(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 February 2019 (07.02.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/028450 A1

(51) International Patent Classification:
A61K 9/00 (2006.01) *A61M 5/158* (2006.01)
A61M 37/00 (2006.01)

(21) International Application Number:
PCT/US2018/045333

(22) International Filing Date:
06 August 2018 (06.08.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/541,157 04 August 2017 (04.08.2017) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) **Title:** METHOD FOR PRODUCING THERAPEUTIC EXOSOMES FROM NANOELECTROPORATION AND OTHER NON-ENDOCYTIC CELL TRANSFECTION

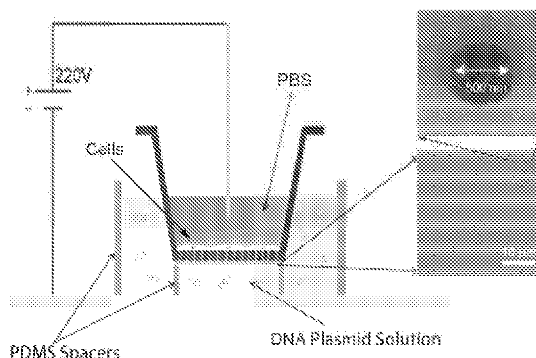

Figure 1. Schematic of a 3D Nanochannel Electroporation (NEP) biochip for donor cell transfection

(57) **Abstract:** Therapeutic extracellular vesicles (EVs) containg high copies of functional nucleic acids and other biomolecules are produced in large quantities by laying donor cells on a surface of a chip, adding various plasmids, other transfection vectors and their combinations to a buffer on the chip, applying a pulsulatic electric field across the cells laid on top of the chip surface and plasmids/vectors buffer solution below the chip surface, and collecting the EVs secreted by the transfected cells. The chip surface has a three-dimensional (3D) nanochannel electroporation (NEP) biochip formed on it, capable of handling large quantities of the donor cells. The buffer is adapted for receiving plasmids and other transfection vectors.

WO 2019/028450 A1

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

METHOD FOR PRODUCING THERAPEUTIC EXOSOMES FROM NANOELECTROPORATION AND OTHER NON-ENDOCYTIC CELL TRANSFECTION

Technical Field

5 **[001]** The present invention relates to methods for producing therapeutic extracellular vesicles (EVs), exosomes in particular, that contain functional messenger RNAs (mRNAs), microRNAs (miRs), short hairpin RNAs (shRNAs), proteins, and other biomolecules by non-endocytic delivery of DNA plasmids and other vectors into donor cells in a way that the strong stimulation caused by delivery triggers donor cells to generate a large number 10 of vesicles within the cell while the non-endocytic delivery of DNA plasmids/vectors leads to fast transcription of RNAs and translation of proteins within cytoplasm, allowing those functional biomolecules to be encapsulated in the vesicles endogenously before they are secreted out from donor cells as EVs.

15 ## Background

[002] Extracellular vesicles (EVs), including exosomes, microvesicles and other vesicles, are secreted by numerous cell types. In the human body, there are >10E12 EVs in 1 mL blood and they also exist in various body fluids. Exosomes are nano-vesicles (40–150 nm), while microvesicles have sizes varied from <100 nm to >1 micron. They contain both 20 coding and non-coding RNAs and their fragments, DNA fragments, proteins, and other cell related biomolecules. EVs and their biomolecule contents have been proposed as biomarkers for disease diagnosis. In addition, they play major roles in cell-cell communications in tumor microenvironment and circulation.

[003] EVs loaded with functional RNAs and proteins have also been suggested as drugs 25 and drug carriers for therapeutic applications. To deliver specific nucleic acids and/or proteins to target tissues or cell types *in vitro* and *in vivo* requires methods that can produce EVs with either endogenous or exogenous therapeutic cargos.

[004] Post-insertion of exogenous small interference RNA (siRNA) and shRNA plasmids into pre-existed exosomes by conventional bulk electroporation (BEP) has been 30 developed in recent years. Although their therapeutic functions have been successfully demonstrated in several mouse models for cancer and non-cancer diseases, this

approach faces many limitations. First, post-insertion of large biomolecules such as DNA plasmids, mRNAs, and proteins into nano-sized exosomes is inefficient. Secondly, the strong electric field generated by BEP would break up many exosomes leading to a low yield of therapeutic exosomes. Furthermore, many large biomolecules such as mRNAs
5 and proteins are difficult and expensive to be synthesized exogenously.

[005] It would be highly desirable if new methods can be developed, that may transfect donor cells with DNA plasmids or other vectors to produce a large number of exosomes or other EVs containing therapeutic RNA and protein targets endogenously.

[006] In a prior US patent application 14/282630, we developed a nanochannel
10 electroporation (NEP) biochip that can deliver DNA plasmids or other charged particulates and molecules into individual cells non-endocytically with good dosage control. Herein, we demonstrate that NEP can produce a large number of therapeutic exosomes containing high copies of functional mRNA and microRNA targets, not achievable by the aforementioned post-insertion methods. In addition to NEP, other non-endocytic delivery
15 methods such as gene gun, micro/nano-injection, etc. may also achieve a similar performance if they can provide proper cell stimulation and fast plasmid/vector delivery.

Summary

[007] The present invention is related to the development of new concept and methods
20 that DNA plasmids and other vectors can be non-endocytically delivered into donor cells with strong cellular stimulation such that a large number of vesicles and transcribed RNAs as well as translated proteins are formed within the transfected cells. Cells would secret many extracellular vesicles (EVs) containing specific RNA and protein targets with therapeutic functions.

25 **[008]** To demonstrate the aforementioned design concept, a three-dimensional (3D) NEP biochip is fabricated, that can transfect many donor cells with pre-specified DNA plasmids to secret 10~100 folds more EVs, including exosomes, containing high copies of intact mRNA and miR targets up to many thousands folds more than those in EVs secreted from the non-transfected donor cells.

30 **[009]** Some aspects of the invention are achieved by a method of producing a large number of therapeutic extracellular vesicles (EVs) containg high copies of functional

nucleic acids and other biomolecules. Such a method comprises the steps of::

laying donor cells on a surface of a chip, the surface having a three dimensional (3D) nanochannel electroporation (NEP) biochip formed thereon;

adding various plasmids, other transfection vectors and their combinations to a 5 buffer on the chip;

applying a pulsulatic electric field across the cells laid on top of the chip surface and plasmids/vectors buffer solution below the chip surface, resulting in strongly stimulating the cells and delivering plasmids/vectors into cells non-endocytically; and

collecting EVs secreted by the transfected cells.

10 **[010]** In some of these methods, the diameter of nanochannels is between 50-900 nm.

[011] In some of these methods, wherein the plasmids and vectors transcribe mRNA, microRNA, shRNA, and other RNAs, and lead to translation of proteins and other biomolecules in the transfected cells.

[012] In some embodiments of the method, the EVs secreted by the transfected cells 15 contain the transcribed mRNA, microRNA, shRNA, and other RNAs, and the translated proteins and other biomolecules.

[013] In some of the embodiments of the method, wherein means to increase the expression of heat shock proteins and other proteins that can promote vesicle formation and exocytosis in the transfected cells are added to the system, wherein the means 20 includes a thermal shock treatment of the cells, or addition of heat shock proteins in cell culture.

[014] In some of the embodiments, means to increase the expression of proteins that promote exosome formation in the transfected cells are added to the system, wherein the means includes co-transfection of CD63, CD9 and other DNA plasmid.

25 **[015]** In some embodiments, multiple DNA plasmids and other vectors are delivered to the transfected cells sequentially to promote co-localization of RNA/protein targets and EV secretion.

[016] In some embodiments, exogenous biomolecules such as DNA plasmids, other transfection vectors, RNAs, proteins/peptides, small molecule drugs are encapsulated 30 within vesicles in cells and secreted out as therapeutic EVs by sequential transfection of donor cells by NEP. In some of these cases, in addition to NEP, other cell transfection

methods that provide strong stimulation to donor cells to facilite EV secrection and non-endocytic plasmid/vector delivery for fast RNA transcription and protein translation are used to produce therapeutic EVs with similar efficacy. In further of these cases, the other cell transfection methods include, gene gun, and micro- or nanoinjection.

5 **[017]** In some of the embodiments, the plasmids and/or other vectors are tethered on nano- or micron-sized gold or other solid particles, and those particles are injected into donor cells under a pneumatuc force using a gene gun to cause strong cell stimulation and non-endocytic plasmis/vector delivery.

[018] In some of the embodiments, the plasmids and/or other vectors are tethered on a 10 nano- or micron-sized tip array, and donor cells are pultruded by those tips to cause strong cell stimulation and non-endocytic plasmis/vector delivery into donor cells.

[019] Other aspects of the invention are achieved by a device for producing a large number of therapeutic extracellular vesicles (EVs) containg high copies of functional nucleic acids and other biomolecules, comprising:a chip having a three-dimensional 15 (3D) nanochannel electroporation (NEP) biochip and a buffer for receiving formed thereon, the buffer adapted for receiving plasmids and other transfection vectors.

[020] Other aspects of the invention comprises cells transfected by any of the foregoing methods.

20

Brief Description of the Drawings

[021] Many aspects of the disclosure can be better understood with reference to the following drawings. The components in the drawings are not necessarily to scale, 25 emphasis instead being placed upon clearly illustrating the principles of the present disclosure. In the drawings:

Figure 1 is a schematic of a 3D Nanochannel Electroporation (NEP) biochip for donor cell transfection;

Figure 2 shows a comparison of BEP and NEP based cell transfection at 1 hr post-30 transfection using Yoyo-1 fluorescence labelled Achaete-Scute Complex Like-1 (Ascl1) DNA plasmid, a neuronal related gene;

Figure 3 shows NEP cell transfection with or without DNA plasmids significantly stimulates the EV secretion from transfected mouse embryonic fibroblast (MEF) cells, with performance much better than lipofectamine (Lipo) and BEP based cell transfection. Ctrl stands for non-transfected MEF cells; NEP stands for NEP cell transfection with DNA plasmids; NEP-PBS stands for NEP cell transfection with PBS buffer only. The DNA plasmids used are Achaete-Scute Complex Like-1 (Ascl1), Pou Domain Class 3 Transcription factor 2 (Pou3f2 or Brn2) and Myelin Transcription Factor 1 Like (Myt1l) at a weight ratio of 2/1/1. A mixture of those DNA plasmids is known to reprogram donor cells into induced neurons (iNs);

Figure 4 shows the effect of heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) inhibitors on EV secretion from NEP transfected MEF cells. After NEP transfection, the cell culture was replaced with fresh medium containing HSP70 inhibitor (VER 155008, 50μM), HSP90 inhibitor (NVP-HSP990, 1μM), or their mixture. Medium was collected at 24 h post-transfection, and EV numbers were detected by dynamic light scattering (DLS) goniometry;

Figure 5 shows the effect of NEP transfection of CD63 DNA plasmid on EV secretion from MEF cells. Cells were transfected with or without CD63 plasmid by NEP. The cell culture medium was collected and replaced with fresh medium every 4 hr. The EV numbers were detected by DLS goniometry;

Figure 6 shows a size distribution measured by DLS of EVs with or without NEP harvested at 24 h post-cell transfection;

Figure 7 demonstrates EV Ascl1 mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection;

Figure 8 shows EV Brn2 mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection;

Figure 9 shows EV Myt1l mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection;

Figure 10 shows that only EVs obtained by NEP contain functional mRNA determined by *in vitro* translation;

Figure 11 shows that EV-mRNAs from NEP are found in exosomes, not in microvesicles.

5 **Figure 12** shows that exosome-mRNAs, not microvesicle-RNAs, from NEP cell transfection can translate proteins.

Figure 13 shows EV-mRNAs secretion profiles from NEP transfected MEF cells;

Figure 14 depicts action potential detection by patch clamp shows that MEF cells transfected every other day with Ascl1/Brn2/Myt1l mRNA containing EVs obtained from 10 NEP could be reprogrammed into functional induced neurons (iNs) after 24 days.

Figure 15 shows EV miR-128 expression determined by qRT-PCR from MEF cells transfected by miR-128 DNA plasmid using various techniques at 24 h post-transfection;

Figure 16 is a comparison of secreted EVs containing miR-128 by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected miR-15 128 by BEP post-insertion;

Figure 17 is a comparison of secreted EVs containing Brn2 mRNA by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected Brn2 mRNA by BEP post-insertion; and

Figure 18 shows increased mRNA co-localization in the same EV by sequential-20 NEP. For NEP transfection, Ascl1, Brn2 and Myt1l plasmids were transfected at the same time as described before. For sequential-NEP, the Myt1l plasmid was transfected first, Brn2 plasmid was transfected 4 h later, while Ascl1 plasmid was transfected 4 h after Brn2 transfection. At 24 h post Myt1l transfection, culture medium was collected for TLN assay. Equal amount of FAM-Ascl1, Cy3-Brn2, and Cy5-Myt1l MBs were encapsulated 25 in tethered lipoplex nanoparticles for EV-mRNA detection. Yellow arrow: EVs containing 3 mRNAs; Blue arrow: EVs containing 2 mRNAs; and Pink arrow: EVs containing 1 mRNA.

30 **Detailed Description**

Example 1- 3D NEP Biochip Schematic and Comparison of EV Secretion and EV mRNA Content Using Different Transfection Methods.

[022] Figure 1 shows the schematic of a 3D NEP biochip with a single layer of donor cells laid on the chip surface. After overnight cell incubation, the DNA plasmids pre-loaded in PBS buffer were injected into individual donor cells via nanochannels using a 220 volts electric field across the nanochannels. Various electroporation conditions such as voltage level, pulse number and pulse length can be chosen.

[023] Using Yoyo-1 fluorescence dye labelled Achaete-Scute Complex Like-1 (Ascl1) DNA plasmid, **Figure 2** shows transfected cells imaged using fluorescence microscopy 1 h after transfection by either BEP or NEP under a wavelength of 488 nm. The fluorescence intensity was calculated by NIS software. Comparison of fluorescence intensity in these two groups is given as bar charts. The results show that BEP at the manufacturer recommended best conditions could deliver nearly 3 folds more plasmids than NEP at 220 volts with five 10-ms pulses to the MEF cells. However, most plasmids were still near the cell surface 1 h after BEP transfection, while the injected plasmids by NEP have already been uniformly diffused within cytoplasm at the same time. This implies that BEP based cell transfection relies mainly on electroporation-mediated endocytosis, while NEP based cell transfection is non-endocytic.

[024] Figure 3 compares EV numbers secreted from the same number of MEF cells (5E6 cells) transfected with the same Ascl1, Brn2 and Myt1l DNA plasmids at a weight ratio of 2/1/1 by either lipofectamine (Lipo), BEP or NEP. All EVs were collected from cell culture medium at 24 h post-transfection and the total EV number was determined by NanoSight^{TM}. For BEP, the transfection voltage was 1250 v with one 30-ms pulse. For NEP, the transfection voltage was 220 with five 10 ms pulses. The concentration of plasmid used was Ascl1/Brn2/Myt1l=200/100/100 ng/µl. For lipofectamine transfection, 5 µg plasmid mixture (Ascl1/Brn2/Myt1l=2/1/1) was used according to manufacturer's instruction. The EVs were collected from cell culture medium by simply centrifugation at 1500 g for 10 mins. The results show that lipofectamine (Lipo) based cell transfection did not change the EV secretion. The EV concentration was around 2E9/ml with or without transfection. Apparently, a slow plasmid endocytosis process by nanoparticle carriers would not stimulate the transfected cells much and, consequently, there was almost no

change on EV secretion. In comparison, BEP based cell transfection led to more EV secretion to ~6E9/ml. A tremendous increase of EV secretion to >1.3E11/ml was observed by NEP cell transfection with or without adding plasmids. This implies that the transfected cells were somewhat stimulated by BEP, but highly stimulated by NEP leading 5 to very significant increase of EVs in the latter case.

[025] During electroporation, Joule heating caused by the imposed electric field could tentatively increase the cell temperature to cause thermal shocking to the transfected cells. It is known that thermal shocking may increase cell secretion of EVs due to chaperone mediated autophage caused by the increase of heat shock proteins in cells 10 (8-10). Indeed, we found that NEP could substantially increase the expression of both heat shock protein 70 (HSP70) by 13.8 folds and heat shock protein 90 (HSP90) by 4.2 folds in the transfected MEF cells vs. the non-transfected MEF cells (Ctrl). When HSP inhibitors were added in cell culture medium after electroporation, EV secretion could be suppressed. **Figure 4** shows 50%, 40% and 70% decrease of EV secretion of NEP 15 transfected MEF cells with HSP 70 inhibitor (VER 155008, , 50 μM) HSP90 inhibitor (NVP-HSP990, 1 μM), and their mixture respectively. Here, the cell culture was replaced with fresh medium containing HSP70 inhibitor (VER 155008), HSP90 inhibitor (NVP-HSP990), or their mixture right after NEP transfection. Medium was collected at 24 h post-transfection and EV numbers were detected by dynamic light scattering (DLS) goniometry. 20 These results imply that any cell stimulation that can increase the expression of heat shock proteins would enhance EV secretion.

[026] Similarly, an increase of proteins that are needed for late endosomal multi-vescular body (MVB) formation in cells may also enhance exosome secretion. **Figure 5** shows the effect of NEP transfection of CD63 DNA plasmid on EV secretion from MEF cells. Cells 25 were transfected with or without CD63 DNA plasmid by NEP. The cell culture medium was collected and replaced with fresh medium every 4 h. The EV numbers were detected by DLS goniometry. The results show a similar EV secretion profile during the first 16 h after NEP transfection in both cases. However, more EVs were secreted between 16 to 44 h after NEP transfection with CD63 DNA plasmid. CD63 protein is essential for the 30 reorganization of endosomal membrane into tetraspanin enriched microdomains, a precursor of exosome secretion.

[027] **Figure 6** shows the EV size distribution measured by DLS goniometry for MEFs (ctrl) and NEP transfected MEFs. NEP stimulation did not change the larger EV (mostly microvesicles) distribution much, but substantially increased the secretion of exosomes with sizes ranging from 40 to 110 nm.

5 **[028]** **Figures 8-9** show that the secreted EVs from NEP cell transfection of Ascl1, Brn2 and Myt1l DNA plasmids contain a large amount of corresponding Ascl1, Brn2 and Myt1l mRNAs or their fragments as determined using quantitative-Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Like the EV numbers, lipofectamine (Lipo) based cell transfection did not change the mRNA expression much, while the BEP based 10 cell transfection could increase the mRNA expression several folds. In comparison, the NEP based cell transfection resulted in thousands folds increase of target mRNAs. Here, the same amount of total RNAs were obtained and reverse transcription was conducted by qRT-PCR according to manufacturer's instruction.

[029] **Figure 10** shows that some of the EV mRNAs were intact and functional because 15 they were able to translate Ascl1, Brn2 and Myt1l proteins. Here, a same amount of total RNA (1 µg) from each transfection method was applied for *in vitro* protein translation using Rabbit Reticulocyte Lysate System (Promega) according to manufacturer's instruction. Samples were separated by SDS-PAGE and the proteins were detected with various antibodies as shown in the Western blotting plot.

20 **[030]** For the collected total EVs, the larger microvesicles were sorted by ultracentifugation at 10,000 g for 30 min. The supernatant was further centrifugated at 100,000 g for 2 h to collect the smaller exosomes. Total RNAs were collected from these two parts as described above. The total mRNA concentration was measured by Nanodrop^{TM}, while the ABM expressions of Ascl1, Brn2 and Myt1l mRNAs were 25 measured by qRT-PCR. **Figure 11** shows that there was more than twice RNA in exosomes than in microvesicles, but most Ascl1, Brn2 and Myt1l mRNAs were presented only in exosomes. **Figure 12** shows that the functional Ascl1, Brn2 and Myt1l mRNAs were also presented in exosomes and those exosomes carry typical exosomal protein markers, CD9, CD63 and Tsg101. In comparison, the larger microvesicles carry the 30 typical protein marker, Arf6.

[031] Figure 13 shows the EV secretion and content profiles as a function of time after NEP transfection with Ascl1, Brn2 and Myt1l DNA plasmids. The Ascl1 plasmid is the smallest one (7k bp) among the three, while the Myt1l plasmid is the largest (9k bp) with the Brn2 plasmid in between (8k bp). EVs in the cell culture medium was collected at the indicated time points, and the culture medium was replaced with fresh medium. The EV numbers were detected by DLS goniometry, while the EV mRNA expressions were detected by qRT-PCR as described before. The results show a quick increase of EV secretion within 4 h post-transfection, and peaked at 8 h with continuous EV secretion for more than 24 h. EVs containing Ascl1 and Brn2 mRNAs also appeared within 4 h post-transfection with profiles matching well with that of EV secretion. EVs containing Myt1l mRNA appeared at a later time, but still within 24 h. This implies that the EV secretion time and the mRNA transcription time need to be matched in cell, which can be achieved by NEP based cell transfection.

[032] To demonstrate that NEP-produced-EVs containing endogenous mRNAs have therapeutic functions, we treated MEF cells with those EVs every other day at a total EV RNA concentration of 1 µg per 100,000 cells. After several days, the treated MEF cells started to reveal neuron-like morphology and at 24 days, the treated cells showed electrophysiological activity as demonstrated by their capacity to undergo induced action potentials as shown in **Figure 14**. In comparison, the NEP-transfected MEF cells also showed a similar electrophysiological activity on Day 21. Cells displayed the necessary voltage-gated currents to fire action potentials. Both transient inward currents and sustained outward currents were observed in response to depolarizing voltage simulations. A typical response to a 20 pA current injection is illustrated in **Figure 14** and indicates that cells fired action potentials in response to depolarizing current.

[033] Whole-cell patch clamp recording was used to measure excitability. Cells were continuously superfused with an extracellular bath solution containing 115 mM NaCl, 2 mM KCl, 1.5 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM HEPES, and 10 mM Glucose (pH 7.4). Glass electrodes (3-4 MΩ) were filled with a pipette solution containing 115 mM K-gluconate, 10mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES), 4 mM NaCl, 0.5 mM ethylene glycol tetraacetic acid (EGTA), , 1.5 mM $MgCl_2$, (pH 7.3). Cells had a patch resistance of >100 MOhm after whole-cell access was gained, and series

resistance was compensated 40-50%. Data were collected using an Axopatch 200B amplifier, Digidata 1322A digitizer, and Clampex 9 software (Molecular Devices, Sunnyvale, CA). For analysis of voltage-gated currents, the basal holding potential was -70 mV and cells were stepped for 400 ms in 10 mV increments from -120 mV to 80 mV.
5 Transient inward currents, due to activity of voltage-gated sodium channels, were isolated from measuring the peak amplitude. Sustained plateau currents, reflective of voltage-gated potassium currents, were measured as the average of the last 50ms of the voltage step in the plateau phase of the current. Action potential induction was measured using current clamp. Current was held at 0 pA and then stepped in 20 pA intervals for 1 sec.
10

Example 2- EV MicroRNA Content Using Different Transfection Methods.

[034] To demonstrate broader therapeutic applicability, we also transfected MEF cells with a DNA plasmid that will transcribe microRNA targets in cells. **Figure 15** shows the EV miR-128 expression for EVs harvested from cell culture medium at 24 h post-
15 transfection (miR-128 plasmid) by various techniques. Total RNAs were obtained according to manufacturer's instruction. The same amount of total RNA (30 ng) was used for miR-128 detection by qRT-PCR using the aforementioned procedures. Again, NEP based transfection was able to produce EVs containing a large amount of miR-128 (more than 4,500 folds increase), not achievable by BEP or lipofectamine based cell transfection.
20

Example 3- Comparison of EVs Containing Endogenous RNAs by NEP Transfection of DNA Plasmid to MEF Cells vs. Existing EVs Loaded with Pre-collected RNAs by BEP Post-insertion.

[035] Here, we compared the efficacy of producing therapeutic EVs using our NEP
25 based cell transfection and the BEP post-insertion approach used by several researchers. For the former, the miR-128 plasmid was co-transfected with CD63-GFP plasmid to MEF cells by NEP to generate EVs containing miR-128 according to aforementioned procedures. For the latter, blank EVs were first harvested from MEF cells transfected with CD63-GFP plasmid 24 h after NEP. In parallel, miR-128 was collected from MEF cells
30 transfected with miR-128 plasmid 24 h post-transfection by NEP. The collected miR-128 (1 µg) was mixed with blank EVs (10E6) and electroporated by BEP (1250 volts, 30 ms)

according to conditions used by other researchers. EVs from the two approaches were tested using a tethered lipoplex nanoparticle (TLN) biochip on a total internal reflection fluorescence (TIRF) microscope. **Figure 16A** shows the TLN-TIRF assay schematic (2, 11). Briefly, a molecular beacon (MB) for the RNA target is designed and encapsulated in cationic liposomal nanoparticles. These cationic lipoplex nanoparticles are tethered on a glass slide, which are able to capture negatively charged EVs by electrical static interactions to form a larger nanoscale complex. This lipoplex-EV fusion leads to mixing of RNAs and MBs within the nanoscale confinement near the biochip interface. TIRF microscopy is capable of detecting a single biomolecule and it measures signals <300 nm near the interface, which is where the tethered liposomal nanoparticles locate.

[036] **Figure 16B** shows the representative TLN-TIRF images of the captured EVs. The green fluorescence is from EVs containing CD63-GFP, while the red fluorescence is from hybridization of miR-128 molecules and the Cy5-miR128 MBs in the captured EVs. It is clear that our NEP approach is able to produce more EVs containing higher copies of miR-128 than the BEP post-insertion approach. **Figures 16C-E** show a quantitative comparison of those two approaches. Although both approaches are able to produce EVs containing miR-128 (~80% of total captured EVs), the EV miR-128 concentration in EVs (~3 times MB fluorescence intensity) is much higher in NEP based direct cell transfection than in BEP based microRNA post-insertion. Furthermore, BEP post-insertion tends to break nearly half of the blank EVs leading to a very low yield of therapeutic EVs.

[037] A similar comparison was also carried out for a much larger RNA, Brn2 mRNA (6272 bases for Brn2 mRNA vs. 21 bases for miR-128) using the same approach as for miR-128. **Figure 17** shows that our NEP approach could produce >70% EVs containing Brn2 mRNA, while only very few existing EVs could be loaded with the same mRNA by BEP post-insertion approach. The concentration of Brn2 mRNA in NEP produced EVs is high, while that in BEP post-insertion is very poor.

Example 4- Improvement of Multiple mRNAs Co-localized in the Same Secreted EVs by Sequential NEP Transfection of DNA Plasmids to MEF Cells.

[038] **Figure 13** implies that different mRNA targets could be transcribed at different times and rates in the transfected cells, even though multiple DNA plasmids were

delivered to the cells at the same time, due to the size difference of plasmids or other reasons. This may lead to individual EVs containing only one or few mRNA targets. For better therapeutic efficacy, it would be valuable if more or all mRNA targets can be encapsulated in the same secreted EVs. By sequentially delivering each DNA plasmid into MEF cells using NEP based on its transcription time, **Figure 18** shows that we could substantially increase the secreted EVs containing all three mRNAs, Ascl1, Brn2 and Myt1l (>50% vs. <25%), needed for iN reprogramming. For NEP transfection, Ascl1, Brn2 and Myt1l plasmids were transfected at the same time as described before. For sequential-NEP, the Myt1l plasmid was transfected first, Brn2 plasmid was transfected 4 h later, while Ascl1 plasmid was transfected 4 h after Brn2 transfection. At 24 h post Myt1l transfection, culture medium was collected for TLN assay. Equal amount of FAM-Ascl1, Cy3-Brn2, and Cy5-Myt1l MBs were encapsulated in tethered lipoplex nanoparticles for EV-mRNA detection. In the figure, the yellow arrow means EVs containing all 3 mRNAs, the blue arrow means EVs containing 2 mRNAs, while the pink arrow means EVs containing only 1 mRNA.

[039] While the invention has been explained in relation to its preferred embodiments, it is to be understood that various modifications thereof will become apparent to those skilled in the art upon reading the specification. Therefore, it is to be understood that the invention disclosed herein is intended to cover such modifications as fall within the scope of the appended claims.

CLAIMS

What is claimed is:

Claim 1. A method of producing a large number of therapeutic extracellular vesicles (EVs) containg high copies of functional nucleic acids and other biomolecules comprising the steps of::

laying donor cells on a surface of a chip, the surface having a three-dimensional (3D) nanochannel electroporation (NEP) biochip formed thereon;

adding various plasmids, other transfection vectors and their combinations to a buffer on the chip;

applying a pulsulatic electric field across the cells laid on top of the chip surface and plasmids/vectors buffer solution below the chip surface, resulting in strongly stimulating the cells and delivering plasmids/vectors into cells non-endocytically; and

collecting EVs secreted by the transfected cells.

Claim 2. The method of claim 1, wherein the diameter of nanochannels is between 50-900 nm.

Claim 3. The method of claim 1 or 2, wherein the plasmids and vectors transcribe mRNA, microRNA, shRNA, and other RNAs, and lead to translation of proteins and other biomolecules in the transfected cells.

Claim 4. The method of any one of the preceding claims, wherein EVs secreted by the transfected cells contain the transcribed mRNA, microRNA, shRNA, and other RNAs, and the translated proteins and other biomolecules.

Claim 5. The method of any one of the preceding claims, wherein means to increase the expression of heat shock proteins and other proteins that promote vesicle formation and exocytosis in the transfected cells are added to the system, wherein the means includes a thermal shock treatment of the cells, or addition of heat shock proteins in cell culture.

Claim 6. The method of any one of the preceding claims, wherein means to increase the expression of proteins that promote exosome formation in the transfected cells are added to the system, wherein the means includes co-transfection of CD63, CD9 and other DNA plasmid.

Claim 7. The method of any one of the preceding claims, wherein multiple DNA plasmids and other vectors are delivered to the transfected cells sequentially to promote co-localization of RNA/protein targets and EV secretion.

Claim 8. The method of any one of the preceding claims, wherein exogenous biomolecules such as DNA plasmids, other transfection vectors, RNAs, proteins/peptides, small molecule drugs are encapsulated within vesicles in cells and secreted out as therapeutic EVs by sequential transfection of donor cells by NEP.

Claim 9. The method of claim 8, wherein, in addition to NEP, other cell transfection methods that provide strong stimulation to donor cells to facilite EV secrection and non-endocytic plasmid/vector delivery for fast RNA transcription and protein translation produce therapeutic EVs with similar efficacy.

Claim 10. The method of claim 9, wherein the other cell transfection methods include, gene gun, and micro- or nanoinjection.

Claim 11. The method of any one of claims 8 to 10, wherein the plasmids and/or other vectors are tethered on nano- or micron-sized gold or other solid particles, and those particles are injected into donor cells under a pneumatuc force using a gene gun to cause strong cell stimulation and non-endocytic plasmis/vector delivery.

Claim 12. The method of any one of claims 8 to 11, wherein the the plasmids and/or other vectors are tethered on a nano- or micron-sized tip array, and donor cells are

pultruded by those tips to cause strong cell stimulation and non-endocytic plasmis/vector delivery into donor cells.

Claim 13. A device for producing a large number of therapeutic extracellular vesicles
5 (EVs) containg high copies of functional nucleic acids and other biomolecules, comprising:

a chip having a three-dimensional (3D) nanochannel electroporation (NEP) biochip and a buffer for receiving formed thereon, the buffer adapted for receiving plasmids and other transfection vectors.

10

Claim 14. Cells transfected by the method of any one of claims 1 to 12.

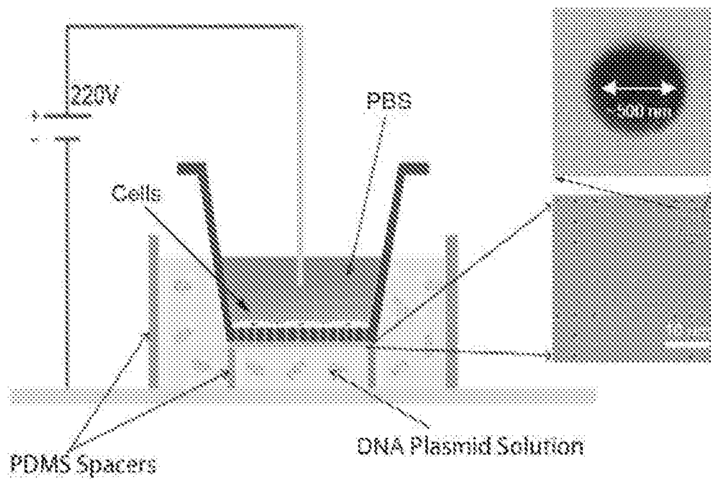


Figure 1. Schematic of a 3D Nanochannel Electroporation (NEP) biochip for donor cell transfection

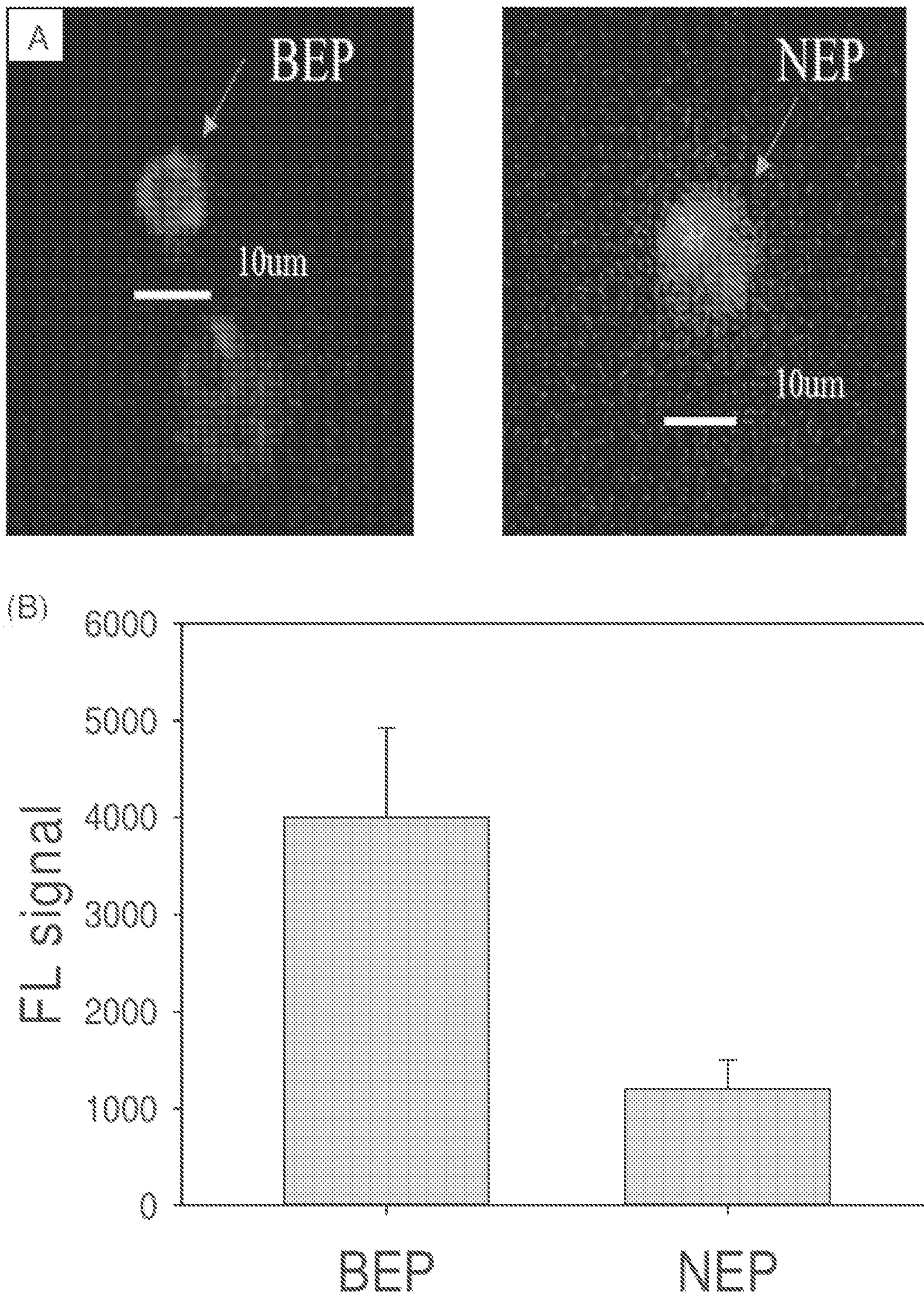


Figure 2. Comparison of BEP and NEP on Yoyo-1 fluorescence labelled DNA plasmid delivery efficiency at 1 h post-transfection. A) Representative cell images for BEP and NEP. B) Comparison of fluorescence intensity in these two cases.

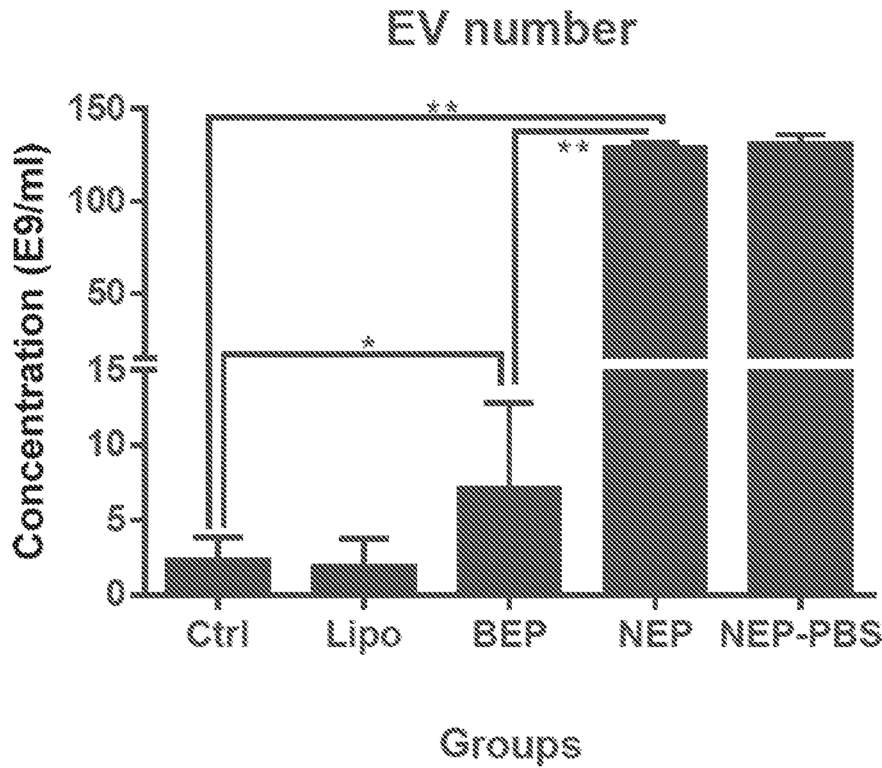


Figure 3. NEP cell transfection with or without DNA plasmids significantly stimulates the EV secretion from transfected mouse embryonic fibroblast (MEF) cells, with performance much better than lipofectamine (Lipo) and BEP based cell transfection. Ctrl stands for non-transfected MEF cells; NEP stands for NEP cell transfection with DNA plasmids; NEP-PBS stands for NEP cell transfection with PBS buffer only. The DNA plasmids used are Achaete-Scute Complex Like-1 (Ascl1), Pou Domain Class 3 Transcription factor 2 (Pou3f2 or Brn2) and Myelin Transcription Factor 1 Like (Myt1l) at a weight ratio of 2/1/1. A mixture of those DNA plasmids is known to reprogram donor cells into induced neurons (iNs). Same number of MEF cells were transfected with DNA plasmids by various techniques, and cell culture mediums were collected 24 h post-transfection. The EV numbers were detected by NanoSight™. For the BEP, the transfection voltage was 1250 volts. For NEP, the transfection voltage was 220 volts with five 10 ms pulses.

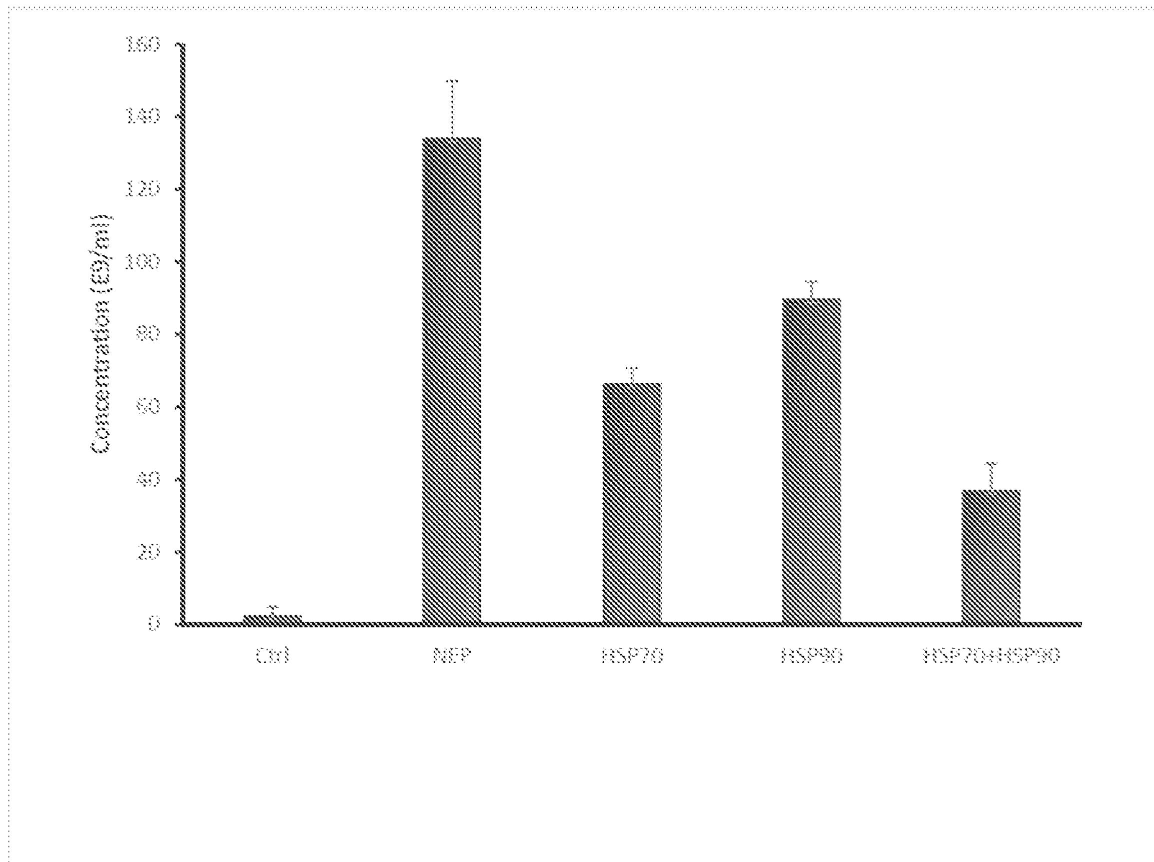


Figure 4. Effect of heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) inhibitors on EV secretion from NEP transfected MEF cells. After NEP transfection, the cell culture was replaced with fresh medium containing HSP70 inhibitor (VER 155008, 50 μM), HSP90 inhibitor (NVP-HSP990, 1 μM), or their mixture. Medium was collected at 24 h post-transfection, and EV numbers were detected by dynamic light scattering (DLS) goniometry.

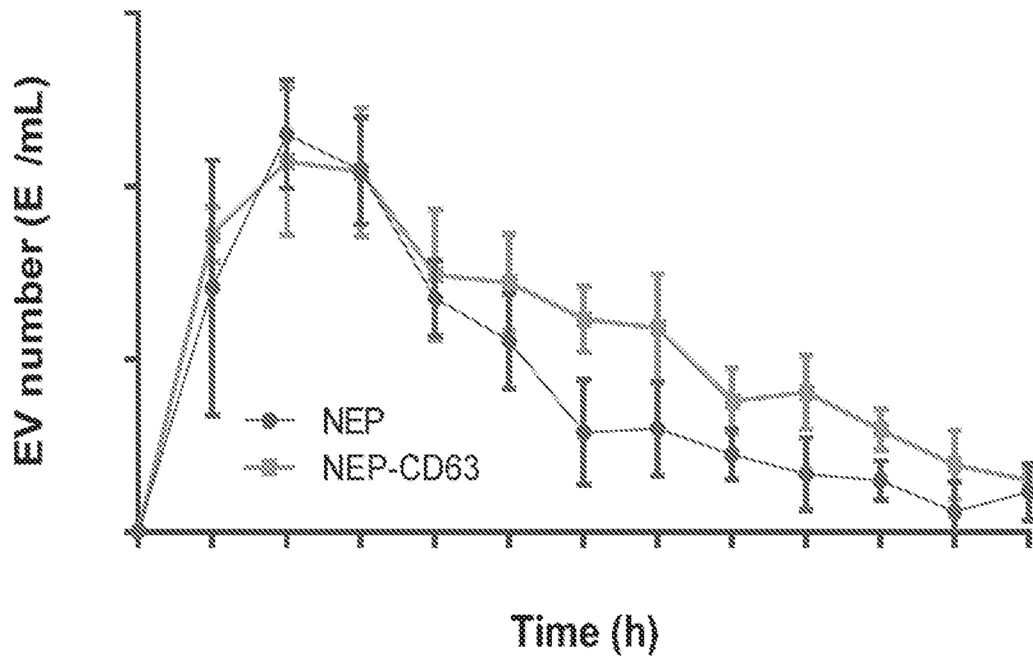
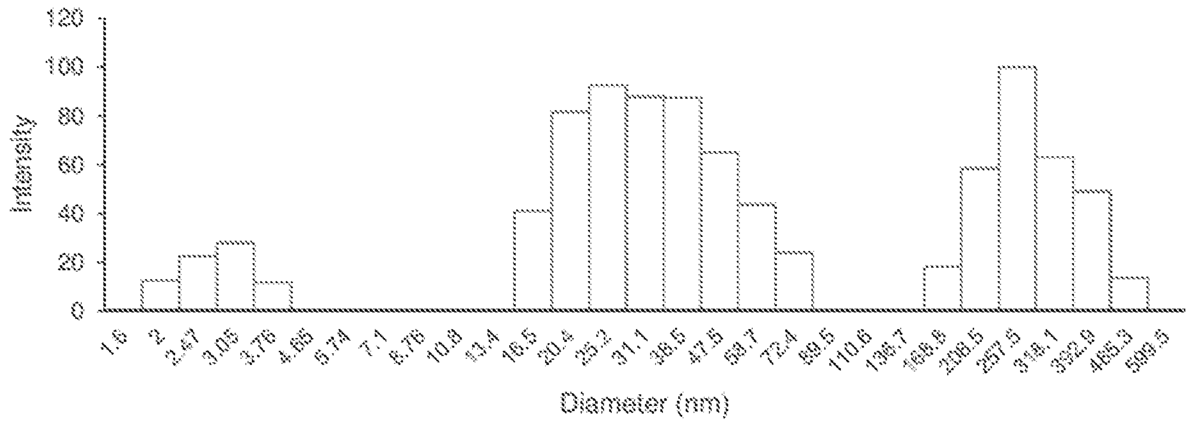


Figure 5. Effect of NEP transfection of CD63 DNA plasmid on EV secretion from MEF cells. Cells were transfected with or without CD63 plasmid by NEP. The cell culture medium was collected and replaced with fresh medium every 4 hr. The EV numbers were detected by DLS goniometry.



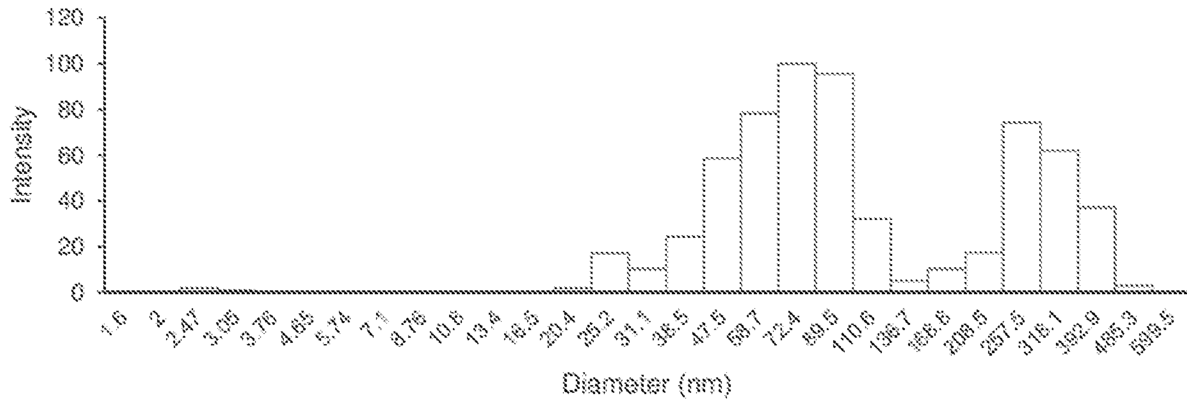
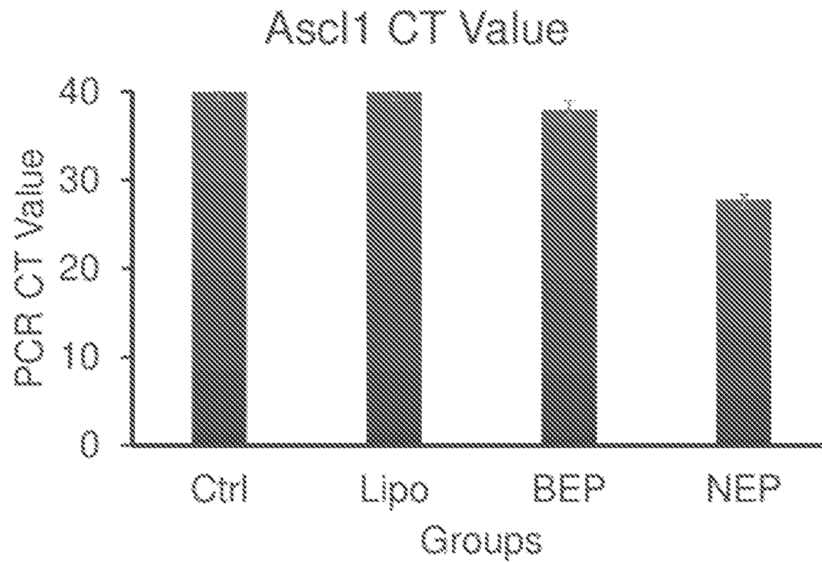


Figure 6. Size distribution measured by DLS goniometry of EVs with or without NEP harvested at 24 h post-cell transfection. The cell culture medium was collected 24 h post NEP transfection, and cell debris was removed by centrifugation at 1500 g for 10 min. EVs in supernatant were measured by DLS.



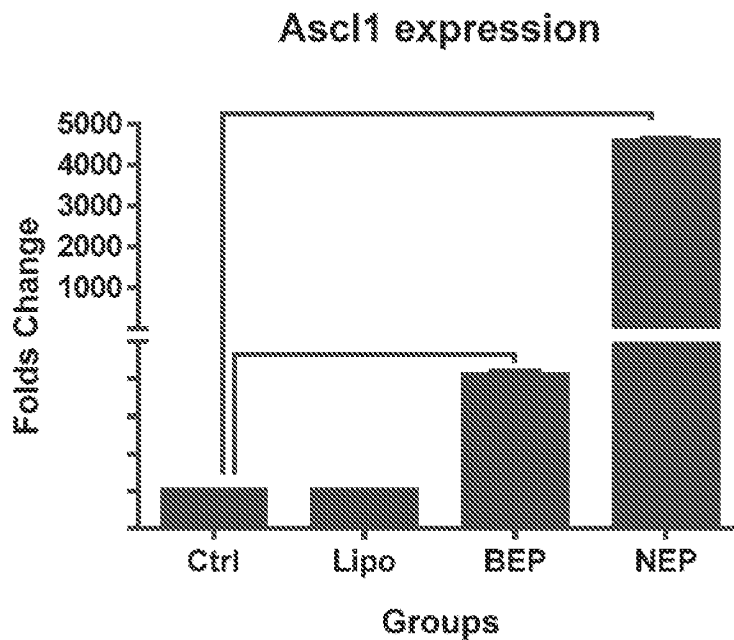


Figure 7. EV Ascl1 mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection. Total RNAs were obtained and reverse transcript according to manufacturer's instruction. The same amount of total RNA (20 ng) was used for Ascl1 detection by qRT-PCR.

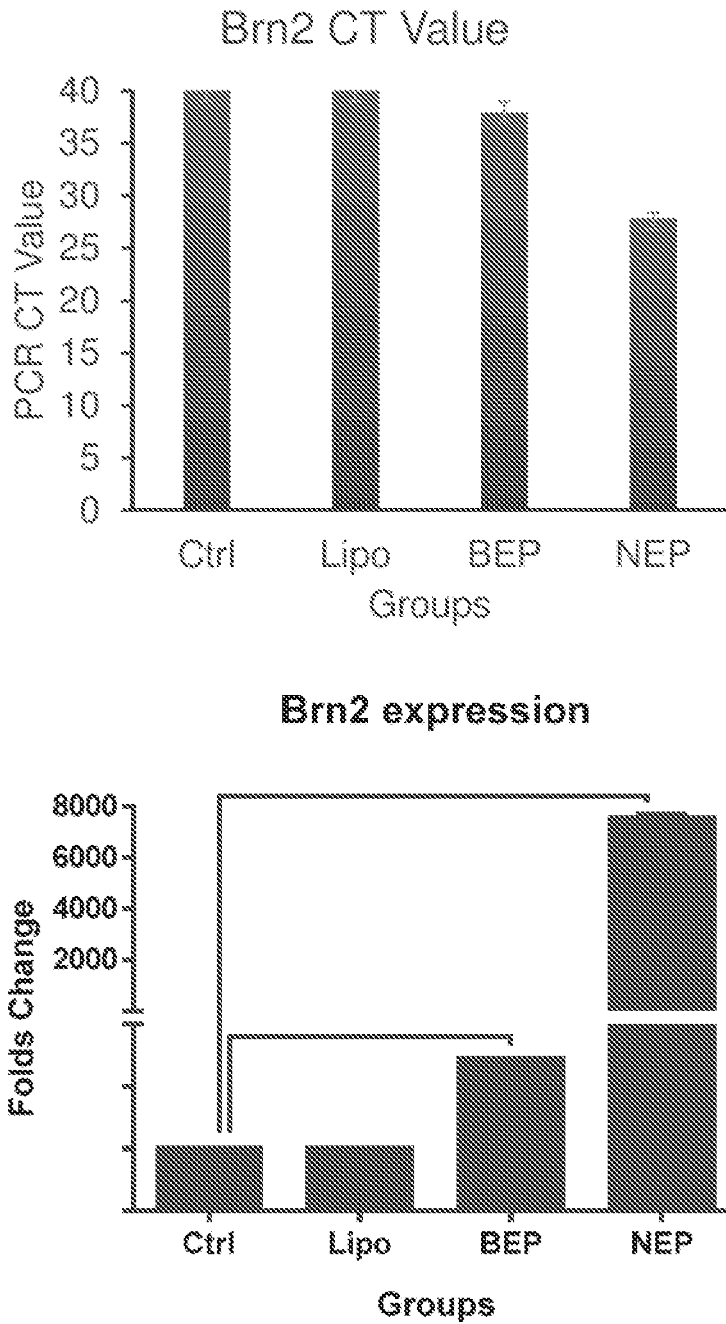


Figure 8. EV Brn2 mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection. Total RNAs were obtained and reverse transcript according to manufacturer's instruction. The same amount of total RNA (20 ng) was used for Ascl1 detection by qRT-PCR.

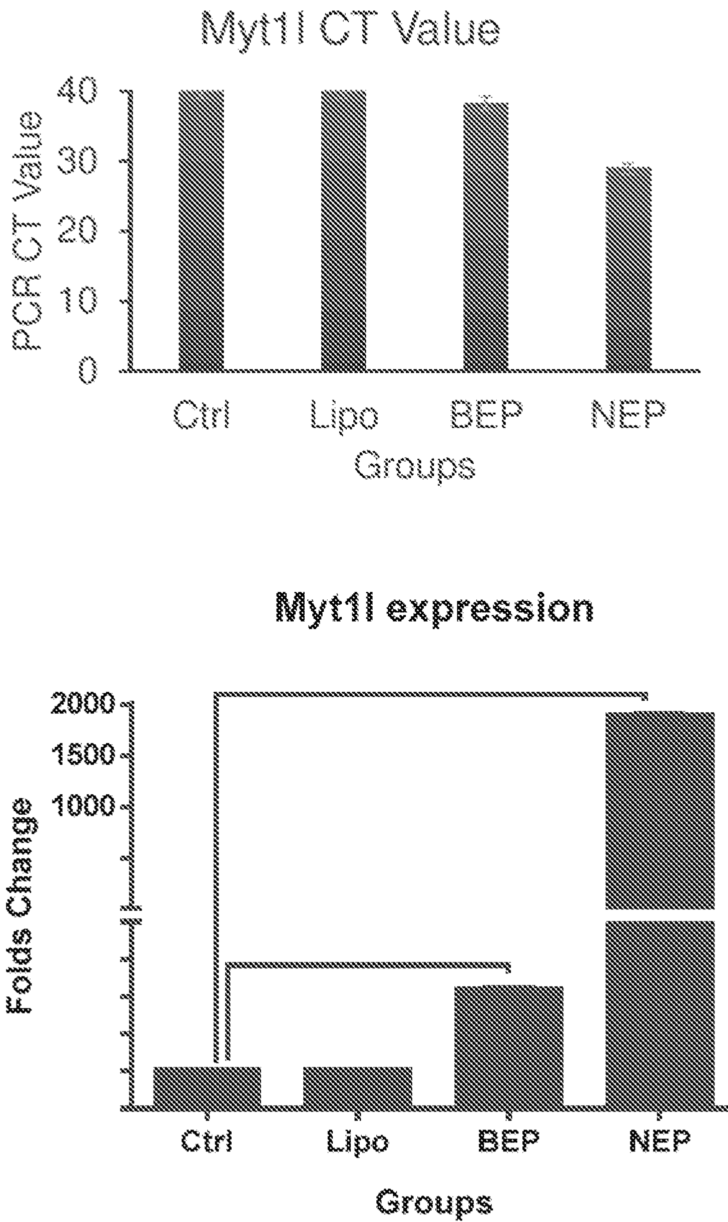


Figure 9. EV Myt1l mRNA expression determined by qRT-PCR from MEF cells transfected by Ascl1/Brn2/Myt1l DNA plasmids at a ratio of 2/1/1 using various techniques at 24 h post-transfection. Total RNAs were obtained and reverse transcript according to manufacturer's instruction. The same amount of total RNA (20 ng) was used for Ascl1 detection by qRT-PCR.

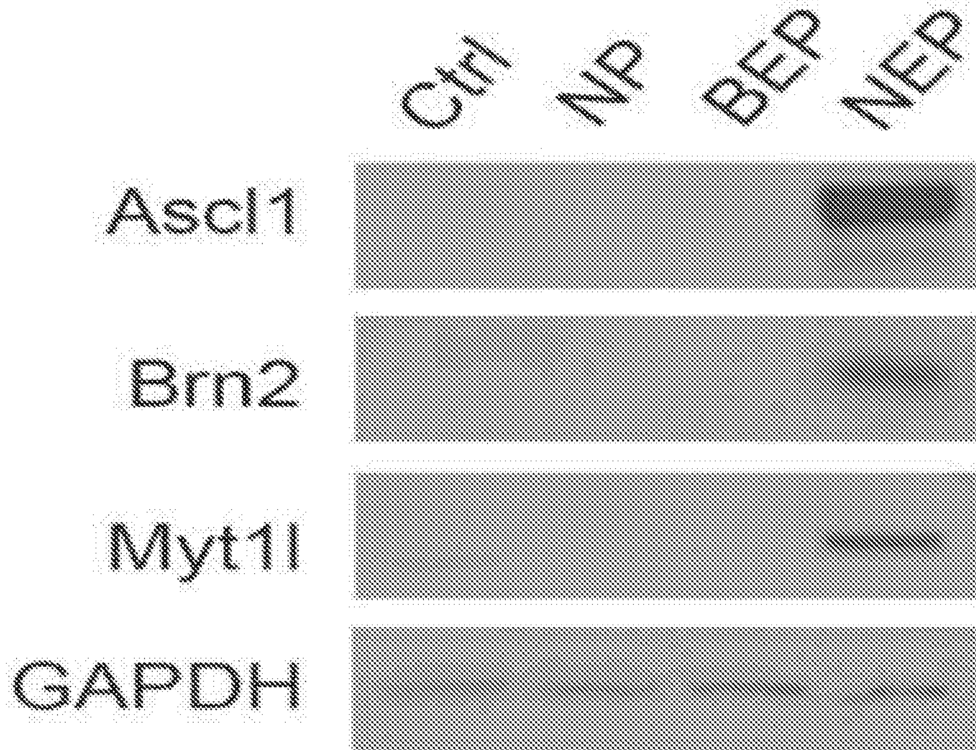


Figure 10. Only EVs obtained by NEP contain functional mRNA determined by *in vitro* translation. Same amount of total RNAs (1 µg) from each transfection group were used for *in vitro* protein translation according to manufacturer's instruction. Samples were separated by SDS-PAGE, and the proteins were detected with antibodies.

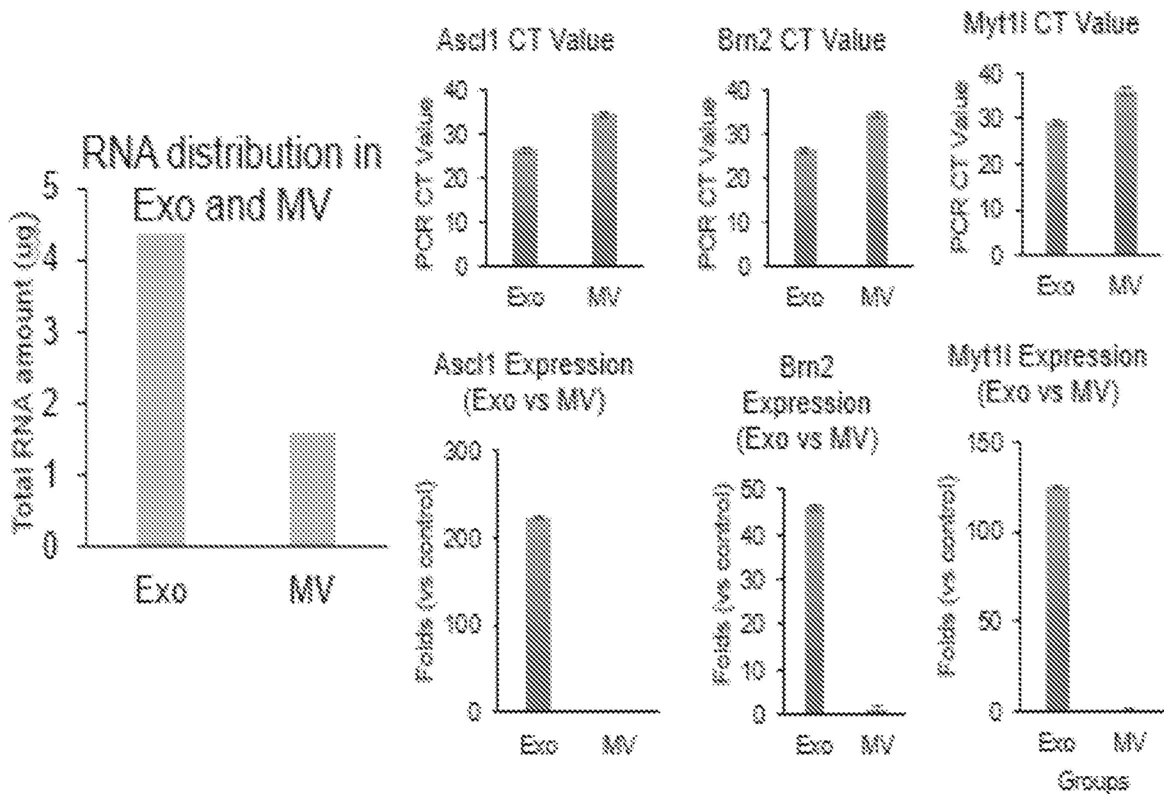


Figure 11. EV-mRNAs from NEP are found in the exosomes (Exo), not in microvesicles (MV). EVs were collected from cell culture medium by simply centrifugation at 1500 g for 10 min. Microvesicles were harvested by ultracentifugation at 10,000 g for 30 min. The supernatant was further centrifugated at 100,000 g for 2 h to collect the exosomes. Total RNAs were collected from these two parts, and the total mRNA concentration was measured by Nanodrop™. The EV mRNA expressions were measured by qRT-PCR.

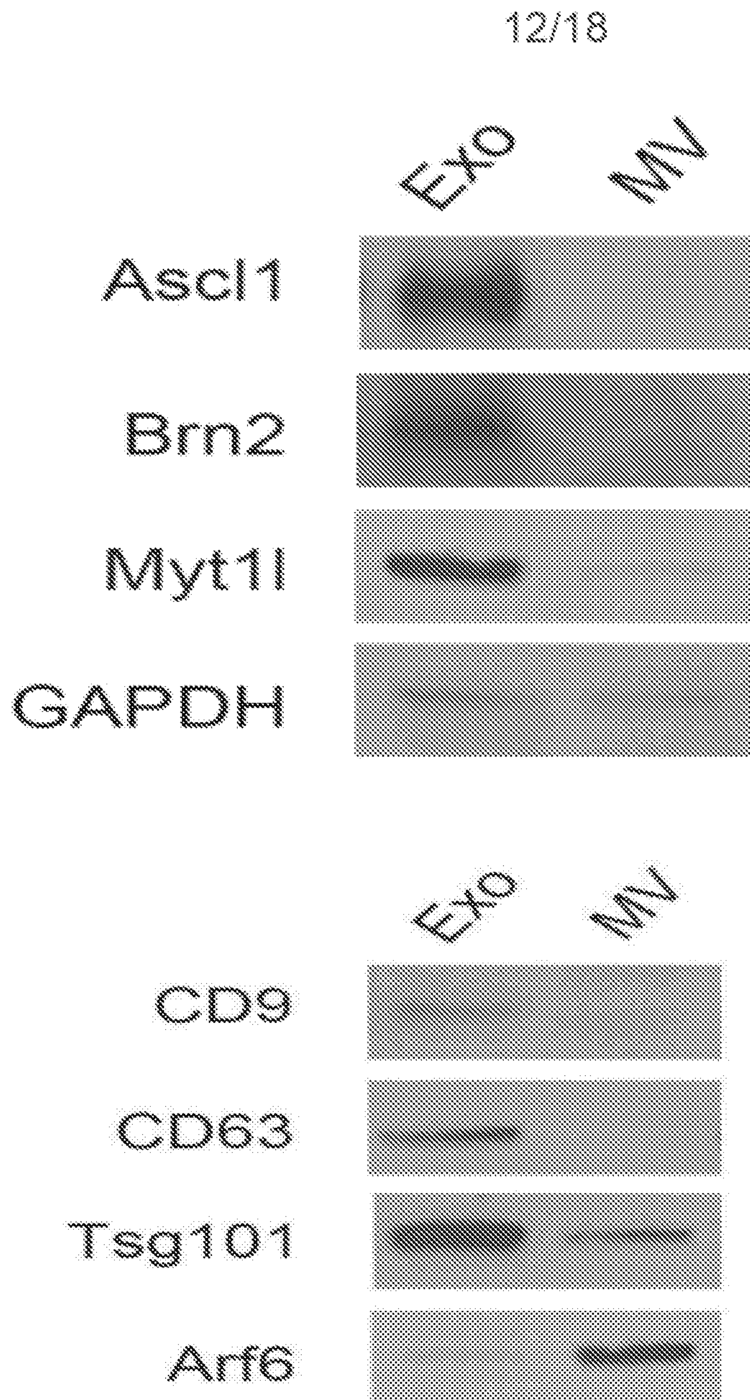


Figure 12. Exosome (Exo)-mRNAs, not microvesicle (MV)-RNAs, from NEP from NEP cell transfection can translate proteins. EVs were collected from cell culture medium by simply centrifugation at 1500 g for 10 mins. Microvesicles were harvested by ultracentifugation at 10,000 g for 30 min. The supernatant was further centrifugated at 100,000 g for 2 h to collect the exosomes. Total RNAs were collected from these two parts as described above, and 1 μg of total RNA was used for *in vitro* translation. The samples were separated by SDS-PAGE, and the proteins, exosome and microvesicle markers were detected by Western blotting.

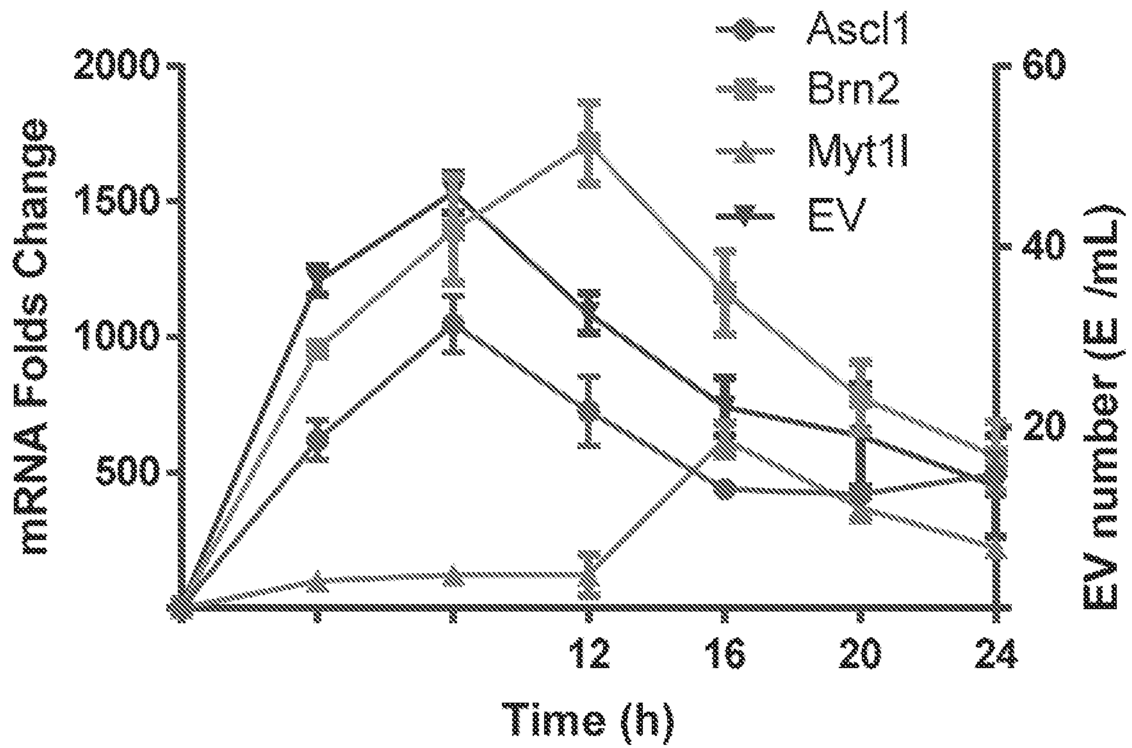


Figure 13. EV-mRNAs secretion profiles from NEP transfected MEF cells. MEF cells were transfected with DNA plasmids by NEP. The cell culture medium was collected at indicated time points, and replaced with fresh medium. The EV numbers were detected by DLS goniometry. The mRNA expressions were detected by qRT-PCR.

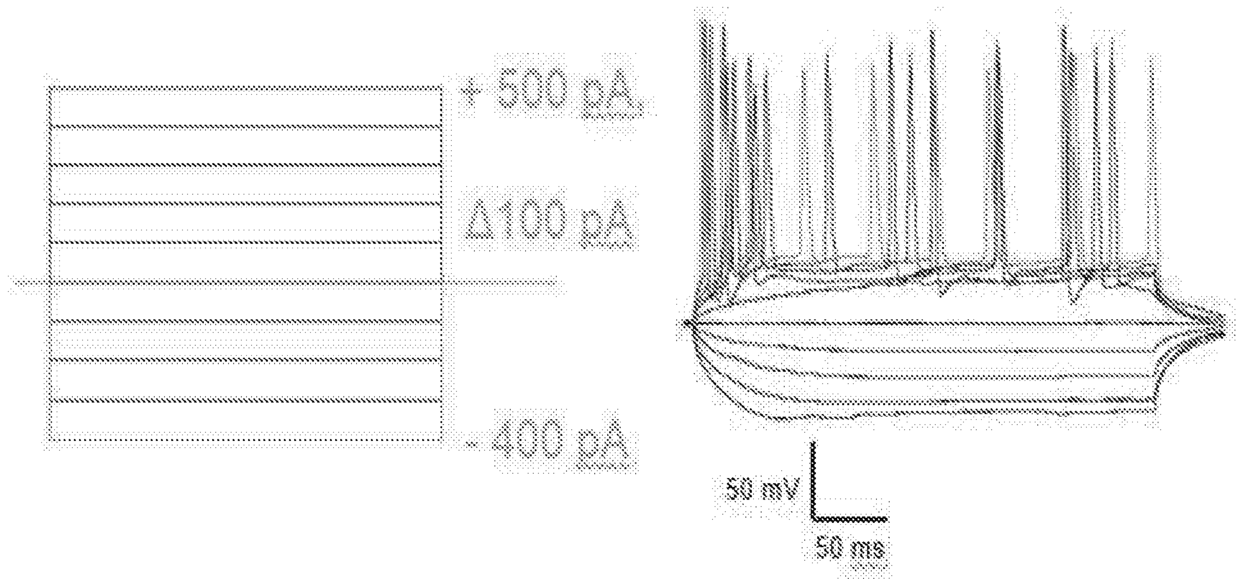


Figure 14. Action potential detection by patch clamp shows that MEF cells transfected every other day with Ascl1/Brn2/Myt1l mRNA containing EVs obtained from NEP could be reprogrammed into functional induced neurons (iNs) after 24 days. NEP transfected MEF cells were reprogrammed into iNs after 21 days

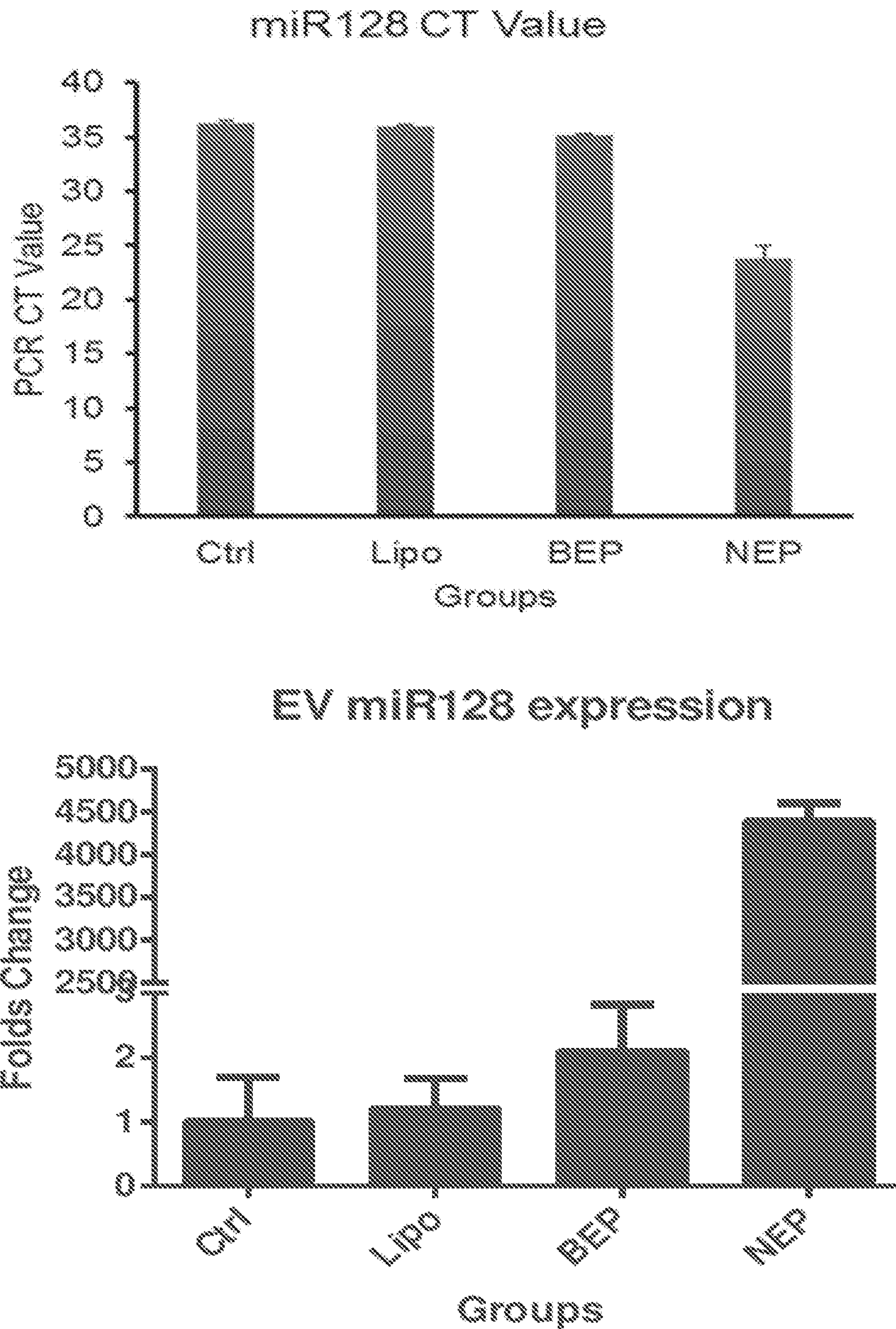


Figure 15. EV miR-128 expression determined by qRT-PCR from MEF cells transfected by miR-128 DNA plasmid using various techniques at 24 h post-transfection. EVs were harvested from cell culture medium at 24 h post-transfection (miR-128 plasmid) by various techniques. Total RNAs were obtained according to manufacturer's instruction. The same amount of total RNA (30 ng) was used for miR-128 detection by qRT-PCR.

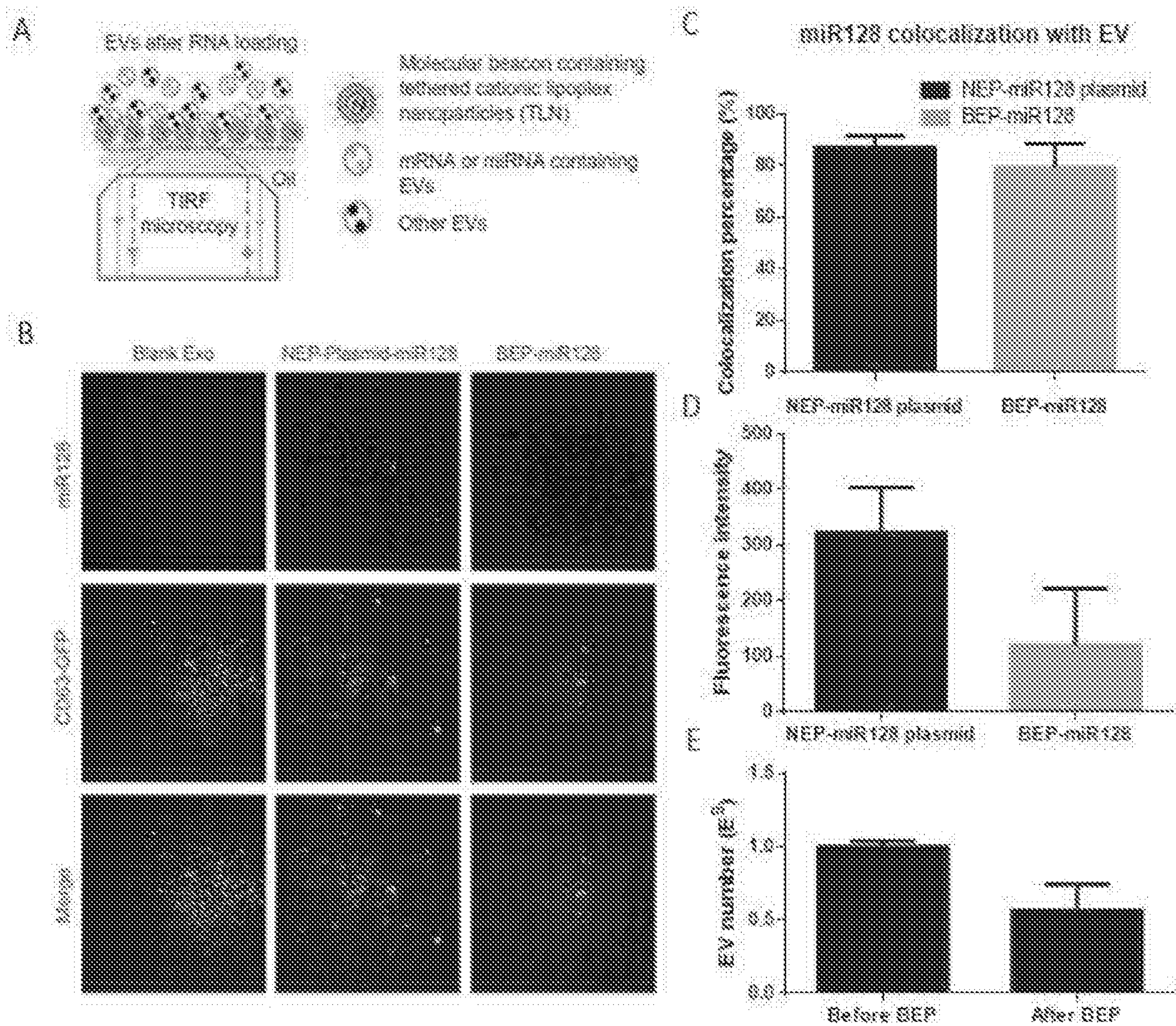


Figure 16. Comparison of secreted EVs containing miR-128 by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected miR-128 by BEP post-insertion.

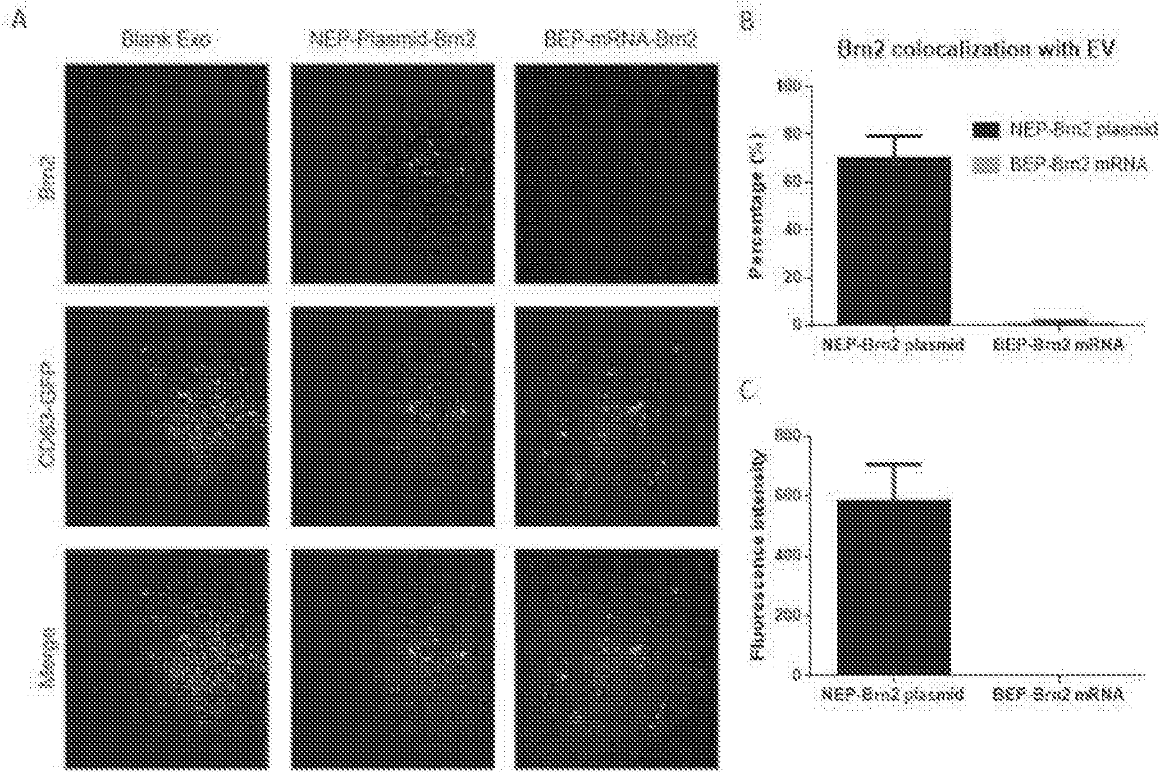


Figure 17. Comparison of secreted EVs containing Brn2 mRNA by NEP transfection of DNA plasmid to MEF cells vs. existing EVs loaded with pre-collected Brn2 mRNA by BEP post-insertion.

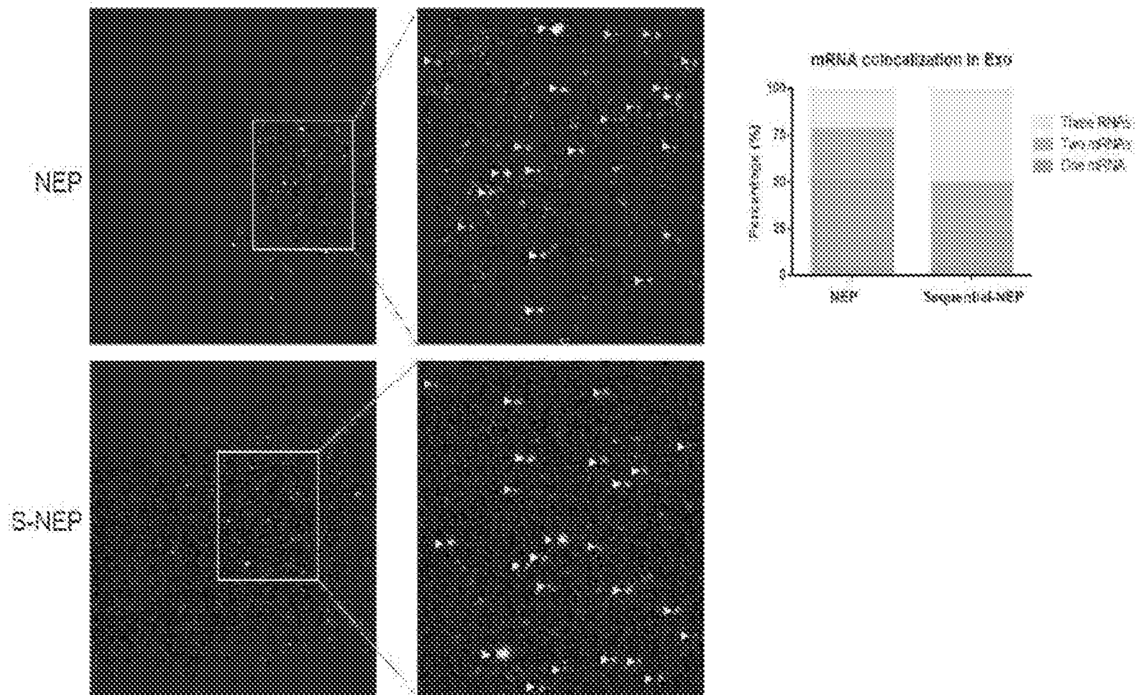


Figure 18. Increased mRNA co-localization in the same EV by sequential-NEP. For NEP transfection, Ascl1, Brn2 and Myt1l plasmids were transfected at the same time as described before. For sequential-NEP, the Myt1l plasmid was transfected first, Brn2 plasmid was transfected 4 h later, while Ascl1 plasmid was transfected 4 h after Brn2 transfection. At 24 h post Myt1l transfection, culture medium was collected for TLN assay. Equal amount of FAM-Ascl1, Cy3-Brn2, and Cy5-Myt1l MBs were encapsulated in tethered lipoplex nanoparticles for EV-mRNA detection. Yellow arrow: EVs containing 3 mRNAs; Blue arrow: EVs containing 2 mRNAs; and Pink arrow: EVs containing 1 mRNA.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/45333

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/00, A61M 37/00, A61M 5/158 (2018.01)
CPC - A61K 9/00, A61M 37/00, A61M 5/158, C12N 15/87

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MIZRAK et al. Genetically engineered microvesicles carrying suicide mRNA/protein inhibit schwannoma tumor growth. Molecular Therapy. 1 January 2013. Volume 21, No 1, pp 101-8. Especially page 101, col 2, para 2; page 105, col 1, last para	1-3, 13
Y	US 2014/0256047 A1 (THE OHIO STATE UNIVERSITY) 11 September 2014 (11.09.2014) Claim 6; Claim 9; para [0003]; para [0011]; para [0042]; para [0059]; para [0071]	1-3, 13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 September 2018	24 OCT 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/45333

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-12, 14
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.