Cytotechnology (2017) 69:801–814
DOI 10.1007/s10616-017-0095-2

ORIGINAL ARTICLE

Extracellular vesicles of ETV2 transfected fibroblasts stimulate endothelial cells and improve neovascularization in a murine model of hindlimb ischemia

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Received: 28 December 2016 / Accepted: 7 April 2017 / Published online: 2 May 2017

Abstract Ischemia are common conditions related to lack of blood supply to tissues. Depending on the ischemic sites, ischemia can cause different diseases, such as hindlimb ischemia, heart infarction and stroke. This study aims to evaluate how extracellular vesicles (EVs) derived from ETV2 transfected fibroblasts affect endothelial cell proliferation and neovascularization in a murine model of hindlimb ischemia. Human fibroblasts were isolated and cultured under standard conditions and expanded to the 3th passage before use in experiments. Human fibroblasts were transduced with a viral vector containing the ETV2 gene. Transduced cells were selected by puromycin treatment. These cells were further cultured for collection of EVs, which were isolated from culture supernatant. Following co-culture with endothelial cells, EVs were evaluated for their effect on endothelial cell proliferation and were directly injected into ischemic tissues of a murine model of hindlimb ischemia. The results showed that EVs could induce endothelial cell proliferation in vitro and improved neovascularization in a murine model of hindlimb ischemia. Our results suggest that EVs derived from ETV2-transfected fibroblasts can be promising non-cellular products for the regeneration of blood vessels.

Keywords Extracellular vesicles · EVs · ETV2 · Fibroblasts · Ischemia · Hindlimb ischemia

Abbreviations

ADSCs	Adipose derived stem cells
ALI	Acute hindlimb ischemia
EPCs	Endothelial progenitor cells
ETV-2	ETS variant 2

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EVs	Extracellular vesicles
HUVECs	Human umbilical vein endothelial cells
MCECs	Mouse cardiac endothelial cells
MSCs	Mesenchymal stem cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
VEGFR2	Vascular endothelial growth factor receptor 2

Introduction

Ischemia is a condition which results from limited blood supply to tissues. Although some kinds of ischemia do not affect patient survival, certain kinds can cause extreme diseases, including hindlimb ischemia, stroke and myocardial infarction. There has been rise of ischemia associated with diabetes and high blood pressure; thus, understanding ways to prevent or treat ischemia is essential. In this study, acute hindlimb ischemia (ALI) was used as a model to investigate treatments for ischemia.

ALI (and ischemia in general) can be treated by stem cell therapy, which has shown some promising results. Unlike traditional therapies, stem cell transplantation can help create new blood vessels via angiogenesis or vasculogenesis. Stem cells, such as bone marrow derived stem cells (BMSCs) and adipose tissue derived stem cells (ADSCs), have been studied in preclinical trials (Hao et al. 2014; Liu et al. 2015; Park et al. 2014; Qin et al. 2014; Yoshida et al. 2016) and clinical trials of ALI therapy (Amann et al. 2008, 2009; Ammar et al. 2015; Gremmels et al. 2014; Gupta et al. 2013; Iafrati et al. 2011; Peeters Weem et al. 2016; Saliutin et al. 2014; Teraa et al. 2015). In addition, menstrual blood cells (Vu et al. 2015), umbilical cord blood derived endothelial progenitor cells (Van Pham et al. 2014), placenta derived mesenchymal stem cells (MSCs) (Xie et al. 2016; Zhang et al. 2014), and Wharton's jelly derived endothelial progenitor cells (Shen et al. 2013) have been studied in preclinical trials of ALI.

Although results have shown promise, stem cell therapy holds several limitations; cell preparation is very time-consuming, stem cell processing and cultures are expensive, and there are risks related to immunogenicity from whole cell transplantation. Thus, next generation therapies using exosomes or extracellular vesicles (EVs) have been suggested as a replacement for whole cell therapy. Exosomes or EVs are cell-derived vesicles which are present in many fluids, including blood, urine and cultured cell medium (Keller et al. 2006; van der Pol et al. 2012). Some reports have suggested that exosomes have a diameter of 30–100 nm, while EVs have a diameter of 100–1000 nm (Booth et al. 2006). Both exosomes and EVs contain RNAs and proteins, and have been identified to play an important role in cell–cell communication. Indeed, tumor cell derived exosomes and EVs in hepatocellular carcinoma were found to promote invasion, metastasis, immune evasion and modulation (Wu et al. 2016), and motility of immortalized hepatocytes (He et al. 2015). Exosomes and EVs from MSCs can suppress angiogenesis by down-regulating VEGF expression in breast cancer cells (Lee et al. 2013), promoting fracture healing in mice (Furuta et al. 2016), accelerating skeletal muscle regeneration (Nakamura et al. 2015), and suppressing myofibroblast differentiation (Fang et al. 2016). The biological activities carried out by exosomes and EVs include transfer of oncogenic proteins and RNAs (He et al. 2015) which inhibit breast cancer, transfer of a group of specific microRNAs (miR-21, miR-23a, miR-125b and miR-145) found to play key roles in suppressing myofibroblast formation (Fang et al. 2016), and transfer of miR-146b which causes inhibition of glioma growth (Katakowski et al. 2013). More interestingly, exosomes and EVs have been shown to be safe when used in animals (Sun et al. 2016; Zhao et al. 2015) and in humans (Besse et al. 2016; Dai et al. 2008; Pitt et al. 2016).

In recent years, the composition of RNAs and proteins inside EVs were modified to produce EVs with particular biological activities. Wen et al. (2016) transfected human BMSCs with a plasmid expressing the siFas and anti-miR-375 genes, and collected EVs from these cells. The authors demonstrated that the EVs could suppress immune reaction by inhibiting peripheral blood mononuclear cell (PBMC) proliferation and enhancing regulatory T cell (Treg) function (Wen et al. 2016). Similarly, Fan et al. (2013) used EVs derived from a MHC class II molecule-expressing murine colon cancer cell line (CT26-CIITA) that had been transduced with the CIITA gene, to induce anti-tumor immunity (Fan et al. 2013).

In this study, fibroblasts were transduced with ETV-2, a master gene regulator of angiogenesis. In fact, ETV2 regulates various signaling pathways and

functions as an essential regulator for vasculogenesis and hematopoiesis. In embryogenesis, ETV2 and GATA2 regulate the expression of SPI1 via binding to its promoter of SPI1 (Shi et al. 2014). It also regulates cardiac development (Schupp et al. 2014), and vascular regeneration (Park et al. 2016). Previously, we and other groups have shown that ETV-2 transduced fibroblasts can be directly converted into endothelial progenitor cells (EPCs) (Ginsberg et al. 2015; Morita et al. 2015; Van Pham et al. 2016). Therefore, this study used fibroblasts as targets for direct conversion using ETV-2 vector.

This study was aimed at investigating the effects of EVs derived from ETV-2 transduced fibroblasts, on endothelial cell proliferation as well as neovascularization in a mice model of hindlimb ischemia. Understanding the role of EVs on proliferation and angiogenesis would potentially aid in the design and rationale for use of EVs for ischemia therapy.

Materials-methods

Dermal fibroblast isolation and proliferation

Foreskin was collected from a donor who completed a consent form at the hospital. Foreskin was stored in PBS solution at 4 °C and transferred to the laboratory for isolation and culture of fibroblasts, as described in previous studies (Nahm et al. 2002). Briefly, the samples were cut into small pieces, placed into wells, and allowed to adhere for 5 min at room temperature (RT). DMEM medium supplemented with 10% FBS and 1 × anti-mitotic-mycotic solution was then added to the wells and the cultures were maintained at 37 °C, 5% CO_2. The cultures were sub-cultured when cells reached 70–80% confluence. Human fibroblast (HFs) were sub-cultured to the 3rd passage prior to use in experiments. Human umbilical vein endothelial cells (HUVECs) were purchased from Life Technologies (Carlsbad, CA, USA).

ETV-2 lenti viral vector preparation

The human ETV2 expression vector (pF1KB9707) was purchased from Addgene (Cambridge, MA, USA). ETV2 was cloned into the vector backbone pSIN4-EF1alphaIRES-Puro (Plasmid #61061; Addgene, Cambridge, MA, USA) to generate pSIN4-EF1a-ETV2-IRES-Puro. All coding sequences in the expression vector were confirmed with the GenomeLab System (Beckman Coulter, Brea, CA, USA). The ETV2 vector was then transfected into HEK293T cells, along with pCMV-VSV-G-RSV-Rev and pCMV-dR8.2 (Addgene). 18 h after transfection, the medium was replaced with fresh culture medium. The lentivirus-containing medium was collected 48 h later, passed through a 0.45-μm filter, and concentrated using centrifugation (8400×*g* at 4 °C for 16 h). The lentivirus pellets were resuspended in PBS at 10^7 IFUs/mL.

Transfection of ETV-2 vector in dermal fibroblasts and cell selection

HFs were plated on 12-well plates at 7×10^4 cells per well and 24 h later were infected with 10 μL of concentrated lentivirus particles with 5 μg/mL protamine. Plates were plated in hypoxia condition with 5% O_2, 5% CO_2, and 37 °C. Another 48 h later, cells were washed twice with PBS and cultured on 6-cm dishes coated with Cellstart (Thermo Scientific, Waltham, MA, USA) in EGM-2 medium under hypoxic conditions. After 1 week, cells were selected with culture medium supplemented with 10 ng/mL puromycin for 36 h. Then, the medium was changed with fresh medium without puromycin. The cells were allowed to proliferate until enough cells could be collected for cell sorting. Only CD31 + cells were sorted by FACSJazz Cell Sorter System (BD Biosciences, San Jose, CA, USA) and used for EVs production. Sorted cells were reconfirmed for CD31 expression by flow cytometry using the FACSCaibur system (BD Biosciences).

Production of EVs

EV isolation was performed with a commercial kit (Thermo-Fisher Scientific, Waltham, MA, USA). Briefly, the cell supernatant was collected and stored at 2–8 °C until use. All supernatant samples were centrifuged at 2000*g* for 30 min to remove cells and debris. The supernatant was transferred to a new tube without disturbing the pellet. The reagent (from the kit) was added to the supernatant at a ratio of 0.5:1 reagent to supernatant. This mixture was carefully mixed and incubated overnight at 2–8 °C. Finally, EVs were collected from the bottom of the tubes after centrifugation at 10,000*g* for 1 h at 2–8 °C. The pellet was re-suspended in PBS for further use in experiments.

EV characterization

EVs were observed under transmission electronic microscope (TEM) to detect and determine the EV diameter. The markers of EVs, CD81 and CD63, were assessed and identified by flow cytometry. Briefly, EV preparations (5–10 μg) were incubated with 5 μl of 4-μm-diameter aldehyde/sulfate latex beads (Thermo-Fisher Scientific) and resuspended into 400 μL PBS containing 2% fetal bovine serum (FBS). Then, EV-coated beads (20 μL) were incubated with the following antibodies: anti-CD63-FITC (Santa Cruz Biotech, Dallas, TX, USA) and anti-CD81-PE (Santa Cruz Biotech), anti-CD9-FITC (Santa Cruz Biotech) for 30 min at 4 °C, then analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Endothelial cell proliferation assay

Cell proliferation was evaluated by xCelligence assay. HUVECs were seeded in E-plates at 5000 cells/well. Before that, 50 uL of medium was added into the plates to read the baseline. The E-plate with cells was left for 30 min and then put into the xCelligence system (ACEABIO, San Diego, CA, USA). Cell proliferation was monitored via cell index and doubling time for 168 h. There were 3 groups tested for their effect on HUVEC proliferation; the first group (G1) was the placebo group, i.e., cell culture medium supplemented with PBS; the second group (G2) and third group (G3) were the treatment groups containing 50 μg/ml of EVs (EXO50) and 100 μg/ml of EVs (EXO100), respectively (the EVs were diluted in PBS).

Murine model of hindlimb ischemia

6- to 12-month old mice were used for the ischemic hindlimb model. All animal protocols and experiments were prepared, based on the "Guide for the Care and Use of Laboratory Animals" from the local research institution, and approved by the Committee of Care and Use of Laboratory Animals. Acute hindlimb ischemic mice were established according to previously published protocols (Vu et al. 2014). Briefly, the mice were anesthetized using 7.5 mg/kg zoletil. Hairy thighs were shaved and an incision, approximately 1 cm long, was made along the thigh skin. The fat thighs were removed and the femoral arteries near the abdomen were dissected from the veins and nerves, and ligated at two positions. Between the two ligated artery positions, a burn was made using an electronic cutting machine (ESU-X, Geister, Tuttlingen, Germany). Finally, the skin was stitched and the wound area was covered in povidone-iodine.

EV injection in acute hind limb ischemic mice

Acute hind limb ischemic mice were divided into 2 groups (15 mice/group). Group I (GI) contained non-treated mice (placebo group; mice were injected with PBS). Group II (GII) contained mice injected with a dose of 100 μL of 100 μg/ml EVs. EVs or PBS were directly injected into the muscle at the burn sites at day 0 after models were produced. All mice were followed up to 4 weeks (30 days) to evaluate the effects of the grafts.

Evaluation of recovery of damaged limbs after EV injection

The degree of ischemic damage was assessed through indicators, such as skin color changes, swelling, and grade of limb necrosis. The latter was evaluated and classified according to the guidelines, as follows, of Goto et al. (2006): Grade 0, normal limb without swelling, necrosis or atrophy of muscle; Grade I, necrosis limited to the toes; Grade II, necrosis extending to the foot; Grade III, necrosis extending to the knee; and Grade IV, necrosis extending to the hip or loss of whole hind limb.

The blood flow recovery was evaluated by changing of peripheral capillary oxygen saturation (spO_2) at toes before burning the blood vessels, and after treating with EVs for 0, 3, or 30 days. The spO_2 was measured by the pulse oximeter (Omron, Osaka, Japan). The trypan blue flow assay was also used to evaluate blood flow. Briefly, 1% trypan blue was injected into the tail vein. If blood vessel recovery occurred at the burn sites, the trypan blue dye would be delivered to the toes and feet, which would stain blue. The time needed for staining of toes and feet after injection of trypan blue was determined based on a stopwatch (in seconds).

Histological analyses of limb tissues were done at 3 and 30 days after transplantation as the previous published study (Vu et al. 2016). The mice were

euthanized and hindlimb muscle was collected. Samples were frozen in optimal cutting temperature compound (Thermo) and cut at cross-sections of 10 μm thickness using a cryostat (Leica, Richmond Hill, ON, Canada). Tissue section slides were then stained with hematoxylin-eosin (H&E) and assessed by microscopy (Carl-Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical analyses of all endpoints were performed using the two-sided Student's t test or one-way analysis of variance. All data are presented as mean ± SD; $p < 0.05$ was considered statistically significant. Data were analyzed with GraphPad Prism 6.0 software.

Results

Dermal fibroblast isolation and proliferation

From the tissue expansion culture, fibroblasts started to migrate out from the tissues and adhere to the flask surface at day 4 after seeding (Fig. 1a). The fibroblasts proliferated from day 10 onward and reached full confluence by day 15 (Fig. 1b). The fibroblast population become homogenous at the 3rd cell culture passage. To evaluate cell homogeneity, the cell population was analyzed with flow cytometry for expression of CD90. The results showed that 100% cells expressed the CD90 marker (Fig. 1c). These cells were used in subsequent experiments.

Fibroblasts were successfully transduced with a lentiviral vector expressing *ETV-2* gene

Per previously published studies, the transduced fibroblasts were cultured until enough cells could be collected for subsequent cell sorting (Fig. 2a), with 6.51% CD31 positive cells (Fig. 2d). ETV2-transduced cells were selected by puromycin resistance for 7 d. The results showed that about 80% cells were eliminated with puromycin treatment (Fig. 2b), with about 29.81% CD31 positive cells (Fig. 2e). The surviving cells were subsequently cultured for an additional 2 weeks, and then used to sort for CD31 + cells (Fig. 2c). The purity of the cell population was verified, with more than 95% of cells positive for CD31 expression (Fig. 2f). Cells were continually expanded for isolation of EVs.

EV isolation and characterization

Extracted EVs were observed by TEM; almost all particles were in the 30–100 nM range in size (Fig. 3a, b). Expression of CD9, CD63 and CD81 was confirmed by flow cytometry. The results showed that 100% EVs expressing CD9 (Fig. 3d), 76.54 ± 8.19% EVs expressing CD63 (Fig. 3e), 48.61 ± 9.14% EVs expressing CD81 (Fig. 3f).

EVs stimulated endothelial cell proliferation and roles of RNAs components

The eXcelligence analysis showed that HUVEC endothelial cells proliferated more rapidly in the group where EVs were added to culture medium, as compared to control (G2, and G3 vs G1) (Fig. 4a). In the first 36 h in the same medium, the proliferation curves in the 3 groups G1, G2 and G3 were similar. However, after replacing the media, different for G1, G2 and G3, the cell proliferation curves became significantly different at 96 h. Figure 4a shows that after 96 h, the proliferation curve of cells in G2 and G3 was clearly higher than in G1, and G3 also was clearly higher than in G2 suggesting that the cell proliferation rate in G3 was faster than for cells in G2 and in G2 was faster than for cells in G1. Interestingly, after 132 h, cells in G1 entered rapid senescence where the proliferation rate suddenly decreased. At the same time, cells in G2 and G3 also entered to senescence but slowlier. The doubling times were analyzed and showed that EV supplement significantly reduced the doubling time of cells- from 56 ± 5.2 h (in placebo, G1) to 41 ± 1.37 h (in G2), to 37 ± 1.75 h (in G3) (Fig. 4b).

EVs efficiently increased the recovery of hindlimb ischemia in a murine model of ischemia

The effect of EVs on the recovery of ischemic hindlimb in mice was observed for 30 days. The grade of limb necrosis was evaluated and classified according to the guidelines, as follows, of Goto et al. (2006) (Figure 5). The results showed that injection of EVs significantly decreased the necrosis of the hindlimb, as compared to the placebo group that received an

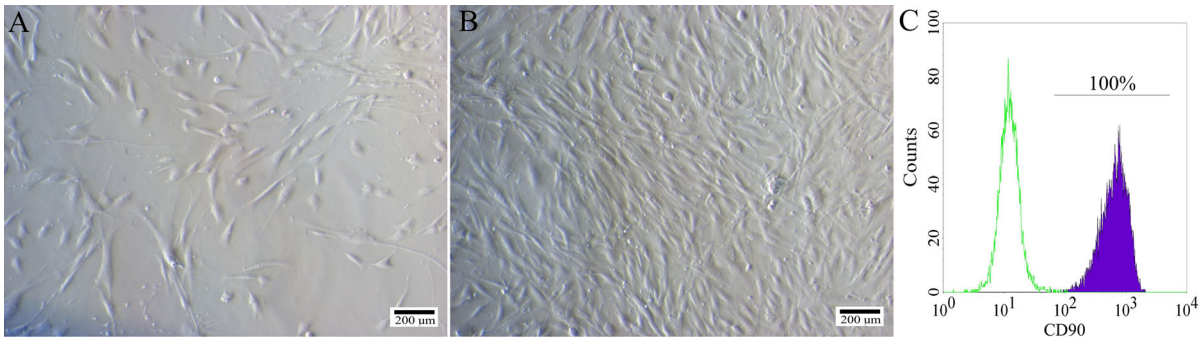

Fig. 1 Dermal fibroblast isolation and expansion. The dermal tissues were cultured to obtain migrated fibroblasts (**a**); proliferating cells reached confluency at day 15 (**b**); cells were a homogenous population, confirmed by CD90 expression (**c**)

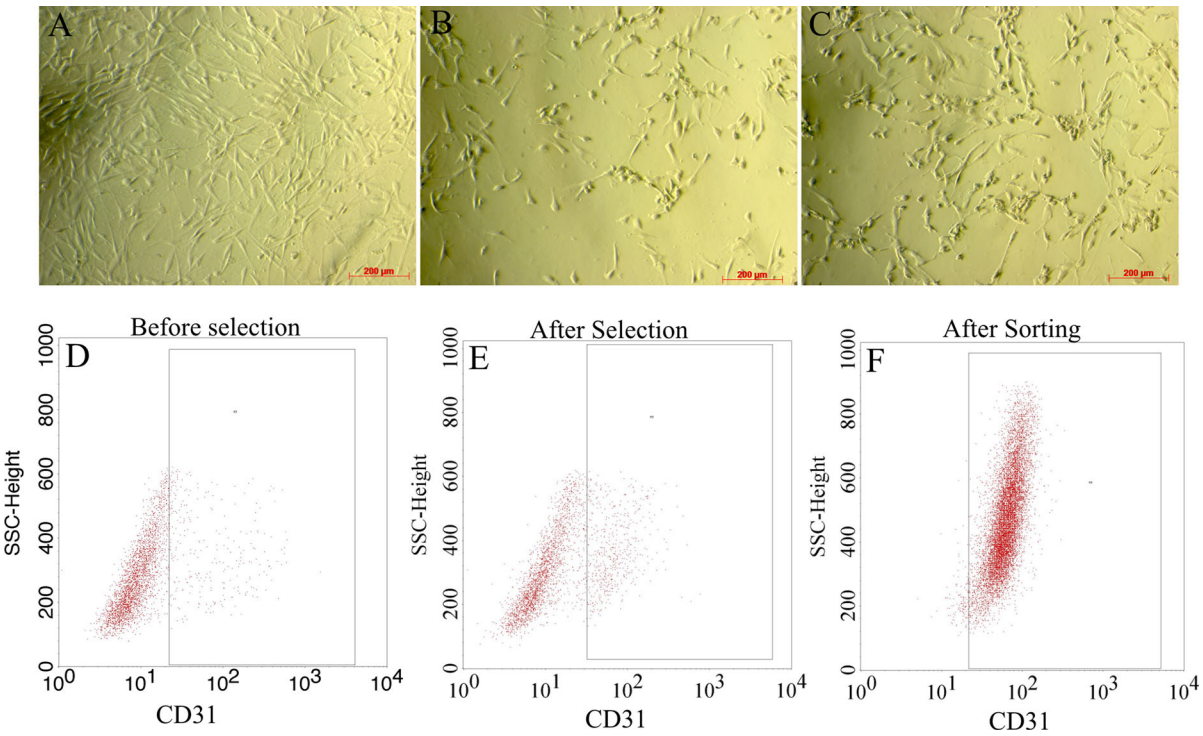

Fig. 2 Transduced fibroblasts were selected and proliferated. ETV2-transduced cells were cultured after transduction (**a**), selected by puromycin resistance for 7 days (**b**), and sorted based on CD31 positive cells (**c**). $CD31^+$ cells were analyzed in transduced samples (**d**), puromycin selected samples; and $CD31^+$ cells sorted samples (**e**)

injection of PBS (Fig. 6a). In the GII (EVs injected) group, 33.33% of mice completed recovered, 16.67% had a necrotic grade I, 33.33% had necrotic grade III, 8.33% had necrotic grade IV, and 8.33% died. In the GI (placebo injected) group, only 16.67% of mice completed recovered, 41.67% had necrotic grade I, 8.33% had necrotic grade IV and 33.33% died. The results show that EV injection increases recovery of mice by twofold (33.33 vs. 16.67%, for GII vs GI, respectively). The death of mice also significantly decreased (33.33 vs. 8.33%; for GI vs. GII, respectively) (Fig. 6a).

The spO_2 results showed that after injection with EVs, spO_2 in GII increased more than in GI; however, there was no statistical difference in spO_2 at day 3 and day 30 (Fig. 6b). The trypan blue assay showed that in surviving mice without loss of legs, the average time for staining of toes and feet with trypan blue was

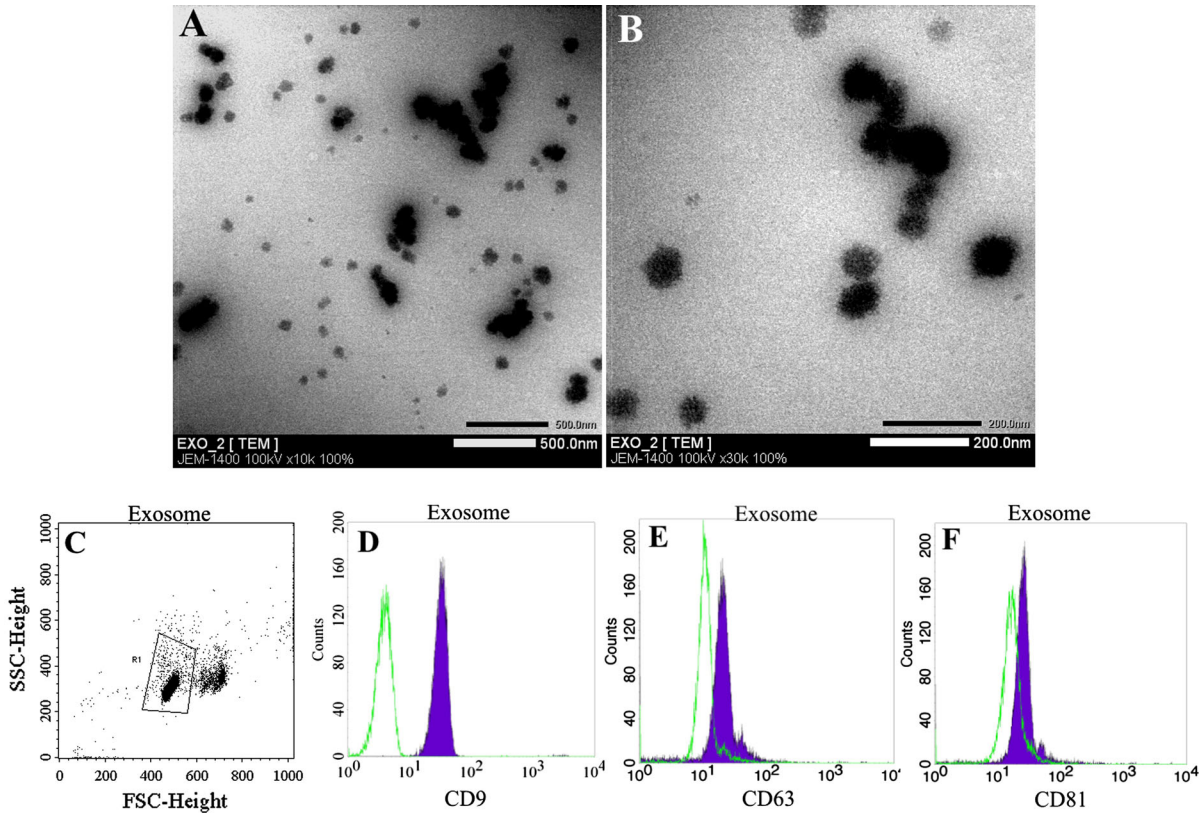

Fig. 3 Extracellular vesicles derived from ETV2-positive fibroblasts. Extracellular vesicles with diameter range of 30–100 nm were determined by TEM (**a**, **b**). The extracellular vesicles were bound to beads and gated with single beads (**c**) for analysis of CD9 (**d**), CD63 (**e**), and CD81 (**f**) expression

significantly different between GI with GII, and with normal mice. In normal mice and in those with normal hindlimb, the feet and toes of the mice become blue 20 s after trypan blue injection into the tail vein. However, ischemic hindlimbs require 72.25 ± 94.35 s to become blue in GI mice and 380 ± 110.9 s in GII mice. After 20 and 30 days, there were no significant differences between GI and GII (24.17 ± 6.646 s vs. 25 s on day 20; and 20 s vs. 21.67 ± 2.887 s on day 30, for GI and GII, respectively) (Fig. 6c).

These observations were supported by histological analysis (Fig. 7). The thigh muscles at treated sites of groups I & II mice were collected to analysis the histological structures and the neo-angiogenesis. The results of H&E staining also showed that in the normal mouse, skeletal muscle was arranged in bundles, in an orderly fashion. Their nuclei were located at the periphery of the muscle fiber; and their cytoplasmic staining was shown in pink and the cell nuclei staining in purple. Meanwhile, tissue necrosis occurred in the group I (Fig. 7a) and group II (Fig. 7c) at day 3. The cytoplasm showed shrinkage and tissue structure was rearranged. In some samples, the cells were not arranged with a particular order. The nuclei were not aligned along the edge of the cell (plasma membrane), but were concentrated near or in the cytoplasm. After 30 days, the tissue structure of both group I (Fig. 7b) and group II (Fig. 7d) that could survive and did not lose the legs were significantly improved. Particularly, the tissue structure of EVs injected mice was similar to those of normal mice at day 30 of treatment. Moreover, blood vessel density were higher in EVs injected mice compared to PBS injected mice.

Discussion

Recently, exosomes as well as EVs from various cells have shown varying biological activities. For

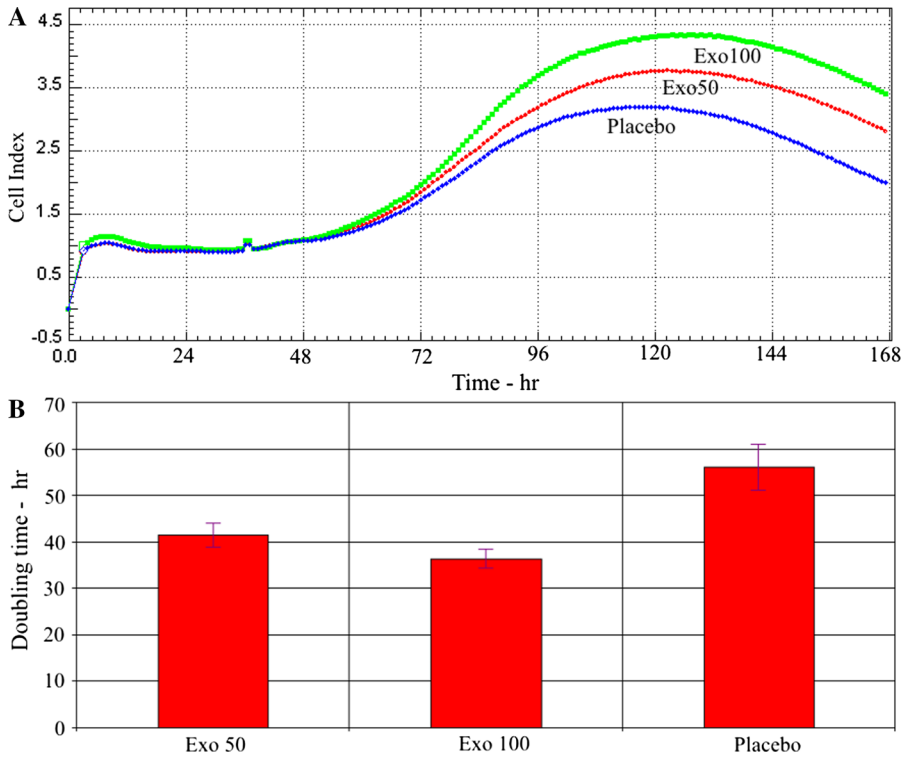

Fig. 4 Extracellular vesicles derived from ETV2 positive fibroblasts stimulated proliferation and inhibited senescence of HUVECs. The proliferation curves of HUVECs in group G1 (placebo), groups G2, G3 (extracellular vesicles supplement, Exo50 and Exo100, respectively) are shown (**a**) and are supported by the doubling time analysis (**b**)

example, EVs from ADSCs promote endothelial cell angiogenesis (Liang et al. 2016). Transfer of miR-125a from ADSCs to endothelial cells via exosomes is the reason for this effect (Liang et al. 2016). Exosomes from BMSCs induce proliferation and migration of normal and chronic wound fibroblasts, enhance in vitro angiogenesis (Shabbir et al. 2015), and promote periodontal tissue regeneration (Kawai et al. 2015). EVs from umbilical cord derived MSCs can suppress myofibroblast differentiation by inhibiting the TGF-beta/SMADs pathway (Fang et al. 2016), EVs from cardiomyocytes isolated from adult Goto–Kakizaki (GK) rats can inhibit the proliferation and migration of mouse cardiac endothelial cells (MCECs) (Wang et al. 2014), and those from embryonic stem cells promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction (Khan et al. 2015). Moreover, EVs from human urine-derived stem cells accelerate skin wound healing by promoting angiogenesis in rats (Fu et al. 2014; Yuan et al. 2016) and prevent kidney complications from type I diabetes (Jiang et al. 2016). EVs from human-induced pluripotent stem cell-derived MSCs have been shown to promote angiogenesis in hindlimb ischemia in mice (Hu et al. 2015), promote collagen synthesis and angiogenesis (Zhang et al. 2015), and induce repair of bone defects in osteoporotic rats (Qi et al. 2016). In mouse models, Geiger et al. (2015) demonstrated that human fibrocyte-derived exosomes successfully accelerated the wound healing process in genetically diabetic mice (Geiger et al. 2015).

Our study herein confirms that EVs derived from ETV-2 transfected fibroblasts can stimulate endothelial cell proliferation and neo-angiogenesis in an ischemia hindlimb mouse model. Firstly, ETV2 expressing fibroblasts were successfully isolated and cultured. The cells were confirmed as dermal fibroblasts from their spindle shape and high expression of CD90, considered as a popular marker of fibroblasts (Johnson et al. 2016; Kisselbach et al. 2009). The cells were capable of being transduced with a lentiviral vector containing the ETV-2 gene. All transduced fibroblasts showed resistance to puromycin; this characteristic is related to the selective puromycin

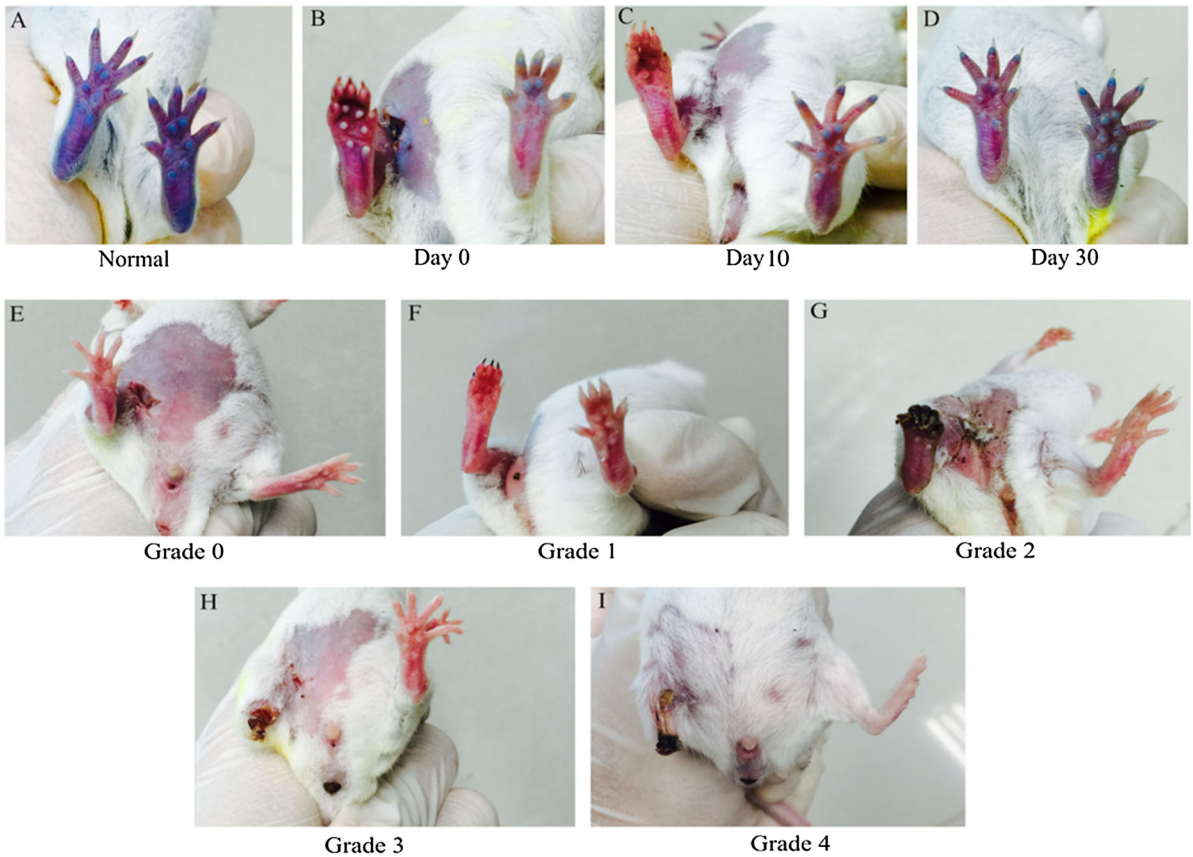

Fig. 5 The grade of limb necrosis was evaluated and classified according to the guidelines, as follows, of Goto et al. (2006). The trypan blue flow assay was used to evaluate the blood flow. The average time after injection of trypan blue at the tail vein to stain toes and feet were determined at normal mice (**a**), and on day 0 (**b**), on day 10 (**c**) and on day 30 (**d**) in animals after induction of hindlimb ischemia. The grades of limb necrosis were classified as Grade 0 (**e**), Grade I (**f**), Grade II (**g**), Grade III (**h**), Grade IV (**i**)

resistance gene encoded by the lentiviral vector. Moreover, expression of ETV-2 by transduced cells was also confirmed by real-time RT-PCR.

Subsequently, EVs from ETV-2 positive fibroblasts were successfully isolated with the use of commercial kits. These EVs contained both RNAs and proteins, and were positive for CD81 and CD63. Importantly, these EVs significantly stimulated endothelial cell proliferation. Indeed, the effects of the EVs included increased HUVEC proliferation rate and decreased doubling time. Our results suggest that materials inside the EVs play an important role in stimulating HUVEC proliferation. In fact, according to a previous study from our laboratory, ETV-2 transduced fibroblasts are capable of directly conversing into endothelial progenitor cells (Van Pham et al. 2016). Before this study, some studies suggested that ETV-2 may have a role in angiogenesis as well as direct reprogramming of fibroblasts to endothelial progenitor cells (Ginsberg et al. 2012, 2015; Morita et al. 2015). The studies showed that about 1–7% of ETV2 transduced fibroblasts go on to become EPCs. These EPCs exhibit full characteristics of natural EPCs, such as expression of CD31 and VEGFR2, and can form blood vessel-like capillaries (Morita et al. 2015; Van Pham et al. 2016). These studies suggest that fibroblasts from our study may be capable of conversion into EPCs.

In concordance with our observations from this study, some recently published studies have shown that exosomes as well as EVs derived from EPCs can induce multiple properties. Human umbilical cord blood EPCs derived EVs can protect against acute kidney injury by inhibition of endothelial cell apoptosis (Burger et al. 2015). These cells can also enhance

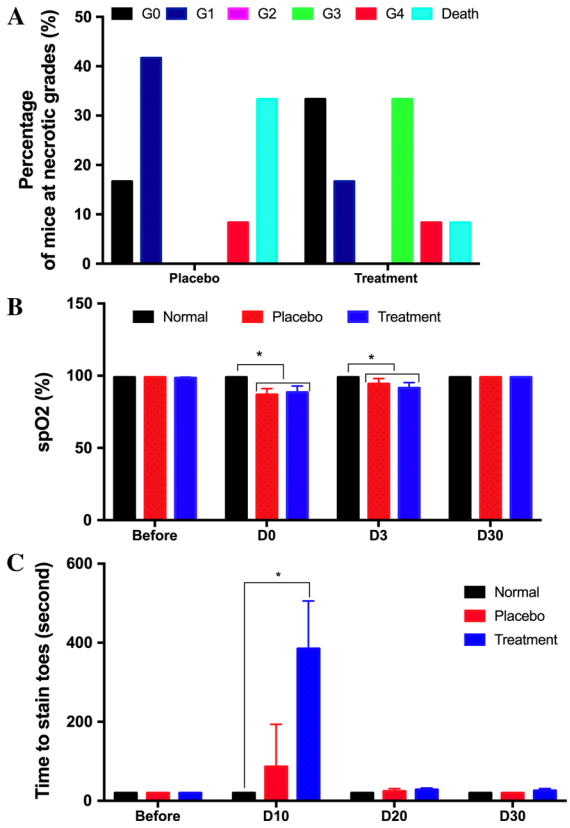

Fig. 6 Extracellular vesicles improve ischemic hindlimb in a murine model. Mice were injected with PBS as considered as Placebo (Placebo); while in the treatment group, mice were injected with a dose of 100 µL of 100 µg/ml EVs (Treatment). Extracellular vesicles efficiently reduced the percentage of mouse deaths due to ischemia, increased the percentage of fully recovered mice, and decreased the percentage of loss-leg mice (**a**). These results were supported by blood flow evaluation by spO_2 (**b**) and trypan blue assay (**c**)

the proliferation, migration and tube formation of vascular endothelial cells in vitro (Li et al. 2016b), while suppressing senescence and inducing angiogenesis in human and mouse endothelial cells (van Balkom et al. 2013). Although in this study, we did not determine what the protein and RNA components of the EVs were. In a previous study in the human endothelial cells, van Balkom et al. (2013) suggested that EVs from ECs containing miR-214 could be later transferred to mouse endothelial cells (van Balkom et al. 2013).

In the last part of our study, EVs from directly converted EPCs were shown to be capable of improving hindlimb ischemia in a mouse model. EVs not only decreased the necrosis grade in mice but also increased the survival of mice. EVs injection doubled the percentage of fully recovered mice (with necrosis grade 0). The blood flow recovery evaluations also supported this observation. At first look, the results from the spO-2- and trypan blue assays appeared to question the efficacy of EVs. However, these results are suitable, given the physiology and pathophysiology of neo-angiogenesis. Indeed, in the group GI (placebo group), only some mice could fully auto-recover their blood vessels which maintained blood flow into the toes and feet; other mice could not survive or had loss of their ischemic legs after 3 days. At day 10, only mice with legs were used for assessment with trypan blue assay. In the GII (treatment group), EV treatment rescued the mice and in fact, increased the percentage of surviving mice with ischemic legs.

Of note, in the first 10 days, neo-angiogenesis at these legs are being carried out with small vessels so that the time needed to stain blue the toes and feet using the trypan blue assay took longer. These results also showed that neovascularization was present and progressed slowly in EV-treated mice. Only after 30 days, blood vessel networks were fully formed; spO_2 and trypan blue assay to stain toes and feet were similar for mice of both GI and GII groups. The histological analysis also supported this observation.

Our results are similar to some very recent studies (Li et al. 2016a, b). Authors used human umbilical cord blood EPCs derived from EVs to treat the wounds. The results showed that EPC-derived EVs could accelerate cutaneous wound healing in diabetic rats (Li et al. 2016b), attenuate vascular repair, and accelerate re-endothelialization by enhancing endothelial function (Li et al. 2016a).

From the results of this study, we suggest two strategies to use the ETV-2 vector for ischemia treatment. The first way is using EVs from directly converted EPCs, which can be isolated from many sources, such as bone marrow, umbilical cord blood and other sources. These EPCs have a short life-span, slow proliferation, easy differentiation into mature cells, and ability for senescence. The directly converted EPCs are a new choice to produce a large-scale of EPCs and then EPC derived EVs. As non-cellular products, EVs are safe for clinical applications (Han et al. 2016; Sun et al. 2016). The second way is to promote in vivo direct conversion of dermal fibroblasts by ETV-2. The converted fibroblasts in vivo

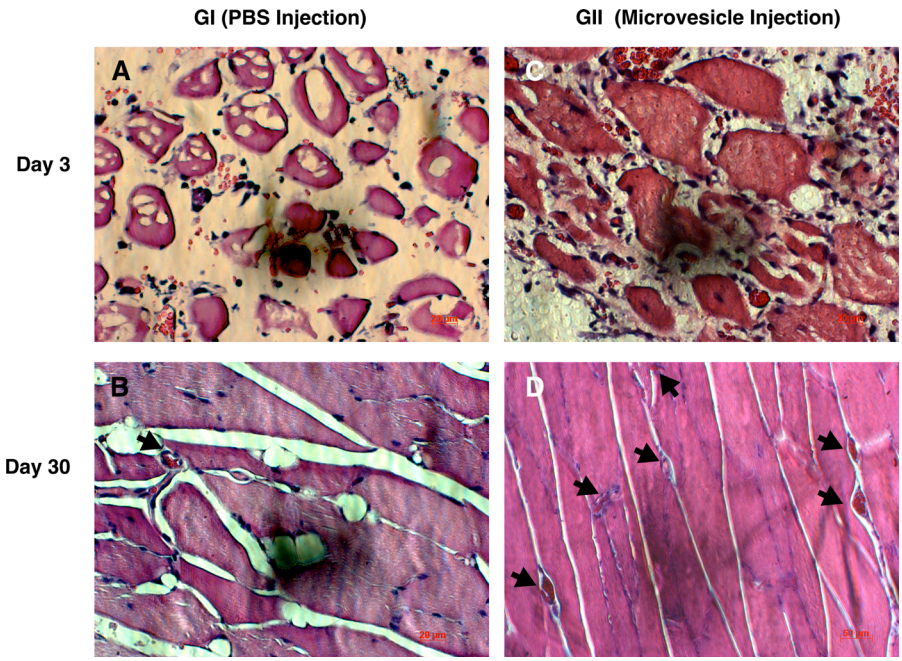

Fig. 7 Histological structure analysis after H&E staining at day 3 and 30 after transplantation. Tissue necrosis occurred in the group I (**a**) and group II (**c**) at day 3. After 30 days, the tissue structure of both group I (**b**) and group II (**d**) that could survive and did not lose the legs were significantly improved, similar to those of normal mice. Moreover, blood vessel density was higher in extracellular vesicles injected mice compared to PBS injected mice. *The arrows* point to blood vessel

produce EVs, which induce endothelial cells at the ischemic site to regenerate and recover/repair blood vessels.

However, there were still some limitations in this study. The effects of empty viral transfected fibroblasts derived EVs on endothelial cells in vitro and neovascularization in a murine model of hindlimb ischemia have not been evaluated yet. Non-viral ETV2 vector also should be investigated to use these EVs for clinical usage.

Conclusion

ETV-2 is a master gene regulator of angiogenesis. ETV-2 plays a role in directly converting fibroblasts into endothelial progenitor cells. EVs from these directly converted endothelial progenitor cells or ETV-2 transduced fibroblasts can stimulate endothelial cell (HUVEC) proliferation in vitro, stimulate angiogenesis in vivo, and improve hindlimb ischemia in a mouse model. Our results show that EVs derived from ETV-2 transduced fibroblasts may be a promising product for ischemia treatment. Moreover, in vivo transfection of ETV-2 into fibroblasts may represent a promising strategy to produce EVs and to stimulate neo-angiogenesis.

Acknowledgements This research was funded by the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under Grant Number 106-YS.06-2013.37.

Funding Vietnam National Foundation for Science and Technology Development (NAFOSTED) under Grant Number 106-YS.06-2013.37.

Authors' contributions PVP was responsible for suggesting the idea for this study, creating the experiment design, vector preparation, data analysis, writing the Discussion, preparing the figures, and revising the manuscript. VBN, TTTD, HTNL, LTP were responsible for performing murine model of hindlimb ischemia, evaluation of recovery of damaged limbs after EVs injection. OTH and MTHT were responsible for performing the essays of fibroblast culture, RT-PCR analysis, flow cytometry analysis, and writing the Methods. OTKN and NKP were were responsible for EVs isolation, characterization; performing the HUVEC cultures, Matrigel assays, and writing the Introduction. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interests The authors declare they have no competing interests.

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