-C chemokine receptor type 4; Nrf-1, nuclear respiratory factor-1; YY1, Yin Yang 1; KLF2, Krüppel-like Factor 2; SP1, specificity protein 1; NF-κB1, nuclear factor-κB subunit 1.

the expression of the other transcription factors, no significant differences were observed between the control group and the TMP-treated group (P>0.05). These results indicated that Nrf-1 may be involved in the transcriptional regulation of CXCR4 by TMP in WERI-Rb1 cells.

Nrf-1 directly binds to the CXCR4 promoter and positively regulates the expression of CXCR4 in WERI-Rb1 cells. To confirm whether Nrf-1 directly binds the CXCR4 promoter in WERI-Rb1 cells, ChIP assays were performed. Cells were sonicated to break up the chromatin molecules into ~0.5 kb fragments (Fig. 4A), followed by incubation with a rabbit Nrf-1 antibody or normal rabbit IgG. A portion of each immunoprecipitation reaction was subjected to western blot assay. Nrf-1 was shown to be readily detectable in the samples incubated with Nrf-1 antibody, but not normal rabbit IgG (Fig. 4B). In addition, the precipitated DNA was subjected to PCR amplification using primers designed to amplify a 200 bp fragment of the CXCR4 promoter region flanking the ATF site. As shown in Fig. 4C, a 200 bp band was only detected in DNA incubated with anti-Nrf-1 and the input DNA. These results indicated that Nrf-1 directly binds to the promoter region of CXCR4.

To further verify that Nrf-1 exerts a crucial role in the regulation of CXCR4 in WERI-Rb1 cells, siRNA transfection was performed to silence Nrf-1 expression. As shown in Fig. 4D, the mRNA expression of CXCR4 was significantly decreased after Nrf-1 silencing. Western blot analysis also showed that silencing Nrf-1 notably inhibited CXCR4 protein expression in WERI-Rb1 cells (Fig. 4E). The relative quantification of Nrf-1 and CXCR4 protein expression is presented in histogram form in Fig. 4F. The protein expression of Nrf-1 was decreased by 42.19±11.46% after siRNA silencing. Accordingly, CXCR4 was also decreased by 35.42±11.98% (P<0.05). These data further suggested that Nrf-1 is involved in the positive transcriptional regulation of CXCR4 in WERI-Rb1 cells.

Nrf-1 and CXCR4 expression are increased in accordance with cell density. Our previous study demonstrated that CXCR4 downregulation by TMP is associated with cell density in WERI-Rb1 cells (19). Therefore, in the present study, the protein levels of Nrf-1 and CXCR4 following culture (24 h) of WERI-Rb1 cells plated at different densities ($1x10^5$, $2.5x10^5$, $5.0x10^5$, $7.5x10^5$, and 10^6 cells/ml) were measured. To verify the expression location of Nrf-1 and CXCR4, and that Nrf-1 and CXCR4 are co-expressed in WERI-Rb1 cells,

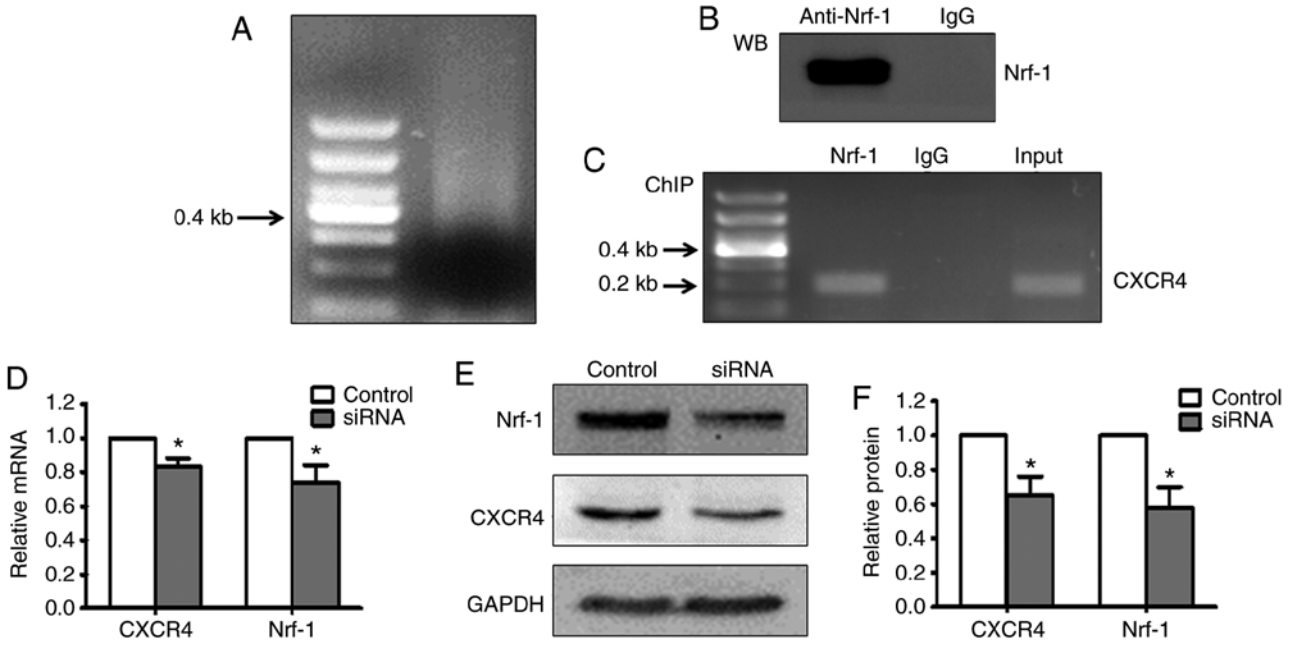

Figure 4. Nrf-1 directly binds to the CXCR4 promoter and regulates the expression of CXCR4 in WERI-Rb1 cells. (A) Sonicated DNA isolated from WERI-Rb1 cells was treated with formaldehyde to cross-link endogenous proteins to DNA (fragments averaged ~0.5 kb in length). (B) Western blot analysis, revealing immunoprecipitation of Nrf-1. (C) DNA was isolated and purified from immunoprecipitated material and amplified by PCR with primers to generate a 200 bp fragment of the CXCR4 promoter spanning the ATF site, showing that the 200 bp fragment was only amplified from the precipitates incubated with anti-Nrf-1 antibodies plus total genomic DNA (input). (D) RT-PCR results, showing that the mRNA of CXCR4 is downregulated after Nrtf-1 siRNA interference in WERI-Rb1 cells. (E) Western blot analysis also showed that expression of CXCR4 is downregulated by Nrf-1 siRNA interference in WERIRb1 cells. (F) Relative quantification of Nrf-1 and CXCR4 protein levels is shown as a histogram. *P<0.05, comparing between the siRNA group and control. ChIP, chromatin immunoprecipitation; TMP, tetramethylpyrazine; CXCR4, C-X-C chemokine receptor type 4.

double immunohistofluorescence staining was performed. The results demonstrated that Nrf-1 was expressed in the nucleus, and that CXCR4 was expressed predominantly on the nuclear membrane, with moderate staining evident in the cytoplasm (Fig. 5A). Western blot analysis demonstrated that Nrf-1 and CXCR4 protein expression was increased in accordance with cell density (Fig. 5B). The relative expression of Nrf-1 and CXCR4 in WERI-Rb1 cells was also quantified by densitometry. The average ratio of Nrf-1 or CXCR4 to GAPDH in WERI-Rb1 cells at the lowest cell density investigated ($1x10^5$ cells/ml) was defined as 1. As shown in Fig. 5C, relative quantification revealed that Nrf-1 in WERI-Rb1 cells was significantly upregulated with increasing cell density, compared with cells at the lowest density ($1x10^5$ cells/ml, 1; $2.5x10^5$ cells/ml, 1.30±0.05; $5x10^5$ cells/ml, 1.58±0.14; $7.5x10^5$ cells/ml, 1.82±0.10; and 10^6 cells/ml, 2.04±0.10) (P<0.05). The expression of CXCR4 was also significantly increased in cells, in essentially a stepwise manner, with increasing cell density ($1x10^5$ cells/ml, 1; $2.5x10^5$ cells/ml, 1.26±0.10; $5x10^5$ cells/ml, 1.47±0.21; $7.5x10^5$ cells/ml, 1.83±0.09; and 10^6 cells/ml, 2.09±0.05) (P<0.05; Fig. 5D). These results demonstrated that the expression pattern of Nrf-1 is similar to that of CXCR4, further indicating that the expression of CXCR4 in WERI-Rb1 cells is regulated by the transcription factor Nrf-1.

Nrf-1 and CXCR4 are downregulated by TMP in vivo. Based on the *in vitro* results, an RB orthotopic xenotransplantation model was established to validate the TMP-mediated inhibition of CXCR4 by the transcription factor Nrf-1. Fundus photographs were taken for all animals aged over 2 weeks. Fig. 6A shows fundus photographs of normal mice eyes (left panel) and mice eyes with RB (center panel; the white arrowheads indicate RB tumors); H&E staining revealed that the cell mass of the RB is located in the vitreous cavity (right panel; the white arrowheads indicate RB tumors). Subsequently, the mice were divided into two groups receiving either vitreous injection of TMP, or vehicle. At 48 h following treatment, total RNA and protein lysates of RB were obtained for RT-qPCR and western blot analyses. As shown in Fig. 6B, the mRNA expression levels of both Nfr-1 and CXCR4 were significantly decreased in retinoblastoma treated with TMP, compared with controls (for Nrf-1: Control, 1; TMP-treated, 0.23±0.06; for CXCR4: Control, 1; TMP-treated, 0.47±0.22) (P<0.05). Similarly, the altered Nfr-1 and CXCR4 protein expression levels were consistent with the changes in mRNA levels (Fig. 6C). The relative quantification of Nrf-1 and CXCR4 protein expression is presented in histogram form in Fig. 6D (for Nrf-1: Control, 1; TMP-treated, 0.66±0.08; for CXCR4: Control, 1; TMP-treated, 0.40±0.04) (P<0.05). These data further demonstrated that CXCR4 is downregulated by TMP through Nrf-1 *in vivo*.

Discussion

TMP is commonly used in the clinic to treat vascular diseases and neurodegenerative diseases with mild side effects (8-11). According to our previous study, TMP possesses a remarkable anti-RB effect (19); however, its underlying molecular mechanism has not been fully elucidated. Currently, TMP

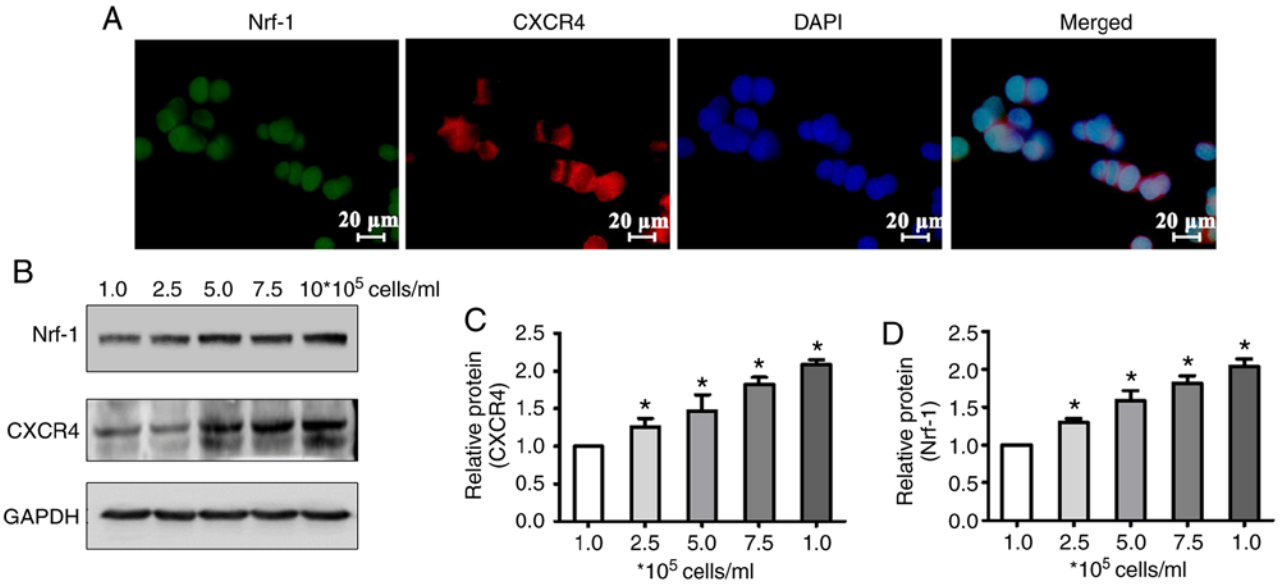

Figure 5. Nrf-1 and CXCR4 expression are increased in accordance with cell density. (A) Immunohistofluorescence double staining showed that Nrf-1 (green) is expressed in the nuclei of WERI-Rb1 cells and CXCR4 (red) is expressed mainly on the nuclear membrane, with moderate staining in the cytoplasm. Scale bars, 20 μm. (B) Western blot analysis showed that Nrf-1 and CXCR4 expression increased with cell density ($1.0x10^5$, $2.5x10^5$, $5.0x10^5$, $7.5x10^5$, and 10^6 cells/ml). (C and D) Relative quantification of protein expression also revealed that Nrf-1 and CXCR4 levels increased in WERI-Rb1 cells concomitantly with an increase in cell density (i.e., $2.5x10^5$, $5.0x10^5$, $7.5x10^5$, and 10^6 cells/ml) compared with the Nrf-1 and CXCR4 levels at the lowest density (i.e., $1x10^5$ cells/ml). *P<0.05 compared with the lowest density (i.e., $1x10^5$ cells/ml). Nrf-1, nuclear respiratory factor-1; TMP, tetramethylpyrazine; CXCR4, C-X-C chemokine receptor type 4.

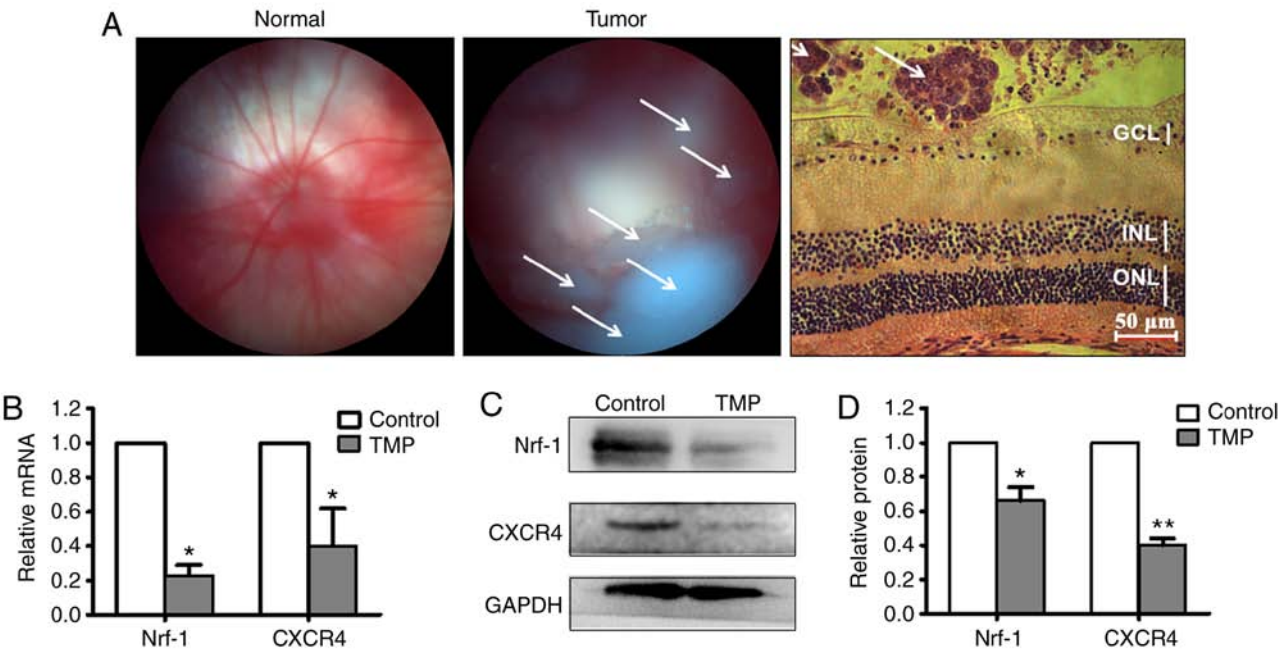

Figure 6. Nrf-1 and CXCR4 are downregulated by TMP *in vivo*. (A) Photographs of nude (nu/nu) mice retinoblastoma xenografts. Fundus photographs of normal mice eyes (left panel) and mice eyes with retinoblastoma (center panel). Hematoxylin and eosin staining showed that the cell mass of retinoblastomas was located in the vitreous cavity (right panel). White arrowheads indicate RB tumors. Scale bars, 50 μm. (B) The mRNA levels of Nrf-1 and CXCR4 were significantly downregulated following TMP treatment. (C and D) Western blot analysis indicated that TMP treatment led to a downregulation of Nrf-1 and CXCR4 protein expression in WERIRb1 cells; relative expression is shown as a histogram in (D). Statistically significant differences between TMP-treated cells and the control are indicated (*P<0.05; **P<0.001). Nrf-1, nuclear respiratory factor-1; TMP, tetramethylpyrazine; CXCR4, C-X-C chemokine receptor type 4; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

is only used in certain Oriental countries, including China and Korea. Therefore, clarifying the anti-RB mechanism of TMP will extend its clinical therapeutic application in medical practice.

In the present study, RB cells and a retinal neurocyte co-culture system were used to mimic the RB ocular physiological environment, and the data obtained revealed that TMP is able to significantly inhibit the viability of WERIRb1

cells and to promote retinal neurocyte survival (Fig. 1). Furthermore, accumulating evidence has confirmed that combining TMP with other treatments could significantly attenuate the multidrug resistance of chemotherapy (15-17). Therefore, TMP may be an ideal chemical for the adjuvant treatment of RB. CXCR4 is the target gene of TMP, which acts as an inhibitor of RB cell growth. In addition to the downregulation of mRNA and protein expression of CXCR4 by TMP, the present study also disclosed that the activity of the CXCR4 promoter was significantly reduced following TMP treatment in WERI-Rb1 cells, suggesting that TMP inhibits CXCR4 expression in WERI-Rb1 cells via transcriptional regulatory mechanisms.

The regulation of transcription is a vital process in all living organisms, which exerts a strong impact on gene expression. Previous studies in various cell lines have reported that the CXCR4 promoter is regulated by several transcription factors, including KLF2, SP1, Nrf-1, YY1, NF-κB1, and so on (32-36). In the present study, however, TMP was only found to mediate the downregulation of Nrf-1. In addition, ChIP assays confirmed that Nrf-1 directly bound to the CXCR4 promoter region, and silencing Nrf-1 notably decreased CXCR4 expression. Furthermore, Nrf-1 and CXCR4 expression were downregulated by TMP in an RB model. These results strongly suggest that TMP downregulates the transcription of CXCR4 via Nrf-1 in WERI-Rb1 cells.

A previously published study by our research group demonstrated that NF-κB and Nrf-1 transcriptionally co-regulate CXCR4 expression in human umbilical vein endothelial cells and the alkali-burn cornea (34). However, in the present study, TMP was found not to affect NF-κB1 expression in WERI-Rb1 cells. Therefore, these results suggested that CXCR4 is regulated by different transcriptional factors in different cells. In addition, a previous study also demonstrated that Nrf-1 expression correlates with that of CXCR4 during retinal development (40). CXCR4 and Nrf-1 are expressed in the postnatal rat retina, and are silenced together in the adult rat retina. Furthermore, in the oxygen-induced retinopathy rat model, retinal hypoxia was able to concurrently induce the upregulation of CXCR4 and Nrf-1 expression. Therefore, the transcriptional mechanism of CXCR4 in WERI-Rb1 cells may be similar to that found in normal retinal tissue. RB is a malignant intraocular tumor derived from the retina; therefore, it is possible to speculate that transcription factors of CXCR4 are specific to host tissue.

In our previous study, it was shown that TMP downregulation of CXCR4 in WERI-Rb1 cells is sensitive to cell density (19). Interestingly, Nrf-1 and CXCR4 expression were found to be significantly increased in parallel with cell density in the present study. Nrf-1, a member of the basic leucine-zipper family of proteins (41,42), was first identified as a transcription factor that regulates mitochondria-associated genes, including p43, CREB, p53, and Stat3 (43,44). A further study also showed that Nrf-1 is able to mediate reactive oxygen species (ROS) and play a vital role in regulating cell metabolism and respiration (45). Therefore, these studies suggest that increasing the cell density induces the upregulation of Nrf-1, which subsequently promotes the transcription of CXCR4 in retinoblastoma cells.

In addition, previous studies demonstrated that Nrf-1 is involved in neural ischemic diseases. For example, the mRNA content of Nrf-1 rapidly increased 3-6 h following an ischemic-tolerant state (46). Nrf-1 was significantly upregulated in the retina of an ischemia-reperfusion surgery model, and decreased after treatment with lithium, a neuroprotective reagent (38). TMP has been used in patients with neural diseases, and our research group previously verified that TMP is able to downregulate CXCR4 expression in cerebral neurocytes, which inhibits increases in somatic Ca^{2+} and decreases glutamate release to protect neurons (12). Therefore, it is possible to speculate that TMP exerts a neuroprotective action by inhibiting Nrf-1 expression, with the subsequent decrease in CXCR4 expression.

In addition, Nrf-1 is known to regulate the expression of other genes, and our previous study demonstrated that Nrf-1 binds the promoter of ligase IV, which regulates DNA repair in retinal neurocytes (38). Therefore, in WERI-Rb1 cells, the Nrf-1-CXCR4 pathway might be one of several complicated signaling pathways that respond to TMP. Further investigation of this subject is required in the future.

In conclusion, the results obtained in the present study have helped to further elucidate the anti-RB molecular mechanism of TMP. The transcription factor Nrf-1 directly binds to the promoter sequence of CXCR4, and TMP downregulates CXCR4 expression at the transcriptional level via Nrf-1, thereby inhibiting the growth of WERI-Rb1 cells. Our study has identified novel potential targets for the treatment of RB, and provides evidence for the clinical application of TMP in adjuvant therapy of retinoblastoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study would be available from the corresponding author upon reasonable request.

Authors' contributions

NW and YY designed the study, performed the experiments, collected and analyzed the data, and wrote the manuscript. NY, YW, XH, and JQ performed experiments and analyzed the data. SC, JZ, XC, CW, and MY assisted in collecting data and assembling the figures. JG assisted in designing the study, and provided professional suggestions. KY and JZ designed the study, analyzed data, and wrote the manuscript. NW and JZ revised the manuscript in terms of its important intellectual content. All authors read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the ARVO statements for the Use of Animals in Ophthalmic and Vision Research, and were approved through the Institutional Animal Ethical Committee of Zhongshan Ophthalmic Center, Ethical Committee of Zhongshan Ophthalmic Center, Sun Yat-Sen University (permit no. 2014-007).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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