

Fibronectin on extracellular vesicles from microvascular endothelial cells is involved in the vesicle uptake into oligodendrocyte precursor cells

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Abstract

We previously reported transplantation of brain microvascular endothelial cells (MVECs) into cerebral white matter infarction model improved the animal's behavioral outcome by increasing the number of oligodendrocyte precursor cells (OPCs). We also revealed extracellular vesicles (EVs) derived from MVECs promoted survival and proliferation of OPCs *in vitro*. In this study, we investigated the mechanism how EVs derived from MVECs contribute to OPC survival and proliferation. Protein mass spectrometry and enzyme-linked immunosorbent assay revealed fibronectin was abundant on the surface of EVs from MVECs. As fibronectin has been reported to promote OPC survival and proliferation via integrin signaling pathway, we blocked the binding between fibronectin and integrins using RGD sequence mimics. Blocking the binding, however, did not attenuate the survival and proliferation promoting effect of EVs on OPCs. Flow cytometric and imaging analyses revealed fibronectin on EVs mediates their internalization into OPCs by its binding to heparan sulfate proteoglycan on OPCs. OPC survival and proliferation promoted by EVs were attenuated by blocking the internalization of EVs into OPCs. These lines of evidence suggest that fibronectin on EVs mediates their internalization into OPCs, and the cargo of EVs promotes survival and proliferation of OPCs, independent of integrin signaling pathway.

Keywords:

Cerebral microvascular endothelial cells, Oligodendrocyte precursor cells, Extracellular vesicles,
Fibronectin, Survival, Proliferation

Abbreviations used:

MVEC, microvascular endothelial cell; OPC, oligodendrocyte precursor cell; EV, extracellular vesicle; ELISA, enzyme-linked immunosorbent assay; HSPG, heparan sulfate proteoglycan; DMEM, Dulbecco's Modified Eagle Medium; BSA, bovine serum albumin; PDL, poly-D-lysine; EGM-2, endothelial cell growth medium-2; FBS, fetal bovine serum; PBS, phosphate buffered saline; BrdU, Bromodeoxyuridine

1. Introduction

Demyelination induced by subcortical ischemia contributes to motor and cognitive deficit [1,2].

There is no established treatment against white matter demyelination. We previously reported transplantation of brain microvascular endothelial cells (MVECs) into the white matter infarction reduced infarction area and improved motor deficit [3]. We also reported transplanted MVECs promoted survival of oligodendrocyte precursor cells (OPCs) [4]. Our *in vitro* analysis revealed extracellular vesicles (EVs) derived from MVECs promoted OPC survival and proliferation [5].

EVs are small vesicles containing proteins, lipids and nucleic acids, and are involved in intercellular communication [6]. We suppose some of these molecules contribute to promotion of OPC survival

and proliferation and elucidation of the mechanism may lead to establishment of the novel therapeutics against white matter infarction.

In this study, we analyzed the molecular mechanism how EVs derived from MVECs (MVEC-EVs) affect OPC behavior, focusing on proteins. Protein mass spectrometry and enzyme-linked immunosorbent assay (ELISA) revealed fibronectin is abundant on the surface of MVEC-EVs. Although fibronectin is reported to promote survival and proliferation of OPCs via integrin signaling pathway [7], we couldn't attenuate the effects of EVs on OPCs by blocking the binding between fibronectin and integrins. Recently, heparan sulfate proteoglycan (HSPG) is reported to be the endocytosis receptor for EVs [8,9]. We found EV uptake was decreased by interfering the interaction between fibronectin on EVs and HSPG on OPCs. The decrease in the EV uptake attenuated their survival/proliferation promoting effect on OPCs. These results indicate fibronectin on the surface of EVs contributes to their internalization into OPCs and thereby promotes OPC survival and proliferation.

2. Materials and methods

2.1. Ethics statement

All experiments were performed in accordance with the guidelines for Animal Experimentation at Gunma University Graduate School of Medicine and were approved by Gunma University Ethics Committee (Permit Number:16-009).

2.2. Animals

We used Sprague-Dawley rats (SLC, Japan) in this study (postnatal day 2 pups for OPC culture, and eight-week-old male rats for MVEC culture). Rats were sacrificed by decapitation under deep anesthesia to obtain the brains.

2.3. Cell culture

OPCs were prepared from postnatal day 2 rat brain cortices by immunopanning as previously described [5,10]. OPCs were plated into poly-D-lysine (PDL, Sigma)-coated dish, supplemented with forskolin (Sigma), ciliary neurotrophic factor, neurotrophin-3 and platelet-derived growth factor-AA (all obtained from PeproTech). After 3 days, OPCs were plated on PDL-coated 8-well slide glasses for survival and proliferation assays or on PDL-coated 35 mm culture dishes for flow cytometry.

MVECs were prepared from adult rat cerebra as previously described [5]. We used MVECs to collect EVs when the cells reached >95% confluent. The contaminating cells were below 10%.

Rat fibroblast-like cell line (Rat-1) was cultured in DMEM (Wako) containing 5% fetal bovine serum (FBS).

2.4. Isolation of EVs from culture media

For EV isolation, cells were cultured in medium containing exosome-depleted FBS (System Biosciences) (MVECs: EGM-2 containing 2% exosome-depleted FBS, Rat-1: DMEM containing 5% exosome-depleted FBS) for 72 h. To isolate EVs, except for mass spectrometry, we used the exosome precipitation solution, ExoQuick-TC (System Biosciences), according to the manufacture's protocol. EV pellet was suspended in serum-free medium or phosphate buffered saline (PBS). EV isolation for mass spectrometry was performed using MagCapture Exosome Isolation Kit PS (Wako) according to the manufacture's protocol. Protein concentration was measured using Protein Quantification Assay Kit (Takara Bio) according to the manufacturer's protocol.

2.5. Protein mass spectrometry

The surface protein peptide library of isolated MVEC-EVs was prepared using XPEP Exosome Mass Spec Kit (System Biosciences) according to the manufacture's protocol. Mass spectrometry was performed with Eksigent Ekspert NanoLC 425 (AB Sciex) coupled to TripleTOF 6600 mass spectrometer (AB Sciex). Peptide mixture was separated by ODS column (Eksigent ChromXP-C18-CL, 3 μm , 120Å, 0.075 mm I.D. \times 150 mm L, AB Sciex) with 2-30% acetonitrile gradient containing 0.1% formic acid for 60 min. Proteins were identified using Mascot sequence matching software (Matrix Science) [11] with SwissProt database.

2.6. ELISA

MVEC-EVs were treated with 5 mIU/ml heparitinase (heparin lyase III, HL) (Sigma) for 3 h at 37°C or 0.25% trypsin (TR) (Sigma) for 10 min at 37°C. Treated EVs were suspended in PBS, and pelleted using ExoQuick-TC. The pellets were resuspended in PBS. Quantification of fibronectin on the surface of EVs was performed using rat fibronectin ELISA kit (abcam) according to the manufacturer's protocol. Samples (50 µl/well) containing 6 µg of EV proteins were added to each well.

2.7. Analysis using integrin function inhibitory peptides

To assess the interaction between fibronectin on EVs and integrins on OPCs, we used peptide mimics (RGD peptides) of fibronectin binding: an antagonist peptide (Gly-Arg-Gly-Asp-Ser [GRGDS]) of integrin function, an inhibitory peptide (Gly-Arg-Gly-Asp-Thr-Pro [GRGDTP]) of fibronectin, type-1 collagen and vitronectin, and a negative control peptide (Gly-Arg-Ala-Asp-Ser-Pro-Lys [GRADSPK]) that does not block integrin binding (all obtained from Sigma). OPCs cultured on 8-well slide glass were washed with PBS, and incubate with serum-free medium containing 300 µg/ml peptides for 30 min. After incubation, MVEC-EVs were added to OPC culture at 50 µg/ml of EV proteins. After 1 day's culture, OPC survival and proliferation were assayed.

2.8. Cell survival assay

Cell death with the typical morphological features of apoptosis including pyknotic nuclei was assessed by staining cell nuclei with Hoechst 33342 (Sigma, 2.5 μ M) for 40 min in 5% CO₂ at 37°C. Samples were observed using a fluorescent microscope (Axioplan2, Zeiss) with cooled CCD camera (DP73, Olympus), and the number of pyknotic cells was counted.

2.9. BrdU incorporation assay

To assess cell proliferation, bromodeoxyuridine (BrdU; 10 μ M) (Roche Diagnostics) was added to the cultures for the last 4 h of culture, followed by fixation and staining with rat monoclonal BrdU antibody (abcam; 1:1000). Propidium iodide (PI, Sigma, 2 μ g/ml) was used for nuclear staining. Samples were observed using a fluorescent microscope with cooled CCD camera, and the number of BrdU-positive cells in each sample was counted.

2.10. Treatments of the surface of EVs and OPCs

To investigate the involvement of fibronectin and glycosaminoglycans (heparan sulfate and heparin) in the uptake of EVs by OPCs, EVs and OPCs were treated with heparitinase, heparin, fibronectin, or RGD peptides (All obtained from Sigma) as follows;

Treatment 1) To remove heparan sulfate on EVs, EVs were treated with 5 mIU/ml heparitinase at 37°C for 3 h, and suspended in PBS.

Treatment 2) To remove heparan sulfate on the surface of OPCs, OPCs were treated with 0.5 mIU/ml heparitinase at 37°C for 3 h prior to addition of EVs.

Treatment 3) To block heparin-binding domain of fibronectin on EVs, EVs were incubated with 50 µg/ml heparin for 30 min. The heparin-bound EVs were collected using ExoQuick-TC.

Treatment 4) To block fibronectin-binding sites on OPCs, the cells were preincubated with 200 µg/ml human plasma fibronectin (Sigma) for 1 h prior to addition of EVs.

Treatment 5) To block the interaction between fibronectin on EVs and integrins on OPCs, OPCs were preincubated with 100 µg/ml antagonist peptide (GRGDS) for 1 h prior to addition of EVs.

2.11. Flow cytometry and image analysis

In these analyses, we used fluorescent dye PKH67-labeled EVs prepared according to the manufacturer's protocol (Sigma). All experiments were performed at 200 µg/ml of EV proteins. PKH67-labeled EVs treated with heparitinase (Treatment 1) or heparin (Treatment 3) were added to untreated OPCs. OPCs treated with heparitinase (Treatment 2) or fibronectin (Treatment 4) or an antagonist peptide (Treatment 5) were cultured in serum-free medium containing untreated PKH67-labeled EVs. Untreated OPCs cultured with untreated PKH67-labeled EVs were also prepared. In any treatment, after 3 h in culture, OPCs were harvested, suspended in PBS containing 0.2% BSA, and loaded on Attune® Acoustic Focusing Cytometer (Thermo Fisher) for measurement

of fluorescent intensity of individual cells. As control, untreated OPCs were measured. Data were analyzed by FlowJo software (ver. 7.6.5, TOMY Digital Biology). Cells were observed using a fluorescent microscope with cooled CCD camera. Fluorescent intensity of individual cells was analyzed by ImageJ software (ver. 1.513, NIH).

2.12. Analysis of the involvement of glycosaminoglycans in EV-OPC interactions

To assess the involvement of glycosaminoglycans present on the surface of EVs or OPCs, in OPC survival and proliferation, we treated EVs or OPCs with heparitinase or heparin (Treatments 1-3 as mentioned above). Untreated or heparitinase-treated (Treatment 2) OPCs were cultured in the serum-free medium with or without 50 µg/ml untreated EVs. Untreated OPCs were cultured in the serum-free medium containing 50 µg/ml EVs treated with heparitinase (Treatment 1) or heparin (Treatment 3). In any treatment, after 1 day in culture, cell survival and proliferation were assayed.

2.13. Statistical analysis

Statistical analysis was performed using EZR software (ver. 1.32) [12]. Differences between groups were assessed with one-way analyses of variance followed by the post hoc Tukey–Kramer test. The error bars represent the standard errors.

3. Results

3.1. Fibronectin is abundant on the surface of the MVEC-EVs

First, in order to investigate what proteins exist on the surface of EVs, we performed protein mass spectrometric analysis of the surface proteins of the MVEC-EVs. Fibronectin was found present on the surface of EVs (Fig. 1A). To quantify the amount of fibronectin on EV surface, we performed ELISA and found fibronectin on the surface of the MVEC-EVs was much more abundant as compared with that on Rat-1-EV surface (Fig. 1). We previously reported Rat-1-EVs also promote OPC survival but much less effectively compared to MVEC-EVs [5]. The difference in the amount of fibronectin between MVEC-EVs and Rat-1-EVs may cause the survival promoting difference.

3.2. Fibronectin on the surface of EVs does not contribute to their survival and proliferation promoting effect on OPCs via binding to integrins on OPCs

Fibronectin binds to integrins [13], and the integrin signaling promotes OPC survival and proliferation [7, 14–17]. Fibronectin on EVs are also reported to activate the integrin pathway and cause biological responses [18, 19]. So, we examined if fibronectin on EVs promotes OPC survival and proliferation by binding to integrins.

Fibronectin binds to integrins by RGD (Arginine - Glycine - Aspartic acid) domain [20]. To assess the involvement of fibronectin-integrin binding in the effects of EVs on OPCs, we blocked the interaction between fibronectin on EVs and integrins on OPCs by using RGD sequence mimics.

As shown in Fig. 2, MVEC-EVs reduced pyknotic OPC deaths and increased proportion of BrdU-positive OPCs compared to control, in accordance with our previous report [5]. Pretreatment of OPCs with RGD sequence mimics, thereby blocking the binding of fibronectin to integrins on OPCs, did not attenuate the effects of EVs on OPCs. These results suggest the survival/proliferation effect of EVs on OPCs is not mediated by integrin signaling.

3.3. Fibronectin on EVs mediates their internalization into OPCs

From the results above, we concluded that EVs did not promote OPC survival or proliferation via stimulation of integrins by fibronectin, but their cargo affected OPCs. So, we examined the role for fibronectin in internalization of EVs into OPCs.

Although the exact mechanism of internalization of EVs into target cells is controversial [8, 9, 21–26], HSPG on the target cell membrane was reported to be the receptor for EV uptake [8, 9]. It is also reported that HSPG exists on EVs [8, 9] and these vesicles capture fibronectin by their HSPG and captured fibronectin, in turn, binds to the HSPG on the target cells. This interaction between fibronectin on EVs and HSPG on target cells may trigger the internalization [9]. To quantify the internalization, we labeled EVs with PKH67, a fluorescent dye, and administered them to OPCs under various conditions described below and performed flow cytometric and imaging analyses.

- 1) Untreated EVs were administered to OPCs that had been treated with heparitinase (Fig. 3A).
- 2) EVs treated with heparitinase were administered to untreated OPCs (Fig. 3B).

- 3) EVs preincubated with heparin were administered to untreated OPCs. (Fig. 3C)
- 4) Untreated EVs were administered to OPCs preincubated with fibronectin (Fig. 3D).
- 5) Untreated EVs were administered to OPC preincubated with GRGDS, an RGD sequence mimic (Fig. 3E).

As shown in Fig. 3A and Supplementary Fig. 1A and B, EV uptake into OPCs was decreased by treating OPCs with heparitinase, suggesting HSPG on OPCs is the receptor for EVs endocytosis. EV uptake was also decreased by treating EVs with heparitinase (Fig. 3B, Supplementary Fig. 1A and B). ELISA revealed the amount of fibronectin was decreased by the same enzymatic treatment (Fig. 1B), indicating a part of fibronectin adheres to HSPG on EVs. Preincubation of OPCs with fibronectin attenuated uptake of EVs by them (Fig. 3D, Supplementary Fig. 1A and B), suggesting fibronectin on EVs is a ligand for internalization. Heparin, a mimic of HSPG, also attenuated EV uptake by OPCs (Fig. 3C, Supplementary Fig. 1A and B). In contrast, preincubation of OPCs with GRGDS peptide did not interfere with EV uptake by them (Fig. 3E). Taken together, these results suggest a part of fibronectin is tethered to HSPG on EVs and the EV uptake by OPCs is mediated by the binding of fibronectin to HSPG on OPCs (Supplementary Fig. 2).

3.4. Decrease in EV uptake by OPCs attenuates their survival/proliferation promoting effect on OPCs

We next investigated whether decrease in EV uptake by OPCs results in attenuation of OPC survival/proliferation promoting effect. As shown in Fig. 4, any intervention which attenuated uptake of EVs by OPCs reduced their survival and proliferation promoting effect on OPCs. These findings suggest that EVs affect OPCs after their internalization into these cells.

4. Discussion

We revealed fibronectin is abundant on the surface of MVEC-EVs. A part of fibronectin is tethered to HSPG on EVs, and this fibronectin contributes to EV internalization into OPCs by binding to HSPG on these cells. Importantly, decrease in EV uptake attenuated their survival/proliferation promoting effect on OPCs.

We also revealed that treating OPCs with heparitinase greatly reduced incorporation of EVs into these cells. We interpret this result as HSPG on OPCs acts as the receptor for fibronectin on EVs. There is, however, a possibility that HSPG on OPCs plays a role for internalization of EVs, independent of fibronectin. HSPG is reported to be the endocytosis receptor [27], and there is a report that EV internalization occurs through endocytosis [21-26]. Although the exact mechanism how HSPG is involved in endocytosis is unknown [28], small GTPases are presumed to play a role for this process. Syndecan-4, a HSPG, activates RhoG [29, 30], which, in turn, induces $\alpha 5 \beta 1$ integrin endocytosis in a dynamin- and caveolin-dependent manner [30]. Syndecan-4 also activates Arf6 [31], which is involved in the clathrin-dependent endocytosis of angiotensin II type 1 receptor [32].

HSPG-mediated clathrin- and caveolin-independent endocytosis is also reported [33]. Further study is needed to elucidate the exact mechanism of HSPG-mediated internalization of EVs.

Although integrin signaling has been reported to promote survival and proliferation of OPCs [7, 13–17], we found the effect of EVs on OPCs was not attenuated by blocking the binding between fibronectin and integrins. One possible reason for this difference may be that the amount of fibronectin on EVs was not enough to directly activate integrins on OPCs. We did not try higher amounts of EVs in this study, but there is a possibility that fibronectin on EVs directly activates integrins on OPCs, if higher amounts of EVs are administered to the cells.

The exact mechanism how MVEC-EVs promote OPC survival and proliferation is still unknown. EVs contain many kinds of miRNA which regulate gene expression [34]. We presume miRNAs are candidates for promoting OPC survival and proliferation. For example, miR-17-92 cluster promotes OPC proliferation by influencing Akt pathway [35]. This miRNA cluster also targets phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is known to be a negative regulator of neural stem cell proliferation and survival [36]. MicroRNA-146a decreased OPC apoptosis by attenuating IRAK1 [37]. We are now trying to identify the molecules in MVEC-derived EVs that promote OPC survival and proliferation.

Recently, EVs are attracting attention as possible tools for treatment of central nervous system diseases [38]. The exact mechanism of EV uptake by neural cells in central nervous system, however, still remains unknown. This is the first report on the uptake pathway of EVs into OPCs. Further

study is needed to elucidate the detailed mechanism of EV uptake by neural cells to develop a novel therapeutic strategy using EVs.

Conflicts of interest

The authors declare no conflict of interests.

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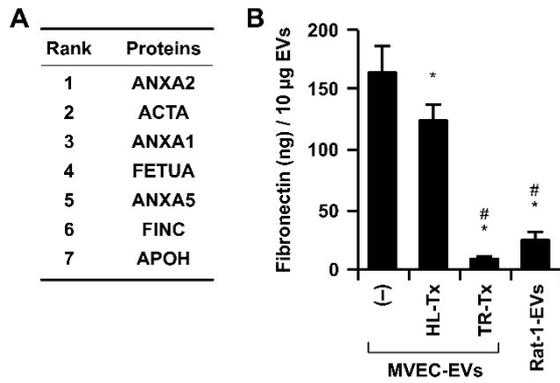


Fig. 1. Fibronectin is abundant on the surface of the MVEC-EVs.

(A) Protein mass spectrometry indicated fibronectin (FINC) was present on the surface of MVEC-EVs. (B) The amount of fibronectin in MVEC-EVs or Rat-1 cells, with or without enzymatic treatments (Tx, heparitinase; HL, trypsin; TR), was quantified by ELISA. Results are shown as mean \pm SE (N = 4 in each condition). * p < 0.05 compared with MVEC-EVs. # p < 0.05 compared with MVEC-EVs treated with HL.

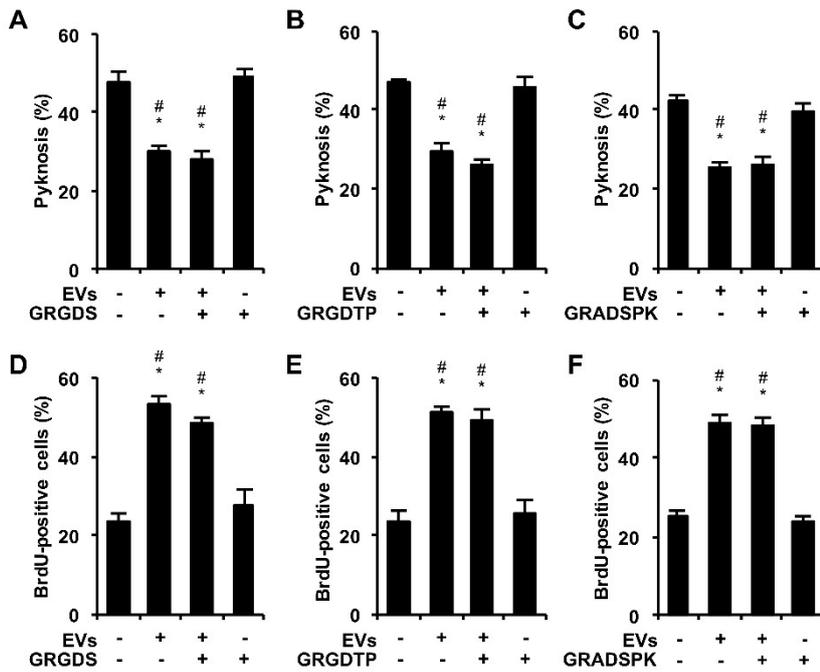


Fig. 2. MVEC-EVs promote OPC survival and proliferation but these effects are not blocked by RGD sequence mimics.

OPCs were preincubated with or without an integrin antagonist peptide (GRGDS), an inhibitory peptide of fibronectin, type 1 collagen or vitronectin (GRGDTP), or a negative control peptide (GRADSPK) for 30 min. MVEC-EVs were added to OPC cultures and maintained for 1 day. Results are shown as mean \pm SE (N = 8 in each condition). * $p < 0.05$ compared with control (EVs(-)/peptide mimic(-)). # $p < 0.05$ compared with EVs(-)/peptide mimic(+) sample. These experiments were repeated three times, and similar results were obtained each time. Typical experiments are shown here.

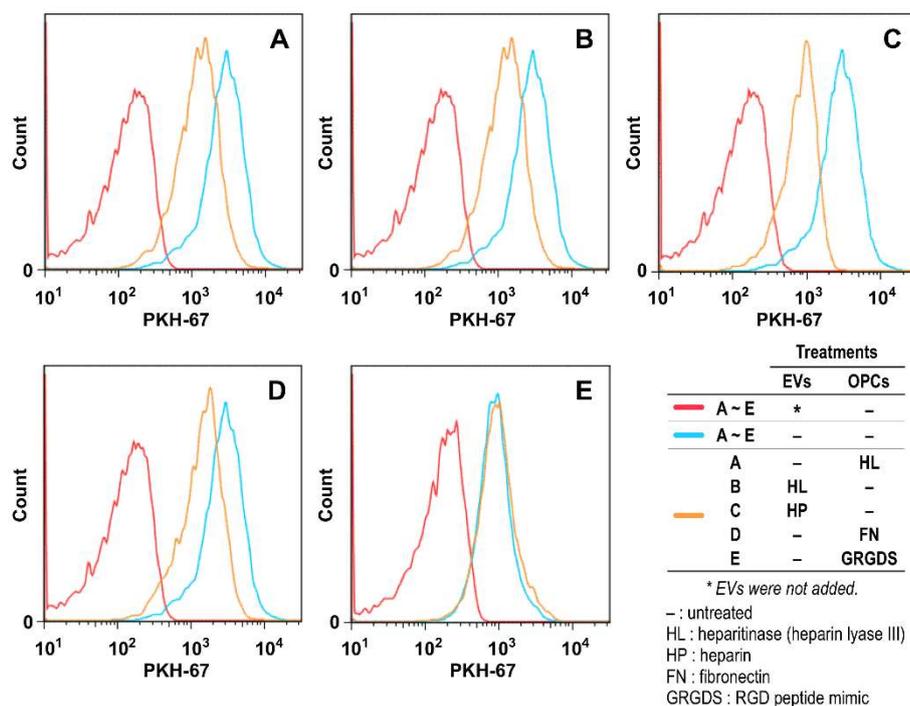


Fig. 3. The internalization of EVs into OPCs is attenuated by blocking the interaction between fibronectin and HSPG.

Flow cytometric analysis revealed EVs uptake into OPCs were attenuated by interfering the interaction between fibronectin and HSPG. (A) OPCs were treated with 0.5 mIU/ml heparitinase (HL) at 37°C for 3 h. (B) EVs were treated with 5 mIU/ml HL at 37°C for 3 h. (C) EVs were preincubated with 50 µg/ml heparin (HP) for 30 min. (D) OPCs were preincubated with 200 µg/ml plasma fibronectin (FN) for 1 h. (E) OPCs were preincubated with 100 µg/ml GRGDS. These experiments were repeated three times, and similar results were obtained each time. Typical experiments are shown here.

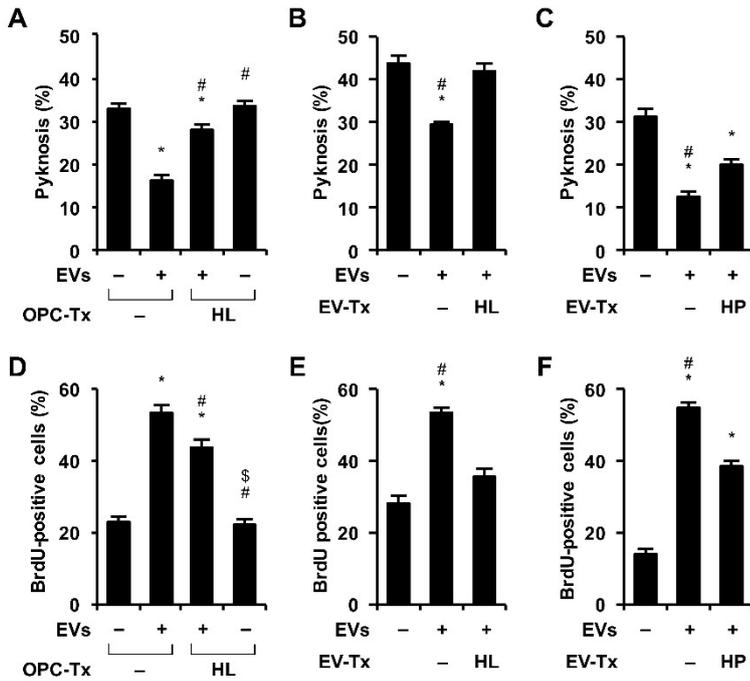


Fig. 4. The promoting effect of EVs on OPC survival and proliferation is diminished by blocking the interaction between fibronectin and HSPG.

(A, D) OPCs were treated (Tx) with or without 0.5 mIU/ml heparitinase (HL) at 37°C for 3 h. EVs were added after the enzymatic treatment. * $p < 0.05$ compared to control (EVs(-)/OPC-Tx(-)). # $p < 0.05$ compared to EVs(+)/OPC-Tx(-). \$ $p < 0.05$ compared to EVs(+)/OPC-Tx(HL). (B, E) EVs were treated with or without 5 mIU/ml HL at 37°C for 3 h. * $p < 0.05$ compared to control (EVs(-)). # $p < 0.05$ compared to EVs treated with HL (EV-Tx(HL)). (C, F) EVs were preincubated with or without 50 μ g/ml heparin (HP) for 30 min. * $p < 0.05$ compared to control (EVs(-)). # $p < 0.05$ compared to EVs conjugated with HP (EV-Tx(HP)). These experiments were repeated three times, and similar results were obtained each time. Typical experiments are shown here. Results are shown as mean \pm SE (N = 8 in each condition).