

1 **Mouse thy1-positive spermatogonia suppress the proliferation of spermatogonial**
2 **stem cells by Extracellular vesicles in vitro**

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18 **Running title**

19 spermatogonia suppress the proliferation of spermatogonial stem cells

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44 **ABSTRACT**

45 The self-renewal of mammalian spermatogonial stem cells (SSCs) supports
 46 spermatogenesis to produce spermatozoa, and this is precisely controlled in a stem
 47 niche microenvironment in the seminiferous tubules. Although studies have revealed
 48 the role of the surrounding factors in SSCs, little is known about whether the division
 49 of SSCs is controlled by extracellular vesicles. Here, extracellular vesicles were found
 50 in the basal compartment of seminiferous tubules in mouse, rat, rabbit and human
 51 testes. In the mice, the testicular extracellular vesicles are secreted by spermatogonia
 52 and are taken up by SSCs. Further, the extracellular vesicles from thy1-positive
 53 spermatogonia were purified by anti-Thy1-coupled magnetic beads, and which
 54 suppress their proliferation of SSCs but not lead to the apoptosis in vitro.

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56 **KEY WORDS**

57 Testicular extracellular vesicles; spermatogonial stem cell; proliferation

58

59 **ABBREVIATIONS**

60 SSCs: spermatogonial stem cells; EVs: extracellular vesicles

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66 1 | INTRODUCTION

67 Spermatogonial stem cells are undifferentiated spermatogonia that are essential
68 for the maintenance of spermatogenesis. Although SSCs are present in low numbers in
69 the mammalian testis (van den Berg et al., 2007), they balance self-renewal and
70 differentiation to maintain themselves and continually produce committed progenitor
71 spermatogonia, which subsequently become differentiating spermatogonia,
72 spermatocytes, spermatids and mature spermatozoa (Ishii et al., 2012). In human,
73 spermatogonia include progenitor A_{dark} -spermatogonia, progenitor
74 A_{pale} -spermatogonia, committed A_{pale} -spermatogonia, and B-spermatogonia. The
75 progenitor A_{dark} -spermatogonia apparently are stems or reserve spermatogonia
76 (Goharbakhsh et al., 2013). In mice, the A_{single} , A_{paired} and A_{aligned} spermatogonia are
77 collectively described as undifferentiated type A spermatogonia based on
78 morphological analysis (Clermont and Bustos-Obregon, 1968). These undifferentiated
79 A-spermatogonia undergo a series of cell divisions to form differentiated
80 spermatogonia (Chiarini-Garcia and Russell, 2002a A_4 , intermediate, and B
81 spermatogonia) before entering meiosis (Chen and Liu, 2015). The A_{single} , A_{paired} and
82 A_{aligned} spermatogonia occurs cyclically with in the highly organized seminiferous
83 epithelium, which proliferate primarily in stages I-IV and XI-XII, and remain
84 relatively quiescent at stages of V-X (Sharma and Braun, 2018). Obviously, the SSC
85 division pattern is a complex process and involves positive and negative regulation
86 (Chen and Liu, 2015).

87 The fate options of SSCs are influenced by extrinsic factors of their stem niche

88 microenvironment comprised of somatic support cell populations that include Sertoli,
 89 Leydig, and myoid cells in mammalian testes (Yang et al., 2013). Sertoli cells, the
 90 only somatic cell types within seminiferous tubules, physically interact with the SSCs,
 91 and likely support SSCs self-renewal by providing growth factors such as glial
 92 cell-derived neurotrophic factor (Airaksinen and Saarma) and fibroblast growth factor
 93 -2 (Hess et al., 2006; Meng et al., 2000). GDNF supplementation to media is also
 94 essential for maintaining the SSCs in culture (Kanatsu-Shinohara et al., 2004). In
 95 previous studies, C-X-C motif chemokine -12 secreted by Sertoli cells and colony
 96 stimulating factor -1 secreted by Leydig and myoid cells were shown to play critical
 97 roles in regulating SSC self-renewal (Chen et al., 2005; Hess et al., 2006; Oatley et al.,
 98 2009). Although several positive regulators of SSC self-renewal have been discovered,
 99 knowledge of the SSCs niche is still incomplete and much remains unknown about
 100 how the microenvironment precisely control the self-renewal division of SSCs,
 101 especially the negative regulators.

102 Extracellular vesicles (EVs) are particles naturally released from the cell that are
 103 delimited by a lipid bilayer and do not contain a functional nucleus, EVs are generally
 104 referred to as exosomes, microvesicles, and apoptosis body. The term exosome are
 105 small (50–100 nm) vesicles that are secreted by a multitude of cell types. Depending
 106 on their origin, they can play a variety of roles in different physiological process. It
 107 was reported that exosomes are the only class of extracellular vesicles known to be
 108 derived from endosomes through the invagination of the endosomal membrane to
 109 form multivesicular bodies (MVBs) with numerous small vesicles. These small

110 vesicles are released as exosomes when MVBs fuse with the plasma membrane
 111 (Simons and Raposo, 2009). In the mouse testis, Chiarini-Garcia and Russell reported
 112 that all type A spermatogonia show MVBs, but were not associated with lysosomes
 113 (Chiarini-Garcia and Russell, 2002b). As MVB could fuse with the cell surface (the
 114 plasma membrane), and release the intraluminal vesicles, which are called exosome ,
 115 so whether the type A spermatogonia could secrete the extracellular vesicles remained
 116 unknown.

117 Here a large number of EVs (testicular EVs) were found near the basement
 118 membrane of seminiferous tubules from rat, rabbit, mouse and human testes across
 119 different stages of spermatogenesis, the rat, rabbit, mouse were laboratory rodents that
 120 helping us to unravel that a number of EVs in testis appear to be conserved among
 121 mammals. We show that the testicular EVs were originated from spermatogonia in the
 122 mouse. By separately labelling the testicular EVs, we found that they can be
 123 specifically taken up by SSCs, the EVs from thy1-positive spermatogonia were
 124 purified by anti-Thy1-coupled magnetic beads, and which suppress their proliferation
 125 of SSCs but not lead to apoptosis in vitro. Therefore, we suggest that spermatogonia
 126 might use negative feedback to regulate SSC proliferation by secreting EVs.

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143 **2 | MATERIALS AND METHODS**

144 **2.1 | Animals**

145 C57BL/6 mice, rabbit, and adult Sprague Dawley rats were purchased from
146 SLAC Laboratory Animal Co., Ltd. (Shanghai, P. R. China), All animal care and
147 experiments of this study were performed in accordance with the guidelines and were
148 approved by the Ethics Committee of International Peace Maternity and Child Health
149 Hospital, School of Medicine, Shanghai Jiaotong University (Permit number: GKLW
150 2017-31).

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152 **2.2 | Human testicular biopsies**

153 The collection of human testicular tissues was in accordance with institutional
154 guidelines, and **all patients provided written informed consent**, the study design
155 was approved by the Ethics Committee of the International Peace Maternity and Child
156 Health Hospital. Informed consent was obtained from the participants. Two testicular
157 tissue biopsies were obtained by puncture from men with obstructive azoospermia
158 (age 30 and 37 years) with normal spermatogenesis. Clinical examinations included
159 the evaluation of secondary sexual characteristics, testicular size and consistency,
160 epididymal distension, presence of the vasa deferentia and varicocele. Both patients
161 had their serum follicle stimulating hormone concentrations measured, with values in

162 the normal range.

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164 **2.3 | Cell culture**

165 Spermatogonial stem cells were established from 6-day-old male F1 progeny of
 166 DBA/2 × C57BL/6 or C57BL/6/Tg14 (act-EGFP-OsbY01) mice as described (Gong
 167 et al., 2017; Kanatsu-Shinohara et al., 2003). There is no difference when it comes to
 168 the percentage and behaviour of the SSCs within the testes of C57BL/6 and DBA/2
 169 mouse (Kubota and Brinster, 2008). They were seeded on mitomycin C-treated mouse
 170 embryonic fibroblast (MEF) feeder cells and cultured in SSC medium consisting of
 171 StemPro-34 SFM medium supplemented with stemPro supplement (Thermo Fisher
 172 Scientific, Waltham, MA, USA), 25 µg/ml insulin, 100 µg/ml transferrin, 60 mM
 173 putrescine, 30 nM sodium selenite, 6 mg/ml D-(+)-glucose, 30 µg/ml pyruvic acid, 1
 174 µl/ml D-L-lactic acid (Sigma-Aldrich, St Louis, USA), 5 mg/ml bovine serum albumin
 175 (Sigma-Aldrich, St Louis, USA), 2 mM L-glutamine, 10 µM 2-mercaptoethanol
 176 (Sigma-Aldrich, St Louis, USA), 1 × MEM vitamins solution (Invitrogen, USA), 1 ×
 177 non-essential amino acid solution (Invitrogen, USA), 2 mM L-glutamine (Invitrogen,
 178 USA), 1 × penicillin/streptomycin solution (Invitrogen, USA), 0.1 mM ascorbic acid,
 179 10 µg/ml d-biotin (Sigma-Aldrich, St Louis, USA), 20 ng/ml recombinant human
 180 epidermal growth factor (Invitrogen, USA), 10 ng/ml human basic FGF (Invitrogen,
 181 USA), 10 ng/ml recombinant human GDNF (Invitrogen, USA) and 1% fetal bovine
 182 serum (FBS) (Gibco/Life Technologies, Thermo Fisher Scientific, Waltham, MA,
 183 USA). The medium was replaced every 2–3 days. For MEF preparation, C57BL/6J

184 mouse embryos were minced, digested with trypsin-EDTA (Invitrogen, USA), and
185 then cultured in Dulbecco's (D) MEM containing 10% FBS supplemented with 2 mM
186 glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (MEF culture medium).

187

188 **2.4 | Isolation and analysis of testicular EVs**

189 Twenty mice at 20 days post-partum were euthanized by cervical dislocation,
190 the testes were dissected and decapsulated, then digested with 0.25 mg/ml collagenase
191 IV (Sigma-Aldrich, St Louis, USA) in DMEM/F12 medium at 37 °C for 20 min with
192 slow shaking (150 cycles per min) until the tubules had dispersed fully. The tubules
193 were allowed to settle for 2 min at room temperature by standing the tube vertically.
194 The supernatant enriched in interstitial testicular cells was discarded, leaving just the
195 settled tubules. These were digested with 2 mg/ml collagenase IV and 2 mg/ml
196 hyaluronidase (Sigma-Aldrich, St Louis, USA) at 37 °C for 20 min, centrifuged at
197 3,000 × g for 30 min to remove the cells, then centrifuged at 10,000 × g for 30 min at
198 4°C to remove cell debris, and the final supernatant was centrifuged at 100,000 × g for
199 90 min in an SWT32 swinging bucket rotor (Beckman Coulter, Brea, CA, USA) at
200 4 °C to precipitate the EVs. The extracellular vesicle pellets were washed with
201 phosphate-buffered saline (PBS), and filtered using 0.22 µm filter (Millipore, USA),
202 and EVs were finally collected by centrifugation at 100,000 × g for 90 min at 4°C and
203 resuspended in 20 µl of PBS. The purified EVs were analyzed using Zeta Particle
204 Metrix equipment (Particle Metrix GmbH, Meerbusch Germany), after which they
205 were stored at -80 °C. The pellets were resuspended in PBS for subsequent analysis.

206

207 **2.5 | Purification of Thy1 positive EVs**

208 The isolation of Thy1 positive EVs is performed by positive selection using
 209 anti-CD90 MicroBeads (Miltenyi Biotec, USA) according to the manufacturer's
 210 instructions. Briefly, the testicular EVs were diluted with 2 ml phosphate-buffered
 211 saline (PBS) containing 0.5% bovine serum albumin, and 2 mM EDTA, and incubated
 212 with anti-CD90 beads (100 μ l) for 3 hours at 4 °C under slow rotation, A microcolumn
 213 (LS separation columns, MACS, Miltenyi Biotec) was placed in MACS magnetic
 214 separator and the column was rinsed thrice with 1 mL rinsing solution (MACS BSA
 215 Stock Solution diluted 1:20 with autoMACS Rinsing Solution, Miltenyi Biotec).
 216 Beads bound to EVs were applied onto a magnetic column and Thy1-negative EVs
 217 that passed through the column were collected.

218

219 **2.6 | Testicular extracellular vesicle labeling and uptake assay**

220 Purified EVs were labeled with Exo-Glow extracellular vesicle labeling kits
 221 (SBI Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions.
 222 Aliquots of 50 μ l of 10 \times Exo-red were added to 500 μ l of resuspended EVs in PBS,
 223 mixed well by flicking the tube and incubated at 37°C for 10 min. The labeling
 224 reaction was stopped by adding 100 μ l of ExoQuick-TC reagent and incubated on ice
 225 for 30 min. The labeled EVs were centrifuged and washed with PBS three times.
 226 SSCs were incubated with the labeled EVs for 1–4 h and visualized using fluorescent
 227 microscopy (Leica, Wetzlar, Germany)

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229 **2.7 | Cluster-forming activity (CFA) assay**

230 CFA assays were performed as described (Yeh et al., 2007). SSCs were
231 harvested from an established cluster culture and seeded at approximately 1×10^4
232 cells/cm² in 96-well culture dishes. After incubation with 0, 2, and 4 µl testicular EVs
233 (5×10^9 particles/µl) per well for 7 days, the medium was changed to SSC culture
234 medium. All clusters in a well were counted visually at the 6th day. Experiments were
235 performed in triplicate.

236

237 **2.8 | Reverse transcription quantitative polymerase chain reaction (RT-qPCR)** 238 **amplification**

239 SSCs were separated from MEFs by gentle pipetting and total RNA was
240 extracted from SSCs using TRIzol (Invitrogen, USA) according to the manufacturer's
241 instructions. Reverse transcription was performed using PrimeScript RT Master Mix
242 (CD201-2, TaKaRa, Otsu, Japan), and qPCR was performed using SYBR Premix Ex
243 Taq II (RR820L, TaKaRa, Japan) in an ABI 7500 Real-Time PCR System (Applied
244 Biosystems, Foster City, CA, USA). The qPCR conditions were 95 °C for 5 min,
245 followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. Transcript levels were
246 normalized to the housekeeping gene *Gapdh*.

247

248 **2.9 | Western blot analysis**

249 Testicular EVs were lysed in RIPA buffer (P0013B; Beyotime Biotechnology,

Shanghai, P. R. China), the protein concentrations were measured using Bradford Protein Assay kits (P0006; Beyotime Biotechnology, China), and exosomal amounts loaded for western blotting were normalized according to the protein concentration. The exosomal lysates were fractionated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), electrotransferred to nitrocellulose membranes blocked with 5% (w/v) nonfat dry milk and incubated with primary antibodies against CD81 (18250-1-AP, Proteintech Group, Wuhan, P. R. China)(diluted as 1:2500), CD9 (ab92726, Abcam, Cambridge, UK) (diluted as 1:2500), Thy1 (ab225, Abcam, Cambridge, UK) (diluted as 1:2500), Gfra1(ab186855, Abcam, Cambridge, UK) (diluted as 1:2500) in 0.1% Tween-20 in tris-buffered saline (TBST) overnight at 4 °C After washing with TBST, the membranes were incubated with secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (SA00001-2, Proteintech, China) (diluted as 1:5000) or HRP-conjugated goat anti-mouse IgG (H+L) (SA00001-1, Proteintech, China) (diluted as 1:5000) in TBST for 1 hour at room temperature. After washing, Immunoblots were visualized using ECL substrate (Thermo Scientific, USA) and ImageQuant LAS4000 Mini software (GE Healthcare Life Sciences, Chicago, IL, USA).

267

2.10 | Transmission electron microscopy (TEM)

Testicular samples from rat, mouse, rabbit and human were fixed in 2.5% glutaraldehyde in 0.1 M HEPES buffer (pH = 5) overnight at 4 °C then postfixed in 1% osmium tetroxide. The samples were then rinsed with PBS followed by dehydration in

272 an ethanol gradient and then embedded in Epon 812 (Sigma-Aldrich, St Louis, USA).
 273 Ultrathin sections obtained using an Ultracut R ultramicrotome (Leica) were stained
 274 with uranyl acetate and lead citrate. For immunoelectron microscopy, mouse testes
 275 were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in 100 mM sodium
 276 phosphate, pH 7.4, at 4 °C by immersion for 3 h, After washing with 100 mM lysine
 277 in 100 mM sodium phosphate, pH 7.4, and 150 mM sodium chloride, they were
 278 dehydrated in a graded series of cold ethanol then embedded in Epon 812. Ultrathin
 279 sections was incubated with primary antibodies against IgG (30000-0-AP, Proteintech,
 280 China), GFR α 1 (ab186855, Abcam, Cambridge, UK) for 12 h at 4 °C. Then, 10 nm of
 281 colloidal gold-conjugated second antibody (A-31566, Thermo Fisher Scientific,
 282 Waltham, MA, USA) was incubated for 2 h at room temperature. After staining with
 283 uranyl acetate and lead citrate, images were captured using a transmission electron
 284 microscope (H-7560; Hitachi, Tokyo, Japan) at 80 kV.

285 EVs were fixed in 4% paraformaldehyde and layered on Formvar-carbon-coated
 286 electron microscopy grids, washed with PBS, and further fixed with 1%
 287 glutaraldehyde for 5 min. Samples were then stained with 4% uranyl acetate for 30
 288 min, after which images of the micrographs were captured using a transmission
 289 electron microscope (H-7560; Hitachi, Japan) at 80 kV.

290

291 **2.11 | Immunofluorescence Staining**

292 After 5 days of culture, OCT4, PLZF and MVH were used as the target molecule for
 293 the identification of SSCs. Briefly, cells were fixed in 4% paraformaldehyde solution

(PFA) for 30 minutes, and washed with PBS three times, each time for 5 minutes. Then, cells used for OCT4 and PLZF staining were treated with 0.5% Triton X-100 for 30 minutes at room temperature, cells used for MVH staining should not be treated with Triton X-100. Following washing with PBS for three times, cells were blocked with goat serum at 37°C for 20 minutes, then incubated overnight at 4°C with rabbit-anti-OCT4 (1:100, Santa Cruz Biotechnology), mouse-anti-PLZF (1:100, Santa Cruz Biotechnology) or rabbit-anti-MVH (1:100, Santa Cruz Biotechnology). After that, the cells were washed with PBS containing 0.05% Tween-20, followed by the incubation with Rhodamine (TRITC)-conjugated goat anti-rabbit IgG(H+L) (ProteinTech, USA) or Rhodamine(TRITC)-conjugated goat anti-mouse IgG(H+L) (ProteinTech, USA) for 30 minutes in darkness at 37°C. Next, the nuclear was stained by Hoechst33342. Finally, images were photographed under a DM2500 fluorescence microscope (DMI3000B; Leica).

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308 **2.12 | EdU (5-Ethynyl-2'-deoxyuridine) Assay**

For analysis of the effect of EVs on SSCs proliferation, the Cell-Light™ EdU Apollo567 In Vitro Kit (RiboBio, Guangzhou, China) was used according to the manufacturer's instructions. SSCs were cultured in 96-well plates for 5 days with different treatment (control, Thy1⁺ EVs and Thy1⁺ EVs), followed by the incubation with 50 μM EdU at 37°C for 2 hours, then cells were washed twice with PBS, each time for 5 minutes. After being fixed by 4% PFA for 30 minutes at room temperature, cells were orderly incubated with 50 μL 2 mg/mL glycine solution for 5

minutes and then treated with 0.5% Trion X-100 solution for 10 minutes on a shaker. Subsequently, cells were washed once with PBS and incubated with 1x Apollo staining solution for 30 minutes in darkness on a shaker. After three times of wash with PBS solution containing 0.5% Triton X-100, cells were incubated with Hoechst33342 for 10 minutes at room temperature to stain cell nucleus. Finally, images were obtained under the Leica fluorescence microscope.

322

2.13 | Cell Apoptosis Assay

Cell apoptosis detection was performed using Annexin V-FITC Apoptosis Detection Kit (eBioscience, BMS500FI-300) according to the manufacturer's instructions. After 5 days of culture, SSCs were collected by trypsin digestion and washed once in PBS by gentle shaking or pipetting up and down, SSCs were resuspended in binding buffer. Then, cells were incubated with Annexin V-FITC for 10 minutes at room temperature, after that, SSCs were washed once in binding buffer and resuspended in binding buffer containing 20 µg/mL propidium iodide (PI), and incubated for 15 minutes at room temperature in darkness. Cell apoptosis were analyzed by Beckman Cytoflex (Beckman Coutler Co.Ltd &Cytoflex).

333

2.14 | Statistical analysis

Experiments were run in triplicate. Two-tailed, unpaired Student t -test was used for statistical analysis, data are presented as the mean ± standard error of the mean (SEM). $P < 0.05$ was considered to be statistically significant. The statistical graphs

338 were generated by GraphPad Prism 6.

339 **3 | RESULTS**

340 **3.1 | Testicular EVs are secreted by spermatogonia**

341 To explore the role of EVs in the development of spermatogenesis, we tracked
 342 the EVs in the testes from mice at 8 days, 14 days, 21 days, and 8 weeks post-partum
 343 (P8, P14, P21, 8w) using TEM. A number of small EVs were found near the basement
 344 membrane of seminiferous tubules at all stage, and not in the adluminal compartment,
 345 The number of smaller vesicles varies greatly in each cluster, some structures contain
 346 up to 150 vesicles, some contain fewer than 20, these testicular EVs had diameters of
 347 30–100 nm (Fig. 1A). In addition, One MVB was detected in the cytoplasm of a type
 348 A spermatogonium, (Fig. 1B), and the EVs are released from cells upon fusion of
 349 MVB with the plasma membrane, The type A spermatogonia was distinguished on
 350 the basis that it contains several compact nucleoli (Fig. 1B arrow); patches of
 351 heterochromatin (Fig. 1B arrowhead) are sparse along the nuclear envelope that
 352 consistent with previous report (Chiarini-Garcia and Russell, 2002b). Together, these
 353 images prompted us to consider whether the testicular EVs were from spermatogonia,
 354 to determine that, we separated the testicular EVs by two-step enzyme digestion and
 355 differential ultracentrifugation. Western blot analysis of them revealed that they were
 356 all detected together with spermatogonial membrane proteins GFR α 1, THY1, CD9
 357 and exosomal protein CD81 (Fig. 1C). Further, immunoelectron microscopy showed
 358 that GFR α 1 protein was detected on testicular EVs in the testis sections from mice at
 359 8w (Fig. 1D). These results indicate that testicular EVs were secreted by

360 spermatogonia.

361

362 **3.2 | EVs are present in mouse, rat, rabbit and human testes**

363 We next asked whether the testicular EVs could be detected in other mammals.

364 To test this, we used TEM to investigate the EVs in rat, mouse, rabbit and human
365 testes. The results shown that testicular EVs were present in the rat, mouse, rabbit and
366 human testes, and they are also closed to the basement membrane of seminiferous
367 tubules and show up in large numbers, these testicular EVs had the same diameters
368 (30–100 nm)(Fig. 2), these results unraveled that a number of EVs in testis appear to
369 be conserved among mammals.

370

371 **3.3 | Testicular EVs suppress the proliferation of SSCs cultured in Vitro**

372 To explore the function of testicular EVs in spermatogenesis, we first separated the
373 testicular EVs from P20 testes by two-step enzyme digestion, and the second digestive
374 supernatant of seminiferous tubules was used to isolate the EVs by an established
375 ultracentrifugation protocol (Thery et al., 2006). To confirm EVs purification, samples
376 were examined using Nanoparticle tracking analysis (NTA), TEM, and western
377 blotting. Both NTA and TEM of the extracellular vesicle fractions revealed that the
378 diameter of EVs ranged from 50 to 120 nm (Fig. 3A, B). Western blot analysis of
379 extracellular vesicle fractions confirmed the presence of the exosomal proteins CD81
380 and CD9, as identified in the ExoCarta database(Mathivanan et al., 2012), and the
381 supernatant was not detected the CD81 and CD9 (Fig. 3C).

382 As the testicular EVs were secreted by spermatogonia that might interact
383 physically with SSCs. Firstly, to assess the identity of the isolated SSCs,
384 immunofluorescence revealed that more than 95% of isolated cells were positive for
385 PLZF, OCT4, and MVH (supplementary Fig. S1), Reverse transcription-PCR and
386 real-time PCR further showed that the isolated cells expressed the transcripts of PLZF,
387 OCT4, MVH, Gfra1, and Etv5 (supplementary Fig. S2), collectively, these data
388 suggest that the isolated cells are mouse SSCs phenotypically. to determine whether
389 the testicular EVs could be taken up by SSCs, we labeled them with Exo-red, an
390 acridine orange based dye that binds to RNA. One hour after exposure to testicular
391 EVs, a remarkable uptake of the EVs by SSCs was observed, while the MEF feeder
392 cells did not exhibit red fluorescence (Fig. 3D), and the supernatant of the labeled
393 testicular EVs treated with Exo-red also did not stain the SSCs (**supplementary** Fig.
394 S3). This indicated that SSCs might specifically take up testicular EVs by
395 receptor-mediated endocytosis. To explore the effects of testicular EVs on the SSCs,
396 the SSCs were exposed to testicular EVs from P8, P20, and P35, the result shown that
397 the inhibitory effect of P20 EVs to the SSC proliferation was much better than P8 EVs
398 (supplementary Fig.S4), so the testicular EVs from P20 were chose to further study.
399 Next, the SSCs were exposed to various volumes of testicular EVs (0, 2.5, 5, and 10
400 μ l), and CFA assays were performed (Yeh et al., 2007). As shown in Figure 3E, F,
401 testicular extracellular vesicle suppressed SSC proliferation in a
402 concentration-dependent manner and reduced the clump size of SSCs (Fig. 3E and
403 supplementary table S1; arrow). These results suggest that testicular EVs can be taken

404 up by SSCs and suppress their proliferation.

405

406 **3.4 | Thy1 positive EVs suppress the proliferation of SSCs cultured in vitro but**
 407 **not lead to apoptosis**

408 To specifically separate the EVs from spermatogonia, we developed an approach
 409 designed to purify EVs bearing the undifferentiated A spermatogonia maker Thy1
 410 (Figure4A), the use of magnetic beads directly conjugated to capture antibody, and the
 411 addition of the beads directly to testicular EVs samples. The Thy1 positive EVs were
 412 proved to be rich in Thy1, CD9 and Gfra1(another undifferentiated A spermatogonia
 413 maker) (Figure 4B, C). the effects of Thy1 positive and negative EVs on proliferation
 414 of SSCs were investigated by an EdU fluorescence assay. Notably, Thy1 positive EVs
 415 significantly repressed the proliferation of the SSCs, and the Thy1 negative EVs have
 416 no effect on the proliferation of the SSCs (Figure 4D, E). We next explored the Thy1
 417 positive and negative EVs on apoptosis by an Annexin V-FITC/PI staining assay, the
 418 SSCs were exposure to Thy1 positive or negative EVs, the results showed that both
 419 Thy1 positive and negative EVs have no effect on the apoptosis in the SSCs (Figure
 420 4F, G). In summary, Thy1 positive EVs suppress the proliferation of SSCs but not
 421 lead to apoptosis.

422

423 4 | DISCUSSION

424 EVs, are known as potent vehicles of intercellular communication by transferring
 425 proteins, lipids and nucleic acids both in prokaryotes and eukaryotes, thereby
 426 influencing various physiological and pathological process, for example, in cancer,
 427 the immune response angiogenesis and tissue regeneration (Merino-Gonzalez et al.,
 428 2016; Yanez-Mo et al., 2015). Whether EVs are involved in spermatogenesis remains
 429 uncharacterized. Here for the first time we have demonstrated one role of extracellular
 430 vesicle in spermatogenesis. First, TEM imaging, immunoelectron microscopy and
 431 western blot analysis revealed that spermatogonia secrete a large number of testicular
 432 EVs close to the basement membrane of seminiferous tubules: a feature that is
 433 common among mouse, rat, rabbit and human testes. Second, Exo-red labelling of
 434 testicular EVs and CFA assays showed that testicular EVs were specifically taken up
 435 by SSCs and repressed their proliferation *in vitro*. Finally, the testicular EVs were
 436 divided into the Thy1 positive EVs and Thy1 negative EVs, only the Thy1 positive
 437 EVs suppress the proliferation of SSCs. Thus, our study provides evidence that
 438 testicular EVs secreted by Thy1 spermatogonia play a significant role in regulating
 439 SSC proliferation

440 We separated the testicular EVs from testes at different stages of
 441 spermatogenesis by two-step enzyme digestion. To exclude EVs from interstitial cell,
 442 only the enzyme-digested supernatants of seminiferous tubules was used. The
 443 expressions of *GFRα1*, *Thy1*, *CD9*, and *c-Kit* in testicular EVs from the P8, P21, P35,
 444 8Ws testes were detected by western blot (*CD81* was expressed in all testicular cells,

as shown in Supplementary Fig. S5). In addition, we purified the EVs from type A spermatogonia by undifferentiated spermatogonia marker Thy1 (Figure 4 A, B, C), suggesting the spermatogonia could secrete the EVs in testis.

What is the significance of secreting EVs close to basement membrane? Here we found that SSC proliferation was repressed when treated by testicular EVs or Thy1 positive EVs *in vitro*. This suggests that testicular EVs might play an important role in negatively regulating SSC proliferation (Fig. 3E, 3F, 4D, 4F), and which also could be related to the culture conditions. According to our TEM analysis, about 83% (40/48) testicular EVs interact physically with spermatogonia, whereas only 17% (8/48) interact with Sertoli cells, so whether the testicular EVs can be taken up by Sertoli cells and influence them needs further study.

ADDITIONAL INFORMATION

F.S., J.W., and Y.L. designed the study and wrote the paper. Y.L., and H.Y. performed the testicular extracellular vesicle-related experiments. F.Q., X.W.G and Y.J.W. performed the SSC-related experiments. Y.L., Y.J.W., A.J.L., Y.Z., and G.S.W. performed data analysis, F.Q. and Y.L. revised the manuscript according to reviewers' suggestions.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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527

528 **Figure legends**

529 Figure. 1. Testicular EVs are secreted by spermatogonia. **A)** TEM images of testicular
530 EVs in cross-sections of seminiferous tubule from mice at 8, 14, and 21 days, and 8
531 weeks post-partum (P8, P14, P21, and P8w), the number of smaller vesicles varies
532 greatly in each cluster, some structures contain up to 150 vesicles, some contain fewer
533 than 20, the diameter of the testicular EVs ranges from 30 to 100nm, arrow: a number
534 of EVs, red line: plasma membrane. **B)** Transmission electron microscopy (TEM)
535 images of type A spermatogonia from 8-week-old mice, multivesicular bodies (MVBs)
536 were detected in the cytoplasm (**b**). arrow: compact nucleoli, arrowhead: patches of
537 heterochromatin. **C)** Western blot analysis of GFR α 1, THY1, CD9, and CD81
538 expressed in testicular EVs from mice at P8, P14, P20, and 8w. **D)** Immunoelectron
539 microscopy images of testicular EVs from 8-week-old mice showing immunogold
540 labeling for GFR α 1 protein was detected in testicular EVs; IgG was used as a
541 negative control, arrow: GFR α 1.

542

543 Figure. 2. Testicular EVs are present in mouse, rat, rabbit and human testes. TEM
544 images of testicular EVs in cross-sections of seminiferous tubules from rat, mouse,
545 rabbit and human testes, red line: plasma membrane.

546

547 Figure 3. Testicular EVs repress the proliferation of SSCs in vitro. **A)** Size distribution
548 of the testicular EVs determined by NTA analysis. **B)** Representative TEM images of
549 isolated testicular EVs. **C)** Western blot analysis for CD81, CD9, and beta-Tubulin

550 using extracts from testicular EVs and supernatant. **D)** EVs uptake was visualized
 551 using fluorescence microscopy after treatment with Exo-red labeled testicular EVs. **E,**
 552 **F)** The effect of increasing concentrations of testicular EVs on SSCs after 7 days were
 553 evaluated from cluster counts. Student's t tests were applied to compare pairs of
 554 means and data are shown as the mean \pm SEM of three independent experiments. *P <
 555 0.05; ** P < 0.01; ***P < 0.001, arrow: the clump size of SSCs.

556

557 Figure. 4. Testicular EVs from Thy1 spermatogonia suppress the proliferation of SSCs
 558 in vitro. (A) Schematic illustration of the direct immunoaffinity capture procedure
 559 Magnetic beads directly conjugated to anti-Thy1 were added directly to testicular EVs
 560 after ultracentrifugation. (B) Western blot analysis for Thy1 and CD9 using extracts
 561 from Thy1 positive EVs and Thy1 negative EVs. (C) Detection of Thy1 in Thy1
 562 positive EVs by immunoelectron microscopy; IgG was used as a negative control. (D,
 563 E) Thy1 positive EVs regulates SSCs proliferation activity. Thy1 positive EVs
 564 enhanced BrdU incorporation, whereas Thy1 negative EVs did not affect cell
 565 proliferation activity. E, Histogram of data expressed as EdU positive cell index.
 566 Results are expressed as mean \pm SEM from three independent experiments. ns > 0.05;
 567 *, P < 0.05; **, P < 0.01; ***, P < 0.005 (Student's t test). (F, G) Evaluation of
 568 apoptosis in SSCs by Annexin V/PI assay (flow cytometry) treated with control Thy1
 569 microbeads, Thy1 positive EVs and Thy1 negative EVs. F. Flow cytometry dot plots.
 570 G. Histogram of data expressed as apoptosis index. The bars represent means \pm SD of
 571 three independent experiments. ns < 0.05.

572

573 **Supplemental data legends**

574 Figure S1. Immunocytochemical analysis of spermatogonial stem cell markers (OCT4,
575 PLZF and MVH) was performed with mouse SSC clumps.

576 Figure S2. RT-QPCR analysis of spermatogonial stem cell makers (PLZF, OCT4,
577 MVH, GFRA1, and ETV5) in the cultured mouse SSC clumps. GAPDH was used as
578 an experimental control.

579 Figure S3. Fluorescence microscopy images of SSCs after treatment with the
580 supernatant of the labeled testicular EVs.

581 Figure S4. The effect of testicular EVs from 8D, 20D and 35D on SSCs after 7 days
582 were evaluated from cluster counts. Student's t tests were applied to compare pairs of
583 means and data are shown as the mean \pm SEM of three independent experiments. *P <
584 0.05; ** P < 0.01; ***P < 0.001.

585 Figure S5. Immunohistochemical analysis of CD81 in the mouse testes.

586

587

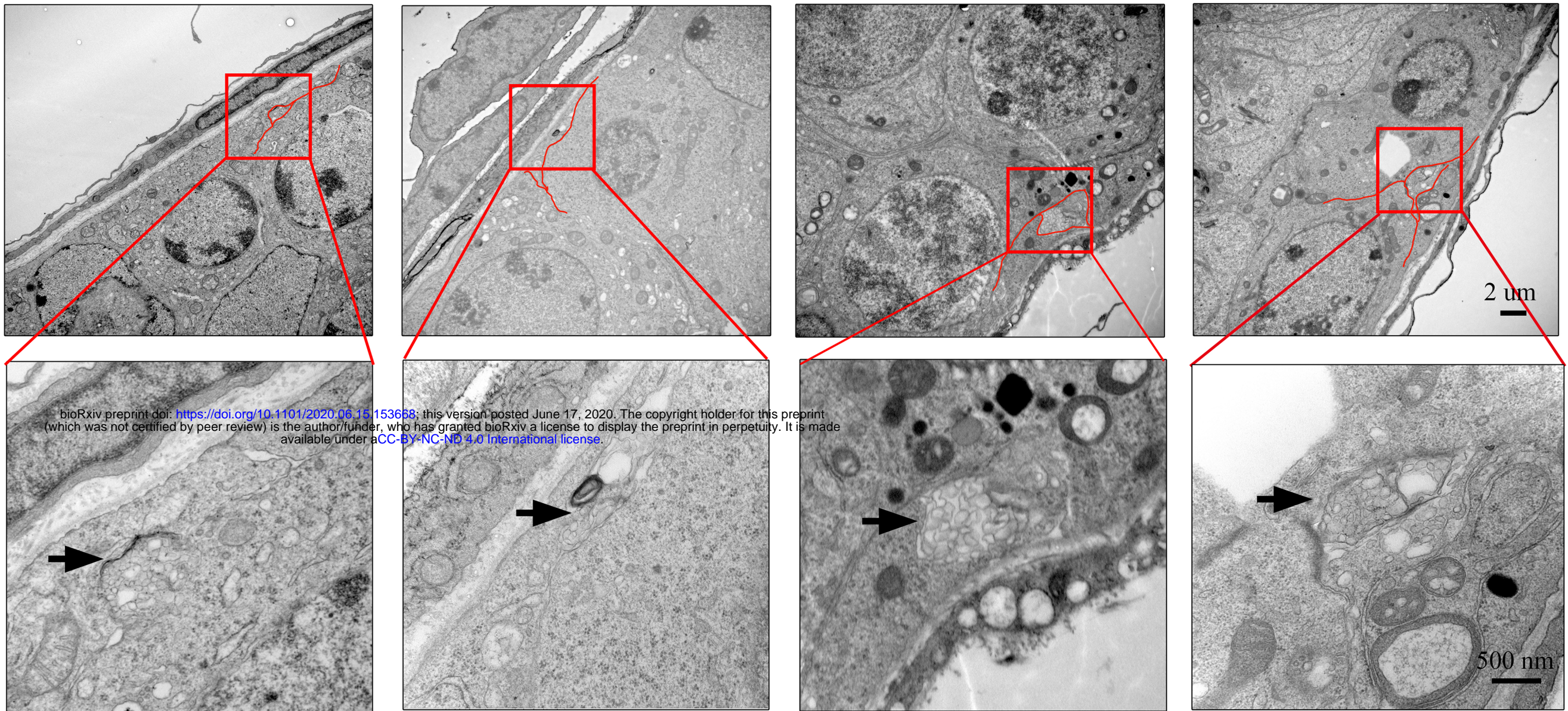
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8 days

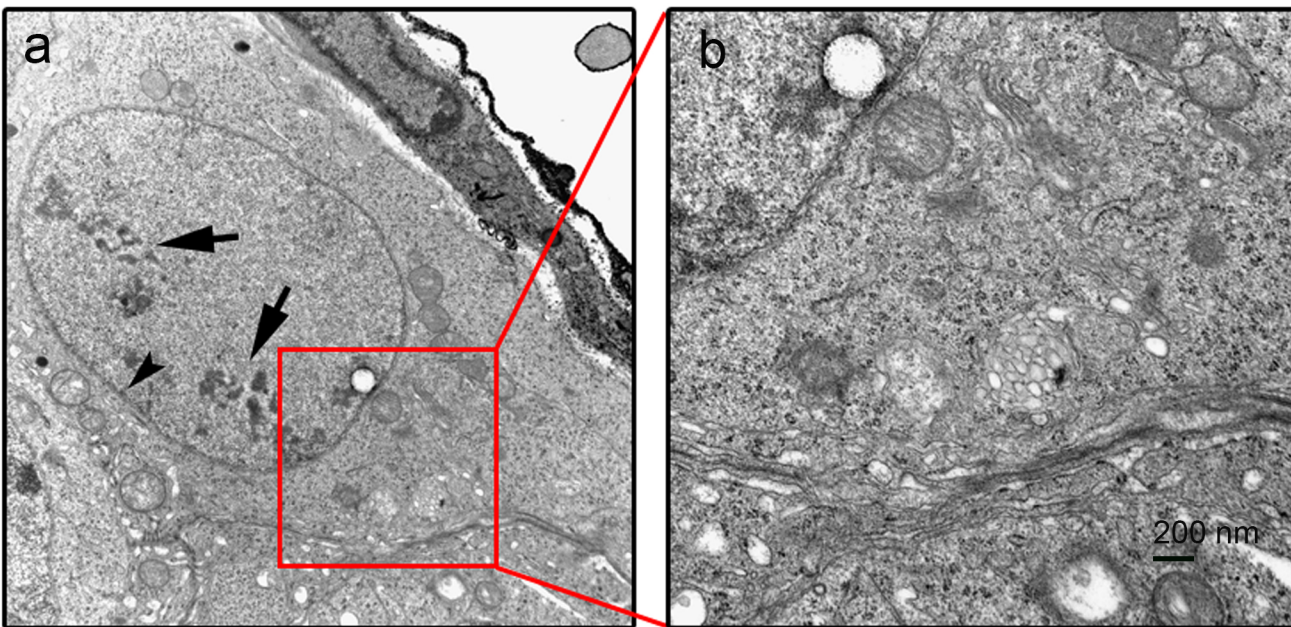
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21 days

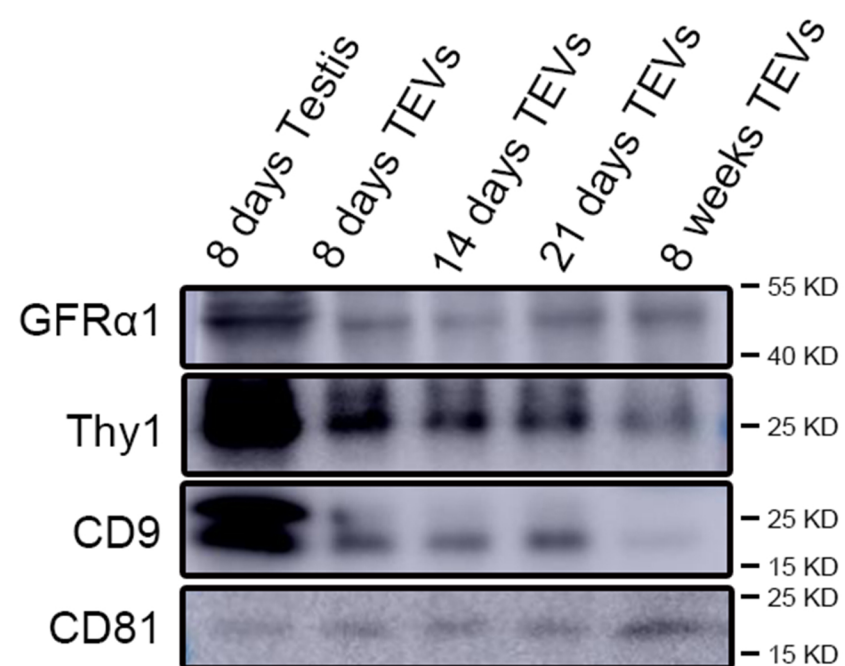
8 weeks



B



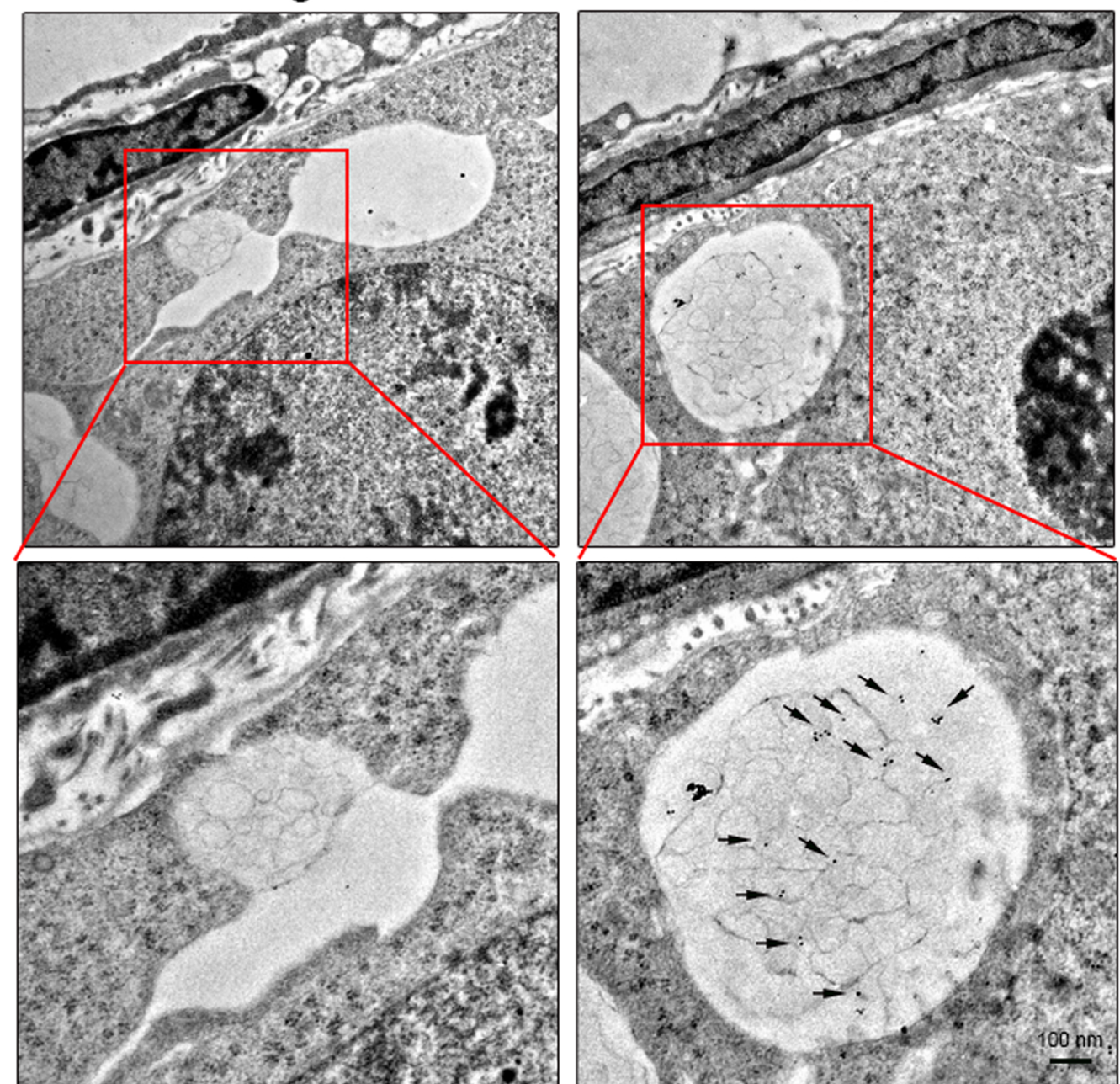
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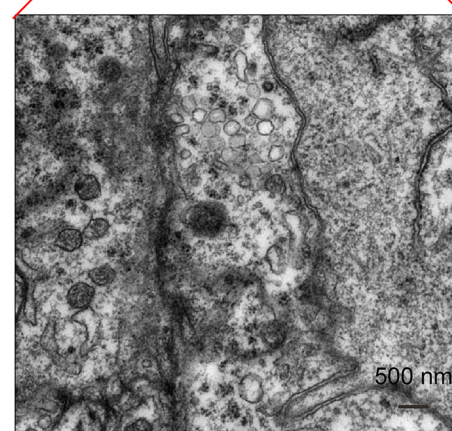
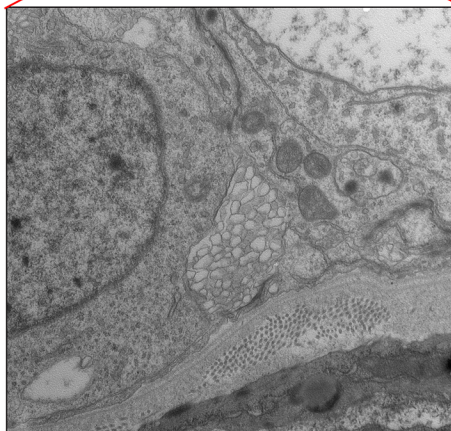
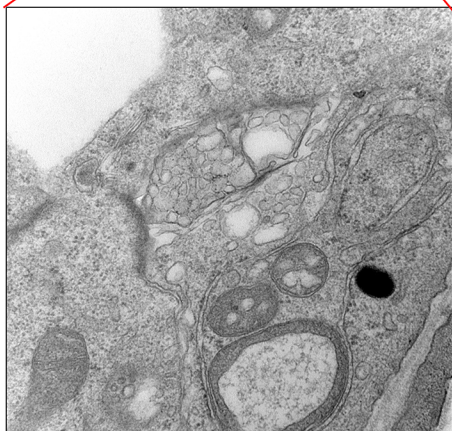
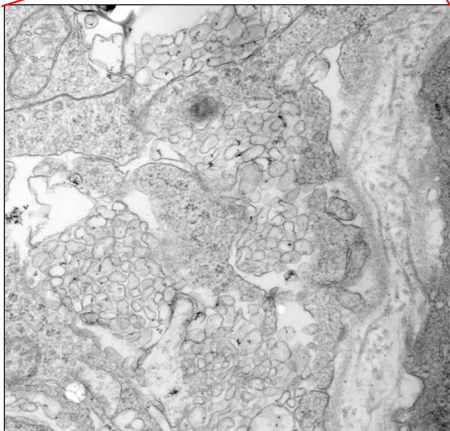
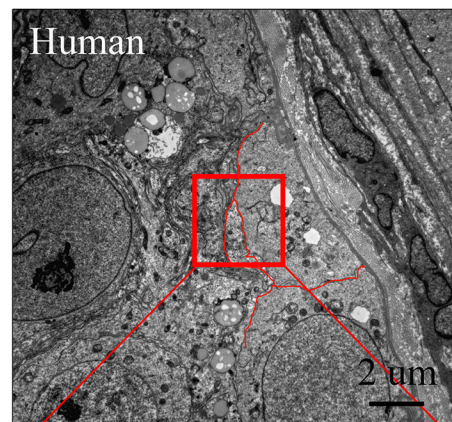
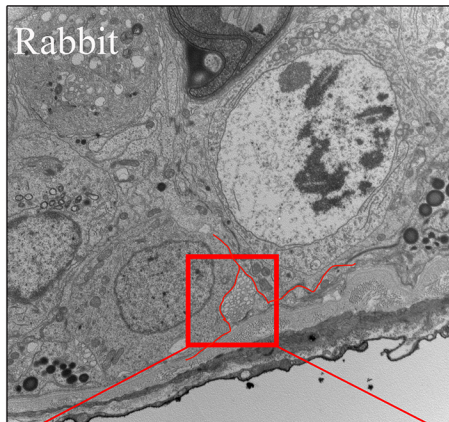
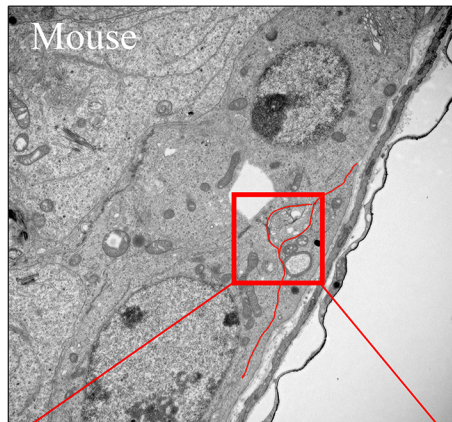
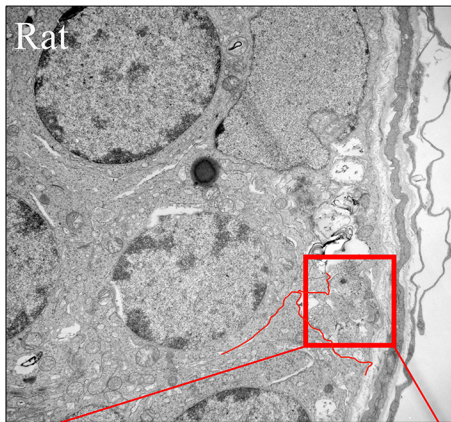


D

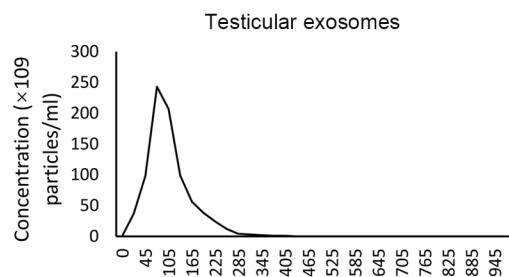
IgG

Gfra1

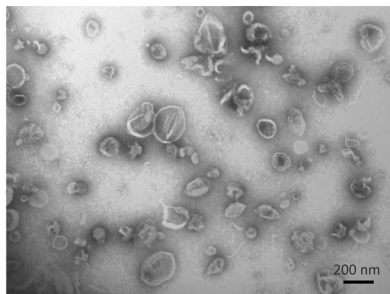




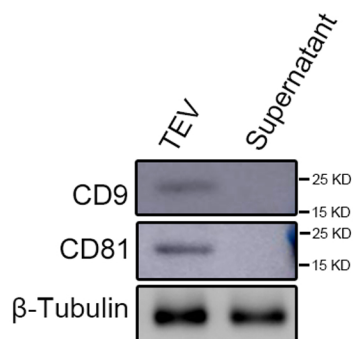
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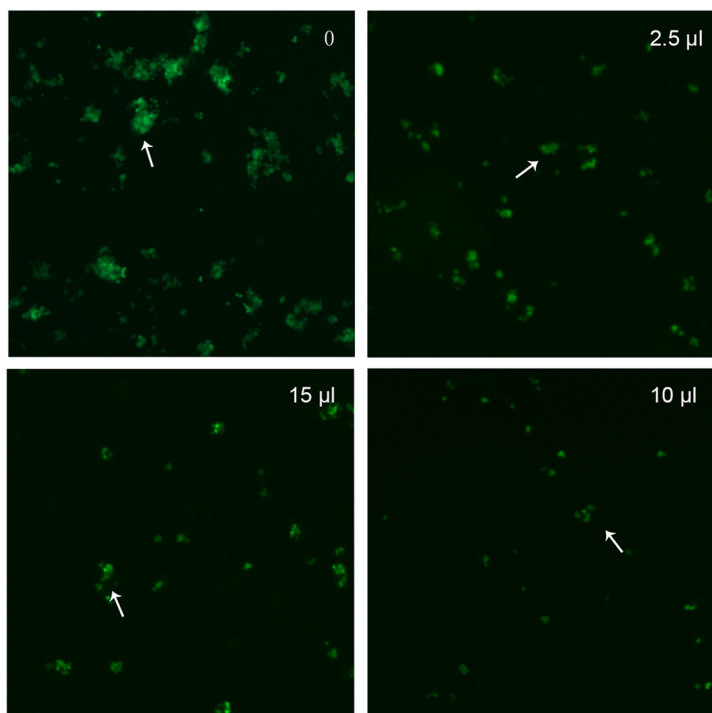
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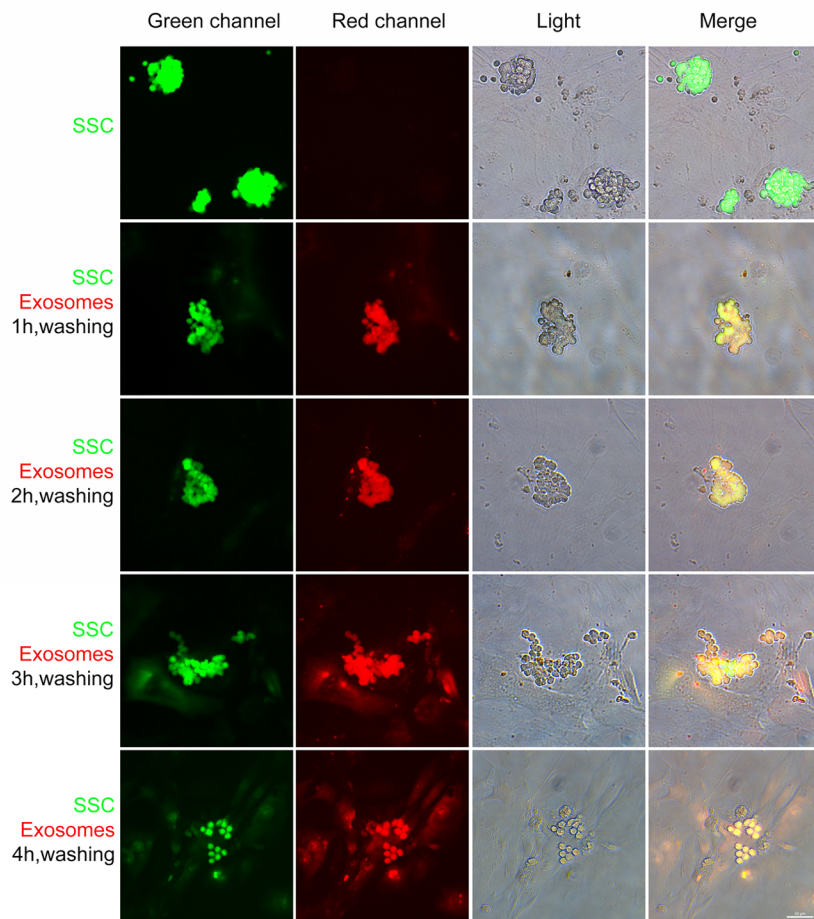
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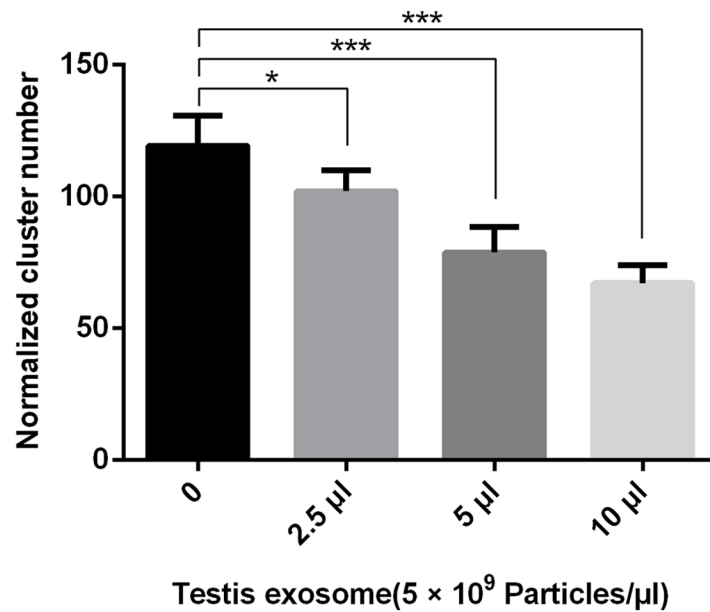
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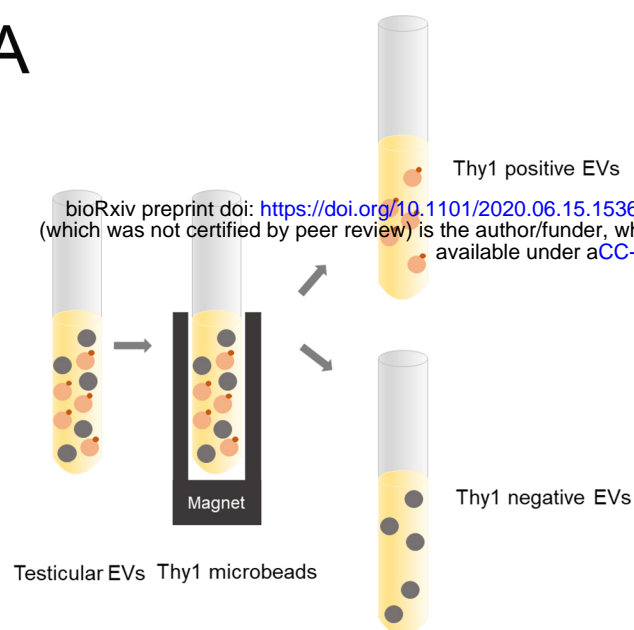
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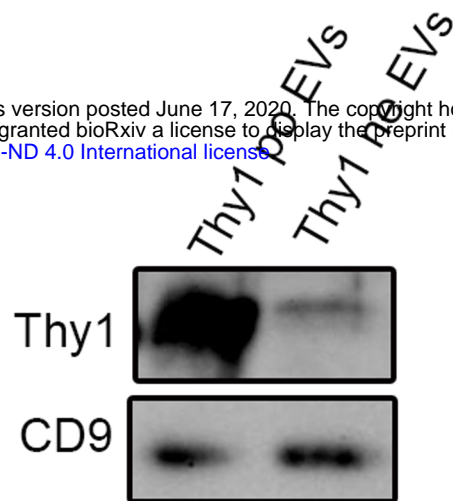
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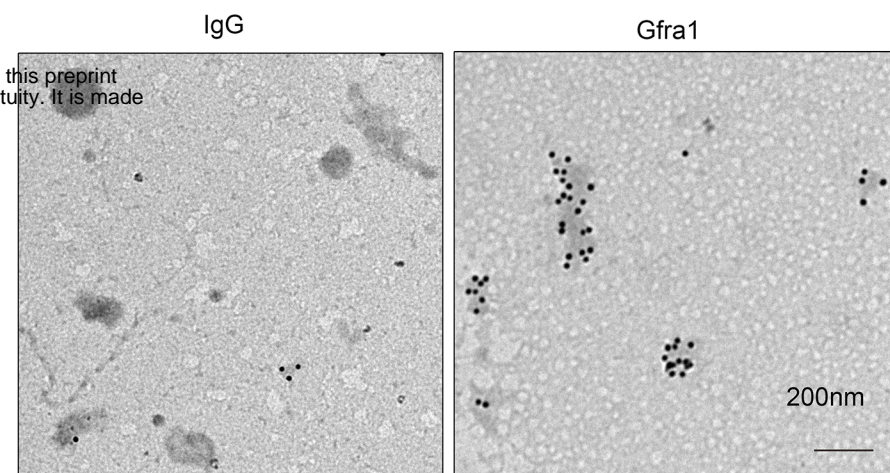
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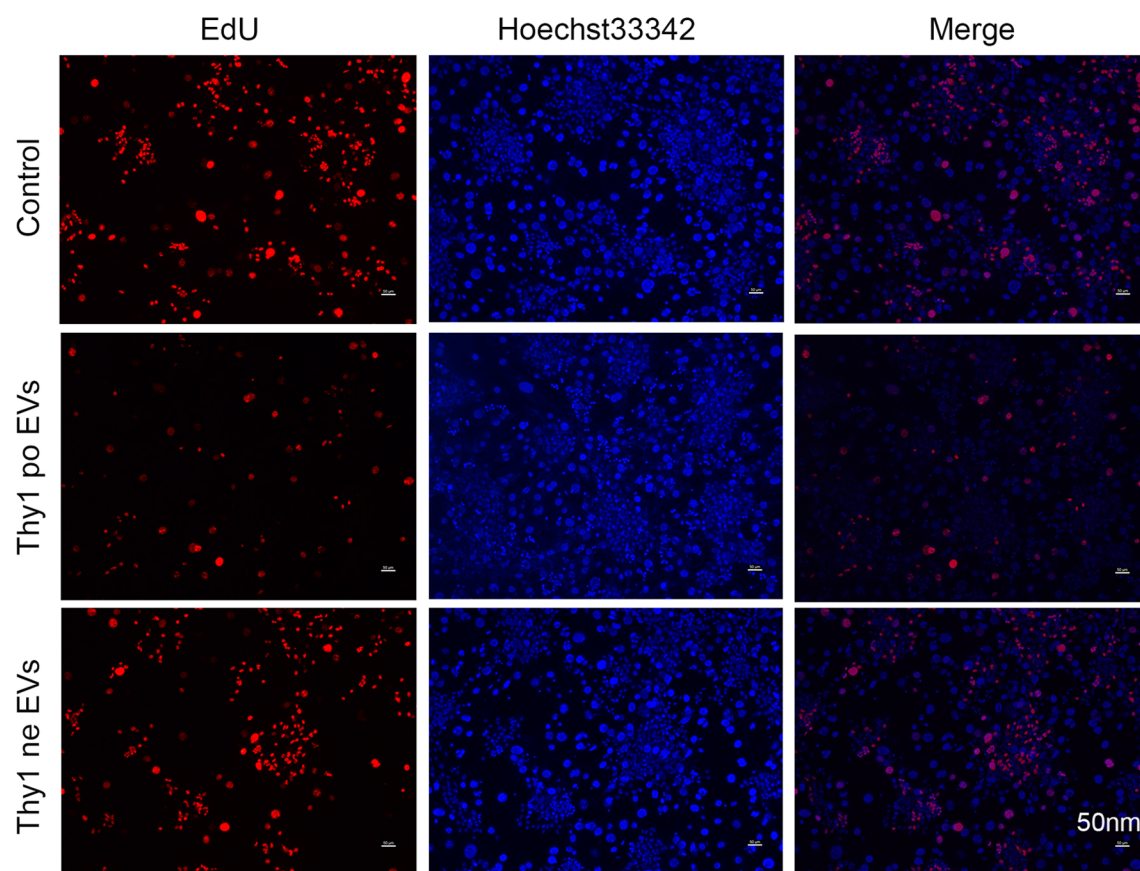
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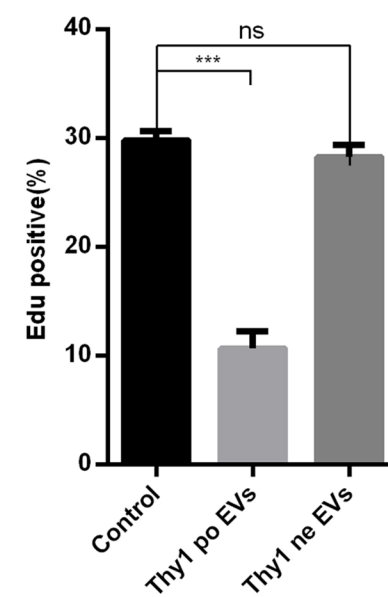
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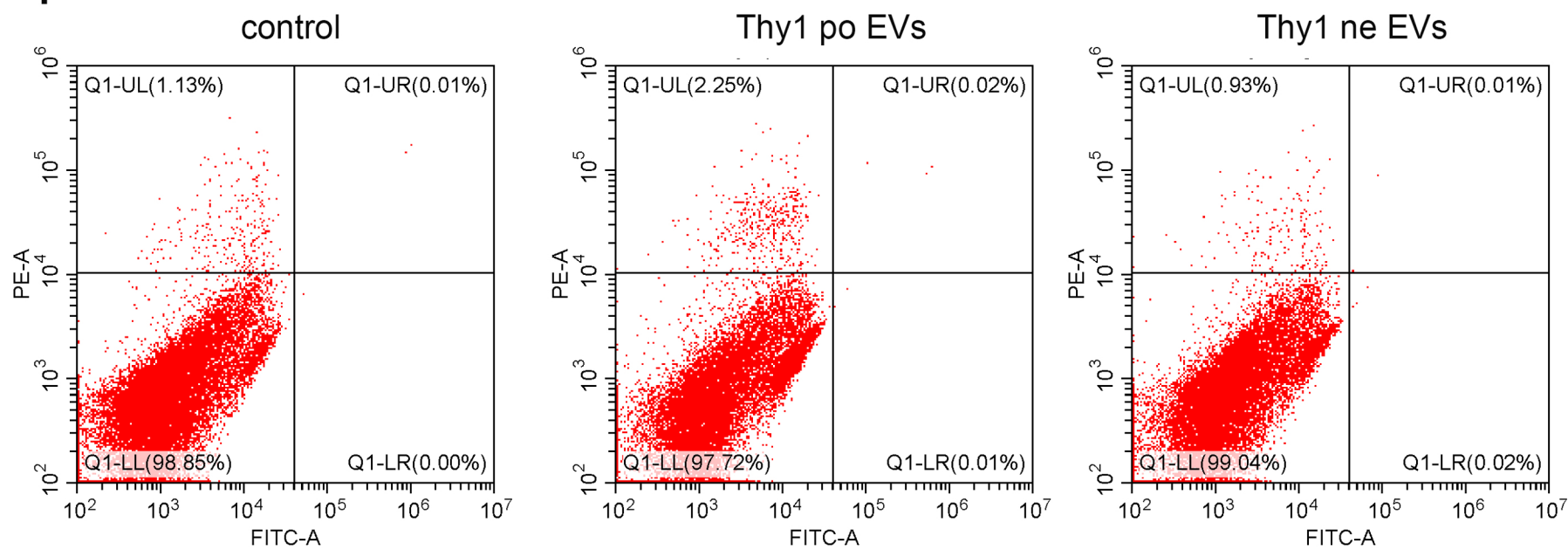
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G

