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Original article

Extracellular vesicles secreted by *Echinococcus multilocularis:* important players in angiogenesis promotion

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ABSTRACT

The involvement of *Echinococcus multilocularis,* and other parasitic helminths, in regulating host physiology is well recognized, but molecular mechanisms remain unclear. Extracellular vesicles (EVs) released by helminths play important roles in regulating parasite-host interactions by transferring materials to the host. Analysis of protein cargo of EVs from *E. multilocularis* protoscoleces in the present study revealed a unique composition exclusively associated with vesicle biogenesis. Common proteins in various *Echinococcus* species were identified, including the classical EVs markers tetraspanins, TSG101 and Alix. Further, unique tegumental antigens were identified which could be exploited as *Echinococcus* EV markers. Parasite- and host-derived proteins within these EVs are predicted to support important roles in parasite−parasite and parasite-host communication. In addition, the enriched host-derived protein payloads identified in parasite EVs in the present study suggested that they can be involved in focal adhesion and potentially promote angiogenesis. Further, increased angiogenesis was observed in livers of mice infected with *E. multilocularis* and the expression of several angiogenesis-regulated molecules, including VEGF, MMP9, MCP-1, SDF-1 and serpin E1 were increased. Significantly, EVs released by the *E. multilocularis* protoscolex promoted proliferation and tube formation by human umbilical vein endothelial cells (HUVECs) *in vitro*. Taken together, we present the first evidence that tapeworm-secreted EVs may promote angiogenesis in *Echinococcus*-infections, identifying central mechanisms of *Echinococcus*-host interactions.

Echinococcosis is one of the most severe zoonoses and is caused by the larval form (metacestode) of Echinococcus genus cestodes. Infections cause serious social and economic burdens associated with the cost of treatment and livestock production losses [1–3]. There are at least eight Echinococcus species. Among them, *Echinococcus granulosus* and *Echinococcus multilocularis* are the major pathogens responsible for cystic echinococcosis (CE, which is globally distributed) and alveolar echinococcosis (AE, which is restricted to the Northern hemisphere), respectively [1,2]. After ingesting parasite eggs, oncospheres hatch in the intestine and subsequently develop into metacestodes, mainly located in liver. Mature metacestodes contain hydatid fluid (HF) and can be filled with bundles of protoscoleces (PSCs) [4]. *E. multilocularis*, which grows into a tumour-like structure in the host liver, can lead to death [1,4], partially attributed to the interplay between parasite and host, but mechanistic issues remain unaddressed. Recent evidence indicates that these parasites secrete extracellular vesicles (EVs) to facilitate modifying the local environment [5–17].

Both prokaryote and eukaryote cells release EVs, small membrane-bound secreted vesicles that can be subdivided into microvesicles (ectosomes, or microparticles, 50 nm to 1 μm in diameter), which pinch off from the surface of the plasma membrane via outward budding, and exosomes (40–160 nm in diameter), which are derived from endosomes [5–7]. EVs can contain nucleic acids, lipids, metabolites and cytosolic and cell-surface proteins, depending on their cellular origin, and are considered as a means to remove excess and/or unnecessary components from cells to maintain cellular homeostasis, or to participate in

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Abbreviations

CE	cystic echinococcosis
AE	alveolar echinococcosis
HF	hydatid fluid
PSCs	protoscoleces
EVs	extracellular vesicles
PBMCs	peripheral blood mononuclear cells
HUVECs	human umbilical vein endothelial cells
HBSS	Hank's balanced salt solution
PBS	phosphate buffered saline
FBS	fetal bovine serum
BSA	bovine serum albumin
VEGF	vascular endothelial growth factor
DAPI	4′, 6- diamidyno-2-fenyloindol
TEM	transmission electron microscopy
Exo	exosome
MVs	microvesicles
ILVs	intraluminal vesicles
HSPs	heat shock proteins
HSP70	heat shock protein 70
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TSG101	tumor susceptibility 101
MMP9	matrix metallopeptidase 9
MCP-1	monocyte chemoattractant protein-1
SDF-1	stromal cell derived factor 1
Serpin E1	plasminogen activator inhibitor-1
ESCRT	endosomal sorting complexes required for transport
HRS	hepatocyte growth factor-regulated tyrosine kinase substrate
STAM1	signal transducing adaptor molecule 1
SRA	serum resistance-associated

intercellular communication, and can also contribute towards emergence of pathology [5–7]. Many parasites employ multipurpose EVs in communication for persistence, development, transfer of virulence factors, adherence to host tissues, evasion of immune responses and modulation of the host [8,9]. For example, the composition of EVs released by Leishmania donovani altered with temperature and pH changes [10], and malaria parasite DNA-harbouring EVs could activate STING-mediated DNA-sensing pathways and initiate a type I interferon responses [11]. *E. granulosus* exosome-like vesicles could be internalized by murine dendritic cells and induce their maturation with increased CD86 and down-regulation of MHC class II [12]. Moreover, some parasites such as Leishmania spp. Plasmodium spp. and *T. cruzi* release EVs that induce pro-inflammatory cytokine responses to promote pathogenesis (etc.), while T. gondii, T. muris, *H. polygyrus*, *N. brasiliensis* and E. caproni EVs inhibit or delay pathogenesis [9]. In addition, EVs may have potential for vaccine, therapeutic or diagnostic purposes [8,9].

Echinococcus metacestodes mainly reside in liver of the mammalian host and interact with a microenvironment composed of immune cells, fibroblasts, endothelia and other cell types [4,13]. Metacestodes orchestrate a series of outcomes that balance infection burden and host survival [14], and metacestode surface molecules and excretory/secretory metabolic products function as key players in these processes [13,15]. Although Echinococcus species lack digestive and excretory systems, they possess endocytic and exocytic intracellular pathways that regulate metabolite uptake and release [12]. In addition, EVs released from Echinococcus spp. could participate in pathogen-host interactions. For example, parasite EVs could be internalized by dendritic cells, peripheral blood mononuclear cells (PBMCs), macrophages and T lymphocytes to modulate immune responses [12,16–19]. Furthermore, among the proteins identified in parasite-derived EVs, some likely act as immunomodulators [20–22], while ncRNA (miRNAs, cirRNAs etcetera) [23,24] have multiple functions, including as virulence factors [25], and inhibiting UBE2N, a ubiquitin-ligase with important roles in inflammatory and immune signal transduction in hepatocytes during *E. multilocularis* infection [26].

Neither the protein composition of *E. multilocularis* PSCs EVs or their impact on human umbilical vein endothelial cells (HUVECs) is known. Pro-angiogenic factors VEGF (vascular endothelial growth factor) and endothelial cells marker CD31 are highly expressed in host tissues proximal to *E. granulosus* lesions and accompanied by angiogenesis, described both by us and others [27–30]. Here we systematically characterized the protein cargo of EVs released by *E. multilocularis* PSCs and investigate their effects on HUVECs to explore their potential pro-angiogenic activity.

1. Methods

1.1. Ethics statement

Experiments involving mice were in strict accordance with Guidelines for the Care and Use of Laboratory Animals produced by the Shanghai Veterinary Research Institute. This study was approved by the Ethics Committee of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (license number IPD-2017-20).

1.2. E. multilocularis *maintenance and culture*

E. multilocularis was maintained in BALB/c mice (Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China). In brief, mice were challenged with 2000 *E. multilocularis* PSCs via abdominal intraperitoneal transplantation passage for six months [31]. Parasite material was isolated from the peritoneal cavity of infected animals and homogenised in Hank's balanced salt solution (HBSS, Thermo Fisher Scientific, Carlsbad, CA, USA). After passing parasite homogenate through a 60-mesh sieve, *E. multilocularis* PSCs were collected and exhaustively washed with phosphate-buffered saline (PBS, Thermo Fisher Scientific, Carlsbad, CA, USA). The viability of these PSCs was determined by methylene blue exclusion and PSCs with viability >95% were used in subsequent experiments. About 10,000 PSCs/ml were cultured in 2 ml DMEM culture medium (Thermo Fisher Scientific, Carlsbad, CA, USA), supplemented with 10% FBS (v/v) (Thermo Fisher Scientific, Carlsbad, CA, USA) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) (Thermo Fisher Scientific, Carlsbad, CA, USA) at 37 °C in 5% CO2 for 24–48 h. Twenty-four hrs before collecting, the medium was replaced with FBS-free DMEM. PSCs were collected and homogenised in lysis buffer (Thermo Fisher Scientific, Carlsbad, CA, USA, cat# 78,430) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific, Carlsbad, CA, USA). After centrifuging at 8000×*g* for 15 min, supernatants were transferred to a new tube without disturbing the pellet as the whole PSCs lysate.

1.3. Extracellular vesicle isolation

A total exosome isolation kit (Thermo Fisher Scientific, Carlsbad, USA) was used for EVs isolation according to the manufacturer's instructions. In brief, 18 ml FBS-free PSC culture medium from three batches of collections was filtered through 0.22 μm PVDF filtered syringes (Millipore, Massachusetts, USA), and 0.5 volumes of total exosome isolation reagent added to collected FBS-free DMEM medium. The mixture was vortexed and incubated at 4 °C overnight, then centrifuged at 10, 000×*g* for 1 h at 4 °C. To remove contaminants, the resulting pellet was washed with PBS, centrifuged (10, 000×*g* for 1 h at 4 °C) and resuspended in PBS. The protein concentration of PSC lysis supernatants and EVs (solubilised in 2× lysis buffer) was determined with a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China); 10 μg of EV suspension was used in all experiments unless specified. EV suspensions were stored at −80 °C until analysis.

1.4. Western blotting

Samples were treated with lysis buffer (Thermo Fisher Scientific, Carlsbad, CA, USA) at 4 °C and centrifuged at 8000×*g* for 15 min. Protein concentration was estimated by BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). 10 μl samples containing 10 μg *E. multilocularis* PSCs EVs or *E. multilocularis* PSCs culture medium were separated by 10% SDS-PAGE (Thermo Fisher Scientific, Carlsbad, CA, USA) and transferred to a 0.45 μm PVDF membrane (Millipore, Burlington, MA, USA). The membrane was blocked for 1 h with 5% non-fat milk (w/v, in 0.1% Tween® 20/PBS) (non-fat milk: Solarbio, Beijing, China; Tween® 20: Sigma−Aldrich, Saint Louis, USA) and incubated with anti-CD63 (Abcam, Cambridge, UK, cat# ab134045) and anti-TSG101 (Abcam, Cambridge, UK, cat# ab83) as primary antibodies (1/2000 in 5% non-fat milk) at 4 °C overnight to detect cross-reactivity with *E. multilocularis* proteins of approximately 50 and 47 kDa respectively, then incubated with a 1/5000 dilution of goat anti-rabbit IgG antibody (Abcam, Cambridge, UK, cat# ab6721) conjugated with HRP as a secondary antibody at room temperature for 1 h and washed again. ECL Western Blot substrate (Thermo Fisher Scientific, Carlsbad, CA, USA) was used to visualize bound antibody.

1.5. Transmission electron microscopy

Extracellular vesicles were fixed in 2.5% glutaraldehyde (w/v) (Sigma−Aldrich, Saint Louis, USA) and placed onto 300-mesh copper grids with carbon-coated formvar film and incubated for 5 min. Excess liquid was removed and the grid negatively stained with saturated uranylacetate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for 1 min, followed by again removing excess liquid. EVs were examined at 100 kV in a FEI Tecnai G2 Spirit transmission electron microscope (FEI Company, Oregon, USA). EV sizes were calculated from eight random fields (Table S01, Fig. S1) using ImageJ software [32], applying a stringent method for enumerating EVs in which only objects that presented a well-defined near-to-circular or tubular morphology were considered.

PSCs were fixed with 2.5% glutaraldehyde (w/v) (Sigma−Aldrich, Saint Louis, USA) at 4 °C for 24 h, washed three times with PBS (pH7.4) and post-fixed in 2% Osmium tetroxide at 4 °C for 2 h. After dehydration in a graded series of ethanol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and embedding with Pon812 embedding kit (SPI, West Chester, PA, USA), 70 nm ultrathin sections were cut on a Leica EM UC7 Ultramicrotome (Illinois, USA), placed on copper grids, and stained with uranyl acetate and lead citrate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Grids were examined with a FEI Tecnai G2 Spirit TEM instrument.

1.6. Proteomic analysis

30 μg PSC lysate, PSC culture medium and purified PSC EVs were sent to the Majorbio proteomic service (Shanghai, China) for mass spectrometry analysis. Briefly, samples were treated with 10 mM tris-2-carboxyethyl-phosphine (Thermo Fisher Scientific, Carlsbad, USA) in 100 mM triethylammonium bicarbonate buffer (Sigma−Aldrich, Saint Louis, USA) at 37 °C for 60 min, followed by alkylation with 40 mM iodoacetamide (Sigma−Aldrich, Saint Louis, USA) for 40 min at room temperature, in darkness. After adding six volumes of cold acetone (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at −20 °C for 4 h, pellets were digested with trypsin (Promega, Madison, USA) and analysed with an EASY-nLC 1200 system (Thermo Fisher Scientific, West Palm Beach, FL, USA) coupled to a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Carlsbad, USA) to separate and identify peptides.

Analysis of spectra was carried out using Xcalibur 4.0. After searching in SwissProt/UniProt *E. multilocularis* (Taxon ID: 6211), *Mus musculus* (Taxon ID: 10,090) and *Bos taurus* (Taxon ID: 9913) databases with PEAKS Studio 8.5, proteins selected by unique peptide >1, 10logP≥20 were analysed further. All searches were conducted with the following parameters: trypsin cleavage at both termini and two missed cleavage sites allowed; 10 ppm for precursor mass tolerance; 0.05 Da for fragment mass tolerance; cysteine carbamidomethylation as static modification, methionine oxidation and acetylation as dynamic modifications. In silico analyses to establish Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) classification was performed using the UniProt database (http://www.uniprot.org/), DAVID bioinformatics resources (https://david.ncifcrf.gov/), KEGG (https://www.kegg.jp/) and KOBAS (http://kobas.cbi.pku.edu.cn/). Proteins identified as uncharacterized, hypothetical, conserved or expressed protein were classified based on the presence of conserved domains using CDD (https://www.ncbi.nlm.nih.gov/cdd/).

1.7. Comparative analysis of extracellular vesicle protein cargo across several cestode species

There are seven [12,16−18,21,22,33] and four [34−37] publications describing EVs protein datasets of Echinococcus spp. and other cestodes, respectively. Due to differences in annotation between these studies, conserved domains identified by CDD (https://www.ncbi.nlm.nih.gov/cdd/) were used for comparative analysis across several cestode species. UpSetR package was used to determine the extent of common and/or unique domains [38].

1.8. Mouse angiogenesis array analysis

Liver tissue (n = 3) surrounding *E. multilocularis*-infected lesions and from uninfected mice were homogenised in PBS with protease inhibitors (Sigma−Aldrich, Saint Louis, USA, cat#4693124001). After homogenisation, 1% Triton X-100 (v/v) was added and samples were frozen at −80 °C. After thawing, tissue lysates were centrifuged at 10,000×*g* for 10 min, and the supernatant collected and protein estimated using BCA protein assay kit. A total of 300 μg tissue lysates from each group was used for angiogenesis array analysis using the mouse angiogenesis array (R&D systems, Minneapolis, USA, cat# ARY015), following the manufacturer's instructions. Membranes were scanned using a fluorescence chemiluminescence imaging system (Chemi Scop2 6300, Clinx Science Instruments Co., Ltd., Shanghai, China), and quantified using NIH ImageJ [32].

1.9. Immunofluorescence and immunohistochemistry

Liver tissues (n = 2) from *E. multilocularis*-uninfected and -infected mice were fixed with 4% paraformaldehyde (w/v) and processed for immunohistochemistry (Wuhan Servicebio Technology Co. Ltd., Wuhan, China). Unless specifically noted, all reagents used are products of Wuhan Servicebio Technology Company. After dehydration with gradient alcohol (5% alcohol for 4 h, 85% alcohol for 2 h, 90% alcohol for 2 h, 95% alcohol for 1 h, anhydrous ethanol II for 30 min, anhydrous ethanol II for 30 min) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), tissue samples were cleared and embedded in paraffin, dissected to 4 μm thickness and affixed to slides. Before staining, slides were processed by deparafinization, rehydration, antigen retrieval and blocking of endogenous peroxidase activity.

For immunofluorescence, slides were blocked in 3% bovine serum albumin (BSA, w/v in 0.3% Triton™ X-100/PBS) for 30 min, incubated with anti-CD63 (1/500 in 1% BSA) primary antibodies at 4 °C overnight followed by incubation with 1/300 dilution of CY3-conjugated goat anti-rabbit IgG at room temperature for 1 h. After washing with PBS (pH7.4), slides were stained with DAPI, dehydrated, mounted and visualized with a fluorescence microscope (Nikon, Tokyo, Japan).

For immunohistochemistry, slides were blocked in 3% BSA for 30 min, and incubated with anti-CD31 (1/100) or anti-VEGF (1/200) as primary antibodies at 4 °C overnight, incubated with 1/200 dilution of HRP-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG antibody at room temperature for 1 h. After washing with PBS pH7.4, slides were stained with DAB (brown) and Harris hematoxylin stain solution (blue), and finally dehydrated, mounted and visualized under an inverted microscope (Nikon, Tokyo, Japan).

Vascular endothelial cells that stained positively for CD31 were quantified to record micro-vessel density (MVD), assessed by counting all stained vessels at 400× magnification in six random fields. The mean number of vessels was defined as the MVD. Positive staining for VEGF expression, indicating angiogenesis, was assessed at 400-times magnification in six random fields. Standardized analysis of pixel density with ImageJ [32] was used to quantitate CD31/VEGF-positive area(s).

1.10. HUVEC culture, cell proliferation and tube formation assays

HUVECs (Sciencell Research Laboratories, Carlsbad, CA, USA) were cultured and maintained according to the supplier's instructions. All of the following assays were conducted using low passage cells (3–5 passages). In brief, cryopreserved primary cells were thawed into an equilibrated fibronectin-coated T-75 flask. Once the culture reached 90% confluence, cells were harvested and seeded in 96-well microtiter plates at 1 × 105 cells/ml in endothelial cell medium and allowed to attach for 24 h at 37 °C under 5% CO2 before stimulating. After adding stimulus (10 μg total protein), plates were incubated for 48 h and cell viability determined with a cell counting kit-8 (Dojindo Laboratories, Kamimashiki-gun, Japan).

The effect of *E. multilocularis* material on HUVEC differentiation was examined by tube formation in Matrigel culture (BD Biosciences, San Diego, USA). HUVECs at > 90% confluence were harvested and diluted to 3 × 105 cells/ml. 100 μL of HUVEC suspension was mixed with 100 μL stimulus (10 μg total protein) diluted with DMEM and placed in 96-well plates at 37 °C, 5% CO2. Capillary networks were visualized under an inverted microscope (Nikon, Tokyo, Japan) and branch lengths in four random fields per well quantified using the angiogenesis module of ImageJ [32]. The experiments were repeated twice with triple replicates for each experiment.

1.11. Statistical analysis

SPSS software (IBM spss statistics professional authorized user, version 26.0) was used for statistical analysis. Differences between or among groups were analyzed using Student's t test/one way ANOVA and considered significant if the P value was <0.05.

2. Results

2.1. E. multilocularis *protoscolex produces exosome-like vesicles*

Many EVs contain cellular components such as nucleic acids and proteins, which influence various biological pathways in recipient cells and tissues. EV pools with a typical cup-shaped morphology, confirmed by TEM analysis, were isolated from cultured *E. multilocularis* PSCs (Fig. 1A), with a diameter varying between 40 and 110 nm, mean 60.6 ± 15.8 nm (Fig. 1B). Immunoblotting indicated that these EVs contain known exosomal markers, including CD63 and tumor susceptibility gene 101 (TSG101) (Fig. 1C).

Vesicle-like structures were also observed in, and on, the syncitial tegument of *E. multilocularis* PSCs by TEM (Fig. 1D). Two different sizes were distinguished: smaller EVs with diameters ~70 nm, falling into the range of exosome-sized vesicles (Exo), and larger EVs with diameters of ~300 nm, the range of microvesicles (MVs). Multivesicular bodies (MVBs) observed in the tegument had either a dense or an electron-lucent matrix surrounding intraluminal vesicles (ILVs) (Fig. 1D). Moreover, highly expressed tetraspanins were identified on the teguments of PSCs and the germinal layers of cysts (Fig. 1E) in liver sections containing metacestodes from mice infected with *E. multilocularis*. These findings suggest that the PSC tegument and cyst germinal layer may be involved in the formation and recycling of EVs.

2.2. Parasite and host-derived proteins in E. multilocularis *EVs*

Proteomic analysis was used to characterize the protein composition of *E. multilocularis* EVs. Thousands of parasite-derived proteins and hundreds of host (mouse)-derived proteins were identified in 30 μg *E. multilocularis* EVs, PSCs culture medium and whole PSCs lysis (Fig. 2A, Tables S01–03). Despite considerable concordance between the different isolates, it was striking that the EV composition was highly distinct from whole parasite lysates and PSCs culture medium. In total, 1082 parasites proteins were identified in PSCs culture medium and 1407 proteins in EVs released by PSCs. Of these proteins, 263 were identified exclusively in PSCs culture medium and 588 only in EVs (Fig. 2A). Among these, several exosomal markers, including heat shock protein 70 (HSP70), TSG101, tetraspanins, 14-3-3 and enolase were identified in *E. multilocularis* PSCs EVs (Table 1, Table S01). Because parasites were cultured with FBS before collecting EVs, bovine proteins were also present in purified EVs, including polyubiquitin-C, actin, myosin heavy chain 9 and so on (Table 1, Fig. S02 and Table S03). However, host (mouse)-derived proteins identified in *E. multilocularis* PSCs EVs were more diverse than bovine-derived proteins, and many were annotated as associated with the immune response (Table 1, Table S04).

GO analysis of those proteins exclusively identified in EVs indicates that parasite-derived proteins associated with vesicle targeting and exocytosis are overrepresented, indicating exocytic vesicle origin, while host-derived proteins were mostly related to actin filament fragmentation and other biological processes (Fig. 2B). Meanwhile, parasite-derived proteins in EVs were enriched in endocytosis pathway components (Fig. 2C), indicating that these EVs are exosomes derived from endosomal structures (Fig. 2D). However, the mouse-derived proteins in *E. multilocularis* PSCs EVs were closely associated with immune response and focal

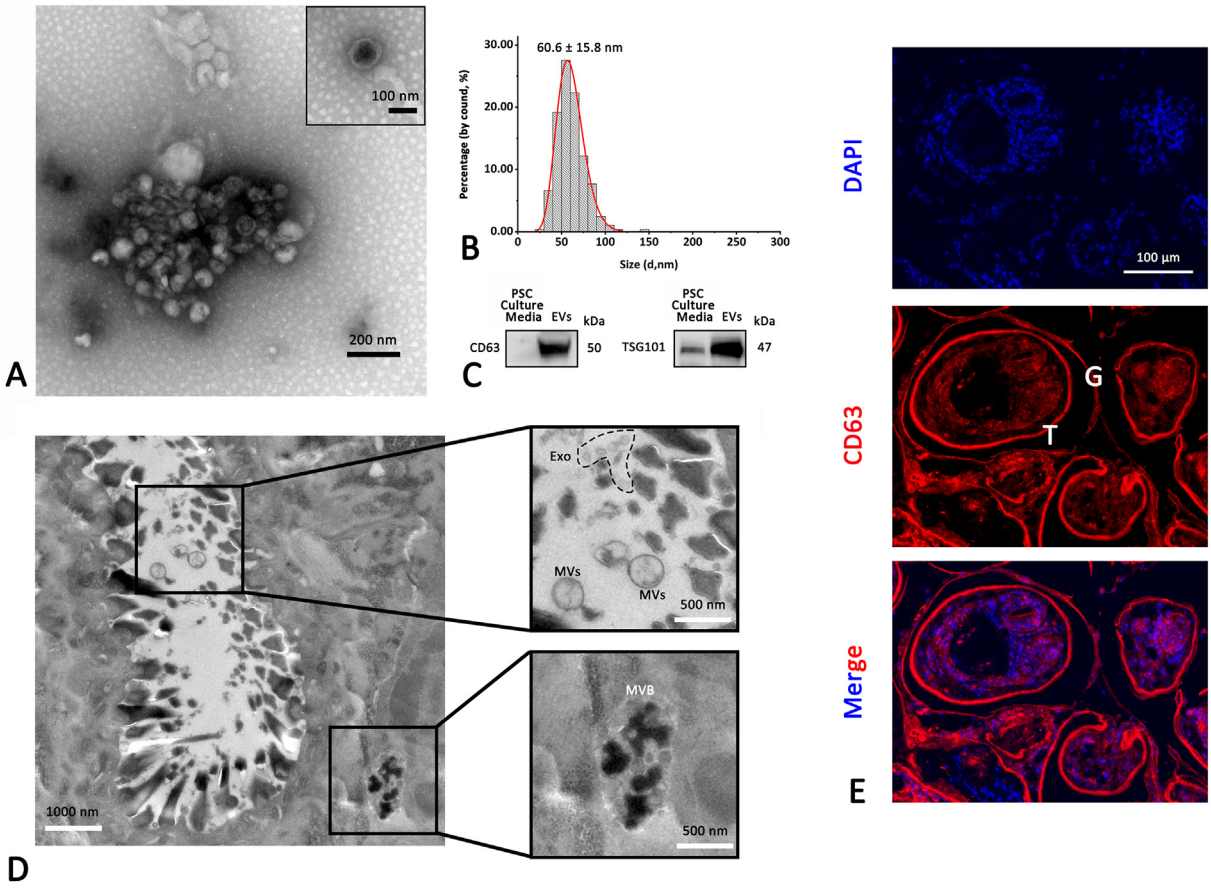

Fig. 1. Characterization of extracellular vesicles secreted by *E. multilocularis* protoscoleces. A) Morphological characterization of purified EVs by TEM. B) Size distribution of isolated EVs determined by TEM. C. Western blot analysis of EV markers, CD63 and TSG101 in *E. multilocularis* PSC lysis and EVs (10 μg total protein). D) TEM of *E. multilocularis* tegument releasing EVs (Exo, exosome; MVs, microvesicles; MVB, multivesicular body). E) Localisation of *E. multilocularis* tetraspanin CD63 in metacestodes (G: germinal layer; T: tegument).

adhesion, and hence potentially promoting angiogenesis (Fig. 2C and D).

2.3. Comparative analysis of EV protein cargo between cestode species

Cestode EVs are an important component in host–parasite interactions, and proteins enriched in EVs are hence candidate effector molecules. To systematically compare parasite- and host-derived proteins in EVs released by different cestode species protein domain classification for all available proteomic datasets was performed (Tables S04–05). Interestingly, the majority of domains in parasite-derived EV proteins appear to be species-specific, with only three parasite-derived domains common to all cestode datasets (seven species in total), including Rab11-like, C2 superfamily and annexin, all of which are domains possessed by classical EVs marker proteins (Fig. 3A and C, Fig. S03 and Table S04). Only two host-derived domains, IgC1L and Ig superfamily, were common to all four species, both of which are associated with the immune response (Fig. 3B and D). As many as 670 parasite-derived domains and 119 host-derived domains are shared between *E. multilocularis* and *E. granulosus* EV proteomes (Tables S05–06). Hence, the common parasite proteins present in these EVs may serve as markers for validating cestode or Echinococcus spp. EVs, such as tetraspanin family proteins, major egg antigen p40 and tegumental antigen, immunoglobulin-like proteins, proteins involved in vesicle trafficking (Rabs, Ubiquitin E3, SNAREs, TSG101, Alix), transporters and channels, structural proteins and others, although the EV proteins in the reported datasets are diverse (Fig. 3E). To discriminate for parasite-specific proteins, species-specific antibodies or mass spectrometric detection are required. However, the enriched proteins derived from host identified in *E. granulosus* PSC EVs and other cestode EVs reported by other studies were not related to the focal adhesion pathway, indicating that this pathway is potentially species-specific.

2.4. Extracellular vesicles from the E. multilocularis protoscolex induce HUVEC proliferation and tube formation

Given that development of Echinococcus spp. in mammalian hosts is associated with formation of new permeable blood vessels, a protein angiogenesis array was used to analyse tissue lysates obtained from Echinococcus-infected and uninfected mice livers. Compared to uninfected mice, Echinococcus-infected livers had increased levels of multiple angiogenesis regulating molecules, including VEGF, matrix metallopeptidase 9 (MMP9), monocyte chemoattractant protein-1 (MCP-1), stromal cell derived factor 1 (SDF-1), and serpin E1 (plasminogen activator inhibitor-1 (Fig. 4A). Angiogenic stimulation in *E. multilocularis*-infected mice was confirmed by immunohistochemical analysis, with the number of CD31+ vessels and VEGF expression levels significantly increased in the livers of infected mice (Fig. 4B).

The impact of *E. multilocularis* PSCs EVs on endothelial cells was further examined by a tube-formation assay using HUVECs. The

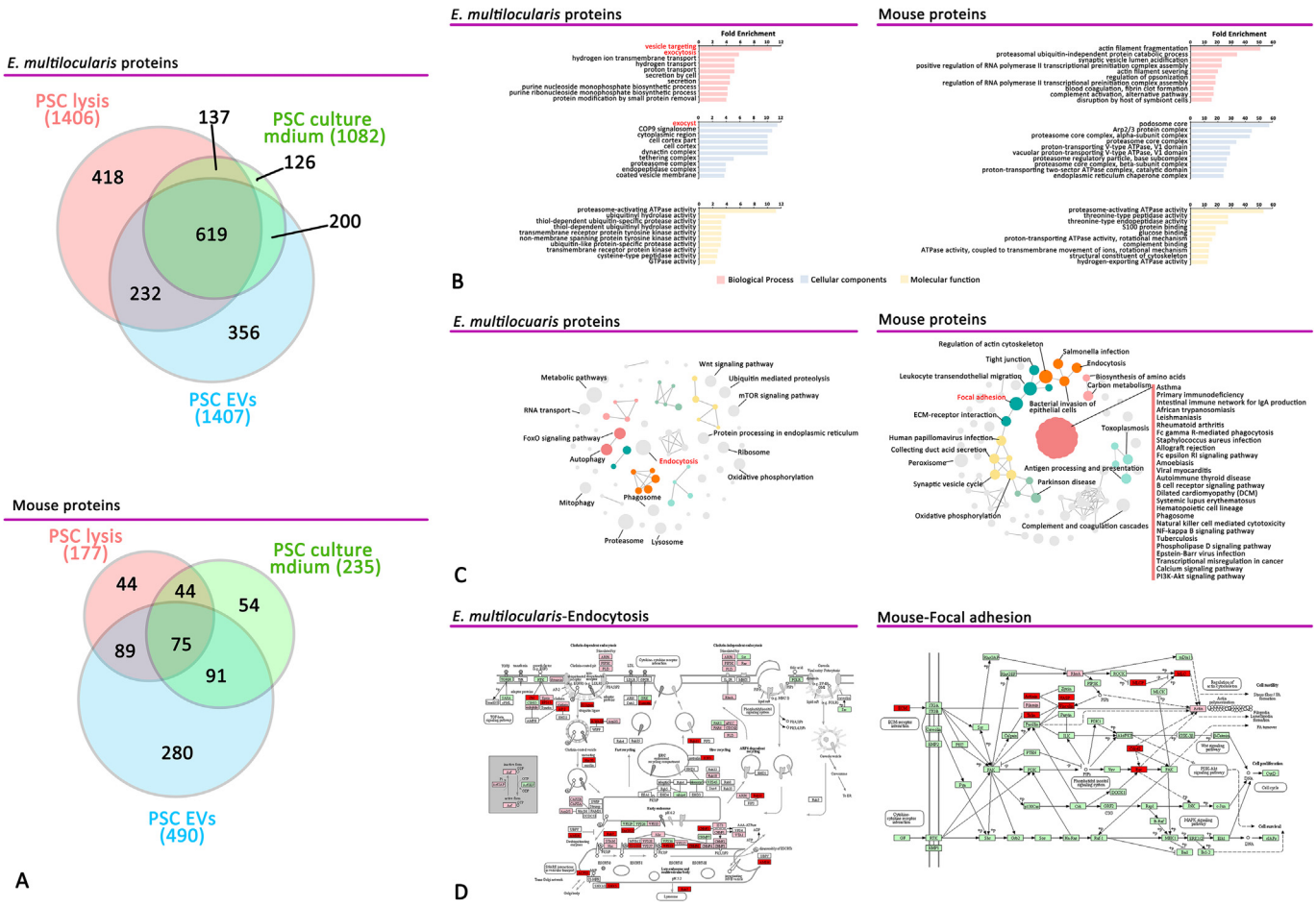

Fig. 2. Profiles of proteins identified in EVs released by *E. multilocularis* PSCs. A) Numbers of parasite and host derived proteins identified in *E. multilocularis* PSC lysis, PSCs culture medium and EVs released by PSCs. B) GO analysis of parasite- and host-derived proteins exclusively identified in EVs released by *E. multilocularis* PSCs. C) KEGG clusters of parasite- and host-derived proteins exclusively identified in EVs released by *E. multilocularis* PSCs. Each node represented an enriched term, and the node color represented different clusters; the node size represented six levels of enriched p-value, node size from small to large [0.05, 1] [0.01, 0.05) [0.001, 0.01) [0.0001, 0.001) [1e-10, 0.0001) [0, 1e-10). D) KEGG pathway of *E. multilocularis* endocytosis and mouse focal adhesion EV-associated proteins. Red, proteins exclusively identified in EVs released by *E. multilocularis* PSCs; pink, proteins identified in EVs released by *E. multilocularis* PSCs and culture medium; green, proteins associated with pathway without corresponding proteins in EVs released by *E. multilocularis* PSCs; white, proteins reported to be associated with this pathway but absent from the *E. multilocularis* genome.

length of HUVEC tubes treated with EVs significantly increased compared to those incubated with DMEM alone or PSC culture medium, while whole PSC lysates significantly disturbed HUVEC tube formation (Fig. 4C). Proliferation of HUVECs was also significantly promoted by EVs shed from PSCs after 48 h (Fig. 4D). These data demonstrate that *E. multilocularis* EVs may contribute to the augmented angiogenesis observed in the livers of *E. multilocularis*-infected mice (Fig. 5).

3. Discussion

EVs released from *E. multilocularis* PSCs contain abundant materials derived from both parasite and host, and can potentially regulate parasite−parasite and parasite-host interactions. Unlike *E. granulosus*, which presents a typical unilocular, subspherical cyst that grows by concentric enlargement, the *E. multilocularis* metacestode is a multivesicular, infiltrating structure with no obvious limiting host-tissue barrier [3,4]. Proliferation of *E. multilocularis* metacestode occurs both endogenously and exogeneously and is attributable to the undifferentiated cells of the germinal layer, leading to the production of numerous PSCs and cysts that can directly contact with host tissues [3,4]. Hence, the contents of the *E. multilocularis* metacesotde, typically germinal cells and PSCs, are potentially more able to interact with host cells and participate in regulation of host−parasite interactions, comparing to *E. granulosus* cysts.

To understand the molecular basis for interactions between parasite and host, we analysed the EVs released by *E. multilocularis* PSCs, and characterized their protein composition and roles in regulating host cells and tissues. Two major EV classes, namely Exo and MVs, both of which can participate in cell−cell communication [7], potentially derived from endocytic compartments and plasma membrane budding respectively, were shed from the *E. multilocularis* tegument observed under TEM. Proteomics was fully consistent with the Exo as the most abundant EVs in the present study, as proteins associated with the endomembrane system were enriched in the EVs released from *E. multilocularis* PSCs. Furthermore, these findings indicate that EV biogenesis in *E. multilocularis* is broadly conserved with mammalian and other parasites [21]. Among the identified EV proteins, tetraspanins and TSG101, enriched in *E. multilocularis* PSCs EVs, are candidates for development as EVs markers.

Biogenesis of EVs proceeds via an endolysosomal pathway with inward budding of the plasma membrane generating an endosome, which in turn fuses with cargo-containing endocytic vesicles. Endosomal membrane further buds inward to form intraluminal vesicles within MVBs, which fuse with the plasma membrane,

Table 1
Top 20 parasite and host-derived proteins exclusively identified in EVs released by *E. multilocularis* PSCs.

Protein ID	Origins	Protein names
Q6VXZ5	*E.multilocularis*	Antigen B subunit 1
A0A068Y074	*E.multilocularis*	Polyubiquitin
A0A068YF78	*E.multilocularis*	Diagnostic antigen gp50
U6HHP6	*E.multilocularis*	Histone H2A
D9J2I8	*E.multilocularis*	Antigen B subunit 3
A0A068XWT9	*E.multilocularis*	Histone H2B
A0A068YDL3	*E.multilocularis*	Telomerase protein component 1
A0A087W0K1	*E.multilocularis*	Dynein light chain
A0A087VYL1	*E.multilocularis*	Expressed conserved protein
A0A068Y8K3	*E.multilocularis*	Ubiquitin modifier activating enzyme 1
A0A068YEV5	*E.multilocularis*	FERM central domain
A0A087W2S3	*E.multilocularis*	fructose-bisphosphatase
A0A068Y336	*E.multilocularis*	Tumor susceptibility locus tag 101 protein
A0A068YJ32	*E.multilocularis*	Ras protein rab
A0A087W1M1	*E.multilocularis*	Ras protein Rab 7a
A0A068Y3A1	*E.multilocularis*	Golgi-associated plant patholocus tagsis
Q24895	*E.multilocularis*	Endoplasmic reticulum chaperone BiP
U6I232	*E.multilocularis*	Tetraspanin
A0A068Y0X1	*E.multilocularis*	Tegumental antigen
A0A068Y706	*E.multilocularis*	Serine/threonine-protein phosphatase
Q6RFG4	*Mus musculus*	Myeloperoxidase
Q8C196	*Mus musculus*	Carbamoyl-phosphate synthase
Q99LC4	*Mus musculus*	Igh protein[a]
P13020	*Mus musculus*	Gelsolin[a]
Q3TWJ4	*Mus musculus*	Actin-related protein 3
Q9CVB6	*Mus musculus*	Actin-related protein 2/3 complex subunit 2
P68134	*Mus musculus*	Actin, alpha skeletal muscle
Q9DAC2	*Mus musculus*	Complement component C8 gamma chain[a]
Q542I3	*Mus musculus*	Pentaxin (Pentraxin)
A0A140T8P5	*Mus musculus*	Immunoglobulin kappa chain variable 8−24[a]
Q91Z25	*Mus musculus*	Actin-related protein 2/3 complex subunit
Q542I8	*Mus musculus*	Integrin beta[a]
Q3THX5	*Mus musculus*	Major vault protein
P50543	*Mus musculus*	Protein S100-A11
P40142	*Mus musculus*	Transketolase
P51437	*Mus musculus*	Cathelicidin antimicrobial peptide[a]
A0A075B5V7	*Mus musculus*	Immunoglobulin heavy variable V1-43[a]
P68372	*Mus musculus*	Tubulin beta-4B chain
Q3UVJ2	*Mus musculus*	Adenylyl cyclase-associated protein
B2RUF9	*Mus musculus*	Olfm4 protein
P0CH28	*Bos taurus (Bovine)*	Polyubiquitin-C
P60712	*Bos taurus (Bovine)*	Actin
F1MQ37	*Bos taurus (Bovine)*	Myosin heavy chain 9
P00974	*Bos taurus (Bovine)*	Pancreatic trypsin inhibitor
E1BNA9	*Bos taurus (Bovine)*	Zinc finger ZZ-type and EF-hand domain containing 1
P04272	*Bos taurus (Bovine)*	Annexin A2rotein I) (p36)
P61585	*Bos taurus (Bovine)*	Transforming protein RhoA
G3N081	*Bos taurus (Bovine)*	Histone H4
A6QPT4	*Bos taurus (Bovine)*	MPO protein
Q5E947	*Bos taurus (Bovine)*	Peroxiredoxin-1
P0CB32	*Bos taurus (Bovine)*	Heat shock 70 kDa protein 1-like
F1ML89	*Bos taurus (Bovine)*	Carbamoyl-phosphate synthase 1
F1N650	*Bos taurus (Bovine)*	Annexin
Q0VCX2	*Bos taurus (Bovine)*	Endoplasmic reticulum chaperone BiP
F1MWU9	*Bos taurus (Bovine)*	Heat shock protein family A (Hsp70) member 6
G3X757	*Bos taurus (Bovine)*	Transitional endoplasmic reticulum ATPase
P48616	*Bos taurus (Bovine)*	Vimentin
G3N0V2	*Bos taurus (Bovine)*	Keratin, type II cytoskeletal 1
P00760	*Bos taurus (Bovine)*	Serine protease 1
F1MAV0	*Bos taurus (Bovine)*	Fibrinogen beta chain

Proteins are listed by relative abundance.
[a] Immune response-related.

releasing these vesicles as EVs [9], and likely involves both ESCRT-dependent and ESCRT-independent pathways. For example, ESCRT components including hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), signal transducing adaptor molecule 1 (STAM1), TSG101, ESCRT accessory protein ALIX and the others [39], were identified in PSC EVs. For ESCRT-independent pathways, tetraspanins also play crucial roles in sorting of multiple proteins, such as the luminal domain of premelanosome protein [40]. Hence, the abundant tetraspanins recovered in *E. multilocularis* EVs point to potential roles for EV ESCRT-independent biogenesis.

Host-derived macromolecules, including proteins and miRNAs, have been reported in EVs released by cestodes [21,24,34] and intracellular parasites [8,9]. The most abundant host-derived proteins in EVs were immunoglobulins and complement factors [34], and were also identified here, rather than as bovine derived proteins Under TEM, EVs from the tegument of *E. multilocularis* were observed resembling EVs released from other platyhelminths [34,42]. In addition, there was evidence that host IgG deposited in the tegument of E. ortleppi [41]. Hence, the presence of host immunoglobulins in these parasites indicates that they may be packed into the membranes of parasite EVs during releasing into the extracellular space, and involve in the regulation of host−parasite interaction [34,42]. Importantly, KEGG analysis revealed that some pathways, including focal adhesion, were enriched in *E. multilocularis* EVs, suggesting a possible angiogenesis-promoting mechanism.

High expression of pro-angiogenic factors in the liver of mice infected with *E. multilocularis* together with previous studies [27−30], underscore the ability of *E. multilocularis* to mediate neovascularization. Analogous to an aggressive tumor, *E. multilocularis* is invasive and causes pathological angiogenesis to provide nutrients and metabolite excretion for parasite growth, maturation and invasion. However, mechanisms underpinning these phenomena are poorly understood. A multifactorial induction of parasite-associated neovascularization could arise through host-, parasite- or host-plus parasite-dependent angiogenic mechanisms [43]. An emerging paradigm is that EVs transfer helminth molecules to HUVECs, participate in blood clot modulation, vasodilation and vascular smooth muscle contraction exemplified by Schistosoma [44], and Leishmania [45]. Furthermore, exosomes are important messengers between tumor cells and vascular endothelial cells in hypoxia-driven pro-angiogenic tumor responses, frequently mediated by

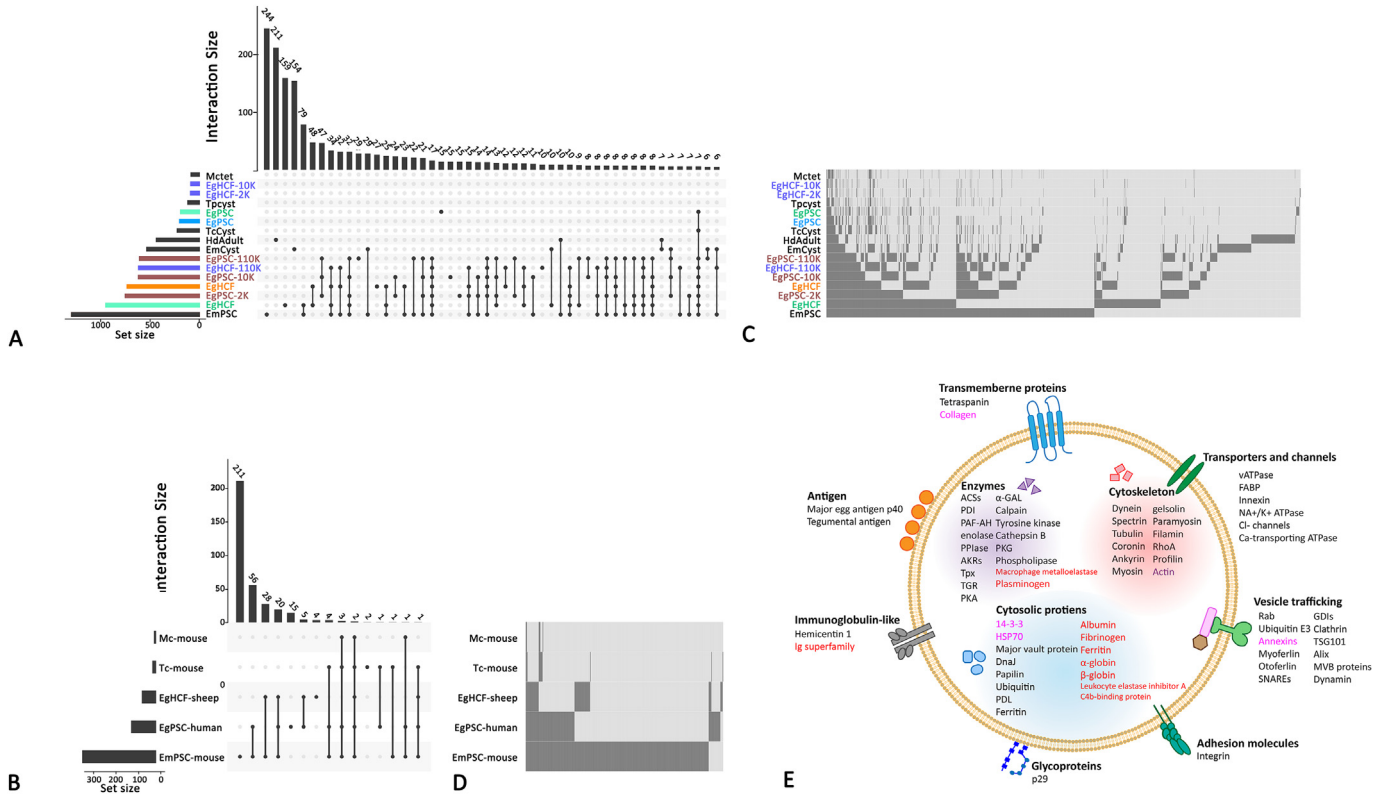

Fig. 3. Protein representation between EVs of multiple-species. A) UpSetR analysis visualizing intersections between unique conserved domains of parasite-derived proteins found in cestodes extracellular vesicles (EVs). The same color indicated that data was retrieved from the same study. B) UpSetR analysis visualizing intersections between unique conserved domains of host-derived proteins found in cestodes EVs. Intersecting datasets are shown as filled circles; the corresponding number of domains in that intersection is presented on top of each histogram. The set size represents the number of domains found in each dataset. C) Conserved domain distribution of parasite-derived proteins in available cestode EVs-contained datasets. The same color indicated that data was retrieved from the same study. D) Conserved domain distribution of host-derived proteins in available cestode EVs-contained datasets (Dark grey, presence; light grey, absence). E. Schematic representation of proteins and domains in EVs shed by *Echinococcus* spp (Black: parasite-derived proteins; Red: host-derived proteins; Purple: parasite- and host-derived common proteins). Please find the complete list of domain information in Table S04-05.

miRNAs, such as miR-21-5p, miR-30 b, miR-100 and others [16,23–25]. However, analogs to these miRNAs are absent from EVs released by Echinococcus spp [16,23–25]. Regardless, we found that *E. multilocularis* PSCs can promote HUVEC proliferation and tube formation through shedding EVs, potentially inducing angiogenesis via related proteins packaged into EVs. However, we cannot exclude impacts from EV-derived miRNAs mediating physiological changes, as exemplified by HUVECs.

Parasite EVs can directly interact with multiple cells in infected hosts and has been extensively characterised in dendritic cells, macrophages, T cells and B cells [46]. These cells exert direct effects on parasites or recipient cells by targeted immunomodulatory strategies to maintain homeostasis, including suppression of type 1 responses, stimulation of Th1- or Th2-responses and induction of type 2 tolerogeneic phenotypes [46]. We identified many proteins in EVs with potential immunological impact. For example, the 14-3-3 protein played vital roles in regulation of inflammatory responses and immunization with recombinant *E. multilocularis* 14-3-3 protein provided 97% protection against a *E. multilocularis* challenge infection [47], HSP70 proteins activated inflammatory pathways [48], cathepsins interacted with macrophages and led to suppression of Th1 responses [48], integrins were involved in activating TGFβ1 to control immune homeostasis [49] and annexin (also found as a host-derived protein here) had the capacity to bind and activate host plasminogen, suggesting a role in parasite invasion [22]. In addition to components commonly found in exosomes generated by most cell types, a series of parasite-specific virulence factors were among the most abundant proteins in the exosomal proteome, such as HSPs and highly immunogenic and tolerogenic antigens. These antigens potentially modulate host defense by suppressing neutrophils and dendritic cell-mediated innate responses and T cell-dependent mechanisms, and influencing the intensity and quality of the global adaptive immune response [21].

In conclusion, we provide deeper understanding of the protein composition of EVs released by *E. multilocularis* PSCs. Both ESCRT-dependent and -independent pathways are involved in the biogenesis of *E. multilocularis* EVs. Moreover, EVs from both parasite and host potentially play important roles in manipulating host development and parasite survival as well as the resulting pathology. It is likely that EVs of multiple cellular origin are involved in vascular development, growth and maturation [50]. In addition, our study indicates that host-derived proteins in EVs released by the parasite are key players to angiogenesis, promoting HUVEC proliferation and tube formation. However, further exploration of the functions of *E. multilocularis* EVs is required to elucidate the precise mechanisms related to angiogenesis and other interactions between the host and *E. multilocularis*.

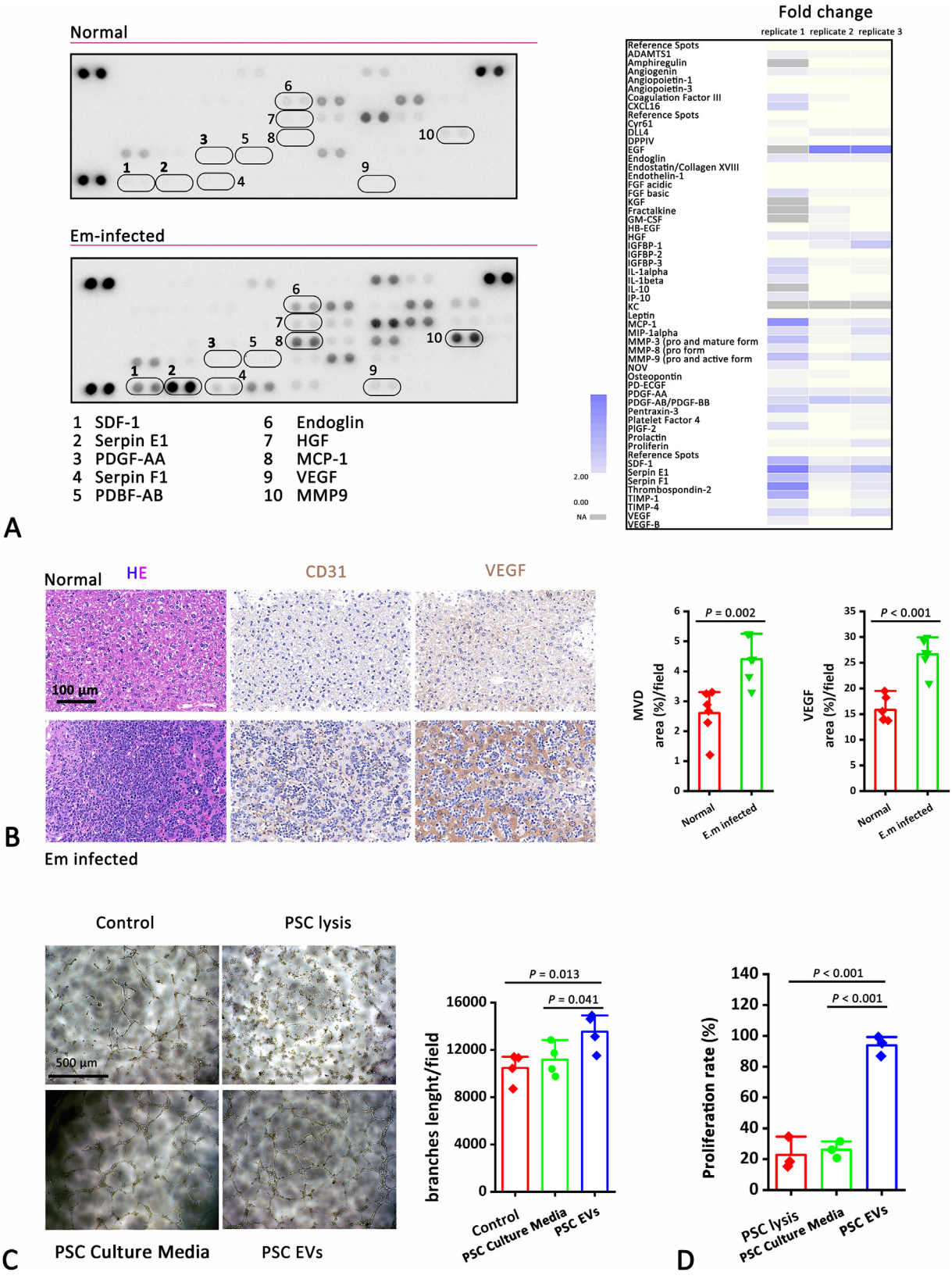

Fig. 4. Angiogenesis induced by *E. multilocularis* infection and EVs released by *E. multilocularis* PSCs. A) The Mouse Angiogenesis Array detected multiple analytes in tissue lysates. A total of 300 µg of tissue lysate was run on each array (n = 3). Factors with fold change (infected/normal)≥2 are called out. B) Immunohistochemical detection of CD31 and VEGF in liver tissues of *E. multilocularis*-infected and non-infected mice. C) Tube formation of HUVECs after incubation with *E. multilocularis* PSC culture medium (10 µg), EVs (10 µg) and whole PSC lysis (10 µg). D) Effects of *E. multilocularis* materials (10 µg) on HUVEC proliferation.

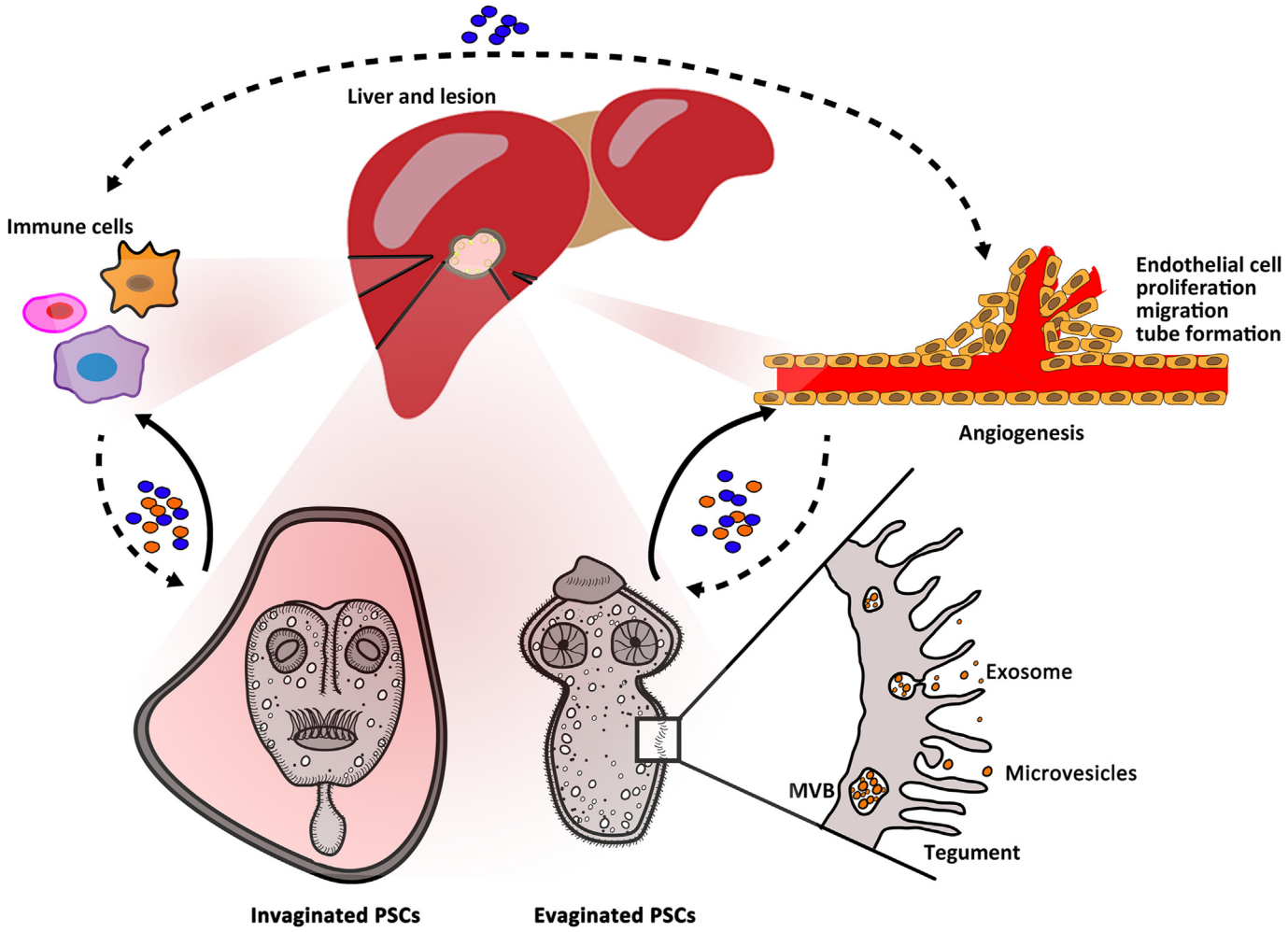

Fig. 5. Model for *E. multilocularis* PSCs EVs and host EVs involvement in angiogenesis. *E. multilocularis* lesions are mostly located in host livers, where immune, fibroblast and vascular cells encyst the *E. multilocularis* metacestode containing invaginated and evaginated PSCs. Host cells and PSCs can release EVs into the lesion environment to regulate host–parasite interactions. Parasite EVs (orange) released by *E. multilocularis* PSCs and cysts can be internalized by various host cells, while host cells may also release EVs (blue) to regulate development of parasites. In addition, communication between host cells can also be facilitated by EVs.

Author contributions

Jianhai Yin, Jianping Cao and Congshan Liu designed and supervised the whole project. Congshan Liu and Jianhai Yin performed experiments, collected, analyzed, and interpreted data. Congshan Liu, Mark C. Field and Jianhai Yin drafted the manuscript and participated in the preparation of its final version. Jianping Cao, Haobing Zhang and Mark C. Field participated in revision and the final version of the manuscript.

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Data availability statement

The data that support the findings of this study are available in the methods and/or supplementary material of this article. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD035601.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2023.105147.

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