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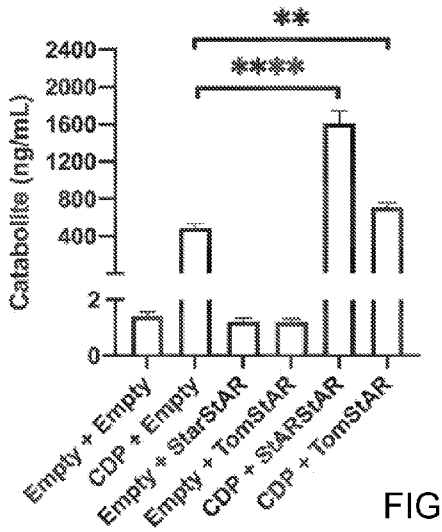

FIG. 1

(57) Abstract: Disclosed herein are compositions, methods, and systems for enhancing cholesterol degradation in a cell, tissue, or organism. In many embodiments, the disclosure describes the use of one or more proteins, or sequences coding therefor, to enhance flow of cholesterol into the mitochondrion, where the cholesterol is degraded by one or more proteins comprising bacteria-related sequences. The compositions, methods, and systems disclosed herein are useful in the prevention or treatment of diseases, disorders, and conditions associated with high levels of cholesterol in the blood or cells of a patient.

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ENHANCING MITOCHONDRIAL-BASED FLOW AND CATABOLISM OF CHOLESTEROL

FIELD

[001] The disclosed processes, methods, and systems are directed to treating a subject with high levels of cholesterol or at risk thereof by enhancing cholesterol shuttling into and degradation within the mitochondria.

CROSS-REFERENCE TO RELATED APPLICATIONS

[002] This application claims benefit of priority pursuant to 35 U.S.C. § 119(e) of U.S. provisional patent application No. 63/122,886 entitled "Enhancing mitochondrial-based flow and catabolism of cholesterol," filed on 8 December 2020, which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on December 7, 2021, is named P289347_WO_01_SL.txt and is 52,905 bytes in size.

BACKGROUND

[004] High cholesterol (or hypercholesterolemia) is the presence of high levels of cholesterol in the blood that may be the result of poor diet, lifestyle, disease (e.g. diabetes) or genetics. High concentrations of cholesterol within the cell is a characteristic of several diseases, for example fatty liver disease, atherosclerosis, etc.

[005] Atherosclerosis involves narrowing of arteries resulting from a buildup of cholesterol-laden lipoproteins within the arterial wall. Often a precursor to leading causes of death including cardiovascular disease (CVD), myocardial infarction, stroke and peripheral vascular disease. Many current therapies for these diseases focus on preventing a second event (or a primary event in high-risk individuals) by reducing the circulating levels of low density lipoproteins (LDLs) and/or increasing high-density lipoproteins (HDLs), because although mammalian cells are able to synthesize cholesterol, they cannot break it down (catabolize it).

[006] What is needed are compositions and methods for reducing lipid and cholesterol levels in subjects suffering from excess lipid and cholesterol.

SUMMARY

[007] Disclosed herein are compositions, methods, and systems for enhancing transport of cholesterol (including various modified cholesterol compounds) into modified mammalian mitochondria, wherein the modification renders the mitochondria capable of catabolizing cholesterol. In many embodiments, cells may be engineered for enhanced cholesterol flow across the mitochondrial membrane. The cells may also include one or more proteins that may aid in transport of cholesterol across the mitochondrial membrane.

[008] Also disclosed are methods of making the disclosed compositions, and methods of using the disclosed compositions and methods for treating a subject having or at risk of developing high cholesterol, and may aid in reducing cholesterol in a subject. In various embodiments, the disclosed compositions may be administered to a subject in need of treatment for high cholesterol. In many embodiments, the nucleic acids expressing the disclosed proteins may be incorporated into a mammalian cell's genome or may be extra-genomic. The disclosed cells may be selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[009] Disclosed herein are compositions, methods, and systems for modifying mammalian mitochondria to enhance cholesterol transport and degradation. In many embodiments, mammalian mitochondria are engineered to comprise one or more recombinant and/or non-endogenous proteins that may help enhance cholesterol flow across the mitochondrial membrane. In many embodiments, the mitochondria may also include one or more proteins that may aid in catabolism of cholesterol. The disclosed modified mitochondria may be useful in reducing intracellular cholesterol concentrations, increasing cholesterol transport across the mitochondrial membrane, and/or reducing concentrations of cholesterol in the blood of a patient or subject at risk of developing, or suffering from high cholesterol or hypercholesterolemia.

[0010] In another aspect, a method of modifying a mammalian mitochondrion includes inserting at least one non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof into the mammalian mitochondrion outer membrane, and where the mitochondrion includes one or more of a cholesterol degrading protein (CDP) includes cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0011] In various aspects of the methods and compositions disclosed herein, the StAR protein may comprise residues 63 to 188 of SEQ ID NO:7, a duplication thereof, or at least a portion of the TOMM20 protein. The CDP may comprise P450 or P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx). The StAR protein and the CDP may comprise at least one mitochondrial signaling protein and/or be expressed from a cell-specific promoter sequence, and/or be expressed from a genome-integrated or from an extra genomic gene, and/or at least about 10% of the total amount of StAR protein and CDP in the cell may be located in a mitochondrial membrane. The disclosed methods and compositions may be useful in treating a subject suffering from or at risk of developing high cholesterol, such as by reducing cholesterol in the subject having high cholesterol. The cell may be selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 depicts the mean catabolite concentration in culture media of cells expressing CDP in the presence and absence of StAR-StAR or Tom-StAR (*n=3* independent experiments).

[0013] FIG. 2 is a representative western blot showing protein expression levels of tubulin, CDP, StAR-StAR, and TomStAR in cells of FIG. 1 (*n=3* independent experiments).

[0014] FIG. 3 bar chart showing cholesterol degradation and consequent production of catabolite is greatly increased in vitro by the expression of StAR in mouse RAW264.7 cell lines expressing CDP.

[0015] FIG. 4 is a Western blot showing protein amounts in cell lines described in FIG. 3.

[0016] FIG. 5 are sequences of various proteins and nucleic acid coding sequences disclosed herein. Specifically, the Human protein sequence for p450-FdxR-Fdx (SEQ ID NO:1) is depicted with the Flag tag in bold; Cholesterol dehydrogenase, CholD (SEQ ID NO:2), is shown; n3-ketosteroid Δ1-dehydrogenase, Δ1-KstD (SEQ ID NO:3), is shown; Anoxic cholesterol metabolism B enzyme, acmB (SEQ ID NO:4) is shown; 3-ketosteroid 9α-hydroxylase, KshAB (SEQ ID NO:5) is shown; 3β-hydroxysteroid dehydrogenase 2, HSD2 (SEQ ID NO:6) is shown; Human StAR protein sequence (SEQ ID NO:7) is shown; Human TOMM20 protein sequence (SEQ ID NO:8) is shown; Human Tom/Star Fusion protein sequence (SEQ ID NO:9; Tom sequence is depicted in bold letters and the linker sequence is depicted in color and underlined) is shown; Human StAR/StAR

fusion protein sequence (with linker sequence in color and underlined (SEQ ID NO:10); StAR mRNA complete sequence (SEQ ID NO:11; start codon shown capitalized) is depicted with the coding sequence corresponding to the underlined amino acids, 63-285, of SEQ ID NO 7 depicted in UPPERCASE lettering; and the TOMM20 mRNA complete sequence, CDS = 123-560 (SEQ ID NO:12; start codon capitalized and stop shown in red) is also shown.

DETAILED DESCRIPTION

[0017] Disclosed herein are compositions, methods, and systems for reducing cholesterol in a subject's cells and/or blood stream by enhancing transport of cholesterol into mitochondria where it is subjected to catalysis by cholesterol catabolizing enzymes.

[0018] Disclosed herein is the development of a unique approach to help manage hypercholesterolemia and other disorders related to the presence of excessive cholesterol. Humans lack enzymes to degrade cholesterol and many mammalian cells take up excess cholesterol without a way to properly store or metabolize it. In some cases, macrophages progress to foam cells as they fill with cholesterol and cholesterol esters. This buildup of cholesterol may lead to formation of arterial plaques that can lead to atherosclerosis. Depending on their location, plaques may cause coronary artery disease, angina (chest pain), intermittent claudication (leg pain with exercise) or chronic kidney disease due to poor circulation. Moreover, many plaques can be unstable and prone to rupture even before they have any significant effect on blood flow. These structures are typically clinically silent until they rupture (complicated plaques) and predispose the underlying tissue to clots which may suddenly occlude the affected blood vessel.

[0019] Applicants show herein that human cells may be engineered to aid in reducing cholesterol. In many embodiments, the cells may be any mammalian cell. The disclosed cells may be engineered to express various proteins that aid in transport and degradation/catabolism of cholesterol. As used herein, an engineered cell may be a cell with one or more non-native and/or exogenous coding sequences or protein, for example an engineered mammalian cell may include one or more mammalian and/or bacteria-related sequences or proteins. Non-native, exogenous, or non-endogenous, as used herein may refer to a sequence or protein that is introduced to the cell, in most cases by recombinant methods. In most embodiments, bacteria-related may refer to a protein or coding sequence with greater than about 50% identity with a similar bacterial sequence. In many embodiments, the disclosed proteins may be fusion proteins and/or modified proteins, for example a protein lacking a sequence found in native proteins and/or duplicating sequences found in native proteins.

[0020] The disclosed proteins may be over-expressed relative to endogenous genes and/or proteins. In one example an endogenous gene or protein may be mammalian p450scc, StAR, or TOMM20 protein. In most embodiments, over-expression of the disclosed proteins may result in 2X, 3X, 4X, 5X, 10X, 20X, 100X, 200X, based on weight or moles, or more of the disclosed gene or protein in a given sample compared to the endogenous gene or protein. In most cases, overexpression is relative to non-induced expression of the endogenous protein. In some cases, over-expression may be relative to induced levels of endogenous protein, wherein expression may be similar or the same. In most embodiments, degradation in reference to degradation of cholesterol, refers to removal of various side chains and/or opening of at least one ring of cholesterol.

[0021] The disclosed coding sequences and proteins for cholesterol degradation may be derived from bacteria and/or mammals. In many embodiments, the disclosed proteins may be referred to as cholesterol degrading proteins (CDP) and may be selected from one or more of cholesterol dehydrogenase (CholD; SEQ ID NO:2), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD; SEQ ID NO:3), anoxic cholesterol metabolism B enzyme (acmB; SEQ ID NO:4), 3-ketosteroid 9α-hydroxylase (KshAB; SEQ ID NO:5), 3β-hydroxysteroid dehydrogenase 2 (HSD2; SEQ ID NO:6), a P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx; SEQ ID NO:1; uniprot numbers P05108, P22570, P10109), and steroidogenic acute regulatory protein (StAR; SEQ ID NO:7). In many embodiments, the disclosed coding sequences and proteins may share greater than about 80% identity to a sequence(s) disclosed herein.

[0022] The disclosed coding sequences and proteins may be delivered to the cell in-vivo or in-vitro, for example by various methods including electroporation, transfection, viral vector, nanoparticle, etc. In some embodiments, the disclosed coding sequences and proteins may be encoded by one or more nucleic acids within a cell, vector, or particle. In some embodiments, the particle may be lipid nanoparticle (or lipo nanoparticle; LNP). In some embodiments, the vector may be viral vector, such as adeno virus or lentivirus. In many embodiments, the coding sequences may include one or more promoter and/or enhancer sequences that may aid in expressing the coded proteins in a specific cell or tissue, or may aid in supporting high expression of the coded proteins, generally, or in a specific cell or tissue.

[0023] The disclosed proteins, which may be referred to collectively or singularly as cholesterol degrading proteins (CDPs) may be directed to the mitochondria of a mammal, for example the outer membrane, inner membrane, intermembrane space, or matrix.

[0024] The term "about" or "approximately" means an acceptable error for a particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured or determined. In certain embodiments, the term "about" or "approximately" means within 1, 2, 3, or 4 standard deviations. In certain embodiments, the term "about" or "approximately" means within 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, or 0.05% of a given value or range. Whenever the term "about" or "approximately" precedes the first numerical value in a series of two or more numerical values, it is understood that the term "about" or "approximately" applies to each one of the numerical values in that series.

[0025] Administration of the disclosed compositions, constructs, cells, methods, and systems may be via various methods. In one embodiment, administration may be "intravenous" administration, referring to introducing the compositions, constructs, and/or cells into a vein of a patient, e.g. by infusion (slow therapeutic introduction into the vein). In other embodiments, administration may be "subcutaneous" wherein introduction is beneath the skin of the patient. "Infusion" or "infusing" refers to the introduction with a solution into the body through a vein for therapeutic purposes. Generally, this is achieved via an intravenous (IV) bag, such as a bag that can hold a solution. The disclosed compositions, constructs, cells, methods, and systems may be "co-administered" to a patient, for example by intravenously administering at least a second therapeutic agent during the same administration. In some embodiments, co-administration is concurrent, in others co-administration is sequential.

[0026] The term "prevention" as used herein means the avoidance of the occurrence or of the re-occurrence of a disease as specified herein, or at least one symptom associated therewith, by the administration of a composition, construct, method, or system according to the invention to a subject in need thereof.

[0027] A "patient" or "subject" includes various animals including a human, monkey, cat, dog, mouse, rat, rabbit or guinea pig. The animal can be a mammal such as a non-primate and a primate (e.g., monkey and human). In one embodiment, a patient is a human, such as a human infant, child, adolescent or adult.

[0028] The terms "treat", "treating" and "treatment" refer to eliminating, reducing, suppressing, or ameliorating, either temporarily or permanently, either partially or completely, at least one symptom, manifestation, or progression of an event, disease, disorder, or condition disclosed herein. As is recognized in the pertinent field, methods, compositions, and systems employed as therapies may reduce the severity of a given disease state, but need not abolish every symptom associated therewith to be regarded as useful. Similarly, a prophylactically administered treatment need not be completely

effective in preventing the onset of a condition to constitute a viable prophylactic composition, agent, method or system. Simply reducing the impact of a disease (for example, as disclosed herein, reducing blood cholesterol concentrations and/or reducing the number or severity of associated symptoms, or by increasing the effectiveness of another treatment, or by producing another beneficial effect), or reducing the likelihood that the disease will occur or worsen in a subject, is sufficient.

[0029] The term "effective amount" refers to an amount of a compound of the invention or other active ingredient sufficient to provide a therapeutic or prophylactic benefit in the treatment or prevention of a disease or to delay or minimize symptoms associated with a disease. Further, a therapeutically effective amount with respect to a compound of the invention means that amount of therapeutic agent alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or prevention of a disease. Used in connection with a compound of the invention, the term can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of disease, or enhances the therapeutic efficacy or synergies with another therapeutic agent.

[0030] The phrase "therapeutically effective amount" means an amount of a composition of the present invention that (i) treats the particular disease, condition, or disorder, (ii) ameliorates or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) prevents or delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein. In the case of excess blood cholesterol, the therapeutically effective amount of the disclosed compositions may reduce the concentration of cholesterol in the patient's blood; reduce intercellular cholesterol; inhibit (i.e., slow to some extent and preferably stop) cholesterol plaque growth and/or development; inhibit (i.e., slow to some extent and preferably stop) lipid buildup in the liver; reduce lipid content in the liver; and/or relieve to some extent one or more of the symptom associated with the high concentrations of blood cholesterol.

[0031] The term "amelioration" as used herein refers to any improvement of a disease state (for example high blood cholesterol) of a patient, by the administration of one or more treatments and/or compositions, according to the present disclosure, to such patient or subject in need thereof. Such an improvement may be seen as a slowing down or stopping the progression of the disease of the patient, and/or decreasing the severity of at least one disease symptom, an increase in frequency or duration of disease symptom-free periods or a prevention of impairment or disability due to the disease.

Target Cells

[0032] Various tissues and cell types made to express CDP may be targeted with the disclosed compositions. In various embodiments, the cells may be endothelial and epithelial cells in all organs and tissues, including, without limitation, smooth, skeletal and cardiac muscle cells, neuronal cells, glandular cells, bone cells, immune cells, hematopoietic cells, and stem cells or precursor cells thereof. In some embodiments, the cell maybe a pluripotent stem cells, for example an induced pluripotent stem cell. In many embodiments, the tissues may be cancerous or pre-cancerous, and may one or more cells of skin and intestinal carcinomas (squamous epithelial cells), adenocarcinoma (epithelial cells of glandular origin, pancreatic) gastrointestinal carcinoid (neuroendocrine cells), pancreatic cancer, small cell carcinoma (neuroendocrine cells), leiomyosarcoma (smooth muscle cells) and lymphomas (lymphocytes found in organs/walls of the gastrointestinal tract).

[0033] Various cells and tissues may be targeted with the disclosed compositions and methods. In many embodiments, the disclosed compositions may be targeted to specific cells by various methods known to those of skill in the art. In some embodiments, LNPs may include one or more molecules that help target specific cells, for example a protein with affinity for a receptor or membrane protein on the target cell.

Cholesterol degrading proteins

[0034] Disclosed herein are various cholesterol, and cholesterol-related genes and proteins. In some embodiments, the disclosed genes and proteins may aid in transport and/or metabolism of cholesterol. In most embodiments the disclosed genes and proteins may be expressed in and/or targeted to the mitochondria. In many embodiments the disclosed genes and proteins may allow cells to degrade and/or catabolize cholesterol to ring opening, whereupon endogenous proteins and enzymes may further metabolize the molecule.

Mitochondria

[0035] Mitochondria are found in most eukaryotic cells and organisms, and consist of a double membrane. Mitochondria supply the cell's energy, and play a role in signaling, cell-cycle, cell growth and differentiation, apoptosis, and cell death. Cells with high demand for energy, such as heart, muscle, brain, and liver cells, may have several thousand mitochondria.

[0036] The double-membrane structure of the mitochondria allows for specialized compartments: the outer membrane, the intermembrane space, the inner membrane,

and the cristae and matrix. These compartments allow for specialized functions – such as ATP synthesis. The matrix is home to the mitochondrion's independent genome.

Steroidogenic Acute Regulatory Protein (StAR)

[0037] Steroids and steroidal hormones are synthesized from cholesterol. The first step in steroidogenesis is the cleavage of cholesterol's side chain by P450scc, which is located on the matrix side of the inner mitochondrial membrane (IMM). Typically, cells that produce steroid hormones store and possess only small amounts of hormone at any one time. Thus, increases in steroid secretion is accomplished by first increasing synthesis of the steroid. However, steroids have broad and powerful action within the cell and organism – thus steroidogenic cells tightly regulate production of steroids and precursors to avoid overproduction and pathologies associated with excess amounts of steroid. Synthesis can be rapidly inducted and rapidly terminated to avoid overproduction. One way cells exert this control (which can increase synthesis 10–100-fold within minutes) is to regulate the flow of cholesterol into the mitochondrial matrix. Cholesterol is transported across the mitochondrial membrane by the StAR protein.

[0038] The StAR protein is synthesized as a 37 kDa protein comprising a mitochondrial targeting leader peptide sequence. Upon insertion into the mitochondrial membrane, the leader peptide is cleaved to yield the 30 kDa protein that is located intra-mitochondrially. Mutations in the StAR gene can result in congenital lipoid adrenal hyperplasia. Subjects suffering from adrenal hyperplasia synthesize very small amounts of steroid.

[0039] StAR may interact with two additional proteins to support importation of cholesterol into mitochondria. TSPO, Translocator Protein, is an 18kDa protein that aids mitochondrial import of steroidogenic cholesterol. Studies have shown that TSPO expression is upregulated in response to exposure of macrophages to modified LDLs. In addition, the ubiquitously expressed PBR (peripheral-type benzodiazepine receptor) is an outer mitochondrial membrane protein involved in regulating cholesterol transport. Inhibition of PBR results in loss of a cells steroidogenic capacity.

[0040] The ability to produce large amounts of steroids from cholesterol can be imparted to non-steroidogenic cells (i.e. cells that do not typically produce steroids or produce very low amounts of steroids) by introducing StAR. Specifically, non-steroidogenic cells transfected with the P450scc enzyme system (i.e. H_2N-P450scc-adrenodoxin reductase-adrenodoxin-COOH) are able to metabolize cholesterol at very low levels. The presence of StAR (i.e. co-expression of StAR and P450scc enzyme system) increases conversion of cholesterol several fold in these cells. Studies have shown that the cells' production of steroids can be further enhanced by anchoring StAR

in the outer mitochondrial membrane (OMM), either by fusion to an OMM protein (e.g. the mitochondrial translocase TOM gene translocase, outer membrane) or duplication of StAR domain, which may help to slow transit of StAR through the OMM. Duplication of the StAR domain may be referred to as StAR-StAR protein. StAR-StAR is a fusion protein that combines 1-188 residues of StAR to 63-285 residues of StAR, thus dimerizing the protease-resistant domain of StAR (residues 63-188).

[0041] StAR proteins for use with the present compositions and methods may include various forms of the StAR protein sequence. In some embodiments, the disclosed StAR proteins may include one or more mutations, truncations, deletions, duplications, fusions, etc. or the disclosed proteins may be wild-type or native StAR proteins. In some embodiments, the disclosed StAR proteins may be modified to reduce transit time through the outer membrane, which may help to enhance mitochondrial import of cholesterol.

[0042] As used herein, the term cholesterol refers to cholesterin or cholesteryl alcohol, a sterol of formula $C_{27}H_{46}O$, with IUPAC names cholest-5-en-3β-ol, and (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-[(2R)-6-methylheptan-2-yl]-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol. As used herein, the term cholesterol may also refer to derivatives of cholesterol, including oxidized cholesterol, $C_{27}H_{46}O_2$, Oxycholesterol, or 5,6-epoxycholesterol, 7-ketocholesterol (7KC), cholestane-3β,5α,6β-triol and 7-α/β hydroxycholesterol, etc.

[0043] The disclosed genes and proteins that may degrade cholesterol may be selected from cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx), and combinations thereof.

[0044] One or more of the disclosed genes, proteins, and enzymes may be packaged into one or more vector, construct, or cassette. In various embodiments, a cassette that includes one or more cholesterol degrading enzymes may be referred to as a cholesterol catabolizing cassette (CCC). In some embodiments the cassette may be a construct and may include a nucleic acid sequence that codes for at least one protein that is about 80% or more identical to a protein disclosed herein or a protein coded for by any of the disclosed genes. In many embodiments, the percent identity with the disclosed protein or nucleotide sequences may be greater than about 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, and less than about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, and 81%.

Sequence identity may be calculated for a protein sequence of greater than about 40 aa (amino acid residues), 50 aa, 60 aa, 70 aa, 80 aa, 90 aa, 100 aa, 110 aa, 120 aa, 130 aa, 140 aa, 150 aa, 160 aa, 170 aa, 180 aa, 190 aa, 200 aa or more and less than about 300 aa, 200 aa, 190 aa, 180 aa, 170 aa, 160 aa, 150 aa, 140 aa, 130 aa, 120 aa, 110 aa, 100 aa, 90 aa, 80 aa, 70 aa, or 60 aa. In many embodiments, sequence identity is calculated over about 80-150 amino acid residues. In most cases an inserted or deleted amino acid, compared to the reference sequence (i.e. SEQ ID NOs: 1-10), counts as a single non-identical amino acid. In some embodiments, for example wherein delivery of the one or more compositions is via an LNP, for example where the composition is one or more nanoplasmid or mRNA, the composition may include one or more cholesterol degrading proteins, for example an enzyme(s) and a cholesterol transporting protein, for example StAR protein or StAR variant. One of skill in the art is capable of creating an mRNA from the protein and nucleic acid sequences disclosed herein. In embodiments where the one or more compositions are comprised in a therapeutic cell that may be administered to a subject, the therapeutic cell may be administered by one or more vectors, constructs, and/or cassettes comprising nucleic acids that code for a cholesterol degrading protein, for example an enzyme and a cholesterol transport protein, for example StAR or a StAR variants. In these cases, the nucleic acids may be transduced into the cell, for example by one or more viral vectors (in one example lentivirus) to aid in integrating or inserting the coding sequence into the genomic sequence or mitochondrial sequence of the cell. In some embodiments, the nucleic acids may not be integrated, and the disclosed proteins are expressed from extra-genomic sequences in the nucleus or mitochondria.

Cholesterol-related Diseases and Disorders

[0045] Excess native LDL and increased LDL:HDL ratio have been shown to play critical roles in cardiovascular disease, atherosclerosis, stroke and coronary heart disease and heart attacks. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have total cholesterol concentration of greater than about 200 mg of cholesterol (i.e. low-density lipoprotein + high-density lipoprotein) per decilitre of blood and/or more than about 5.2 mmol/L total cholesterol. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have an LDL concentration of greater than about 100 mg/dl and/or more than about 2.6 mM LDL. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have an HDL concentration of less than about 50 mg/dl and/or less than about 1.3 mM HDL. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have a triglyceride concentration of greater than

about 150 mg/dl and/or greater than about 1.7 mM triglycerides. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have an LDL:HDL ratio of about 4.0 (i.e. 4 to 1) or more than 4.0.

[0046] Acetylated LDL is an in vitro chemically modified form of LDL and does not exist in vivo. Both acetylated LDL and oxidized LDL, are taken up by macrophages, transforming those cells into foam cells. In most cases, all components of LDL are susceptible to oxidation, producin an oxidized form of LDL (oxLDL). The uptake of oxLDL by arterial macrophages is pivotal in the formation of plaques. Unlike unmodified LDL, oxLDL is taken up by arterial wall macrophages in an unregulated manner via LDL scavenger receptors. Oxysterols are 10-100X more reactive than native cholesterol, with the most toxic of these being 7-ketocholesterol (7KC), which is also the most abundant in oxLDL.

[0047] Studies find that higher levels of circulating 7KC are associated with greater future risk of cardiovascular events and increased total mortality. 7KC is a pro-inflammatory, pro-oxidant, pro-apoptotic, and fibrogenic molecule that alters endothelial cell function by disrupting cell membranes and critical ion transport pathways for vasodilatory response. In some embodiments, a subject or patient at risk for, or suffering from, high cholesterol may have a 7KC concentration higher than about 109.8 nmol/L nmol/L.

[0048] A subject or patient suffering from, or at risk of developing high cholesterol may be referred to as hypercholesterolemic. In some embodiments, hypercholesterolemia may be assessed by measuring the percentage of plasma oxysterols, for example the percentage of 7-KC in plasma oxysterols. In hypercholesterolemic patients, 7 KC may account for about 57% of the plasma oxysterols. 7KC is followed by 7-α/β hydroxycholesterol (at 21% of plasma oxysterols), which is a direct product of 7KC metabolism. In arterial plaques, 55% of oxysterols are reported to be 7KC, with the second and third most abundant being cholestane-3β,5α,6β-triol and 7-α/β hydroxycholesterol at 13% and 12%, respectively.

[0049] As noted above, NASH (nonalcoholic steatohepatitis) is another disease associated with excess cholesterol. Altered cholesterol homeostasis and transport contribute to the accumulation of free cholesterol in the liver, which in turn contributes to NAFLD (Non-alcoholic fatty liver disease) via damage to hepatocytes and the activation of non-parenchymal cells. Particularly, the overload of free cholesterol in and around the mitochondria induces mitochondrial dysfunction and promotes inflammation, fibrosis and hepatocyte death.

[0050] Other cholesterol-associated diseases include pulmonary alveolar proteinosis (PAP), eye disease, neurodegenerative diseases, Niemann Pick Type C (NPC), and Lysosomal Acid Lipase (LAL) deficiency. Because cholesterol content plays a role in regulating surfactant fluidity and function in lunged animals, and that fluidity can change rapidly, especially under extremes of temperature, reduced cholesterol clearance is a primary defect driving PAP pathogenesis. In the case of eye disease, oxysterols and, in particular 7KC, cause degeneration of retinal cells. Thus, increased oxysterol levels may play a role in various eye diseases including macular degeneration (AMD), choroidal neovascularization (CNV), glaucoma, and cataracts.

[0051] Increased oxysterol levels may also result in alterations in brain cholesterol metabolism. Cholesterol metabolism may be an integral part of several brain disorders including Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), Parkinson's disease, and dementia progression. Various oxysterols derived from the auto-oxidation of cholesterol, including 7KC have been identified in post-mortem brains of patients with Alzheimer's disease. Chronic epilepsy may also share many of these pathologies. Specifically, a link has been suggested between epilepsy and atherosclerosis. Thus, treatment of atherosclerosis, such as the presently disclosed compositions, cells, and methods may lessen the effects of epilepsy. Further, 7KC is highly cytotoxic to neuronal cells and has been suspected to be involved in the progression of various neurological diseases. Surprisingly, oxysterols, unlike cholesterol, can cross the blood brain barrier (BBB) and accumulate in brain tissue, ultimately causing neurodegeneration.

[0052] Various other diseases may be linked with increased cholesterol levels. For example, patients with Niemann Pick Type C (NPC) are inable to clear cholesterol, causing the accumulation of cholesterol and oxysterols in mostly the liver, spleen, and brain. A positive correlation between the 7KC profile and the severity of the disease has been reported. In addition, patients with Lysosomal Acid Lipase (LAL) deficiency accumulate cholesterol esters and triglycerides in lysosomes, and can present with hypercholesterolemia, hyperlipidemia, and/or atherosclerosis. These patients also have very high levels of oxysterols, including 7KC, in their plasma. Increased formation of oxysterols further increases oxidative stress worsens the condition.

[0053] The disclosed compositions and methods are useful in treating diseases or conditions associated with excess cholesterol and/or fat deposits in cells, tissues, and organs. In some embodiments, the disease or condition may be associated with excess cholesterol and/or the presence of one or more oxidized cholesterol species, such as 7-ketocholesterol. In some embodiments, the disease or condition may be one or more of fatty liver disease, atherosclerosis, heart failure, stroke, ischemia, coronary heart

disease, eye disease, neurodegenerative and neurological disease, diseases of the eye, such as macular degeneration, pulmonary dysfunction, etc.

[0054] The disclosed compositions, cells, methods, and therapies may aid in treating, reducing, or reversing various diseases, disorders, or conditions related to excess cholesterol. In one embodiment, the disease, disorder, or condition may be one or more of early type II lesions (i.e. macrophage foam cell formation), type III lesions or pre-atheromas (i.e. having small pools of extracellular lipids), type IV lesions or atheromas (i.e. having a core of extracellular lipids), type V lesions or fibroatheromas (i.e. atheromas with fibrous thickening).

[0055] The disclosed compositions, constructs, cells, methods, therapies, and systems may aid in reducing the concentration of cholesterol in the blood of a patient. In some embodiments, a decrease in the concentration of cholesterol in the blood (e.g. weight per volume, such as mg/dl, or molarity, such as mmol/L) may be greater than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% and less than about 100%, 95%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, or 2%. In many embodiments, the decrease in cholesterol levels upon treatment may be reflected in an increase in the concentration or amount of one or more cholesterol catabolites, for example an increase of greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 1.5X, 2.0X, 2.5X, 3.0X, 3.5X, 4.0X, 4.5X, 5.0X, 10X, 20X, 30X, 40X, 50X, 60X, 70X, 80X, 90X, 100X, 200X, 300X, 400X, 500X, 600X, 700X, 800X, or more, and less than about 2000X, 1500X, 1000X, 500X, 400X, 300X, 200X, 100X, 90X, 80X, 70X, 60X, 50X, 40X, 30X, 20X, 10X, 5X, 4X, 3X, 2X, 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, or 10% over the concentration of catabolite prior to treatment.

EXAMPLES

Example 1 – Co-expression of Cholesterol Degrading Enzymes and StAR Protein

[0056] In vitro experiments were performed to test the cholesterol degrading activity of cholesterol degrading proteins in combination with StAR. In these experiments, Human 293T kidney epithelial cells were seeded at 3 x 10^5 293T cells/well in a 12-well plate in DMEM and 10% FBS. At 75% confluency, cells were transfected Lipofectamine 3000. Next, equimolar amounts of a CDP or empty plasmid and either a StARStAR, TomSTaR, or empty plasmid were mixed with P3000 in 50 µL of Opti-MEM to have the following conditions:

1. Empty plasmid + Empty plasmid

2. CDP plasmid + Empty plasmid
3. StARStAR plasmid + Empty plasmid
4. TomStAR plasmid + Empty plasmid
5. CDP plasmid + StARStAR plasmid
6. CDP plasmid + TomStAR plasmid

[0057] 3X µL of Lipofectamine Reagent was mixed with 50 µL of Opti-MEM. Next, the DNA/P3000 tube was combined with the Lipofectamine tube and incubated at room temperature for 15 minutes. The transfection mixture was added in a dropwise fashion to the cells and incubated for 48 hours.

[0058] At the end of the incubation period, the cell media was assayed for catabolite concentration. Total cellular protein was extracted using RIPA buffer and total protein concentrations were quantified using a standard BCA kit.

Results:

[0059] As shown in Fig. 1, below, the expression of CDP in 293T kidney epithelial cells resulted in a ~300-fold increase catabolite concentration in culture media from baseline levels (media of cells expressing only empty plasmid i.e. no CDP). Co-expression of this fusion protein with CDP increased catabolite production ~4-fold compared to CDP alone. Tom20 has 50N-terminal residues embedded in the outer mitochondrial membrane (OMM) and 95 residues in the cytoplasm. Fusion of StAR residues 63-285 to the carboxyl terminus of Tom20 (i.e. Tom-StAR) affixes StAR to the cytoplasmic side of the OMM and, when co-expressed with CDP, increased catabolite production by ~30% compared to cells expressing CDP alone. Expression of either StAR-StAR or TomStAR alone did not affect catabolite concentrations. Transfection of cells with StAR-StAR cDNA resulted in robust expression of the StAR-StAR protein detected as a ~50kD band by SDS-PAGE and western blot, see Fig. 2, below. Interestingly, co-expression of CDP (detected as a FLAG-tagged protein with anti-FLAG antibody) with StAR-StAR resulted in a significant downregulation of steady-state StAR-StAR protein levels. Without wishing to be bound to a theory, these data suggest that continued StAR-StAR-mediated transport of the CDP-substrate, cholesterol, into the mitochondrial matrix result in the destabilization and degradation/consumption of StAR-StAR protein. Downregulation of Tom-StAR was not detected in cells co-expressing CDP and was perhaps due, without wishing to be bound to a theory, to the lower efficiency of Tom-StAR in transporting cholesterol to the mitochondrial matrix. This reduced efficiency was evidenced by significantly lower levels of CDP-derived catabolite in CDP and Tom-StAR co-expressing cells *versus* those co-expressing CDP and StAR-

StAR (see Figure 1 above). The level of StAR-StAR protein was higher in cells expressing only StAR-StAR compared to those expressing StAR-StAR and CDP, suggesting that the level of StAR-StAR activity and its consequent degradation might be limited by increased levels of cholesterol accumulation in the mitochondrial matrix in the absence of CDP. We speculate that continued conversion of cholesterol by CDP to its catabolite increases the cholesterol concentration gradient along the inner mitochondrial membrane, thus further stimulating StAR-StAR-mediated cholesterol transport into the mitochondrion.

[0060] Figure 1 shows results from this experiment in bar graph format. Specifically, FIG. 1 shows the mean catabolite concentration in media of cells expressing CDP in the presence and absence of StAR-StAR or Tom-StAR (*n=3* independent experiments) as indicated below the graph.

[0061] Figure 2 is a representative western blot showing CDP protein levels in cells in the presence and absence of StAR-StAR or Tom-StAR with tubulin as control (*n=3* independent experiments). Lanes are as follows: 1, Empty plasmid + Empty plasmid; 2, CDP plasmid + Empty plasmid; 3, StARStAR plasmid + Empty plasmid; 4, TomStAR plasmid + Empty plasmid; 5, CDP plasmid + StARStAR plasmid; 6, CDP plasmid + TomStAR plasmid.

Example 2 – Co-expression of Cholesterol Degrading Enzymes and StAR Protein in Mouse cells

[0062] In vitro experiments were performed as described in Example 2. In these experiments, Mouse RAW264.7 cells were seeded at 3 x 10^5 293T cells/well in a 12-well plate in DMEM and 10% FBS. At 75% confluency, cells were transfected Lipofectamine 3000. Next, equimolar amounts of a CDP or empty plasmid and either a StARStAR or empty plasmid were mixed with P3000 in 50 µL of Opti-MEM to have the following conditions: Empty plasmid + Empty plasmid; CDP plasmid + Empty plasmid; or CDP plasmid + StARStAR plasmid.

[0063] Cholesterol catabolism greatly increased in cells co-expressing CDP and StAR. Specifically catabolite production, in pg/cell were as follows: Empty + Empty, 2.83E-03; CDP + Empty, 1.26E-01; and CDP + StARStAR, 1.07E+00. These results correspond to a 44.52-fold increase in the presence of CDP, and an additional 8.49-fold increase in the presence of StARStAR protein.

[0064] Claim 1. A nucleic acid composition comprising:

a coding sequence for Steroidogenic Acute Regulatory (StAR) protein; and

a coding sequence for a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-

KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0065] Claim 2. The nucleic acid of claim 1, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[0066] Claim 3. The nucleic acid of claim 1 or claim 2, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[0067] Claim 4. The nucleic acid of any of claim 1 to claim 3, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

[0068] Claim 5. The nucleic acid of any of claim 1 to claim 4, wherein the CDP comprises P450.

[0069] Claim 6. The nucleic acid of any of claim 1 to claim 5, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0070] Claim 7. The nucleic acid of any of claim 1 to claim 6, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[0071] Claim 8. The nucleic acid of any of claim 1 to claim 7, wherein the nucleic acid further includes a cell-specific promoter sequence.

[0072] Claim 9. The nucleic acid of any of claim 1 to claim 8, wherein the nucleic acid is comprised in a lipo nanoparticle.

[0073] Claim 10. The nucleic acid of any of claim 1 to claim 8, wherein the nucleic acid is comprised in a viral vector.

[0074] Claim 11. The nucleic acid of any of claim 1 to claim 10, for use in treating a subject having high cholesterol.

[0075] Claim 12. The nucleic acid of any of claim 1 to claim 11, for use in transforming a mammalian cell.

[0076] Claim 13. The nucleic acid of claim 12, wherein the mammalian cell is transformed in-vitro.

[0077] Claim 14. The nucleic acid of claim 12, wherein the mammalian cell is transformed in-vivo.

[0078] Claim 15. The nucleic acid of any of claim 1 to claim 14, wherein the nucleic acid is comprised in a lipo nanoparticle.

[0079] Claim 16. The nucleic acid of any of claim 1 to claim 15, wherein the nucleic acid is comprised in a cell of subject suffering from or at risk of developing high cholesterol.

[0080] Claim 17. The nucleic acid of any of claim 1 to claim 16, for use in reducing cholesterol in a subject having high cholesterol.

[0081] Claim 18. A mammalian cell comprising:

a coding sequence for an engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

a coding sequence for a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0082] Claim 19. The mammalian cell of claim 18, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[0083] Claim 20. The nucleic acid of claim 18 or claim 19, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[0084] Claim 21. The mammalian cell of any of claim 18 to claim 20, wherein the StAR protein comprises at least a portion of a TOMM20 protein sequence.

[0085] Claim 22. The mammalian cell of any of claim 18 to claim 21, wherein the CDP comprises P450.

[0086] Claim 23. The mammalian cell of any of claim 18 to claim 22, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0087] Claim 24. The mammalian cell of any of claim 18 to claim 23, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[0088] Claim 25. The mammalian cell of any of claim 18 to claim 24, wherein the coding sequence of the StAR or CDP further includes a cell-specific promoter sequence.

[0089] Claim 26. The mammalian cell of any of claim 18 to claim 25, wherein the coding sequence of the StAR or CDP is integrated into the mammalian cell's genome.

[0090] Claim 27. The mammalian cell of any of claim 18 to claim 25, wherein the coding sequence of the StAR or CDP is not integrated into the genome of the mammalian cell.

[0091] Claim 28. The mammalian cell of any of claim 18 to claim 27, for use in treating a subject suffering from or at risk of developing high cholesterol.

[0092] Claim 29. The mammalian cell of any of claim 18 to claim 28, for use in reducing cholesterol in a subject.

[0093] Claim 30. The mammalian cell of any of claim 18 to claim 29, wherein the mammalian cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[0094] Claim 31. A mammalian cell comprising:

an engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[0095] Claim 32. The mammalian cell of claim 31, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[0096] Claim 33. The mammalian cell of claim 31 or claim 32, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[0097] Claim 34. The mammalian cell of any of claim 31 to claim 33, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

[0098] Claim 35. The mammalian cell of any of claim 31 to claim 34, wherein the CDP comprises P450.

[0099] Claim 36. The mammalian cell of any of claim 31 to claim 35, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00100] Claim 37. The mammalian cell of any of claim 31 to claim 36, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[00101] Claim 38. The mammalian cell of any of claim 31 to claim 37, wherein at least 10% of the total amount of StAR protein and CDP in the cell are located in a mitochondrial membrane.

[00102] Claim 39. The mammalian cell of any of claim 31 to claim 38, wherein the StAR protein or the CDP is coded for by a gene integrated into the genome of the mammalian cell.

[00103] Claim 40. The mammalian cell of any of claim 31 to claim 39, wherein the StAR protein or the CDP is coded for by an extra genomic gene.

[00104] Claim 41. The mammalian cell of any of claim 31 to claim 39, for use in treating a subject suffering from or at risk of developing high cholesterol.

[00105] Claim 42. The mammalian cell of any of claim 31 to claim 40, for use in reducing cholesterol in a subject.

[00106] Claim 43. The mammalian cell of any of claim 18 to claim 29, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[00107] Claim 44. A method of enhancing cholesterol degradation in a mammalian cell comprising:

expressing non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

expressing one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00108] Claim 45. The method of claim 44, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[00109] Claim 46. The method of claim 44 or claim 45, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[00110] Claim 47. The method of any of claim 44 to claim 46, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

[00111] Claim 48. The method of any of claim 44 to claim 47, wherein the CDP comprises P450.

[00112] Claim 49. The method of any of claim 44 to claim 48, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00113] Claim 50. The method of any of claim 44 to claim 49, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[00114] Claim 51. The method of any of claim 44 to claim 50, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

[00115] Claim 52. The method of any of claim 44 to claim 51, for use in treating a subject suffering from or at risk of developing high cholesterol.

[00116] Claim 53. The method of any of claim 44 to claim 52, for use in reducing cholesterol in a subject having high cholesterol.

[00117] Claim 54. The method of any of claim 44 to claim 53, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

[00118] Claim 55. The method of any of claim 44 to claim 54, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

[00119] Claim 56. The method of any of claim 44 to claim 54, wherein the StAR protein or the CDP is from an extra genomic gene.

[00120] Claim 57. The method of any of claim 44 to claim 56, for use in treating a subject suffering from or at risk of developing high cholesterol.

[00121] Claim 58. The method of any of claim 44 to claim 57, for use in reducing cholesterol in a subject.

[00122] Claim 59. The method of any of claim 44 to claim 58, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[00123] Claim 60. The method of any of claim 44 to claim 59, wherein the cholesterol is LDL.

[00124] Claim 61. The method of any of claim 44 to claim 59, wherein the cholesterol is 7KC.

[00125] Claim 62. A method of treating a patient at risk of developing or suffering from high cholesterol comprising:

administering to the patient at least one modified cell, the modified cell comprising

a non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00126] Claim 63. The method of claim 62, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[00127] Claim 64. The method of claim 62 or claim 63, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[00128] Claim 65. The method of claim 62 to claim 64, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

[00129] Claim 66. The method of claim 62 to claim 65, wherein the CDP comprises P450.

[00130] Claim 67. The method of claim 62 to claim 66, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00131] Claim 68. The method of claim 62 to claim 67, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[00132] Claim 69. The method of claim 62 to claim 68, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

[00133] Claim 70. The method of claim 62 to claim 76, for use in reducing cholesterol in a subject having high cholesterol.

[00134] Claim 71. The method of claim 62 to claim 71, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

[00135] Claim 72. The method of claim 62 to claim 72, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

[00136] Claim 73. The method of claim 62 to claim 72, wherein the StAR protein or the CDP is from an extra genomic gene.

[00137] Claim 74. The method of claim 62 to claim 73, wherein the patient has a total cholesterol of greater than 200 mg/dl.

[00138] Claim 75. The method of claim 62 to claim 74, wherein the patient suffers from non-alcoholic fatty liver disease.

[00139] Claim 76. The method of claim 62 to claim 74, wherein the patient suffers from non-alcoholic steatohepatitis.

[00140] Claim 77. The method of claim 62 to claim 76, wherein the modified cell is derived from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[00141] Claim 78. The method of claim 62 to claim 77, wherein the method reduces the total cholesterol concentration in the patient's blood by greater than 5%.

[00142] Claim 79. A method of modifying a mammalian mitochondrion comprising:

inserting at least one non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof into the mammalian mitochondrion outer membrane; and

wherein the mitochondrion comprises one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00143] Claim 80. The method of claim 79, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

[00144] Claim 81. The method of claim 79 or claim 80, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

[00145] Claim 82. The method of any of claim 79 to claim 81, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

[00146] Claim 83. The method of any of claim 79 to claim 82, wherein the CDP comprises P450.

[00147] Claim 84. The method of any of claim 79 to claim 83, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

[00148] Claim 85. The method of any of claim 79 to claim 84, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

[00149] Claim 86. The method of any of claim 79 to claim 85, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

[00150] Claim 87. The method of any of claim 79 to claim 86, for use in treating a subject suffering from or at risk of developing high cholesterol.

[00151] Claim 88. The method of any of claim 79 to claim 87, for use in reducing cholesterol in a subject having high cholesterol.

[00152] Claim 89. The method of any of claim 79 to claim 88, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

[00153] Claim 90. The method of any of claim 79 to claim 90, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

[00154] Claim 91. The method of any of claim 79 to claim 89, wherein the StAR protein or the CDP is from an extra genomic gene.

[00155] Claim 92. The method of any of claim 79 to claim 91, for use in treating a subject suffering from or at risk of developing high cholesterol.

[00156] Claim 93. The method of any of claim 79 to claim 92, for use in reducing cholesterol in a subject.

[00157] Claim 94. The method of any of claim 79 to claim 93, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

[00158] While multiple embodiments are disclosed, still other embodiments of the present invention will become apparent to those skilled in the art from the following detailed description. As will be apparent, the invention is capable of modifications in various obvious aspects, all without departing from the spirit and scope of the present invention. Accordingly, the detailed description is to be regarded as illustrative in nature and not restrictive.

[00159] All references disclosed herein, whether patent or non-patent, are hereby incorporated by reference as if each was included at its citation, in its entirety. In case

of conflict between reference and specification, the present specification, including definitions, will control.

[00160] Although the present disclosure has been described with a certain degree of particularity, it is understood the disclosure has been made by way of example, and changes in detail or structure may be made without departing from the spirit of the disclosure as defined in the appended claims.

CLAIMS

We claim:

1. A nucleic acid composition comprising:

a coding sequence for Steroidogenic Acute Regulatory (StAR) protein; and

a coding sequence for a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

2. The nucleic acid of claim 1, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

3. The nucleic acid of claim 1 or claim 2, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

4. The nucleic acid of any of claim 1 to claim 3, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

5. The nucleic acid of any of claim 1 to claim 4, wherein the CDP comprises P450.

6. The nucleic acid of any of claim 1 to claim 5, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

7. The nucleic acid of any of claim 1 to claim 6, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

8. The nucleic acid of any of claim 1 to claim 7, wherein the nucleic acid further includes a cell-specific promoter sequence.

9. The nucleic acid of any of claim 1 to claim 8, wherein the nucleic acid is comprised in a lipo nanoparticle.

10. The nucleic acid of any of claim 1 to claim 8, wherein the nucleic acid is comprised in a viral vector.

11. The nucleic acid of any of claim 1 to claim 10, for use in treating a subject having high cholesterol.

12. The nucleic acid of any of claim 1 to claim 11, for use in transforming a mammalian cell.

13. The nucleic acid of claim 12, wherein the mammalian cell is transformed in-vitro.

14. The nucleic acid of claim 12, wherein the mammalian cell is transformed in-vivo.

15. The nucleic acid of any of claim 1 to claim 14, wherein the nucleic acid is comprised in a lipo nanoparticle.

16. The nucleic acid of any of claim 1 to claim 15, wherein the nucleic acid is comprised in a cell of subject suffering from or at risk of developing high cholesterol.

17. The nucleic acid of any of claim 1 to claim 16, for use in reducing cholesterol in a subject having high cholesterol.

18. A mammalian cell comprising:

a coding sequence for an engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

a coding sequence for a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

19. The mammalian cell of claim 18, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

20. The nucleic acid of claim 18 or claim 19, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

21. The mammalian cell of any of claim 18 to claim 20, wherein the StAR protein comprises at least a portion of a TOMM20 protein sequence.

22. The mammalian cell of any of claim 18 to claim 21, wherein the CDP comprises P450.

23. The mammalian cell of any of claim 18 to claim 22, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

24. The mammalian cell of any of claim 18 to claim 23, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

25. The mammalian cell of any of claim 18 to claim 24, wherein the coding sequence of the StAR or CDP further includes a cell-specific promoter sequence.

26. The mammalian cell of any of claim 18 to claim 25, wherein the coding sequence of the StAR or CDP is integrated into the mammalian cell's genome.

27. The mammalian cell of any of claim 18 to claim 25, wherein the coding sequence of the StAR or CDP is not integrated into the genome of the mammalian cell.

28. The mammalian cell of any of claim 18 to claim 27, for use in treating a subject suffering from or at risk of developing high cholesterol.

29. The mammalian cell of any of claim 18 to claim 28, for use in reducing cholesterol in a subject.

30. The mammalian cell of any of claim 18 to claim 29, wherein the mammalian cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

31. A mammalian cell comprising:

an engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

a cholesterol degrading protein (CDP) comprising one or more of cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

32. The mammalian cell of claim 31, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

33. The mammalian cell of claim 31 or claim 32, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

34. The mammalian cell of any of claim 31 to claim 33, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

35. The mammalian cell of any of claim 31 to claim 34, wherein the CDP comprises P450.

36. The mammalian cell of any of claim 31 to claim 35, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

37. The mammalian cell of any of claim 31 to claim 36, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

38. The mammalian cell of any of claim 31 to claim 37, wherein at least 10% of the total amount of StAR protein and CDP in the cell are located in a mitochondrial membrane.

39. The mammalian cell of any of claim 31 to claim 38, wherein the StAR protein or the CDP is coded for by a gene integrated into the genome of the mammalian cell.

40. The mammalian cell of any of claim 31 to claim 39, wherein the StAR protein or the CDP is coded for by an extra genomic gene.

41. The mammalian cell of any of claim 31 to claim 39, for use in treating a subject suffering from or at risk of developing high cholesterol.

42. The mammalian cell of any of claim 31 to claim 40, for use in reducing cholesterol in a subject.

43. The mammalian cell of any of claim 18 to claim 29, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

44. A method of enhancing cholesterol degradation in a mammalian cell comprising:

expressing non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

expressing one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

45. The method of claim 44, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

46. The method of claim 44 or claim 45, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

47. The method of any of claim 44 to claim 46, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

48. The method of any of claim 44 to claim 47, wherein the CDP comprises P450.

49. The method of any of claim 44 to claim 48, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

50. The method of any of claim 44 to claim 49, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

51. The method of any of claim 44 to claim 50, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

52. The method of any of claim 44 to claim 51, for use in treating a subject suffering from or at risk of developing high cholesterol.

53. The method of any of claim 44 to claim 52, for use in reducing cholesterol in a subject having high cholesterol.

54. The method of any of claim 44 to claim 53, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

55. The method of any of claim 44 to claim 54, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

56. The method of any of claim 44 to claim 54, wherein the StAR protein or the CDP is from an extra genomic gene.

57. The method of any of claim 44 to claim 56, for use in treating a subject suffering from or at risk of developing high cholesterol.

58. The method of any of claim 44 to claim 57, for use in reducing cholesterol in a subject.

59. The method of any of claim 44 to claim 58, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

60. The method of any of claim 44 to claim 59, wherein the cholesterol is LDL.

61. The method of any of claim 44 to claim 59, wherein the cholesterol is 7KC.

62. A method of treating a patient at risk of developing or suffering from high cholesterol comprising:

administering to the patient at least one modified cell, the modified cell comprising

a non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof; and

one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

63. The method of claim 62, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

64. The method of claim 62 or claim 63, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

65. The method of claim 62 to claim 64, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

66. The method of claim 62 to claim 65, wherein the CDP comprises P450.

67. The method of claim 62 to claim 66, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

68. The method of claim 62 to claim 67, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

69. The method of claim 62 to claim 68, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

70. The method of claim 62 to claim 76, for use in reducing cholesterol in a subject having high cholesterol.

71. The method of claim 62 to claim 71, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

72. The method of claim 62 to claim 72, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

73. The method of claim 62 to claim 72, wherein the StAR protein or the CDP is from an extra genomic gene.

74. The method of claim 62 to claim 73, wherein the patient has a total cholesterol of greater than 200 mg/dl.

75. The method of claim 62 to claim 74, wherein the patient suffers from non-alcoholic fatty liver disease.

76. The method of claim 62 to claim 74, wherein the patient suffers from non-alcoholic steatohepatitis.

77. The method of claim 62 to claim 76, wherein the modified cell is derived from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

78. The method of claim 62 to claim 77, wherein the method reduces the total cholesterol concentration in the patient's blood by greater than 5%.

79. A method of modifying a mammalian mitochondrion comprising:

inserting at least one non-native engineered Steroidogenic Acute Regulatory (StAR) protein or portion thereof into the mammalian mitochondrion outer membrane; and

wherein the mitochondrion comprises one or more of a cholesterol degrading protein (CDP) comprising cholesterol dehydrogenase (CholD), 3-ketosteroid Δ1-dehydrogenase (Δ1-KstD), anoxic cholesterol metabolism B enzyme (acmB), 3-

ketosteroid 9α-hydroxylase (KshAB), 3β-hydroxysteroid dehydrogenase 2 (HSD2), P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

80. The method of claim 79, wherein the StAR protein comprises residues 63 to 188 of SEQ ID NO:7.

81. The method of claim 79 or claim 80, wherein the StAR protein comprises a duplication of residues 63-188 of SEQ ID NO:7.

82. The method of any of claim 79 to claim 81, wherein the StAR protein comprises at least a portion of the TOMM20 protein.

83. The method of any of claim 79 to claim 82, wherein the CDP comprises P450.

84. The method of any of claim 79 to claim 83, wherein the CDP comprises P450-ferredoxin reductase-ferredoxin fusion protein (P450-FdxR-Fdx).

85. The method of any of claim 79 to claim 84, wherein the StAR protein and the CDP comprise at least one mitochondrial signaling protein.

86. The method of any of claim 79 to claim 85, wherein the StAR protein and CDP are expressed from a cell-specific promoter sequence.

87. The method of any of claim 79 to claim 86, for use in treating a subject suffering from or at risk of developing high cholesterol.

88. The method of any of claim 79 to claim 87, for use in reducing cholesterol in a subject having high cholesterol.

89. The method of any of claim 79 to claim 88, wherein at least about 10% of the total amount of StAR protein and CDP in the cell is located in a mitochondrial membrane.

90. The method of any of claim 79 to claim 90, wherein the StAR protein or the CDP is expressed from a gene integrated into the mammalian cell's genome.

91. The method of any of claim 79 to claim 89, wherein the StAR protein or the CDP is from an extra genomic gene.

92. The method of any of claim 79 to claim 91, for use in treating a subject suffering from or at risk of developing high cholesterol.

93. The method of any of claim 79 to claim 92, for use in reducing cholesterol in a subject.

94. The method of any of claim 79 to claim 93, wherein the cell is selected from a liver cell, neuronal cell, bone cell, muscle cell, endothelial cell, epithelial cell, immune cell, hematopoietic cell, and stem cell or precursor cell thereof.

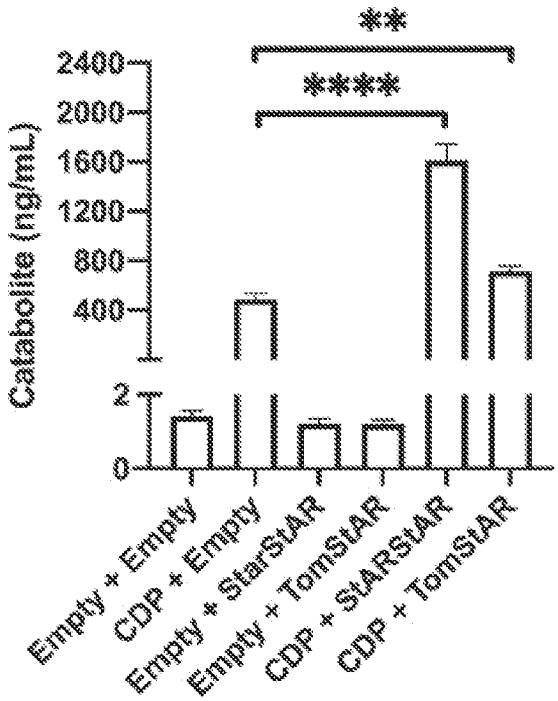
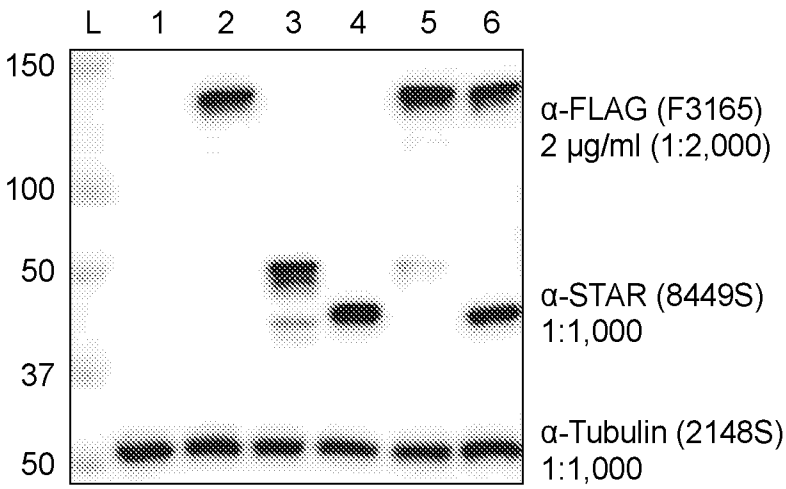


FIG. 1



Lane 1: Empty + Empty
Lane 2: CDP + Empty
Lane 3: Empty + StARStAR
Lane 4: Empty + TomStAR
Lane 5: CDP + StARStAR
Lane 6: CDP + TomStAR

FIG. 2

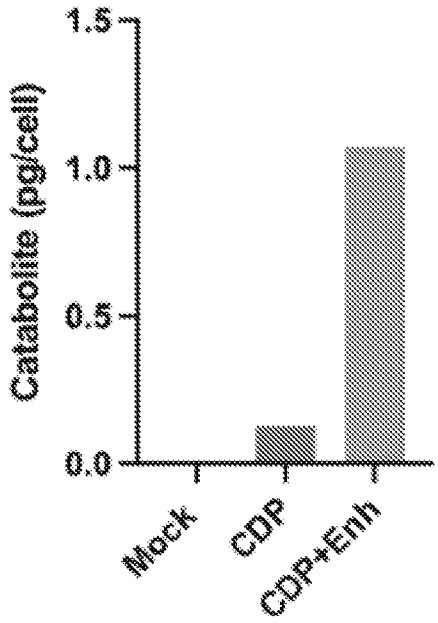
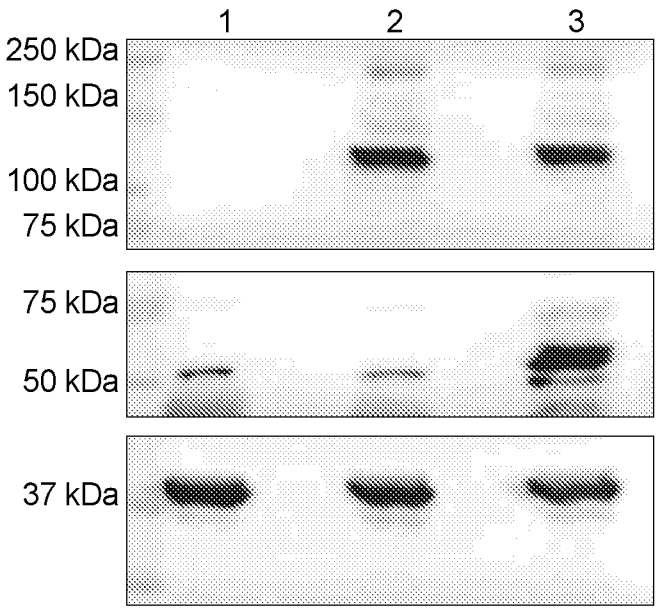


FIG. 3



α-FLAG (F3165) 1:4000, 0.25 sec exposure
Densitometry:
Mock: 0.00
CDP: 1.11
CDP+stAR-StAR: 1.13

α-FLAG (D10H12) 1:1000, 2 sec exposure
Densitometry:
Mock: 0.00
CDP: 0.00
CDP+stAR-StAR: 0.90

α-Tubulin (2148S) 1:1000, 0.2 sec exposure

Lane 1: mass marker
Lane 1: Mock RAW264
Lane 2: CDP RAW264
Lane 3: CDP+StAR-StAR RAW264

FIG. 4

Human Protein Sequence for p450-FdxR-Fdx (w/Flag tag in bold) SEQ ID NO:1
MLAKGLPPRSVLVKGCQTFLSAPREGLGRLRVPTGEGAGISTRSPRPFNEIPSPGDNGWLNLYH
FWRETGTHKVHLHHVQNFQKYGPIYREKLGNVESVYVIDPEDVALLFKSEGPNPERFLIPPWVAY
HQYYQRPIGVLLKKSAAWKKDRVALNQEVMAPEATKNFLPLLDAVSRDFVSVLHRRIKKAGSGNY
SGDISDDLFRFAFESITNVIFGERQGMLEEVVNPEAQRFIDAIYQMFHTSVPMLNLPPDLFRLFRTK
TWKDHVAAWDVIFSKADIYTQNFYWELRQKGSVHHDYRGILYRLLGDSKMSFEDIKANVTEMLA
GGVDTTSMTLQWHLYEMARNLKVQDMLRAEVLAARHQAQGDMATMLQLVPLLKASIKETLRLHP
ISVTLQRYLVNDLVLRDYMIPAKTLVQVAIYALGREPTFFFDPENFDPTRWLSKDKNITYFRNLGFG
WGVRQCLGRRIAELEMTIFLINMLENFRVEIQHLSDVGTTFNLILMPEKPISFTFWPFNQEATQQT
DGTSSTQEKTPQICVVGSGPAGFYTAQHLLKHPQAHVDIYEKQPVPFGLVRFGVAPDHPEVKNVI
NTFTQTAHSGRCAFWGNVEVGRDVTVPELREAYHAVVLSYGAEDHRALEIPGEELPGVCSARAF
VGWYNGLPENQELEPDLSCDTAVILGQGNVALDVARILLTPPEHLERTDITKAALGVLRQSRVKTV
WLVGRRGPLQVAFTIKELREMIQLPGARPILDPVDFLGLQDKIKEVPRPRKRLTELLLRTATEKPGP
AEAARQASASRAWGLRFFRSPQQVLPSPDGRRAAGVRLAVTRLEGVDEATRAVPTGDMEDLPC
GLVLSSIGYKSRPVDPSVPFDSKLGVIPNVEGRVMDVPGLYCSGWVKRGPTGVIATTMTDSFLTG
QMLLQDLKAGLLPSGPRPGYAAIQALLSSRGVRPVSFSDWEKLDAEEVARGQGTGKPREKLVDP
QEMLRLLGHTDGASSSSEDKITVHFINRDGETLTTKGKVGDSLLDVVVENNLDIDGFGACEGTLA
CSTCHLIFEDHIYEKLDAITDEENDMLDLAYGLTDRSRLGCQICLTKSMDNMTVRVPETVADARQS
IDVGKTSDYKDDDDK

Cholesterol dehydrogenase, CholD SEQ ID NO:2
MPTLYKKAGFQLGSLENLYFQGHHHHHHGTMAGFLNCCPGCCMEPGASDYKDDDDKMLRRMG
DASLTTELGRVLVTGGAGFVGANLVTTLLDRGHWVRSFDRAPSLLPAHPQLEVLQGDITDADVCA
AAVDGIDTIFHTAAIIELMGGASVTDEYRQRSFAVNVGGTENLLHAGQRAGVQRFVYTSSNSVVM
GGQNIAGGDETLPYTDRFNDLYTETKVVAERFVLAQNGVDGMLTCAIRPSGIWGNGDQTMFRKL
FESVLKGHVKVLVGRKSARLDNSYVHNLIHGFILAAAHLVPDGTAPGQAYFINDAEPINMFEFARP
VLEACGQRWPKMRISGPAVRWVMTGWQRLHFRFGFPAPLLEPLAVERLYLDNYFSIAKARRDLG
YEPLFTTQQALTECLPYYVSLFEQMKNEARAEKTAATVKPG

3-ketosteroid Δ1-dehydrogenase, Δ1-KstD SEQ ID NO:3
MQDWTSECDVLVVGSGGGALTGAYTAAAQGLTTIVLEKTDRFGGTSAYSGASIWLPGTQVQERA
GLPDSTENARTYLRALLGDAESERQDAYVETAPAVVALLEQNPNIEFEFRAFPDYYKAEGRMDTG
RSINPLDLDPADIGDLAGKVRPELDQDRTGQDHAPGPMIGGRALIGRLLAAVQSTGKAELRTESVL
TSLIVEDGRVVGAEVESGGETQRIKANRGVLMAAGGIEGNAEMREQAGTPGKAIWSMGPFGANT
GDAISAGIAVGGATALLDQAWFCPGVEQPDGSAAFMVGVRGGLVVDSAGERYLNESLPYDQFG
RAMDAHDDNGSAVPSFMIFDSREGGGLPAICIPNTAPAKHLEAGTWVGADTLEELAAKTGLPADA
LRSTVEKFNDAAKLGVDEEFHRGEDPYDAFFCPPNGGANAALTAIENGPFYAARIVLSDLGTKGG
LVTDVNGRVLRADGSAIDGLYAAGNTSASLSGRFYPGPGVPLGTAMVFSYRAAQDMAK

Anoxic cholesterol metabolism B enzyme, acmB SEQ ID NO:4
MPTLYKKAGFQLGSGTENLYFQGTMSIETNTYDVIVVGSGAGAMLAAARAHDLGLSVLVVEKSDK
YGGTSAVSGGAVWIPNNSQMQIKDSFDEALTYLKAATQGLVAEDRLLAYLESAPQMVEYINANMT
LQYFPCHRYPDYYQHLPGAKPGGRTMEPMLFDAALLGDEFANLRMAYTGTLLMGKASMTATEA
HVMLAKEPGWMLQVIKSLGRYYLDLPWRLKSRHDRKRGLGNAMAAGLRHALLERKVPLWLNTP
FESLITEGAENKRVTGIVVKRNGQTLQLTARRGVVLGAGGFERNQQMREQYLPKPTNAAWSATP
PHNTGDTIRAAMDIGARAELMDWAWWVPSIHVPGEAAQTGLFAERNLPGCIVVNGKGQRFINEA
SPYLEFGAAMYENHARSGSAVPAWLIFDGKFRYNYPMGPLMPGQIQPDRKAWLGKVYWRDDTL
EGLAKQIGVDAAGLKQSVELNNQYAQDGKDREFDKGGNVFDRYYGDYNVKPNPCLAPIGKPPYY
AMRVDAGDIGTKGGLLTDKDARVLDESDRPIEGLYCIGNNSASVMGKAYPGAGGTLGPAMTFGF
RAANHIAASKYPYDVPDYAG

FIG. 5

3-ketosteroid 9α-hydroxylase, KshAB SEQ ID NO:5
MAPYSLLVTRLQKALGVRQYHVASVLCQRAKVAMSDYKDDDDKGGGGSMSIDTARSGSDDDVE
IREIQAAAAPTRFARGWHCLGLLRDFQDGKPHSIEAFGTKLVVFADSKGQLNVLDAYCRHMGGD
LSRGEVKGDSIACPFHDWRWNGKGKCTDIPYARRVPPIAKTRAWTTLERNGQLYVWNDPQGNP
PPEDVTIPEIAGYGTDEWTDWSWKSLRIKGSHCREIVDNVVDMAHFFYIHYSFPRYFKNVFEGHT
ATQYMHSTGREDVISGTNYDDPNAELRSEATYFGPSYMIDWLESDANGQTIETILINCHYPVSNN
EFVLQYGAIVKKLPGVSDEIAAGMAEQFAEGVQLGFEQDVEIWKNKAPIDNPLLSEEDGPVYQLR
RWYQQFYVDVEDITEDMTKRFEFEIDTTRAVASWQKEVAENLAKQAEGSTATPGSGATNFSLLK
QAGDVEENPGPMAPYSLLVTRLQKALGVRQYHVASVLCQRAKVAMSGYPYDVPDYAGGGGSM
TAVQAPVTSRATVLTVSAVVQETADAVSLVFDVPDDRREDFTYRPGQFLTLRIPSDRTGSVARCY
SLASSPFTGEPPKVTVKRTAGGYGSNWLCDNIVAGRSIEVLPPAGVFTPADLTEKLVLFAGGSGIT
PVMSILESALHSGNRDVVLIYGNRDEKSVIFAEKLRELAARHAGALTVVHWLESVQGLPSPQQLAT
LISPFADHRAYMCGPGPFMDTVREGLLLAGVPKDRIHAEVFTSLSGDPFADVPLVEIDESDADATS
ATVQLDGEEHDLVWPRSATLVDVMLSKGLDVPYSCREGECGSCACTVVEGDVDSLPSAILDEED
IANGYVLACQARPKSDHVRIEF

3β-hydroxysteroid dehydrogenase 2, HSD2 SEQ ID NO:6
MGWSCLVTGAGGLLGQRIVRLLVEEKELKEIRALDKAFRPELREEFSKLQNRTKLTVLEGDILDEP
FLKRACQDVSVVIHTACIIDVFGVTHRESIMNVNVKGTQLLLEACVQASVPVFIYTSSIEVAGPNSY
KEIIQNGHEEEPLENTWPTPYPYSKKLAEKAVLAANGWNLKNGDTLYTCALRPTYIYGEGGPFLS
ASINEALNNNGILSSVGKFSTVNPVYVGNVAWAHILALRALRDPKKAPSVRGQFYYISDDTPHQSY
DNLNYILSKEFGLRLDSRWSLPLTLMYWIGFLLEVVSFLLSPIYSYQPPFNRHTVTLSNSVFTFSYK
KAQRDLAYKPLYSWEEAKQKTVEWVGSLVDRHKETLKSKTQ

Human StAR protein sequence SEQ ID NO:7
MLLATFKLCAGSSYRHMRNMKGLRQQAVMAISQELNRRALGGPTPSTWINQVRRRSSLLGSRLE
ETLYSDQELAYLQQGEEAMQKALGILSNQEGWKKESQQDNGDKVMSKVVPDVGKVFRLEVVVD
QPMERLYEELVERMEAMGEWNPNVKEIKVLQKIGKDTFITHELAAEAAGNLVGPRDFVSVRCAKR
RGSTCVLAGMATDFGNMPEQKGVIRAEHGPTCMVLHPLAGSPSKTKLTWLLSIDLKGWLPKSIIN
QVLSQTQVDFANHLRKRLESHPASEARC.

Human TOMM20 protein sequence SEQ ID NO:8
MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFKNRLRERRKKQKLAKERAGLSKLPDLKDAEA
VQKFFLEEIQLGEELLAQGEYEKGVDHLTNAIAVCGQPQQLLQVLQQTLPPPVFQMLLTKLPTISQ
RIVSAQSLAEDDVE

Human Tom/Star Fusion Protein Sequence (Tom sequence in **bold**; underlined is linker sequence) SEQ ID NO:9
MVGRNSAIAAGVCGALFIGYCIYFDRKRRSDPNFKNRLRERRKKQKLAKERAGLSKLPDLKDA
EAVQKFFLEEIQLGEELLAQGEYEKGVDHLTNAIAVCGQPQQLLQVLQQTLPPPVFQMLLTKLP
TISQRIVSAQSLAEDDVEGSDDDDKLEETLYSDQELAYLQQGEEAMQKALGILSNQEGWKKESQ
QDNGDKVMSKVVPDVGKVFRLEVVVDQPMERLYEELVERMEAMGEWNPNVKEIKVLQKIGKDT
FITHELAAEAAGNLVGPRDFVSVRCAKRRGSTCVLAGMATDFGNMPEQKGVIRAEHGPTCMVLH
PLAGSPSKTKLTWLLSIDLKGWLPKSIINQVLSQTQVDFANHLRKRLESHPASEARC

Human StAR/StAR Fusion Protein Sequence(underlined is linker sequence) SEQ ID NO:10
MLLATFKLCAGSSYRHMRNMKGLRQQAVMAISQELNRRALGGPTPSTWINQVRRRSSLLG
SRLEETLYSDQELAYLQQGEEAMQKALGILSNQEGWKKESQQDNGDKVMSKVVPDVGKVF
RLEVVVDQPMERLYEELVERMEAMGEWNPNVKEIKVLQKIGKDTFITHELAAEAAGNLVGP
RDFVSVRGSDDDDKLEETLYSDQELAYLQQGEEAMQKALGILSNQEGWKKESQQDNGDKV
MSKVVPDVGKVFRLEVVVDQPMERLYEELVERMEAMGEWNPNVKEIKVLQKIGKDTFITHE
LAAEAAGNLVGPRDFVSVRCAKRRGSTCVLAGMATDFGNMPEQKGVIRAEHGPTCMVLHP
LAGSPSKTKLTWLLSIDLKGWLPKSIINQVLSQTQVDFANHLRKRLESHPASEARC

FIG. 5 con't

STAR1 mRNA complete sequence, SEQ ID NO:11; Underlined amino acids 63-285 of SEQ ID NO:7 corresponding to UPPERCASE in following nucleotide sequence:

gcagctgcgggactcagaggcgaagcttgaggggctcaggaaggacgaagaaccacccttgagagaagaggcagcagcagcggc
ggcagcagcagcggcagcgaccccaccactgccacatttgccaggaaacaATGctgctagcgacattcaagctgtgcgctgggagc
tcctacagacacatgcgcaacatgaaggggctgaggcaacaggctgtgatggccatcagccaggagctgaaccggagggccctggg
gggccccacccctagcacgtggattaaccaggttcggcggcggagctctctactcggttctcggCTGGAAGAGACTCTCTAC
AGTGACCAGGAGCTGGCCTATCTCCAGCAGGGGGAGGAGGCCATGCAGAAGGCCTTGGGC
ATCCTTAGCAACCAAGAGGGCTGGAAGAAGGAGAGTCAGCAGGACAATGGGGACAAAGTGA
TGAGTAAAGTGGTCCCAGATGTGGGCAAGGTGTTCCGGCTGGAGGTCGTGGTGGACCAGCC
CATGGAGAGGCTCTATGAAGAGCTCGTGGAGCGCATGGAAGCAATGGGGGAGTGGAACCC
CAATGTCAAGGAGATCAAGGTCCTGCAGAAGATCGGAAAAGATACATTCATTACTCACGAGC
TGGCTGCCGAGGCAGCAGGAAACCTGGTGGGGCCCCGTGACTTTGTGAGCGTGCGCTGTG
CCAAGCGCCGAGGCTCCACCTGTGTGCTGGCTGGCATGGCCACAGACTTCGGGAACATGCC
TGAGCAGAAGGGTGTCATCAGGGCGGAGCACGGTCCCACTTGCATGGTGCTTCACCCGTTG
GCTGGAAGTCCCTCTAAGACCAAACTTACGTGGCTACTCAGCATCGACCTCAAGGGGTGGCT
GCCCAAGAGCATCATCAACCAGGTCCTGTCCCAGACCCAGGTGGATTTTGCCAACCACCTG
CGCAAGCGCCTGGAGTCCCACCCTGCCTCTGAAGCCAGGTGTTGAagaccagcctgctgttcccaactg
tgcccagctgcactggtacacacgctcatcaggagaatccctactggaagcctgcaagtctaagatctccatctggtgacagtgggatgg
gtggggttcgtgtttagagtatgacactaggattcagattggtgaaagtttttagtaccaagaaaacagggatgaggctcttggattaaaagg
taacttcattcactgattagctatgacatgagggttcaggcccctaaaataattgtaaaacttttttttctgggcccttatgtacccacctaaaacc
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ctggcagtacatgtgcacaaagcagaatctcagagggtctcctgcagcccctgctcctcccggccgctgcacagcaacaccacagaa
caagcagcaccccacagtgggtgccttccagaaatatagtccaagctttctctgtggaaaaagacaaaactcattagtagacatgtttccct
attgctttcataggcaccagtcagaataaagaatcataattcacacaaacatcagtctttgttttaatattgtactgttaaaaaaatctatgcag
ctgggtgcagtggctcacgcctgtaatcccagcattttgggaggctgaggtaggcggatcacaaggtcaggagatcgagaccatcctgg
ccaacatggtgaaaccccgtctctactaaaatacaaaaaattagctgggtgtggtggcgcaaacctgtagtcgtagctacttgggaggctg
aggcaggggaatcacttgaaccccggaggcggaggttgtagtgggccgagattgtgccactgcgctccagcgtgggcgacagagtga
gactccatctcaaaaaaaaaaaaaaaaaaatctatgctagtagattacaacttcacactagaggagttctggacaaagcttttaattagtc
aaactaaattaaggctcattaaaaggaaaggaactactgggaaattatgcaattcaataatttagactctgttaccaggatcttttcataaaaa
tttaatttccataatcataacctaaatgagttcttaaagaattctataagcaatagctgattaatgggccctggaagatgaagattataactgttt
atttacctaattaaaaggaaaggcagtgccaaatatgagaggataaacaatattagttaacatttctgttatttatgatgccaattagtagtaa
gataattccacagctgtcaactttgtttggggctggcaacttctctgcttaaacaggctaaaagtttagtattctgggagaagtggctggaaga
aggggtaatatggtgaaagcaaattcccttcccaggagtcaagagaatttatgtgaggtggctcccgcctgtaatctcagcactttgggag
gccaaggcaggcaatcacctgagggcaggagaccagcctgactaacatggagaaaccccatctctactaaaaatac

FIG. 5 con't

TOMM20 mRNA complete sequence, CDS = 123-560 SEQ ID NO:12

ctttctgtgttcctggcccgcggccgtcgggtgtgagctgcgccgaccgctctgagggttcgtggcccaccgctccttcgcggtccctgccgc
caccgtccacgctcagcgttgtagagaa**agATGgtgggtcggaacagcgccatcgccgccggtgtatgcgggggccctttttcatt**
gggtactgcatctacttcgaccgcaaaagacgaagtgaccccaacttcaagaacaggcttcgagaacgaagaaagaaaca
gaagcttgccaaggagagagctgggctttccaagttacctgaccttaaagatgctgaagctgttcagaagttcttccttgaaga
aatacagcttggtgaagagttactagctcaaggtgaatatgagaagggcgtagaccatctgacaaatgcaattgctgtgtggtgg
acagccacagcagttactgcaggtcttacagcaaactcttccaccaccagtgttccagatgcttctgactaagctcccaacaatt
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gtttacttgtgtagatccatttgtctatttttatttatttttcccagtgaaaagtgtattttgatagagaacttttcattctataaatacactatgagttacta
aaatatcatggatttgtttattcctgaaacatagttacatagttaaactgtacatatgacatggcttatgttaaaaatacccagtgctcagttttga
aagataggcaaaaaaaaaaaagtataggagaaactgaagaatgtacacttttttagagggcacattttgctgtaaatctggaaatttgata
gacttgactgtgtttgtgaaaactgagcattaaaggttttgattgatcctttctttccatttaatctctgagacgtaaatatgtgaggtgtgctgctgt
gctgggttaacagcttccttccctttctgtgtagcagtcttgaaatgttctgtttaaatcagtaggcttaatgtgttctgggtatttatctccttgtatttta
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cctgaggaatgtatgtgggcaaccaggccctgcatttttttatattctgaatttgcatgcttgcctgacttagtatttctgaattgatgttttttttaatg
gtataactatcttgatttcactgaaattatatggttctgtcactactctgtaaattaatccgaaacttttaaggtaactgggatgatctgcttgtaaa
aatgctigttgccttttgctttatcttcagtgtacctccttaatcctgcttcaacttgattatcttgtgaaacgatgagagtaagttgcaacctigtgac
tgaaaacttgaaaagagtggagcaggtgggacctcttattctcaaatagtgacatattctccgtagtcacagtttcagaactgagtaaggat
cctiggtacttggtggcatctgttgaactgaggagcatttctcattgtaaagattgccittgttctgtctaaaagtctggagaaatcccaaagactt
ttcctatgtactaggcatttattttgattgacttacaaactcttcttaatcattatcaatctcggttttttttgtggtgcagtggaaggagaaataggtct
agtttctgcctctgattagccgcacagccttgaacaaatcacatttcatctttgaacttacctctactgttagactaggcgactcacatttgagga
cttttctcgggtatcttgagggtttgtgatcctgaacccttaaacagtgcttttttgttacacaggagggctttttttgggggggatgaccagtacaga
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cagcagcccagacatgtatagaggggcagttagagggagaacaggggtgggaaagggagcaaggggcagatagctcagcaagga
aagaatgggctcagaaaaaggagggctggctggaggagtgaggggcagcttaagtttggggagggtagaaacgccgtttccttggga
actggagtgcagtatgagctgggtgtcacttggctctgaacatactggcttigctgtaatgcttgaaaaggcgttggtatcttcattttacagttc
attaacccaagtacgttttctattiaaatgacaacttiggtgctttaaaatgaggtaccacttttttaaagctagctgtgtcgagttaaagaaaaa
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ggccaaatggtgctcagcaggtgagaacaaaaaaaaccccagatctcagtgaactaatacacagcttgagcgtttccatgtgctaatgttg
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gtacttaaaagcgaaagaacattgaaaacatagacttactggctgtagcaatgctggcctgttaactgataactagaacttaggttcacgttt
atgtaaagtgtgtaaaacctagtagagcttgcatagtcggcactcagtaaatgtttggttcctttgcccctggtaagtttatttttaccatcctccc
acctgccattctgactttattaaatcaacatgtggaccagagtgttaatgagatgttattgcagaagagattgagaaaattggtatatcatgca
gataacatacaaaatcttttttgtaacgtaaaaaatgcagttttattattgcttgtgcctcaactgtttaagtgaatattaaagggcttggagaaaa

FIG. 5 con't

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/62389

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 15/52, A61K 35/12, A61K 48/00, C12N 9/02 (2022.01)

CPC - C12N 15/52, A61K 35/12, A61K 48/00, C12N 9/0004, C12Y 101/03006

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,903,183 B1 (STOCCO et al.) 7 June 2005 (07.06.2005) Abstract; Col. 4, ln 1-21; Col. 5, ln 45-67; Col. 6, ln 1-2	1-3, 18-20, 31-33
Y	US 2020/0179452 A1 (UNIVERSITY OF SOUTH ALABAMA FOUNDATION FOR RESEARCH AND COMMERCIALIZATION) 11 June 2020 (11.06.2020) Abstract; para [0007]; claim 7	1-3, 18-20, 31-33
Y	US 2006/0110730 A1 (BOSE et al.) 25 May 2006 (25.05.2006) para [0007]; para [0010]	3, 20, 33

☐ 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
"D" document cited by the applicant in the international application
"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
14 March 2022	MAR 28 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Kari Rodriquez Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/62389

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/62389

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

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

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

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

3. ☒ Claims Nos.: 4-17, 21-30, 34-43, 47-61, 65-78, 82-94
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-3,18-20 and 31-33 directed to a nucleic acid composition comprising a coding sequence for Steroidogenic Acute Regulatory (StAR) protein; and a coding sequence for a cholesterol degrading protein (CDP).

Group II: Claims 44-46, 62-64 and 79-81, directed to a method of enhancing cholesterol degradation in a mammalian cell comprising expressing non-native engineered StAR protein; and expressing one or more of a CDP.

----------continued on extra sheet----------

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3, 18-20, 31-33

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 21/62389

--continued from- Box III: Observations where unity of invention is lacking:

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I requires a nucleic acid composition comprising a coding sequence for an engineered StAR protein; and a coding sequence for a CDP, not required by Group II.

Group II requires a mammalian cell comprising a coding sequence for an engineered StAR protein; and a coding sequence for a CDP, not required by Group I.

Common Technical Features

The features shared by the inventions listed as Group I & II a coding sequence for Steroidogenic Acute Regulatory (StAR) protein; and a coding sequence for a cholesterol degrading protein (CDP).

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 6,903,183 B1 to Stocco et al. (hereinafter 'Stocco') in view of US 2020/0179452 A1 to University of South Alabama Foundation For Research And Commercialization (hereinafter 'USAFRC').

Stocco discloses a coding sequence for Steroidogenic Acute Regulatory (StAR) protein (Abstract- "Compositions and methods relating to the regulation of transport of cholesterol into the mitochondria of a cell and, therefore, for the regulation of steroidogenesis are provided. Compositions include nucleic acid molecules encoding a steroidogenic acute regulatory protein (StAR), StAR protein"); but does not teach a coding sequence for a cholesterol degrading protein (CDP).
However, Stocco teaches that the disclosed protein can be used for regulating cholesterol (Abstract).
USAFRC discloses expressing a cholesterol degrading protein (Abstract- " subjects may be administered one or more mammalian cells modified to express at least one sterol degrading enzyme derived from a bacterium"; claim 7- "wherein the protein is selected from cholesterol dehydrogenase (CholD)"). Since USAFRC teaches that the disclosed enzymes can be used for regulating cholesterol (para [0007]- "herein are methods to humanize and express the enzymes that aid in catalyzing cholesterol degradation"), it would have been obvious to one of ordinary skill in the art, to have combined the teachings of Stocco with USAFRC and use a combination of a coding sequence for StAR protein; and a coding sequence for a CDP, to achieve a desired cholesterol regulation, using routine experimentation.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I and II therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (continued)
Claims 4-17, 21-30, 34-43, 47-61, 65-78 and 82-94 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).