

## Description

### Title of Invention: ENGINEERED EXOSOMES FOR SKIN REJUVENATION

#### Field of the Invention

- [0001] The present disclosure relates to engineered exosomes, in particular to an exosome carrying different fusion proteins anchored external to the membrane of the exosome. The present disclosure also relates to a composition comprising the engineered exosome, a nucleic acid construct comprising the polynucleotide encoding the fusion proteins and uses of the engineered exosome in skin rejuvenation.

#### Background

- [0002] Sun, skin disorders, aging and even heredity can all contribute to skin irregularities on the face and elsewhere on the body. These include textural irregularities like wrinkles and acne scars, pigmentation changes like freckles, sunspots or visible blood vessels. In addition, skin may lose tone, feel less firm and lose the healthy glow that is evident in younger skin.
- [0003] Skin rejuvenation refers to any procedure that aims to improve the appearance of the skin. Different treatment modalities are available to treat the different aspects of skin damage. Laser resurfacing, mechanical resurfacing, chemical peels and injectable products can improve the appearance of fine lines and wrinkles of the entire face or those that develop in specific regions of the face, such as the upper lip and around the eyes. These treatments can also be used to address pigmentation disorders, such as sun and age spots, and they can be used to improve the appearance of acne scars or other skin conditions.
- [0004] Regenerative medicine-based products can be classified into following two categories: cell-based and cell-free therapies. Both these categories can be autologous or allogeneic. The most common regenerative medicine-based products include cell-based methods utilizing autologous adult stem cells, such as mesenchymal stem cells (MSCs), fibroblasts, emerging blood-derived cellular therapies, such as platelet-rich plasma (PRP) products, and the clinical application induced pluripotent stem cells (iPSCs) is progressing, with cell therapies for several ailments being under clinical investigations, especially those in line with the use of iPSC-derived products with less risk, such as the iPSC-derived MSCs (iPSC-MSCs). Cell-free products are mostly based on the secretory components of MSCs, such as MSC-derived exosomes (MSC-exo), MSC-derived conditioned media (MSC-CM), and MSC-derived extracellular vesicles (MSC-EVs). Autologous transplantation is performed by isolating and culturing MSCs from the patient's own tissue sources, such as adipose MSCs (AD-

MSCs) , bone marrow MSCs (BM-MSCs) , umbilical cord MSCs (UC-MSC) , fetal dermis MSCs (FD-MSC) , endometrial and menstrual blood (eMSC/MenSC) . Autologous PRP is obtained by collecting and centrifuging the patient's own blood. The molecular mechanisms underlying the action of regenerative medicine-based therapies continue to be studied. However, the unique properties of MSCs, such as self-renewal, multidirectional differentiation, regulation of inflammation, immunomodulation, angiogenesis, and the hematopoietic abilities of PRP exhibited through the release of cytokines and various growth factors, are considered to play important roles in skin regeneration.

[0005] There exists a need for additional and improved methods and compositions for skin rejuvenation.

### **Summary of the Invention**

[0006] In one aspect of the invention, provided is an engineered exosome, comprising (a) a KGF polypeptide, fused to a first anchoring polypeptide, (b) an EGF polypeptide, fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide, wherein (a) , (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.

[0007] In some embodiments, the engineered exosome further comprises (d) a PDGF-BB polypeptide, fused to a fourth anchoring polypeptide, and the PDGF-BB polypeptide is exposed on the outer surface of the membrane of the exosome.

[0008] In some embodiments, the KGF polypeptide is a KGF-1 polypeptide, preferably the KGF-1 polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 1.

[0009] In some embodiments, the EGF polypeptide is a human EGF polypeptide or an ortholog or paralog thereof, preferably the EGF polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 2.

[0010] In some embodiments, the FGF-2 polypeptide is human FGF-2 or an ortholog or paralog thereof; more preferably the FGF-2 polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 3.

[0011] In some embodiments, the PDGF-BB polypeptide is a human PDGF-BB dimer; more preferably the PDGF-BB dimer comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 4.

- [0012] In some embodiments, the anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins.
- [0013] In preferable embodiments, the anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the first, second, third, and fourth, when present, anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, third, and fourth, when present, anchoring polypeptides is a TM3 domain of CD63. In some embodiments, each of the anchoring polypeptides is a TM3 domain of CD63; preferably, the TM3 domain of CD63 comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 9.
- [0014] In some embodiments, the KGF polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide, when present, is fused to the N or C-terminus of respective anchoring polypeptide, optionally through a peptide linker, preferably, the peptide linker consists of glycine and serine, e.g., (G4S)<sub>n</sub>, in which n is an integer from 1 to 3.
- [0015] In some embodiments, the exosome is not derived from a mesenchymal stem cell; preferably, the exosome is not derived from a stem cell.
- [0016] In some embodiments, the exosome (i) enhances fibroblast proliferation, (ii) up-regulates COL2A1, COL3A1, fibronectin, TIMP1, and/or COL1A1B genes, (iii) downregulates MMP7, MMP9, and/or MMP16B genes, and/or (iv) downregulates melanin synthesis.
- [0017] Another aspect of the disclosure relates to a composition comprising the exosome as described herein, and a carrier; preferably, the composition is a liquid formulation; more preferably, the composition is formulated for topical or subcutaneous administration.
- [0018] In some embodiments, the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide), an EGF polypeptide, a FGF-2 polypeptide (e.g., a human FGF-2 polypeptide), or a PDGF-BB polypeptide (e.g., a human PDGF-BB dimer polypeptide), not attached to the membrane of the exosome.
- [0019] Another aspect of the disclosure relates to a nucleic acid construct comprising a polynucleotide encoding (a) a KGF polypeptide fused to a first anchoring polypeptide,

(b) an EGF polypeptide fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide.

- [0020] In some embodiments, the nucleic acid construct comprising a polynucleotide further encoding (d) a PDGF-BB polypeptide, fused to a fourth anchoring polypeptide.
- [0021] In some embodiments, the KGF polypeptide is a KGF-1 polypeptide, preferably the KGF-1 polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 1.
- [0022] In some embodiments, the EGF polypeptide is a human EGF polypeptide or an ortholog or paralog thereof, preferably the EGF polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 2.
- [0023] In some embodiments, the FGF-2 polypeptide is a human FGF-2 or an ortholog or paralog thereof; more preferably the FGF-2 polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 3.
- [0024] In some embodiments, the PDGF-BB polypeptide is a human PDGF-BB dimer; more preferably the PDGF-BB dimer comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 4.
- [0025] In some embodiments, a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 5 or a degenerate sequence thereof.
- [0026] In some embodiments, a polynucleotide encoding the EGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 6 or a degenerate sequence thereof.
- [0027] In some embodiments, a polynucleotide encoding the FGF-2 polypeptide has a nucleotide sequence as shown in SEQ ID NO. 7 or a degenerate sequence thereof.
- [0028] In some embodiments, a polynucleotide encoding the PDGF-BB polypeptide has a nucleotide sequence as shown in SEQ ID NO. 8 or a degenerate sequence thereof.
- [0029] In some embodiments, the anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins.
- [0030] In preferable embodiments, the anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the first, second, third, and fourth, when present, anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, third,

and fourth, when present, anchoring polypeptides is a TM3 domain of CD63. In some embodiments, a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO. 10 or a degenerate sequence thereof.

- [0031] In some embodiments, the polynucleotide is a single polynucleotide comprising a fragment of nucleotides encoding the polypeptides (a) , (b) , (c) and, optionally, (d) , with each of the polypeptides (a) , (b) , (c) and, optionally, (d) linked to the anchoring polypeptide; preferably the polypeptides (a) , (b) , (c) and, optionally, (d) are spaced by self-cleavage peptides; optionally, the self-cleavage peptides are 2A peptides, e.g., T2A, E2A, P2A or any combination thereof.
- [0032] Another aspect of the disclosure relates to a vector comprising the nucleic acid construct as described in any of the nucleic acid construct described herein.
- [0033] Another aspect of the disclosure relates to a cell transduced with the vector as described herein, wherein the polynucleotide is integrated into the genome of the cell.
- [0034] In some embodiments, the cell is not a mesenchymal stem cell; preferably, the cell is not a stem cell; preferably, the cell is a mammalian cell; or more preferably, the cell is a HEK293 or CHO cell.
- [0035] Another aspect of the disclosure relates to a method of producing an engineered exosome as disclosed herein, comprising (a) transducing the cell as described above with the vector as described above; (b) culturing the cell in a condition allowing secretion of an exosome from the cell; and (c) collecting and purifying the exosome.
- [0036] In some embodiments, the method further comprises adapting the cell to a serum-free condition during step (b) .
- [0037] A further aspect of the disclosure relates to use of the engineered exosomes, or the composition as disclosed herein manufacturing a medicament for skin rejuvenation; preferably, the skin rejuvenation includes treatment of any of sun damage, sensitive skin, scars such as from acne or stretch marks such as striae gravidarum or adolescent striae, chickenpox, or injury, wrinkles such as static or dynamic wrinkles, dark circle under eyes, hyperpigmentation such as darkened skin or freckles, loosen skin, uneven or dull skin tone, or any of combination thereof.
- [0038] A further aspect of the disclosure relates to a method for skin rejuvenation; preferably, the skin rejuvenation includes treatment of any of sun damage, sensitive skin, scars such as from acne or stretch marks such as striae gravidarum or adolescent striae, chickenpox, or injury, wrinkles such as static or dynamic wrinkles, dark circle under eyes, hyperpigmentation such as darkened skin or freckles, loosen skin, uneven or dull skin tone, or any of combination thereof.
- [0039] A further aspect of the disclosure relates to the engineered exosomes, or the composition as disclosed herein for use in for skin rejuvenation; preferably, the skin rejuvenation includes treatment of any of sun damage, sensitive skin, scars such as from

acne or stretch marks such as striae gravidarum or adolescent striae, chickenpox, or injury, wrinkles such as static or dynamic wrinkles, dark circle under eyes, hyperpigmentation such as darkened skin or freckles, loosen skin, uneven or dull skin tone, or any of combination thereof.

[0040] These and other aspects and advantages of the disclosure will be apparent from the detailed description provided in the following.

Brief Description of the Figures

[0041] Figure 1. Construction of stable cell lines, which can secrete engineered exosomes loading with functional proteins. Mammalian cells, e.g. HEK293 cells were cultured and infected with lentivirus, which packaged with functional genes, EGF, FGF-2, KGF-1 and PDGF-BB. Then antibiotics was used for stable cell line selection. After three passages selection, the expression of the functional genes was identified in both cell pellet and exosomes. After confirmation of the expression, an adaptation of stable cell line to a serum-free condition was performed. Finally, collect the cultured supernatant from the serum-free stable cell line and the identify the exosomes purified via ultracentrifuge.

[0042] Figure 2. Identification of engineered exosomes derived from the stable cell line. (A) The particle diameter and particle concentration of exosomes derived from the stable cell lines (NO. 6 and NO. 8) were analyzed by NanoFCM. (B) The exosomes from stable cell lines (NO. 6 and NO. 8) were further inspected by transmission electron microscopy (TEM) . (C) Immunoblotting analysis of exosomes with antibodies against CD63, the fused exosomes scaffold protein.

[0043] Figure 3. Engineered exosomes exhibit enhanced cell proliferation activity on fibroblast. Two fibroblast cell lines, HSF (human skin fibroblast) (A) and HFF (human foreskins fibroblast) (B) were cultured and incubation with exosomes (NO. 6 and NO. 8) at different concentrations (0.5 $\mu$ g, 1.0 $\mu$ g, 2.0 $\mu$ g) . Functional protein, KGF (KGF-1) was used as a positive control. After 48h incubation, the cell viability was evaluated via CCK8 assay.

[0044] Figure 4. Engineered exosome regulated skin regenerated associated genes on skin fibroblast. The skin fibroblasts (HSF) were cultured and incubated with exosomes (NO. 6 or NO. 8) for 48h. Then, RNA was extracted from the cell pellets and skin regenerated associated genes including COL2A1 (A) , COL3A1 (B) , Fibronectin (C) , TIMP1 (D) and MMP7 (E) were detected via RT-qPCR.

[0045] Figure 5. Engineered exosomes regulated skin regenerated associated genes in zebrafish model. The zebrafish embryos (4dpf, days post fertilization) were prepared to have a yolk sac. injection with exosomes (NO. 6 or NO. 8) . Twenty-four hours after injection, the gene expression analysis in zebrafish was performed via RT-qPCR to

detect the skin regenerated associated genes, including COL1A1B (A) , MMP9 (B) and MMP16B (C) .

- [0046] Figure 6. Engineered exosomes contribute to downregulate the melanin synthesis. (A) The human melanocytes were cultured and incubated with exosomes (NO. 6 or NO. 8) for 48h. Then, cell pellets were used for melanin extraction and the concentration of melanin was measured via Melanin Assay Kit (Fluorometric) . (B) In zebrafish model, the zebrafish embryos (4hpf, hours post fertilization) were cultures in the dark condition within exosomes (NO. 8) for 45h at 28°C. Then, images were captured under the dissecting microscope and analyzed to evaluate the melanin. The whitening efficacy (%) = (Mock-NO. 8) /Mock\*100%.

### **Detailed Description of the Invention**

- [0047] Definition
- [0048] The words “a” and “an” when used in the present specification in concert with the word comprising, including the claims, denote “one or more. ”
- [0049] As used herein, the terms “or” and “and/or” are utilized to describe multiple components in combination or exclusive of one another. For example, “x, y, and/or z” can refer to “x” alone, “y” alone, “z” alone, “x, y, and z, ” “ (x and y) or z, ” “x or (y and z) , ” or “x or y or z. ” It is specifically contemplated that x, y, or z may be specifically excluded from an embodiment.
- [0050] Throughout this application, the term “about” is used according to its plain and ordinary meaning in the area of cell and molecular biology to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.
- [0051] “Homology” or “identity” or “similarity” refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. An “unrelated” or “non-homologous” sequence shares less than 40%identity, though preferably less than 25%identity, with one of the sequences of the present disclosure.
- [0052] A polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%or 99%) of “sequence identity” to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences.

This alignment and the percent homology or sequence identity can be determined using software programs known in the art.

[0053] The term “linker” as used herein refers to a short fragment of amino acid (AA) or nucleotide sequence containing two or more amino acids or nucleotides which may be same or different.

[0054] As used herein, “cell line” refers to a population of cells formed by one or more sub-cultivations of a primary cell culture. Each round of subculturing is referred to as a passage. When cells are subcultured, they are referred to as having been passaged. A specific population of cells, or a cell line, is sometimes referred to or characterized by the number of times it has been passaged. For example, a cultured cell population that has been passaged ten times may be referred to as a P10 culture. The primary culture, i.e., the first culture following the isolation of cells from tissue, is designated P0. Following the first subculture, the cells are described as a secondary culture (P1 or passage 1) . After the second subculture, the cells become a tertiary culture (P2 or passage 2) , and so on. It will be understood by those of skill in the art that there may be many population doublings during the period of passaging; therefore, the number of population doublings of a culture is greater than the passage number. The expansion of cells (e.g., the number of population doublings) during the period between passaging depends on many factors, including but not limited to seeding density, substrate, medium, growth conditions, and time between passaging.

[0055] The terms “reduce,” “inhibit,” “diminish,” “suppress,” “decrease,” “prevent” and grammatical equivalents (including “lower,” “smaller,” etc. ) when in reference to the expression of any symptom in an untreated subject relative to a treated subject, mean that the quantity and/or magnitude of the symptoms in the treated subject is lower than in the untreated subject by any amount that is recognized as clinically relevant by any medically trained personnel. In one embodiment, the quantity and/or magnitude of the symptoms in the treated subject is at least 10%lower than, at least 25%lower than, at least 50%lower than, at least 75%lower than, and/or at least 90%lower than the quantity and/or magnitude of the symptoms in the untreated subject.

[0056] As used herein, the term “therapeutically effective amount” is synonymous with “effective amount” , “therapeutically effective dose” , and/or “effective dose” and refers to the amount of compound that will elicit the biological, cosmetic, or clinical response being sought by the practitioner in an individual in need thereof. As one example, an effective amount is the amount sufficient to reduce hair loss. The appropriate effective amount to be administered for a particular application of the disclosed methods can be determined by those skilled in the art, using the guidance provided herein. For example, an effective amount can be extrapolated from in vitro and in vivo assays as described in the present specification. One skilled in the art will

recognize that the condition of the individual can be monitored throughout the course of therapy and that the effective amount of an exosome or composition disclosed herein that is administered can be adjusted accordingly.

- [0057] As used herein, the terms “treatment,” “treat,” or “treating” refers to intervention in an attempt to alter the natural course of the individual or cell being treated and may be performed either for prophylaxis or during the course of pathology of a disease or condition. Treatment may serve to accomplish one or more of various desired outcomes, including, for example, preventing occurrence or recurrence of disease, alleviation of symptoms, and diminishment of any direct or indirect pathological consequences of the disease, lowering the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.
- [0058] The term “subject,” as used herein, may be used interchangeably with the term “individual” or “patient” and generally refers to an individual in need of a therapy. The subject can be a mammal, such as a human, dog, cat, horse, pig, or rodent.
- [0059] “Carrier”, as used herein, refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic agent is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsions, tablets, pills, capsules, powders, sustained-release formulations, and the like. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.
- [0060] The term “N-terminal amino acid residue” or “N-terminus” refers to the first amino acid residue (amino acid number 1) of a polypeptide or peptide. The term “C-terminal amino acid residue” or “C-terminus” refers to the last amino acid residue (amino acid number n, wherein n = the total number of residues in the peptide or polypeptide) of a polypeptide or peptide.
- [0061] The term “KGF” refers to keratinocyte growth factor, which is an epithelial mitogen and a member of the fibroblast growth factor (FGF) family. The FGF family contains

22 members in vertebrates which are essential for the regulation of numerous developmental processes. KGF include two functionally similar variants, i.e., KGF-1 (also known as FGF-7) and KGF-2 (also known as FGF-10). Characterization of recombinant human fibroblast growth factor FGF-10 reveals functional similarities with keratinocyte growth factor (FGF-7). These two growth factors interact with the same high affinity receptor, the KGFR isoform of FGFR2, which differs from FGFR2 in the second half of the third immunoglobulin loop and is encoded by an alternative exon. The ability of KGF-1 and KGF-2 to bind the KGFR with high affinity distinguishes them from other members of the FGF-10 family. KGF-2 is highly related to KGF-1, it binds to the same receptor as KGF-1 and shares 57% sequence homology. Human KGF-2 is 96% identical to the rat KGF-2 and specifically stimulates growth of normal human epidermal keratinocytes. The amino acid sequence of human KGF-1 is available from UniProtKB/Swiss-Prot: P21781.1, and a mature chain of human KGF-1 is shown in SEQ ID NO. 1 in the present disclosure. The amino acid sequence of human KGF-2 is available from UniProtKB/Swiss-Prot: O15520.1, and a mature chain thereof is between amino acids 38 and 208.

[0062] The term “FGF-2” is also known as basic FGF ( “bFGF” ). Both FGF1 and FGF2 are known to be highly released by damaged endothelial cells and macrophages at wound sites, and if FGF2 activity is blocked, wound angiogenesis is almost completely impaired. FGF2 is also known to induce scar-free healing. However, owing to the short half-life of free FGFs, the use of delivery systems has been proposed. Among the FGFs, application studies of wound healing and skin regeneration have primarily been conducted on FGF2. Because acidic gelatin is highly negatively charged, it can hold FGF2 well by forming an ionic complex with gelatin. The amino acid sequence of human KGF-2 is available from UniProtKB/Swiss-Prot: P09038.3, and a mature chain thereof is between amino acids 143 and 288, which is shown as SEQ ID NO. 3 in the present disclosure.

[0063] The term “EGF” refers to epidermal growth factor, a growth factor that stimulates cell growth, proliferation and differentiation by binding to its receptor EGFR. It has been proven to be a potent mitogen by stimulating mRNA, DNA and protein synthesis of epithelial cells. It is a single-chain polypeptide consisting of 53 amino acids that is derived from the cleavage of a large precursor, prepro-EGF. EGF is now known as the prototype of the group I EGF family that also includes transforming growth factor- $\alpha$  (TGF- $\alpha$ ), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen. Structurally, they all contain one or more EGF repeats (EGF motif) in their extracellular domain, which is a sequence of 35–40 amino acids spaced by six conserved cysteines in the following pattern: CX7CX3–5CX10–12CXCX5GXRC (C, cysteine; G, glycine; R, arginine; X, other amino acids). One glycine and one arginine in this

sequence are also conserved in all EGF-related growth factors but not in proteins that contain EGF motifs without growth factor activity. The six cysteines pair and form three intramolecular disulfide bonds with the following interactions: C1–C3, C2–C4 and C5–C6 (numbered according to their order in the sequence), which are important for maintaining their biological activities. Functionally, these growth factors share the ability to bind the same receptor, the EGF receptor (EGFR, ErbB1), activate its intrinsic tyrosine kinase activity, and couple the receptor to downstream signaling pathways controlling cell proliferation, differentiation, survival, or motility. The amino acid sequence of human EGF is available from GenBank: AAS83395.1, which is also shown as SEQ ID NO. 2 in the present disclosure.

[0064] The term “PDGF” refers to platelet-derived growth factor, which is a potent mitogen and chemoattractant for many cells of mesenchymal origin, such as fibroblasts, activated macrophages, and smooth muscle cells. PDGF plays critical roles in angiogenesis, embryogenesis, inflammation, and cell differentiation; therefore, it can be beneficial in wound healing. The PDGF family of growth factors consists of four different polypeptide chains encoded by four different genes: the classical PDGF-A and PDGF-B chains, and the more recently discovered PDGF-C and PDGF-D. The four PDGF chains assemble into disulphide-bonded dimers via homo- or heterodimerization, and five different dimeric isoforms have been described so far: PDGF-AA, PDGFAB, PDGF-BB, PDGF-CC and PDGF-DD. Two distinct PDGF receptors, alpha and beta, mediate the effects of the PDGFs on target cells. The PDGF-A and -C chains selectively bind the alpha receptor, whereas PDGF-D preferentially binds the beta receptor and PDGF-B displays a similar affinity for both receptors. Receptor activation requires PDGF-induced receptor dimerization, leading to transphosphorylation on tyrosine. PDGF AA induces only alpha/alpha receptor dimers, PDGF AB induces alpha/alpha and alpha/beta dimers, and PDGF BB induces all 3 combinations. Although PDGF-C binds only to the alpha receptor it can produce alpha-beta heterodimer formation by transactivation of beta receptor. A potential application of PDGF-BB protein is wound healing induction in diabetics. Furthermore, compositions comprising PDGF can facilitate the healing of injured or depleted bones by promoting the growth of the connective tissue, growth of the bone, and by stimulation of collagen synthesis in the injured bone region. The amino acid sequence of human PDGF-B is available from GenBank: CAA02294.1, with amino acids 82 to 190 showing the mature peptide, which is also shown in the present disclosure in SEQ ID NO. 4.

[0065] The COL2A1 (collagen type II alpha 1) gene provides instructions for making one component of type II collagen, called the pro-alpha1 (II) chain. Type II collagen adds structure and strength to the connective tissues that support the body's muscles, joints, organs, and skin. Type II collagen is found primarily in cartilage, a tough but flexible

tissue that makes up much of the skeleton during early development. Most cartilage is later converted to bone, except for the cartilage that continues to cover and protect the ends of bones and is present in the nose and external ears. Type II collagen is also part of the clear gel that fills the eyeball (the vitreous) , the inner ear, and the center portion of the discs between the vertebrae in the spine (nucleus pulposus) . To construct type II collagen, three pro- $\alpha$ 1 (II) chains twist together to form a triple-stranded, rope-like procollagen molecule. Procollagen molecules are then processed by enzymes in the cell. Once processed, the molecules leave the cell and arrange themselves into long, thin fibrils that link to one another (cross-link) in the spaces around cells. The cross-linkages result in the formation of very strong, mature type II collagen fibers.

[0066] The COL3A1 (collagen type III alpha 1 chain) gene provides instructions for making type III collagen. Collagens are a family of proteins that strengthen and support many tissues in the body. Type III collagen is found in the skin, lungs, intestinal walls, and the walls of blood vessels. The components of type III collagen, called pro- $\alpha$ 1 (III) chains, are produced from the COL3A1 gene. Each molecule of type III procollagen is made up of three copies of this chain. The triple-stranded, rope-like procollagen molecules are processed by enzymes outside the cell to create mature type III collagen. The collagen molecules then arrange themselves into long, thin fibrils that form stable interactions (cross-links) with one another and with other types of collagen in the spaces between cells. The cross-links result in the formation of very strong collagen fibers.

[0067] The term “fibronectin” refers to fibronectin 1 gene or its product. This gene encodes fibronectin, a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. The encoded preproprotein is proteolytically processed to generate the mature protein. Fibronectin is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense, and metastasis. The gene has three regions subject to alternative splicing, with the potential to produce 20 different transcript variants, at least one of which encodes an isoform that undergoes proteolytic processing.

[0068] TIMP1, TIMP metalloproteinase inhibitor 1, belongs to the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases (MMPs) , a group of peptidases involved in degradation of the extracellular matrix. In addition to its inhibitory role against most of the known MMPs, the encoded protein is able to promote cell proliferation in a wide range of cell types and may also have an anti-apoptotic function. Transcription of this gene is highly inducible in response to many cytokines and hormones. In addition, the expression from some but

not all inactive X chromosomes suggests that this gene inactivation is polymorphic in human females.

[0069] COL1A1B gene is specific to zebrafish. In tetrapods, collagen type I is a trimer mainly composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain, encoded by COL1A1 and COL1A2 genes, respectively. In contrast, in zebrafish three type I collagen genes exist, colla1a, colla1b and colla2 coding for  $\alpha 1$  (I),  $\alpha 3$  (I) and  $\alpha 2$  (I) chains. During embryonic and larval development, the three collagen type I genes showed a similar spatial-temporal expression pattern, indicating their co-regulation and interdependence at these stages. In both embryonic and adult tissues, the presence of the three  $\alpha$  (I) chains was demonstrated, although in embryos  $\alpha 1$  (I) was present in two distinct glycosylated states, suggesting a developmental-specific collagen composition. Even though in adult bone, skin and scales equal amounts of  $\alpha 1$  (I),  $\alpha 3$  (I) and  $\alpha 2$  (I) chains are present, the presented data suggest a tissue-specific stoichiometry and/or post-translational modification status for collagen type I.

[0070] Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular matrix (ECM) remodeling endopeptidases that have the capacity to degrade almost every component of the ECM. The degradation of the ECM is of great importance, since it is related to embryonic development and angiogenesis. It is also involved in cell repair and the remodeling of tissues. When the expression of MMPs is altered, it can generate the abnormal degradation of the ECM. This is the initial cause of the development of chronic degenerative diseases and vascular complications generated by diabetes. In addition, this process has an association with neurodegeneration and cancer progression. Within the ECM, the tissue inhibitors of MMPs (TIMPs) inhibit the proteolytic activity of MMPs. TIMPs are important regulators of ECM turnover, tissue remodeling, and cellular behavior. Therefore, TIMPs (similar to MMPs) modulate angiogenesis, cell proliferation, and apoptosis. An interruption in the balance between MMPs and TIMPs has been implicated in the pathophysiology and progression of several diseases. The MMPs belong to a family of endopeptidases that contains 23 members. These contain zinc, are dependent on calcium, and can degrade and remodel the proteins that form the ECM. They also participate in different biological and physiological processes that are regulated by hormones, growth factors, and cytokines. Based on their sub-cellular distribution and specificity for components of the ECM, the MMPs are divided into membrane-type matrix metalloproteases (MT-MMPs), collagenases, gelatinases, stromelysins, and matrilysins. Collagenases (MMP-1, MMP-8, MMP-13, and MMP-18) degrade triple-helical fibrillar collagen, which is fundamental in bone and ligaments. Gelatinases (MMP-2 and MMP-9) are involved in different cellular process including angiogenesis and neurogenesis; these proteases alter the molecules of the basal lamina, subsequently leading to cell death.

Stromelysins (MMP-3, MMP-10, and MMP-11) are small proteases that degrade segments of the ECM. Matrilysins (MMP-7 and MMP-26) process cell surface molecules and digest ECM components.

[0071] The tetraspanin protein family members, such as CD63, CD81, and CD9, ubiquitously expressed on exosomes and extensively used as exosome biomarkers, are involved in physiological processes, for instance cell adhesion, cell motility, and signal transduction. CD63, the first characterized tetraspanin, has two extracellular loops of unequal sizes and two short cytoplasmic domains, is involved in the signal transduction processes of various types of immune cells. Sequential domain deletion has identified the transmembrane helix 3 (TM3) being necessary and sufficient for membrane anchoring and exosome targeting. The amino acid sequence of human CD63 is available from e.g., GenBank: AHI51903.1, the amino acid sequence of TM3 of which corresponds to aa. 70 to 133, which is also shown as SEQ ID NO. 9 in the present disclosure.

[0072] The term “anchoring polypeptide” is a polypeptide that is anchored on the exosome membrane when the exosome is generated by a cell. A transmembrane protein is a typical anchoring polypeptide in the context of the present disclosure. By “anchoring” or its grammatical variants, it means that at least a fragment of the polypeptide is embedded in the exosome membrane. The anchoring polypeptide may be fully or partly embedded in the exosome membrane. In this disclosure, the anchoring polypeptide is fused with a polypeptide heterologous to the exosome naturally produced by the same cell, such as the KGF-1 polypeptide. Exemplary anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins. A detailed review regarding GPI anchors in exosome is available from, e.g., Michel Vidal, Exosomes and GPI-anchored proteins: Judicious pairs for investigating biomarkers from body fluids, *Advanced Drug Delivery Reviews*, Volumes 161–162, 2020 (incorporated herein by reference in its entirety). In a preferable embodiment of the present disclosure, the anchoring polypeptide comprises or consists of the transmembrane helix 3 (TM3) of CD63 protein.

[0073] Engineered Exosomes

[0074] One aspect of the present disclosure relates to an engineered exosome, comprising (a) a KGF polypeptide, fused to a first anchoring polypeptide, (b) an EGF polypeptide,

fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.

[0075] In preferable embodiments, the engineered exosome provided by the present disclosure comprises (a) a KGF polypeptide, fused to a first anchoring polypeptide, (b) an EGF polypeptide, fused to a second anchoring polypeptide, (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide, fused to a fourth anchoring polypeptide, wherein (a), (b), (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the KGF polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.

[0076] In the present disclosure, the KGF polypeptide may be a KGF-1 polypeptide or a KGF-2 polypeptide. In preferable embodiments, the KGF polypeptide is a KGF-1 polypeptide. In some embodiments, the KGF-1 polypeptide is a human EGF polypeptide or an ortholog or paralog thereof. In some embodiments, the KGF-1 polypeptide comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 1. In some embodiments, the KGF-1 polypeptide comprises an amino acid sequence as shown in SEQ ID NO. 1. In some embodiments, the KGF-1 polypeptide consists essentially of an amino acid sequence as shown in SEQ ID NO. 1.

[0077] In preferable embodiments, the EGF polypeptide is a human EGF polypeptide or an ortholog or paralog thereof. In some embodiments, the EGF polypeptide comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 2. In some embodiments, the EGF polypeptide comprises an amino acid sequence as shown in SEQ ID NO. 2. In some embodiments, the EGF polypeptide consists essentially of an amino acid sequence as shown in SEQ ID NO. 2.

[0078] In preferable embodiments, the FGF-2 polypeptide is a human FGF-2 polypeptide or an ortholog or paralog thereof. In some embodiments, the EGF polypeptide comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 3. In some embodiments, the FGF-2 polypeptide comprises an amino acid sequence as shown in SEQ ID NO. 3. In some embodiments, the FGF-2 polypeptide consists essentially of an amino acid sequence as shown in SEQ ID NO. 3.

- [0079] In preferable embodiments, the PDGF-BB polypeptide is a human PDGF-BB dimer or an ortholog or paralog thereof. In some embodiments, the PDGF-BB polypeptide is a human PDGF-BB dimer. In some embodiments, the PDGF-BB polypeptide comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 4. In some embodiments, the PDGF-BB polypeptide comprises an amino acid sequence as shown in SEQ ID NO. 4. In some embodiments, the PDGF-BB polypeptide consists essentially of an amino acid sequence as shown in SEQ ID NO. 4.
- [0080] In some embodiments, the anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functional fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins. In preferable embodiments of the present disclosure, the first, second, third, and fourth anchoring polypeptides comprise a TM3 domain of CD63. In preferable embodiments, each of the first, second, third, and fourth anchoring polypeptides comprises a TM3 domain of CD63. In some embodiments, the anchoring polypeptides are full-length CD63 proteins, e.g., full-length human CD63 (see e.g., UniProtKB/Swiss-Prot: F8VZE2, P08962, Q5TZP3, Q8N6Z9, or Q9UCG6). In some embodiments, the anchoring polypeptides are truncated CD63 proteins that comprise a TM3 domain and at least one of TM1, TM2, and TM4 domains. For example, the anchoring polypeptide may consist of TM2 and TM3 of CD63; TM3 and TM4 of CD63; or TM1, TM2, and TM3 of CD63. In some embodiments, the anchoring polypeptides consist of TM3 domain of CD63. In the present disclosure, the first, second, third, and fourth anchoring polypeptides can be different or same. In preferable embodiments, the first, second, third, and fourth anchoring polypeptides are same and consist of TM3 domain of CD63.
- [0081] In preferable embodiments, the TM3 domain of CD63 comprises an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 9. In preferable embodiments, the TM3 domain of CD63 comprises an amino acid sequence as shown in SEQ ID NO. 9. In preferable embodiments, the TM3 domain of CD63 consists essentially of an amino acid sequence as shown in SEQ ID NO. 9.

- [0082] Therefore, in preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) an EGF polypeptide, fused to a second anchoring polypeptide, and (c) FGF-2 polypeptide, fused to a third anchoring polypeptide, wherein each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.
- [0083] In preferable embodiments, provided is an engineered exosome comprising (a) a human KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) a human EGF polypeptide, fused to a second anchoring polypeptide, and (c) a human FGF-2 polypeptide, fused to a third anchoring polypeptide, wherein each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the human KGF-1 polypeptide, the human EGF polypeptide and the human FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.
- [0084] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, wherein each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.
- [0085] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or

100%identity to the amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%identity to the amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, wherein each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63 comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%identity to the amino acid sequence as shown in SEQ ID NO. 9, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.

- [0086] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, wherein each of the first, second, and third anchoring polypeptides consists of a TM3 domain of CD63 having an amino acid sequence as shown in SEQ ID NO. 9, wherein (a), (b) and (c) are anchored on a membrane of the exosome via the first, second and third anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide and the FGF-2 polypeptide are exposed on an outer surface of the membrane of the exosome.
- [0087] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) an EGF polypeptide, fused to a second anchoring polypeptide, (c) FGF-2 polypeptide, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide, fused to a fourth anchoring polypeptide, wherein each of the first, second, third and fourth anchoring polypeptides comprises a TM3 domain of CD63, wherein (a), (b), (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide, the FGF-2 polypeptide and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.
- [0088] In preferable embodiments, provided is an engineered exosome comprising (a) a human KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) a human EGF polypeptide, fused to a second anchoring polypeptide, (c) a human FGF-2 polypeptide,

fused to a third anchoring polypeptide, and (d) a human PDGF-BB polypeptide, fused to a fourth anchoring polypeptide, wherein each of the first, second, third and fourth anchoring polypeptides comprises a TM3 domain of CD63, wherein (a) , (b) , (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the human KGF-1 polypeptide, the human EGF polypeptide, the FGF-2 polypeptide and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.

[0089] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, (c) a FGF-2 polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 4, fused to a fourth anchoring polypeptide, wherein each of the first, second, third and fourth anchoring polypeptides comprises a TM3 domain of CD63, wherein (a) , (b) , (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.

[0090] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, (c) a FGF-2 polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%or 100%identity to the amino acid sequence as shown in SEQ ID NO. 4, fused to a fourth anchoring polypeptide, wherein each of the first, second, third and

fourth anchoring polypeptides comprises a TM3 domain of CD63 comprising an amino acid sequence having at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to the amino acid sequence as shown in SEQ ID NO. 9, wherein (a), (b), (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.

[0091] In preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 1, fused to a first anchoring polypeptide, (b) an EGF polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 2, fused to a second anchoring polypeptide, (c) a FGF-2 polypeptide comprising an amino acid sequence having an amino acid sequence as shown in SEQ ID NO. 3, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide comprising an amino acid sequence as shown in SEQ ID NO. 4, fused to a fourth anchoring polypeptide, wherein each of the first, second, third and fourth anchoring polypeptides consists of a TM3 domain of CD63 having an amino acid sequence as shown in SEQ ID NO. 9, wherein (a), (b), (c) and (d) are anchored on a membrane of the exosome via the first, second, third and fourth anchoring polypeptide, respectively, and wherein the KGF-1 polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide are exposed on an outer surface of the membrane of the exosome.

[0092] In any of the embodiments described above, the KGF polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and, when present, the PDGF-BB polypeptide, is fused to the C-terminus of the first, second, third, and fourth anchoring polypeptides, respectively, directly or through a peptide linker. The peptide linker could be any peptide linker that is available in the art useful for linking different domains or functional regions in a fusion protein. In preferable embodiments, the peptide linker consists of glycine and serine, e.g., (G4S)<sub>n</sub>, in which n is an integer from 1 to 3.

[0093] In the present disclosure, the exosome is preferably not derived from a mesenchymal stem cell. More preferably, the exosome is not derived from a stem cell. In preferable embodiments, the exosome provided by the present invention is derived from a non-stem cell, such as a CHO or HEK293 cell. In the present disclosure, the exosome is preferably purified and/or isolated from the cell from which it is derived.

[0094] In some embodiments, the exosome provided by the present disclosure enhances fibroblast proliferation. In some embodiments, the exosome provided by the present disclosure upregulates COL2A1, COL3A1, fibronectin, TIMP1, and/or COL1A1B genes. In some embodiments, the exosome provided by the present disclosure down-

regulates MMP7, MMP9, and/or MMP16B genes. In some embodiments, the exosome provided by the present disclosure downregulates melanin synthesis. In some embodiments, the exosome provided by the present disclosure enhances fibroblast proliferation; upregulates COL2A1, COL3A1, fibronectin, TIMP1, and/or COL1A1B genes; downregulates MMP7, MMP9, and/or MMP16B genes; and downregulates melanin synthesis.

- [0095] Methods and Compositions for Skin Rejuvenation
- [0096] Aspects of the present disclosure are directed to methods and compositions for skin rejuvenation. In preferable embodiments, the skin rejuvenation includes treatment of any of sun damage, sensitive skin, scars such as from acne or stretch marks such as striae gravidarum or adolescent striae, chickenpox, or injury, wrinkles such as static or dynamic wrinkles, dark circle under eyes, hyperpigmentation such as darkened skin or freckles, loosen skin, uneven or dull skin tone, or any of combination thereof; the method comprising administering to a subject in need thereof a pharmaceutically or cosmetically effective amount of the engineered exosomes or the composition disclosed herein.
- [0097] In preferable embodiments, provided are method of anti-wrinkles, including static and dynamic wrinkles, e.g., on neck and/or face, comprising administering to a subject in need thereof a pharmaceutically or cosmetically effective amount of the engineered exosomes or the composition disclosed herein. In preferable embodiments, provided are method of treating hyperpigmentation, e.g., on neck and/or face.
- [0098] In preferable embodiments, provided are method of treating a scar, including a scar from acne or a stretch mark such as striae gravidarum or adolescent striae, e.g., on abdomen and/or legs, comprising administering to a subject in need thereof a pharmaceutically or cosmetically effective amount of the engineered exosomes or the composition disclosed herein.
- [0099] In the present disclosure, the composition provided comprises the engineered exosome and a carrier. In preferable embodiments, the composition is a liquid formulation. In preferable embodiment, the composition is formulated for topical or subcutaneous administration. Contemplated are compositions comprising the engineered exosomes, including, for example, soaps, shampoos, ointments, and other such formulations.
- [0100] In preferable embodiments, the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide) , an EGF polypeptide, a FGF-2 polypeptide, or a PDGF polypeptide (such as PDGF-BB polypeptide) , not attached to the membrane of the exosome (i.e., free polypeptides) . For example, no additional KGF polypeptide (e.g. a KGF-1 polypeptide) , an EGF polypeptide, a FGF-2 polypeptide, or a PDGF polypeptide (such as PDGF-BB polypeptide) is added or supplemented to the com-

position except for the polypeptides anchored to the membrane of the engineered exosomes.

- [0101] In some embodiments, the composition is a cosmetic composition. In some embodiments, the composition is a non-cosmetic composition. In some embodiments, the composition is a pharmaceutical composition.
- [0102] Nucleic Acid Constructs, Vectors, Cells, and Methods of Production
- [0103] Aspects of the present disclosure relate also to nucleic acid constructs that encode the polypeptides anchored on the exosomes.
- [0104] In some embodiments, provided is a nucleic acid construct comprising a polynucleotide encoding (a) a KGF polypeptide fused to a first anchoring polypeptide, (b) an EGF polypeptide fused to a second anchoring polypeptide, and (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide.
- [0105] In some embodiments, provided is a nucleic acid construct comprising a polynucleotide encoding (a) a KGF polypeptide fused to a first anchoring polypeptide, (b) an EGF polypeptide fused to a second anchoring polypeptide, (c) a FGF-2 polypeptide, fused to a third anchoring polypeptide, and (d) a PDGF-BB polypeptide, fused to a fourth anchoring polypeptide.
- [0106] In some embodiments, provided are a set of three nucleic acid constructs, with a first nucleic acid construct comprising a polynucleotide encoding (a) , a second nucleic acid construct comprising a polynucleotide encoding (b) , and a third nucleic acid construct comprising a polynucleotide encoding (c) , wherein (a) , (b) , and (c) are defined as above.
- [0107] In some embodiments, provided are a set of four nucleic acid constructs, with a first nucleic acid construct comprising a polynucleotide encoding (a) , a second nucleic acid construct comprising a polynucleotide encoding (b) , a third nucleic acid construct comprising a polynucleotide encoding (c) , and a fourth nucleic acid construct comprising a polynucleotide encoding (d) , wherein (a) , (b) , (c) , and (d) are defined as above.
- [0108] In some embodiments, provided are a set of two nucleic acid constructs, with one nucleic acid construct comprising a polynucleotide encoding two of (a) , (b) and (c) , and the other nucleic acid construct comprising a polynucleotide encoding the remaining, wherein (a) , (b) , and (c) are defined as above.
- [0109] In some embodiments, provided are a set of two nucleic acid constructs, with one nucleic acid construct comprising a polynucleotide encoding two of (a) , (b) , (c) and (d) , and the other nucleic acid construct comprising a polynucleotide encoding the other two, wherein (a) , (b) , (c) and (d) are defined as above.
- [0110] In some embodiments, provided are a set of three nucleic acid constructs, with a first nucleic acid construct comprising a polynucleotide encoding two of (a) , (b) , (c)

and (d) , a second nucleic acid construct comprising a polynucleotide encoding one of the remaining polypeptide, and a third nucleic acid construct comprising a polynucleotide encoding the other of the remaining polypeptide, wherein (a) , (b) , (c) and (d) are defined as above.

- [0111] In preferable embodiments, provided is a single nucleic acid construct comprising a polynucleotide encoding (a) , (b) , and (c) , wherein (a) , (b) , and (c) are defined as above.
- [0112] In preferable embodiments, provided is a single nucleic acid construct comprising a polynucleotide encoding (a) , (b) , (c) and (d) , wherein (a) , (b) , (c) and (d) are defined as above.
- [0113] In some embodiments, the anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functionally fragment thereof. Exemplary membrane proteins of exosome include but are not limited to lamp2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs) , lactadherin (C1C2 domain) , vesicular stomatitis virus glycoprotein (VSVG) , prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins.
- [0114] In some embodiments, each of the anchoring polypeptide may be located at the N terminus of the polypeptides to be presented on the surface of the exosome (e.g., EGF, KGF, FGF-2 and PDGF-BB) , to ensure that the latter are exposed on the surface of the exosome. In some embodiments, each of the anchoring polypeptide may be located at the C terminus of the polypeptides to be presented on the surface of the exosome, to ensure that the latter are exposed on the surface of the exosome. For example, when lamp2b is used as one of the anchoring polypeptides, the polypeptides (e.g., EGF, KGF, FGF-2 or PDGF-BB) to be presented on the surface of the exosome may be located at N terminus of lamp2b. For example, when TM3 domain of CD63 is used as one of the anchoring polypeptides, the polypeptides (e.g., EGF, KGF, FGF-2 or PDGF-BB) to be presented on the surface of the exosome may be located at C terminus of the TM3 domain of CD63. In some embodiments, when different anchoring polypeptides are used, the polypeptides to be presented on the surface of the exosome may be located at either N or C terminus of the anchoring polypeptides, dependent on the species of the anchoring polypeptides used.
- [0115] In preferable embodiments, the anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, third, and fourth anchoring polypeptides is a TM3 domain of CD63.

- [0116] In the preferable embodiments, the polynucleotide may comprise, from 5' to 3', a fragment of nucleotides encoding:
- [0117] (i) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] ,
- [0118] (ii) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] ,
- [0119] (iii) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] ,
- [0120] (iv) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] ,
- [0121] (v) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] , or
- [0122] (vi) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] ,
- [0123] wherein, [ ] represents a separate polypeptide, and ] - [ represents a linker or a bond.
- [0124] In preferable embodiments, the self-cleavage peptide is a 2A peptide, e.g., T2A, E2A, P2A or any combination thereof. For example, the self-cleavage peptide is a T2A peptide. The self-cleavage peptide is cleaved after the polynucleotide is translated, resulting in three or four independent fusion proteins, each comprising a single polypeptide to be presented onto the surface of the exosome and a single anchoring polypeptide.
- [0125] In preferable embodiments, provided is a single nucleic acid construct comprising a polynucleotide encoding (a) , (b) , and (c) , wherein (a) , (b) , and (c) are defined as above, and wherein each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63, the polynucleotide may comprise, from 5' to 3' , a fragment of nucleotides encoding one of
- [0126] (i) [TM3] - [KGF] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] ,
- [0127] (ii) [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] ,
- [0128] (iii) [TM3] - [EGF] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] ,
- [0129] (iv) [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] ,
- [0130] (v) [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [EGF] , and
- [0131] (vi) [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [KGF] ,
- [0132] wherein, [ ] represents a separate polypeptide, and ] - [ represents a linker or a bond; and wherein TM3 represents TM3 domain of CD63; and T2A represents self-cleavage peptide T2A.
- [0133] In preferable embodiments, the polynucleotide may comprise, from 5' to 3' , a fragment of nucleotides encoding:

- [0134] (a) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0135] (b) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,
- [0136] (c) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,
- [0137] (d) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,
- [0138] (e) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0139] (f) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0140] (g) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0141] (h) [1<sup>st</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0142] (i) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0143] (j) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,
- [0144] (k) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,

- [0145] (l) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [FGF-2] ,
- [0146] (m) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0147] (n) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] ,
- [0148] (o) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] ,
- [0149] (p) [1<sup>st</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] ,
- [0150] (q) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0151] (r) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0152] (s) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0153] (t) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [EGF] ,
- [0154] (u) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [PDGF-BB] ,
- [0155] (v) [1<sup>st</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] ,

- [0156] (w) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] , or
- [0157] (x) [1<sup>st</sup> anchoring polypeptide] - [FGF-2] - [self-cleavage peptide] - [2<sup>nd</sup> anchoring polypeptide] - [EGF] - [self-cleavage peptide] - [3<sup>rd</sup> anchoring polypeptide] - [PDGF-BB] - [self-cleavage peptide] - [4<sup>th</sup> anchoring polypeptide] - [KGF] ,
- [0158] wherein, [ ] represents a separate polypeptide, and ] - [ represents a linker or a bond.
- [0159] In preferable embodiments, the self-cleavage peptide is a 2A peptide, e.g., T2A, E2A, P2A or any combination thereof. For example, the self-cleavage peptide is a T2A peptide.
- [0160] In other embodiments, the single polynucleotide encodes at least an anchoring polypeptide that is located at the C terminus of the polypeptide to be presented onto the surface of the exosome. In some embodiments, each of the first, second, third, and fourth anchoring polypeptides are different and thus the anchoring polypeptides may locate at N or C terminus of the polypeptides KGF, EGF, FGF-2, and, optionally, PDGF-BB.
- [0161] In preferable embodiments, provided is a single nucleic acid construct comprising a polynucleotide encoding (a) , (b) , (c) and (d) , wherein (a) , (b) , (c) and (d) are defined as above, and wherein each of the first, second, third, and fourth anchoring polypeptides comprises a TM3 domain of CD63, the polynucleotide may comprise, from 5' to 3' , a fragment of nucleotides encoding one of
- [0162] [TM3] - [KGF] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] ,
- [0163] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] ,
- [0164] [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] ,
- [0165] [TM3] - [KGF] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] ,
- [0166] [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] ,
- [0167] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] ,
- [0168] [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] ,
- [0169] [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] ,

- [0170] [TM3] - [EGF] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] ,
- [0171] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] ,
- [0172] [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [FGF-2] ,
- [0173] [TM3] - [EGF] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] ,
- [0174] [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] ,
- [0175] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] ,
- [0176] [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] ,
- [0177] [TM3] - [EGF] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF] ,
- [0178] [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] ,
- [0179] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [EGF] ,
- [0180] [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [EGF] ,
- [0181] [TM3] - [FGF-2] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] ,
- [0182] [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [PDGF-BB] ,
- [0183] [TM3] - [PDGF-BB] - [T2A] - [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [KGF] ,
- [0184] [TM3] - [FGF-2] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [KGF] , or
- [0185] [TM3] - [FGF-2] - [T2A] - [TM3] - [EGF] - [T2A] - [TM3] - [PDGF-BB] - [T2A] - [TM3] - [KGF]
- [0186] wherein, [ ] represents a separate polypeptide, and ] - [ represents a linker or a bond; and wherein TM3 represents TM3 domain of CD63; and T2A represents self-cleavage peptide T2A.
- [0187] In this section, the KGF, EGF, FGF-2, and PDGF-BB polypeptide have the meaning and preferable embodiments given above in reference to the section titled Engineered Exosomes.

- [0188] In some embodiments, a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 5 or a degenerate sequence thereof.
- [0189] In some embodiments, a polynucleotide encoding the EGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 6 or a degenerate sequence thereof.
- [0190] In some embodiments, a polynucleotide encoding the FGF-2 polypeptide has a nucleotide sequence as shown in SEQ ID NO. 7 or a degenerate sequence thereof.
- [0191] In some embodiments, a polynucleotide encoding the PDGF-BB polypeptide has a nucleotide sequence as shown in SEQ ID NO. 8 or a degenerate sequence thereof.
- [0192] In some embodiments, a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO. 10 or a degenerate sequence thereof.
- [0193] Also provided are vectors that comprise the nucleic acid construct (s) described above. In some embodiments, the vector is a viral vector. In preferable embodiments, the vector is a lentiviral vector or an adeno-associated viral vector.
- [0194] The vectors provided herein facilitate integration of the polynucleotides encoding the polypeptides anchored on the membrane of the engineered exosomes into the genome of the cells producing the exosomes.
- [0195] Also provided are cells transduced with the vectors. In preferable embodiments, the cell is not a mesenchymal stem cell. In preferable embodiments, the cell is not a stem cell. In preferable embodiments, the cell is a non-stem cell, such as a HEK293 or CHO cell.
- [0196] The present disclosure also provides a process of producing the engineered exosome provided herein, comprising transducing the cell described above, such as HEK293 cell, with the vector described above; culturing the cell in a condition allowing secretion of the engineered exosome from the cell; and collecting and purifying the engineered exosome.
- [0197] In some embodiments, the process comprises an adaption of the cell from serum-containing condition to a serum-free condition during culturing. The adaption may comprise a sequential adaptation with decreasing full medium and increasing serum-free medium.
- [0198] Sequences Listings

| SEQ ID NO. | Description    | Sequences                                                                                                                                                                         |
|------------|----------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1          | Human KGF-1 AA | CNDMTPEQMATNVNCSSPERHTRSVDYMEGGDIRVRL<br>FCRTQWYLRIKRGKVKGTQEMKNNYNIMEIRTVAVGI<br>VAIKGVESEFYLAMNKEGKLYAKKECNEDCNFKELILEN<br>HYNTYASAKWTHNGGEMFVALNQKGIPVRGKKTKKEQ<br>KTAHFLPMAIT |
| 2          | Human EGF AA   | NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGVI                                                                                                                                            |

|   |                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
|---|-----------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|   |                       | GERCQYRDLKWWE LR                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
| 3 | Human FGF-2<br>AA     | PALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRHPDGR<br>VDGVREKSDPHIKLQLQAEERGVSISIKGVCANRYLAMK<br>EDGRLLASKCVTDECFFFERLESNNYNTYRSRKYTSWY<br>VALKRTGQYKLGSKTGPQGKAILFLPMSAKS                                                                                                                                                                                                                                                                                                                                                                                                  |
| 4 | Human PDGF-<br>BB AA  | SLGSLTIAEPAMIAECKTRTEVFEISRRLIDRTNANFLVWP<br>PCVEVQRCSGCCNNRNVQCRPTQVQLRPVQVRKIEIVRK<br>KPIFKKATVTLEDHLACKCETVAAARPVT                                                                                                                                                                                                                                                                                                                                                                                                                                           |
| 5 | Human KGF-1<br>DNA    | TGCAACGATATGACACCCGAGCAGATGGCCACCAATGT<br>GAATTGTTCCAGCCCTGAGAGGCACACCAGATCCTAC<br>GATTACATGGAGGGCGGCGACATCAGGGTGAGAAGAC<br>TGTTTTGTAGAACACAGTGGTACCTGAGGATCGACAA<br>GAGAGGCAAGGTGAAGGGCACCCAGGAGATGAAGAA<br>TAATTACAACATCATGGAGATCAGAACCGTGGCCGTGG<br>GCATCGTGGCCATCAAGGGCGTGGAGAGCGAGTTTTTA<br>CCTGGCCATGAACAAGGAGGGCAAGCTGTACGCCAAG<br>AAGGAGTGCAATGAGGACTGTAAC TTAAAGGAGCTGA<br>TCCTGGAGAACCACTACAACACCTACGCCTCCGCCAA<br>GTGGACCCACAACGGCGGCGAGATGTTCTGTGGCCCTG<br>AATCAGAAGGGCATCCAGTGAGGGGCAAGAAGACA<br>AAGAAGGAGCAGAAGACCGCCCACTTTCTGCCTATGG<br>CCATCACC |
| 6 | Human EGF<br>DNA      | AATTCCGATTCCGAGTGCCCTCTGTCCCACGATGGCTA<br>CTGTCTGCACGATGGCGTGTGCATGTACATCGAGGCCC<br>TGGACAAGTACGCCTGCAACTGCGTGGTGGGCTACAT<br>CGGCGAGAGGTGCCAGTACAGGGACCTGAAGTGGTG<br>GGAGCTGAGG                                                                                                                                                                                                                                                                                                                                                                                 |
| 7 | Human FGF-2<br>DNA    | CCAGCTCTGCCTGAGGATGGCGGCAGCGGCGCTTTCC<br>CTCCCGACATTTTAAGGATCCCAAGAGACTGTACTGT<br>AAGAATGGCGGCTTCTTCCTGAGGATCCACCCAGATGG<br>CAGGGTGGACGGCGTGAGGGAGAAGTCCGACCCTCA<br>CATCAAGCTGCAGCTGCAGGCCGAGGAGAGGGGCGTG<br>GTGAGCATCAAGGGCGTGTGTGCCAATAGGTACCTGG<br>CCATGAAGGAGGACGGCAGACTGCTGGCCAGCAAGTG<br>CGTGACCGACGAGTGTTTTTTCTTCGAGAGACTGGAG<br>AGCAACA ACTACAACACCTACAGATCCAGAAAAGTACA<br>CATCCTGGTACGTGGCCCTGAAGAGAACAGGCCAGTA<br>CAAGCTGGGCTCCAAGACCGGCCCTGGCCAGAAGGCC<br>ATCCTGTTTCTGCCCATGAGCGCCAAGAGC                                                             |
| 8 | Human PDGF-<br>BB DNA | AGCCTGGGATCCCTGACAATCGCCGAGCCCCGCCATGAT<br>CGCCGAGTGCAAGACAAGGACAGAGGTGTTTGAGATC<br>TCCAGAAGACTGATCGACAGAACAAATGCCAATTCCT<br>GGTGTGGCCTCCTTGCGTGGAGGTGCAGAGGTGCTCC<br>GGCTGCTGTAACAATAGAAACGTGCAGTGCAGACCCA                                                                                                                                                                                                                                                                                                                                                     |

|    |                               |                                                                                                                                                                                    |
|----|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|    |                               | CCCAGGTGCAGCTGAGGCCCGTGCAGGTGAGAAAGAT<br>CGAGATCGTGAGGAAGAAGCCTATCTTTAAGAAGGCC<br>ACCGTGACCCTGGAGGACCACCTGGCCTGCAAGTGCG<br>AGACAGTGGCCGCCGCTAGACCCGTGACC                           |
| 9  | CD63 TM3 AA                   | YCLMITFAIFLSLIMLVEVAAAIAGYVFRDKVMSEFNNTF<br>RQQMENYPKNNHTA                                                                                                                         |
| 10 | CD63 TM3 DNA                  | TACTGTCTGATGATCACCTTCGCCATCTTTCTGTCCCTG<br>ATCATGCTGGTGGAGGTGGCCGCCCATCGCCGGATA<br>CGTGTTTACAGAGACAAGGTGATGAGCGAGTTCAACAAT<br>AATTTTAGACAGCAGATGGAGAATTACCCTAAGAATAA<br>CCACACCGCC |
| 11 | Linker AA                     | GGGSGGGSGGGSGGGS                                                                                                                                                                   |
| 12 | Linker DNA                    | GGCGGCGGCGGCTCCGGAGGAGGAGGAAGCGGAGGA<br>GGCGGCAGC                                                                                                                                  |
| 13 | T2A AA                        | GSGEGRGSLTCDVEENPGP                                                                                                                                                                |
| 14 | T2A DNA                       | GGCAGCGGCGAAGGCCGCGGCAGCCTGCTGACCTGCG<br>GCGATGTGGAAGAAAACCCGGGCCCCG                                                                                                               |
| 15 | COL1A2<br>Forward primer      | GGCCCTCAAGGTTTCCAAGG                                                                                                                                                               |
| 16 | COL1A2<br>Reverse primer      | CACCCTGTGGTCCAACAACCTC                                                                                                                                                             |
| 17 | COL3A1<br>Forward primer      | GGAGCTGGCTACTTCTCGC                                                                                                                                                                |
| 18 | COL3A1<br>Reverse primer      | GGGAACATCCTCCTTCAACAG                                                                                                                                                              |
| 19 | Fibronectin<br>Forward primer | AGGAAGCCGAGGTTTAACTG                                                                                                                                                               |
| 20 | Fibronectin<br>Reverse primer | AGGACGCTCATAAGTGTCACC                                                                                                                                                              |
| 21 | TIMP1<br>Forward primer       | CTTCTGCAATTCCGACCTCGT                                                                                                                                                              |
| 22 | TIMP1<br>Reverse primer       | ACGCTGGTATAAGGTGGTCTG                                                                                                                                                              |
| 23 | MMP7<br>Forward primer        | GAGTGAGCTACAGTGGGAACA                                                                                                                                                              |
| 24 | MMP7<br>Reverse primer        | CTATGACGCGGGAGTTTAACAT                                                                                                                                                             |
| 25 | COL1A1B<br>Forward primer     | GGGATGCTGTTGAAAGGTGAA                                                                                                                                                              |
| 26 | COL1A1B<br>Reverse primer     | GGTGGTCCGGTAAATCCTGG                                                                                                                                                               |
| 27 | MMP9<br>Forward primer        | TGTACCGCTATGGTTACACTCG                                                                                                                                                             |
| 28 | MMP9                          | GGCAGGGACAGTTGCTTCT                                                                                                                                                                |

|    |                         |                     |
|----|-------------------------|---------------------|
|    | Reverse primer          |                     |
| 29 | MMP16<br>Forward primer | AGCACTGGAAGACGGTTGG |
| 30 | MMP16<br>Reverse primer | CTCCGTTCCGCAGACTGTA |

AA: Amino Acid Sequence

Examples

- [0199] Example 1. Construction of engineered exosomes derived stable cell lines.
- [0200] Materials and Methods
- [0201] Materials: The HEK293 cell line (human embryonic kidney 293 cells, CRL-1573<sup>TM</sup>) was purchased from ATCC, which were maintained in DMEM (high-glucose) containing 10% (vol/vol) FBS, supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The CHO-K1 cell line (Chinese Hamster Ovary Cell) was purchased from BeNa Culture Collection (Beijing, China) . CHO-K1 cells were maintained in F-12K (31765035, Thermo Fisher Scientific, United States) containing 10% (vol/vol) FBS, supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The HSF (human skin fibroblast) and HFF (human foreskins fibroblast) were purchased from Mingzhou Biotechnology (Ningbo, China) and cultured with DMEM (high-glucose) containing 10%FBS. Cells were incubated in a humidified atmosphere containing 5%CO<sub>2</sub> at 37 °C. Antibody used in this study was anti-CD63 antibodies (Cat. No. MA5-32085, Invitrogen) .
- [0202] pGOI Plasmids Construction: The amino acid sequences of all the target genes, including KGF-1, EGF, FGF-2, and PDGF-BB were derived from Uniprot and the corresponding DNA sequences (see SEQ ID NO. 5, 6, 7, and 8, respectively) were synthesized by General Biotechnology (Chuzhou, China) with plasmid pCDH-CMV-MCS-EF1a-GFP+BSD (System Biosciences) . The T2A peptide (see SEQ ID NO. 13 and 14) was used for dissociating all the target proteins into individual protein upon translation. The 4 plasmids (i.e., pGOI, pGag/Pol, pRev, and pVSV-G) of 3<sup>rd</sup>-generation system were used for lentivirus production, with pCDH-CMV-MCS-EF1a-GFP+BSD (blasticidin resistance) as lentivirus packaging plasmid. The lentiviruses were packaged by WZ Biotechnology (Jinan, China) .
- [0203] Payload genes constructed in two pGOI plasmids:
- [0204] No. 6: CD63-TM3-Linker-KGF-1-T2A-CD63-TM3-Linker-EGF-T2A-CD63-TM3-Linker-FGF-2, in which each CD63-TM3 is the TM3 domain of CD63, encoded by DNA sequence shown in SEQ ID NO. 10, each linker is encoded by DNA sequence shown in SEQ ID NO. 12, and each T2A is encoded by DNA sequence shown in SEQ ID NO. 14.

[0205] No. 8: CD63-TM3-Linker-KGF-1-T2A-CD63-TM3-Linker-EGF-T2A-CD63-TM3-Linker-FGF-2-T2A-CD63-TM3-Linker-PDGF-BB, in which each CD63-TM3 is the TM3 domain of CD63, encoded by DNA sequence shown in SEQ ID NO. 10, each linker is encoded by DNA sequence shown in SEQ ID NO. 12, and each T2A is encoded by DNA sequence shown in SEQ ID NO. 14.

[0206] Generation of stable cell lines: The stable HEK293 cell line expressing target proteins, including KGF-1, EGF, FGF-2, and PDGF-BB, was generated by transfection with the plasmids or infection with the corresponding lentiviruses. Forty-eight hours after transfection or infection, cells were selected by the addition of antibiotics, e.g. blasticidin (Solarbio Life Sciences) to a final concentration of 6 µg/ml. A single cell colony with green fluorescent protein (GFP) expression was selected and cultured in complete medium with 6 µg/ml blasticidin. The stable cell line was monitored for the expression of GFP and the corresponding targeted proteins.

[0207] Adaptation of cell culture to a SFM (serum-free medium) : After three initial passages in FM (full medium) from HEK293 stable cell line, adaptation for serum-free culture was started from the 4<sup>th</sup> passage. Cells were subcultured with medium composition in Table 1. To establish the fully adapted serum-free culture, cells should be subcultured in a SFM (HyClone<sup>TM</sup> peak expression, SH31193.02, Cytiva Life Sciences) for at least three times.

[0208] Table 1. Culture Medium for Adaptation

| Steps | Sequential adaptation |
|-------|-----------------------|
| 0     | 100% FM               |
| 1     | 50% FM + 50% SFM      |
| 2     | 25% FM + 75% SFM      |
| 3     | 12.5% FM + 87.5% SFM  |
| 4     | 5% FM + 95% SFM       |
| 5     | 2.5% FM + 97.5% SFM   |
| 6     | 100% SFM              |

[0209] FM: Full medium; SFM: Serum-free medium

[0210] Exosomes isolation: The stable cell line was seeded in T150 flasks for 24h, rinsed extensively with PBS and incubated in SFM for another 48h. The cultured cell-free extracellular medium containing exosomes was harvested by centrifugation at 300×g for 10 min to remove the cells. Then centrifuge at 10,000×g for 30 min to remove dead cells and cell debris. Finally, the clear supernatant was centrifuged for 70 min at 100,

000×g to pellet the exosomes for twice. And the exosome pellet was resuspended. All centrifugation steps were carried out at 4 °C.

- [0211] Figure 1 shows schematically the flowchart of exosome production process derived from HEK293 cells, according to an exemplary embodiment of the present disclosure.
- [0212] Example 2. Characterization of Exosomes
- [0213] Analysis of the particle concentration and size distribution of exosomes
- [0214] The particle concentration and size distribution of exosomes from stable cell line NO. 6 or NO. 8 (transduced with pGOI No. 6 and 8, respectively) were analyzed by the NanoFCM (NanoFCM Inc., Xiamen, China) . The NanoFCM analysis used two single photon counting avalanche photodiodes (APDs) to detect individual particle side scatter (SSC) and fluorescence simultaneously. Firstly, the exosomes pellet was prepared for analysis. Then, 200 nm PE and AF488 fluorophore-conjugated polystyrene beads were used for particle concentration and Silica Nanosphere Cocktail (NanoFCM Inc., Xiamen, China) for particle size distribution. The detector recorded particles passing by during a 1-min interval in each test. Each sample was diluted to reach a particle count within the optimal range of 3000-9, 000 particles per minute. NanoFCM software (NanoFCM Profession V2.0) was used to convert flow rate and side scattering intensity to vesicle concentration and size.
- [0215] Figure 2A shows the particle diameter and particle concentration of exosomes derived from the stable cell lines No. 6 and 8 were analyzed by NanoFCM. The exosomes have a mean particle diameter of 71.3 nm for No. 6, and 72.9 nm for No. 8.
- [0216] Identification of target proteins on exosomes via Western blotting (WB) or ELISA
- [0217] For the identification of target proteins expression on exosomes, the purified exosomes were lysed with RIPA lysis buffer (Beyotime) supplemented with 1 mM protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Beyotime) and phosphatase inhibitor (Beyotime) , then heat denatured, separated by SDS-PAGE, and transferred onto PVDF membrane (Millipore, MA, USA) . The proteins were detected by incubation with primary antibody against CD63, the scaffold protein of exosomes, followed by incubation with an HRP-conjugated secondary antibody (Invitrogen) . Enhanced chemiluminescence reagent (Millipore, MA, USA) was then used for the visualization of the membranes. For the detection of target proteins concentration, the purified exosomes were prepared and the target proteins on exosomes were analyzed via an appropriate ELISA kit (Solarbio, SEKH-0051; SEKH-0220; SEKH-0052; SEKH-0290) according to manufacturer's instructions.
- [0218] Transmission electron microscopy (TEM) analysis of exosomes
- [0219] TEM was used to confirm the presence of exosomes. Approximately, 20  $\mu$ l of exosomes were added separately to copper grids. All excess fluids were removed using filter paper, and the samples were negatively stained with 2%uranyl acetate for 30 s.

The grids were rinsed in deionized water and allowed to dry overnight. The samples were then air-dried using an electric incandescent lamp and viewed using an electron microscope (Hitachi, S-3000N) .

[0220] Figure 2B shows the transmission electron microscopy (TEM) image of the exosomes produced from stable cell lines No. 6 and 8. Figure 2C shows the immunoblotting analysis of exosomes from cell line No. 8 with antibody against CD63-TM3, the exosome fused scaffold proteins. As expected, the engineered exosome-associated target proteins, KGF (KGF-1) , FGF-2, PDGF-BB, and EGF were present in purified exosomes derived from stable cell line No. 8.

[0221] The concentration of functional proteins within exosomes were measured via ELISA kit. Table 2 shows the results of concentrations of different proteins as determined by ELISA.

[0222] Table 2. Concentrations of effective factors determined by ELISA

| Concentration of effective factors (pg/ml exosomes) |         |        |         |         |
|-----------------------------------------------------|---------|--------|---------|---------|
|                                                     | KGF-1   | EGF    | FGF-2   | PDGF-BB |
| NO.6                                                | 3235.51 | 155.28 | 1327.82 | /       |
| NO.8                                                | 3041.06 | 221.56 | 1259.25 | 368.73  |

/: The target is not included in this plasmid.

[0223] Example 3. Engineered exosomes exhibit enhanced cell proliferation activity on fibroblast.

[0224] Exosomes incubated with fibroblast HSF or HFF

[0225] HSF or HFF cells at a density of 4000 per well of a 96-well plate were incubated with exosomes from NO. 6 or NO. 8 at different concentrations (0.5  $\mu$ g, 1.0  $\mu$ g, 2.0  $\mu$ g) for 48h. KGF was used as a positive control. After 48 hours, cell counting kit (CCK8) analysis was performed to detect the cell viability (MCE, HY-K0301) .

[0226] Figure 3 shows the engineered exosomes exhibit enhanced cell proliferation activity on fibroblast, both of HSF and HFF. What's more, the cell proliferation activity was enhanced at a dose dependent manner especially for exosome NO. 8 treatment on HSF cells.

[0227] Example 4. Quantification of skin regenerated associated genes in fibroblast after treated with exosomes via RT-qPCR

[0228] Total RNA from fibroblast treated the same as above was isolated using TRIzol LS reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The RNA tested was reverse transcribed and measured by qPCR using a Hifair® III One Step RT-qPCR SYBR Green Kit (Yeasen, HB220624) . The RNA copy number was normalized to that of cellular 18S rRNA. The primers specific for skin regenerated associated genes were shown in Table 3.

[0229] Table 3. Primers for RT-qPCR in fibroblasts

|             |                |                        |               |
|-------------|----------------|------------------------|---------------|
| COL1A2      | Forward primer | GGCCCTCAAGGTTTCCAAGG   | SEQ ID NO. 15 |
|             | Reverse primer | CACCCTGTGGTCCAACAACCTC | SEQ ID NO. 16 |
| COL3A1      | Forward primer | GGAGCTGGCTACTTCTCGC    | SEQ ID NO. 17 |
|             | Reverse primer | GGGAACATCCTCCTTCAACAG  | SEQ ID NO. 18 |
| Fibronectin | Forward primer | AGGAAGCCGAGGTTTAACTG   | SEQ ID NO. 19 |
|             | Reverse primer | AGGACGCTCATAAGTGTACC   | SEQ ID NO. 20 |
| TIMP1       | Forward primer | CTTCTGCAATTCCGACCTCGT  | SEQ ID NO. 21 |
|             | Reverse primer | ACGCTGGTATAAGGTGGTCTG  | SEQ ID NO. 22 |
| MMP7        | Forward primer | GAGTGAGCTACAGTGGGAACA  | SEQ ID NO. 23 |
|             | Reverse primer | CTATGACGCGGGAGTTTAACAT | SEQ ID NO. 24 |

[0230] Figure 4A to 4E shows the fold change of gene copy number of the tested genes.

Both NO. 6 and NO. 8 exosomes treatment can significantly increase the expression of proteins involved in the maintenance of the extracellular matrix, such as collagen (COL1A2, COL3A1), fibronectin, and TIMP1, and inhibit the expression of MMP7.

[0231] Example 5. The evaluation of anti-wrinkle efficacy on zebrafish model

[0232] The zebrafish embryos (4dpf, days post fertilization) were prepared to have a yolk sac. injection with exosomes (NO. 6 or NO. 8). Twenty-four hours after injection, the gene expression analysis in zebrafish was performed via RT-qPCR to detect the skin regenerated associated genes (Hunter Biotech). The specific primers were shown in Table 4 below.

[0233] Table 4. Primers for RT-qPCR in zebrafish

|         |                |                        |               |
|---------|----------------|------------------------|---------------|
| COL1A1B | Forward primer | GGGATGCTGTTGAAAGGTGAA  | SEQ ID NO. 25 |
|         | Reverse primer | GGTGGTCCGGTAAATCCTGG   | SEQ ID NO. 26 |
| MMP9    | Forward primer | TGTACCGCTATGGTTACACTCG | SEQ ID NO. 27 |
|         | Reverse primer | GGCAGGGACAGTTGCTTCT    | SEQ ID NO. 28 |
| MMP16   | Forward primer | AGCACTGGAAGACGGTTGG    | SEQ ID NO. 29 |
|         | Reverse primer | CTCCGTTCCGCAGACTGTA    | SEQ ID NO. 30 |

[0234] Figure 5 shows the engineered exosomes regulate skin regenerated associated genes in zebrafish model. The treatment with NO. 8 exosome can remarkably increase the in vivo synthesis of collagen (COL1A1B) and inhibit the expression of MMP9 and MMP16B, which could degrade the extracellular matrix. The NO. 6 exosome can obviously increase the in vivo synthesis of collagen (COL1A1B) and inhibit the expression of MMP9.

[0235] Example 6. Engineered exosomes contribute to downregulate the melanin synthesis.

[0236] Analysis of melanin synthesis in vitro

[0237] The human melanocytes were cultured and incubated with exosomes derived from NO. 6 or NO. 8 stable cell lines for 48h. Afterwards, cell pellets were used for melanin

extraction and the concentration of melanin was measured via Melanin Assay Kit (Fluorometric) (ABN-KA6030, Abnova) .

[0238] The evaluation of hyperpigmentation inhibition efficacy on zebrafish model

[0239] The zebrafish embryos (4hpf, hours post fertilization) were cultures in 6-well plate with 3ml exosomes (NO. 8) for 45h at 28 °C. All the wells (15 zebrafish embryos per well) were cultured with light-avoidance. Afterwards, images were captured under the dissecting microscope and analyzed to evaluate the inhibition efficacy of hyperpigmentation. The calculation is as following: The whitening efficacy (%) = (Mock-NO. 8) /Mock\*100%.

[0240] Figures 6A and 6B show that the engineered exosomes NO. 8 significantly down-regulated the melanin synthesis both in the melanocytes and zebrafish model. And in the zebrafish model, the whitening efficacy when treated with NO. 8 reached 48% (P<0.001) .

## Claims

- [Claim 1] An engineered exosome, comprising  
(a) a KGF polypeptide, fused to a first anchoring polypeptide,  
(b) an EGF polypeptide, fused to a second anchoring polypeptide, and  
(c) a FGF-2 polypeptide, fused to a third anchoring polypeptide,  
wherein (a) , (b) and (c) are anchored on a membrane of the exosome  
via the first, second and third anchoring polypeptide, respectively, and  
wherein the KGF polypeptide, the EGF polypeptide and the FGF-2  
polypeptide are exposed on an outer surface of the membrane of the  
exosome.
- [Claim 2] The engineered exosome of claim 1, wherein the engineered exosome  
further comprises (d) a PDGF-BB polypeptide, fused to a fourth  
anchoring polypeptide, and the PDGF-BB polypeptide is exposed on  
the outer surface of the membrane of the exosome.
- [Claim 3] The engineered exosome of claim 1, wherein the KGF polypeptide is  
a KGF-1 polypeptide, preferably the KGF-1 polypeptide comprises  
an amino acid sequence having at least 80%identity to the amino acid  
sequence as shown in SEQ ID NO. 1.
- [Claim 4] The engineered exosome of claim 1, wherein the EGF polypeptide  
is a human EGF or an ortholog or paralog thereof, preferably the  
EGF polypeptide comprises an amino acid sequence having at least  
80%identity to the amino acid sequence as shown in SEQ ID NO. 2.
- [Claim 5] The engineered exosome of claim 1, wherein the FGF-2 polypeptide is  
a human FGF-2 or an ortholog or paralog thereof; more preferably the  
FGF-2 polypeptide comprises an amino acid sequence having at least  
80%identity to the amino acid sequence as shown in SEQ ID NO. 3.
- [Claim 6] The engineered exosome of claim 2, wherein the PDGF-BB  
polypeptide is a human PDGF-BB dimer or an ortholog or paralog  
thereof; more preferably the PGDF-BB dimer comprises an amino acid  
sequence having at least 80%identity to the amino acid sequence as  
shown in SEQ ID NO. 4.
- [Claim 7] The engineered exosome of claim 1 or 2, wherein the anchoring  
polypeptides are membrane proteins of exosome, membrane-targeting  
sequences, or an anchoring functional fragment thereof; preferably,  
the membrane proteins of exosome include lamp2b, tetraspanins such  
as CD63, CD9 and CD81, platelet-derived growth factor receptors  
(PDGFRs) , lactadherin (C1C2 domain) , vesicular stomatitis virus

glycoprotein (VSVG) , prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof; preferably the membrane-targeting sequences include glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins; ; preferably, the anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, each of the first, second, third, and optionally, fourth anchoring polypeptides comprises a TM3 domain of CD63; more preferably, each of the anchoring polypeptides is a TM3 domain of CD63; preferably, the TM3 domain of CD63 comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 9.

- [Claim 8] The engineered exosome of claim 1 or 2, wherein the KGF polypeptide, the EGF polypeptide, the FGF-2 polypeptide, and the PDGF-BB polypeptide is fused to the C-terminus of the first, second, third, and fourth anchoring polypeptides, respectively, optionally through a peptide linker, preferably, the peptide linker consists of glycine and serine, e.g., (G4S)<sub>n</sub>, in which n is an integer from 1 to 3.
- [Claim 9] The engineered exosome of claim 1, wherein the exosome is not derived from a mesenchymal stem cell; preferably, the exosome is not derived from a stem cell.
- [Claim 10] The engineered exosome of claim 1, wherein the engineered exosome: (i) enhances fibroblast proliferation, (ii) upregulates COL2A1, COL3A1, fibronectin, TIMP1, and/or COL1A1B genes, (iii) downregulates MMP7, MMP9, and/or MMP16B genes, and/or (iv) downregulates melanin synthesis.
- [Claim 11] A composition comprising the exosome of any of claims 1 to 10, and a carrier; preferably, the composition is a liquid formulation; more preferably, the composition is formulated for topical or subcutaneous administration; the composition is optionally a cosmetic composition or non-cosmetic composition; and wherein the composition is optionally a pharmaceutical composition.
- [Claim 12] The composition of claim 11, wherein the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide) , an EGF polypeptide, a FGF-2 polypeptide (e.g., a human FGF-2 polypeptide) , or a PDGF-BB polypeptide (e.g., a human PDGF-BB dimer polypeptide) , not attached to the membrane of the exosome.
- [Claim 13] A nucleic acid construct comprising a polynucleotide encoding

- (a) a KGF polypeptide fused to a first anchoring polypeptide,  
 (b) an EGF polypeptide fused to a second anchoring polypeptide, and  
 (c) a FGF-2 polypeptide fused to a third anchoring polypeptide.
- [Claim 14] The nucleic acid construct of claim 11, wherein the polynucleotide further encodes (d) a PDGF-BB polypeptide fused to a fourth anchoring polypeptide.
- [Claim 15] The nucleic acid construct of claim 13 or 14, wherein
- (i) the KGF polypeptide is a KGF-1 polypeptide, preferably the KGF-1 polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 1;
  - (ii) the EGF polypeptide is a human EGF or an ortholog or paralog thereof, preferably the EGF polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 2;
  - (iii) the FGF-2 polypeptide is a human FGF-2 or an ortholog or paralog thereof; more preferably the FGF-2 polypeptide comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 3;
  - (iv) the PDGF-BB polypeptide is a human PDGF-BB dimer; more preferably the PDGF-BB dimer comprises an amino acid sequence having at least 80%identity to the amino acid sequence as shown in SEQ ID NO. 4;
  - (v) a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 5 or a degenerate sequence thereof;
  - (vi) a polynucleotide encoding the EGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 6 or a degenerate sequence thereof;
  - (vii) a polynucleotide encoding the FGF-2 polypeptide has a nucleotide sequence as shown in SEQ ID NO. 7 or a degenerate sequence thereof;
  - (viii) a polynucleotide encoding the PDGF-BB polypeptide has a nucleotide sequence as shown in SEQ ID NO. 8 or a degenerate sequence thereof;
  - (ix) the anchoring polypeptides are membrane proteins of exosome, membrane-targeting sequences, or an anchoring functional fragment thereof; preferably, the membrane proteins of exosome include lam-p2b, tetraspanins such as CD63, CD9 and CD81, platelet-derived growth factor receptors (PDGFRs), lactadherin (C1C2 domain), vesicular stomatitis virus glycoprotein (VSVG), prostaglandin F2 receptor negative regulator (PTGFRN) and any combination thereof;

preferably the membrane-targeting sequences include glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins; ; preferably, the anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, each of the first, second, third, and optionally, fourth anchoring polypeptides comprises a TM3 domain of CD63; more preferably, each of the first, second, third, and optionally, fourth anchoring polypeptides is a TM3 domain of CD63; and/or

(x) a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO. 10 or a degenerate sequence thereof.

- [Claim 16] The nucleic acid construct of any of claims 13 to 15, wherein the polynucleotide is a single polynucleotide comprising a fragment of nucleotides encoding the polypeptides (a) , (b) , (c) and, optionally, (d) , with each of the polypeptides (a) , (b) , (c) and, optionally, (d) linked to the anchoring polypeptide; preferably the polypeptides (a) , (b) , (c) and, optionally, (d) are spaced by self-cleavage peptides; optionally, the self-cleavage peptides are 2A peptides, e.g., T2A, E2A, P2A or any combination thereof.
- [Claim 17] A vector comprising the nucleic acid construct of any of claims 13 to 16; preferably, the vector is a viral vector; more preferably the vector is a lentiviral vector or an adeno-associated viral vector.
- [Claim 18] A cell transduced with the vector of claim 17, wherein the polynucleotide is integrated into the genome of the cell; preferably, the cell is not a mesenchymal stem cell; preferably, the cell is not a stem cell; preferably, the cell is a mammalian cell; or more preferably, the cell is a HEK293 or CHO cell.
- [Claim 19] A method of producing an engineered exosome of any of claims 1 to 10, comprising  
(a) transducing the cell of claim 18 with the vector of claim 17;  
(b) culturing the cell in a condition allowing secretion of an exosome from the cell; and  
(c) collecting and purifying the exosome.
- [Claim 20] The method of claim 19, further comprising adapting the cell to a serum-free condition during step (b) .
- [Claim 21] Use of the engineered exosome of any of claims 1 to 10 or the composition of claims 11 or 12 in manufacturing a medicament for skin rejuvenation; preferably, the skin rejuvenation includes treatment of any

of sun damage, sensitive skin, scars such as from acne or stretch marks such as striae gravidarum or adolescent striae, chickenpox, or injury, wrinkles such as static or dynamic wrinkles, dark circle under eyes, hyperpigmentation such as darkened skin or freckles, loosen skin, uneven or dull skin tone, or any of combination thereof.