

Description

Title of Invention: ENGINEERED EXOSOMES FOR TREATMENT OF ALOPECIA AND/OR CANITIES

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 preventing or treating alopecia and/or canities.

Background

[0002] Hair loss, also known as alopecia or baldness, refers to a loss of hair from part of the head or body. The severity of hair loss can vary from a small area to the entire body. Alopecia may be caused by psychological distress or may be drug induced, among other causes. Common types of alopecia include androgenetic alopecia (including male-pattern hair loss and female-pattern hair loss) , alopecia areata, and a thinning of hair known as telogen effluvium. Causes of male-pattern hair loss include a combination of genetics and male hormones, causes of female pattern hair loss are unclear, the cause of alopecia areata is autoimmunity, and the cause of telogen effluvium is typically a physically or psychologically stressful event.

[0003] The hair follicle is a regenerating organ where stem cells enable a massive large-scale renewal. The hair follicle is composed of an outer root sheath, an inner root sheath, and the hair shaft. The proliferating undifferentiated matrix cells give rise to the inner root sheath and the hair shaft and are surrounded by a dermal papilla of specialized mesenchymal cells. The dermal papilla instructs the formation of the follicle, but the characteristics of the follicle are acquired by epithelial information. The lower portion of the follicle goes through a growth cycle that involves the phases of anagen (active growth) , catagen (destruction) and telogen (quiescence) . These different phases last for varying time periods depending on the hair follicle location and function. The matrix cells proliferate rapidly during the anagen phase, migrate upwards then differentiate into the cell types of the inner root sheath and hair shaft. During the catagen phase, the lower follicle undergoes apoptotic death, and the dermal papilla moves upwards until it reaches the area beneath the bulge. It remains there during telogen. Once the dermal papilla recruits stem cells from the bulge, anagen begins anew and the follicle can regenerate through proliferation and differentiation.

- [0004] The management of alopecia generally involves use of medication or surgery. Medicines currently used in treatment of alopecia are minoxidil, finasteride, and dutasteride. The management of alopecia by surgery involves hair transplantation. Hair transplantation is usually carried out under local anesthesia. A surgeon will move healthy hair from the back and sides of the head to areas of thinning. The procedure can take between four and eight hours, and additional sessions can be carried out to make hair even thicker. Conventional hair transplantation process suffers when there are not enough hairs in the non-bald areas. Stem cell therapy represents an emerging strategy for treatment and management of alopecia. However, in the context of conventional hair transplantation, administration of hair follicle stem cells into the bald scalp zone has a very low efficiency since there are no required growth factors for the growth of new follicles in the skin of such a zone.
- [0005] Canities (natural hair whitening) is linked to a specific and gradual depletion of the hair melanocytes which affects both the melanocytes of the hair bulb and the precursor cells for melanocytes. Other cell types present in the hair follicles are not affected. In addition, this depletion of melanocytes is not observed in the epidermis. The cause of this gradual and specific depletion of melanocytes and melanocyte precursors in the hair follicle has not been identified to date.
- [0006] There exists a need for additional and improved methods and compositions for treatment of alopecia, including androgenetic alopecia and alopecia areata. It is also necessary to combat the disappearance of melanocytes from human hair follicles, a process which affects both the active melanocytes of the bulbs and the quiescent melanocytes of the upper region of the hair follicles, in order to combat canities.

Summary of the Invention

- [0007] In one aspect of the invention, provided is an engineered exosome, comprising (a) a KGF polypeptide, fused to a first anchoring polypeptide, (b) a VEGF-A polypeptide, fused to a second anchoring polypeptide, and (c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, 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 VEGF-A polypeptide and the Wnt family member polypeptide are exposed on an 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 or 4.
- [0009] In some embodiments, the VEGF-A polypeptide is a human VEGF₂₀₆ isomer or an ortholog or paralog thereof, preferably the VEGF-A polypeptide comprises an amino

acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 2 or 5.

- [0010] In some embodiments, the Wnt family member polypeptide is Wnt10a, 10b, 3a, 1a, 4, 5a, 7a, or 7b polypeptide; preferably the Wnt family member polypeptide is Wnt10b polypeptide; more preferably the Wnt10b polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 3 or 6.
- [0011] 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.
- [0012] In preferable embodiments, the first, second, and third anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, and third anchoring polypeptides is a TM3 domain of CD63. In some embodiments, each of the first, second, and third 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: 13.
- [0013] In some embodiments, the KGF polypeptide, the VEGF-A polypeptide and the Wnt family member polypeptide is fused to the C-terminus of the first, second, and third anchoring polypeptides, respectively, optionally through a peptide linker, preferably, the peptide linker consists of glycine and serine, e.g., (G4S)_n, in which n is an integer from 1 to 3.
- [0014] In some embodiments, the exosome is not derived from a mesenchymal stem cell; preferably, the exosome is not derived from a stem cell.
- [0015] In some embodiments, the exosome promotes hair regrowth, and/or improves alopecia, e.g. androgenetic alopecia, alopecia areata, or telogen effluvium. In some embodiments, the exosome prevents and/or limits and/or stops the development of canities and maintains and/or promotes the natural repigmentation of head hair and/or body hair. In some embodiments, the exosome: (i) promotes hair regrowth, and/or improves alopecia, e.g. androgenetic alopecia, alopecia areata, or telogen effluvium; and (ii) prevents and/or limits and/or stops the development of canities and maintains and/or promotes the natural repigmentation of head hair and/or body hair.

- [0016] Another aspect of the disclosure relates to a composition comprising the exosome of any of claims 1 to 8, and a carrier; preferably, the composition is a liquid formulation; more preferably, the composition is formulated for topical or subcutaneous administration.
- [0017] In some embodiments, the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide), a VEGF-A polypeptide, or a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway (e.g., a Wnt10b polypeptide), not attached to the membrane of the exosome.
- [0018] 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) a VEGF-A polypeptide fused to a second anchoring polypeptide, and (c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, fused to a third anchoring polypeptide.
- [0019] 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 or 4.
- [0020] In some embodiments, the VEGF-A polypeptide is a human VEGF₂₀₆ isomer or an ortholog or paralog thereof, preferably the VEGF-A polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 2 or 5.
- [0021] In some embodiments, the Wnt family member polypeptide is Wnt10a, 10b, 3a, 1a, 4, 5a, 7a, or 7b polypeptide; preferably the Wnt family member polypeptide is Wnt10b polypeptide; more preferably the Wnt10b polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 3 or 6.
- [0022] In some embodiments, a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO: 7, 10 or a degenerate sequence thereof.
- [0023] In some embodiments, a polynucleotide encoding the VEGF-A polypeptide has a nucleotide sequence as shown in SEQ ID NO: 8, 11 or a degenerate sequence thereof.
- [0024] In some embodiments, a polynucleotide encoding the Wnt family member polypeptide has a nucleotide sequence as shown in SEQ ID NO: 9, 12 or a degenerate sequence thereof.
- [0025] 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 com-

ination thereof. Exemplary membrane-targeting sequences include but are not limited to glycosylphosphatidylinositol (GPI) anchors and lipid-anchored proteins.

- [0026] In preferable embodiments, the first, second, and third anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, and third 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: 14 or a degenerate sequence thereof.
- [0027] In some embodiments, the polynucleotide is a single polynucleotide comprising a fragment of nucleotides encoding (a), (b), and (c); preferably the polynucleotide comprises, from 5' to 3', a fragment of nucleotides encoding:
- [0028] (i) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt],
- [0029] (ii) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A],
- [0030] (iii) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt],
- [0031] (iv) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF],
- [0032] (v) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A], or
- [0033] (vi) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF],
- [0034] wherein, [] represents a separate polypeptide, and] - [represents a linker or a bond;
- [0035] wherein, Wnt represents a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway.
- [0036] In some embodiments, the self-cleavage peptide is a 2A peptide, e.g., T2A, E2A, P2A or any combination thereof.
- [0037] In other embodiments, each of the first, second, and third anchoring polypeptide may be located at the C terminus of Wnt, KGF, and/or VEGF, 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., Wnt, KGF, and VEGF) to be

presented on the surface of the exosome may be located at N terminus of lamp2b. 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. For example, in one embodiment, the single polynucleotide comprising a fragment of nucleotides encoding (a) , (b) , and (c) comprises, from 5' to 3' , a fragment of nucleotides encoding: [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [VEGF-A] - [2nd anchoring polypeptide] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF] , in which the second anchoring polypeptide is located at the C terminus of VEGF-A polypeptide.

- [0038] 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.
- [0039] 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.
- [0040] 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.
- [0041] 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.
- [0042] In some embodiments, the method further comprises adapting the cell to a serum-free condition during step (b) .
- [0043] A further aspect of the disclosure relates to use of the engineered exosomes, or the composition as disclosed herein manufacturing a medicament for treatment of alopecia and/or canities; preferably, the alopecia is androgenetic alopecia, alopecia areata, or telogen effluvium; preferably, the canities is age-related hair graying, premature canities, or canities subita.
- [0044] A further aspect of the disclosure relates to a method of treating alopecia and/or canities; preferably, the alopecia is androgenetic alopecia, alopecia areata, or telogen effluvium; preferably, the canities is age-related hair graying, premature canities, or canities subita; comprising administering to a subject an effective amount of the engineered exosomes, or the composition as disclosed herein.
- [0045] A further aspect of the disclosure relates to the engineered exosomes, or the composition as disclosed herein for use in treatment of alopecia and/or canities; preferably, the alopecia is androgenetic alopecia, alopecia areata, or telogen effluvium; preferably, the canities is age-related hair graying, premature canities, or canities subita.

[0046] 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

[0047] 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, KGF, VEGFA and Wnt10b. Then blasticidin 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.

[0048] Figure 2. The identification of engineered exosomes used for androgenetic alopecia. (A) The particle diameter and particle concentration of exosomes derived from the stable cell line were analyzed by NanoFCM. (B) The exosomes were inspected by transmission electron microscopy (TEM) . (C) Immunoblotting analysis of exosomes with antibody against CD63-TM3, the exosome fused scaffold proteins.

[0049] Figure 3. In vivo evaluation of the engineered exosome 35# for hair loss treatment. (A) Schematic representation of the androgenetic alopecia mouse model and the regimen. After dorsal hair depilation, mice skin was treated every 3 days with 35#exosome, control exosome (Ctrl Exo.) , PBS (negative control) , or 5%minoxidil (positive control) . Observation continued over an 18-day treatment period, then animals were sacrificed at D18 for histologic analysis. (B) Observation of hair coverage. Mice were divided into four groups (n = 4) and representative images of mice at day15 were showed. (C) Histological analysis of mice dorsal skin regarding the hair follicle quantity, length, and growth phase ration. Data points represent mean $\pm$ SD (n = 4) . Error bars indicate SD. ns, no significant difference; *P < 0.05, **P < 0.01, and ***P < 0.001.

[0050] Figure 4. In vivo evaluation of the engineered exosome 35# on anti-graying hair. (A) Schematic representation of the hydroquinone-induced hair graying model and the regimen. After dorsal hair depilation, female C57BL/6 mice were randomly divided into groups (n = 4) and were topically applied by hydroquinone cream 2 times a day. Negative model control group was treated with PBS via topical application by smearing. 35#exosome groups were treated with different administration route, e.g., microneedling followed by smearing, Nanocrystalline infusion followed by smearing, subcutaneously injection. Observation continued over a 28-day treatment period. (B) Observation of dorsal hair. Representative images of mice on day14 were showed.

Detailed Description of the Invention

- [0051] Definition
- [0052] The words “a” and “an” when used in the present specification in concert with the word comprising, including the claims, denote “one or more.”
- [0053] 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.
- [0054] 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.
- [0055] “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.
- [0056] 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.
- [0057] 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.
- [0058] 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.

[0059] 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.

[0060] 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.

[0061] 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.

[0062] 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. The subject can be a patient, e.g., have or be suspected of having or at risk for having a disease or medical condition related to hair loss. For subjects having or suspected of having a medical condition directly or indirectly associated with hair loss, the medical condition may be of one or more types.

- [0063] “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.
- [0064] 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.
- [0065] 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.

[0066] The term “VEGFA” or vascular endothelial growth factor A is the main member of vascular endothelial growth factor (VEGF) family of cytokines, which plays a key role in vasculogenesis, angiogenesis, and lymphangiogenesis, alongside placental growth factor (PlGF), VEGF-B/C/D in mammals, and VEGF-E/F in other organisms. Initially designated as “VEGF” in early 1989 by N. Ferrara et al., VEGF-A is the most extensively studied member of the VEGF family. Distinct VEGF-A isoforms result from alternative splicing of the *Vegfa* gene at exon 8, resulting in VEGF_{xxx}a or VEGF_{xxx}b isoforms. Alternative splicing events at exons 5–7, in addition to recently identified posttranslational read-through events, produce VEGF-A isoforms that differ in their bioavailability and interaction with the co-receptor Neuropilin-1. To date 16 distinct VEGFA isoforms have been identified most commonly from six transcripts: VEGF₁₁₁, VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆. In the rat, a similar profile of VEGF-A splice variants has been described, each encoding one fewer amino acid than the human species. The shorter isoforms VEGF₁₁₁ and VEGF₁₂₁ both lack exons 6 and 7, and as a consequence are not tethered to the extracellular matrix (ECM) and are freely diffusible. In contrast the longer isoforms VEGF₁₄₅, VEGF₁₈₉ and VEGF₂₀₆ containing both exons 6a and 7 can bind with high affinity to heparin sulphate glycoproteins.

[0067] The term “Wnt/ β -catenin signaling pathway” refers to the canonical Wnt pathway which involves the nuclear translocation of β -catenin and activation of target genes via TCF/LEF transcription factors. The canonical Wnt pathway mainly controls cell proliferation. The noncanonical Wnt pathways are independent of β -catenin-T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), such as the Wnt/ Ca^{2+} pathway and noncanonical Wnt planar cell polarity. The Wnt/ β -catenin signalling pathway is necessary for embryonic development and adult tissue homeostasis regeneration. Abnormal regulation of the pathway is closely associated with different diseases, suggesting that the Wnt/ β -catenin signaling pathway is an attractive target for disease treatment. The term “Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway” refers to any Wnt family member or a functional fragment thereof involved in Wnt/ β -catenin signaling pathway, including but are not limited to, Wnt10a, Wnt10b, Wnt3a, Wnt1a, Wnt4, Wnt5a, Wnt7a, or Wnt7b polypeptide.

[0068] 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.

[0069] 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.

[0070] Engineered Exosomes

[0071] One aspect of the present disclosure relates to an engineered exosome, comprising (a) a KGF polypeptide, fused to a first anchoring polypeptide, (b) a VEGF-A polypeptide, fused to a second anchoring polypeptide, and (c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, 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 VEGF-A polypeptide and the Wnt family member polypeptide are exposed on an outer surface of the membrane of the exosome.

[0072] In the present disclosure, the KGF polypeptide is 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 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.

- [0073] In preferable 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: 4. In some embodiments, the KGF-1 polypeptide comprises an amino acid sequence as shown in SEQ ID NO: 4, which is human KGF-1 polypeptide. In some embodiments, the amino acid sequence of the KGF-1 polypeptide is shown in SEQ ID NO: 4.
- [0074] In the present disclosure, the VEGF-A polypeptide may be any of VEGF-A isomers found available in the art. In preferable embodiments, the VEGF-A polypeptide is a human VEGF₂₀₆ isomer or an ortholog or paralog thereof. In some embodiments, the VEGF-A 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.
- [0075] In preferable embodiments, the VEGF-A 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: 5. In some embodiments, the VEGF-A polypeptide comprises an amino acid sequence as shown in SEQ ID NO: 5, which is human VEGF₂₀₆ isomer. In some embodiments, the amino acid sequence of the human VEGF₂₀₆ isomer is shown in SEQ ID NO: 5.
- [0076] In the present disclosure, the Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway could be any Wnt family member involved in Wnt/ β -catenin signaling pathway. In preferable embodiments, the Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway is Wnt10a, 10b, 3a, 1a, 4, 5a, 7a, or 7b polypeptide.
- [0077] In preferable embodiments, the Wnt family member polypeptide is Wnt 10b. In preferable embodiments, the Wnt10b 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 preferable embodiments, the Wnt10b 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: 6. In some embodiments, the Wnt10b polypeptide comprises an amino acid sequence as shown in SEQ ID NO: 6, which is human Wnt10b polypeptide. In some embodiments, the amino acid sequence of the Wnt10b polypeptide is shown in SEQ ID NO: 6.
- [0078] 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, the first, second, and third anchoring polypeptides comprise a TM3 domain of CD63. In preferable embodiments, each of the first, second, and third 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, and third anchoring polypeptides can be different or same. In preferable embodiments, the first, second, and third anchoring polypeptides are same and consist of TM3 domain of CD63.

[0079] 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: 13. In preferable embodiments, the TM3 domain of CD63 comprises an amino acid sequence as shown in SEQ ID NO: 13. In preferable embodiments, the amino acid sequence of the TM3 domain of CD63 is shown in SEQ ID NO: 13.

[0080] Therefore, in preferable embodiments, provided is an engineered exosome comprising (a) a KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) a VEGF-A polypeptide, fused to a second anchoring polypeptide, and (c) a Wnt10b 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 VEGF-A polypeptide and the Wnt10b polypeptide are exposed on an outer surface of the membrane of the exosome.

[0081] In preferable embodiments, provided is an engineered exosome comprising (a) a human KGF-1 polypeptide, fused to a first anchoring polypeptide, (b) a human VEGF-A polypeptide, fused to a second anchoring polypeptide, and (c) a human Wnt10b 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 VEGF-A polypeptide and the human Wnt10b polypeptide are exposed on an outer surface of the membrane of the exosome.

[0082] 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: 4, fused to a first anchoring polypeptide, (b) a VEGF-A 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: 5, fused to a second anchoring polypeptide, and (c) a Wnt10b 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: 6, 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 VEGF-A polypeptide and the Wnt10b 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 KGF-1 polypeptide comprising an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO: 4, fused to a first anchoring polypeptide, (b) a VEGF-A 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: 5, fused to a second anchoring polypeptide, and (c) a Wnt10b 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: 6, 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: 13, 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 VEGF-A polypeptide and the Wnt10b 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 as shown in SEQ ID NO: 4,

fused to a first anchoring polypeptide, (b) a VEGF-A polypeptide comprising an amino acid sequence as shown in SEQ ID NO: 5, fused to a second anchoring polypeptide, and (c) a Wnt10b polypeptide comprising an amino acid sequence as shown in SEQ ID NO: 6, 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: 13, 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 VEGF-A polypeptide and the Wnt10b polypeptide are exposed on an outer surface of the membrane of the exosome.

- [0085] In any of the embodiments described above, the KGF polypeptide, the VEGF-A polypeptide and the Wnt family member polypeptide is fused to the C-terminus of the first, second, and third 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)_n, in which n is an integer from 1 to 3.
- [0086] 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.
- [0087] In some embodiments, the exosome provided by the present disclosure promotes hair regrowth, and/or improves alopecia, e.g. androgenetic alopecia, alopecia areata, or telogen effluvium. For example, the exosome provided by the present disclosure increases the number of hair follicle, the length of hair follicle, and/or the anagen/telogen ratio.
- [0088] In some embodiments, the exosome provided by the present disclosure prevents and/or limits and/or stops the development of canities and maintains and/or promotes the natural repigmentation of head hair and/or body hair.
- [0089] In some embodiments, the exosome provided by the present disclosure promotes hair regrowth, and/or improves alopecia, e.g. androgenetic alopecia, alopecia areata, or telogen effluvium; and prevents and/or limits and/or stops the development of canities and maintains and/or promotes the natural repigmentation of head hair and/or body hair.
- [0090] Methods and Compositions for Treatment or Prevention of Alopecia
- [0091] Aspects of the present disclosure are directed to methods and compositions for treatment or prevention of alopecia, canities, or both. Alopecia (also “hair loss”) describes any loss of hair from any part of the head of body of a subject. Various types

of alopecia are recognized in the art and contemplated herein including, but not limited to, androgenetic alopecia (e.g., male-pattern hair loss, female-pattern hair loss), alopecia areata, and telogen effluvium. In some embodiments, disclosed are methods for treatment of alopecia areata. Canities (natural hair whitening) is linked to a specific and gradual depletion of the hair melanocytes which affects both the melanocytes of the hair bulb and the precursor cells for melanocytes. Various types of canities are recognized in the art and contemplated herein including, but not limited to, canities is age-related hair graying, premature canities, or canities subita. In some embodiments, disclosed are methods for treatment of age-related hair graying.

[0092] As disclosed herein, the engineered exosomes provided by the disclosure are useful in the treatment and prevention of alopecia, canities, or both. Accordingly, embodiments of the disclosure are directed to methods for treatment or prevention of alopecia, canities, or both, comprising providing to a subject an effective amount of the engineered exosomes. In some embodiments, methods of the disclosure comprise administration of the engineered exosomes to a subject for treatment or prevention of alopecia, canities, or both. Administration of such compositions include, for example, topical administration, transdermal administration, and intradermal administration.

[0093] The engineered exosomes may be provided to a subject in combination with one or more therapeutics or therapies. When the engineered exosome is intended for use in treatment of alopecia, in one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) diphenylcyclopropanone. In one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) baricitinib. In one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) minoxidil. In one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) finasteride. In one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) spironolactone or dutasteride. In one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) hair transplant surgery.

[0094] When the engineered exosome is intended for use in treatment of canities, in one example, the engineered exosomes of the disclosure are administered to a subject in combination with (e.g., simultaneous with, prior to, or subsequent to) hair dyeing.

[0095] 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 sub-

cutaneous administration. Contemplated are compositions comprising the engineered exosomes, including, for example, soaps, shampoos, ointments, and other such formulations.

- [0096] In preferable embodiments, the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide), a VEGF-A polypeptide, or a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway (e.g., a Wnt10b polypeptide), not attached to the membrane of the exosome. For example, no additional KGF polypeptide (e.g. a KGF-1 polypeptide), a VEGF-A polypeptide, or a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway (e.g., a Wnt10b polypeptide) is added to the composition except for the polypeptides anchored to the membrane of the engineered exosomes.
- [0097] 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. In some embodiments, the composition comprises a further active agent for treatment of alopecia. The further active agent for treatment of alopecia is preferably selected from a group consisting of diphenylcyclopropanone, baricitinib, minoxidil, finasteride, spironolactone, dutasteride or any combination thereof. In preferable embodiments, the further active agent for treatment of alopecia is diphenylcyclopropanone, baricitinib, or minoxidil. In some embodiments, the composition comprises a further active agent for treatment of canities.
- [0098] Nucleic Acid Constructs, Vectors, Cells, and Methods of Production
- [0099] Aspects of the present disclosure relate also to nucleic acid constructs that encode the polypeptides anchored on the exosomes.
- [0100] In some embodiments, provided is a nucleic acid construct comprising a polynucleotide encoding (a) a KGF polypeptide fused to a first anchoring polypeptide, (b) a VEGF-A polypeptide fused to a second anchoring polypeptide, and (c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, fused to a third anchoring polypeptide.
- [0101] 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.
- [0102] In some embodiments, provided are a set of two nucleic acid constructs, with one nucleic acid construct comprising a polynucleotide encoding (a) and (b), and the other nucleic acid construct comprising a polynucleotide encoding (c), wherein (a), (b), and (c) are defined as above.

- [0103] In some embodiments, provided are a set of two nucleic acid constructs, with one nucleic acid construct comprising a polynucleotide encoding (a) and (c) , and the other nucleic acid construct comprising a polynucleotide encoding (b) , wherein (a) , (b) , and (c) are defined as above.
- [0104] In some embodiments, provided are a set of two nucleic acid constructs, with one nucleic acid construct comprising a polynucleotide encoding (b) and (c) , and the other nucleic acid construct comprising a polynucleotide encoding (a) , wherein (a) , (b) , and (c) are defined as above.
- [0105] 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.
- [0106] 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.
- [0107] In some embodiments, each of the first, second, and third anchoring polypeptide may be located at the N terminus of Wnt, KGF, and/or VEGF, to ensure that the latter are exposed on the surface of the exosome. In some embodiments, each of the first, second, and third anchoring polypeptide may be located at the C terminus of Wnt, KGF, and/or VEGF, 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., Wnt, KGF, and VEGF) 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., Wnt, KGF, and VEGF) 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.
- [0108] In preferable embodiments, the first, second, and third anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain. In preferable embodiments, each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63. In preferable embodiments, each of the first, second, and third anchoring polypeptides is a TM3 domain of CD63.

- [0109] In the preferable embodiments, the polynucleotide may comprise, from 5' to 3', a fragment of nucleotides encoding:
- [0110] (i) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt] ,
- [0111] (ii) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A] ,
- [0112] (iii) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt] ,
- [0113] (iv) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF] ,
- [0114] (v) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A] , or
- [0115] (vi) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF] ,
- [0116] wherein, [] represents a separate polypeptide, and] - [represents a linker or a bond; wherein, each of the polypeptide is arranged from N to C termini; and wherein, Wnt represents a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway.
- [0117] 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 independent fusion proteins, each comprising a single polypeptide to be presented onto the surface of the exosome and a single anchoring polypeptide.
- [0118] In other embodiments, the single polynucleotide comprising a fragment of nucleotides encoding (a) , (b) , and (c) comprises, from 5' to 3' , a fragment of nucleotides encoding: [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [VEGF-A] - [2nd anchoring polypeptide] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF] , in which the second anchoring polypeptide is located at the C terminus of VEGF-A polypeptide. This change is also applicable to any of the polynucleotides listed in (i) to (vi) as above. In some embodiments, each of the first, second, and third anchoring polypeptides are different and thus the anchoring polypeptides may locate at N or C terminus of the polypeptides Wnt, KGF and VEGF-A.

- [0119] 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
- [0120] (i) [TM3] - [KGF] - [T2A] - [TM3] - [VEGF-A] - [T2A] - [TM3] - [Wnt] ,
- [0121] (ii) [TM3] - [KGF] - [T2A] - [TM3] - [Wnt] - [T2A] - [TM3] - [VEGF-A] ,
- [0122] (iii) [TM3] - [VEGF-A] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [Wnt] ,
- [0123] (iv) [TM3] - [VEGF-A] - [T2A] - [TM3] - [Wnt] - [T2A] - [TM3] - [KGF] ,
- [0124] (v) [TM3] - [Wnt] - [T2A] - [TM3] - [KGF] - [T2A] - [TM3] - [VEGF-A] , and
- [0125] (vi) [TM3] - [Wnt] - [T2A] - [TM3] - [VEGF-A] - [T2A] - [TM3] - [KGF] ,
- [0126] wherein, [] represents a separate polypeptide, and] - [represents a linker or a bond; wherein, each of the polypeptide is arranged from N to C termini; and wherein, Wnt represents a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway; TM3 represents TM3 domain of CD63; and T2A represents self-cleavage peptide T2A.
- [0127] In this section, the KGF, VEGF-A, and Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, have the meaning and preferable embodiments given above in reference to the section titled Engineered Exosomes.
- [0128] In some embodiments, a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO: 7, 10 or a degenerate sequence thereof.
- [0129] In some embodiments, a polynucleotide encoding the VEGF-A polypeptide has a nucleotide sequence as shown in SEQ ID NO: 8, 11 or a degenerate sequence thereof.
- [0130] In some embodiments, a polynucleotide encoding the Wnt family member polypeptide has a nucleotide sequence as shown in SEQ ID NO: 9, 12 or a degenerate sequence thereof.
- [0131] In some embodiments, a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO: 14 or a degenerate sequence thereof.
- [0132] 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.
- [0133] 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.
- [0134] 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.

[0135] 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.

[0136] 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.

[0137] Sequences Listings

SEQ ID NO:	Description	Sequences
1	Mouse KGF-1 (FGF-7) AA	CNDMSPEQTATSVNCSSPERHTRS DYMEGGDIRVRRLF CRTQWYLRIDKRGKVKG TQEMKNSYNIMEIRT VAVGIVA IKGVESEYYLAMNKEGKLYAKKECNEDCNFKELILENH YNTYASAKWTHSGGEMFVALNQKGIPVKGKKTKEQK TAHFLPMAIT
2	Mouse VEGF-A AA	APTTEGEQKSHEVIKFMDVYQRSYCRPIETLVDIFQEYPD EIEYIFKPSCVPLMRCAGCCNDEALECVPTSES NITMQIM RIKPHQSQHIGEMSFLQHSRCECRPKKDRTKPEKKSVRG KGKGQKRKRKKS RFKSWSVHCEPC SERRKHLFVQDPQT CKCCKNTDSRCKARQLELNERTCRCDKPRR
3	Mouse Wnt10b AA	NEILGLKLPGEPPLTANTVCLTSLGLSKRQLGLCLRSPDV TASALQGLHI AVHECQHQLRDQRWNCSALEGGGRLPHH SAILKRGFRESAFSFSMLAAGVMHAVATACSLGKLVSCG

		CGWKGSGEQDRLRAKLLQLQALSRGKTFPISQSPVPVGS VPSPGPQDTWEWGGCNHDMDFGEKFSRDFLDSREAPRD IQARMRIHNNRVGRQVV TENLKRKCKCHGTSGSCQFKT CWRAAPEFRAIGAALRERLSRAIFIDTHNRNSGAFQPRLR PRRLSGELVYFEKSPDFCERDPTLGSPGTRGRACNKTSRL LDGCGSLCCGRGHNVLRQTRVERCHCRFWCCYVLCD ECKVTEWVNVCK
4	Human KGF-1 AA	CNDMTPEQMATNVNCSSPERHTRS YDYMEGGDIRVRRL FCRTQWYLRIKRGKVKG TQEMKNNYNIMEIRTVAVGI VAIKGVESEFYLAMNKEGKLYAKKECNEDCNFKELILEN HYNTYASAKWTHNGGEMFVALNQKGIPVRGKTKKEQ KTAHFLPMAIT
5	Human VEGF-A AA	APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLV DIFQE YPDEIEYIFKPSCVPLMR CGGCCNDEGLECVPTESNITM QIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEKK SVRGKGKGQKRKRKKSRYKSWSVYVGARCC LMPWSLP GPHPCGPCSERRKHLFVQDPQTCKCCKNTDSRCKARQ LELNERTCRCDKPRR
6	Human Wnt10b AA	NEILGLKLPGEPPLTANTVCLTSLGLSKRQLGLCLRNPDV TASALQGLHIAVHECQHQLRDQRWNC SALEGGGRLPHH SAILKRGFRESAFSFSMLAAGVMHAVATACSLGKLVSCG CGWKGSGEQDRLRAKLLQLQALSRGKSFP HSLPSPGPGS SPSPGPQDTWEWGGCNHDMDFGEKFSRDFLDSREAPRD IQARMRIHNNRVGRQVV TENLKRKCKCHGTSGSCQFKT CWRAAPEFRAVGAALRERLGRAIFIDTHNRNSGAFQPRL RPRRLSGELVYFEKSPDFCERDPTMGSPGTRGRACNKTS RLLDGCGSLCCGRGHNVLRQTRVERCHCRFWCCYVL CDECKVTEWVNVCK
7	Human KGF-1 DNA	TGCAACGATATGACACCCGAGCAGATGGCCACCAATGT GAATTGTTCCAGCCCTGAGAGGCACACCAGATCCTAC GATTACATGGAGGGCGGCGACATCAGGGTGAGAAGAC TGTTTTGTAGAACACAGTGGTACCTGAGGATCGACAA GAGAGGCAAGGTGAAGGGCACCCAGGAGATGAAGAA TAATTACAACATCATGGAGATCAGAACCGTGGCCGTGG GCATCGTGGCCATCAAGGGCGTGGAGAGCGAGTTTTA CCTGGCCATGAACAAGGAGGGCAAGCTGTACGCCAAG AAGGAGTGCAATGAGGACTGTAAC TTTAAGGAGCTGA TCCTGGAGAACCACTACAACACCTACGCCTCCGCCAA GTGGACCCACAACGGCGGCGAGATGTTCTGTGGCCCTG AATCAGAAGGGCATCCAGTGAGGGGCAAGAAGACA AAGAAGGAGCAGAAGACCGCCCACTTTCTGCCTATGG CCATCACC
8	Human VEGF-A DNA	GCGCCGATGGCGGAAGGCGGCGGCCAGAACCATCATG AAGTGGTGAAATTTATGGATGTGTATCAGCGCAGCTAT

		TGCCATCCGATTGAAACCCTGGTGGATATTTTTCAGGA ATATCCGGATGAAATTGAATATATTTTAAACCGAGCTG CGTGCCGCTGATGCGCTGCGGCGGCTGCTGCAACGAT GAAGGCCTGGAATGCGTGCCGACCGAAGAAAGCAAC ATTACCATGCAGATTATGCGCATTAAACCGCATCAGGG CCAGCATATTGGCGAAATGAGCTTTCTGCAGCATAACA AATGCGAATGCCGCCCGAAAAAAGATCGCGCGCGCCA GGAAAAAAAAGCGTGCGCGGCAAAGGCCAAAGGCCA GAAACGCAAACGCAAAAAAGCCGCTATAAAAGCTG GAGCGTGTATGTGGGCGCGCGCTGCTGCCTGATGCCGT GGAGCCTGCCGGGCCCGCATCCGTGCGGCCCGTGAG CGAACGCCGCAAACATCTGTTTGTGCAGGATCCGCAG ACCTGCAAATGCAGCTGCAAAAAACACCGATAGCCGCT GCAAAGCGCGCCAGCTGGAAGTGAACGAACGCACCT GCCGCTGCGATAAACCGCGCCGC
9	Human Wnt10b DNA	AATGAGATCCTGGGCCTGAAGCTGCCCGGCGAGCCTC CTCTGACCGCCAATACAGTGTGCCTGACCCTGTCCGGC CTGAGCAAGAGACAGCTGGGCCTGTGCCTGAGAAATC CCGATGTGACCGCCAGCGCCCTGCAGGGACTGCACAT CGCCGTGCACGAGTGTGAGCACCAGCTGAGAGACCAG AGATGGAATTGCAGCGCCCTGGAGGGCGGCGGCAGAC TGCCTCATCACAGCGCCATCCTGAAGAGAGGCTTCAG GGAGAGCGCCTTCAGCTTCTCCATGCTGGCCGCCGGC GTGATGCACGCCGTGGCTACAGCCTGCAGCCTGGGCA AGCTGGTGTCTGCGGCTGCGGCTGGAAGGGCAGCGG AGAGCAGGACAGGCTGAGAGCCAAGCTGCTGCAGCT GCAGGCCCTGTCCAGAGGCAAGAGCTTCCCTCACAGC CTGCCTAGCCCTGGCCCTGGCTCCAGCCCAAGCCCTG GACCTCAGGACACCTGGGAGTGGGGCGGCTGTAATCA CGATATGGATTTTCGGCGAGAAGTTTCCAGAGATTTCC TGGATAGCAGAGAGGCTCCTAGAGATATCCAGGCCAG AATGAGGATCCACAACAACAGGGTGGGCAGACAGGTG GTGACAGAGAACCTGAAGAGAAAGTGTAAGTGCCAC GGCACAAGCGGCTCCTGTGAGTTCAAGACATGCTGGA GAGCCGCTCCTGAGTTCAGAGCCGTGGGCGCCGCCCT GAGAGAGAGACTGGGCAGAGCCATCTTTATCGATACC CACAATAGAAACTCCGGCGCCTTCCAGCCTAGACTGA GGCCCAGGAGACTGTCCGGCGAGCTGGTGTACTTCGA GAAGAGCCCTGACTTCTGTGAGAGGGACCCTACCATG GGCAGCCCTGGCACAAGAGGCAGGGCCTGTAATAAGA CCAGCAGACTGCTGGACGGCTGCGGCTCCCTGTGCTG TGGCAGGGGACACAACGTGCTGAGACAGACCAGGGT GGAGAGATGTCACTGTAGGTTCCACTGGTGTGTTACG TGCTGTGCGATGAGTGCAAGGTGACCGAGTGGGTGAA

		CGTGTGCAAG
10	Mouse KGF-1 DNA	TGTAATGATATGAGCCCTGAGCAGACCGCCACAAGCGT GAATTGTTCCAGCCCTGAGAGACACACCAGATCCTAC GACTACATGGAGGGCGGCGACATCAGAGTGAGAAGAC TGTTCTGTAGAACCCAGTGGTACCTGAGAATCGACAA GAGGGGCAAGGTGAAGGGCACCCAGGAGATGAAGAA TAGCTACAATATCATGGAGATCAGGACAGTGGCCGTGG GCATCGTGGCCATCAAGGGCGTGGAGAGCGAGTACTA CCTGGCCATGAACAAGGAGGGCAAGCTGTACGCCAAG AAGGAGTGTAATGAGGACTGTAACCTCAAGGAGCTGA TCCTGGAGAATCACTACAATACATACGCCAGCGCCAAG TGGACCCACTCCGGCGGAGAGATGTTTGTGGCCCTGA ACCAGAAGGGCATCCCAGTGAAGGGCAAGAAGACCA AGAAGGAGCAGAAGACCGCCCACTTCCTGCCCATGGC CATCACA
11	Mouse VEGF-A DNA	GCTCCTACAACCGAGGGCGAGCAGAAGTCCCACGAGG TGATCAAGTTTATGGACGTGTACCAGAGATCCTACTGT AGGCCTATCGAGACACTGGTGGACATCTTCCAGGAGT ACCCTGACGAGATCGAGTACATCTTCAAGCCCTCCTGT GTGCCTCTGATGAGATGCGCCGGCTGCTGTAACGATGA GGCCCTGGAGTGTGTGCCTACAAGCGAGAGCAACATC ACAATGCAGATCATGAGAATCAAGCCTCATCAGTCCCA GCACATCGGCGAGATGTCCTTTCTGCAGCACTCCAGGT GCGAGTGCAGACCTAAGAAGGACAGAACAAGCCCG AGAAGAAGAGCGTGAGGGGCAAGGGCAAGGGCCAGA AGAGAAAGAGGAAGAAGTCCAGATTTAAGAGCTGGTC CGTGCACTGTGAGCCTTGCTCCGAGAGGAGAAAGCAC CTGTTTCGTGCAGGACCCTCAGACATGTAAGTGCAGCT GTAAGAACACAGACAGCAGATGTAAGGCCAGGCAGCT GGAGCTGAACGAGAGAACCTGTAGATGTGACAAGCCC AGAAGA
12	Mouse Wnt10b DNA	AACGAGATCCTGGGCCTGAAGCTGCCCCGGCGAGCCTC CTCTGACCGCCAATACAGTGTGCCTGACACTGTCCGGC CTGAGCAAGAGGCAGCTGGGCCTGTGCCTGAGAAGCC CTGACGTGACCGCCAGCGCCCTGCAGGGACTGCACAT CGCCGTGCACGAGTGCCAGCACCAGCTGAGAGACCAG AGGTGGAAGTGCAGCGCCCTGGAGGGCGGCGGAAGG CTGCCTCATCACAGCGCCATCCTGAAGAGAGGCTTTAG GGAGTCCGCCTTCTCCTTTCCATGCTGGCCGCCGGCG TGATGCACGCCGTGGCTACAGCCTGCTCCCTGGGCAA GCTGGTGAGCTGTGGCTGTGGCTGGAAGGGCTCCGGC GAGCAGGACAGGCTGAGGGCTAAGCTGCTGCAGCTGC AGGCCCTGTCCAGAGGCAAGACATTTCATATCTCCAG CCCAGCCCAGTGCCCGGCTCCGTTCCAAGCCCTGGCC

		CTCAGGACACCTGGGAGTGGGGCGGATGTAACCACGA TATGGACTTTGGCGAGAAGTTCAGCAGGGATTTCCTGG ATTCCAGAGAGGCTCCTAGGGACATCCAGGCCAGAAT GAGGATCCACAATAATAGAGTGGGCAGACAGGTGGTG ACAGAGAACCTGAAGAGGAAGTGCAAGTGCCACGGC ACAAGCGGCTCCTGTCAGTTCAAGACATGTTGGAGGG CCGCTCCTGAGTTCAGGGCCATCGGCGCCGCCCTGAG AGAGAGACTGAGCAGAGCCATCTTTATCGACACCCAC AATAGGAACTCCGGCGCCTTCCAGCCTAGACTGAGGC CCAGGAGGCTGAGCGGCGAGCTGGTGTACTTTGAGAA GAGCCCTGATTTTGTGAGAGAGACCCAACCCTGGGC AGCCCAGGCACCAGAGGCAGGGCTTGCAACAAGACC AGCAGGCTGCTGGACGGCTGCGGCTCCCTGTGCTGTG GCAGGGGACACAACGTGCTGAGACAGACAAGGGTGG AGAGATGCCACTGTAGATTCCACTGGTGTGCTACGTG CTGTGTGATGAGTGCAAGGTGACCGAGTGGGTGAATG TGTGTAAG
13	CD63 TM3 AA	YCLMITFAIFLSLIMLVEVAAAIAGYVFRDKVMSEFNNNF RQQMENYPKNNHTA
14	CD63 TM3 DNA	TACTGTCTGATGATCACCTTCGCCATCTTTCTGTCCCTG ATCATGCTGGTGGAGGTGGCCGCCGCCATCGCCGGATA CGTGTTTCAGAGACAAGGTGATGAGCGAGTTCAACAAT AATTTTAGACAGCAGATGGAGAATTACCCTAAGAATAA CCACACCGCC
15	Linker AA	GGGSGGGSGGGGS
16	Linker DNA	GGCGGCGGCGGCTCCGGAGGAGGAGGAAGCGGAGGA GGCGGCAGC
17	T2A AA	GSGEGRGSLTCDGVEENPGP
18	T2A DNA	GGCAGCGGCGAAGGCCGCGGCAGCCTGCTGACCTGCG GCGATGTGGAAGAAAACCCGGGCCCCG
AA = Amino Acid		

Examples

- [0138] Example 1. Construction of engineered exosomes derived stable cell lines.
- [0139] Materials and Methods
- [0140] Materials: The HEK293 cell line (human embryonic kidney 293 cells, CRL-1573TM) 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. Cells were

incubated in a humidified atmosphere containing 5%CO₂ at 37 °C. Antibody used in this study was anti-CD63 antibodies (Cat. No. MA5-32085, Invitrogen) .

- [0141] pGOI Plasmid Construction: The amino acid sequences of all the target genes, including KGF-1, VEGF-A and Wnt10b were derived from Uniprot and the corresponding DNA sequences (see SEQ ID NO: 10, 11 and 12, 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: 17 and 18) 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 3rd-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) . Payload genes constructed in the pGOI plasmid: CD63-TM3-Linker-KGF-1-T2A-CD63-TM3-Linker-VEGF-A-T2A-CD63-TM3-Linker-Wnt10b, in which each CD63-TM3 is the TM3 domain of CD63, encoded by DNA sequence shown in SEQ ID NO: 14, each linker is encoded by DNA sequence shown in SEQ ID NO: 16, and each T2A is encoded by DNA sequence shown in SEQ ID NO: 18.
- [0142] Generation of stable cell lines: The stable HEK293 cell line expressing target proteins, including KGF-1, VEGF-A and Wnt10b, was generated by infection with the corresponding lentiviruses. Forty-eight hours after 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.
- [0143] Adaptation of cell culture to a SFM (serum-free medium) : After three initial passages in FM (full medium) from the stable cell line, adaptation for serum-free culture was started from the 4th 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 (HyCloneTM peak expression, SH31193.02, Cytiva Life Sciences) for at least three times.
- [0144] 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

[0145] FM: Full medium; SFM: Serum-free medium

[0146] 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. The exosome obtained is referred to as 35#exosome in the following examples.

[0147] Figure 1 shows schematically the flowchart of exosome production process derived from HEK293 cells, according to an exemplary embodiment of the present disclosure.

[0148] Example 2. Characterization of Exosomes

[0149] Analysis of the particle concentration and size distribution of exosomes

[0150] The particle concentration and size distribution of exosomes from stable cell line 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.

[0151] Figure 2A shows the particle diameter and particle concentration of exosomes derived from the stable cell line were analyzed by NanoFCM. The exosomes have a mean particle diameter of 75.4 nm.

- [0152] Identification of target proteins on exosomes via Western blotting (WB)
- [0153] 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.
- [0154] Transmission electron microscopy (TEM) analysis of exosomes
- [0155] TEM was used to confirm the presence of exosomes. Approximately, 20 μ 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).
- [0156] Figure 2B shows the transmission electron microscopy (TEM) image of the exosomes. Figure 2C shows the immunoblotting analysis of exosomes with antibody against CD63-TM3, the exosome fused scaffold proteins. As expected, the engineered exosome-associated target proteins, Wnt10b, VEGF-A and KGF-1 were present in purified exosomes derived from stable cell line.
- [0157] Example 3. The evaluation of the engineered exosome for hair loss treatment in mouse model
- [0158] Animals and in vivo studies
- [0159] All experiments were performed using 6-week-old male C57BL/6 mice. Animals were purchased from Cavens (Changzhou, China). Figure 3A shows schematic representation of the androgenetic alopecia mouse model and the regimen. After dorsal hair depilation, mice skin was treated every 3 days with 35#exosome, control exosome (Ctrl Exo.), PBS (negative control), or 5% minoxidil (positive control). Observation continued over an 18-day treatment period, then animals were sacrificed at D18 for histologic analysis. For in vivo therapy experiment, the mice were shaved firstly, then hair was removed by using depilatory cream to observe the pink skin. On the next day of depilation, 30 mg/EA DHT (dihydrotestosterone) suspension was injected subcutaneously into 5 spots of the dorsal depilation area. Animals were randomly divided into 4 groups (n = 4) to study hair regrowth. After hair depilation, mice dorsal skin was treated every 3 days with 35#exosome, control exosome and PBS (negative control), or 5% minoxidil daily (positive control). Exosome treatment dose: 8.0×10^9 exosomes

in 100 μ l of PBS were subcutaneously injected into 5 spots (20 μ l per site) on the dorsal skin. 200 μ l of minoxidil was topically applied daily. The dorsal skin was photographed at 0, 9, 12, and 15 days. Image J was used to analyze the depilation area and the hair growth area of the mice. Hair coverage $\%$ = (new hair area/hair removal area) * 100%. Observation continued over an 18-day treatment period, then animals were sacrificed for further analysis.

- [0160] Skin tissue histology
- [0161] Mice were euthanized, and the whole dorsal skin was removed. Skin tissue was fixed in 4% paraformaldehyde and then cut into sections by Cryostat, followed by hematoxylin and eosin (HE) staining. Five fields were randomly selected from each section. Counting the number of hair follicles in the dermis and calculating the value of anagen/resting follicles. Measure the distance from the hair follicle in the dermis to the epidermis to calculate the average follicle length.
- [0162] Figure 3B shows observation of hair coverage. Mice were divided into four groups (n = 4) and representative images of mice on day 15 were showed. The results showed that treatment with 35#exosome can almost fully restore hair coverage on day 15, with a similar efficacy to the positive control minoxidil. Figure 3C shows histological analysis of mice dorsal skin regarding the hair follicle quantity, length, and growth phase ration. Data points represent mean $\pm$ SD (n = 4) . Error bars indicate SD. Ns, no significant difference; *P < 0.05, **P < 0.01, and ***P < 0.001. The 35#exosome treatment significantly increased the number and length of hair follicles, as well as the prolonged anagen/telogen phase ratio, which can promote the hair follicle normalization and further enhance the telogen-anagen transition.
- [0163] Example 4. Evaluation of the engineered exosome for canities treatment in mouse model
- [0164] All experiments were performed using 6-week-old female C57BL/6 mice. Fig. 4A is a schematic representation of the hydroquinone-induced hair graying model and the regimen. For in vivo therapy experiment, the mice were shaved firstly, then hair was removed by using depilatory cream. Animals were randomly divided into 4 groups (n = 4) to study anti-graying hair. On the next day of depilation, 2.5% concentration hydroquinone cream was topically applied on the dorsal depilation area. Negative model control group was treated with PBS via topical application by smearing. 35#exosome groups were treated with different administration route, e.g., microneedling followed by smearing, nanocrystalline infusion followed by smearing, subcutaneously injection. Exosome treatment dose: 1E11 exosomes in 200 μ l volume were administered via different routes. Observation of mice dorsal hair continued over a 28-day treatment period. Table 2 shows a summary of the treatment regimen.
- [0165] Table 2. Summary of treatment regimen in different groups.

Group	Treatment	Administration route	Frequency	Dosage(particles)	Volume (mL)
G1	PBS	Smearing	Q3D*10	/	0.2mL
G2	35# exosome	Microneedling + smearing	Q3D*10	1E11	0.2mL
G3	35# exosome	Nanocrystalline infusion + smearing	Q3D*10	1E11	0.2mL
G4	35# exosome	Subcutaneously injection	Q3D*10	1E11	0.2mL

[0166] Fig. 4B shows the observation of dorsal hair. Representative images of mice on day14 were showed. The results showed that mice treated with 35#exosome via different administration routes, e.g., microneedling followed by smearing, nanocrystalline infusion followed by smearing and subcutaneously injection, all have more pigmented hairs than mock treatment.

Claims

- [Claim 1] An engineered exosome, comprising
(a) a KGF polypeptide, fused to a first anchoring polypeptide,
(b) a VEGF-A polypeptide, fused to a second anchoring polypeptide,
and
(c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, 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 VEGF-A polypeptide and the Wnt family member polypeptide are exposed on an outer surface of the membrane of the exosome.
- [Claim 2] 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 or 4.
- [Claim 3] The engineered exosome of claim 1, wherein the VEGF-A polypeptide is a human VEGF₂₀₆ isomer or an ortholog or paralog thereof, preferably the VEGF-A polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 2 or 5.
- [Claim 4] The engineered exosome of claim 1, wherein the Wnt family member polypeptide is Wnt10a, 10b, 3a, 1a, 4, 5a, 7a, or 7b polypeptide; preferably the Wnt family member polypeptide is Wnt10b polypeptide; more preferably the Wnt10b polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 3 or 6.
- [Claim 5] The engineered exosome of claim 1, 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 first, second, and third

- anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63; more preferably, each of the first, second, and third anchoring polypeptides is a TM3 domain of CD63; more 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. 13.
- [Claim 6] The engineered exosome of claim 1, wherein the KGF polypeptide, the VEGF-A polypeptide and the Wnt family member polypeptide is fused to the C-terminus of the first, second, and third anchoring polypeptides, respectively, optionally through a peptide linker, preferably, the peptide linker consists of glycine and serine, e.g., (G4S) *n*, in which *n* is an integer from 1 to 3.
- [Claim 7] 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 8] The engineered exosome of claim 1, wherein the exosome:
(i) promotes hair regrowth, and/or improves alopecia, e.g. androgenetic alopecia, alopecia areata, or telogen effluvium; and/or
(ii) prevents and/or limits and/or stops the development of canities, and maintains and/or promotes the natural repigmentation of head hair and/or body hair.
- [Claim 9] A composition comprising the exosome of any of claims 1 to 8, 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 10] The composition of claim 9, wherein the composition does not contain a KGF polypeptide (e.g. a KGF-1 polypeptide), a VEGF-A polypeptide, or a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway (e.g., a Wnt10b polypeptide), not attached to the membrane of the exosome.
- [Claim 11] A nucleic acid construct comprising a polynucleotide encoding
(a) a KGF polypeptide fused to a first anchoring polypeptide,
(b) a VEGF-A polypeptide fused to a second anchoring polypeptide,
and

- (c) a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway, fused to a third anchoring polypeptide.
- [Claim 12] The nucleic acid construct of claim 11, 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 or 4;
 - (ii) the VEGF-A polypeptide is a human VEGF₂₀₆ isomer or an ortholog or paralog thereof, preferably the VEGF-A polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 2 or 5;
 - (iii) the Wnt family member polypeptide is Wnt10a, 10b, 3a, 1a, 4, 5a, 7a, or 7b polypeptide; preferably the Wnt family member polypeptide is Wnt10b polypeptide; more preferably the Wnt10b polypeptide comprises an amino acid sequence having at least 80% identity to the amino acid sequence as shown in SEQ ID NO. 3 or 6;
 - (iv) a polynucleotide encoding the KGF polypeptide has a nucleotide sequence as shown in SEQ ID NO. 7, 10 or a degenerate sequence thereof;
 - (v) a polynucleotide encoding the VEGF-A polypeptide has a nucleotide sequence as shown in SEQ ID NO. 8, 11 or a degenerate sequence thereof;
 - (vi) a polynucleotide encoding the Wnt family member polypeptide has a nucleotide sequence as shown in SEQ ID NO. 9, 12 or a degenerate sequence thereof;
 - (vii) 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 first, second, and third anchoring polypeptides comprise a full-length CD63 or a truncated CD63 that retains TM3 domain; more preferably, each of the first, second, and third anchoring polypeptides comprises a TM3 domain of CD63; more preferably, each of the first,

second, and third anchoring polypeptides is a TM3 domain of CD63;
and/or

(viii) a polynucleotide encoding the TM3 domain of CD63 has a nucleotide sequence as shown in SEQ ID NO. 14 or a degenerate sequence thereof.

[Claim 13]

The nucleic acid construct of claim 11 or 12, wherein the polynucleotide is a single polynucleotide comprising a fragment of nucleotides encoding (a), (b), and (c); preferably the polynucleotide comprises, from 5' to 3', a fragment of nucleotides encoding:

(i) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt],

(ii) [1st anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A],

(iii) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [Wnt],

(iv) [1st anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF],

(v) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [KGF] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [VEGF-A], or

(vi) [1st anchoring polypeptide] - [Wnt] - [self-cleavage peptide] - [2nd anchoring polypeptide] - [VEGF-A] - [self-cleavage peptide] - [3rd anchoring polypeptide] - [KGF],

wherein, [] represents a separate polypeptide, and] - [represents a linker or a bond;

wherein, Wnt represents a Wnt family member polypeptide involved in Wnt/ β -catenin signaling pathway;

optionally, the self-cleavage peptide is a 2A peptide, e.g., T2A, E2A, P2A or any combination thereof.

[Claim 14]

A vector comprising the nucleic acid construct of any of claims 11 to 13; preferably, the vector is a viral vector; more preferably the vector is a lentiviral vector or an adeno-associated viral vector.

- [Claim 15] A cell transduced with the vector of claim 14, 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 16] A method of producing an engineered exosome of any of claims 1 to 8, comprising
- (a) transducing the cell of claim 15 with the vector of claim 14;
 - (b) culturing the cell in a condition allowing secretion of an exosome from the cell; and
 - (c) collecting and purifying the exosome.
- [Claim 17] The method of claim 16, further comprising adapting the cell to a serum-free condition during step (b) .
- [Claim 18] Use of the engineered exosome of any of claims 1 to 8 or the composition of claims 9 or 10 in manufacturing a medicament for treatment of alopecia and/or canities; preferably, the alopecia is androgenetic alopecia, alopecia areata, or telogen effluvium; preferably, the canities is age-related hair graying, premature canities, or canities subita.