

ORGANOID-DERIVED EXOSOME PRODUCTS, METHODS OF MAKING, AND
METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Patent Application No. 63/543,729, filed October 11, 2023, which is incorporated herein by reference in its entirety.

SUMMARY

This disclosure describes, in one aspect, a method of preparing exosomes. Generally, the method includes providing a multi-tissue organoid, culturing the multi-tissue organoid in culture medium, collecting at least a portion of the culture medium, and isolating exosomes from the culture medium.

In one or more embodiments, the exosomes express at least one marker identifying the exosomes as being isolated from culture medium in which a multi-tissue organoid is cultured.

In another aspect, this disclosure describes exosomes that express at least one marker identifying the exosomes as being isolated from culture medium in which a multi-tissue organoid is cultured.

In another aspect, this disclosure describes a method of treating a subject having, or at risk of having, a condition treatable with exosomes isolated from culture medium in which a multi-tissue-organoid is cultured. Generally, the method includes administering to the subject a pharmaceutical composition that includes an effective amount of exosomes isolated from culture medium in which a multi-tissue-organoid is cultured.

In one or more embodiments, the pharmaceutical composition is administered before the subject manifests a symptom or clinical sign of the condition.

In one or more embodiments, the pharmaceutical composition is administered after the subject manifests a symptom or clinical sign of the condition.

The above summary is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is

provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

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BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Biogenesis, structure, and isolation of exosomes. (A) The biogenesis pathway of an exosome begins with late endosomes, so called multivesicular bodies, containing multiple internal vesicles that merge with the cell membrane and release its internal vesicles into the extracellular matrix. (B) Simple illustration of an exosome. Exosomes are formed by a bilayer of
 10 lipids and contain components such as transmembrane proteins (including MHC molecules, exosomal marker proteins, tetraspanins, etc.), saccharides, cytosolic proteins and nucleic acids. (C) Detailed isolation schema for the isolation of exosomes. Exosomes were collected and isolated by a series of centrifugation/ultracentrifugation steps.

FIG. 2. Characterization of exosomes. (A) NANOSIGHT (Malvern Panalytical Ltd.,
 15 Malvern, United Kingdom) based size characterization – A representative NANOSIGHT analysis of exosomes isolated from organoid culture medium at 10 weeks shows size peaks at 128 nm and 180 nm and concentration of 5.41×10^{12} particles per ml. (B) Western blots of organoid-derived exosomes at six (sample 1) and eight (sample 2) weeks in culture showing positive bands for exosome markers CD63, flotillin-2, and flotillin-1, and CD9.

20 FIG. 3. Characterization of exosomes. Gating strategy to define the CD9⁺ exosomes isolated from MTO culture media using multiparametric flow cytometer.

FIG. 4. Organoid-derived exosomes increases the proliferation of C20A4 Chondrocyte Cells. (A) Gating Strategy for proliferation marker (Ki-67) on C20A4 Chondrocyte Cells (Isotype control). Singlets were gated based on the forward and side scatter parameters (Area vs
 25 Height). Chondrocytes were then gated on the singlet population followed by the live cells (L/D - Ghost Dye). Ki-67⁺ population were detected on the live cells based on the isotype control. (B) Flow cytometry-based detection of Ki-67⁺ population using the isotype control. TNF α -treated cells showed decreased proliferation (70.9%) compared to the normal media control (85.5%) after 48 hours in the media control and TNF α control overlay.

30 FIG. 5. Organoid-derived exosomes increases the proliferation of C20A4 Chondrocyte Cells. (A) Detection of percentage change in Ki-67⁺ population compared to the media control in

C20A4 chondrocytes. Increased proliferation is observed on cells treated with exosomes from Week 5, Week 9, Week 11, and Week 13 of MTO culture media. Detection of % change in Ki-67⁺ population compared to the TNF α control after 48 hours is also represented. (B) Detection of percent change in Ki-67⁺ population compared to the media control in TC28a2 chondrocyte cell line. Increase in proliferation is observed on cells treated with exosomes from Week 5, Week 9, Week 11, and Week 16 of MTO culture media. Detection of % change in Ki-67⁺ population compared to the TNF α control after 48 hours is represented as well. Exosome treatment show a clear reversal of TNF α -mediated inhibition of proliferation.

FIG. 6. Exosomes from MTO culture medium alters the colony forming ability of the chondrocytes. TC28a2 chondrocyte colonies after treatment with exosomes from different weeks of MTO culture media in the presence and absence of TNF α .

FIG. 7. Exosomes from MTO culture medium enhances the colony forming ability of the chondrocytes. (A) The crystal violet retained by the cells (FIG. 6) was washed with 10% acetic acid and the color intensity was measured at 595 nm. (B) Percentage change in TC28a2 chondrocytes. Detection of percentage change in TC28a2 population compared to the media control. An increase in proliferation is observed on cells treated with exosomes from early weeks of MTO culture media. Detection of percentage change in TC28a2 population compared to the TNF α control is depicted on the right side. TNF α -mediated inhibition of proliferation is clearly reversed by exosome treatment.

FIG. 8. Exosomes inhibit the inflammation by inducing expansion of the Treg population and their activation through CD69 expression. Detailed gating strategy to determine the effect of exosomes on regulatory T cell population. Singlets were gated based on the forward and side scatter parameters (Area vs Height). Lymphocytes were then gated on the singlet population followed by the live cells (L/D - Ghost Dye). CD4⁺ T cell population were detected on the live cells which were then gated to look at the double positive population for CD25 and FOXP3 (Treg Cells).

FIG. 9. Exosomes inhibit the inflammation by inducing the Treg population and CD69 expression. Effect of exosomes on human regulatory T cell population (Treg). Exosomes from different weeks of the MTO culture media show an increased expansion of Treg cells compared to the responder-stimulator alone control.

FIG. 10. Exosomes inhibit the inflammation by inducing expansion of the Treg population and their activation through CD69 expression. Effect of exosomes on early activation marker CD69 in human PBMC MLR culture. Exosomes from different weeks of the MTO culture media show an increased expression of CD69, one of the early activation markers of T cells that provides an anti-inflammatory niche, in both MLR cultures compared to the corresponding responder-stimulator alone controls.

FIG. 11. Schematic illustration of exemplary mechanisms of action of MTO exosomes for the treatment of osteoarthritis. Exosomes isolated from MTO culture media increase chondrocyte proliferation. The presence of exosomes increases the level of regulatory T cell (Treg) population and CD4⁺CD69⁺ T cells in human MLR cultures.

FIG. 12. Therapeutic trial of MTO-derived exosomes in the mouse model of traumatic brain injury. (A) Histopathology of brain hippocampus showed a prominent increase in GFAP (marker for astrocytes) staining intensity in all exosome treated mice versus staining in sham-treated TBI controls and sham operated mice. (B) TBI-exosome treated mice showed Barnes Maze performance that was comparable to that of the sham operated mice, while the female TBI sham-control mouse showed the expected poor memory performance. Results indicated a therapeutic response to MTO-derived exosomes in the mouse model of TBI with an associated bilateral astrocyte response in the hippocampus.

FIG. 13. Gating strategy for immunomodulatory molecule (CD9) on MTO exosomes.

FIG. 14. Expression of immunomodulatory molecules on Week 8 exosomes from Batch-13 MTO culture medium. (A) Expression of CD63 compared to unstained control. (B) Expression of CD81 compared to unstained control. (C) Expression of CD9 compared to unstained control.

FIG. 15. Expression of immunomodulatory molecules on Week 9 exosomes from Batch-13 MTO culture medium. (A) Expression of CD63 compared to unstained control. (B) Expression of CD81 compared to unstained control. (C) Expression of CD9 compared to unstained control.

FIG. 16. Expression of immunomodulatory molecules on Week 10 exosomes from Batch-13 MTO culture medium. (A) Expression of CD63 compared to unstained control. (B) Expression of CD81 compared to unstained control. (C) Expression of CD9 compared to unstained control.

FIG. 17. Expression of immunomodulatory molecules on Week 11 exosomes from Batch-13 MTO culture medium. (A) Expression of CD63 compared to unstained control. (B) Expression of CD81 compared to unstained control. (C) Expression of CD9 compared to unstained control.

5 FIG. 18. Expression of immunomodulatory molecules on exosomes isolated from different weeks of Batch-13 MTO culture medium.

FIG. 19. Expression of immunomodulatory molecules on Batch-13 MTO culture exosomes compared to exosomes isolated from parental iPSC cell culture medium. (A) CD63. (B) CD81. (C) CD9. Top three tracings: separate Batch-13 samples; fourth tracing: iPSC
10 exosomes; bottom: unstained.

FIG. 20. Expression of immunomodulatory molecules on Batch-14 MTO culture exosomes compared to exosomes isolated from parental iPSC cell culture medium. (A) CD63. (B) CD81. (C) CD9. Top three tracings: separate Batch-14 samples; fourth tracing: iPSC exosomes; bottom: unstained.

15 FIG. 21. Expression of immunomodulatory molecules on Batch-15 MTO culture exosomes compared to exosomes isolated from parental iPSC cell culture medium. (A) CD63. (B) CD81. (C) CD9. Top three tracings: separate Batch-15 samples; fourth tracing: iPSC exosomes; bottom: unstained.

FIG. 22. Batch-to-batch characterization of immunological molecules expressed by MTO
20 exosomes compared to iPSC exosomes. (A) MTO derived exosomes from three separate batches and baseline iPSC derived exosomes were profiled and compared for the expression of CD9, CD63 and CD81 by flowcytometry. Percentage expression of immunomodulatory molecules on exosomes isolated from different batches of MTO culture medium. (B) Mean fluorescent intensity (MFI) of Immunomodulatory molecules on exosomes isolated from different batches of
25 MTO culture medium.

FIG. 23. Organoid-derived exosomes increase the proliferation of chondrocytes. Effect of exosomes on the proliferation of chondrocytes – Ki-67 based assay representing Ki67 expression in mitotic cells. TC28a2 chondrocyte cells were treated with exosomes of week 10 MTO culture medium. Proliferation marker, Ki-67 level was detected on control and exosome treated cells
30 through immunohistochemistry. In the magnified image, the cells marked by the arrow are found to be in the active stage of cell cycle showing positive signal for Ki67.

FIG. 24. Organoid-derived exosomes increase the proliferation of chondrocytes. Effect of Week 10 exosomes from MTO Batches 13 to 17 compared to iPSC exosomes on the proliferation of chondrocytes – Ki-67 based assay. Ki67 level was detected on TC28a2 chondrocytes treated with TNF α (50 ng/million cells) and exosomes from Batches 13 to 17 on week 10 of the MTO culture medium. The cells were also treated with control iPSC exosomes and the level of expression of Ki67 was detected. The merged images of DAPI and Ki67 show the reversal of the TNF α -mediated decrease in proliferation on TC28a2 chondrocytes upon treatment with week 10 exosomes compared to the iPSC exosomes.

FIG. 25. Organoid-derived exosomes increase the proliferation of chondrocytes. Characterization of chondrocyte proliferation using exosomes from 10-week MTOs. (A) Effect of Week 10 exosomes from batches 13 to 17 on the proliferation of chondrocytes – Ki-67+ Cell Percentage. (B) Percentage increase in Ki-67+ Cells on MTO exosome treatment compared to iPSC exosome treated chondrocytes. Frequency increase in the Ki67+ proliferating chondrocytes in MTO exosome treatment compared to the IPSC exosome treatment.

FIG. 26. Organoid-derived exosomes increase the proliferation of chondrocytes. Exosome treatment revert the TNF α mediated inhibition in Ki67+ cell percentage to near normal (control) levels in C20a4 (left) and TC28a2 (right) chondrocyte cell lines.

FIG. 27. Exosomes inhibit the inflammation by inducing the regulatory T cell population, Treg and Tr1. (A) Detailed gating strategy to determine the effect of exosomes on regulatory T cell population (Treg and Tr1) and their activation status (CD69 expression). Singlets were gated based on the forward and side scatter parameters (Area vs. Height). Lymphocytes were then gated on the singlet population followed by the live cells (L/D - Ghost Dye). CD4⁺ T cell population were detected on the live cells which were then gated to identify CD25-FOXP3 double positive Treg cells and CD49b-LAG3 double positive Tr1 cells. Activation marker, CD69 expression was measured in regulatory T cell (Treg and Tr1) populations.

FIG. 28. Exosomes inhibit the inflammation by inducing expansion of regulatory T cell populations, Treg and Tr1. (A) Exosomes isolated from Multi-Tissue Organoid (MTO) cultures were tested for their immunomodulatory functions. MTO Exosomes from week 10 to 12 from eight separate batches (Batch 26 to Batch 32) were tested at a dose of 100,000 exosome particles/million cells on a mixed lymphocyte reaction (MLR) culture in the presence of peripheral blood mononuclear cells (PBMCs) and their effect on the frequency (%) of Treg cells

was determined. MTO exosomes from multiple batches show a significant increase in the frequency of immunomodulatory Treg cells compared to untreated media control. Potency of MTO exosomes to increase the frequency of Treg cells is consistent across the tested batches (Batch 26 to Batch 32). (B) MTO Exosomes from week 10 to 12 of eight separate batches (Batch 26 to Batch 32) were tested at a dose of 500,000 exosome particles/million cells on a mixed lymphocyte reaction (MLR) culture in the presence of human peripheral blood mononuclear cells (PBMCs) and their effect on the frequency (%) of Tr1 cells was determined. Potency of MTO exosomes to increase the frequency of Tr1 cells is consistent across the tested batches (Batch 26 to Batch 32).

FIG. 29. Exosomes inhibit the inflammation by inducing the regulatory T cell population, Treg and Tr1. (A) MTO exosomes show a significant increase in the frequency of activated (CD69⁺) Treg and Tr1 cells compared to the untreated media control. (B) MTO exosomes contains significantly elevated levels of anti-inflammatory CD9 and CD81 compared to their parental IPSC culture medium exosomes.

FIG. 30. Graphic representation of Dynamic Weight Bearing data shown in Tables 10-12, showing percent of animals in each group that score excellent or good at each time point.

FIG. 31. Graphical representation of data shown in Tables 10-12, indicating the percent of rats in each group that achieved an excellent score at any time point in the study.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

This disclosure describes, in one aspect, exosomes generated by multi-tissue organoids. In another aspect, this disclosure describes methods of preparing exosomes generated from a multi-tissue organoid. In another aspect, this disclosure describes methods of using exosomes obtained from multi-tissue organoids. As used herein, unless otherwise specified, exosomes described as “generated by,” “prepared from,” or “derived from” multi-tissue organoids are exosomes produced by cells of a multi-tissue organoid and may be isolated from culture medium in which one or more multi-tissue organoids are being grown, isolated from cells of a multi-tissue organoid, or both.

Methods of preparing multi-tissue organoids are described, for example, in US Patent Application Publication No. 2020/0248139 A1, US Patent Application Publication No. 2021/0301251 A1, and U.S. Patent Application Publication No. 2024-0271105-A1.

In one or more embodiments, the multi-tissue organoids can be generated by culturing iPSCs in culture medium (e.g., a chemically defined maintenance cell culture medium) that includes a minimum of at least 0.01% hyaluronic acid such as, for example, at least 0.01% hyaluronic acid, at least 0.02% hyaluronic acid, at least 0.05% hyaluronic acid, at least 0.1% hyaluronic acid, at least 0.2% hyaluronic acid, at least 0.3% hyaluronic acid, at least 0.4% hyaluronic acid, at least 0.5% hyaluronic acid, at least 0.6% hyaluronic acid, at least 0.7% hyaluronic acid, at least 0.8% hyaluronic acid, at least 0.9% hyaluronic acid, at least 1.0% hyaluronic acid, at least 2.0% hyaluronic acid, at least 3.0% hyaluronic acid, at least 4.0% hyaluronic acid, or at least 5.0% hyaluronic acid.

In one or more embodiments, the multi-tissue organoids can be generated by culturing iPSCs in culture medium (e.g., a chemically defined maintenance cell culture medium) that includes a maximum of no more than 10% hyaluronic acid such as, for example, no more than 5% hyaluronic acid, no more than 4% hyaluronic acid, no more than 3% hyaluronic acid, no more than 2% hyaluronic acid, no more than 1% hyaluronic acid, no more than 0.5% hyaluronic acid, no more than 0.4% hyaluronic acid, no more than 0.3% hyaluronic acid, no more than 0.2% hyaluronic acid, or no more than 0.1% hyaluronic acid.

In one or more embodiments, the multi-tissue organoids can be generated by culturing iPSCs in culture medium (e.g., a chemically defined maintenance cell culture medium) that includes hyaluronic acid at a concentration that falls within a range having endpoints defined by any minimum amount of hyaluronic acid described above and any maximum amount of hyaluronic acid described above that is greater than the selected minimum amount.

Structure, Isolation, and Measurement of Organoid-Derived Exosomes

Exosomes are nanoscopic particles released by cells to communicate. Their molecular and biophysical properties change depending on their parental cell type, with biological implications in health and disease. Accordingly, therapeutic intervention mediated by exosomes offers a broad range of applications spanning from immune modulation to regenerative medicine.

Exosomes are nano-spherical membrane structures formed by a bilayer of lipids, as illustrated in FIG. 1A. They are membrane bound vesicles derived from endosomes that act as intercellular messengers during physiological and pathological conditions. Exosomes are typically defined by their size and surface markers. Exosomes range in a size from about 30 nm

to about 200 nm. Exosomes typically express one or more surface markers which may include CD9, CD63, CD81, and other tetraspanins. Further, exosomes can possess various transmembrane components such as proteins, lipids, and saccharides, as well as various cytosolic proteins and nucleic acids. The cargo contained within an exosome (or a population of
5 exosomes) can include a variety of proteins, polypeptides, glycoproteins, lipids, and/or various types of RNA (FIG. 1B). The particular transmembrane components and/or cytosolic components of an exosome is determined, at least in part, by the cell and/or tissue from which the exosome is derived.

The biogenesis pathway of an exosome begins with late endosomes, so called
10 multivesicular bodies, containing multiple internal vesicles that merge with the cell membrane and release its internal vesicles into the extracellular matrix (FIG. 1A). This biogenesis of exosomes via multivesicular bodies differentiates exosomes from other extracellular vesicles such as apoptotic bodies and microvesicles.

Extracellular vesicles vary in their size, origin within the donor cell and function.
15 Microvesicles vary greatly in size (100 nm to 1000 nm), with their cargo predominantly consisting of cytoplasmic content, such as mRNA, miRNA and proteins, as they are directly released from the cell membrane. Apoptotic bodies, the largest of the extracellular vesicles (800–5000 nm), are released during the execution phase of the apoptotic process, potentially carrying organelles, receptors, and similar cytoplasmic content.

In contrast, the cargo of exosomes typically includes DNA, messenger RNA (mRNA),
20 microRNA (miRNA), proteins, and growth factors. Exosomes are present in most bodily fluids including serum, uterine and peritoneal fluid, urine, and breast milk. The lipid bilayer of exosomes protects the cargo from degradation and allows for distant communication throughout the body. Exosome cargo is known to regulate biological processes. Thus, the therapeutic
25 efficacy of an exosome can be determined, at least in part, by the cellular or tissue source from which they originate, which, in-turn, is associated with the cargo they carry.

This disclosure describes generating exosomes from multi-tissue organoids (MTOs). In one or more embodiments, the exosomes are prepared from the culture medium in which MTOs are being grown. In one or more embodiments, three biological replicate batches of MTOs can
30 be generated and cell culture medium collected from each time segment. Time segments can correspond to the emergence and maturation stages of cartilage in MTOs. For example, the

“early” time segment can correspond to weeks 1-6, the “middle” time segment can correspond to weeks 7-11, and the “late” time segment can correspond to weeks 12-22. Exosomes can be collected and isolated by any suitable method including, but not limited to, centrifugation and/or ultracentrifugation, and tangential flow filtration (TFF).

5 Thus, in one or more embodiments, exosomes may be isolated from the culture medium after the MTOs have been in culture for a minimum of at least one week such as, for example, at least two weeks, at least three weeks, at least four weeks, at least five weeks, at least six weeks, at least seven weeks, at least eight weeks, at least nine weeks, at least ten weeks, at least eleven weeks, or at least twelve weeks.

10 In one or more embodiments, exosomes may be isolated from the culture medium after the MTOs have been in culture for a maximum of no more than one week such as, for example, no more than 52 weeks, no more than 26 weeks, no more than 22 weeks, no more than 20 weeks, no more than 19 weeks, no more than 18 weeks, no more than 17 weeks, no more than 16 weeks, no more than 15 weeks, no more than 14 weeks, no more than 13 weeks no more than 12 weeks,
15 no more than 11 weeks, no more than 10 weeks, no more than nine weeks, no more than eight weeks, no more than seven weeks, no more than six weeks, no more than five weeks, no more than four weeks, no more than three weeks, or no more than two weeks.

 In one or more embodiments, exosomes may be isolated from the culture medium after the MTOs have been in culture for a period that falls within a range having endpoints defined by
20 any minimum period of time described above and any maximum period of time described above that is greater than the selected minimum period of time.

Isolation of Exosomes

 In one or more embodiments, the multi-tissue organoid (MTO) culture media can be
25 centrifuged at, for example, 300×g at 4 °C for 10 minutes to pellet and remove detached cells. The supernatant can be centrifuged at, for example, 2000×g for 10 minutes and further recentrifuged at 10000×g for 30 minutes to remove cell debris. Furthermore, the supernatants can be concentrated by ultrafiltration with a molecular weight cut-off of, for example, 100 kDa. The supernatant can then be ultracentrifuged at, for example, 100,000×g_{avg} at 4 °C for 90 minutes to
30 pellet exosomes. The supernatant can be removed, and exosome-containing pellets resuspended in an appropriate buffer (e.g., PBS, phosphate-buffered saline) and pooled. A second round of

ultracentrifugation—e.g., 100,000× g_{avg} at 4 °C for 90 minutes—can be performed and the resulting exosome pellet resuspended in an appropriate volume (e.g., 200 μ l) of an appropriate buffer (e.g., PBS) for further characterization and preclinical studies.

A detailed schema of one embodiments of exosome isolation is depicted in FIG. 1B.

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Characterization of Multi-Tissue Organoid-Derived Exosomes

Nanoparticle tracking analysis (NTA) is a powerful characterization technique that combines the properties of both laser light scattering microscopy and Brownian motion to obtain size distributions of particles in liquid suspension. Certain NTA instruments are equipped with one or more lasers and an optical microscope connected to a digital camera. NTA enables one to characterize particles in solution ranging from about 10 nm to about 2000. Particles are visualized by the light they scatter upon laser illumination, and their Brownian motion is monitored. NTA software enables sizing of single particles by tracking their mean squared displacement and thereby calculating their theoretical hydrodynamic diameter. Based on knowing the analyzed sample volume, NTA also allows for an estimation of particle concentration.

Exosomes isolated from multi-tissue organoid (MTO) culture medium were subjected to NTA characterization. NANOSIGHT (Malvern Panalytical Ltd., Malvern, UK)-based characterization of the exosome samples isolated from Week 6 of the MTO culture medium is depicted in FIG. 2A. The data suggests that the samples show size peaks at 128 nm and 180 nm and concentration of 5.41×10^{12} particles per ml.

The exosomes were subsequently studied to determine the level of expression of exosome marker proteins such as CD63 (25kDa), flotillin-2 (49kDa), flotillin-1 (47kDa), and CD9 (24 kDa). Marker-based characterization of exosomes can be used to select exosome populations that will be effective and potent for selected therapeutic applications. Two different exosome samples isolated from two different weeks of MTO culture medium were used for this study. The samples were subjected to Western blot analysis and specific antibodies and secondary antibodies were used. The exosomes isolated from Week 6 and Week 8 of the MTO culture medium contained CD63, flotillin-2, flotillin-1, and CD9 marker proteins (FIG. 2B). The presence of CD9 on the exosomes was also confirmed by a flow cytometry based approach (FIG. 3). The singlets were gated out using their side and forward scatter properties. Week 10 MTO exosomes from multiple

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batches were studied for the expression of CD9, CD63 and CD81 compared to their unstained counterpart (FIG. 22A). Together, these data demonstrate that the approach and isolation methods described herein provide high quality exosomes from MTO culture medium.

While described above in an exemplary embodiment in which exosome were screened
5 based on the presence of CD63, flotillin-2, flotillin-1, and CD9 marker proteins. The compositions and methods herein can involve identifying exosomes by screening for any suitable exosome marker. Exemplary exosome markers include, but are not limited to, CD63, flotillin-2, flotillin-1, CD9, and CD81.

10 Therapeutic Applications of Multi-Tissue Organoid-Derived Exosomes

Exosomes have the potential to share a therapeutic profile that is similar to their cells of origination, but can offer advantages over cellular therapies. For example, exosomes typically show low/no immunogenicity and can be easier to handle, store, and/or ship compared to cellular therapies. Exosomes also reduce or eliminate certain issues associated with cell transplantation
15 such as, for example, immune rejection, low cell survival/engraftment, off-target engraftment and tumorigenicity.

Once isolated, exosomes can be included in a pharmaceutical composition for administering to a subject. Thus, this disclosure describes pharmaceutical compositions that includes exosomes derived from multi-tissue organoids. This disclosure also describes methods
20 of treating a subject having, or at risk of having, a condition treatable with exosomes derived from multi-tissue organoids.

The exosomes generated from multi-tissue organoids described herein may be formulated with a pharmaceutically acceptable carrier. As used herein, "carrier" includes any solvent, dispersion medium, vehicle, coating, diluent, antibacterial, and/or antifungal agent, isotonic
25 agent, absorption delaying agent, buffer, carrier solution, suspension, colloid, and the like. The use of such media and/or agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the MTO-generated exosomes, its use in the therapeutic compositions is contemplated. In one or more embodiments, the supplementary active ingredients also can be incorporated into the compositions. As used
30 herein, "pharmaceutically acceptable" refers to a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with MTO-generated

exosomes without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

MTO-generated exosomes may therefore be formulated into a pharmaceutical composition. The pharmaceutical composition may be formulated in a variety of forms adapted to a preferred route of administration. Thus, a composition can be administered via known routes including, for example, oral, parenteral (e.g., intradermal, transcutaneous, subcutaneous, intramuscular, intravenous, intraperitoneal, etc.), or topical (e.g., intranasal, intrapulmonary, intramammary, intravaginal, intrauterine, intradermal, transcutaneous, rectally, etc.). A pharmaceutical composition can be administered to a mucosal surface, such as by administration to, for example, the nasal or respiratory mucosa (e.g., by spray or aerosol). A composition also can be administered via a sustained or delayed release.

Thus, a pharmaceutical composition that includes MTO-generated exosomes may be provided in any suitable form including but not limited to a solution, a suspension, an emulsion, a spray, an aerosol, or any form of mixture. The composition may be delivered in formulation with any pharmaceutically acceptable excipient, carrier, or vehicle. For example, the formulation may be delivered in a conventional topical dosage form such as, for example, a cream, an ointment, an aerosol formulation, a non-aerosol spray, a gel, a lotion, and the like. The formulation may further include one or more additives including, but not limited to, an adjuvant, a skin penetration enhancer, a colorant, a fragrance, a flavoring, a moisturizer, a thickener, and the like.

A formulation may be conveniently presented in unit dosage form and may be prepared by methods well known in the art of pharmacy. Methods of preparing a composition with a pharmaceutically acceptable carrier include the step of bringing the MTO-derived exosomes into association with a carrier that constitutes one or more accessory ingredients. In general, a formulation may be prepared by uniformly and/or intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

The amount of MTO-derived exosomes administered can vary depending on various factors including, but not limited to, the weight, physical condition, and/or age of the subject, and/or the route of administration. Thus, the absolute number of MTO-derived exosomes

included in a given unit dosage form can vary widely, and depends upon factors such as the species, age, weight, and physical condition of the subject, and/or the method of administration. Accordingly, it is not practical to set forth generally the amount that constitutes an amount of MTO-derived exosomes effective for all possible applications. Those of ordinary skill in the art, however, can readily determine the appropriate amount with due consideration of such factors.

In one or more embodiments, the method can include administering sufficient MTO-derived exosomes to provide a dose of, for example, from about 500,000 exosomes to about 100 trillion exosomes to the subject, although in one or more embodiments the methods may be performed by administering MTO-derived exosomes in a dose outside this range.

In one or more embodiments, the method can include administering sufficient MTO-derived exosomes to provide a minimum dose of, for example, at least 500,000 exosomes, at least one million exosomes, at least 10 million exosomes, at least 20 million exosomes, at least 50 million exosomes, at least 100 million exosomes, at least 500 million exosomes, at least one billion exosomes, at least five billion exosomes, at least 10 billion exosomes, at least 50 billion exosomes, or at least 100 billion exosomes.

In one or more embodiments, the method can include administering sufficient MTO-derived exosomes to provide a maximum dose of, for example, no more than 100 trillion exosomes, no more than 50 trillion exosomes, no more than 10 trillion exosomes, no more than one trillion exosomes, no more than 500 billion exosomes, no more than 100 billion exosomes, no more than 50 billion exosomes, no more than 10 billion exosomes, or no more than 1 billion exosomes. Exosomes are said to be present in amounts “no more than” a reference amount when exosomes are not absent but are present in an amount up to the reference amount.

In one or more embodiments, the method can include administering sufficient MTO-derived exosomes to provide a dose characterized by a range having endpoints defined by any a minimum dose identified above and any maximum dose identified above that is greater than the selected minimum dose. Thus, in one or more embodiments, the method can include administering sufficient MTO-derived exosomes to provide a dose of, for example, from one billion exosomes to one trillion exosomes, from one million exosomes to 10 trillion exosomes, from 100 million exosomes to 500 billion exosomes, from 50 billion exosomes to 10 trillion exosomes, etc.

In certain embodiments, the method can include administering sufficient MTO-derived exosomes to provide a dose equal to any minimum dose or any maximum dose listed above. Thus, for example, the method can include administering sufficient MTO-derived exosomes to provide a dose of one million exosomes, one billion exosomes, 10 billion exosomes, 50 billion
5 exosomes, 100 billion exosomes, one trillion exosomes, etc.

A single dose may be administered all at once, continuously for a prescribed period of time, or in multiple discrete administrations. When multiple administrations are used, the amount of each administration may be the same or different. For example, a dose of 100 billion per day may be administered as a single administration of 100 billion exosomes, continuously over 24
10 hours, as two or more equal administrations (e.g., two administrations of 50 billion exosomes), or as two or more unequal administrations (e.g., a first administration of 75 billion exosomes followed by a second administration of 25 billion exosomes). When multiple administrations are used to deliver a single dose, the interval between administrations may be the same or different.

In one or more embodiments, MTO-generated exosomes may be administered, for
15 example, from a single dose to multiple doses per week, although in one or more embodiments the method can involve a course of treatment that includes administering doses of the MTO-generated exosomes at a frequency outside this range. When a course of treatment involves administering multiple doses within a certain period, the amount of each dose may be the same or different. For example, a course of treatment can include a loading dose initial dose, followed
20 by a maintenance dose that is lower than the loading dose. Also, when multiple doses are used within a certain period, the interval between doses may be the same or be different.

In one or more embodiments, MTO-generated exosomes may be administered from a once per week to a single once-off dose, although in one or more embodiments the methods may be performed by administering MTO-derived exosomes at a frequency outside of this range.

25 Thus, in one or more embodiments, MTO-generated exosomes may be administered at a minimum frequency of at least once per week, at least once per month, at least once per year, at least once every two years, at least once every three years, at least once five years, at least once every 10 years, or as a single once-off dose.

In one or more embodiments, MTO-generated exosomes may be administered at a
30 maximum frequency of no more than once every five years, no more than one every three years,

no more than one every two years, no more than once per year, no more than one per month, or no more than once per week.

In one or more embodiments, MTO-generated exosomes may be administered at a frequency characterized by a range having endpoints defined by any a minimum frequency identified above and any maximum frequency identified above that is more frequent than the
5 selected minimum frequency. For example, in one or more embodiments, MTO-generated exosomes may be administered at a frequency of from a once-off dose to once per week, from once every three years to once per week, from once every five years to once per month, etc.

In certain embodiments, MTO-generated exosomes may be administered at a frequency
10 equal to any minimum frequency or any maximum frequency listed above. Thus, for example, MTO-generated exosomes may be administered as a single once-off dose or at a frequency of once every three years, once every five years, etc.

In one or more embodiments, a course of treatment can have a duration of a single dose to the remaining life of the subject. Thus, in one or more embodiments, the course of treatment
15 with MTO-generated exosomes can have a minimum duration of a once-off dose, at least six months, at least one year, at least three years, at least five years, or until complete recovery.

In one or more embodiments, the course of treatment with MTO-generated exosomes can have a maximum duration of the remaining life of the subject, no more than 10 years, no more than five years, no more than three years, no more than one year, no more than six months, or no
20 more than three months.

In one or more embodiments, the duration of a course of treatment can be characterized by a range having endpoints defined by any a minimum duration identified above and any maximum duration identified above that is greater than the selected minimum duration. For example, in one or more embodiments, the duration of a course of treatment can be from a single
25 once-off dose to the remaining life of the subject, from six months to five years, from three months to three years, etc.

In certain embodiments, the duration of the course of treatment can be equal to any minimum duration or any maximum duration listed above. Thus, for example, the duration of the course of treatment can be a single once-off dose, six months, three years, until complete
30 recovery, or for the remaining life of the subject.

The pharmaceutical compositions described above can be used to treat a condition treatable using MTO-generated exosomes. Treating a condition can be prophylactic or, alternatively, can be initiated after the subject exhibits one or more symptoms or clinical signs of the condition. As used herein, the term “symptom” refers to any subjective evidence of disease or of a patient’s condition. As used herein, the term “sign” or “clinical sign” refers to an objective physical finding relating to a particular condition capable of being found by one other than the patient.

Treatment that is prophylactic—e.g., initiated before a subject manifests a symptom or clinical sign of the condition such as, for example, while an infection remains subclinical—is referred to herein as treatment of a subject that is “at risk” of having the condition. As used herein, the term “at risk” refers to a subject that may or may not actually possess the described risk. Thus, for example, a subject “at risk” of having a condition is a subject possessing one or more risk factors associated with the condition such as, for example, genetic predisposition, ancestry, age, sex, geographical location, lifestyle, or medical history. Treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence.

Accordingly, a composition can be administered before, during, or after the subject first exhibits a symptom or clinical sign of the condition. Treatment initiated before the subject first exhibits a symptom or clinical sign associated with the condition may result in decreasing the likelihood that the subject experiences clinical evidence of the condition compared to a subject to which the composition is not administered, decreasing the severity of symptoms and/or clinical signs of the condition, and/or completely resolving the condition. Treatment initiated after the subject first exhibits a symptom or clinical sign associated with the condition may result in decreasing the severity of symptoms and/or clinical signs of the condition compared to a subject to which the composition is not administered, and/or completely resolving the condition.

Thus, the method includes administering an effective amount of MTO-generated exosomes to a subject having, or at risk of having, a condition treatable using MTO-generated exosomes. In this aspect, an “effective amount” is an amount effective to reduce, limit progression, ameliorate, or resolve, to any extent, a symptom or clinical sign related to the condition.

Osteoarthritis as an Exemplary Therapeutic Application of Exosomes Therapies

Osteoarthritis is one exemplary condition treatable with MTO-generated exosomes.

Osteoarthritis affects about 3.3% to 3.6% of the global population. It causes moderate to severe disability in 43 million people worldwide. Osteoarthritis affects 240 million people globally, about 10% of men and 18% of women over 60 years of age, carrying with it substantial morbidity, including disability and reduced quality of life, and contributing to mortality. In addition, the socioeconomic burden of osteoarthritis on patients and society is considerable.

Currently available treatments for osteoarthritis are mainly palliative (non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular hyaluronan and corticosteroid injections) and do not arrest the progression of the disease, repair the ongoing damage, or eliminate the disabling pain associated with osteoarthritis. Ultimately, as a final resort, these patients require total knee or hip arthroplasty (total knee or hip replacement) to gain relief.

Traditional non-surgical treatments for osteoarthritis can improve symptoms but cannot restore articular cartilage, regenerate cartilage, or modify degenerative processes. Although surgical arthroplasty results in long-term functional improvement and improves quality of life, such treatments are only suitable for the end stage of the diseases, and instability and infection are the most common limitations, necessitating further revision surgery. Cell-based therapy, especially using mesenchymal stem cells, has facilitated rapid advances in regenerative medicine for osteoarthritis/cartilage injury in recent years. However, the clinical applications of stem cells have raised considerable concerns including, but not limited to, teratomas, immune rejection, inconsistency between batches, and dose-dependent effect.

Administering MTO-generated exosomes to subjects having osteoarthritis can be applied earlier in the course of osteoarthritis than surgical treatments, or even prophylactically to subjects at risk for developing osteoarthritis. MTO-generated exosome therapy can reduce the likelihood or severity of osteoarthritis, slow or reverse the progression of disease, regenerate damaged cartilage, reduce inflammation and pain, and improve the subject's quality of life.

Effect of Multi-Tissue Organoid-Derived Exosomes on the proliferation of Chondrocytes

To determine the effect of multi-tissue organoid derived exosomes on chondrocyte proliferation and alleviation of osteoarthritis, chondrocytes were cultured in the presence or absence of TNF α with exosomes purified from the culture supernatant from organoid cultures at

different stages of organoid development. Following 48 hours of culture with exosomes, chondrocyte proliferation was analyzed by enumerating the numbers of Ki-67⁺ chondrocytes. Ki-67 is a nonhistone nuclear protein that is a convenient and reproducible biomarker for cell proliferation. Ki-67 protein is present during the active phases of the cell cycle but is absent during the inactive phases of the cell cycle.

The chondrocyte is the only specialized cell type present in articular cartilage. Chondrocytes produce secretions to support and repair the cartilage matrix. Human chondrocytes in mature articular cartilage are post-mitotic and terminally differentiated cells. Studies on human chondrocytes have been hampered by the difficulty in obtaining sufficient numbers of primary human chondrocytes from a single joint and by variabilities among donors due to factors such as age and medical conditions. Human chondrocyte cell lines (e.g., TC28a2 and C20A4) are widely used as a model cell line for studying normal and pathological cartilage repair mechanisms related to chondrocyte biology and physiology.

Chondrocyte cell lines TC28a2 and C20A4 were cultured in the presence and absence of exosomes isolated from multi-tissue organoid (MTO) culture medium at various time points during organoid development. Exosomes isolated from different weeks of the batch 4 MTO culture medium was used. The exosomes were dosed based on their protein cargo measurement. For the proliferation assay, exosomes with an overall cargo content of 50 µg of protein per million cells were used.

TNF α , an inhibitor of chondrocyte proliferation, was also used (10 ng/ml) as a control along with a normal complete media (1% FBS) control to study the effect of exosomes. In many chronic autoimmune and inflammatory diseases involving the cartilage, several cytokines are released as part of the pathophysiologic response, including TNF α . TNF α is a proinflammatory cytokine and, along with other cytokines, is a catabolic factor for cartilage. TNF α promotes the release of matrix metalloproteinases (MMPs) from synovial fibroblasts resulting in cartilage destruction and inhibits chondrogenesis through the nuclear factor- κ B (NF- κ B) pathway by downregulating the production of SOX9. One function performed by TNF α that is of interest is the ability to inhibit proliferation and induce inflammation. TNF α also inhibits the ability of mesenchymal stem cells (MSCs) to differentiate into chondroblasts affecting chondrogenesis.

As noted above, chondrocytes were treated with exosomes isolated from different time points of MTO development in the presence and absence of TNF α . After 48 hours of the

treatment period, the cells were collected and stained for the expression of Ki-67 using a Ki-67-PE staining kit. An isotype control (IgG1) was also performed for each sample. Isotype controls are primary antibodies that lack specificity to the target but match the class and type of the primary antibody used in the application. Isotype controls are used as negative controls to help
 5 differentiate non-specific background signal from specific antibody signal. The detailed gating strategy (FIG. 4) was followed to determine the Ki-67⁺ cell percentage on different treatment conditions.

Exosomes from different the MTO culture media collected at different time points in MTO development were used on C20A4 chondrocyte cells and the effect on proliferation was
 10 determined through Ki-67-based proliferation assay. Ki-67⁺ population was clearly gated with the help of isotype control. Cells treated with TNF α (10 ng/ml) show a significant decrease in the proliferation of C20A4 cells (FIG. 4B).

Proliferation percentage of exosome-treated cells in the presence and absence of TNF α were compared to the normal media control (85.5%) and TNF α alone treated control (70.9%).
 15 Table 1 and Table 2 show the proliferation percentage of C20A4 (Table 1) and TC28a2 (Table 2) chondrocytes upon various conditions.

Table 1 – Multi-Tissue Organoid-derived exosomes increase C20A4 chondrocyte proliferation

Sample	Ki-67 subset / Frequency of Parent (%)
C20A4 (untreated)	85.5
C20A4/TNF α /M	70.9
C20A4/TNF α /Exo1	78.5
C20A4/TNF α /Exo2	77.3
C20A4/TNF α /Exo3	78.0
C20A4/TNF α /Exo4	86.3
C20A4/TNF α /Exo5	73.8
C20A4/M	85.5
C20A4/Exo1	89.4
C20A4/Exo2	91.5

C20A4/Exo3	90.4
C20A4/Exo4	89.9
C20A4/Exo5	80.8

M: Media control – no exosomes

Exo1: Week 5

Exo2: Week 9

Exo3: Week 11

Exo4: Week 13

Exo5: Week 15

Table 2 - Organoid-derived exosomes increase TC28a2 chondrocyte proliferation

Sample	Ki-67 subset / Frequency of Parent (%)
TC28a2 (untreated)	67.3
TC28a2/TNF α /M	52.2
TC28a2/TNF α /Exo1	53.1
TC28a2/TNF α /Exo2	64.7
TC28a2/TNF α /Exo3	59.3
TC28a2/TNF α /Exo4	58.3
TC28a2/TNF α /Exo5	53.7
TC28a2/M	67.3
TC28a2/Exo1	71.6
TC28a2/Exo2	70.5
TC28a2/Exo3	71.3
TC28a2/Exo4	67.1
TC28a2/Exo5	74.1

M: Media control – no exosomes

Exo1: Week 5

Exo2: Week 9

Exo3: Week 11

Exo4: Week 13

Exo5: Week 15

In both cases, exosomes reversed the TNF α inhibition of chondrocyte proliferation. The effect of MTO-derived exosomes on the change in proliferation of chondrocytes cell lines C20A4 and TC28a2 compared to the TNF α control cells are presented in FIG. 5A and FIG. 5B.

Exosomes derived from MTO culture media at Week 5, Week 9, Week 11, Week 13, and Week 15 of MTO development reversed the TNF α -mediated inhibition of proliferation on C20A4 and TC28a2 chondrocytes (FIG. 5A and FIG. 5B). Exosomes from Week 13 (Exo4) reversed the TNF α -mediated inhibition of proliferation to the maximum level (15.4%) on C20A4 cells (FIG. 5A). In the case TC28a2, exosomes from Week 9 (Exo2) were most effective reversing the TNF α -mediated inhibition of chondrocyte proliferation (FIG. 5B).

The exosome-mediated increases in proliferation reversed the TNF α -mediated inhibition to near normal level. In both chondrocyte cell lines, exosomes from Week 16 (Exo5) showed the lowest level of reversal of TNF α -mediated inhibition: C20A4 (2.9%, FIG. 5A) and TC28a2 (1.5%, FIG. 5B). Exosomes secreted from earlier weeks of MTO may contain cargo that might induce more proliferation compared to exosomes from the later weeks.

Exosomes from MTO culture medium alters the colony forming ability of the chondrocytes

The colony formation assay is an in vitro cell survival and proliferation assay based on the ability of a single cell to grow into a colony of at least 50 cells. The assay essentially tests every cell in the population for its ability to retain their reproductive integrity over a prolonged period of time. This is an important feature as it reveals phenotypic effects that require time and possibly several cell divisions to develop. When analyzing therapeutic use of exosomes this is particularly important. Through this assay the long-term effect of an exosome on proliferation can be determined.

TC28a2 chondrocytes were used for this assay. The cells were treated with exosomes (50 μ g/million cells) collected from different weeks of the batch3 MTO culture supernatants. The colonies were compared with the media control and TNF α -treated control wells to determine the effect of exosomes on the colony forming ability of the TC28a2 chondrocytes. Since the TC28a2 chondrocytes grow as a dispersed colony (FIG. 6), quantification of the crystal violet retained by the cells on each condition was measured. The results of the quantification were depicted in FIG. 7A. Based on the optical density measured at 595 nm, percentage change in proliferation was determined keeping the values of the control wells (media alone and TNF α) as 100%. The change in proliferation percentage in the exosome-treated wells compared to the respective controls were determined and the data is presented in FIG. 7B.

Treatment with exosomes show a significant increase in the proliferation as there was an increase in chondrocyte percentage upon treatment with exosomes compared to the TNF α control wells. Exosomes from early stage of the MTO culture media (Week 3; Exo1) show a very high increase in proliferation with as high as 48.0%. The decrease in chondrocyte percentage compared to media control in FIG. 7B could be due to over proliferation and necrosis of the cells.

Altogether, the Ki-67⁺ proliferation assay and colony formation assay show that exosomes isolated from MTO culture media collected at up to Week 15 of multi-tissue organoid development reversed TNF α -mediated decreases in chondrocyte proliferation.

Exosomes inhibit inflammation by inducing the Treg population and CD69 expression

Regulatory T (Treg) cells are a specialized subset of immunosuppressive CD4 T cells that express the X-chromosome-encoded, lineage-specific transcription factor FOXP3. Treg cells maintain peripheral tolerance by providing essential suppression of autoreactive CD4 T cells that have escaped negative selection in the thymus. In addition, Treg cells act as negative regulators of inflammation in various biological contexts, including infection, metabolic disease, tissue repair, and cancer. Treg cells respond to inflammation by sharply increasing their suppressive function. Activated Treg cells upregulate immunosuppressive molecules and tissue homing receptors and undergo polycomb-mediated repression of Foxp3-bound genes, which may inhibit pro-inflammatory functions.

Gating strategies for defining the Treg population are depicted in FIG. 8 and FIG. 27. In short, area versus height on forward and side scattered population was used to gate for the singlet population. The lymphocytes were then selected, and the live population was gated based on the Live-Dead ghost dye. CD4⁺ T cells were gated from the live population and the Tregs were defined by the expression of both CD25 and FOXP3 (FIG. 8; FIG. 27).

After five days of MLR culture with human PBMCs induced by mismatched PBMCs in the presence or absence of exosomes, exosomes from the MTO culture medium induce the proliferation on CD25⁺FOXP3⁺ regulatory T cells (FIG. 9; FIG. 28A). Human PBMC responder sample (Sample Code: Human 23) that was used in the study stimulated with mismatched human PBMC sample (Sample code: STC3) show a marked increase in the level of Treg population in the presence of exosomes.

These data indicate that exosomes modulate the immune system, providing an anti-inflammatory niche that, in turn, may be effective to regulate osteoarthritis progression. The CD4⁺CD25⁺FOXP3⁺ Treg cells are actively engaged in mitigating autoimmunity and aberrant or excessive immune responses. The transcription factor Forkhead box P3 (denoted “FOXP3” in humans, and “Foxp3” in mice) is a well-characterized marker of Treg cells, and its expression typically is considered a requisite for Treg cell differentiation and function. FOXP3 deficiency leads to the scurfy phenotype in mice and to immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome in humans. Moreover, Treg-cell function is impaired in several autoimmune and inflammatory diseases, including colitis, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus. Thus, the manipulation of Treg cell function by MTO exosomes might provide a practical approach to the treatment of autoimmune and inflammatory diseases.

Expression of the C-type lectin receptor CD69 (encoded in NK gene cluster), an early activation antigen of lymphocytes, also was examined. CD69 is involved in regulating immune responses in murine models of asthma, arthritis, colitis, myocarditis, pathogen clearance, and tumors. CD69 activation induces TGF- β expression and suppresses the production of pro-inflammatory cytokines IL-17 and IFN- γ .

Treating human PBMCs with exosomes isolated from the MTO culture medium increased expression of Treg (FIG. 28A) and Tr1 (FIG. 28B) regulatory T cells. The level of expression was consistent across multiple batches of MTO exosome treatment compared to untreated media control PBMCs. The activation marker, CD69, also increased in Treg and Tr1 cells upon MTO exosome treatment compared to the untreated media control (FIG. 10, FIG. 29A). Activation of CD69 leads to ERK phosphorylation and thus stabilizes TGF- β on the cell surface of lymphocytes. After allogeneic bone-marrow transplantation, CD4⁺CD69⁺CD25⁻ T cells inhibit development of graft-versus host disease. CD69⁺ T cells are able to induce indoleamine 2,3-dioxygenase (IDO) in tumor-associated macrophages and hence downregulate inflammatory immune responses. Therefore CD4⁺CD69⁺CD25⁻ T cells have been introduced as novel regulatory cell type whose effector functions depend mainly on TGF- β .

Exosomal membranes are enriched in endosome-specific tetraspanins (CD9 and CD81). CD9 and CD81 regulate systemic inflammation, including functions that are related to modulating CD19 and MHC class II molecules in B cells, with CD4 in T cells, and with integrins

in B cells and T cells. The many involvements of CD81 stem from its ability to stabilize and facilitate the organization of its specific partner molecules such as CD9 in response to stimuli. To determine the expression level of these immune suppressive markers on MTO-derived exosomes, exosomes from IPSC-derived medium and MTO-derived medium were incubated with

5 antibodies against CD9 and CD81. The level of expression of CD9 and CD81 was determined via flow cytometry and the results were presented (FIG. 29B). Exosomes isolated from week 12 MTO culture medium is found to be showing high levels of CD9 and CD81 compared to the exosomes from regular IPSC culture medium. High levels of CD9 and CD81 with the anti-inflammatory potential being a part of the MTO exosomal cargo could contribute to the anti-

10 inflammatory effects of MTO-derived exosomes.

Expression profile of immunomodulatory molecules on early, mid, and late weeks of MTO exosomes

FIGS. 13-18 provide data showing increased expression percentage and mean fluorescent

15 intensity (MFI) of immune suppressive markers CD9, CD81, and CD63 in exosomes collected from Week 8 to Week 11 from Batch 13 of the MTO culture medium compared to the exosomes collected from iPSC culture medium (control). Exosomes isolated from Early (Week 6), Mid (Week 12) and Late (Week 14-Week 19) weeks of MTO culture medium from different batches were analyzed for the expression profile of Immunosuppressive markers such as CD9, CD63 and

20 CD81.

FIG. 3A shows the gating strategy for assessing CD9 expression on MTO exosomes. FIGS. 19-22 show expression profiles of immunosuppressive markers CD9, CD63, and CD82 in different batches of MTOs. The profiling suggests that exosomes isolated from MTO culture medium show an increased expression of the markers compared to the exosomes isolated from

25 control iPSC culture medium across various batches. All these profilings show an increased expression of immunomodulatory markers on exosomes isolated from around week 10 of the MTO culture medium (FIG. 18).

Effect of Exosomes from week 10 of MTO culture medium on proliferation of chondrocytes (Ki-

30 67 based immunofluorescent assay)

TC28a2 chondrocyte cells were treated with exosomes of week 10 MTO culture medium. Ki-67 level was detected on control and exosome treated cells and the levels were compared, the cells were treated with TNF α to suppress the inherent active proliferative state of the cells. The nucleus of the cells were labelled with DAPI (Red) and Ki-67 level marked by the expression ranging from green (low) to orange (High) spectrum of colors based on the intensity of Ki-67 expression, with blue to purple being no expression of Ki-67 (FIG. 23). In the close-up image on FIG. 23, the cells marked by the arrow are found to be in the active stage of cell cycle also showing positive signal for Ki-67. The Ki-67 level was detected on Tc28a2 chondrocytes treated with TNF α (50 ng/million cells) and exosomes from Batches 13 to 17 on week 10 of the MTO culture medium. The cells were also treated with control iPSC exosomes and the level of expression of Ki-67 was detected.

The merged images of DAPI and Ki-67 show the reversal of the TNF α -mediated decrease in proliferation on TC28a2 chondrocytes upon treatment with Week 10 exosomes from batches 13 – 17 compared to the iPSC exosomes (FIG. 24). The percentage proliferative Ki-67 positive cells on control and exosome treated chondrocytes were calculated and results are presented in FIG. 25. FIG. 25B shows the percentage increase in Ki-67⁺ cells on MTO derived exosome treatment compared to iPSC exosome treated chondrocytes.

To arrive at a quantifiable measure of effect of MTO exosomes, a flow-based assay was also performed using C20A4 and TC28a2 chondrocytes that were treated with exosomes isolated from different weeks of MTO in the presence or absence of TNF α . After 48 hours of the treatment period, the cells were collected and stained for the expression of Ki-67. Isotype controls are used as negative controls to help differentiate non-specific background signal from specific antibody signal. The detailed gating strategy (FIG. 4B) was followed to determine the Ki-67⁺ cell percentage on different treatment conditions. Cells treated with TNF α (10 ng/ml) shows a significant decrease in the proliferation of chondrocyte cell lines (FIG. 4B).

The percentage of exosome treated cells undergoing proliferation in the presence and absence of TNF α were compared to the normal media control (85.5%) and TNF α alone treated control (70.9%) (FIG 4B). MTO Exosomes of weeks 5, 9, 11, 13 and 15 were found to be overturning the TNF α (10 ng/ml) mediated inhibition of proliferation on C20A4 and TC28a2 chondrocytes (FIG. 4, FIG. 5). Exosomes from week 13 (Exo4) was found to be reversing the TNF α (10 ng/ml) mediated inhibition of proliferation to the maximum level (15.4%) on C20A4

cells (left panel). In the case of TC28a2 (right panel), exosomes from week 9 were found to exhibit maximum increase in proliferation (12.5%) after TNF α -mediated inhibition. This increase in proliferation brought the TNF α -mediated inhibition to near normal level. Exosomes from week 16 shows the lowest level of increase in proliferation from the TNF α treated C20A4 (2.9%) and TC28a2 (1.5%) chondrocytes (FIG. 5). Exosomes secreted from earlier weeks (13 weeks or less) of MTO might contain the necessary cargo to induce more proliferation compared to exosomes from later weeks of MTO culture medium. In summary and when taken together, exosomes reversed the TNF α inhibition in proliferation of TC28a2 (right panel) and C20A4 (left panel) similar to the proliferation observed in the media controls (FIG 26).

The overall possible mechanism of action of MTO exosomes for the treatment of osteoarthritis is depicted in FIG. 11. From the Ki-67⁺ proliferation assay and colony formation assay, it is evident that exosomes isolated from MTO culture media at up to Week 15 of MTO development reverses the TNF α -mediated decrease in chondrocyte proliferation. Apart from the role of inhibiting the proliferation, TNF α also induces inflammation that can lead to osteochondral defects. TNF α also may interfere with the healing process of chondral and osteochondral defects that occurs naturally or in low inflammatory environment after a cartilage repair procedure.

A significant correlation between inflammation and disease severity has been observed in human and animal models of osteoarthritis. This disclosure provides data showing that MTO exosomes contain high levels of anti-inflammatory molecules such as CD9 and CD81 in their cargo compared to exosomes from regular iPSC culture medium. Exosomes purified from MTO-development inhibits TNF α -induced inflammation in human peripheral blood mononuclear cells. Regulatory T cell (Treg) function is impaired in several autoimmune and inflammatory diseases, including osteoarthritis. The presence of exosomes increases the level CD4⁺FoxP3⁺ Treg population and CD4⁺CD69⁺ T cells in human MLR cultures. CD69⁺ T cells can induce indoleamine 2,3-dioxygenase (IDO) in osteoarthritis models and down-regulate inflammatory immune responses. Ki-67-based proliferation assay and colony formation assay demonstrates that exosomes isolated from MTO culture medium induce the proliferation of chondrocytes. Taken together, exosomes from MTO culture medium could be a potential agent in improving the proliferation of chondrocytes and/or modulate the immune system to provide an anti-inflammatory niche that, in turn, could inhibit the progression of osteoarthritis. Thus, from the

Ki-67⁺ proliferation assay and the colony formation assay it is evident that exosomes isolated from MTO culture media up to Week 15 can reverse TNF α mediated decrease in chondrocyte proliferation.

5 MTO-derived exosomes could be a potential agent in improving the proliferation of chondrocytes while modulating the immune system providing an anti-inflammatory niche which, in turn, could result in an effective outcome towards regulating the progression of osteoarthritis. Apart from the role of inhibiting the proliferation, TNF α is involved in inducing the inflammation that can lead to osteochondral defects. TNF α might have the potential to interfere with the healing process of chondral and osteochondral defects occurring naturally or in low
10 inflammatory environment after a cartilage repair procedure. Since these exosomes are derived from a fetal-like source of hyaline cartilage, they could provide both effective regenerative cues for articular cartilage while promoting an immune modulated anti-inflammatory environment making them a promising agent for the treatment of osteoarthritis.

Thus, exosomes isolated from MTO culture media increase chondrocyte proliferation,
15 increases the level of regulatory T cell (Treg and Tr1) population, and increase regulatory T cell activation (CD69 expression) in human MLR cultures. Altogether, exosomes act through increasing the proliferation of chondrocytes and modulating the immune system. Exosome-mediated immune modulation provides an anti-inflammatory environment that may regulate the progression of osteoarthritis. The use of exosomes to inhibit TNF α interference with cartilage
20 repair surgery could be beneficial, and this could translate into a promising therapy that improves the outcome of currently available cartilage procedures.

A rat monoiodoacetate model was used to assess the efficacy of MTO-derived exosomes for the in vivo treatment of osteoarthritis. Four groups of 14 rats (7M, 7F) had osteoarthritis induced in their right femoro-tibial joint (equivalent of human knee joint). A fifth group of
25 unoperated rats (5M, 5F) were used as normal control group. Groups 1-4 received weekly intra-articular injections into the right femoro-tibial joint beginning on day 0, consisting of saline (Group 1), 2.20×10^{11} exosomes in saline (Group 2), TCA + Tramadol (Group 3), and FGF-18 (Group 4). On Day 7 and Day 38 rats were assessed by dynamic weight bearing analysis. In normal rats the weight bearing should be approximately equal between left and right hind limbs
30 which is indicated by a score of 0.50 (as in the Naïve rats in Group 5). Scores for each rat in all five groups are shown in FIG. 30. Scores were categorized as Excellent if above 0.45 and Good

if above 0.40. In this categorization analysis, vehicle-treated rats showed an initial good response at D7 but had worse scores at D38. In contrast, the exosome-treated group began with a worse response than saline controls at D7 but showed marked improvement at D38 and had 9/14 animals with Excellent or Good scores compared to the vehicle-treated rat with only 5/14 with
5 Excellent or Good scores. The exosome-treated rats were approaching the levels apparent in the TCA + Tramadol group which represents the standard of care for osteoarthritis. Exosome treatment also showed a far superior therapeutic response than FGF-18 (FIG. 31), which has been shown to be highly effective in some models of osteoarthritis. Overall, the data indicates a therapeutic effect for MTO-derived exosomes for the treatment of osteoarthritis.

10 While described above in the context of an exemplary embodiment in which osteoarthritis is the condition treated using MTO-generated exosomes, the compositions and methods described herein can involve treating other conditions. Exemplary alternative conditions treatable using MTO-generated exosomes include, but are not limited to, cartilage injury, autoimmune diseases (e.g., immune dysregulation, polyendocrinopathy, enteropathy, X-linked
15 syndrome, etc.), inflammatory diseases (e.g., colitis, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, immune-mediated inflammatory disease, etc.), neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, traumatic brain injury, etc.), stroke, and conditions involving mitochondrial dysfunction (e.g., aging, diabetes, metabolic disorders, obesity, etc.).

20 MTO-Derived Exosome Proteomics

Protein and peptide contents of exosomes isolated from conditioned MTO cell culture medium harvested from weeks 8 to 15 in culture, were analyzed by mass spectrometry. Over 4,000 proteins, over 40,000 peptides, and over 50,000 protein motifs were identified in the
25 exosomes. Examples of proteins found in the exosomes are listed in Tables 3, 4, 5, 6, 7, and 8. Table 3 shows examples of cartilage extracellular matrix (ECM) proteins that are present in the exosomes. Release of these ECM components by the exosomes may play a role in formation of cartilage ECM and thereby promote cartilage regeneration. Table 3 lists additional proteins that are markers characteristic of chondrocytes and indicate that the source of the exosomes is cells in
30 the chondrocytic lineage. Table 4 lists examples of proteins present in the exosomes that have been shown to have chondrogenic and/or chondrotrophic properties that are relevant to potential

cartilage regenerative effects of the MTO-derived exosomes. Table 5 lists several surface proteins present in the MTO-derived exosomes that are relevant to the identity of exosomes. Some of these surface markers also have important immunomodulatory effects (noted below), which may be relevant to anti-inflammatory actions of the MTO-derived exosomes. Table 6 lists
5 several proteins present in the MTO-derived exosomes that are immunomodulatory and/or anti-inflammatory. Table 7 lists examples of proteins present in MTO-derived exosomes that are of mitochondrial origin and which may assist in mitochondrial function. Table 8 lists examples of proteins present in MTO-derived exosomes that may stimulate cell growth and tissue regeneration, and may have anti-aging activities.

10

Table 3. Cartilage Extracellular Matrix Components Detected in MTO-Derived Exosomes and Additional Chondrocyte Marker Proteins		
Marker ID	Corresponding Gene ID	Annotation
Aggrecan	ACAN	Cartilage ECM component
Type 2 collagen	COL2A1	Cartilage ECM component
Type 6 collagen 1A1	COL6A1	Cartilage ECM component
Type 6 collagen 1A2	COL6A2	Cartilage ECM component
Type 6 collagen 1A3	COL6A3	Cartilage ECM component
Type 11 collagen	COL11A1	Cartilage ECM component

Type 12 collagen	COL12A1	Cartilage ECM component
Hyaluronan synthase	HAS2	Chondrocyte marker
CD44	CD44	Chondrocyte marker--hyaluronic acid receptor
CD151	CD151	Chondrocyte marker
Link protein 1	HAPLN1	Cartilage ECM component
CD49c (integrin alpha chain)	ITGA3	Chondrocyte marker
Perlecan	HSPG2	Cartilage ECM component
Decorin	DCN	Cartilage ECM component
Biglycan	BGN	Cartilage ECM component
Fibromodulin	FMOD	Cartilage ECM component
Lumican	LUM	Cartilage ECM component
Chondroitin sulfate proteoglycan 4	CSPG4	Cartilage ECM component
CD166	ALCAM	Chondrocyte marker
Matrilin 2	MATN2	Cartilage ECM component
MCAM/CD146	AP002956.1	Chondrocyte marker
CD49e (integrin alpha 5)	ITGA5	Chondrocyte marker
Tenascin-C	TNC	Cartilage ECM component
NT5E	CD73	Chondrocyte marker

Table 4. Chondrogenic/Chondrotrophic Proteins Detected in MTO-Derived Exosomes		
Marker ID	Corresponding Gene ID	Annotation
Agrin	AGRN	Chondrotrophic heparin sulfate basal lamina glycoprotein
TGF beta1	TGFb1	Chondrogenic
Bone Morphogenetic Protein 7	BMP7	Chondrogenic
LDL Receptor Related Protein 4	LRP4	Chondrotrophic co-factor with agrin
Alpha Dystroglycan	DAG1	Chondrotrophic co-factor with agrin
PDGFR β	PDGFRB	Chondrotrophic

Table 5. Exosome Surface Marker Proteins Detected in MTO-Derived Exosomes		
Marker ID	Corresponding Gene ID	Annotation
Tetraspanin	CD63	Involved in the regulation of inflammation and extracellular matrix (ECM) remodeling
Syntenin-1	SDCBP	Involved in vesicle trafficking and signaling related to ECM components
Tetraspanin	CD9	Anti-inflammatory
Programmed Cell Death 6 Interacting Protein	PDCD6IP	Involved in endosomal vesicle formation
Epithelial Cell Adhesion Molecule	EPCAM	Anti-inflammatory
Annexin 5	ANXA5	Anti-inflammatory
Tumor Susceptibility 101	TSG101	Promotes exosome secretion and up-regulates Nrf2 signaling pathway that counters oxidative stress.
Flotillin 1	flot1	Endocytosis and membrane trafficking
Flotillin 2	flot2	Membrane dynamics and signaling pathways

Table 6. Anti-inflammatory and Immunomodulatory Proteins Detected in MTO-Derived Exosomes		
Marker ID	Corresponding Gene ID	Annotation
Tetraspanin CD63	CD63	Regulation of inflammation and ECM remodeling
CD276	CD276	Immune regulation
Tetraspanin CD151	CD151	Interacts with integrins and plays a role in cell adhesion and migration, which are processes involved in cartilage repair
CD109	CD109	Negatively regulates signaling by TGF-beta
CD47	CD47	Don't eat me signal
CD46	CD46	Cofactor activity for inactivation of complement C3b and C4b by factor I
CD55	CD55	Binding to complement proteins accelerates their decay thereby preventing damage to host cells.
Tetraspanin CD82	CD82	Involved in cell adhesion and signal transduction
CD70	CD70	TNF ligand
CD200	CD200	Role in immunosuppression and chondroprotective
LFA-3	CD58	Immunosuppressive
CD83	CD83	Immune checkpoint molecule that promotes resolution of inflammation

Table 7. Mitochondrial Proteins Detected in MTO-Derived Exosomes		
Marker ID	Corresponding Gene ID	Annotation
60 kDa heat shock protein, mitochondrial	HSPD1	Cell differentiation; cell growth cell organization and biogenesis; cell proliferation; cellular homeostasis
ATP synthase subunit alpha, mitochondrial	ATP5F1A	cellular homeostasis; conjugation; metabolic process
Stress-70 protein, mitochondrial	HSPA9	cell growth; cell organization and biogenesis; cellular homeostasis; conjugation; metabolic process
ATP synthase subunit beta, mitochondrial	ATP5F1B	cellular homeostasis; conjugation; defense response; metabolic process
Serine hydroxymethyltransferase, mitochondrial	SHMT2	cell growth; cell organization and biogenesis; cellular homeostasis; coagulation; metabolic process
Isocitrate dehydrogenase [NADP], mitochondrial	IDH2	cellular homeostasis
Glutamate dehydrogenase 1, mitochondrial	GLUD1	cellular homeostasis; metabolic process
Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADVL	cellular homeostasis; coagulation; metabolic process

Table 8. Growth/Regenerative/Anti-Aging Factors Detected in MTO-Derived Exosomes		
Marker ID	Corresponding Gene ID	Annotation
Agrin	AGRN	Prevention of age-associated muscle weakness and atrophy, and promotion of adult neurogenesis
Transforming growth factor-beta-induced protein	TGFB1	Cell communication; cell differentiation; cell growth; defense response; metabolic process
Platelet-derived growth factor receptor beta	PDGFRB	Cell differentiation; cell growth; cell organization and biogenesis; cellular homeostasis; defense response; development; metabolic process
Insulin-like growth factor 2 mRNA-binding protein	IGF2BP1	Cell differentiation; cell growth; coagulation; conjugation; defense response; metabolic process
Epidermal growth factor receptor	EGFR	Cell communication; cell differentiation; cell growth; cell organization and biogenesis;
Insulin-like growth factor 1 receptor	IGF1R	Cell growth; cell organization and biogenesis; cellular homeostasis; development; metabolic process
Fibroblast growth factor 2	FGF2	Development; cell growth
Platelet-derived growth factor C	PDGFC	Cell differentiation; defense response; development; metabolic process
Heparin binding growth factor	HDGF	Cell proliferation and differentiation
Transforming growth factor beta-1 proprotein	TGFB1	Cell growth; cell organization and biogenesis; cellular homeostasis; coagulation; conjugation; defense response; development; metabolic process

In the preceding description and following claims, the term “and/or” means one or all of
5 the listed elements or a combination of any two or more of the listed elements; the terms

“comprises,” “comprising,” and variations thereof are to be construed as open ended—i.e., additional elements or steps are optional and may or may not be present; unless otherwise specified, “a,” “an,” “the,” and “at least one” are used interchangeably and mean one or more than one; and the recitations of numerical ranges by endpoints include all numbers subsumed
5 within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

In the preceding description, particular embodiments may be described in isolation for clarity. Reference throughout this specification to “one embodiment,” “an embodiment,” “certain embodiments,” or “some embodiments,” etc., means that a particular feature, configuration, composition, or characteristic described in connection with the embodiment is included in at
10 least one embodiment of the disclosure. Thus, the appearances of such phrases in various places throughout this specification are not necessarily referring to the same embodiment of the disclosure. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more embodiments. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any
15 suitable manner in one or more embodiments. Thus, features described in the context of one embodiment may be combined with features described in the context of a different embodiment except where the features are necessarily mutually exclusive.

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be
20 conducted simultaneously.

As used herein, the terms “preferred” and “preferably” refer to embodiments of the invention that may afford certain benefits under certain circumstances. However, other embodiments may also be preferred under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not
25 useful and is not intended to exclude other embodiments from the scope of the invention.

EXAMPLES

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in
30 accordance with the scope and spirit of the invention as set forth herein.

Generating hiPSC-derived multi-tissue organoids (MTOs):

iPSC line referred to as 1024 (ATCC-BYS0110, American Type Culture Collection, Manassas, VA) was expanded in culture on vitronectin (VTN-N, Thermo Fisher Scientific, Inc., Waltham, MA) in Essential 8 Medium (E8, Thermo Fisher Scientific, Inc., Waltham MA) for
5 two or three passages. iPSCs were harvested using sodium citrate buffer and briefly centrifuged for five minutes at room temperature, 250×g. Multi-tissue organoid (MTO) induction was initiated by resuspending expanded iPSCs (~20 million cells) in 40 µl of buffered hyaluronic acid (HA). Cells in HA were then transferred to a G-REX 100 bioreactor (Wilson Wolf Manufacturing Corp., New Brighton, MN) containing E8 media and incubated at 37 °C in 5%
10 CO₂. E8 culture medium containing 1% antibiotic-antimycotic (Thermo Fisher Scientific, Inc., Waltham, MA) was changed every 3-4 days over the entirety of multi-tissue organoid (MTO) culture.

Isolation of Exosomes

15 Organoid culture supernatant was centrifuged at 300×g at 4 °C for 10 minutes to remove detached cells. The supernatant was centrifuged at 2000×g for 10 minutes and further recentrifuged at 10,000×g for 30 minutes to remove cell debris. Furthermore, the supernatant was concentrated by ultrafiltration using AMICON-ULTRA 15 centrifugal filter units with a molecular weight cut-off of 100 kDa (Merck Millipore, Tullagreen, Cork, Ireland). The
20 supernatant was then ultracentrifuged (OPTIMA L-80XP, Beckman Coulter, Brea, CA) at 100,000 g_{avg} at 4 °C for 90 minutes to pellet exosomes. The supernatant was removed, and exosome-containing pellets resuspended in 1 mL of ice-cold PBS and pooled. A second round of ultracentrifugation was performed at 100,000 g_{avg} at 4 °C for 90 minutes, and the resulting exosome pellet resuspended in 200 µl of PBS for further characterization and preclinical studies.

25

Exosome measurement with the NANOSIGHT and characterization

Particle concentration and size distribution was determined using a NANOSIGHT NS500 instrument (Malvern Panalytical, Ltd., Malvern, UK) equipped with a 488 nm laser, and a high sensitivity sCMOS camera. Exosome samples were diluted 50 times in 0.02-µm-filtered PBS to
30 obtain a concentration within the range of 10⁸–10⁹ particles/ml. The exosome analyses were performed with the NTA software (version 3.1 Build 3.1.54, Malvern Panalytical, Ltd., Malvern,

UK) using 60 seconds of video captures per sample (in triplicate). Camera level was set to 14 and detection threshold was set to 3.

Western Blot Analysis

5 Purified exosomes were characterized by semi-quantitative Western blot analysis by measuring the presence of CD63 and flotillin-1 content in the exosomes using specific antibodies. Briefly, SDS-sample buffer was added to the exosome sample and the sample was incubated for five minutes at 95 °C and separated on 4-12% precast acrylamide gels (Invitrogen, Carlsbad, CA). After transfer to PVDF membranes (Millipore, Bedford, MA) and blocked
10 overnight (5% milk and 0.05% Tween-20 in PBS), primary antibody was added for one hour, followed by PBS washing and the application of secondary HRP-conjugated antibody. Chemiluminescence detection of bands was performed with ECL Plus reagent (GE Healthcare, Buckinghamshire, UK).

15 Analysis of Exosomes by Flow Cytometry

Purified exosomes were stained with a known exosome marker CD63. Exosome pellets were resuspended in anti-CD63-PerCP in PBS and incubated at 4°C for 30 minutes. After the incubation period, exosomes were washed with PBS (10 ml) to remove the excess antibody. The solution was transferred to a centrifuge (AMICON-Ultra 15 Centrifugal Filter Units, Merck
20 KGAA, Darmstadt, Germany) and spun at 3200×g for 30 minutes. The collected exosomes were resuspended in 200 µl of PBS and the samples were used for flow cytometric analysis for the expression of CD63. The stained exosomes were washed twice with 3 ml of 1× BD Perm/Wash buffer, and then resuspended in 300 µl of the stain buffer prior to flow cytometric analysis. A minimum of 200,000 events were acquired on three-laser flow cytometer (FACSCANTO II
25 equipped with FACSDIVA 6.1.3, BD Biosciences, Franklin Lakes, NJ). Relative percentages and mean fluorescence intensity (MFI) of each of the populations were determined using the FlowJo 10.1. software (TreeStar).

Chondrocyte Cell lines

30 The following cell lines were used for the study. T/C-28a2 cell line (MilliporeSigma, Burlington, MA) was established by transfecting primary cultures (day 5) of costal cartilage from

a 15-year-old female with a retroviral vector expressing simian virus SV40 large T antigen. C-20/A4 cell line (MilliporeSigma, Burlington, MA) was established by transfecting primary cultures of rib chondrocytes from a 5-year-old male with vectors expressing origin-defective simian virus SV40 large T antigen.

5

Ki-67 based chondrocyte Proliferation Assay

Chondrocytes were treated with exosomes isolated from different weeks of multi tissue organoid (MTO) in the presence and absence of TNF α , an inhibitor of chondrocyte proliferation. After 48 hours of the treatment period, the cells were collected and stained for the expression of

10 Ki-67 using a Ki-67-PE staining kit (BD Biosciences, Franklin Lakes, NJ). In short, cells were trypsinized after treatment, then added to a 12×75 mm test tube, and first stained with a Live-Dead Ghost Dye for 20 minutes at 4 °C in the dark. The cells were then washed twice with PBS. The permeabilization procedure was performed according to the manufacturer's protocol. 250 μ l of CYTOFIX/CYTOPERM (BD Biosciences, Franklin Lakes, NJ) solution was added per tube

15 and vortexed well. After incubating for 20 minutes at 4°C, the cells were washed twice in 1× BD PERM/WASH buffer (BD Biosciences, Franklin Lakes, NJ), 3 ml per tube. The permeabilized cells were thoroughly resuspended in 100 μ l of 1× PERM/WASH buffer (BD Biosciences, Franklin Lakes, NJ) before the Ki-67 intracellular staining. A titrated anti-Ki-67 fluorochrome-conjugated antibody was added and incubated at 4°C for 30 minutes in the dark. A test tube

20 without the isotype antibody was used as a negative control. The stained cells were washed twice with 3 ml of 1× PERM/WASH buffer (BD Biosciences, Franklin Lakes, NJ), and then resuspended in 300 μ l of the stain buffer prior to flow cytometric analysis using a FACSCANTO system (BD Biosciences, Franklin Lakes, NJ).

25 Colony Formation Assay

TC28a2 chondrocytes were used for this assay. The cells were treated with exosomes (50 μ g/million cells) collected from different weeks of the batch 3 multi-tissue organoid (MTO) culture supernatants. After 72 hours of exosome treatment in the presence and absence of TNF α (10 ng/ml), cells were counted and replated at 2,000 viable cells/well in six-well plates and

30 allowed to grow without treatment for seven days. The cells were then fixed and stained. After the seven-day period, cells were washed twice with 2 ml of 1X PBS and fixed with 400 μ l of

methanol at room temperature for 20 minutes. Cells were then stained with 1 ml of crystal violet solution (0.5% w/v crystal violet, 25% methanol) at room temperature for 40 minutes. Plates were washed with dH₂O and allowed to dry overnight.

Plates were scanned the next day and the retained dye was quantified. 1 mL of 10% acetic acid was added to each well and incubated on a shaker for one hour at room temperature. 100 µl of each extraction was then read, in triplicate, on a plate reader (BioTek Instruments, Winooski, VT) at 595 nm. 10% acetic acid as such was also read as a blank. The blank was subtracted out of all samples. Technical triplicates were averaged. The mean and standard deviation (SD) of three biological replicates were computed. The colonies were compared with the media control and TNF α -treated control wells to determine the effect of exosomes on the colony forming ability of the TC28a2 chondrocytes.

CFSE-Based Chondrocyte Proliferation Assay

Chondrocytes were labeled with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Cat# C34554, Carlsbad, CA) and were cultured with exosomes isolated from the MTO culture medium. Following four days of culture with exosomes, proliferation of the chondrocytes was analyzed by flow cytometry by enumerating the numbers of CFSE^{lo} chondrocytes and by measuring the levels of CFSE.

Mixed Lymphocyte Reaction (MLR) Culture for Treg Determination

To understand the effect of exosomes from multi-tissue organoid culture media on Treg cells, a mixed lymphocyte reaction (MLR) culture was performed as indicated in Table 9. Human PBMC responder cells were stimulated in the presence and absence of exosomes. Human PBMC responder sample (Sample ID: Human PBMC-23) was stimulated with mismatched human PBMC samples (Sample IDs: Human PBMC STC3) in the presence and absence of exosomes. Exosomes isolated from Batch 3 and Batch 4 were used at a dose of 100 µg/million cells. Activation markers and proliferation percentage (CFSE based assay) were analyzed on CD4⁺ T cells, CD8⁺ T cells, and CD20⁺ B cells. The effect of exosomes on regulatory T cell population (Treg) was studied. A minimum of 200,000 events were acquired on three-laser CANTO II (BD Biosciences, Franklin Lakes, NJ) with FACSDIVA 6.1.3 (BD Biosciences, Franklin Lakes, NJ). Relative percentages and mean fluorescence intensity (MFI) of each of the

populations were determined using the FlowJo 10.1. software (BD Biosciences, Franklin Lakes, NJ).

Table 9 – Plate Map of MLR assay

S.NO	Responder (CFSE Labeled)	Stimulator (Irradiated and labelled with VPD450)	Stimulator (Irradiated and labelled with VPD450)	Exosome (Batch 3 & 4)
1	H23_PBMCs	None (Unstimulated)	None (Unstimulated)	-
2	H23_PBMCs	PHA (Phytohaemagglutinin) (10 ug)	PHA (10 ug)	-
3	H23_PBMCs	H22_PBMCs	STC3_PBMCs	-
4	H23_PBMCs	H22_PBMCs	STC3_PBMCs	(B3-Week 11) 100 ug
5	H23_PBMCs	H22_PBMCs	STC3_PBMCs	(B3-Week 15) 100 ug
6	H23_PBMCs	H22_PBMCs	STC3_PBMCs	(B3-Week 18) 100 ug
7	H23_PBMCs	H22_PBMCs	STC3_PBMCs	(B4-Week 13) 100 ug
8	H23_PBMCs	H22_PBMCs	STC3_PBMCs	(B4-Week 16) 100 ug

5

Analysis of exosome components

Total RNA and protein from the exosomes purified from the MTO culture supernatant were isolated using Total Exosome RNA & Protein Isolation Kit (Thermo Fisher Scientific, Inc., Waltham, MA). RNA sequencing library was prepared using the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA). After library preparation, the constructed library was sequenced using Illumina HiSeq2000/2500, and the reading length was 1×50 bp. Curated reads were analyzed using the RNA analysis pipeline as described earlier (O'Grady et al., *BMC Biology* 20:72, 2022). Briefly, for genome-wide visualization of RNA-Seq coverage, reads were aligned and mapped to version GRCh38 of the human genome with STAR aligner V. 2.5.2b (Dobin et al., *Bioinformatics* 29(1):15-21, 2013) and visualized with J-Circos (An et al., *Bioinformatics* 31(9):1463-1465, 2015). For quantification, reads were first mapped to the set of NCBI RefSeq rRNA sequences using STAR aligner V. 2.5.2b (Dobin et al., *Bioinformatics* 29(1):15-21, 2013). Unmapped reads were then mapped to the GRCh38.90 human transcriptome from Ensembl (cDNA + noncoding RNA) using Salmon v.0.8.2 (Patro et al., *Nat Biotechnol* 32(5):462-464, 2014). Finally, remaining reads were once again mapped to the GRCh38.90

10

15

20

human transcriptome using Salmon v.0.8.2. Genes with an abundance level of at least 1 transcript per million (TPM) averaged across the replicates were considered to be reliably detected. To investigate differential gene expression, mRNA and lncRNA read counts were summed to the gene level using tximport and compared using DESeq2 (Love et al., *Genome Biology* 15:550, 2014).

Confocal and imaging analysis

For the evaluation of distribution and interactions between different proteins, TC28a2 cells were initially seeded in glass bottom dishes (ibidi GmbH, Gräfelfing, Germany) at a density of 1×10^5 cells per well, grown for 24 hours in complete medium. The cells were treated with exosomes (50 μ g/million cells) collected from week 10 of batch13-batch17 MTO culture supernatants. Control cells were treated with iPSC culture medium exosomes. After 24 hours of exosome treatment in the presence of TNF α (10 ng/ml), the cells were stained to look at Ki-67 expression and finally analyzed by means of confocal microscopy. The protein-protein interactions were investigated under confocal microscope (TCS SP8, Leica Camera, Hesse, Germany), and the cellular samples were processed with a standard immunofluorescence protocol: fixation with formaldehyde 3.7% for 10 minutes, permeation with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 10 minutes, block with bovine serum albumin (BSA) 2% for two hours. Ki-67 protein was directly investigated through a mouse IgG anti-Ki-67, conjugated with PE dye (Ki-67 Monoclonal Antibody, PE, eBioscience).

Expression pattern of Immunomodulatory Molecules on MTO Exosomes

Exosomes from the MTO show elevated levels of immune suppressive markers. MTO exosomes were incubated with antibodies against CD9, CD63, and CD81, known exosomal markers with immunoinhibitory potential. The level of expression of CD9 and CD81 was determined via flow cytometry and the results are presented (FIG. 13-22). Exosomes isolated from different weeks of Batch-13, Batch-14, and Batch-15 MTO culture medium show high levels of CD9, CD63, and CD81 compared to the exosomes from regular iPSC culture medium. A detailed gating strategy and expression profile of the immunoinhibitory markers such as CD9, CD63, and CD81 from different weeks of MTO exosomes are provided in FIGs. 13-22.

Dynamic Weight Bearing Assessment

Prior to testing the force sensor was cleaned lightly with 70% isopropyl alcohol and wiped clean of any debris. The lid was placed on the dynamic weight bearing chamber and the camera checked for proper alignment and adjusted as necessary. Rats were then placed into the dynamic weight bearing testing chamber and allowed to move freely for two minutes.

Approximately one minute of video was used for data analysis. Animals were assessed for amount of weight placed on the left and right hind foot (% normalized to individual body weight) and were reported as the ratio of right hind (MIA-injected)/left hind (unoperated)+right hind foot weight bearing.

The rat moniodoacetate model was used to assess the efficacy of MTO-derived exosomes for the treatment of osteoarthritis (OA). Four groups of 14 rats (7M, 7F) had osteoarthritis induced in their right femoro-tibial joint (equivalent of human knee joint). A fifth group of unoperated rats (5M, 5F) were used as normal control group. Groups 1-4 received weekly intra-articular injections into the right femoro-tibial joint beginning on day 0, consisting of saline (Group 1), 2.2×10^{11} exosomes in saline (Group 2), TCA (triamcinolone acetonide) + Tramadol (Group 3), and FGF-18 (Group 4). On Days 7, 38, and 72 rats were assessed by dynamic weight bearing analysis. In normal rats the weight bearing should be approximately equal between left and right hind limbs, which is indicated by a score of 0.50 (as in the Naïve rats in Group 5, Table 10). Scores for each rat in all five groups are reported below. Scores were categorized as Excellent if above 0.45 and Good if above 0.40. In this categorization analysis, Vehicle treated rats showed an initial good response at Day 7 but had worse scores at Day 38. In contrast, the Exosome treated group began with a worse response than saline controls at Day 7 but showed marked improvement at Day 38 and Day 72. On Day 72 the Exosome treated rats were close to the declining levels apparent in the TCA+Tramadol group (which represents the standard of care for osteoarthritis) (FIG. 30). Exosome treatment also showed a far superior therapeutic response than treatment with FGF-18 (FIG. 30), which has been shown to be highly effective in some models of osteoarthritis. FIG. 31 shows the percent of animals in each group that achieved an excellent Dynamic Weight Bearing score at any time point in the study, demonstrating a superior therapeutic effect versus saline controls. Overall, the data indicates a therapeutic effect for MTO-derived exosomes for the treatment of osteoarthritis.

Table 10. Day 7

Treatment Group	Group 1 Vehicle	Group 2 Exosomes	Group 3 TCA+Tram	Group 4 FGF18	Group 5 Naïve
Fraction of weight bearing on right (treated) hind limb	0.4449	0.4954	0.5287	0.4533	0.5593
	0.4429	0.4816	0.5180	0.4424	0.5212
	0.4409	0.4619	0.5080	0.4412	0.5140
	0.4391	0.4437	0.4977	0.4228	0.4995
	0.4388	0.4422	0.4919	0.3391	0.4958
	0.4229	0.3908	0.4916	0.3338	0.4919
	0.4076	0.3833	0.4858	0.3191	0.4825
	0.4042	0.3346	0.4672	0.2067	0.4687
	0.3764	0.3280	0.4607	0.1795	0.4599
	0.3678	0.3245	0.4520	0.1788	0.4478
	0.3419	0.3030	0.4422	0.1684	
	0.3341	0.2971	0.4330	0.1495	
	0.2756	0.2800	0.4174	0.1334	
	0.1901	0.2604	0.3944	0.1106	
Decimal fraction at or above 0.40	0.57	0.36	0.93	0.29	1.00
Percent at or above 0.40	57.14	35.71	92.86	28.57	100.00

Table 11. Day 38

Treatment Group	Group 1 Vehicle	Group 2 Exosomes	Group 3 TCA+Tram	Group 4 FGF18	Group 5 Naïve
Fraction of weight bearing on right (treated) hind limb	0.6470	0.6290	0.6845	0.3164	0.6529
	0.5193	0.5599	0.5392	0.3032	0.6034
	0.4560	0.5318	0.5317	0.3031	0.5261
	0.4380	0.5029	0.5248	0.2700	0.5096
	0.4092	0.4932	0.5073	0.2383	0.5018
	0.3863	0.4801	0.5025	0.2372	0.4984
	0.3860	0.4716	0.4897	0.1743	0.4709
	0.3628	0.4269	0.4528	0.1655	0.4467
	0.3408	0.3967	0.4430	0.1385	0.4395
	0.3324	0.3681	0.4411	0.1293	0.4156
	0.3056	0.3481	0.4410	0.1212	
	0.2811	0.1935	0.4125	0.0927	
	0.2287	0.1860	0.3646	0.0645	

	0.1981	0.1622	0.2772	0.0074	
Decimal fraction at or above 0.40	0.36	0.64	0.86	0	1.00
Percent at or above 0.40	35.71	64.29	85.71	0	100

Table 12. Day 72

Treatment Group	Group 1 Vehicle	Group 2 Exosomes	Group 3 TCA+Tram	Group 4 FGF18	Group 5 Naïve
Fraction of weight bearing on right (treated) hind limb	0.5822	0.5232	0.5230	0.4964	0.7625
	0.5297	0.5170	0.5222	0.4576	0.5655
	0.4861	0.4882	0.5041	0.4081	0.5240
	0.4297	0.4675	0.4757	0.4078	0.5198
	0.4152	0.4649	0.4631	0.4056	0.5120
	0.4016	0.4534	0.4598	0.3970	0.5093
	0.3902	0.4507	0.4541	0.3954	0.5048
	0.3829	0.4441	0.4540	0.3750	0.4687
	0.3586	0.4154	0.4426	0.3545	0.4085
	0.3448	0.3517	0.4212	0.3286	0.3873
	0.3196	0.3326	0.3743	0.3112	
	0.2873	0.2179	0.3698	0.2932	
	0.2873	0.2120	0.3476	0.2274	
	0.2524	0.1371	0.3464	0.2143	
Decimal fraction at or above 0.40	0.4286	0.6429	0.7143	0.5000	0.9000
Percent at or above 0.40	42.86	64.29	71.43	50.00	90.00

Therapeutic trial of MTO-derived exosomes in the mouse model of traumatic brain injury

- 5 On day 0, mild TBI was mechanically induced in 6 mice (3M, 3F), and in two additional mice (1M, 1F) a craniotomy was performed without TBI induction to serve as sham operated controls. Two TBI mice (1M, 1F) received IV tail vein saline injections on Days 7, 14, and 21 (sham treated TBI controls), and 4 TBI mice (2M, 2F) received 10^{10} MTO-derived exosomes in saline IV in the tail vein on Days 7, 14, and 21 (exosome treated). Barnes Maze memory
- 10 assessments were conducted on Days 26, 27, and 28. Mice were sacrificed on Day 28 and brains were fixed and processed for histopathological assessments. Data shown in FIG. 12.

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are
5 incorporated by reference in their entirety. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details
10 shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless otherwise indicated
15 to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding
20 techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective
25 testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

What is claimed is:

1. A method of preparing exosomes, the method comprising:
 - providing a multi-tissue organoid;
 - 5 culturing the multi-tissue organoid in culture medium for at least one week;
 - collecting at least a portion of the culture medium; and
 - isolating exosomes from the culture medium.
2. The method of claim 1, wherein the exosomes express at least one marker identifying the
10 exosomes as being isolated from culture medium in which a multi-tissue organoid is cultured.
3. A composition comprising exosomes that express at least one marker identifying the exosomes as being isolated from culture medium in which a multi-tissue organoid is cultured.
- 15 4. A method of treating a subject having, or at risk of having, a condition treatable with exosomes isolated from culture medium in which a multi-tissue-organoid is cultured, the method comprising:
 - administering to the subject a pharmaceutical composition comprising an effective
amount of exosomes isolated from culture medium in which a multi-tissue-organoid is cultured.
 - 20
5. The method of claim 4, wherein the condition is osteoarthritis.
6. The method of claim 4, wherein the condition is an autoimmune disease.
- 25 7. The method of claim 4, wherein the condition is an inflammatory disease.
8. The method of claim 7, wherein the inflammatory disease comprises immune-mediated inflammatory disease.
- 30 9. The method of claim 4, wherein the condition is a neurodegenerative disease.

10. The method of claim 4, wherein the condition comprises cartilage injury.
11. The method of claim 4, wherein the condition comprises traumatic brain injury.
- 5 12. The method of claim 4, wherein the condition comprises stroke.
13. The method of claim 4, wherein the condition comprises aging.
14. The method of claim 4, wherein the condition comprises mitochondrial dysfunction.
- 10 15. The method of any one of claims 4-14, wherein the pharmaceutical composition is administered before the subject manifests a symptom or clinical sign of the condition.
16. The method of claim 15, wherein the pharmaceutical composition is administered in an
- 15 amount effective to:
- reduce the likelihood that the subject develops the condition;
 - reduces the severity of symptoms or clinical signs of the condition; or
 - reduces the duration that the subject experiences symptoms or clinical signs of the
- condition;
- 20 compared to a subject to whom the pharmaceutical composition is not administered.
17. The method of any one of claims 4-14, wherein the pharmaceutical composition is administered after the subject manifests a symptom or clinical sign of the condition.
- 25 18. The method of claim 17, wherein the pharmaceutical composition is administered in an amount effective to ameliorate at least one symptom or clinical sign of the condition.

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