Abstract. The survival of young children (under 5 years of age) with malignant retinoblastoma remains poor, and clarification of the mechanism underlying tumour development is urgently needed. The present study aimed to reveal the role of exosomes (EXOs) from retinoblastoma cells in tumour development. The in vitro data indicated that EXOs derived from WERI-Rb1 cells significantly inhibited the antitumour activity of macrophages and induced bone marrow mesenchymal stem cells to promote tumour growth via an increase in monocyte chemotactic protein 1 (also known as C-C motif chemokine ligand 2) levels. In vivo data from a xenotransplantation model also showed that EXOs infiltrated the spleen, which induced a decrease in leukocytes and natural killer (NK) cells. Accordingly, the proportion of tumour-associated macrophages was increased and the proportion of NK cells was decreased in tumours injected with EXOs compared with those injected with the control. EXOs were absorbed by Kupffer cells, and more metastases were observed in the liver. Thus, these results suggested that EXOs derived from retinoblastoma promoted tumour progression by infiltrating the microenvironment. Moreover, microRNAs (miRs), including miR-92a, miR-20a, miR-129a and miR-17, and C-X-C chemokine receptor type 4 and thrombospondin-1 were detectable in EXOs, which might account for EXO-mediated tumour deterioration.

Introduction

Retinoblastoma is the most common intraocular malignant tumour that threatens the health of children, affecting 1:15,000-1:20,000 live births (1). Current treatments for retinoblastoma include chemotherapy, plaque radiotherapy, external beam radiotherapy, cryotherapy and surgery (2). However, despite the clinical response to such comprehensive strategies, tumour metastasis and extraocular invasion in the advanced stage still cause death in children (3,4). Therefore, investigation of the underlying mechanism of retinoblastoma tumorigenesis and development is necessary to improve therapeutic efficacy.

A growing body of literature has reported that the microenvironment regulates tumour progression and metastasis (5,6). Macrophages are plastic cells and have multiple bioactivities in response to environmental signals. Interferon and TLR ligands could orient macrophage function toward the M1 phenotype (high levels of proinflammatory), while IL-4 and IL-13 activate the M2 phenotype (tissue remodelling and promotion of tumour progression) (7). The tumour microenvironment can induce macrophages to adopt a tumour-promoting state (7). IL-6, TNF-α and monocyte chemotactic protein-1 (MCP-1) are highly expressed in resident tumour-associated macrophages (TAMs) or in macrophages derived from peripheral reservoirs, such as the bone marrow (BM) and spleen (8,9). TAMs contribute to matrix breakdown and tumour cell motility (10). However, the mechanism of activation of these cells by tumour cells is not well defined.

Exosomes (EXOs) are endocytosis-derived small membrane vesicles (40-150 nm) that are composed of proteins, lipids, microRNA (miRNA/miR) and mRNA surrounded by a phospholipid bilayer and are secreted into the extracellular space (11). A growing number of studies have demonstrated that EXOs and other extracellular vesicles secreted by tumour cells play an important role in cell-to-cell communication (12-16). EXOs can bind to target cells, fuse with the membrane and transfer their contents to mediate intercellular communication. Additionally, tumour-derived EXOs can facilitate tumour malignancy (17). Moreover, previous reports have verified that tumour-derived EXOs contribute to the establishment of a premetastatic niche and generate oncogenic microenvironments at distant metastatic sites by modulating stromal cells and remodelling the extracellular matrix (18,19).

Nevertheless, very little is known concerning the possible functions of retinoblastoma cell EXOs in tumour progression. Based on the aforementioned evidence, the aim of the present study was to determine the effects of retinoblastoma
cell EXOs to elucidate the possible underlying mechanisms of retinoblastoma deterioration.

Materials and methods

**Cell culture.** WERI-Rb1 human retinoblastoma cells (American Type Culture Collection) were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% foetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Murine macrophages RAW264.7 (The Cell Bank of Type Culture Collection (FBS; Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS. Bone marrow mesenchymal stem cells (BMSCs) were acquired from C57 mice and BMSCs from passages 4-6 were cultured in DMEM with 10% FBS for use (8x10⁶/ml). These cells were incubated in a humidified atmosphere with a mixture of 1% O₂, 5% CO₂ and 94% N₂ at 37°C.

**Primary BMSC isolation.** BMSCs were isolated from the bone marrow of C57 BL/6 mice using previously described methods (20). Briefly, 4–6 week old C57 BL/6 mice were anaesthetized by intraperitoneal injection of Nembutal (30 mg/kg; cat. no. P3761, Sigma-Aldrich; Merck KGaA) and then sacrificed by cervical dislocation, and their titibas and femurs were separated aseptically. DMEM was used to flush the marrow out with a sterile 25-gauage needle and the extruded BM cells were filtered through a 70-µm cell strainer (BD Biosciences) and centrifuged at 1,000 x g for 5 min at 37°C. The BM cells were resuspended with DMEM containing 10% FBS and plated into flasks. The cells were treated with 0.25% trypsin/1 mM EDTA for 2 min until the cells reached 90% confluence. The culture medium was changed every 3 days. After 3-4 passages, isolated cells were used for experiments.

**EXO isolation, characterization and treatment.** EXOs were purified from WERI-Rb1-derived conditioned medium (collected from 48 h cell cultures with RPMI-1640 and 10% exosome-free FBS) by ultracentrifugation, as outlined previously (21). The FBS was depleted of EXOs through ultracentrifugation at 110,000 x g overnight at 4°C. After 48 h, the conditioned media were centrifuged at 1,000 x g for 5 min, followed by 10,000 x g for 30 min, and then ultracentrifugation of the supernatants was performed at 100,000 x g for 90 min. All centrifugation was performed at 4°C. The EXOs were washed with phosphate-buffered saline (PBS), and then ultracentrifugation was carried out at 100,000 x g for 90 min at 4°C, following which, EXOs were resuspended in PBS. The size and number of EXOs were counted by Nano-Flow cytometry, according to a previous method (22), which is the imaging of individual fluorescently labelled EXOs passing through nanochannels in a pressure-driven flow (23). Lastly, the protein concentration was measured using a Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology).

EXOs were viewed by transmission electron microscopy (FEI Tecnai Spirit G2; Thermo Fisher Scientific, Inc.), according to a previous method (21). Briefly, freshly isolated EXO pellets were transferred to a copper grid coated with carbon in a 30-µl drop of 1% glutaraldehyde for 5 min and then the grid was washed with distilled water. The grids were stained with 4% uranyl-acetate solution for 10 min at 37°C, and then treated with a 50-µl drop of methyl cellulose for 5 min on ice. Then, the grid was washed with distilled water and observed under transmission electron microscopy at 80 Kx.

For EXO labelling, EXOs were fluorescently labelled using the PKH26 membrane dye (Sigma-Aldrich; Merck KGaA), according to a previous method (24). Briefly, EXOs were resuspended into 1 ml Diluent C and 6 µl PKH26 dye was added for 5 min at room temperature. Then, the reaction was quenched by adding 2 ml 10% BSA (cat. no. 0332; Amresco, LLC) followed by ultracentrifugation at 100,000 x g for 2 h to remove excess dye.

**BMSCs and WERI-Rb1 co-culture.** For the co-culture experiments, BMSCs (4x10⁵) treated with PBS or EXOs were plated in 24-well plates. WERI-Rb1 cells (4x10⁵) were suspended in EXO-free medium and seeded into Transwell chambers with 0.4-µm pore size inserts (BD Biosciences). The viability of BMSCs and WERI-Rb1 cells were assessed using a CCK-8 assay after 24 h. BMSC RNA was extracted for RT-qPCR analysis.

**Cell viability assay.** The viabilities of WERI-Rb1 cells and RAW264.7 macrophages treated with EXOs or PBS were detected using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.). Each group of cells was incubated with CCK-8 reagent for 3 h at 37°C. Finally, the absorbance was assessed at 450 nm and recorded.

**Reverse transcription-quantitative (RT-q)PCR analysis.** Total RNA from WERI-Rb1 cells, RAW264.7 cells and BMSCs was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was subjected to reverse transcription using a PrimeScript™ RT Reagent kit (Takara Biotechnology Co., Ltd.), following the manufacturer's protocol. Total RNA from exosomes was extracted with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and Dr GenTLE™ Precipitation Carrier (Takara Biotechnology Co., Ltd.). Total RNA (1 µg) was subjected to reverse transcription using a Mir-X™ miRNA First-Strand Synthesis Kit (Takara Biotechnology Co., Ltd.), following the manufacturer's protocol. RT-qPCR was performed with a SYBR Prime Script RT-PCR Kit on a Roche 480 system (Roche Diagnostics) following the manufacturer's protocols. PCR conditions were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. The relative target gene expression was quantitated using the 2⁻ΔΔCq method (25) and normalized to the endogenous expression of b-actin, GAPDH or miR-16. The primers for mRNA and miRNA detection are listed in Tables S1 and SII, respectively.

**Immunofluorescence assay.** Cells or tissue sections (tumour, spleen and liver) were fixed with 4% paraformaldehyde (10 min at 37°C), and then blocked with 1% BSA and 0.2% Triton X-100 for 30 min at 37°C. Samples were incubated with primary antibodies overnight at 4°C, including anti-CD49b for natural killer (NK) cells (1:100; cat. no. 14-5971-85; Invitrogen; Thermo Fisher Scientific, Inc.), anti-CD45 for leukocytes (1:100; cat. no. sc-1178; Santa Cruz Biotechnology, Inc.),
anti-CD68 for macrophages (1:100; cat. no. BA3638; Wuhan Boster Biological Technology Co., Ltd.), anti-Ki-67 for proliferative cells (1:500; cat. no. MA5-14520; Thermo Fisher Scientific, Inc.), anti-Vimentin for invasive tumour cells (1:100; cat. no. sc-6260; Santa Cruz Biotechnology, Inc.). After washing with PBS, samples were stained with the appropriate Alexa Fluor-conjugated secondary antibody for 1 h at 37°C (Alexa Fluor 555 anti-rabbit IgG, 1:500, cat. no. 4413; Alexa Fluor 488 anti-rabbit IgG, 1:500, cat. no. 4412; Alexa Fluor 488 anti-mouse IgG, 1:500, cat. no. 4408; all purchased from Cell Signaling Technology, Inc.). Images were captured using fluorescence microscopy (Axio Imager Z1; Carl Zeiss AG; magnification, x100).

Immunofluorescence staining for cells was quantified by analysing the fraction or number of positively-stained pixels per total field using ImageJ software (version 1.51; National Institutes of Health). This protocol was used in a previous study (26). Briefly, the images were binarized to black and white with a common threshold level such that white pixels represented positive cells and were quantified by histogram analysis using GraphPad Prism system 7 (GraphPad Software, Inc.). At least five random sections per mouse were used for quantification.

Western blotting. Whole proteins of cells and EXOs were extracted using a RIPA lysate kit (Beyotime Institute of Biotechnology). The protein concentration was determined with a BCA assay (Beyotime Institute of Biotechnology). Total protein (30 µg) from each sample was separated via sodium dodecyl sulfate-polyacrylamide gel by electrophoresis, and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% BSA for 2 h at 37°C, then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: Anti-GAPDH (1:10,000; cat. no. 10494-1-AP; Proteintech Group, Inc.), anti-Tubulin (1:1,000; cat. no. sc-5274; Santa Cruz Biotechnology, Inc.), anti-CDC63 (1:1,000; cat. no. EXOAB-CDB63A-1; Systems Biosciences, LLC), anti-tumour susceptibility gene 101 protein (1:1,000; cat. no. ab125011; Abcam), anti-C9 (1:1,000; cat. no. 13403; Cell Signalling Technology, Inc.), anti-heat shock 70 kDa protein 1A (1:1,000; cat. no. 4873; Cell Signalling Technology, Inc.), anti-proliferating cell nuclear antigen (PCNA; 1:1,000; cat. no. 13110; Cell Signalling Technology, Inc.), anti-Bax (1:1,000; cat. no. 2772; Cell Signalling Technology, Inc.), anti-Bcl-2 (1:1,000; cat. no. A00040-1; Wuhan Boster Biological Technology, Ltd.), anti-Caspase-3 (1:1,000; cat. no. 9662; Cell Signalling Technology, Inc.), anti-C-X-C chemokine receptor type 4 (CXC4; 1:1,000; cat. no. ab124824; Abcam), anti-MMP2 (1:1,000; cat. no. 10373-2-AP; Proteintech Group, Inc.) and anti-thrombospondin-1 (TSP-1; 1:1,000; cat. no. ab1823; Abcam). Membranes were then incubated with HRP-conjugated anti-mouse IgG (1:3,000; cat. no. 7076s; Cell Signalling Technology, Inc.) or anti-rabbit IgG (1:10,000; cat. no. 7074s; Cell Signalling Technology, Inc.) for 1 h at 37°C, following which the blots were visualized with an enhanced chemiluminescence system (Thermo Fisher Scientific, Inc.). GAPDH or Tubulin were used as loading controls. The band intensity was semi-quantified by ImageJ software (version 1.51; National Institutes of Health).
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EXOs derived from WERI-Rb1 cells affect the antitumour activity of macrophages and BMSCs in vitro. To reveal the effect of EXOs on immune cells, RAW264.7 cells, which are well-characterized with regard to their macrophage-specific immune functions (31), were incubated with EXOs from WERI-Rb1 cells labelled with PKH26 (red). At 6 h after incubation, EXOs could be taken up by RAW264.7 cells. PKH26 was partially colocalized with CD68 (green, a specific marker of macrophages; Fig. 2A). Moreover, the cells changed from round to spindle-shaped with ramified morphology (Fig. 2B), which indicated that macrophages were activated. However, EXOs significantly increased the levels of IL-6 and MCP-1 (relative fold-change: IL-6, 2.30±1.11-fold; MCP-1, 1.43±0.43-fold; *P<0.05). The increase in IL-6 and MCP-1 expression implied that the antitumour activity of macrophages was inhibited by EXOs, which was consistent with a previous study (6). There was no significant difference in TNF-α expression between the PBS and EXO groups in BMSCs (relative fold-change: 0.66±0.26-fold; ns). Moreover, the viability of WERI-Rb1 cells was significantly increased (PBS, 0.14±0.03; EXO, 0.21±0.02; *P<0.05) in the co-culture system, whereas the viability of BMSCs was not affected by EXOs (PBS, 0.42±0.05; EXO, 0.46±0.02; ns) (Fig. 2G).

Taken together, these findings demonstrated that EXOs derived from WERI-Rb1 cells could inhibit the antitumour activity of macrophages and induce the BMSCs to promote retinoblastoma cell growth in vitro.

EXOs derived from WERI-Rb1 cells increase retinoblastoma growth. To confirm the in vitro results, a xenotransplantation model was established and then mice were regularly injected with EXOs or PBS around the tumour. EXOs in tumour tissues were observed after 2 days and were markedly increased in the loose tumour tissue at day 10. It was observed that EXOs were mainly located in loose tissue outside the tumour, and few were detected in the dense tissue within the tumour (Fig. 3A). However, quantification of tumour volume growth showed that EXO treatment significantly increased retinoblastoma tumour volume 10 days after transplantation (PBS, 66±172 mm3; EXO, 259±224 mm3; *P<0.05; Fig. 3B and C). Moreover, the protein levels of PCNA were significantly elevated by EXOs in the tumour (PBS, 0.77±0.18; EXO, 1.08±0.31; *P<0.05; Fig. 3D). Furthermore, the protein levels of apoptosis-related Bcl-2, Bax...
and caspase-3 were detected. As presented in Fig. 3E, the ratio of Bcl-2 to Bax was significantly increased (PBS, 0.58±0.23; EXO, 1.44±0.87; *P<0.05), whereas the relative expression of cleaved caspase-3 was decreased (PBS, 0.28±0.18; EXO, 0.17±0.14; *P<0.05) in the EXO group. In summary, EXOs derived from WERI-Rb1 cells could inhibit tumour apoptosis and promote tumour growth in vivo.

EXOs derived from WERI-Rb1 cells could promote the malignancy of retinoblastoma. Ki-67 is widely used to measure tumour proliferation and assess the prognosis of patients with cancer (34). Vimentin, an intermediate filament protein, is often used as a marker of invasive tumour cells due to its expression during activation of the epithelial-mesenchymal transition (35). Therefore, tissue sections were double stained with antibodies against Ki-67 and Vimentin. As shown in Fig. 4A and B, the relative Vimentin-positive area was significantly increased (PBS, 0.28±0.18; EXO, 0.17±0.14; *P<0.05) in the EXO group. In summary, EXOs derived from WERI-Rb1 cells could inhibit tumour apoptosis and promote tumour growth in vivo.

Figure 2. EXOs derived from WERI-Rb1 cells affect the antitumour activity of macrophages and BMSCs in vitro. (A) EXOs labelled with PKH26 were engulfed by RAW264.7 cells. Scale bar, 20 µm. (B) Morphology changes of RAW264.7 cells following treatment with EXOs. Scale bar, 50 µm. (C) RT-qPCR analysis of IL-6, MCP-1 and TNF-α expression in RAW264.7 cells incubated with EXOs derived from WERI-Rb1 cells for 24 h. (D) Cell Counting Kit-8 assay showed that RAW264.7 cell viability was significantly decreased by EXOs. (E) WERI-Rb1 cells and BMSCs co-culture system, BMSCs treated with PBS or EXOs were co-cultured with WERI-Rb1 cells for 24 h. (F) RT-qPCR analysis of IL-6, MCP-1 and TNF-α expression in BMSCs from the co-culture system. Data are from three independent experiments. *P<0.05 vs. PBS group. EXO, exosome; ns, no significance; RT-qPCR, reverse transcription-quantitative PCR; BMSC, bone marrow mesenchymal stem cells; MCP-1, monocyte chemotactic protein-1; RB, retinoblastoma.

and metastasis during tumour progression (36,37), were significantly increased (CXCR4: PBS, 0.225±0.088; EXO, 0.567±0.238; MMP2: PBS, 0.330±0.189; EXO, 0.802±0.433; *P<0.05; Fig. 4C and D). Furthermore, TAMs, a significant subset of tumour-infiltrating immune cells, play an important role in tumour growth and metastasis (38,39). Therefore, TAMs were evaluated in whole tumour sections using staining with an anti-CD68 antibody (Fig. 4E). Accordingly, the CD68-positive area was significantly increased in tumour tissues treated with EXOs compared with that in control tissue (PBS, 1.18±0.41%; EXO, 4.50±3.53%; *P<0.05; Fig. 4F). NK cells labelled with CD49b (red) surrounded tumour cells and were decreased in the EXO-treated group (PBS, 14.94±3.77; EXO, 3.66±2.29; ***P<0.001; Fig. 4G and H). Therefore, these results also partially supported the aforementioned results that demonstrated that exosomes derived from WERI-Rb1 cells promoted tumour growth and malignancy.

EXOs derived from WERI-Rb1 cells promote innate immunity in vivo. Although athymic nude mice with immunodeficiency were used in the present study, the mice still exhibited intact
innate and adaptive immunity. Thus, the spleen was analysed to determine whether EXOs affect immune processes. Of note, it was found that EXOs could diffuse into the spleen after 2 days and continue to accumulate at day 10 (Fig. 5A and B). EXOs were also observed in CD68-positive macrophages in spleen tissues (Fig. 5C).

Additionally, NK cells can limit tumourigenesis in the absence of functioning T-lymphocytes (40,41). Therefore,
spleen tissues were double stained with antibodies against Ki-67 and CD45 to determine the number and proliferative activity of leukocytes (Fig. 5D). CD45-positive leukocytes, which include granulocytes, monocytes and lymphocytes, control the innate immune response (42). The present data showed that EXOs significantly decreased the number of leukocytes in the spleen (PBS, 109.30±52.11; EXO, 37.54±21.42; *P<0.05; Fig. 5E). More specifically, EXOs also significantly decreased the ratio of cells co-expressing Ki-67 and CD45 (white triangle), which indicated that compared with the control, EXOs inhibited leukocyte proliferation in the spleen (PBS, 28.87±10.85; EXO, 8.47±5.39; **P<0.01; Fig. 5F). Similarly, immunofluorescence staining of CD49b, a pan-marker of NK cells (43), indicated that NK cells were significantly decreased in the spleens of the EXO-injected groups compared with the sham operation and PBS-injected groups (sham, 41.28±6.24; PBS, 37.81±18.08; EXO, 14.84±5.84; *P<0.05, ***P<0.001; Fig. 5G and H). Collectively, these data demonstrated that EXOs inhibited the innate immune response in vivo.
EXOs derived from WERI-Rb1 cells accelerate retinoblastoma metastasis. To explore the effects of EXOs on retinoblastoma metastasis, the liver, which was the closest organ to the subcutaneous tumour, was analysed. As shown in Fig. 6A, EXOs were observed in the cells around the liver sinusoids 2 days following the EXO injection and only appeared in the cells near the liver sinusoids. At day 10, EXOs (red) appeared in distant liver tissues and were mainly engulfed by Kupffer cells (CD68-positive), demonstrating that EXOs could spread throughout liver tissues and accumulate in macrophages (white arrows; Fig. 6B). Additionally, macroscopic images of liver metastatic nodules shown in Fig. 6C indicated that EXO injection led to more metastatic lesions (white arrowheads, metastatic liver nodules). Three livers from the EXO-injection
The group were cirrhotic, which indicated liver deterioration (green arrowheads, liver cirrhosis). Then, the excised livers were stained with Ki-67. It was found that livers from the EXO-injected group exhibited increased Ki-67-positive areas, which was consistent with metastatic nodules in the liver tissues (white arrowheads; PBS, 0.405±0.353%; EXO, 3.926±1.533%; "P<0.01; Fig. 6D and E). Thus, EXOs derived from WERI-Rb1 cells may exhibit a promoting effect on tumour metastasis.

**Constitution of EXOs derived from WERI-Rb1 cells.** Since EXOs derived from WERI-Rb1 cells significantly affected tumour malignancy, innate immunity and metastasis as aforementioned, the expression of a selection of miRNAs and proteins involved in tumour deterioration in EXOs were analysed via RT-qPCR and western blotting. As shown in Fig. 7A, miR-92a, miR-20a, miR-129 and miR-17 were detected. Moreover, the chemokine receptor CXCR4 was not only expressed in WERI-Rb1 cells but was also detected in EXOs (Fig. 7B). TSP-1, a matricellular protein, plays multiple roles in tumour development and was not observed in WERI-Rb1 cells, which was consistent with our previous study (44). However, of note, TSP-1 was detected in EXOs. CXCR4 and TSP-1 are involved in proliferation and TAM recruitment (45-51). Thus, the contents of EXOs may play a key role in EXO-mediated tumour progression.
Discussion

A growing body of evidence has suggested that EXOs play an important role in tumour invasion and metastasis (13,14,52). EXOs derived from retinoblastoma cells may have multiple effects on retinoblastoma development. In the present study, it was found that EXOs could inhibit the antitumour effects of macrophages and induce BMSCs to promote tumour growth. In vivo, through a xenotransplantation model in nude mice, the data showed that EXOs significantly increased tumour growth. Remarkably, EXOs injected around the tumour infiltrated the tumour tissue, spleen and liver. The changes in TAMs, leukocytes and NK cells induced antitumour activity that was inhibited by EXO infiltration of these tissues. Analysis of the contents of EXOs showed that EXOs were rich in miRNAs and proteins involved in tumour deterioration. Thus, these data provided direct evidence, both in vitro and in vivo, to support the notion that EXOs derived from retinoblastoma promote tumour progression by infiltrating the microenvironment.

Notably, the present study showed that EXOs derived from WERI-Rb1 cells slightly increased the growth of those cells in vitro (Fig. 1F), but had a more significant effect in vivo (Fig. 3C). After incubation with EXOs, WERI-Rb1 cell viability was slightly increased. The expression levels of PCNA, a specific cell proliferation marker, were the same in cells treated with EXOs compared with the control (Fig. 1G). However, EXOs extracted from WERI-Rb1 cells significantly enhanced PCNA protein levels and inhibited tumour apoptosis in vivo (Fig. 3E and F). The ratio of Ki-67-positive cells following EXO treatment was significantly higher than that after control treatment (Fig. 4B). Tumour growth in the EXO-injected group was increased ~4.0-fold 10 days after treatment (Fig. 3B and C). Therefore, the inconsistency between tumour proliferative activity in vitro and in vivo indicated that EXOs promote tumour growth by affecting the peripheral microenvironment.

Indeed, it was found that the viability of macrophages was significantly inhibited by EXOs in vitro. The inflammatory cytokines IL-6 and MCP-1 were increased in macrophages incubated with EXOs (Fig. 2). Both IL-6 and MCP-1 could further contribute to tumour growth and metastasis (6). Similarly, IL-6 and MCP-1 levels were also increased in BMSCs, leading to elevated WERI-Rb1 cell viability. Moreover, EXOs could exacerbate the microenvironment in xenograft tumours. EXOs infiltrated and accumulated in the tumour, spleen and liver. TAMs were enhanced and NK cells were decreased in tumours injected with exosomes (Fig. 4). Similarly, PKH26-labelled EXOs were directly taken up by macrophages in the spleen. CD45-positive leukocytes and NK cells, a type of lymphocyte that predominantly mediates innate antitumour immunity, were significantly decreased in spleens with EXO treatment (Fig. 5). Moreover, EXOs appeared in distant liver tissues and were mainly engulfed by Kupffer cells (Fig. 6B). In accordance with the roles of TAMs, leukocytes and NK cells, EXOs derived from WERI-Rb1 cells accelerated the proliferation, malignancy and metastasis of retinoblastoma in vivo. Therefore, the present findings indicated that EXOs modify the tumour microenvironment and promote tumour progression (Fig. 8).

The contents of EXOs include small non-coding RNA molecules, such as miRNAs, piwi-interacting RNAs, tRNA-derived small RNAs, and a series of proteins (53). It was found that miRNAs (miR-92a, miR-20a, miR-129 and miR-17), CXCR4 and TSP-1 were detectable in EXOs derived from retinoblastoma cells. According to previous studies, miRNAs (miR-92a, miR-20a, miR-129 and miR-17), CXCR4 and TSP-1 are involved in TAM recruitment and proliferation (45-51). For example, miR-92a stimulates the secretion of the proinflammatory cytokine IL-6 in TAMs, which in turn promotes tumour cell proliferation, invasion and metastasis by interacting with the surrounding microenvironment (46). CXCR4 accelerates TAM recruitment (36,48). However, the role of TSP-1 expression in tumorigenesis is complex and controversial. Some studies have reported that TSP-1 could inhibit tumour angiogenesis and suppress tumour growth in various types of cancer, such as cervical cancer, lung cancer.
and melanoma (54,55). Other studies have suggested that TSP-1 can be detected in multiple types of cancers, including prostate cancer, breast cancer and cutaneous melanoma, and is associated with tumour proliferation, migration and metastasis, leading to a poor prognosis (56‑59). Specifically, Xiao et al (49) reported that EXO-transferred TSP-1 can activate TAMs and promote malignant migration in oral squamous cell carcinoma, which is consistent with the present results. Based on the aforementioned previous studies, the function of elevated TSP1 expression in retinoblastoma EXOs may promote retinoblastoma progression. However, a limitation of the present study was that only a select few miRNAs and proteins were investigated. The overall identification of protein and miRNA profiles of EXOs will be performed in future studies.

Overall, the present study elucidated the role and function of EXOs derived from retinoblastoma cells in retinoblastoma growth and metastasis. The findings of this study suggested that retinoblastoma EXOs may be a therapeutic target that can be exploited to inhibit retinoblastoma by regulating its composition; alternatively, inhibitors that interfere with EXO uptake could be developed. Thus, the present study warrants future clinical exploration of the suppression of retinoblastoma with EXOs in the paediatric population.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions
SC and XC designed and performed the study, analysed the data and drafted the manuscript. JQ, PC, XH and YW assisted in performing the experiments and analysed the data. JieZ, MY, CW, NW and YY participated in collecting data and organizing the figures. JG was involved in designing the study and provided professional advice. SC and JinZ reviewed the manuscript and organized the figures. KY and JinZ designed the study, analysed the data, administered the project and supervised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments adhered strictly to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of the Zhongshan Ophthalmic Center [approval no. SYXK (YUE) 2018-101, 2018-168, 2019-009].

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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