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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 January 2016 (21.01.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/008937 A1

(51) International Patent Classification:
A61K 35/12 (2015.01) *C12N 5/00* (2006.01)

(21) International Application Number:
PCT/EP2015/066190

(22) International Filing Date:
15 July 2015 (15.07.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
14306154.7 16 July 2014 (16.07.2014) EP

(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, F-75013 Paris (FR). **UNIVERSITE DE MONTPELLIER** [FR/FR]; 163 Rue Auguste Broussonnet, F-34090 Montpellier (FR). **CENTRE HOSPITALIER UNIVERSITAIRE DE MONTPELLIER** [FR/FR]; 191, Avenue Doyen Gaston Giraud, F-34000 Montpellier (FR).

(72) Inventors: **VIGNAIS, Marie-Luce**; Inserm U1183, IRMB, 80 avenue Augustin Fliche, F-34295 Montpellier Cedex 5 (FR). **BRONDELLO, Jean-Marc**; Inserm U1183, IRMB, 80 avenue Augustin Fliche, F-34295 Montpellier Cedex 5 (FR). **JORGENSEN, Christian**; Inserm U1183, IRMB, 80 Avenue Augustin Fliche, F-34295 Montpellier Cedex 5 (FR). **CAICEDO PALIZ, Andrés Bernardo**; Inserm U1183, IRMB, 80 avenue Augustin Fliche, F-34295 Montpellier Cedex 5 (FR).

(74) Agent: **HIRSCH, Denise**; Inserm Transfert, 7 rue Watt, F-75013 Paris (FR).

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

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

Published:

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

WO 2016/008937 A1

(54) Title: METHODS FOR THE INTERCELLULAR TRANSFER OF ISOLATED MITOCHONDRIA IN RECIPIENT CELLS

(57) Abstract: The present invention relates to methods for the intercellular transfer of isolated mitochondria in recipient cells. In particular, the present invention relates to a method for the intercellular transfer of an amount of mitochondria isolated from a population of donor cells into a population of recipient cells comprising the step of i) centrifuging the population of recipient mammalian cells in presence of the isolated mitochondria at centrifugation force ranging from 1000g to a 2000g at a temperature ranging from 1°C to 8°C and for a time ranging from 5min to 30min, ii) resting the centrifuged cells at a temperature ranging from 30°C to 40°C for a time ranging from 90min to 180min, and iii) repeating the cycling of steps i) and ii) for a sufficient number of times for reaching transfer efficiency.

METHODS FOR THE INTERCELLULAR TRANSFER OF ISOLATED MITOCHONDRIA IN RECIPIENT CELLS

FIELD OF THE INVENTION:

The present invention relates to methods for the intercellular transfer of isolated mitochondria in recipient cells.

BACKGROUND OF THE INVENTION:

Through their involvement in the central cell tasks of nutrient uptake and energy production, mitochondria are at the core of a number of biological functions and corresponding disorders [1,2]. Mitochondria are also actively involved in cancer progression and resistance to therapy [3]. Intercellular mitochondria transfer has recently been described as a phenomenon occurring both *in vitro* and *in vivo*, leading to cellular reprogramming and to phenotypes as diverse as protection against tissue injury and resistance to therapeutic agents [4-11]. A number of these mitochondria transfers were shown to originate, through the formation of nanotube structures, from mesenchymal stem/stromal cells (MSCs) [4,6-9]. These various studies clearly showed that MSC mitochondria could convey new properties to recipient cells. However, despite the obvious interest of these findings, the precise characterization of MSC mitochondria effects on the recipient cells remains partly elusive because of the lack of suitable study systems. Technical approaches to artificially transfer mitochondria from donor to recipient cells have been sought in the past. This was achieved by direct injection of mitochondria into oocytes [12,13]. The specific contribution of mitochondrial DNA (mtDNA) was also studied by preparing transmitochondrial cybrids that are the result of the fusion of enucleated cells, whose mtDNA is to be analyzed, with ρ° cells, that are deficient in mtDNA [14]. However, these techniques are complex and difficult to put into practice for large cell populations.

Tumors are complex organizations between cancer cells and stromal components. MSCs are recruited to the tumor microenvironment where they can modify cancer cell growth and metastatic potential as well as response to therapy [15-18]. In addition to the long-known cytokine-dependent communications between the stromal and cancer cells [19], current data indicate that metabolite exchange and direct cell-cell contacts also greatly contribute to these effects, through cancer cell metabolic reprogramming [2,20,21]. As we show in this manuscript,

and as recently published by others [5], MSCs can transfer mitochondria to cancer cells. This opens new routes for cancer cell metabolic reprogramming since MSCs are part of the cancer cell microenvironment, with functional consequences for tumor progression and resistance to anti-cancer drugs.

With the known contribution of mitochondria in essential biological processes, the involvement of mitochondria dysfunction in a number of diseases [1] and the current evidence that mitochondria activity can interfere with cell responses to therapeutic agents, experimental procedures that allow the manipulation of mitochondria and the study of their properties in different cell contexts are of the utmost interest. Unfortunately, up to now, these approaches were complex and time consuming.

SUMMARY OF THE INVENTION:

The present invention relates to methods for the intercellular transfer of mitochondria, isolated from donor cells, to recipient cells. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors propose here a new method that they named MitoCeption, to transfer mitochondria isolated from cell type A to cell type B. They validated this method by showing a dose-dependent transfer of mitochondria isolated from MSCs to MDA-MB-231 cancer cells and further showed the biological consequences of this transfer on cancer cell metabolism and functional properties. The added values of this novel methodology are its efficiency and quickness, as tested in different cell types, thus opening new avenues for the study of the activity of mitochondria in different cell contexts but also offer new therapeutic perspectives. The MitoCeption technique, by allowing the manipulation of the mitochondrial pool, will therefore lead not only to the understanding of the mitochondria functions but also to a reappraisal of their possible use as therapeutic targets [22].

Accordingly, an aspect of the present invention relates to a method for the intercellular transfer of an amount of mitochondria isolated from a population of donor cells into a population of recipient cells comprising the step of i) centrifuging the population of recipient mammalian cells in presence of the isolated mitochondria at centrifugation force ranging from 1000g to a 2000g at a temperature ranging from 1°C to 8°C and for a time ranging from 5min

to 30min, ii) resting the centrifuged cells at a temperature ranging from 30°C to 40°C for a time ranging from 90min to 180min, and iii) repeating the cycling of steps i) and ii) for a sufficient number of times for reaching transfer efficiency.

As used herein, the term "donor cell" refers to a cell from which the mitochondria of the invention are isolated.

The terms "recipient cell", "acceptor cell" and "host cell" are interchangeably used herein to describe a cell receiving and encompassing the isolated mitochondria.

In some embodiments, the donor cells and the recipient cells may be different or identical. In some embodiments, the donor cells and the recipient cells come from different or the same species. In some embodiments, the donor cells and the recipient cells come from different or the same tissues.

In some embodiments, the cells (i.e. donor cells and recipient cells) are mammalian cells. In some embodiments, the cells are isolated from a mammalian subject who is selected from a group consisting of: a human, a horse, a dog, a cat, a mouse, a rat, a cow and a sheep. In some embodiments, the cells are human cells. In some embodiments, the cells are cells in culture. The cells may be obtained directly from a mammal (preferably human), or from a commercial source, or from tissue, or in the form for instance of cultured cells, prepared on site or purchased from a commercial cell source and the like. The cells may come from any organ including but not limited to the blood or lymph system, from muscles, any organ, gland, the skin, brain... In some embodiments, the cells are selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, hepatocytes, B-cells, T-cells, erythrocytes, macrophages, monocytes, fibroblasts, muscle cells, vascular smooth muscle cells, hepatocytes, splenocytes, pancreatic β cells…

In some embodiments, the cells are cancer cells. Typically, the cancer cells are isolated from a cancer selected from the group consisting of breast cancer, prostate cancer, lymphoma, skin cancer, pancreatic cancer, colon cancer, melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head-neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma, lung carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma,

testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic insulinoma, malignant carcinoid carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia vera, essential thrombocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, soft-tissue sarcoma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma.

In some embodiment, the cells are stem cells. As used herein, the term "stem cell" refers to an undifferentiated cell that can be induced to proliferate. The stem cell is capable of self-maintenance or self-renewal, meaning that with each cell division, one daughter cell will also be a stem cell. Stem cells can be obtained from embryonic, post-natal, juvenile, or adult tissue. Stem cells can be pluripotent or multipotent. The term "progenitor cell," as used herein, refers to an undifferentiated cell derived from a stem cell, and is not itself a stem cell. Some progenitor cells can produce progeny that are capable of differentiating into more than one cell type. Stem cells include pluripotent stem cells, which can form cells of any of the body's tissue lineages: mesoderm, endoderm and ectoderm. Therefore, for example, stem cells can be selected from a human embryonic stem (ES) cell; a human inner cell mass (ICM)/epiblast cell; a human primitive ectoderm cell, a human primitive endoderm cell; a human primitive mesoderm cell; and a human primordial germ (EG) cell. Stem cells also include multipotent stem cells, which can form multiple cell lineages that constitute an entire tissue or tissues, such as but not limited to hematopoietic stem cells or neural precursor cells. Stem cells also include totipotent stem cells, which can form an entire organism. In some embodiment, the stem cell is a mesenchymal stem cell. The term "mesenchymal stem cell" or "MSC" is used interchangeably for adult cells which are not terminally differentiated, which can divide to yield cells that are either stem cells, or which, irreversibly differentiate to give rise to cells of a mesenchymal cell lineage, e.g., adipose, osseous, cartilaginous, elastic and fibrous connective tissues, myoblasts) as well as to tissues other than those originating in the embryonic mesoderm (e.g., neural cells) depending upon various influences from bioactive factors such as cytokines. In some embodiments, the stem cell is a partially differentiated or differentiating cell. In some embodiments, the stem cell

is an induced pluripotent stem cell (iPSC), which has been reprogrammed or de-differentiated. Stem cells can be obtained from embryonic, fetal or adult tissues.

As used herein, the term "isolated mitochondria" refers to mitochondria separated from other cellular components of the donor cells.

Typically, the isolated mitochondria are functional mitochondria or dysfunctional (i.e. in opposition to functional) mitochondria. As used herein, the term "functional mitochondria" refers to mitochondria that consume oxygen. In some embodiments, functional mitochondria have an intact outer membrane. In some embodiments, functional mitochondria are intact mitochondria. In another embodiment, functional mitochondria consume oxygen at an increasing rate over time. In another embodiment, the functionality of mitochondria is measured by oxygen consumption. In another embodiment, oxygen consumption of mitochondria may be measured by any method known in the art such as, but not limited to, the MitoXpress fluorescence probe (Luxcel). In some embodiments, functional mitochondria are mitochondria which display an increase in the rate of oxygen consumption in the presence of ADP and a substrate such as, but not limited to, glutamate, malate or succinate. Each possibility represents a separate embodiment of the present invention. In another embodiment, functional mitochondria are mitochondria that produce ATP. In another embodiment, functional mitochondria are mitochondria capable of manufacturing their own RNAs and proteins and are self-reproducing structures. In another embodiment, functional mitochondria produce a mitochondrial ribosome and mitochondrial tRNA molecules. As used herein, the term "a mitochondrial membrane" refers to a mitochondrial membrane selected from the group consisting of: the mitochondrial inner membrane, the mitochondrial outer membrane or a combination thereof. As used herein, the term "intact mitochondria" refers to mitochondria comprising an outer and an inner membrane, an inter-membrane space, the cristae (formed by the inner membrane) and the matrix. In another embodiment, intact mitochondria comprise mitochondrial DNA. In another embodiment, intact mitochondria contain active respiratory chain complexes I-V embedded in the inner membrane. In another embodiment, intact mitochondria consume oxygen. As used herein, the term "mitoplasts" refers to mitochondria devoid of outer membrane. In some embodiments, intactness of a mitochondrial membrane may be determined by any method known in the art. In a non-limiting example, intactness of a mitochondrial membrane is measured using the tetramethylrhodamine methyl ester (TMRM) or the tetramethylrhodamine ethyl ester (TMRE) fluorescent probes. Each possibility represents

a separate embodiment of the present invention. Mitochondria that were observed under a microscope and show TMRM or TMRE staining have an intact mitochondrial outer membrane.

In some embodiments, the isolated mitochondria are modified mitochondria. As used herein, the term "modified mitochondria" refers to mitochondria harboring at least one modification in their composition. Typically, modified mitochondria refer to mitochondria isolated from a genetically modified source. As used herein, a genetic modified source refers to a cell harboring a foreign gene or foreign gene product. In some embodiments, the cells from which the modified mitochondria are derived are transfected with DNA comprising an expression cassette. An "expression cassette" refers to a natural or recombinantly produced polynucleotide that is capable of expressing a desired gene(s). The term "recombinant" as used herein refers to a polynucleotide molecule that is comprised of segments of polynucleotides joined together by means of molecular biology techniques. The cassette contains the coding region of the gene of interest along with any other sequences that affect expression of the gene. A DNA expression cassette typically includes a promoter (allowing transcription initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include, but is not limited to, transcriptional enhancers, non-coding sequences, splicing signals, transcription termination signals, and polyadenylation signals. An RNA expression cassette may include a translation initiation codon (allowing translation initiation) and a sequence encoding one or more proteins.

Preparation of isolated mitochondria may be done by any method well known in the art. Typically preparation of isolated mitochondria requires changing buffer composition or additional washing steps, cleaning cycles, centrifugation cycles or even sonication cycles. The mitochondria according to the invention may be obtained by methods disclosed herein or by any other method known in the art. Commercially available mitochondria isolation kits include, for example, Mitochondria Isolation Kit, MITOISOl (Sigma- Aldrich) and Pierce Mitochondria Isolation Kit for Cultured Cells – (Thermo Fisher Scientific), among others. In some embodiments, the mitochondria have been isolated by centrifugation. In other embodiments, the mitochondria have been isolated by mitochondrial membrane potential-dependent cell sorting. In some embodiments, the preparation of isolated mitochondria does not contain intact cells. In some embodiments, the preparation does not comprise mitochondrial clumps or aggregates or cellular debris or components larger than 5 μ m. In some embodiments, the

preparation is devoid of particulate matter greater than 5 μ m. As used herein, the term "particulate matter" refers to intact cells, cell debris, aggregates of mitochondria, aggregates of cellular debris or a combination thereof. Typically, the mitochondria preparation is performed on ice to maintain their integrity.

In some embodiments, the mitochondria harbour a tracking probe. Typically, the tracking probe is a fluorescent mitochondrial tracking probe to mitochondria. Alternatively the tracking probe is selected from the group consisting of a non-oxidation dependent probe, an accumulation dependent probe, or a reduced oxidative state probe. In some embodiments, the probe is a MitoTracker Probe selected from the group consisting of MitoTracker Orange CMTMRos, MitoTracker Orange CM-H2TMRos, MitoTracker Red CMXRos, MitoTracker Red CM-H2XRos, MitoTracker Red 580, and MitoTracker Deep Red 633. The tracking probe is very suitable for sorting the mitochondria based upon binding of the tracking probe, for determining the percentage of functional mitochondria based on the percentage of mitochondria which bind the tracking probe, and/or for following and quantifying the rate and efficacy of the mitochondria transfer.

Typically, recipient cells are placed in an appropriate carried medium. As used throughout this application, the term "carrier medium" is a fluid carrier such as cell culture media, cell growth media, buffer which provides sustenance to the cells. The carrier medium can be refreshed and/or removed as needed. It should be noted that this invention is preferably operated without the presence of proteases. In some embodiments, a protease inhibitor may be present in the cell culture chamber.

In some embodiments, the volume of isolated mitochondria is added to the recipient cells at the desired concentration. Typical the ratio is 0.12; 0.25; 0.5; 1; or 2. These values represent the ratio of the number of mitochondria donor cells versus the number of mitochondria recipient cells.

The centrifugation step may be performed with any centrifugation system well known in the art. Typically, the centrifugation force is 1500g.

In some embodiments, the centrifugation step is performed at a temperature ranging from 1°C to 8°C. In some embodiments, the centrifugation step is performed at a temperature

of is 1; 1,1; 1,2; 1,3; 1,4; 1,5; 1,6; 1,7; 1,8; 1,9; 2; 2,1; 2,2; 2,3; 2,4; 2,5; 2,6; 2,7; 2,8; 2,9; 3; 3,1; 3,2; 3,3; 3,4; 3,5; 3,6; 3,7; 3,8; 3,9; 4; 4,1; 4,2; 4,3; 4,4; 4,5; 4,6; 4,7; 4,8; 4,9; 5; 5,1; 5,2; 5,3; 5,4; 5,5; 5,6; 5,7; 5,8; 5,9; 6; 6,1; 6,2; 6,3; 6,4; 6,5; 6,6; 6,7; 6,8; 6,9; 7; 7,1; 7,2 ; 7,3; 7,4; 7,5; 7,6; 7,7; 7,8; 7,9; or 8 °C. Typically, the centrifugation step is performed at a temperature of 4°C.

In some embodiments, the centrifugation step is performed for a time ranging from 5min to 30min. In some embodiments, the centrifugation step is performed for 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20; 21; 22; 23; 24; 25; 26; 27; 28; 29; or 30min. Typically, the centrifugation step is performed for 15min.

In some embodiments, the resting step is performed at a temperature ranging from 30°C to 40°C. In some embodiments, the centrifugation step is performed at a temperature of is performed at a temperature of 30; 31; 32; 33; 34; 35; 36; 37; 38; 39; or 40 °C. Typically, the resting step is performed at a temperature of 37°C.

In some embodiments, the resting step is performed for a time ranging from 30 min to 180 min. In some embodiments, the centrifugation step is performed for 30; 40; 50; 60; 70; 80; 90; 100; 110; 120; 130; 140; 150; 160; 170; or 180min. Typically, the centrifugation step is performed for 120 min (i.e. 2h).

In some embodiments, the cycle of step i) (i.e. centrifugation step) and step ii) (i.e. resting step), is performed at least 1; 2; 3; 4; 5; 6; 7; 8; 9; 10 times. The skilled artisan is able to determine when the transfer efficiency is reached by any method well known in the art. For example detection and quantification of tracking probe are performed. In some embodiments, functional assays may also be performed to determine in which manner the transfer of mitochondria occurred. For example, detection and quantification of the mitochondrial mtDNA may be performed by any method well known in the art and typically involve PCR. Functional assays may also include metabolic assays. For example, the assay is based on the differential measurement of biomarkers associated with changes in cell membrane integrity and cellular ATP levels. Typically, the assay is performed in a single-well, with bioluminescent and fluorescent readouts. Bioluminescent signal is proportional to ATP concentration. Other examples include the citrate synthase assay. Citrate synthase is indeed the initial enzyme of the tricarboxylic acid (TCA) cycle. This enzyme is an exclusive marker of the mitochondrial matrix

and catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid and CoA with a thiol group (CoA-SH). A colorimetric assay can thus be based on the reaction between 5', 5'-Dithiobis 2-nitrobenzoic acid (DTNB) and CoA-SH to form TNB, which exhibits maximum absorbance at 412 nm. The intensity of the absorbance is proportional to the citrate synthase activity.

The method of the present invention may find various applications.

For instance the method of the invention may be suitable for improving energy metabolism of cells obtained from donors (e.g. cells harbouring dysfunctional mitochondria, cells harbouring mutated mtDNA…). Thus, transfer of exogenous mitochondria to target cells may lead to subsequent repopulation of cells in which failure of mitochondrial function occurred as a result of inherited defect or progression of disease process or aging. Thus, direct transfer of exogenous functional mitochondria into the cells provides a new therapeutic approach permitting changes in the bioenergetic profile of recipient cells affected with mitochondrial dysfunction, consequently leading to alleviation of defects in energy production (ATP) presented e.g. in genetically inherited mitochondrial diseases. Other functional assays include cellular bioenergetic assay performed with any appropriate system (e.g. Seahorse Extracellular Flux (XF) Analyzer). Typically such an assay provides a non-invasive profile of the metabolic activity of the cells in minutes, offering a physiologic cell based assay for determination of basal oxygen consumption, glycolysis rates, ATP turnover and respiratory capacity in a single experiment to assess mitochondrial function. The assay can also measure fatty acid oxidation and metabolism of glucose and amino acids for kinetic metabolic information. Other functional assays may also consist in determining the capability of the recipient cells to proliferate or migrate.

Accordingly the invention provides a method of treating a condition which benefits from increased mitochondrial function in a subject in need thereof, said method comprising preparing a population of recipient cells by the transfer method as above described and administered the subject with a therapeutically effective amount of the prepared recipient cells.

In some embodiments, a condition that benefits from increased mitochondrial function is a disease or disorder associated with nonfunctional or dysfunctional mitochondria. As used herein, "a disease or disorder associated with nonfunctional or dysfunctional mitochondria" is

a disease or disorder that is caused by or is aggravated by mitochondria that are not functioning as healthy mitochondria or are not functioning at all or are structurally impaired. In some embodiments, the disease or disorder associated with nonfunctional or dysfunctional mitochondria is selected from the group consisting of: a mitochondrial disease caused by ageing, a mitochondrial disease caused by damage to mtDNA, a mitochondrial disease caused by damage to nuclear genes and a mitochondrial disease caused by a toxin. In some embodiments, the damage is selected from the group consisting of: mutation, deletion, truncation, cross-linking and a combination thereof. Non limiting examples of a disease or disorder associated with nonfunctional or dysfunctional mitochondria include Diabetes mellitus and deafness (DAD), Leber's hereditary optic neuropathy (LHON), visual loss beginning in young adulthood, eye disorder characterized by progressive loss of central vision due to degeneration of the optic nerves and retina, Wolff-Parkinson-White syndrome, multiple sclerosis-type disease, Leigh syndrome, subacute sclerosing encephalopathy, neuropathy, ataxia, retinitis pigmentosa, ptosis, dementia, myoneurogenic gastrointestinal encephalopathy (MNGIE), gastrointestinal pseudoobstruction, myoclonic epilepsy with ragged red fibers (MER F), short stature, hearing loss, lactic acidosis, mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptoms (MELAS) and mitochondrial neurogastrointestinal encephalomyopathy.

In some embodiments, the functional mitochondria are derived from the subject in need thereof. In some embodiments, the functional mitochondria are derived from a different subject than the subject in need thereof. In some embodiments, the functional mitochondria are derived from the same subject to whom they are administered. In some embodiments, the functional mitochondria are derived from a different subject than the subject to whom they are administered. In some embodiments, the functional mitochondria of the invention are from a source selected from autologous, allogeneic and xenogeneic. As used herein, mitochondria of an autologous source refer to mitochondria derived from the same subject to be treated. As used herein, mitochondria of an allogeneic source refer to mitochondria derived from a different subject than the subject to be treated from the same species. As used herein, mitochondria of a xenogeneic source refer to mitochondria derived from a different subject than the subject to be treated from a different species. In some embodiments, the functional mitochondria of the invention are derived from a donor. In some embodiments, the donor is an allogeneic donor. In some embodiments, the donor is an autologous donor.

In some embodiments, the functional mitochondria of the invention comprise at least one protein, or a gene encoding at least one protein, capable of inhibiting, ameliorating or preventing said disease or disorder associated with nonfunctional or dysfunctional mitochondria.

The transfer method of the present invention is also particularly suitable in regenerative medicine, and also for preparing recipients cells (e.g. mesenchymal stem cells) that can be used for reducing inflammation or limiting the impact of ageing.

The term "therapeutically effective amount" as used herein refers to the amount of composition of the invention effective to treat or ameliorate a condition that benefits from increased mitochondrial function in a subject in need thereof.

The term "subject in need thereof, as used herein, refers to a subject afflicted with, or at a risk of being afflicted with, a condition which benefits from increased mitochondrial function. In some embodiments, "a subject in need thereof is a subject afflicted with a condition which may benefit from pro-apoptotic activity. In a non-limiting example, a condition that may benefit from pro-apoptotic activity is cancer. In some embodiments, a subject in need thereof is mammalian. In some embodiments, a subject in need thereof is human. In some embodiments, a subject in need thereof is selected from the group consisting of: a human, a horse, a dog, a cat, a mouse, a rat, a cow and a sheep.

Any suitable route of administration to a subject may be used including but not limited to topical and systemic routes. In some embodiments, the cells are administered systematically. In some embodiments, the cells are formulated for systemic administration. In some embodiments, administration systemically is through a parenteral route. In some embodiments, compositions of cells for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions, each representing a separate embodiment of the present invention. Non-limiting examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate.

In some embodiments, the transfer method of the present invention is also suitable for screening purposes. In particular, the recipient cells as prepared by the transfer method of the

present invention may be contacted by test substances and the ability of the test substances to improve or alter the mitochondrial function of the cells may be determined. In some embodiments, donor cells are mesenchymal stem cells and recipient cells are cancer cells so that the test substance may be tested for its ability to kill the cancer recipient cells by e.g. inducing apoptosis in said cells. Therefore it is possible to mimic the physiopathological situation wherein mesenchymal stem cells of the tumoral microenvironment modify cancer cell growth and metastatic potential as well as response to therapy. In some embodiments, the recipient cells may receive dysfunctional mitochondria so that substances can be tested for their capacity to restore a mitochondrial function or limit the mitochondrial dysfunction.

The transfer method of the present invention is also particularly suitable for improving protocols for differentiating cells in IPS (induced pluripotent stem cells).

The transfer method of the present invention is also particularly suitable for research purposes. For example, the transfer method of the present invention is suitable for studying embryogenesis by e.g. transferring mitochondria into oocytes or embryonic stem cells.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Exchange of mitochondria between hMSCs and MDA-MB-231 cancer cells. **(A)** Coculture (24h) of hMSCs (prestained with red MitoTracker) and MDA-MB-231 cells (prestained with green CellTracker). Upper panel, fluorescence and phase contrast (scale bar, 50 μm). MitoTracker stained mitochondria in the MSC protusion are indicated by arrows. Lower panels, 3D reconstructions of stacks of confocal images (scale bar, 20 μm). **(B)** FACS analysis of the transfer of mitochondria from hMSCs to MDA-MB-231 cancer cells. The coculture was performed for 24 hours with MitoTracker-prelabeled MSCs and unlabeled MDA-MB-231 cells.

Figure 2. Cell transfer of isolated mitochondria by MitoCeption. **(A)** Timeline for the generation of MitoCepted cells. **B (1/2)** Imaging of MSC mitochondria after MitoCeption to MDA-MB-231 cells. Prior to isolation, MSC mitochondria were labeled (red MitoTracker in upper panels, green MitoTracker in lower panels) and transferred by MitoCeption into MDA-MB-231 cells labeled beforehand with green CellTracker (upper panels) and red MitoTracker (lower panels). 3D reconstructions of confocal image stacks are shown, with opaque (left) and transparent (right) views. Scale bars, 10 μm (upper panels), 5 μm (lower panels). **B (2/2)** MDA-MB-231 cell imaging after the transfer by MitoCeption of mitochondria isolated from MSCs to MDA-MB-231 cells. Red Mito Tracker-labeled MSC mitochondria were transferred to MDA-MB-231 cells prelabeled with a green CellTracker. (a) 2D view of the culture after the mitochondria transfer. (b-h) Stacks of confocal images were made on the cell shown in (a) with a multiphoton microscope. (b-d) A 3D reconstruction of the cell was made. Shown are the cell isosurface views (Imaris) (b), with either a xy plane section (c) or yz plane section (d), scales 5μm. (e-h) Four individual confocal sections are shown, with the corresponding orthogonal views, starting at 0μm (cell bottom) and spanning the cell height. The level of each confocal image is shown on the corresponding orthogonal views. Scale bars, 5μm. **(C)** FACS analysis of MDA-MB-231 cells MitoCepted with increasing amounts of MitoTracker-labeled mitochondria isolated from MSCs. Indicated are the ratios of the numbers of mitochondria "donor" cells over "recipient" cells. Plot of the MFI values measured in MDA-MB-231 cells after the transfer of MSC mitochondria (n=8) (3 MSC donors). **(D)** Quantification of mitochondrial DNA of MSC origin (upper panel, n=6), MDA origin (lower panel, n=6) and total mtDNA (lower panel, n=7) in MDA-MB-231 cells after acquisition of MSC mitochondria (3 MSC donors). Relative values are plotted. In the lower panel, values are relative to the mtDNA concentrations found in MDA-MB-231 cells without MSC mitochondria.

Figure 3. Effects of MSC mitochondria on MDA-MB-231 metabolism and functional capacities. **(A,B)** XF-24 Extracellular Flux analysis. All measures were performed 48 hours after the transfer of MSC mitochondria to MDA-MB-231 cells. **(A)** OXPHOS activity. Oxygen consumption rates (OCR, pMoles/min) for the control (red) or the MDA-MB-231 cells MitoCepted with 0.12 (blue), 0.25 (purple) and 0.5 (pink) MSC mitochondria (relative amounts) were measured during 4 min in basal conditions and after the addition of the mitochondrial inhibitors: oligomycin, FCCP, antimycin A and rotenone. Measurements were performed in quadruplicates in 6 different experiments (2 MSC donors). One of the 6 experiments taken into account in the graphs is represented. The basal and maximal

mitochondrial respiration rates were calculated and expressed as fold of the control MDA-MB-231 cells. Values are shown as mean ± S.E.M. **(B)** Glycolysis. Extracellular acidification rates (ECAR) were measured in basal conditions and after the addition of glucose and oligomycin. Measurements were performed in quadruplicates in 3 different experiments (2 MSC donors). **(C)** ATP measurement. The level of total ATP in MDA-MB-231 cells MitoCepted with increasing quantities of MSC mitochondria was measured by a chemoluminescent assay. Values were calculated as mean ± S.E.M (n = 8) of Relative Luciferase Units (RLU) and expressed as fold of the control MDA-MB-231 cells. **(D)** Effects of MitoCepted MSC mitochondria on MDA-MB-231 proliferative and invasive capacities. MDA-MB-231 cells were MitoCepted with different amounts of mitochondria isolated from MSCs and tested the following day. Left panel: cell invasion assay. Cells were seeded in a 3D-collagen matrix and let to migrate for 72 hours. The invasion index represents the weighted migration, it is based on the number of cells that migrated at 25 and 50 μm (n= 20, 2 MSC donors). Right panel: proliferation assay. Cell were seeded (in quadruplicates) and counted 5 days later (n = 6, 3 MSC donors). Values are normalized to cell numbers found for the MDA-MB-231 cells without MSC mitochondria.

Figure 4: A dot plot representation of the MFI values obtained for the "no centrifugation" and "centrifugation" conditions, with the mean values and standard deviations (SD) indicated.

Figure 5: A dot plot representation of the MFI values obtained for the "none", "one" and "two" centrifugation conditions, with the mean values and standard deviations (SD) indicated.

Figure 6 Selected images from 3D-collagen cocultures of CellTracker stained MSCs (red) and MDA-MB-231 cancer cells (green) analyzed by real-time confocal imaging. (a) Cells displayed highly dynamic movements and were found to make physical contacts that could last for several hours. **(b)** During the time-lapse imaging, starting 24 hours after the beginning of the coculture, transfer of MSC cell components (marked by CellTracker vital dye) was observed at the early time-points (T0 to T8). Interestingly, the MDA-MB-231 cell with MSC cell components (indicated by the arrow) demonstrated a high migration capacity within the 3D collagen matrix in the 24 hours following the transfer.

Figure 7. Transfer of hMSC mitochondria to murine cancer cells in coculture. After the coculture of murine TSA-pc cancer cells with human MSCs (prestained with red MitoTracker), human mitochondria were stained with the Ab-2 antibody. In the overlay, murine cells are identified by their specific Hoechst staining pattern (scale bar, 20 μm). MSCs were observed to make long cell protrusions containing mitochondria (lower panel).

Figure 8. FACS quantification of the mitochondria transfer between hMSCs and MDA-MB-231 cancer cells. The coculture was performed with one cell type prelabeled with a MitoTracker and the other cell type unlabeled. The coculture was performed for 24 hours. As a negative control, mixing the two cell types immediately prior to the FACS analysis did not lead to a shift of the MDA-MB-231 cell population, nor did the incubation of the MDA-MB-231 cells with a conditioned medium of the MitoTracker-stained MSCs (not shown).

Figure 9. MSC mitochondria acquired through MitoCeption have the capacity to transfer to cancer cells in coculture. To compare the properties of transferred versus endogenous mitochondria, we transferred mitochondria isolated from MSCs to other MSCs and asked whether these novel mitochondria also demonstrated the capacity to transfer to MDA-MB-231 cells in coculture conditions. For this purpose, red MitoTracker stained MSC mitochondria were transferred, through the MitoCeption protocol, to MSCs prestained with a green MitoTracker. After the coculture (24h) between these MSCs and MDA-MB-231 cancer cells, red MitoTracker-labeled MSC mitochondria were observed spread throughout the MSC mitochondria network, thus validating the MitoCeption protocol. Furthermore, as the endogenous MSC mitochondria, the exogenous MSC mitochondria demonstrated the capacity to transfer to neighboring MDA-MB-231 cells. A confocal section (top panel) and 3D reconstructions of confocal image stacks (lower panels) are shown. Scale bars, 10 μm.

Figure 10. Quantification of the amount of transferred MSC relative to the endogenous MDA-MB-231 mitochondria. MSCs and MDA-MB-231 cancer cells were MitoTracker labeled at day 1. At day 2, the MitoTracker labeled MSCs were incubated with unlabeled MDA-MB-231 cancer cells. Alternatively, mitochondria were isolated from the MitoTracker labeled MSCs and MitoCepted to unlabeled MDA-MB-231 cancer cells. At day 3, cell cultures were stopped and MDA-MB-231 cancer cells were analyzed by FACS for the MitoTracker staining resulting from either (1) the initial MitoTracker labeling, (2) the coculture

with the labeled MSCs or (3) the MitoCeption with MitoTracker labeled MSC mitochondria. The ratios of the values obtained in conditions (2) versus (1) and (3) versus (1) were calculated. They are indicative of MSC mitochondrial mass relative to the endogenous MDA-MB-231 mitochondrial mass (%) following MSC mitochondria acquisition by MDA-MB-231 cells.

EXAMPLES:

EXAMPLE 1: QUANTITATIVE MITOCEPTION AS A TOOL TO ASSESS MITOCHONDRIA EFFECTS ON CELL METABOLISM AND FUNCTIONS.

Methods:

Cell culture

Human MSCs were isolated from bone marrow aspirates from three healthy donors, each of whom gave informed consent. All the isolation and culture procedures were conducted in the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. The cells were grown in Minimum Essential Eagle Medium alpha (αMEM) supplemented with glutamine and FCS 10% and used at an early passage. Cancer cells (MDA-MB-231 and TSA-pc) were grown in DMEM supplemented with glutamine and FCS 10%. The cocultures were performed in DMEM/FCS 5% with MSCs seeded 24 hours before the addition of the cancer cells. When indicated, MSCs were MitoTracker labeled the day before. All cells were trypsinized without EDTA in order to prevent membrane damage and MitoTracker leakage. When indicated, cells were stained with the green CellTracker CMFDA or the red CellTracker CMTPX (Molecular Probes). For intracellular mitochondria staining, both the MitoTracker green FM and the MitoTracker Red CMXRos (Molecular Probes) were used.

Mitochondria isolation

Mitochondria were prepared using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) with the following the manufacturer's instructions. To obtain mitochondria preparations with reduced contamination from other cytosol compounds, centrifugation for the recovery of mitochondria was performed at 3,000*g* for 15 minutes.

Mitochondrial artificial transfer

General considerations:

1. In order to follow and quantify the rate and efficacy of the mitochondria transfer, mitochondria can be MitoTracker labeled beforehand in the donor cells. Likewise, the recipient cells can also be labeled (CellTracker) beforehand if cells are to be analyzed by microscopy after the mitochondria transfer. Once the protocol has been validated by the user with his specific cells, mitochondria labeling can be left out, allowing a wider range of possible experiments with the MitoCepted cells. (All remarks in italics in this protocol are related to fluorescent labeling of cells or mitochondria).

2. Of great importance, cell exposure to EDTA should be avoided at all steps of the protocol. Therefore cell trypsination is recommanded with trypsin and no EDTA; the cocktail of protease inhibitors should, as well, be devoid of EDTA.

3. The mitochondria preparation should be performed on ice to maintain their integrity. We set up a protocol for mitochondria transfer (MitoCeption) that relies on the centrifugation of the mitochondria suspension on the cultured cells at the adequate centrifugation force, with a number of centrifugations that can be adjusted as a function of the system of mitochondria donor/recipient cells.

Day 1 : MSC and MDA-MB-231 cultures

Seed 100 000 human MSCs per 35 mm-wells (6-well plate).

Prepare extra wells for FACS controls of the transfer (Mitotracker stained MSC mitochondria) or quantification of MSC mtDNA in the target cells after the transfer.

Optional: label MDA-MB-231 cells with CellTracker (5-chloromethyl-fluorescein diacetate)

Day 2 : MSC mitochondria labeling (MitoTracker)

MSC MitoTracker Labeling (protect cells from light using an aluminum foil)

Rinse MSC cells with PBS (1ml) and add 750 μl DMEM/FCS 1%.

Dilute the MitoTracker stock solution in DMEM/FCS 1% (concentration for MitoTracker green (5-chloromethyl-fluorescein diacetate) 1 μM).

Take 250 μl of the MitoTracker dilution and add it to the cells (final MitoTracker concentration: 250 nm in 1 ml). Incubate for 1h at 37°C.

Remove the MitoTraker solution, rinse cells with 1 ml of PBS and add 2 ml of DMEM/FCS 5%. Perform the medium changes every 2h, 3 times.

Seed 100 000 MDA-MB-231 cells in 22 mm-wells (12-well plate, P12).

Optional: cells can be prestained with a green CellTracker (protect cells from light).

Day 3 : Mitochondria Isolation and MitoCeption

Adjust centrifuge temperature to 4°C.

Trypsinize and count MDA-MB-231 cells from a control well of the 12-well plate (for adjustment of the amount of isolated mitochondria to add in the MitoCeption protocol, expected: 110-150 000 MDA-MB-231 cells).

In case MSCs have been MitoTracker labeled, check by FACS the rate of MitoTracker mitochondria staining. Detach MSCs with trypsin (no EDTA). Resuspend cells in 5 ml final of αMEM/FCS 10%, centrifuge at 900g (1300 rpm) for 5 min, and add 300 µl of PBS/FCS 10% to the cell pellet.

Prepare the reagents for 2 extractions minimum (Fisher mitochondria isolation kit, reference 1057-9663), following manufacturer instructions. Number of cells to be used in one extraction: 4 to 10.10^6. Use protease inhibitors without EDTA.

Wash MSCs with 2ml of PBS and add 300 µl of trypsin (no EDTA). Incubate cells for 5 min at 37°C. Recover the cells of each well by adding 1ml of αMEM/FCS 10%, 3 times (final volume: 6 ml).

Centrifuge cells at 900 g (1300 rpm) for 5 min

Discard the supernatant and collect all pellets in 20 ml αMEM/FCS 5%, in a 50 ml Falcon tube.

Count cells, take the corresponding cell volume to perform the 2 extractions, putting cells in 2 independent 15 ml Falcon tubes.

In case MSCs were green MitoTracker labeled, plate $6x10^5$ cells in one well of a P12 for control FACS analysis performed at the same time as the MitoCepted cell samples.

Centrifuge the Falcon tubes at 900 g for 5 min, remove the supernatant, leaving roughly 1 ml of medium in each tube, resuspend the cell pellets and transfer cells of each Falcon to a 2ml Eppendorf tube.

Centrifuge the Eppendorf tubes at 900 g for 5 min, at 4°C.

Remove all residual media from the tube, it could affect mitochondrial isolation. Be careful not to let the cells dry. (It may be useful to centrifuge tubes for a few seconds to eliminate excess liquid.)

Add 200 µl of Mitochondria Isolation Reagent A. Vortex at medium speed for 5 seconds and let tubes on ice for exactly 2 minutes. Note: Do not exceed 2 minute incubation.

Add 2.5 µl of Mitochondria Isolation Reagent B. Vortex at maximum speed for 10 seconds, then let tubes on ice.

Start timer for 5 minutes, vortexing at maximum speed every 30 seconds for 10 seconds. Put tubes on ice in the meantime.

Add 200 µl of Mitochondria Isolation Reagent C. Shake the tubes strongly by hand (roughly 30 times) (do not vortex).

Centrifuge tube at 700 g for 10 minutes at 4°C.

Transfer the supernatants to new 2 ml Ependorf tubes. Centrifuge at 3,000g (15 min at 4°C) to get the mitochondria pellets.

In the meantime, change the medium of the mitochondria recipient cells in the 12-well culture plate. Final: 3 ml of DMEM/FCS 5%, in each well.

Discard the supernatant of the mitochondria preparation. The pellet contains the isolated mitochondria.

Add 1 ml of DMEM/FCS 5% to each mitochondria pellet (the volume needs to be adjusted as a function of the number of cells used for the mitochondria isolation).

Add the volume of isolated mitochondria to the non labeled MDA-MB-231 cells at the desired concentration. Typical range would be 0. 12 ; 0.25 ; 0.5 ; 1 ; 2. These values represent the ratio in the number of cells from which mitochondria are isolated to the number of cells used in the MitoCeption assay.

Add the mitochondria slowly, close to the bottom of the well, covering all the surface at least once, use clock-wise movements. Be careful thereafter to move the culture plates smoothly, avoiding shocks.

Centrifuge the 12-well plates containing the mitochondria recipient cells and the seeded mitochondria at 1,500 g for 15 min at 4°C.

Cover the plates with aluminum foil, and place them in the 37°C cell incubator immediately after centrifugation.

If needed, two hours later, remove the plates from the 37°C incubator and centrifuge them another time at 4°C for 15 min.

Day 4: Cell analysis

Evaluation of the efficacy of the mitochondria transfer on the basis of MSC mtDNA concentration in the cancer cells.

If donor cell mitochondria were MitoTracker-labeled beforehand, MitoCeption efficacy can be checked by FACS (trypsin with no EDTA).

Biological characterization (mitochondrial activity, proliferation, invasion).

Metabolic measurements, Extracellular Flux Analysis

The XF24 Flux analyzer (SeaHorse Bioscience) was used to measure oxygen consumption rates (OCR) on 100,000 MDA-231 cells placed in XF media (nonbuffered DMEM with glucose 2.5 mM, L-glutamine 2 mM and sodium pyruvate 1 mM) under basal conditions and in response to mitochondrial inhibitors: oligomycin (1 μM), FCCP (0.33 μM) and a mixture of rotenone (100 nM) and antimycin A (1 μM) (Sigma). Measurements of O_2 concentrations, in close vicinity to the seeded cells, were made over 4 min and OCR values were reported in pmol/min after normalisation to cell numbers.

The basal respiration rate was calculated as the difference between the values of basal OCR and OCR after rotenone/antimycin A dependent inhibition of mitochondrial complexes I and III. The maximal respiration rate was measured following addition of the uncoupler FCCP (uncoupled rate), indicative of the maximal electron transport activity and substrate oxidation achievable by the cells. The spare respiratory capacity (SRC) is calculated as the difference between the uncoupled and basal rates. It is indicative of the bioenergetic limits of the cell, under the assay conditions. The rate of mitochondrial ATP synthesis can be estimated from the decrease in OCR, following inhibition of ATP synthase with oligomycin.

ECAR measurement was performed in XF media supplemented with 2mM L-glutamine, in response to 10 mM glucose, 1 μM oligomycin and 200 mM 2-deoxyglucose (2-DG). The glycolytic capacity of the cells was calculated as the difference between the values of ECAR upon glucose addition and ECAR after 2-DG dependant inhibition of the glycolytic enzyme hexokinase. The glycolytic reserve was calculated as the difference between the value of ECAR upon glucose addition and ECAR following oligomycine-dependant inhibition of mitochondrial ATP synthase. It is indicative of the metabolic phenotype of the cells and their ability to shift from mitochondrial respiration to glycolysis in response to ATP demand.

ATP measurement

Measurements of the ATP produced by the control or MitoCepted MDA-MB-231 cells were performed on 50,000 cells, 48 hours after the transfer of MSC mitochondria, using the

ATPlite luminescent detection assay, according to the manufacturer instructions (Perkin Elmer). Measurements were expressed as Relative Luciferase Units (RLU) and calculated as fold of RLU measured in control MDA-MB-231 cells.

Proliferation and invasion assays

MDA-MB-231 cells with different amounts of MitoCepted MSC mitochondria were seeded in DMEM/FCS 5%, in quadruplicates, at the density of 10,000 cells per P24 well (5,000 cells/cm^2), and counted manually 5 days later.

Invasion assays of MDA-MB-231 cells were performed in 96-well View plates (PerkinElmer) pre-coated with 0.2% BSA (Sigma-Aldrich) and containing red fluorescent polystyrene microspheres at the bottom of the wells (10^4 beads per well; FluoSpheres; Invitrogen). In brief, cells were suspended in 1.7 mg/ml serum-free liquid bovine collagen at 10^5 cells/ml. 100-µl aliquots were dispensed into the plates. Plates were centrifuged at 300 g and incubated in a 37°C/5% CO_2 tissue-culture incubator. Once collagen had polymerized, FCS was added on top of the collagen to a final concentration of 5%. After 72-h incubation at 37°C, cells were fixed in 4% formaldehyde (Sigma-Aldrich) containing Hoechst 33342 (5 μg/ml, Invitrogen). Confocal z slices were collected from each well at 0, 25 and 50 μm from the bottom (0 μm) using a high content microscope (Arrayscan VTI Live; Zeiss) with a 40× PlanFluor objective (0.5 NA; Zeiss). Nuclear staining in each slice was quantified automatically (Thermo Scientific Cellomics BioApplications-Image) to determine the percentage of invaded cells.

FACS analysis

FACS experiments were performed using a Becton Dickinson FACSCanto II flow cytometer with 488-nm laser excitation and analyzed with CellQuest Pro software. Data are expressed as the mean percentage of positive cells for the indicated fluorescence intensity.

PCR analysis

Real time quantitative PCR was done on a Light Cycler 480 instrument (Roche, Meylan, France) using the SYBR green Master Mix (Roche, Meylan, France) and following the manusfacturer instructions. Nuclear DNA was quantified using the following primers nu-1: 5'-aca caa ctg tgt tca cta gc -3'; nu-2: 5'- cca act tca tcc acg ttc a -3', targetting the nuclear β-galactosidase gene. Mitochondrial DNA was quantified by amplication of a DNA domain

within the D-loop mt-1: 5'- tta act cca cca tta gca cc -3' ; mt-2: 5'- gag gat ggt ggt caa ggg a- 3'. To specifically quantify mtDNA from the MDA-MB-231 cancer cell, the reverse primer mt-2MDA: 5'- tta agg gtg ggt agg ttt gta ga -3' was used instead of mt-2. To specifically quantify mtDNA from MSCs, the reverse primer mt-2MSC: 5'- tta agg gtg ggt agg ttt gta gc -3' was used instead of mt-2.

Imaging

Fluorescence and time-lapse analysis was done with a Carl Zeiss LSM 5 live duo (LSM 510 META and 5 live) confocal laser system using a Zeiss 40X plan NeoFluar Oil objective. Time-lapse analysis was performed in an incubation chamber providing controlled temperature, CO_2 concentration and hygrometry. Pictures were taken every 30 minutes for 24 to 36 hours. After imaging, all time points were compiled and exported as a Quicktime (avi) file using the MetaMorph software. For phase-contrast microscopy, photographs were taken on a Zeiss Primo Vert inverted-phase microscope coupled to a digital Canon 1000D power shot camera. For phase-contrast coupled to fluorescence microscopy, photographs were taken on a Carl Zeiss AxioVert 200M inverted-phase microscope coupled to a Micromax YHS 1300 camera. The 3D reconstructions were done using the Imaris Bitplane program. The digitalized images were mounted with the Adobe Photoshop software.

Statistical analyses.

Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc.). Multiple samples were analyzed by one-way analysis of variance (ANOVA) and Dunnett post hoc test to evaluate statistical differences among the samples. Differences were considered statistically significant for $p < 0.05$ (*P < 0.05, **P < 0.01, ***P < 0.001). All data are presented as mean values with s.e.m.

Results and discussion:

Through their long-known involvement in the central cell tasks of nutrient uptake and energy production and also recently recognized role in cellular signaling, mitochondria are at the core of essential biological functions and corresponding disorders, including cancers [1-3]. Mitochondria transfer between cells was recently described as a phenomenon occurring both *in vitro* and *in vivo*, through nanotube formation, leading to cellular reprogramming and to phenotypes as diverse as protection against tissue injury and resistance to therapy [4-9]. As we show here and as recently reported by others, isolated mitochondria can also be directly

internalized by cells, as observed both *in vitro* and *in vivo* [10,11]. These novel findings have several important implications. First, they highlight the existence of unexpected means of cell communication and signaling whose mechanisms and effects still need to be fully established. Second, in a methodological point of view, direct acquisition of isolated mitochondria provides worthwhile means to study mitochondria functions in heterologous cell environments, alternative to the previously described methods of mitochondria injection and transmitochondrial cybrid formation [12-14].

We developed a model system based on the interactions between mesenchymal stem cells (MSCs) and MDA-MB-231 cancer cells as MSCs are known to be recruited to tumor sites, with resulting consequences on cancer cell growth and metastatic potential [15-18]. In addition to the known communications through metabolite exchange and cytokines [2,19-21], we show herein that MSCs can transfer mitochondria to cancer cells. To distinguish the effects of MSC mitochondria from other signaling contributions, we designed a method (MitoCeption) for quantitatively transferring MSC mitochondria, in amounts comparable to those occurring in coculture. We exploited differences in mtDNA sequences between MSCs and cancer cells to specifically follow and quantify mtDNAs of both the transferred and the endogenous mitochondria. These tools enabled us to demonstrate that few transferred MSC mitochondria have the capacity to boost the endogenous pool of mitochondria and to enhance both the energetic metabolism and functional properties of the targeted cancer cells.

Preliminary coculture experiments had shown that MSCs could make strong physical interactions and transfer cellular components to MDA-MB-231 cancer cells (**Fig. 6**). To test whether mitochondrial organelles were involved in this transfer, we used MitoTracker prestained MSCs in the coculture. Phase contrast and fluorescence microscopy showed that MSCs could form long protrusions targeting cancer cells and containing mitochondria (**Fig. 1a, upper panel**). Analysis by confocal microscopy and 3D cell reconstruction confirmed the passage of MSC mitochondria in the MDA-MB-231 cells (**Fig. 1a, lower panels**). As a control, cancer cell staining was not observed if, instead of MSCs, MSC conditioned medium was used. Moreover, transfer of human MSC mitochondria was confirmed in a heterologous coculture with murine TSA-pc cancer cells using an antibody (Ab-2) that recognizes mitochondria of human, but not murine origin (**Fig. 7**).

To quantify the extent of the mitochondria transfer from hMSCs to MDA-MB-231 cells, we analyzed the cocultures by FACS. MSCs was prestained with the MitoTracker 24 hours beforehand and then cocultured with the unlabeled MDA-MB-231 cells for 24 hours, at which point cells were analyzed by FACS for MitoTracker labeling. FACS data showed an uptake of MitoTracker fluorescence by MDA-MB-231 cells that confirmed the acquisition of the MitoTracker labeled MSCs by MDA-MB-231 cells (**Fig. 1b**). The other cell combinations showed that the MDA-MB-231 cancer cells could also transfer mitochondria to MSCs, albeit with a lower efficacy, and that MSCs demonstrated the capacity to transfer mitochondria between one another, which was not observed among MDA-MB-231 cells (**Fig. 8**). These results highlighted the complex interactions between MSCs and cancer cells, with the occurrence of a mitochondria transfer between these two cell types, in addition to the known cytokine-mediated cross-talk. Because nanotubes can allow the passage of other cell components and because cytokines induce various cell responses, we designed a protocol for the quantitative transfer to cancer cells of mitochondria isolated from hMSCs, to specifically determine the effects of MSC mitochondria in cancer cells.

The MitoCeption protocol that we designed allows the transfer of mitochondria isolated from cell type A to cell type B so that, at the end, cell type B contains both its own and the exogenous mitochondria (**Fig. 2a**). To follow the mitochondria transfer process, MSCs were MitoTracker labeled beforehand and 24 hours after the transfer, cancer cells were analyzed by confocal imaging. The 3D reconstructions from confocal images confirmed that the transferred MSC mitochondria did localize inside the MDA-MB-231 cancer cells and that the transferred MSC mitochondria were located close to the endogenous MDA-MB-231 mitochondria network (**Fig. 2b**). In a preliminary evaluation of the transfer protocol, we transferred MSC mitochondria to other MSCs. We could show that these mitochondria were spread out among the endogenous MSC mitochondria and that they also demonstrated the capacity to transfer to MDA-MB-231 cells in coculture conditions (**Fig. 9**).

The efficiency of the mitochondria transfer was quantified both by flow cytometry on the basis of the MSC mitochondria MitoTracker labeling and by quantification of MSC mitochondrial DNA (mtDNA). FACS analysis showed a dose-dependent uptake of MSC mitochondria by MDA-MB-231 cancer cells (**Fig. 2c**). Interestingly, the MSC mitochondrial mass detected in cancer cells after the coculture with a 1:1 ratio between MSCs and cancer cells was in the same range as that detected after transfer by MitoCeption (condition 0.5), of the order

of a few percents (**Fig. 10**). Transfers of mitochondria by our MitoCeption protocol were also obtained between cancer cells as well as from MSCs to non adherent cells, as tested with Jurkat cells (data not shown).

Quantification of the transferred MSC mitochondria was also performed measuring mtDNA concentrations. Mutations present in the D-loop of MDA-MB-231 mtDNA were used to design primers enabling the specific quantification of mtDNAs originating either from MSCs or from the cancer cells. Noteworthingly, MSC donors could also be distinguished between one another by primers targeting SNPs (data not shown). As expected, following MSC mitochondria transfer by MitoCeption, MSC mtDNA concentrations were found to increase in a dose dependent fashion (**Fig. 2d, upper panel**). The total concentration of mtDNA in the MDA-MB-231 cells was also measured, using previously published primers. Data showed that MSC mitochondria acquisition by cancer cells resulted in a dose-dependent increase in total mtDNA concentration (**Fig. 2d, lower panel**). However, because the concentration of mtDNA of MSC origin in the MDA-MB-231cells was measured to be many fold lower, it could not account for this increase. Indeed, using PCR primers specific for MDA-MB-231 mtDNA, we showed that acquisition of MSC mitochondria by the MDA-MB-231 cells did lead to an increased concentration of the endogenous MDA-MB-231 mtDNA (**Fig. 2d, lower panel**). Interestingly, our data suggested that the low amount of MSC mtDNA, in the range of one hundredth of the endogenous cancer cell mtDNA pool, was sufficient to induce endogenous mtDNA replication and the functional effects shown below.

We next investigated the effect of MSC mitochondria on the metabolic activity of the MDA-MB-231 cancer cells. Increasing amounts of MSC mitochondria were transferred, using the MitoCeption protocol, to MDA-MB-231 cells that were analyzed 48 hours later. For OXPHOS activity, the oxygen consumption rate (OCR) was measured under basal conditions and in response to the oligomycin, FCCP, rotenone and antimycin A mitochondrial inhibitors. Both basal and maximal OCR values were significantly increased, in a dose-dependent manner, with respectively 1.49 and 1.28 fold increases for the 0.5 MSC to MDA-MB-231 Cell Ratio condition (0.5 MMCR) (**Fig. 3a**). Moreover, the spare respiratory capacity was also enhanced following MitoCeption with MSC mitochondria, with a 1.31 fold increase for the 0.5 MMCR condition ($p=0.06$, data not shown), suggesting that MSCs can modify the metabolic activity of cancer cells by increasing their respiratory capacity. In contrast, the production of extracellular lactate by cancer cells (ECAR values) diminished upon acquisition of MSC mitochondria as

did the glycolytic reserve (resp. 0.64 and 0.6 fold of the control for the 0.5 MMCR condition) (**Fig. 3b**). Importantly, acquisition of MSC mitochondria by MDA-MB-231 cells resulted in significant increases in ATP production (1.30 fold increase for the 0.12 MMCR condition) (**Fig. 3c**). We then checked the effect of MSC mitochondria on MDA-MB-231 cells migration and proliferation capacities. Using a 3D-collagen invasion assay, we showed that acquisition of MSC mitochondria by the cancer cells increased their invasion capacity reaching 1.6 fold within the 3 day migration time-frame (condition 0.25 MMCR) (**Fig. 3d, left panel**). MDA-MB-231 cell proliferation was measured over a 5 day period following MSC mitochondria acquisition and was also found to be increased in a dose response fashion with a 1.35 fold stimulation for the 0.1 MMCR condition (**Fig. 3d, right panel**).

The cell-to-cell transfer of mitochondria constitutes a new communication process whose biological implications are just beginning to emerge. It is expected to lead to widespread effects, spanning from tissue regeneration to resistance to therapy. We propose here a protocol, that we named MitoCeption, to mimic this transfer of mitochondria. Furthermore, we show that the transferred mitochondria can be followed by specific mtDNA quantification. We thus show that minute amounts of transferred MSC mitochondria nonetheless give rise to increased OXPHOS, ATP production and functional capacities in the recipient cancer cells, presumably due to a boosting effect on cancer cell mitochondrial activity.

In this novel field of intercellular mitochondria signaling, a number of questions still remain unanswered. For instance, we do not know how long the transferred mitochondria are maintained in the recipient cells, whether the acquisition of exogenous mitochondria is a reiterative process and if, in a combinatorial manner, different cell types can target a same cell. We believe that the methods proposed herein provide tools for answering these questions.

EXAMPLE 2:

1. Human MSCs transfer mitochondria to human T cells

Since physical interactions between human MSCs and Th17 lymphocytes are required for their modulation, we investigated whether a mitochondrial transfer could be involved in this process. Polyclonal T cell lines were used, isolated from the PBMC of a Crohn's Disease patient. The isolation and culture of diverse T cell lines ($CCR6^+$ and $CCR6^-$) have been already described, CCR6 is expressed almost exclusively by $CD4^+$ Th17-T cells and not by Th1 nor Th2 cells. The isolated T cells were used at 10 to 15 days after the latest propagation phase, a time window corresponding to T cells in a resting phase. The phenotype of the T cells was periodically controlled on the basis of their specific cytokine production profile and the presence/absence of the lineage-specific transcription factors upon CD3 and CD28 activation. Human MSCs, isolated from the bone marrow of healthy donors, were obtained from the EFS (Etablissement Français du Sang, Grenoble). Approval for the use of clinical biopsies and blood samples from rheumatoid arthritis patients has been obtained from the ethics committee of the University Hospitals of Montpellier (n° DC-2008-417 – coordinator: Ch. Jorgensen). The preliminary experiments described below were performed with MSCs isolated from two different donors and used at an early passage as previously described.

MitoTracker labeled MSCs were cocultured for 24 hours with either $CCR6^+$ (Th17) or $CCR6^-$ (Th1 and Th2) T cells, after which time T cells (that are not adherent) were recovered from the coculture with the MSCs (MSCs are adherent). T cells were analyzed by FACS for the acquisition of the MitoTracker, indicative of a transfer of mitochondria from MSCs. Both $CCR6^+$ or $CCR6^-$ cells displayed an increase in MitoTracker fluorescence intensity in the T cell analysis gate. MSCs alone only gave a very low background signal, dismissing the possibility that the signal observed for T cells following the coculture was that of the MitoTracker labeled MSCs. We also checked that the observed T cell labeling was not due to the passive diffusion of the MitoTracker dye from the stained MSCs by incubating the T cells with a conditioned medium of the MitoTracker stained-MSCs. This 24 hour incubation did not lead to an increase in fluorescence of the T cells further supporting our conclusions of an active mitochondria transfer from MSCs to T cells. FACS analysis confirmed the specific transfer of MSC mitochondria to both $CD3^+$ $CD4^+$ $CCR6^+$ and $CD3^+$ $CD4^+$ $CCR6^-$ T cell populations.

Next, we assessed the kinetics of the transfer of MSC mitochondria to T cells (n = 2). Undetectable at 30 min of coculture, the mitochondria transfer was observed as early as 1 h after the onset of the coculture and increased thereafter (both in the percentage of positive cells

and in the amount of MitoTracker labelling per cell, MFI), reaching a plateau at about 4 h. To verify the need of tight contacts between T cells and MSCs for the transfer of mitochondria, cocultures were set up in parallel under continuous circular agitation (n=2). These incubation conditions greatly reduced the mitochondria transfer efficacy. These observations underline the importance of cell-cell contacts for the active transfer of MSC mitochondria to T cells and further dismiss the possibility of a passive diffusion of the fluorescent dye from MSCs to T cells.

Resting T cells in a restimulated T cell line could be considered as memory-like cells. To test whether effector cells, those involved in the immune response, are also targets of MSC mitochondria transfer, we performed similar experiments utilizing T cells recently activated with anti-CD3 and anti-CD28 monoclonal antibodies (mAb). At 24 hours of coculture, the profile of MSC mitochondria acquisition (MitoTracker staining) was very similar to that of the non activated T cells. However, shorter time points showed higher MitoTracker staining in activated versus non activated T cells (n=3; data not shown) suggesting that, indeed, activation of T cells enhances acquisition of MSC mitochondria.

As these first experiments were performed with *in vitro* cultured T cell lines, we further checked the occurrence of the mitochondria transfer process with freshly isolated lymphocytes. For this purpose, we isolated peripheral blood mononucleated cell (PBMCs) from a healthy donor and cocultured these cells with MSCs as described above. After a 4 hour coculture with MitoTracker labeled MSCs, PBMCs were harvested and labeled with a set of fluorochrome-conjugated mAbs, allowing the identification of T cell subsets (CD3, CD4, CD8, CD45RO). The transfer of MSC mitochondria was also observed in these freshly isolated lymphocytes. Remarkably, a higher percentage of memory $CD4^+$ and $CD8^+$ T cells ($CD45RO^+$) acquired mitochondria as compared to the naïve counterparts. Similarly to the T cell lines, the transfer was completely abolished by the mechanical shaking of the cells. Taken together, our results suggest that the mitochondria transfer is operating in primary T cells and that the ability to acquire mitochondria varies depending on the subset ($CD4^+$ vs. $CD8^-$) and their differentiation status (naïve, effector, memory).

2. Mouse MSCs transfer mitochondria to mouse T cells

Next, we investigated whether the phenomenon of mitochondrial transfer could also be observed in the mouse system. For this purpose, we isolated mouse MSCs from the bone marrow and labeled them with the MitoTracker 24 h before coculture, as previously described for the human cells. Total lymphocytes from the lymph nodes of syngeneic Balb/c mice were used as target cells. Lymphocytes were seeded over monolayers of labeled MSCs and cultured for periods of 4 and 24 hours. Lymphocytes were then recovered and the uptake of labeled mitochondria by $CD4^+$ and $CD8^+$ T cells was analyzed by FACS. The profiles obtained were similar to those of human cells. Both T cell subsets were able to acquire mitochondria from mouse MSCs, although to different extents. While 66% of the $CD3^+$ $CD4^+$ T cells were Mitotracker+, only 15% of the $CD3^+$ $CD8^+$ T cells had incorporated the dye. These results indicate that mouse MSCs can transfer mitochondria to mouse T cells.

3. Mitochondria isolated from MSCs can be transferred by MitoCeption to T cells

In addition to direct cell-cell contacts, cells in coculture can also interact by the well-characterized cytokine cross-talk. In order to specifically identify the effects of MSC mitochondria on the target cell phenotype, we developed an innovative technique, called MitoCeption (EXAMPLE 1). This technique allows the transfer of mitochondria isolated from MSCs to the target cells without the need of a coculture. We showed that this technique is also applicable to the transfer of MSC mitochondria to T cells as shown by FACS analysis and confocal microscopy on the basis of the MitoTracker labeling of MSC mitochondria. In addition, because mitochondrial DNA contains different SNPs corresponding to different haplotypes, mitochondrial DNA from different donors can be specifically identified. It thus allows the specific tracking of the mitochondrial DNA from either MSCs and T cells and, consequently, the monitoring of MSC mitochondria transfer to T cells, on the basis of the mitochondrial DNA concentrations.

EXAMPLE 3: MitoCeption of MSC mitochondria to T cells:

Methods:

Cell culture

Human MSCs were isolated from bone marrow aspirates from healthy donors, who gave informed consent. All the isolation and culture procedures were conducted in the authorized cell therapy unit (Biotherapy Team of General Clinic Research Center, French health minister agreement TCG/04/0/008/AA) at the Grenoble University Hospital. The cells were grown in Minimum Essential Eagle Medium alpha (αMEM) supplemented with L-Glutamine 1% and FCS 10% and used at an early passage. For intracellular mitochondria staining, the MitoTracker green FM (Molecular Probes) was used. Alternatively, the MitoTracker Red CMXRos (Molecular Probes) could be used as well. After labeling, MSCs were washed several times in order to prevent excess MitoTracker probe inside the cell.

T cells were isolated from fresh blood (obtained from the EFS Montpellier) using Ficoll-Hypaque. Cells were grown in IMEM supplemented with L-Glutamine 1%, penicillin (100 U/ml), streptomycin (100µg/ml), Yssel's medium 10% and Human Serum AB+ 1%.

Mitochondria isolation

Mitochondria were prepared using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) with the following the manufacturer's instructions. To obtain mitochondria preparations with reduced contamination from other cytosol compounds, centrifugation for the recovery of mitochondria was performed at 3,000*g* for 15 minutes.

Mitochondrial artificial transfer

General considerations:

1. In order to follow and quantify the rate and efficiency of the mitochondria transfer, mitochondria can be MitoTracker labeled beforehand in the donor cells. Likewise, the recipient cells can also be labeled (CellTracker) beforehand if cells are to be analyzed by microscopy after the mitochondria transfer. Once the protocol has been validated by the user with his specific cells, mitochondria labeling can be left out, allowing a wider range of possible experiments with the MitoCepted cells. (All remarks in italics in this protocol are related to fluorescent labeling of cells or mitochondria).

2. Of great importance, cell exposure to EDTA should be avoided at all steps of the protocol. Therefore cell trypsinization is recommended with trypsin and no EDTA; the cocktail of protease inhibitors should, as well, be devoid of EDTA.

3. The mitochondria preparation should be performed on ice to maintain their integrity.

We set up a protocol for mitochondria transfer (MitoCeption) that relies on the centrifugation of the mitochondria suspension on the cultured cells at the adequate centrifugation force, with a number of centrifugations that can be adjusted as a function of the system of mitochondria donor/recipient cells.

Day 1: MSC and T cells cultures

Seed 300 000 human MSCs on 100 mm petri dish.

Prepare extra plate for FACS controls of the transfer (Mitotracker stained MSC mitochondria) or quantification of MSC mtDNA in the target cells after the transfer.

Day 2: MSC mitochondria labeling (MitoTracker)

MSC MitoTracker Labeling (protect cells from light using an aluminum foil).

Rinse MSC cells with PBS (10 ml). Dilute the MitoTracker stock solution in αMEM/FCS 1% (concentration for MitoTracker green (5-chloromethyl-fluorescein diacetate) 1 μM).

Take 8 ml of the MitoTracker dilution and add it to the cells (final MitoTracker concentration: 250 nm in 1 ml). Incubate for 45min. at 37°C.

Remove the MitoTraker solution, rinse cells with 10 ml of PBS and add 10 ml of αMEM/FCS 10%. Perform the medium changes every 2h, 3 times.

Optional: T cells can be prestained with a CellTracker (protect cells from light) if cells are to be analyzed by fluorescence microscopy after MitoCeption of the fluorescent MSC mitochondria.

Day 3: Mitochondria Isolation and MitoCeption

Adjust centrifuge temperature to 4°C.

In case MSCs have been MitoTracker labeled, check by FACS the rate of MitoTracker mitochondria staining. Detach MSCs with trypsin (no EDTA). Resuspend cells in 5 ml final of αMEM/FCS 10%, centrifuge at 900g (1300 rpm) for 5 min, and add 300 μl of PBS/FCS 10% to the cell pellet.

Prepare the reagents for the number of desired extractions (Fisher mitochondria isolation kit, reference 1057-9663), following manufacturer instructions. Number of cells to be used in one extraction: from 2.10^5 to 5.10^6.

Wash MSCs with 10 ml of PBS, wash with 2 ml of trypsin (no EDTA) for 10 sec. and add finally 1 ml of trypsin (no EDTA). Incubate cells for 5 min at 37°C. Recover the cells of each petri dish by adding 10 ml of αMEM/FCS 10%. Centrifuge cells at 1200 rpm for 5 min.

Discard the supernatant and collect all pellets in 10 to 20 ml αMEM/FCS 10%, in a 50 ml Falcon tube.

Count MSC cells, take the corresponding volume of re-suspended cells to perform the extractions, centrifuge them at 1200 rpm for 5 min, discard the supernatant and add 1 ml to transfer the cells to 1.5 ml Eppendorf tubes, keep the tubes in ice.

In case MSCs were green MitoTracker labeled, plate $6x10^5$ cells in one well of a P12 (12-well plate) for control FACS analysis performed at the same time as the MitoCepted cell samples.

Count T cells, check for viability with trypan blue and plate 500 000 T cells in each well of a P24 (24-well plate), final volume for MitoCeption will be 2 ml of αMEM/FCS 5%.

Centrifuge the Eppendorf tubes containing the MSCs at 900 g for 5 min, at 4°C.

Remove all residual medium from the tube as it could affect mitochondrial isolation (it may be useful to centrifuge tubes for a few seconds to eliminate excess liquid.). Be careful not to let the cells dry.

Add 200 µl of Mitochondria Isolation Reagent A (for 10^6 MSCs, 400 µl for 2 to 3.10^6 MSCs). Vortex at medium speed for 5 seconds and let tubes on ice for exactly 2 minutes. Note: Do not exceed 2 minute incubation.

Add 2.5 µl of Mitochondria Isolation Reagent B (for 10^6 MSCs, 5 µl for 2 to 3.10^6 MSCs). Vortex at maximum speed for 10 seconds, then let tubes on ice.

Start timer for 5 minutes, vortexing at maximum speed every 30 seconds for 10 seconds. Put tubes on ice in the meantime.

Add 200 µl of Mitochondria Isolation Reagent C (for 10^6 MSCs, 400 µl for 2 to 3.10^6 MSCs). Shake the tubes strongly by hand (roughly 30 times) (do not vortex).

Centrifuge tube at 700 g for 10 minutes at 4°C.

Transfer the supernatants to new 1.5 ml Eppendorf tubes. Centrifuge at 3,000g (15 min at 4°C) to get the mitochondria pellets.

Discard the supernatant of the mitochondria preparation. The pellet contains the isolated mitochondria.

Add 1 ml of αMEM/FCS 5% to the mitochondria pellet isolated from 10^6 MSCs (the volume needs to be adjusted as a function of the number of cells used for the mitochondria isolation).

Add the volume of isolated mitochondria to the non-labeled T cells at the desired concentration. Typical range would be 1/25. This value represents the ratio of the number of cells from which mitochondria are isolated to the number of cells to which mitochondria are transferred by MitoCeption. A range of 1/25 thus means mitochondria isolated from 1,000 MSC used for MitoCeption of 25,000 T cells.

Add the mitochondria slowly, close to the bottom of the well, covering all the surface of the well at least once, use clock-wise movements. Be careful thereafter to move the culture plates smoothly, avoiding shocks.

Centrifuge the 24-well plates containing the mitochondria recipient cells and the seeded mitochondria at 1,500 g for 15 min at 4°C.

Cover the plates with aluminum foil, and place them in the 37°C cell incubator immediately after centrifugation.

Day 4: Cell analysis

If donor cell mitochondria were MitoTracker-labeled beforehand, MitoCeption efficiency can be checked by FACS (trypsin with no EDTA).

Evaluation of the efficiency of the mitochondria transfer can also be done on the basis of MSC mtDNA concentration in the recipient cells.

Thereafter, the biological characterization (mitochondrial activity, proliferation, invasion) of the cells containing the exogenous mitochondria can be performed.

FACS analysis

FACS experiments are performed using a Becton Dickinson FACSCanto II flow cytometer with 488-nm laser excitation and analyzed with the FACS DIVA software. Data are expressed as the mean fluorescence intensity for the cell population after Mitotracker mitochondria transfer by MitoCeption, after subtraction of the background MFI value (obtained with cells without added mitochondria).

To perform the analysis, aspirate the medium from the culture plate containing the MitoCepted cells, add 1 ml of fresh medium and collect the cells. Centrifuge cells 3 minutes at

1200 rpm, discard the supernatant, add 200 µl PBS and transfer the cells to a 96-well plate. Perform four washes with 200 µl PBS and after the last wash resuspend the cells with PBS or PBS/FCS 10%.

Statistical analyses.

Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc.). An unpaired t-test was performed to evaluate statistical differences among the samples. Differences were considered statistically significant for $p < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

The figure 4 shows a dot plot representation of the MFI values obtained for the "no centrifugation" and "centrifugation" conditions, with the mean values and standard deviations (SD) indicated. Performing the centrifugation step of the MitoCeption protocol enabled to raise the mean MFI value from 100 (in the "no centrifugation" condition) to 144 (in the "centrifugation" condition). This difference was highly significant with a p value < 0.001 (unpaired t test).

Conclusion

The transfer to T cells of mitochondria isolated from mesenchymal stem cells (MSCs) is increased when the culture plates are centrifuged after the seeding of the isolated mitochondria on top of the T cells. Therefore, the centrifugation step in the MitoCeption protocol is important for increasing the efficiency of the MSC mitochondria transfer to non adherent cells such as human primary T cells.

EXAMPLE 4: MitoCeption of MDA-MB-231 mitochondria to MDA-MB-231 cells:

Methods:

Cell culture

The MDA-MB-231 cancer cells were grown in DMEM-F12/ FCS (10%). Staining of the intracellular mitochondria was done with the green MitoTracker FM (Molecular Probes). After labeling, MDA-MB-231 cells were washed several times to eliminate excess

MitoTracker. The cells were thereafter trypsinized without EDTA in order to prevent membrane damage and MitoTracker leakage.

Mitochondria isolation

Mitochondria were prepared using the Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) with the following the manufacturer's instructions. To obtain mitochondria preparations with reduced contamination from other cytosol compounds, centrifugation for the recovery of mitochondria was performed at 3,000*g* for 15 minutes.

Mitochondrial artificial transfer

General considerations:

1. In order to follow and quantify the rate and efficiency of the mitochondria transfer, mitochondria can be MitoTracker labeled beforehand in the donor cells. Likewise, the recipient cells can also be labeled (CellTracker) beforehand if cells are to be analyzed by microscopy after the mitochondria transfer. Once the protocol has been validated by the user with his specific cells, mitochondria labeling can be left out, allowing a wider range of possible experiments with the MitoCepted cells. (All remarks in italics in this protocol are related to fluorescent labeling of cells or mitochondria).

2. Of great importance, cell exposure to EDTA should be avoided at all steps of the protocol. Therefore cell trypsinization is recommended with trypsin and no EDTA; the cocktail of protease inhibitors should, as well, be devoid of EDTA.

3. The mitochondria preparation should be performed on ice to maintain their integrity. We set up a protocol for mitochondria transfer (MitoCeption) that relies on the centrifugation of the mitochondria suspension on the cultured cells at the adequate centrifugation force, with a number of centrifugations that can be adjusted as a function of the system of mitochondria donor/recipient cells.

Day 1: MDA-MB-231 cultures

Seed 10^6 MDA-MB-231 cells in a 100 mm dish 72h before the MitoTracker labeling.

Rinse MDA cells with PBS (10 ml). Dilute the MitoTracker stock solution in DMEM/FCS 1% (concentration for MitoTracker green (5-chloromethyl-fluorescein diacetate) 1 μM).

Take 8 ml of the MitoTracker dilution and add it to the cells (final MitoTracker concentration: 250 nm in 1 ml). Incubate for 45 min. at 37°C.

Remove the MitoTraker solution, rinse cells with 10 ml of PBS and add 10 ml of DMEM/FCS 10%. Perform the medium changes every 2h, 3 times.

At the same time seed 10^5 MDA-MB-231 cells in 22 mm-wells (12-well plate, P12) to be MitoCepted.

Optional: label MDA-MB-231 cells with CellTracker (5-chloromethyl-fluorescein diacetate)

Day 2: Mitochondria Isolation and MitoCeption

Adjust centrifuge temperature to 4°C.

The staining of the MDA-MB-231 cells with the MitoTracker can be checked by FACS. Detach the MDA-MB-231 cells with trypsin (no EDTA). Resuspend cells in 5 ml final of DMEM/FCS 10%, centrifuge at 900g (1300 rpm) for 5 min, and add 300 µl of PBS/FCS 10% to the cell pellet.

Trypsinize and count the MDA-MB-231 cells from a control well of the 12-well plate (for adjustment of the amount of isolated mitochondria to add in the MitoCeption protocol, expected: 1.1 to 1.5 10^5 MDA-MB-231 cells).

Prepare the reagents for the number of desired extractions (Fisher mitochondria isolation kit, reference 1057-9663), following manufacturer instructions. Number of cells to be used in one extraction: from 4 to 10.10^6. Use protease inhibitors without EDTA.

Wash the MitoTracker labeled MDA-MB-231 cells with 10 ml PBS and add 1ml trypsin (no EDTA). Incubate cells for 5 min at 37°C. Recover the cells by adding 10ml DMEM/FCS 10%.

Centrifuge cells at 900 g (1300 rpm) for 5 min.

Discard the supernatant and resuspend the cell pellet in 20 to 30 ml DMEM/FCS 10%, in a 50 ml Falcon tube.

Count the MDA-MB-231 cells, take the needed volume of cells to perform the mitochondria extraction, centrifuge them at 1200 rpm for 5 min, discard the supernatant and add 1 ml to transfer the cells to 1,5 ml Eppendorf tubes, keep the tubes in ice.

As a control, plate 6.10^5 MitoTracker labeled MDA-MB-231 cells in one well of a P12 plate for FACS analysis that will be performed at the same time as the MitoCepted cell samples.

Centrifuge the Eppendorf tubes at 900 g for 5 min, at 4°C.

Remove all residual medium from the tube as it could affect mitochondrial isolation. Be careful not to let the cells dry. (It may be useful to centrifuge tubes for a few seconds to eliminate excess liquid.)

Add 200 µl of Mitochondria Isolation Reagent A (for 10^6 MDA-MB-231 cells, 400 µl for 2 to 3.10^6 MDA-MB-231 cells). Vortex at medium speed for 5 seconds and let tubes on ice for exactly 2 minutes. Note: Do not exceed 2 minute incubation.

Add 2.5 µl of Mitochondria Isolation Reagent B (for 10^6 MDA-MB-231 cells, 5 µl for 2 to 3.10^6 MDA-MB-231 cells). Vortex at maximum speed for 10 seconds, then let tubes on ice

Start timer for 5 minutes, vortexing at maximum speed every 30 seconds for 10 seconds. Put tubes on ice in the meantime.

Add 200 µl of Mitochondria Isolation Reagent C (for 10^6 MDA-MB-231 cells, 400 µl for 2 to 3.10^6 MDA-MB-231 cells). Shake the tubes strongly by hand (roughly 30 times) (do not vortex).

Centrifuge tube at 700 g for 10 minutes at 4°C.

Transfer the supernatants to new 1.5 ml Eppendorf tubes. Centrifuge at 3,000g (15 min at 4°C) to get the mitochondria pellets.

In the meantime, change the medium of the mitochondria recipient cells in the 12-well culture plate. Final: 3 ml of DMEM/FCS 5%, in each well.

Discard the supernatant of the mitochondria preparation. The pellet contains the isolated mitochondria.

Add 1 ml of DMEM/FCS 5% to the mitochondria pellet isolated from 10^6 MDA-MB-231 cells (the volume needs to be adjusted as a function of the number of cells used for the mitochondria isolation).

Add the volume of mitochondria isolated from the labeled MDA-MB-231 cells at the desired concentration. In the experiments shown, mitochondria were isolated from a given number of MDA-MB-231 cells and transferred, by MitoCeption, to the same number of (mitochondria receiver) MDA-MB-231 cells.

Add the mitochondria slowly, close to the bottom of the well, covering all the surface at least once, use clock-wise movements. Be careful thereafter to move the culture plates smoothly, avoiding shocks.

Centrifuge the 12-well plates containing the mitochondria recipient cells and the seeded mitochondria at 1,500 g for 15 min at 4°C.

Cover the plates with aluminum foil, and place them in the 37°C cell incubator immediately after centrifugation.

Day 4: Cell analysis

If donor cell mitochondria were MitoTracker-labeled beforehand, MitoCeption efficiency can be checked by FACS (trypsin with no EDTA).

Thereafter, the biological characterization (mitochondrial activity, proliferation, invasion) of the cells containing the exogenous mitochondria can be performed.

FACS analysis

FACS experiments are performed using a Becton Dickinson FACSCanto II flow cytometer with 488-nm laser excitation and analyzed with the FACS DIVA software. Data are expressed as the mean fluorescence intensity for the cell population after Mitotracker mitochondria transfer by MitoCeption, after subtraction of the background MFI value (obtained with cells without added mitochondria).

To perform the analysis, aspirate the medium from the culture plate containing the MitoCepted cells and rinse the cells with PBS. Trypsinize the cells and after centrifugation (5 min, 1200 rpm), add 300 µl PBS or PBS/FCS 10%.

Statistical analyses

Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc.). Multiple samples were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test to evaluate statistical differences among the samples. Differences were considered statistically significant for $p < 0.05$ (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). All data are presented as mean values with s.e.m.

Results

The figure 5 shows a dot plot representation of the MFI values obtained for the "none", "one" and "two" centrifugation conditions, with the mean values and standard deviations (SD) indicated. Performing the centrifugation step of the MitoCeption protocol enabled to raise the mean MFI value to 133 in the "one" centrifugation condition and 124 in the "two" centrifugation condition. The difference between the centrifugation conditions and the "no" centrifugation condition was significant (ANOVA and Tukey's multiple comparisons test). However, performing the centrifugation step two times did not show a significant effect on the efficacy of mitochondria transfer by MitoCeption.

Conclusion

The transfer to the MDA-MB-231 cancer cells of mitochondria isolated from MDA-MB-231 cells is increased when the culture plates are centrifuged after the seeding of the isolated mitochondria on top of the MDA-MB-231 cells. On the other hand, performing this centrifugation step does not seem to be necessary for this cell type. Therefore, the centrifugation step in the MitoCeption protocol is important for increasing the efficiency of the MDA-MB-231 mitochondria transfer to MDA-MB-231 cells. The number of centrifugation steps to yield maximal efficacy in the mitochondria transfer rate is likely to depend on the cell type used.

REFERENCES:

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

1. Nunnari, J. & Suomalainen, A. Mitochondria: in sickness and in health. *Cell* 148, 1145-59 (2012).

2. Chandel, N.S. Mitochondria as signaling organelles. *BMC Biol* 12, 34 (2014).

3. Schulze, A. & Harris, A.L. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* 491, 364-73 (2012).

4. Islam, M.N. et al. Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 18, 759-65 (2012).

5. Pasquier, J. et al. Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med* 11, 94 (2013).

6. Spees, J.L., Olson, S.D., Whitney, M.J. & Prockop, D.J. Mitochondrial transfer between cells can rescue aerobic respiration. *Proc Natl Acad Sci U S A* 103, 1283-8 (2006).

7. Ahmad, T. et al. Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *Embo J* (2014).

8. Acquistapace, A. et al. Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer. *Stem Cells* 29, 812-24 (2011).

9. Plotnikov, E.Y. et al. Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture. *J Cell Mol Med* 12, 1622-31 (2008).

10. Masuzawa, A. et al. Transplantation of autologously derived mitochondria protects the heart from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 304, H966-82 (2013).

11. Kitani, T. et al. Direct human mitochondrial transfer: a novel concept based on the endosymbiotic theory. *Transplant Proc* 46, 1233-6 (2014).

12. Takeda, K. et al. Microinjection of cytoplasm or mitochondria derived from somatic cells affects parthenogenetic development of murine oocytes. *Biol Reprod* 72, 1397-404 (2005).

13. Van Blerkom, J., Sinclair, J. & Davis, P. Mitochondrial transfer between oocytes: potential applications of mitochondrial donation and the issue of heteroplasmy. *Hum Reprod* 13, 2857-68 (1998).

14. Kaipparettu, B.A., Ma, Y. & Wong, L.J. Functional effects of cancer mitochondria on energy metabolism and tumorigenesis: utility of transmitochondrial cybrids. *Ann N Y Acad Sci* 1201, 137-46 (2010).

15. Ohkouchi, S. et al. Mesenchymal Stromal Cells Protect Cancer Cells From ROS-induced Apoptosis and Enhance the Warburg Effect by Secreting STC1. *Mol Ther* 20, 417-23 (2012).

16. Karnoub, A.E. et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 449, 557-63 (2007).

17. Rattigan, Y., Hsu, J.M., Mishra, P.J., Glod, J. & Banerjee, D. Interleukin 6 mediated recruitment of mesenchymal stem cells to the hypoxic tumor milieu. *Exp Cell Res* 316, 3417-24 (2010).

18. Houthuijzen, J.M., Daenen, L.G., Roodhart, J.M. & Voest, E.E. The role of mesenchymal stem cells in anti-cancer drug resistance and tumour progression. *Br J Cancer* 106, 1901-06 (2012).

19. Torsvik, A. & Bjerkvig, R. Mesenchymal stem cell signaling in cancer progression. *Cancer Treat Rev* 39, 180-8 (2013).

20. Zhang, W. et al. Stromal control of cystine metabolism promotes cancer cell survival in chronic lymphocytic leukaemia. *Nat Cell Biol* 14, 276-86 (2012).

21. Bonuccelli, G. et al. Ketones and lactate "fuel" tumor growth and metastasis: Evidence that epithelial cancer cells use oxidative mitochondrial metabolism. *Cell Cycle* 9, 3506-14 (2010).

22. Smith, R.A., Hartley, R.C., Cocheme, H.M. & Murphy, M.P. Mitochondrial pharmacology. *Trends Pharmacol Sci* 33, 341-52 (2012).

CLAIMS:

1. A method for the intercellular transfer of an amount of mitochondria isolated from a population of donor cells into a population of recipient cells comprising the step of i) centrifuging the population of recipient mammalian cells in presence of the isolated mitochondria at centrifugation force ranging from 1000g to a 2000g at a temperature ranging from 1°C to 8°C and for a time ranging from 5min to 30min, ii) resting the centrifuged cells at a temperature ranging from 30°C to 40°C for a time ranging from 90min to 180min, and iii) repeating the cycling of steps i) and ii) for a sufficient number of times for reaching transfer efficiency.

2. The method of claim 1 wherein the donor cells and the recipient cells are different or identical.

3. The method of claim 1 wherein the donor cells and the recipient cells come from different or the same species.

4. The method of claim 1 wherein the donor cells and the recipient cells come from different or the same tissues.

5. The method of claim 1 wherein the cells are mammalian cells.

6. The method of claim 1 wherein the cells are isolated from a mammalian subject who is selected from a group consisting of: a human, a horse, a dog, a cat, a mouse, a rat, a cow and a sheep.

7. The method of claim 1 wherein the cells are cells in culture.

8. The method of claim 1 wherein the cells are selected from the group consisting of epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, hepatocytes, B-cells, T-cells, erythrocytes, macrophages, monocytes, fibroblasts, muscle cells, vascular smooth muscle cells, hepatocytes, splenocytes, and pancreatic β cells.

9. The method of claim 1 wherein the cells are cancer cells.

10. The method of claim 9 wherein the cancer cells are isolated from a cancer selected from the group consisting of breast cancer, prostate cancer, lymphoma, skin cancer,

pancreatic cancer, colon cancer, melanoma, malignant melanoma, ovarian cancer, brain cancer, primary brain carcinoma, head-neck cancer, glioma, glioblastoma, liver cancer, bladder cancer, non-small cell lung cancer, head or neck carcinoma, breast carcinoma, ovarian carcinoma, lung carcinoma, small-cell lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, bladder carcinoma, pancreatic carcinoma, stomach carcinoma, colon carcinoma, prostatic carcinoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, myeloma, multiple myeloma, adrenal carcinoma, renal cell carcinoma, endometrial carcinoma, adrenal cortex carcinoma, malignant pancreatic insulinoma, malignant carcinoid carcinoma, choriocarcinoma, mycosis fungoides, malignant hypercalcemia, cervical hyperplasia, leukemia, acute lymphocytic leukemia, chronic lymphocytic leukemia, chronic granulocytic leukemia, acute granulocytic leukemia, acute myelogenous leukemia, chronic myelogenous leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, polycythemia vera, essential thrombocytosis, Hodgkin's disease, non-Hodgkin's lymphoma, soft-tissue sarcoma, osteogenic sarcoma, primary macroglobulinemia, and retinoblastoma.

11. The method of claim 1 wherein the cells are stem cells.

12. The method of claim 11 wherein the stem cell is a mesenchymal stem cell or an induced pluripotent stem cell (iPSC).

13. Typically, the isolated mitochondria are functional mitochondria or dysfunctional mitochondria.

14. In some embodiments, the isolated mitochondria are modified mitochondria. As used herein, the term "modified mitochondria" refers to mitochondria harboring at least one modification in their composition. Typically, modified mitochondria refer to mitochondria isolated from a genetically modified source. As used herein, a genetic modified source refers to a cell harboring a foreign gene or foreign gene product. In some embodiments, the cells from which the modified mitochondria are derived are transfected with DNA comprising an expression cassette. An "expression cassette" refers to a natural or recombinantly produced polynucleotide that is capable of expressing a desired gene(s). The term "recombinant" as used herein refers to a polynucleotide molecule that is comprised of segments of polynucleotides joined

together by means of molecular biology techniques. The cassette contains the coding region of the gene of interest along with any other sequences that affect expression of the gene. A DNA expression cassette typically includes a promoter (allowing transcription initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include, but is not limited to, transcriptional enhancers, non-coding sequences, splicing signals, transcription termination signals, and polyadenylation signals. An RNA expression cassette may include a translation initiation codon (allowing translation initiation) and a sequence encoding one or more proteins.

15. The method of claim 1 wherein the mitochondria harbour a tracking probe, such as a fluorescent mitochondrial tracking probe.

16. The method of claim 15 wherein the tracking probe is a MitoTracker Probe selected from the group consisting of MitoTracker Orange CMTMRos, MitoTracker Orange CM-H2TMRos, MitoTracker Red CMXRos, MitoTracker Red CM-H2XRos, MitoTracker Red 580, and MitoTracker Deep Red 633.

17. The method of claim 1 wherein the centrifugation force is 1500g.

18. The method of claim 1 wherein the centrifugation step is performed at a temperature of 4°C.

19. The method of claim 1 wherein the centrifugation step is performed for a time of 15min.

20. The method of claim 1 wherein the resting step is performed at a temperature of 37°C.

21. The method of claim 1 wherein the resting step is performed for a time of 120 min.

22. The method of claim 1 wherein the cycle of step i) and step ii) is performed at least 1; 2; 3; 4; 5; 6; 7; 8; 9; or 10 times.

23. The method according to any one of claim 1 to 22 for improving energy metabolism of cells obtained from donors such as cells harbouring dysfunctional mitochondria, or cells harbouring mutated mtDNA.

24. A method of treating a condition which benefits from increased mitochondrial function in a subject in need thereof, said method comprising preparing a population of recipient

cells by the transfer method according to any one of claims 1 to 23 with an amount of functional mitochondria and administered the subject with a therapeutically effective amount of the prepared recipient cells.

25. The method of claim 24 wherein the condition is selected from the group consisting of a mitochondrial disease caused by ageing, a mitochondrial disease caused by damage to mtDNA, a mitochondrial disease caused by damage to nuclear genes and a mitochondrial disease caused by a toxin.

26. The method of claim 24 wherein the condition is selected from the group consisting of Diabetes mellitus and deafness (DAD), Leber's hereditary optic neuropathy (LHON), visual loss beginning in young adulthood, eye disorder characterized by progressive loss of central vision due to degeneration of the optic nerves and retina, Wolff-Parkinson-White syndrome, multiple sclerosis-type disease, Leigh syndrome, subacute sclerosing encephalopathy, neuropathy, ataxia, retinitis pigmentosa, ptosis, dementia, myoneurogenic gastrointestinal encephalopathy (MNGIE), gastrointestinal pseudoobstruction, myoclonic epilepsy with ragged red fibers (MER F), short stature, hearing loss, lactic acidosis, mitochondrial myopathy, encephalomyopathy, lactic acidosis, stroke-like symptoms (MELAS) and mitochondrial neurogastrointestinal encephalomyopathy.

27. The method of claim 24 wherein the functional mitochondria comprise at least one protein, or a gene encoding at least one protein, capable of inhibiting, ameliorating or preventing said disease or disorder associated with nonfunctional or dysfunctional mitochondria.

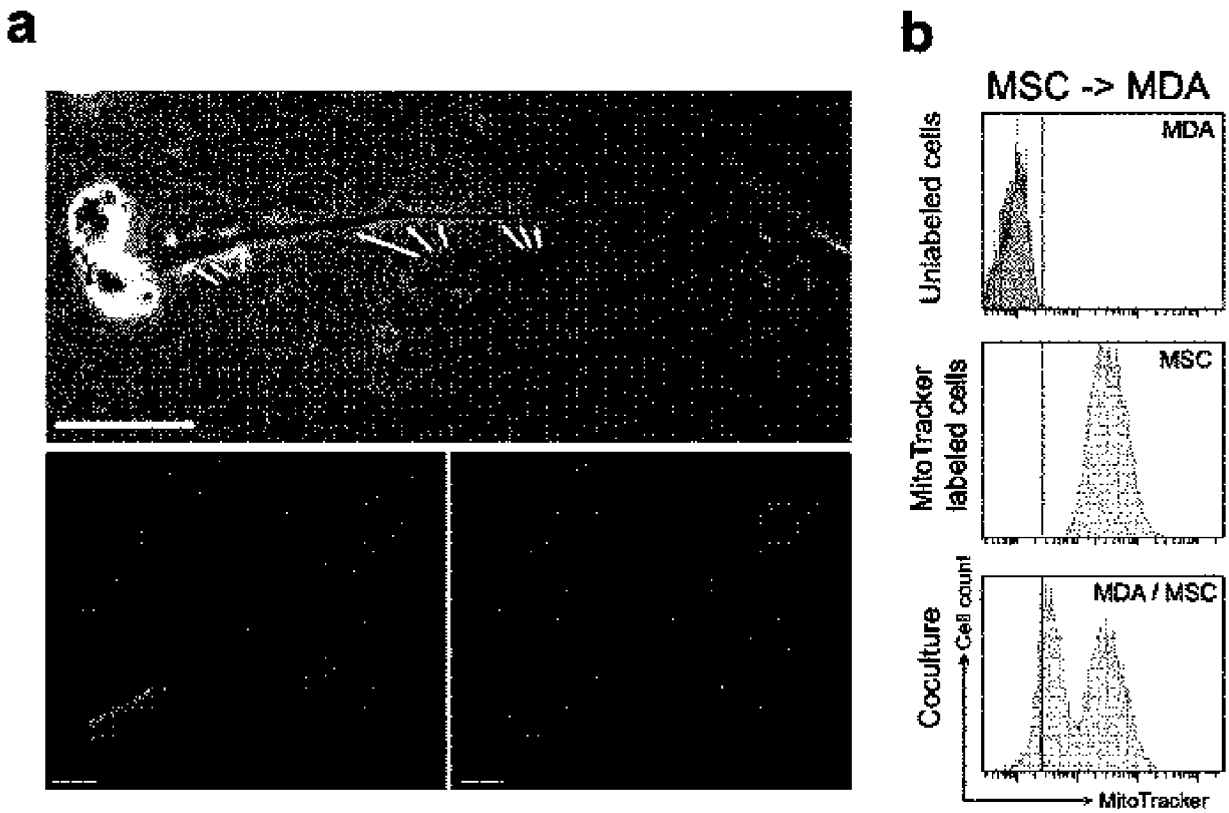


Figure 1

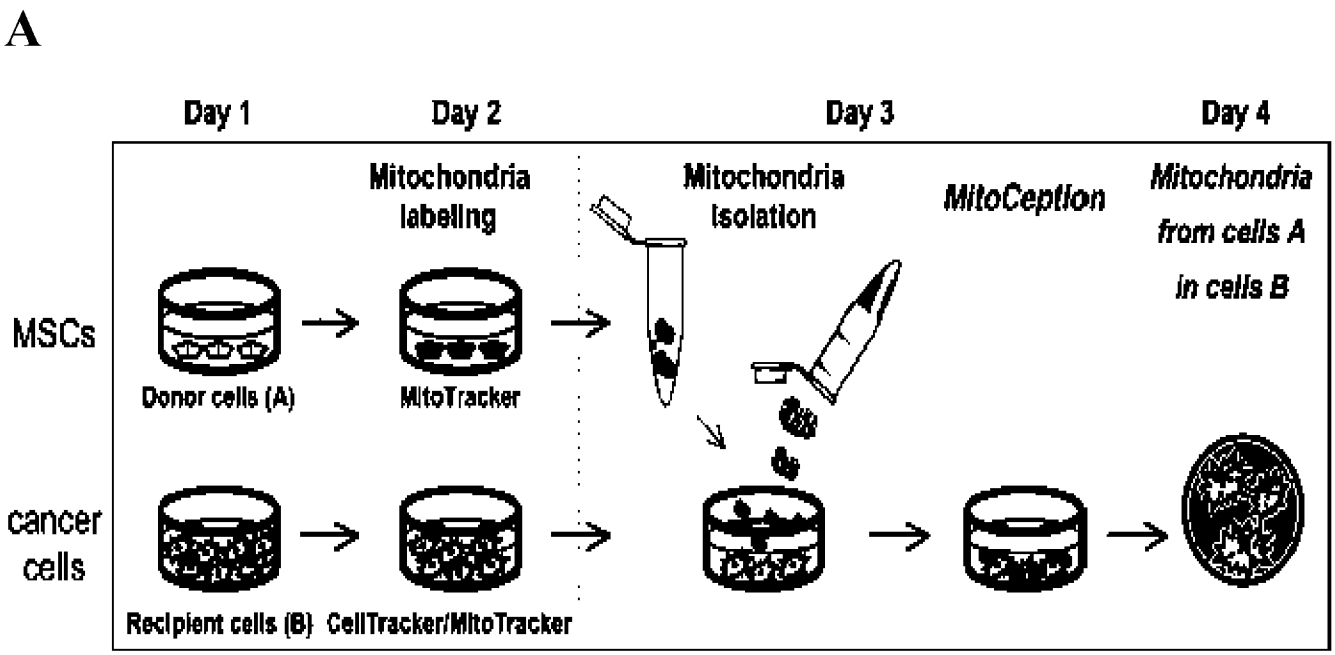


Figure 2A

B

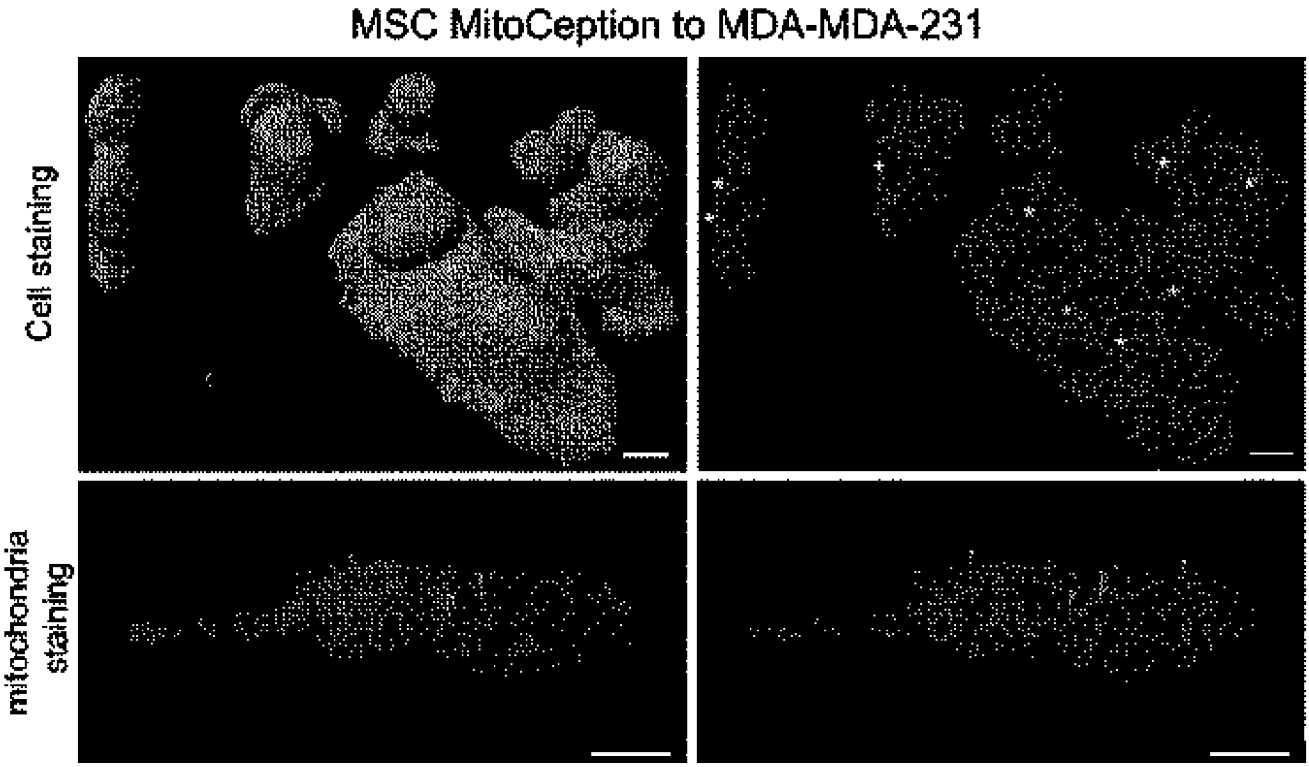


Figure 2B (1/2)

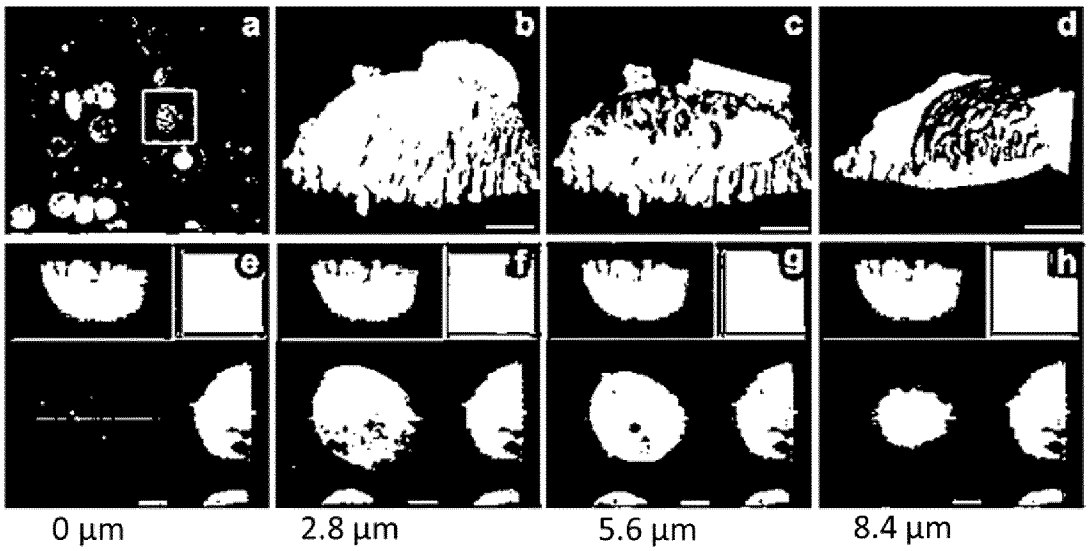


Figure 2B (2/2)

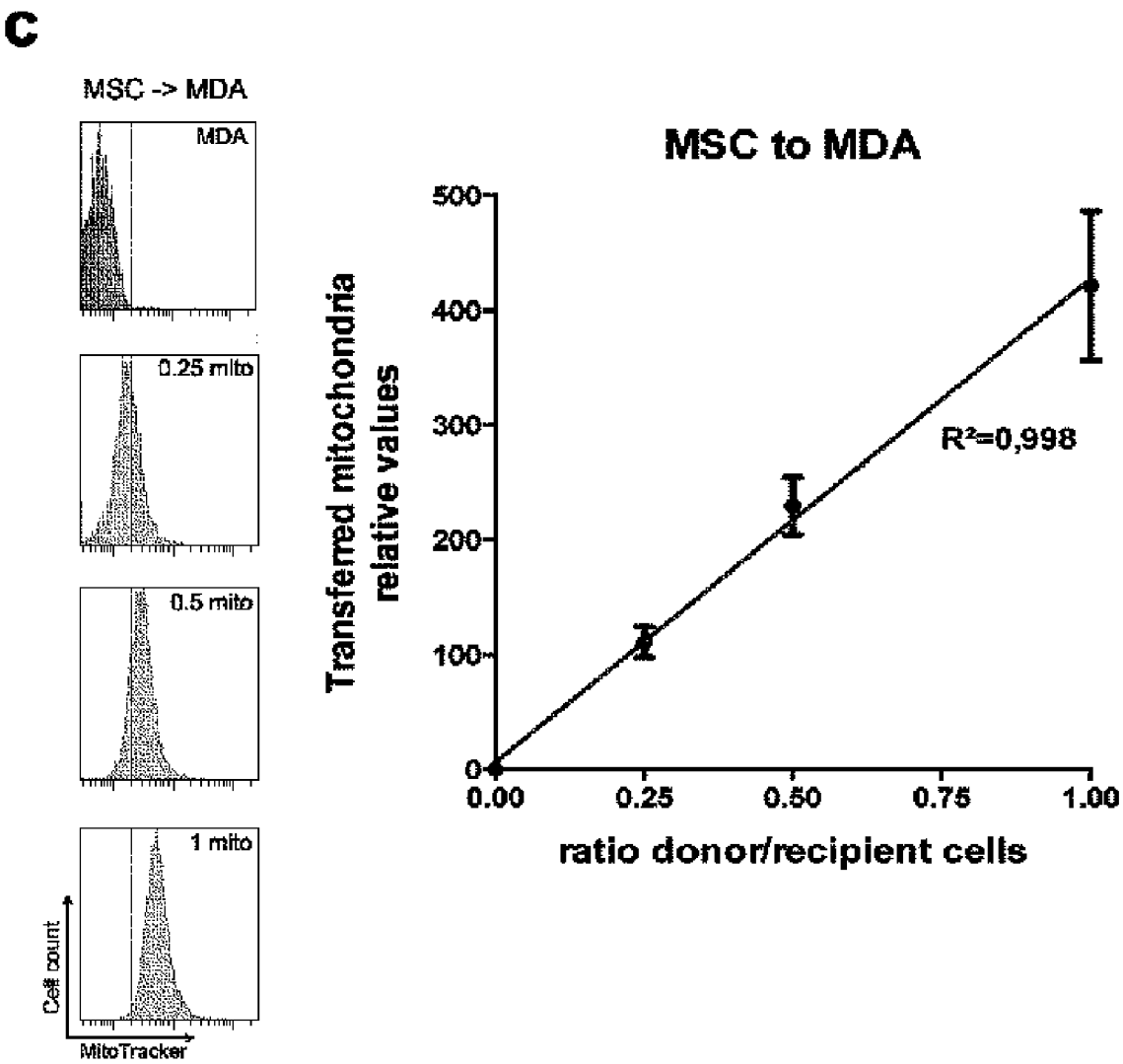
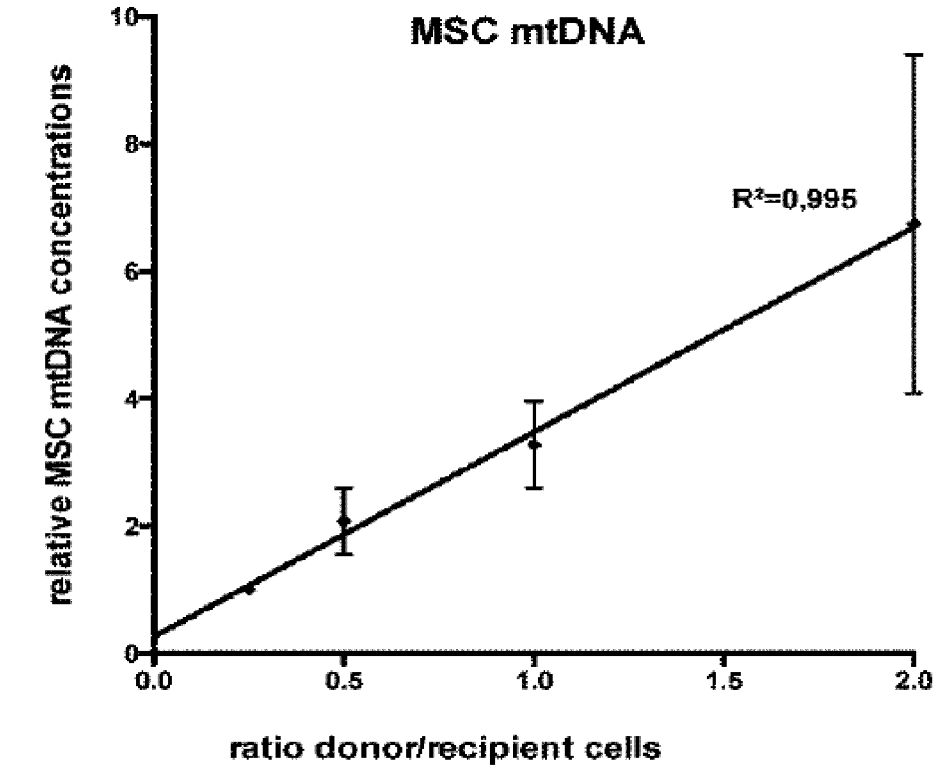

C
MSC -> MDA
MDA
0.25 mito
0.5 mito
1 mito
Cell count
MitoTracker
MSC to MDA
Transferred mitochondria relative values
500
400
300
200
100
0
0.00
0.25
0.50
0.75
1.00
ratio donor/recipient cells
R²=0,998

Figure 2C

D


MSC mtDNA
relative MSC mtDNA concentrations
R²=0,995
ratio donor/recipient cells

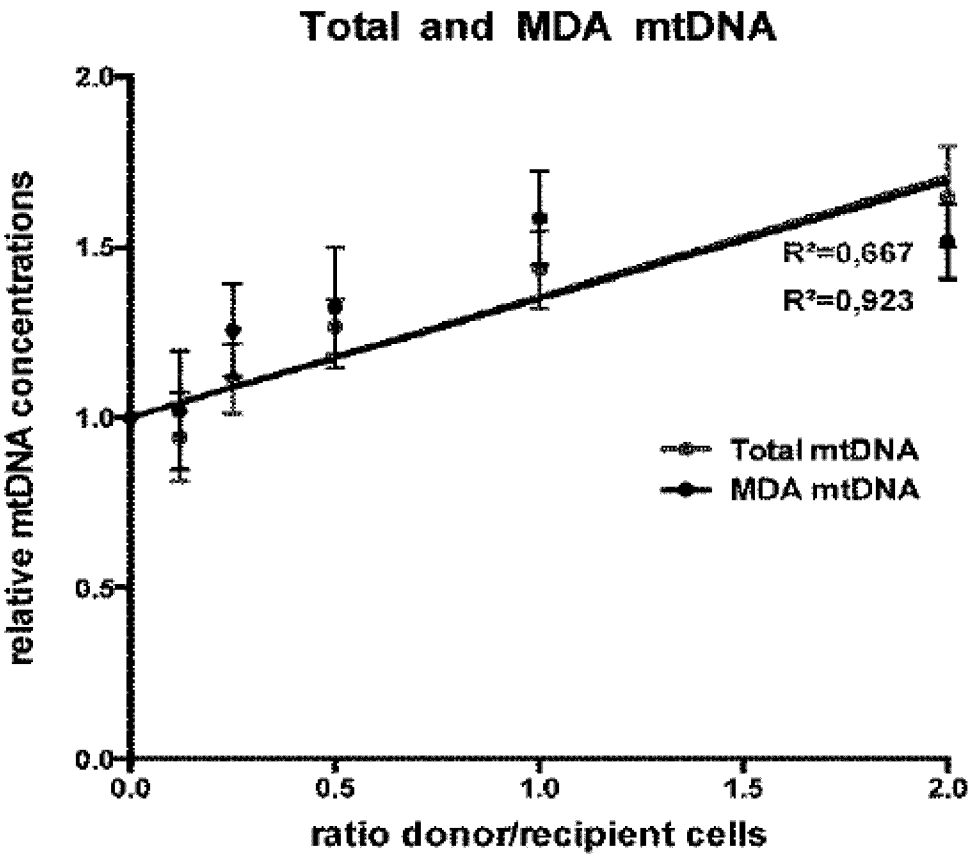

Total and MDA mtDNA
relative mtDNA concentrations
R²=0,667
R²=0,923
Total mtDNA
MDA mtDNA
ratio donor/recipient cells

Figure 2D

A

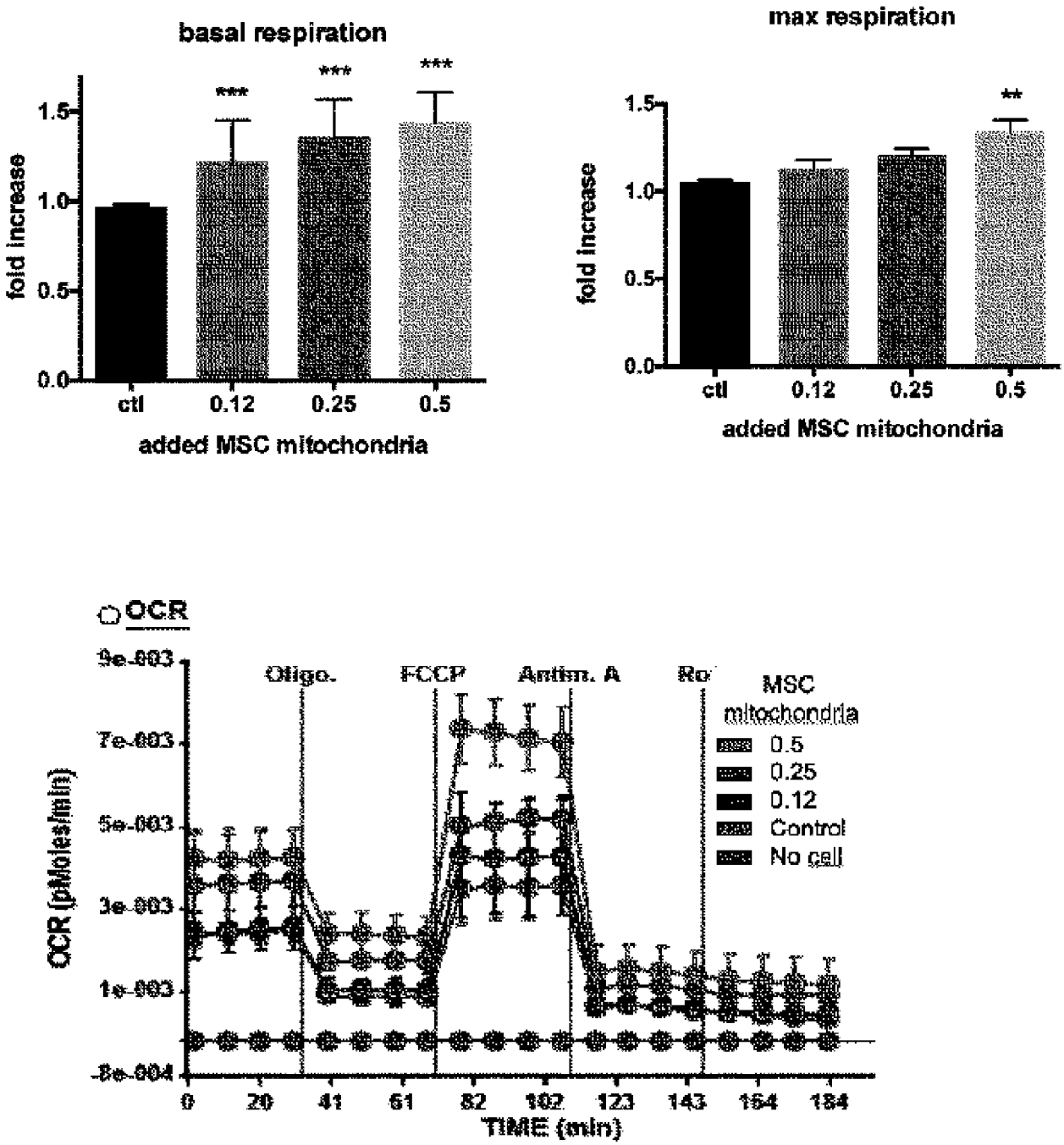
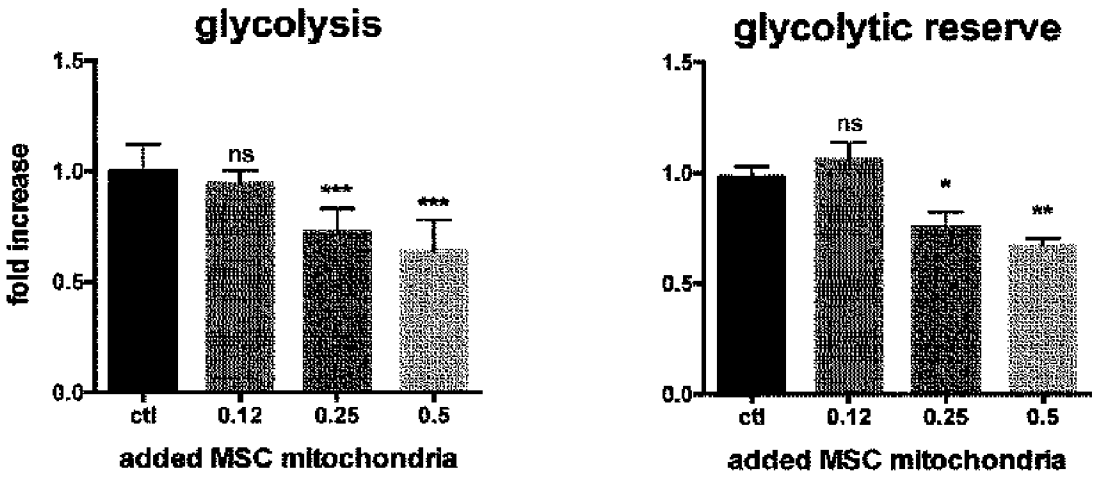

basal respiration
fold increase
1.5
1.0
0.5
0.0
***
***
***
ctl
0.12
0.25
0.5
added MSC mitochondria
max respiration
fold increase
1.5
1.0
0.5
0.0
**
ctl
0.12
0.25
0.5
added MSC mitochondria
OCR
OCR (pMoles/min)
9e-003
7e-003
5e-003
3e-003
1e-003
-3e-004
Oligo.
FCCP
Antim. A
Ro
MSC mitochondria
0.5
0.25
0.12
Control
No cell
0
20
41
61
82
102
123
143
164
184
TIME (min)

Figure 3A

B


glycolysis
fold increase
1.5
1.0
0.5
0.0
ns
***
***
ctl
0.12
0.25
0.5
added MSC mitochondria
glycolytic reserve
1.5
1.0
0.5
0.0
ns
*
**
ctl
0.12
0.25
0.5
added MSC mitochondria

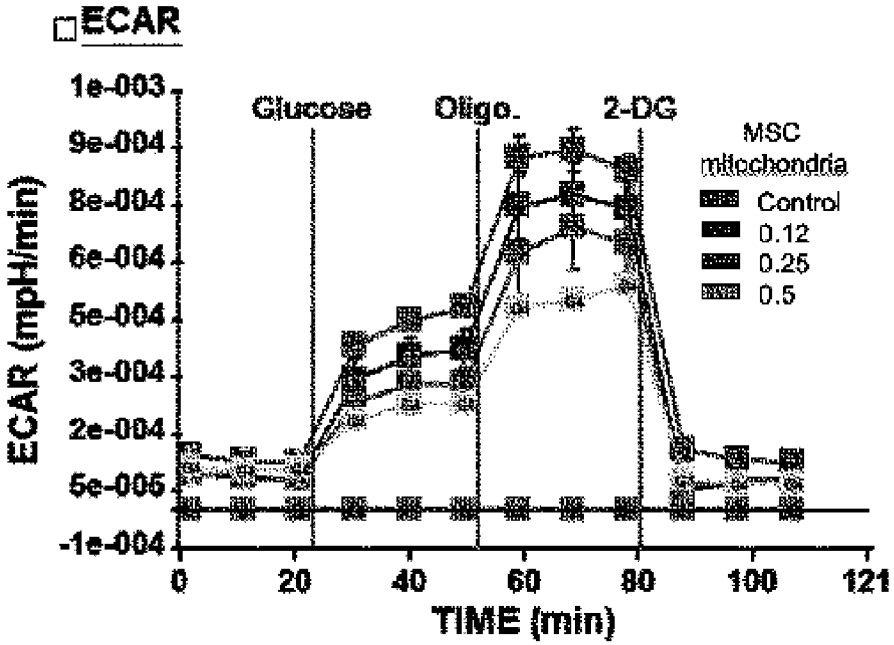

ECAR
ECAR (mpH/min)
1e-003
9e-004
8e-004
6e-004
5e-004
3e-004
2e-004
5e-005
-1e-004
Glucose
Oligo.
2-DG
MSC mitochondria
Control
0.12
0.25
0.5
0
20
40
60
80
100
121
TIME (min)

Figure 3B

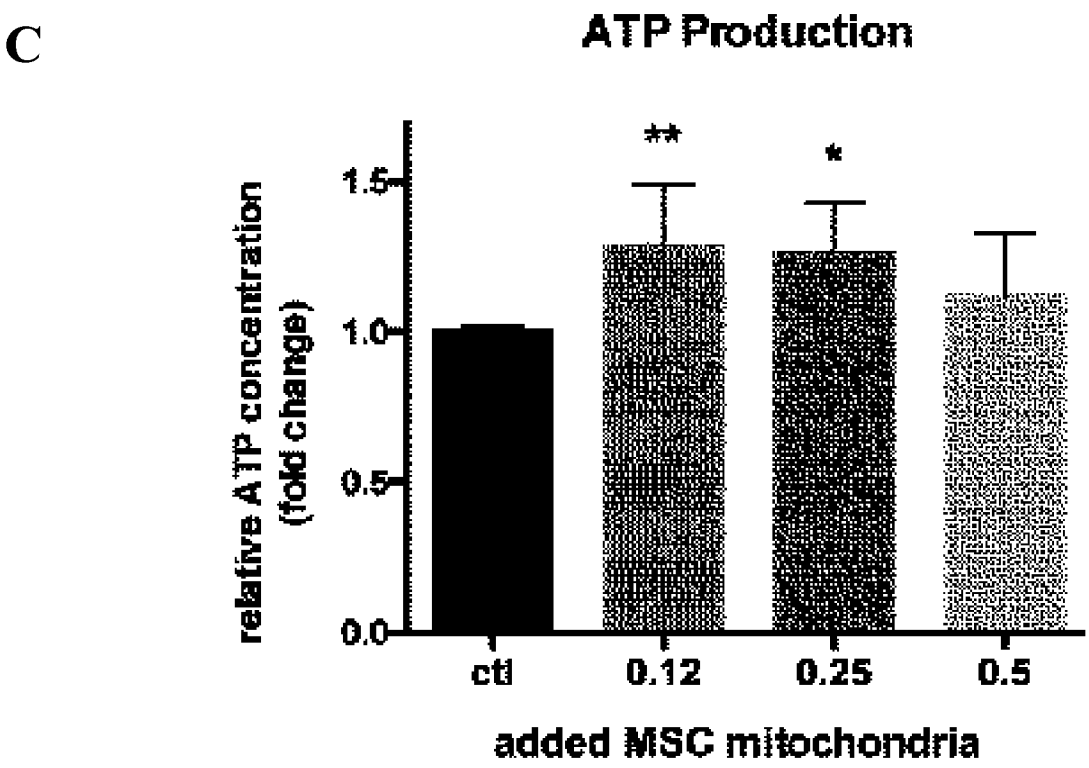


Figure 3C

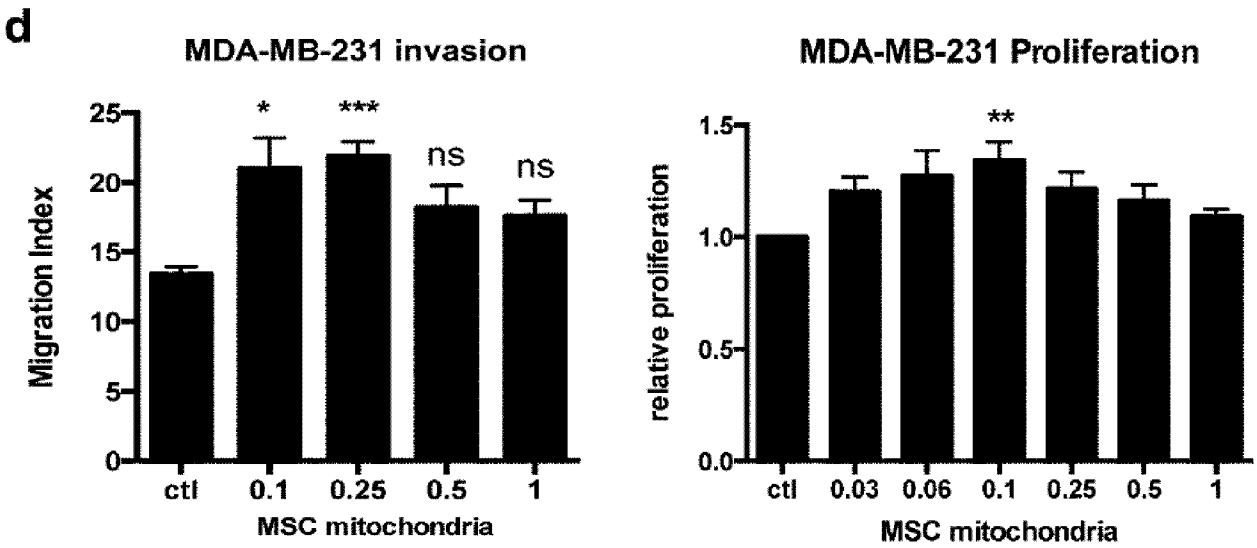


Figure 3D

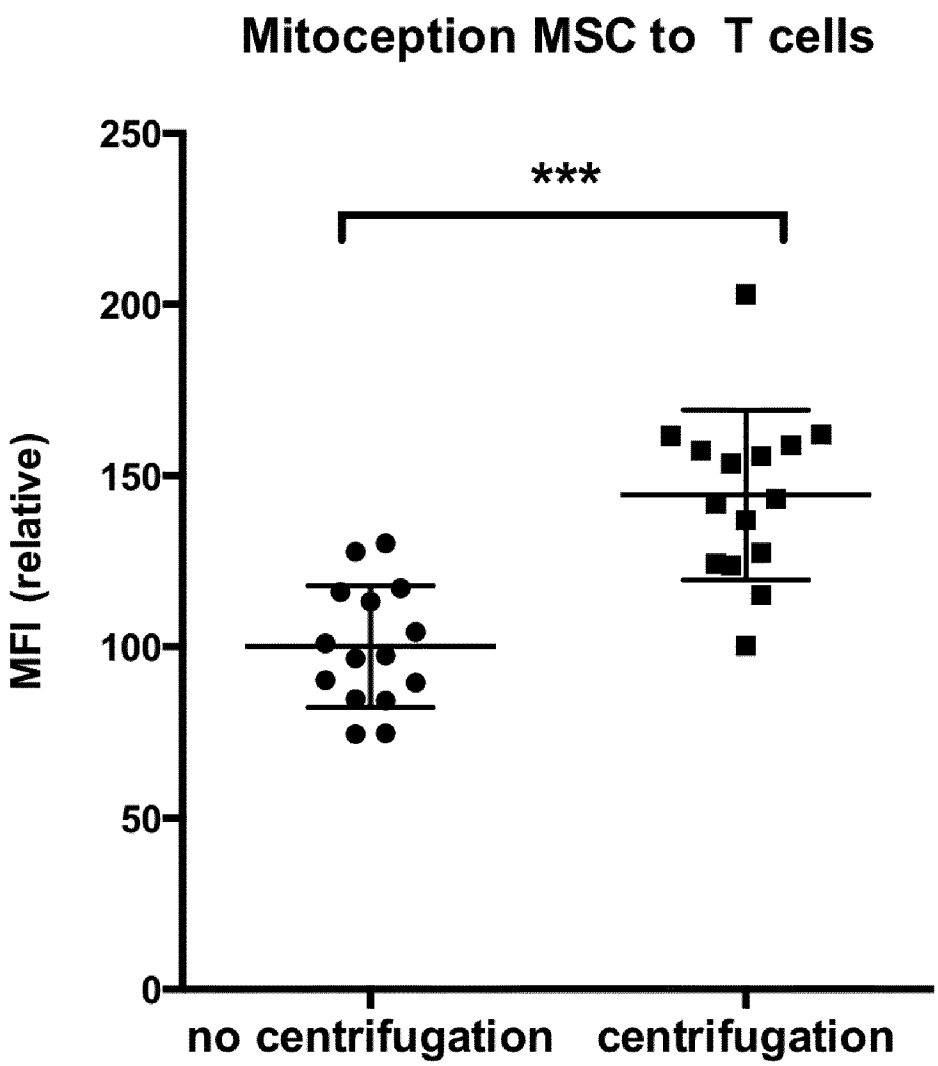

Mitoception MSC to T cells
250
200
150
100
50
0
MFI (relative)
***
no centrifugation
centrifugation

Figure 4

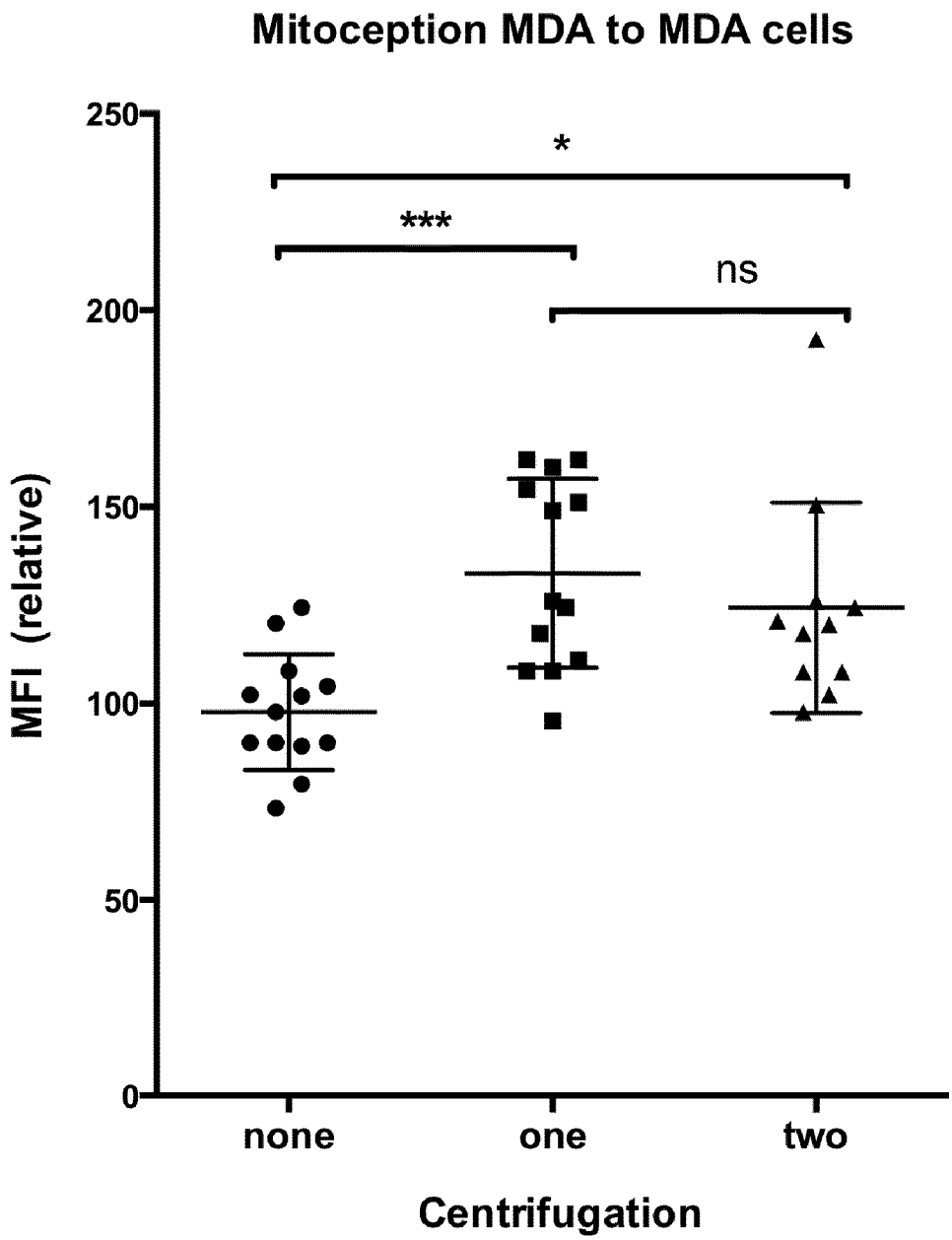

Mitoception MDA to MDA cells
250
200
150
100
50
0
MFI (relative)
*
***
ns
none
one
two
Centrifugation

Figure 5

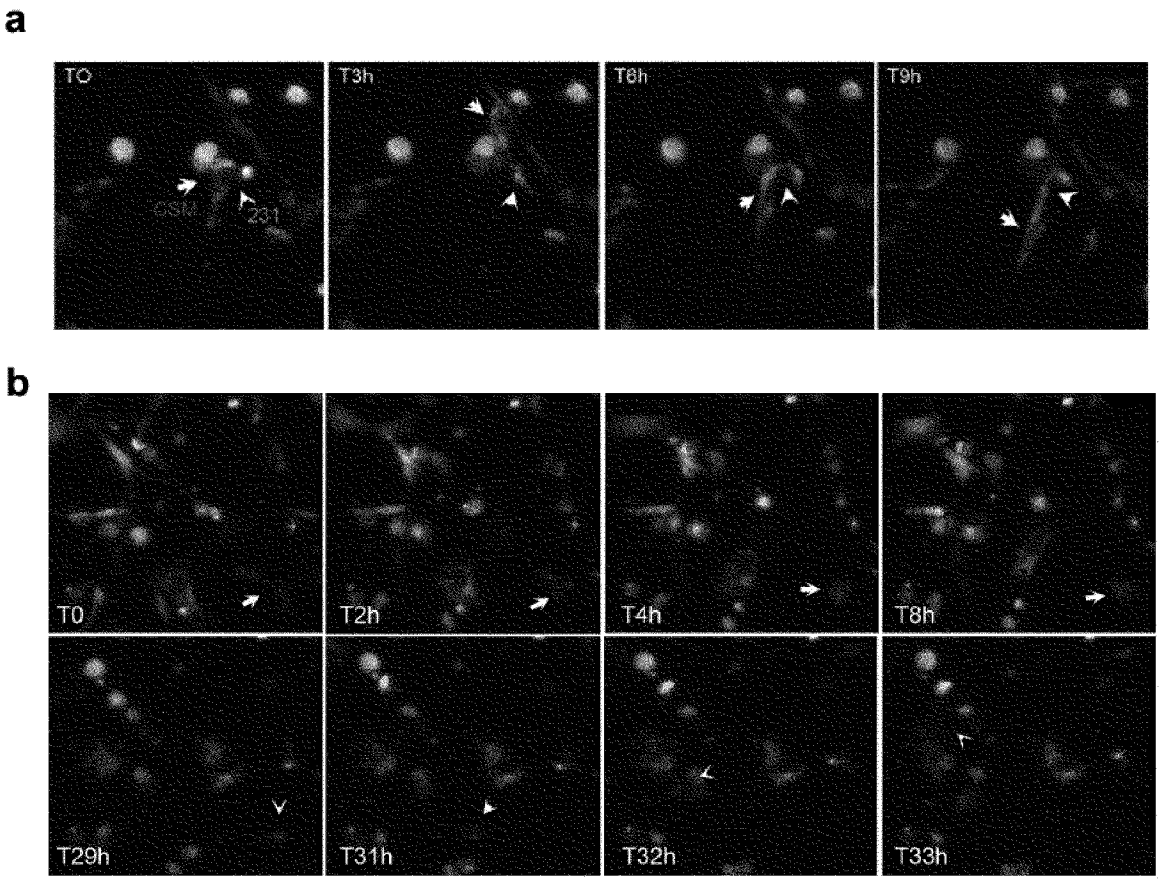


Figure6

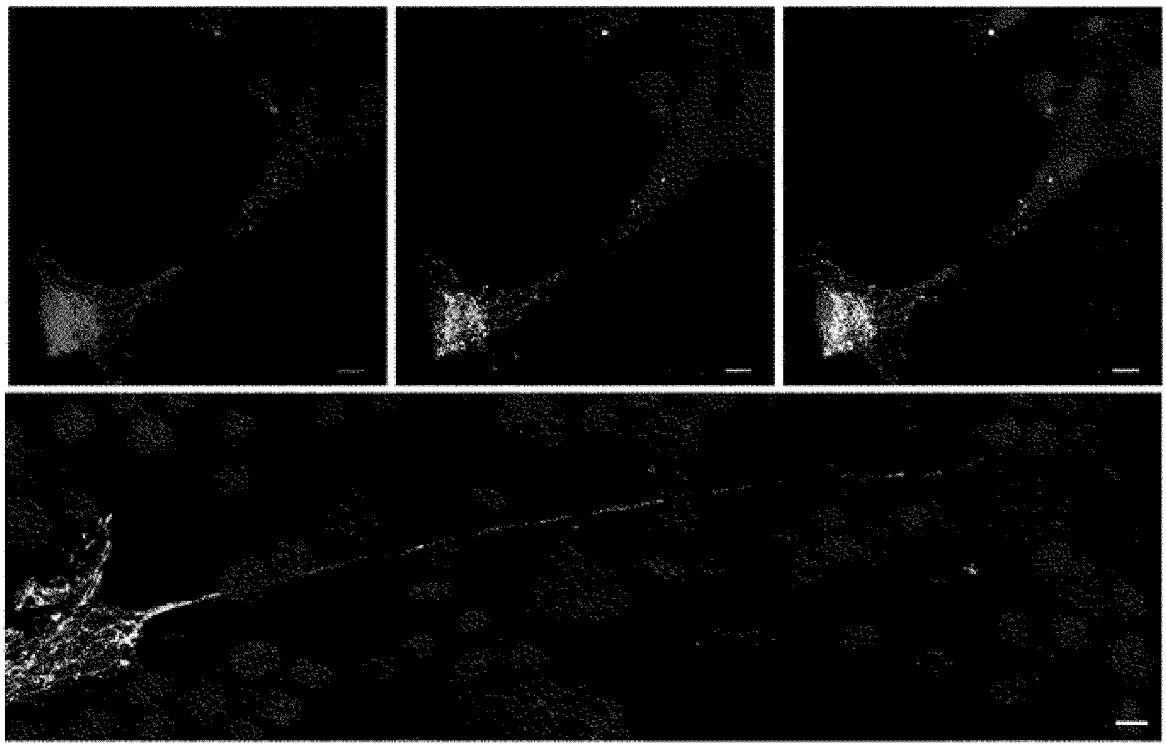


Figure 7

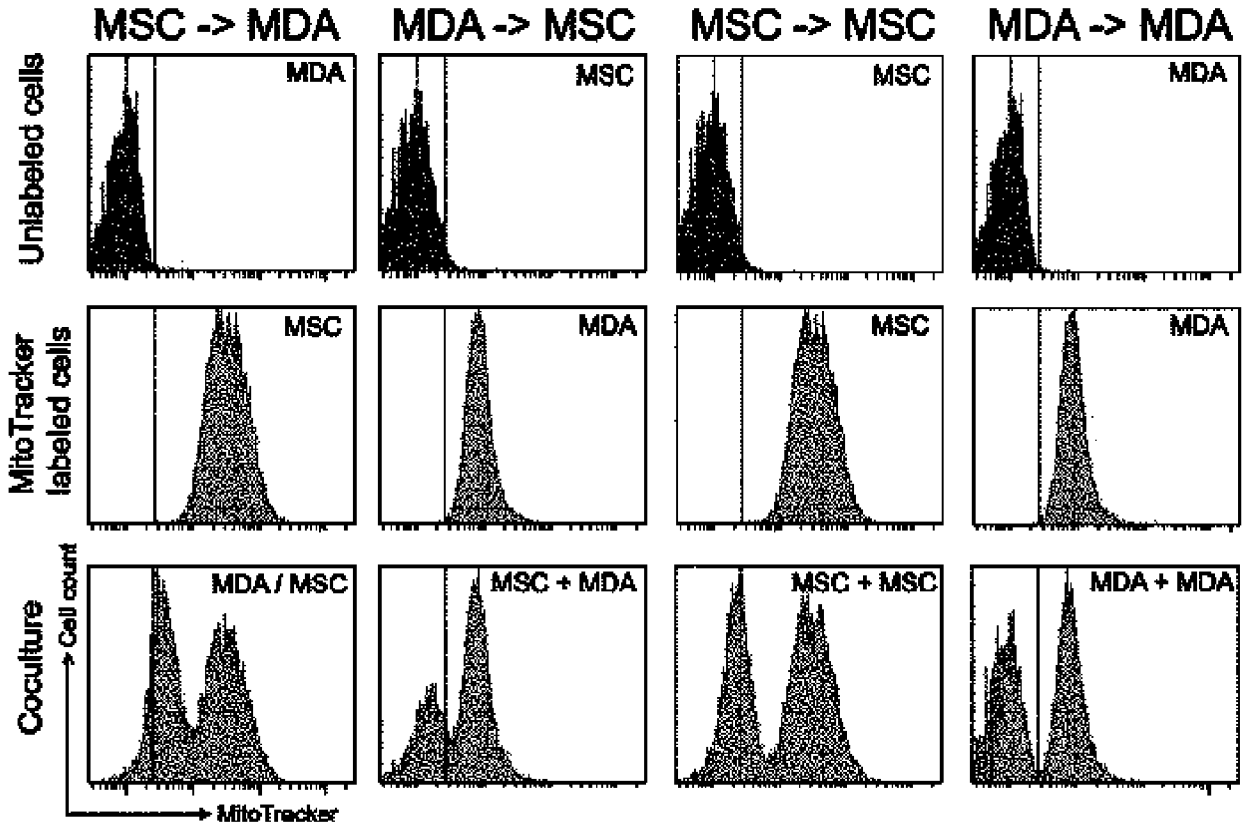

MSC -> MDA
MDA -> MSC
MSC -> MSC
MDA -> MDA
Unlabeled cells
MitoTracker labeled cells
Coculture
Cell count
MitoTracker
MDA
MSC
MDA / MSC
MSC + MDA
MSC + MSC
MDA + MDA

Figure 8

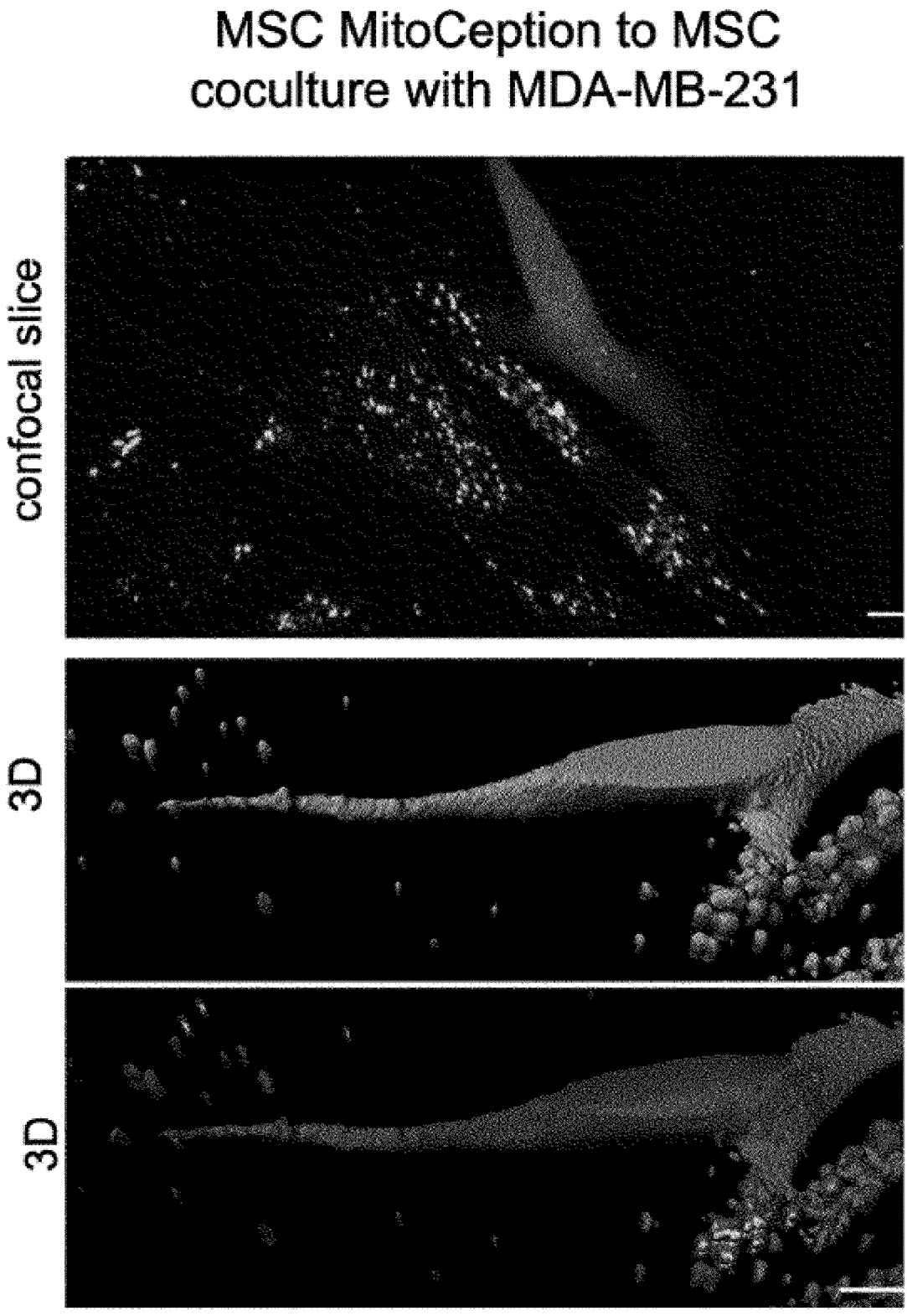

MSC MitoCeption to MSC coculture with MDA-MB-231
confocal slice
3D
3D

Figure 9

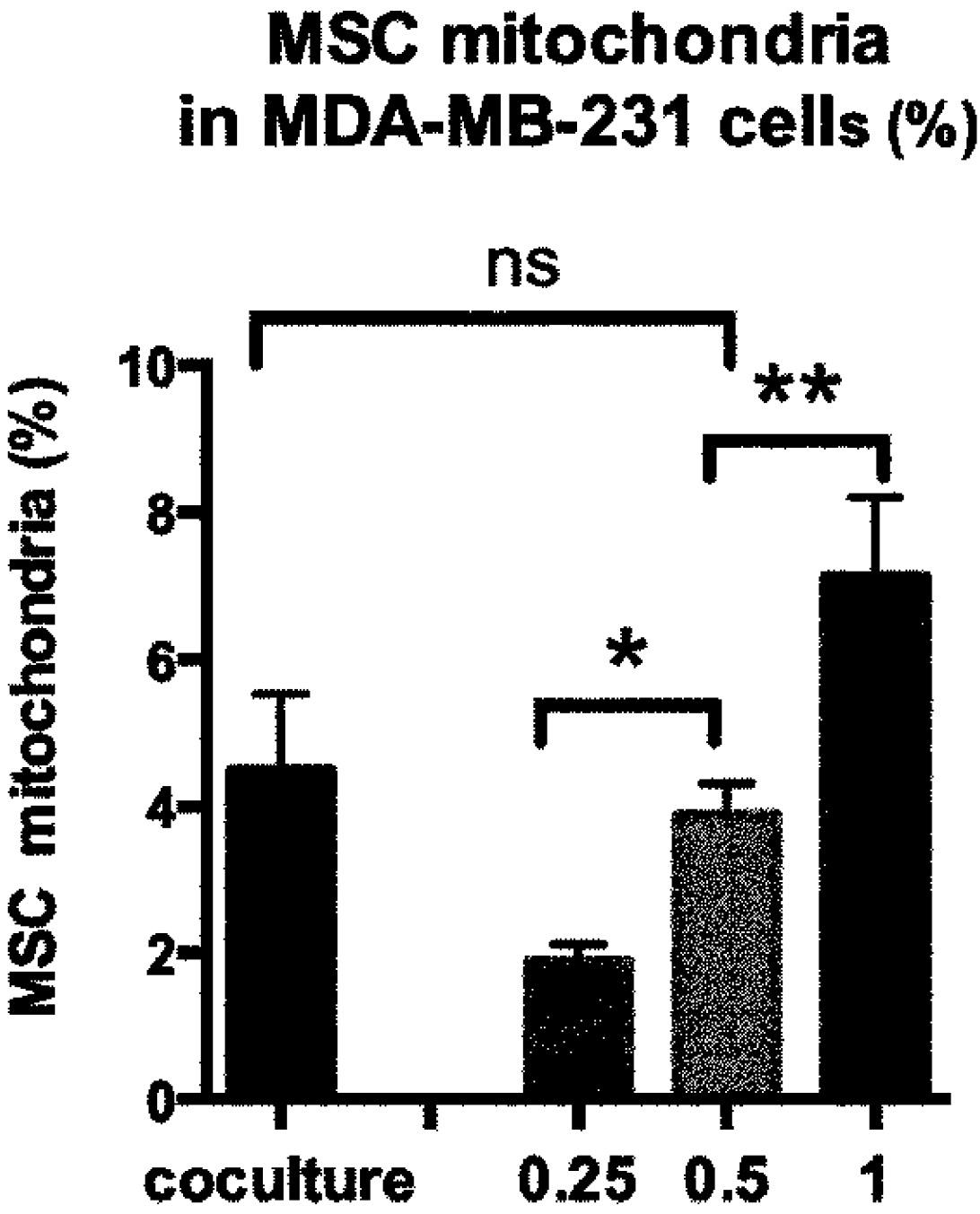

MSC mitochondria
in MDA-MB-231 cells (%)
ns
**
*
MSC mitochondria (%)
10
8
6
4
2
0
coculture
0.25
0.5
1

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066190

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K35/12 C12N5/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOMOYA KITANI ET AL: "Internalization of isolated functional mitochondria: involvement of macropinocytosis", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, 9 June 2014 (2014-06-09), pages 1-10, XP055135790, ISSN: 1582-1838, DOI: 10.1111/jcmm.12316 the whole document -----	24-27
X	US 2013/149778 A1 (CHANG JUI-CHIH [TW] ET AL) 13 June 2013 (2013-06-13) the whole document -----	24-27

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2015	30/09/2015

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Manu, Dominique

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/066190

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2013149778 A1	13-06-2013	US 2013149778 A1	13-06-2013
		US 2014178993 A1	26-06-2014