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(54) **Title**: REVERTED STELLATE CELLS AND USES THEREOF

(57) **Abstract**: The present invention relates to a composition for treating fibrosis comprising EGF and/or FGF2, and optionally at least one dietary fatty acid. The present invention also relates to a process for reverting activated stellate cells, preferably hepatic stellate cells, and to reverted stellate cells obtained by such process. The present invention also relates to *in vitro* methods for determining the pro- or anti-fibrotic activity of an agent.

REVERTED STELLATE CELLS AND USES THEREOF

FIELD OF INVENTION

The present invention relates to compositions and methods for treating fibrosis. In particular, the present invention relates to a composition comprising EGF and/or FGF2, and to the use thereof for reverting stellate cells or for treating fibrosis.

BACKGROUND OF INVENTION

In response to injury, tissue can heal and restore its normal function through a controlled sequence of events known as wound-healing. However, when the insult is of chronic nature it can impair the wound-healing response and subsequently develop into tissue fibrosis, a condition characterized by the excessive accumulation of matrix proteins and associated with severe morbidity and mortality. Different cellular sources, including tissue-specific fibroblasts, bone-marrow derived progenitor cells, pericytes and epithelial cells have been suggested to give rise to myofibroblasts, the major source of extracellular matrix (ECM) components in the fibrotic organ.

In the liver however, the resident hepatic stellate cells (HSCs) have unambiguously been identified as the predominant source of myofibroblasts, irrespective of the underlying disease etiology (Mederacke et al. Nat Commun. 2012, 4). In the normal liver, quiescent HSCs (qHSCs) reside in a virtual space (of Disse) between the hepatocytes and liver sinusoidal endothelial cells and are characterized by the abundance of cytoplasmic lipid droplets containing up to 80% of total vitamin A body reserve (Friedman, Physiological Reviews. 2008, 88: 125-172). Besides their well-known role in the regulation of retinoid and ECM homeostasis, there is evidence that HSCs can regulate the sinusoidal blood flow and stimulate angiogenesis (Reynaert et al. The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology. 2008, 291: 693-698; Taura et al. Gastroenterology. 2008, 135: 1729-1738). Following chronic liver injury, qHSCs undergo a process of activation, during which they transdifferentiate into cells with a

fibrogenic, myofibroblast-like phenotype characterized by increased ACTA2 expression which leads to an enhanced contractility, and an augmentation in ECM production and secretion (Friedman, Physiological Reviews. 2008, 88: 125-172; Taura et al. Gastroenterology. 2008, 135: 1729-1738).

Initially, this activation process was considered to be unidirectional while the principal ability of the fibrotic liver to revert to normal state upon cessation of injury (Ellis et al. Journal of Hepatology. 2012, 56: 1171-1180) was mainly attributed to apoptotic clearance of activated HSCs (aHSCs) that undergo apoptosis (Iredale et al. J Clin Invest. 1998, 102: 538-549; Issa et al. Gut. 2001, 48: 548-557).

However, different studies strongly imply that the activated phenotype of HSCs can be modulated and reverted to quiescent-like state *in vivo* in rodents (Kisseleva et al. PNAS. 2012; Troeger et al. Gastroenterology. 2012, 143: 1073-1083.e1022). Although these studies describe HSCs deactivation after cessation of injections which cause liver fibrosis in mouse models, no component was identified to induce such HSCs deactivation *in vivo*.

As activation of hepatic stellate cells is the central event in hepatic fibrosis, reversal of HSC activation could contribute to fibrosis cure. Stellate cell-like cells are present in various tissues and organs that can be affected by fibrosis, such as for example lung, kidney, pancreas, intestine, and the like. Therefore, for treating fibrosis, it could be of interest to develop compositions and/or methods for reversing HSC-like cells to a non-activated state.

The patent application US 2013/0101553 discloses reversal of hepatic stellate cells in mouse models and identified markers of activated HSCs, such as Hspa1a/b, PPARα and PPARγ. Moreover, US 2013/0101553 suggests that compounds upregulating the expression of these markers may reduce symptoms of fibrosis. However, US 2013/0101553 does not exemplify the effect of any of the claimed compounds in the treatment of fibrosis.

Different studies also demonstrated the reversal of HSCs *in vitro*. Hazra et al. discloses that depletion of PPARγ is a key feature in the activation of HSCs and demonstrates that restoration of this component reverses the activated HSCs to a quiescent phenotype

(Journal of Biological Chemistry. 2004, 279: 11392-11401). She et al. extends these findings and describes the use of an adipocyte differentiation mixture comprising isobutylmethylxanthine, dexamethasone, and insulin, or the ectopic expression of PPARγ or SREBP-1c, to reverse activated HSCs to a quiescent phenotype (Journal of Biological Chemistry. 2005, 280: 4959-4967).

Nevertheless, to the Applicant knowledge, there is currently no molecule restoring a quiescent phenotype of stellate cells which has been validated in human by clinical trials. Therefore, there is still a need for compounds that allow reversing activated stellate cells, thereby treating fibrosis.

Identification of a potential pro-fibrotic activity of agents could be useful to prevent or minimize drug-related fibrotic adverse effects. However, actual conditions for *in vitro* stellate cells culture are limited by cell activation as soon as they are cultured. Consequently, identification of anti- and pro-fibrotic agents is currently performed *in vivo* in animal models. Therefore, a compound allowing reversing activated stellate cells may also be of interest for developing culture media for maintaining HSCs in a non-activated state in culture. Such culture media could thus be used for screening agents for their pro- or anti-fibrotic activity.

The inventors herein surprisingly demonstrate that the presence of EGF and/or FGF2 in a culture medium reverses activated stellate cells to a quiescent-like state (see Examples).

Therefore, the present invention relates to a composition comprising EGF and/or FGF2 and to the use thereof for reverting stellate cells or for treating fibrosis.

SUMMARY

The present invention thus relates to a composition for treating fibrosis comprising EGF and/or FGF2. In one embodiment, the composition further comprises at least one dietary fatty acid. In one embodiment, said at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid,

arachidonic acid, myristic acid, and retinol, preferably oleic acid, palmitic acid or retinol, or a mixture thereof.

In one embodiment, said fibrosis is selected from the group comprising liver fibrosis, pulmonary fibrosis, heart fibrosis, Crohn's Disease, progressive kidney disease, pancreatic fibrosis, scleroderma/systemic sclerosis, post-surgery adhesions, peritoneal adhesions, retroperitoneal fibrosis, pleural fibrosis, pericardial fibrosis, uterine fibroid, and graft fibrosis.

In a particular embodiment, said fibrosis is liver fibrosis, wherein said liver fibrosis is cirrhosis. In another particular embodiment, fibrosis is pulmonary fibrosis, wherein said pulmonary fibrosis is idiopathic pulmonary fibrosis or cystic fibrosis.

The present invention also relates to an *in vitro* process for reverting stellate cells comprising a step of incubating stellate cells into a composition comprising EGF and/or FGF2. In one embodiment, said composition further comprises at least one dietary fatty acid. In one embodiment, said at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, arachidonic acid, myristic acid, and retinol, preferably oleic acid, palmitic acid or retinol, or a mixture thereof.

In one embodiment, said stellate cells are selected from the group comprising hepatic stellate cells (HSCs), pancreatic stellate cells (PaSCs), lung stellate cells, intestinal stellate cells, kidney stellate cells, spleen stellate cells, adrenal gland stellate cells, ductus deferens stellate cells and vocal cords stellate cells, preferably HSCs, PaSCs, lung stellate cells and intestinal stellate cells, more preferably HSCs.

The present invention further relates to an isolated reversed stellate cells population obtained by the *in vitro* process as described hereinabove.

Another object of the present invention is a composition comprising EGF, FGF2, oleic acid, palmitic acid and retinol, wherein the concentration of EGF ranges from 0.1 to 100 ng/mL, the concentration of FGF2 ranges from 0.05 to 80 ng/mL, the concentration of oleic acid ranges from 1 to 1000 nmol/mL, the concentration of palmitic acid ranges

from 1 to 1000 nmol/mL and the concentration of retinol ranges from 0.01 to 100 nmol/mL. Another object of the present invention is a culture medium comprising the composition as described hereinabove.

The present invention further relates to an in vitro method for determining the pro-fibrotic activity of a tested agent comprising the steps of *in vitro* incubating stellate cells in a culture medium as described hereinabove; adding the tested agent in the culture medium; and determining the phenotype of said stellate cells, preferably the activated or quiescent phenotype of said stellate cells.

The present invention also relates to a kit of part comprising two parts, wherein the first part comprises a composition as described hereinabove and the second part comprises a stellate cells population.

DEFINITIONS

In the present invention, the following terms have the following meanings:

- "**About**" preceding a value means plus or less 10% of said value.
- "**Culture medium**" refers to a composition comprising components for supporting the growth and maintenance of cells in culture. Usually, a culture medium comprises a carbon source, various salts and water.
- "**Fibrosis**" refers to the formation of excess fibrous connective tissue in an organ or tissue in a reparative or reactive process. In one embodiment, fibrosis involves excessive collagen mRNA production and deposition (mostly Type I collagen). In another embodiment, fibrosis is caused, at least in part, by injury, e.g. chronic injury (e.g. an insult, a wound, a toxin, a disease). In another embodiment, fibrosis is associated with an inflammatory, an autoimmune or a connective tissue disorder. Tissues that may be affected by fibrosis include, without limitation, liver tissue, lung tissue, heart tissue, kidney tissue, skin tissue, gut tissue, peritoneal tissue, bone marrow, and the like.

- "**Subject**" refers to a mammal, preferably a human. In one embodiment, a subject may be a "patient", i.e. a warm-blooded animal, more preferably a human, who/which is awaiting the receipt of, or is receiving medical care or was/is/will be the object of a medical procedure, or is monitored for the development of fibrosis.

- "**Treating**" refers to both therapeutic treatment and prophylactic or preventative measures; wherein the object is to prevent or slow down (lessen) the targeted fibrosis. Those in need of treatment include those already with the disease as well as those prone to have the disease or those in whom the disease is to be prevented. A subject is successfully "treated" for fibrosis if, after receiving a therapeutic amount of the composition, pharmaceutical composition or medicament of the present invention, the subject shows observable and/or measurable reduction in or absence of one or more of the following: reduction in the number of pathogenic cells; reduction or absence of fibrotic area; reduction in the percent of total cells that are pathogenic; and/or relief to some extent, of one or more of the symptoms associated with the specific fibrosis; reduced morbidity and mortality, and improvement in quality of life issues. The above parameters for assessing successful treatment and improvement in the respiratory disease are readily measurable by routine procedures familiar to a physician.

- "**Therapeutically effective amount**" means level or amount of the composition, pharmaceutical composition or medicament of the invention that is aimed at, without causing significant negative or adverse side effects to the target, (1) delaying or preventing the onset of fibrosis; (2) slowing down or stopping the progression, aggravation, or deterioration of one or more symptoms of fibrosis; (3) bringing about ameliorations of the symptoms of fibrosis; (4) reducing the severity or incidence of fibrosis; or (5) curing fibrosis. A therapeutically effective amount may be administered prior to the onset of the fibrosis, for a prophylactic or preventive action. Alternatively or additionally, the therapeutically effective amount may be administered after initiation of the fibrosis, for a therapeutic action.

- "**Pharmaceutically acceptable carrier or excipient**" refers to an excipient or carrier that does not produce an adverse, allergic or other untoward reaction when administered to an animal, preferably a human. It includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and

absorption delaying agents and the like. For human administration, injected preparations should meet sterility, pyrogenicity, general safety and purity standards as required by regulatory offices, such as, for example, FDA Office or EMA.

DETAILED DESCRIPTION

The inventors herein demonstrated that the presence of epithelial growth factor (EGF) and/or fibroblast growth factor 2 (FGF2) in a culture medium leads to the reversion of activated stellate cells, such as, for example, stellate cells in culture, to a quiescent-like state (see Examples).

This invention thus relates to a composition comprising EGF and/or FGF2. In one embodiment, the composition comprises EGF. In another embodiment, the composition comprises FGF2. In another embodiment, the composition comprises EGF and FGF2.

In one embodiment of the present invention, the concentration of EGF in the composition of the invention ranges from 0.1 to 100 ng/mL, preferably from 1 to 75 ng/mL, more preferably from 5 to 50 ng/mL, more preferably the concentration of EGF in the composition of the invention is of about 20 ng/mL.

In one embodiment of the present invention, the concentration of FGF2 in the composition of the invention ranges from 0.05 to 80 ng/mL, preferably from 0.1 to 50 ng/mL, more preferably from 1 to 30 ng/mL, more preferably the concentration of FGF2 in the composition of the invention is of about 10 ng/mL.

The inventors herein also demonstrated that the presence of dietary fatty acids such as oleic, palmitic acids and retinol (also named retinoic acid) leads to the reversion of activated stellate cells to a quiescent-like state (see Examples).

Therefore, in one embodiment, the composition of the invention further comprises at least one dietary fatty acid.

In one embodiment of the present invention, the concentration of the at least one dietary fatty acid ranges from 1 to 1000 nmol/mL, preferably from 10 to 500 nmol/mL, more

preferably from 50 to 200 nmol/mL, more preferably the concentration of the at least one dietary fatty acid is of about 100 nmol/mL.

In one embodiment, the composition of the invention comprises EGF and at least one dietary fatty acid. In another embodiment, the composition of the invention comprises FGF2 and at least one dietary fatty acid. In another embodiment, the composition of the invention comprises EGF, FGF2 and at least one dietary fatty acid.

In one embodiment, the at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, arachidonic acid, myristic acid, retinol or a mixture thereof. In a particular embodiment, the at least one dietary acid is selected from oleic acid, palmitic acid and retinol or a mixture thereof. In one preferred embodiment, the composition of the invention comprises oleic acid, palmitic acid and retinol.

In one embodiment of the present invention, the concentration of oleic acid in the composition of the invention ranges from 1 to 1000 nmol/mL, preferably from 10 to 500 nmol/mL, more preferably from 50 to 200 nmol/mL, more preferably the concentration of oleic acid in the composition of the invention is of about 100 nmol/mL.

In one embodiment of the present invention, the concentration of palmitic acid in the composition of the invention ranges from 1 to 1000 nmol/mL, preferably from 10 to 500 nmol/mL, more preferably from 50 to 200 nmol/mL, more preferably the concentration of palmitic acid in the composition of the invention is of about 100 nmol/mL.

In one embodiment of the present invention, the concentration of retinol in the composition of the invention ranges from 0.01 to 100 nmol/mL, preferably from 0.1 to 50 nmol/mL, more preferably from 0.5 to 20 nmol/mL, more preferably the concentration of retinol in the composition of the invention is of about 5 nmol/mL.

In one embodiment of the present invention, the concentration of retinol in the composition of the invention ranges from 0.01 to 95.5 IU/mL, preferably from 0.095 to

47.7 IU/mL, more preferably from 0.48 to 19.1 IU/mL, more preferably the concentration the concentration of retinol in the composition of the invention is of about 4.8 IU/mL.

In one embodiment, the composition of the invention comprises EGF and oleic acid. In another embodiment, the composition of the invention comprises EGF and palmitic acid. In another embodiment, the composition of the invention comprises EGF and retinol. In another embodiment, the composition of the invention comprises EGF, oleic acid and palmitic acid. In another embodiment, the composition of the invention comprises EGF, oleic acid and retinol. In another embodiment, the composition of the invention comprises EGF, palmitic acid and retinol. In another embodiment, the composition of the invention comprises EGF, oleic acid, palmitic acid and retinol.

In one embodiment, the composition of the invention comprises FGF2 and oleic acid. In another embodiment, the composition of the invention comprises FGF2 and palmitic acid. In another embodiment, the composition of the invention comprises FGF2 and retinol. In another embodiment, the composition of the invention comprises FGF2, oleic acid and palmitic acid. In another embodiment, the composition of the invention comprises FGF2, oleic acid and retinol. In another embodiment, the composition of the invention comprises FGF2, palmitic acid and retinol. In another embodiment, the composition of the invention comprises FGF2, oleic acid, palmitic acid and retinol.

In one embodiment, the composition of the invention comprises EGF, FGF2 and oleic acid. In another embodiment, the composition of the invention comprises EGF, FGF2 and palmitic acid. In another embodiment, the composition of the invention comprises EGF, FGF2 and retinol. In another embodiment, the composition of the invention comprises EGF, FGF2, oleic acid and palmitic acid. In another embodiment, the composition of the invention comprises EGF, FGF2, oleic acid and retinol. In another embodiment, the composition of the invention comprises EGF, FGF2, palmitic acid and retinol. In another embodiment, the composition of the invention comprises EGF, FGF2, oleic acid, palmitic acid and retinol.

In one preferred embodiment, the composition of the invention comprises:

- from 0.1 to 100 ng/mL of EGF, preferably from 1 to 75 ng/mL, more preferably from 5 to 50 ng/mL, more preferably about 20 ng/mL,
- from 0.05 to 80 ng/mL of FGF2, preferably from 0.1 to 50 ng/mL, more preferably from 1 to 30 ng/mL, more preferably about 10 ng/mL,
- from 1 to 1000 nmol/mL of oleic acid, preferably from 10 to 500 nmol/mL, more preferably from 50 to 200 nmol/mL, more preferably about 100 nmol/mL,
- from 1 to 1000 nmol/mL of palmitic acid, preferably from 10 to 500 nmol/mL, more preferably from 50 to 200 nmol/mL, more preferably about 100 nmol/mL, and
- from 0.01 to 100 nmol/mL of retinol, preferably from 0.1 to 50 nmol/mL, more preferably from 0.5 to 20 nmol/mL, more preferably about 5 nmol/mL.

In one embodiment, the composition according to the invention is in form of solution. In another embodiment, the composition according to the invention is in form of powder to be dissolved or resuspended in water.

Another object of the present invention is the use of the composition as described hereinabove as a culture medium.

The invention also relates to a culture medium comprising a composition according to the invention.

In one embodiment, the composition or the culture medium of the invention further comprises:

- a carbon source for cell growth, which may be a sugar such as glucose, or a less energy-rich source like succinate,
- various salts, which may vary among cell species and growing conditions, for example essential elements such as magnesium, nitrogen, phosphorus, or sulfur, and
- water.

In one embodiment, the composition or the culture medium of the invention is suitable for eukaryote cell culture, preferably for mammalian cell culture. In one embodiment, the composition for use as a culture medium or the culture medium of the invention comprises

the composition according to the invention and a culture medium of the prior art such as for example MEM, DMEM, IMDM, RPMI 1640, 199/109 medium, HamF10/HamF12 or McCoy's 5A.

In one embodiment, the composition further comprises any supplementary factors known by the person skilled in the art that may be used in cell culture. Examples of supplementary factors include, but are not limited to, FBS; glycine; amino acids, such as glutamine, asparagine, glutamic acid, aspartic acid, serine, proline or alanine, preferably the L-configuration of amino acids; and antibiotics, such as streptomycin or penicillin.

In one embodiment, the composition of the invention has a pH ranging from 6.8 to 8.0, preferably from 7.0 to 7.8, more preferably from 7.2 to 7.6.

In one embodiment, the composition of the invention is free of biological contamination such as for example bacteria, fungi (such as, for example, molds or yeasts), viruses, protozoa or mycoplasmas. In another embodiment, the composition according to the invention is sterile and stored in sterile conditions.

The invention also relates to the use of the composition as described hereinabove for reverting stellate cells, preferably for *in vitro* reverting stellate cells.

As used herein, reverting stellate cells, or reversion of stellate cells, means allowing activated stellate cells to return to a non-activated state, preferably a quiescent-like state. In one embodiment, activated HSCs are HSCs that have transdifferentiated into cells with a fibrogenic, myofibroblast-like phenotype may be characterized by the loss of vitamin A containing lipid droplets. In another embodiment, activated HSCs may be characterized by an increased ACTA2, COL1A1 and LOX expression. In another embodiment, activated HSCs may be characterized by the loss of vitamin A containing lipid droplets and an increased ACTA2, COL1A1 and LOX expression.

Alpha smooth muscle actin (ACTA1), collagen type I alpha 1 (COL1α1) and lysyl oxidase (LOX) are known genes related to hepatic stellate cells activation (Mannaerts et al. PLoS ONE. 2013, 8(12):e84071).

In one embodiment of the invention, a quiescent-like state or quiescent-like phenotype may be characterized by the presence of vitamin A containing lipid droplets. In another embodiment, a quiescent-like state or quiescent-like phenotype may be characterized by a low expression of ACTA2, COL1A1 and LOX. In another embodiment, a quiescent-like state or quiescent-like phenotype may be characterized by the presence of vitamin A containing lipid droplets and a low expression of ACTA2, COL1A1 and LOX. As used herein, the term "low level" or "low expression" refers to levels lower than level in activated stellate cells. In one embodiment, a low level is at least 10% lower than the level in activated stellate cells, preferably at least 20%, 30%, 40% or 50% lower than the level in activated stellate cells.

In one embodiment, stellate cells are selected from the group comprising hepatic stellate cells (HSCs), pancreatic stellate cells (PaSCs), lung stellate cells, intestinal stellate cells, kidney stellate cells, spleen stellate cells; preferably HSCs, PaSCs, lung stellate cells and intestinal stellate cells, and more preferably HSCs and PaSCs. In a preferred embodiment, stellate cells are HSCs.

In one embodiment, the composition according to the invention is used for reverting HSCs.

Another object of the invention is an *in vitro* process for reverting stellate cells, preferably HSCs.

In one embodiment, the process of the invention comprises a step of culture of stellate cells within a culture medium of the invention or within a composition of the invention. In one embodiment, the composition is refreshed every day, or every two days, or every three days.

In one embodiment, the process of the invention optionally comprises a preceding step of culture of stellate cells in a composition that does not contain EGF, FGF2 and/or dietary components, which leads stellate cells to be activated.

In another embodiment, stellate cells used for the process according to the invention are freshly isolated quiescent stellate cells or not yet cultured stellate cells.

This invention also relates to a reversed stellate cell population (rSC), preferably a reversed hepatic stellate cell population (rHSC).

In one embodiment, the reversed stellate cell population is an isolated reversed stellate population.

In one embodiment, the reversed stellate cells population is characterized by a non-activated phenotype, preferably a quiescent-like phenotype.

In one embodiment, the reversed stellate cell population expresses a low level of ACTA2, COL1A1 and LOX. In another embodiment, the reversed stellate cell population is characterized by the presence of lipid containing droplets. In another embodiment, the reversed stellate cells population expresses a low level of ACTA2, COL1A1 and LOX, and is characterized by the presence of lipid containing droplets.

In one embodiment, the reversed stellate cell population is obtained by the *in vitro* process for reverting stellate cells as described hereinabove.

According to one embodiment, the reversed stellate cell population of the invention comprises reversed stellate cells and non-reversed, or activated, stellate cells. In a particular embodiment, the reversed stellate cell population at least 70%, preferably at least 75, 80, 85, 90, 95 or 100% of reversed stellate cells.

This invention also concerns a composition comprising a population of stellate cells and a composition according to the invention, wherein the stellate cells expand in said composition.

Another object of the invention is a kit of part comprising two parts, wherein the first part comprises a composition or a culture medium according to the invention and the second part comprises a stellate cells population, preferably a HSCs population.

In one embodiment, the first part of the kit of the invention comprises a composition or a culture medium according to the invention, wherein the composition or a culture medium is in the form of a solution. In another embodiment, the first part of the kit of the invention comprises a composition or a culture medium according to the invention, wherein the

composition or a culture medium is in the form of a powder to be dissolved or resuspended in water.

In one embodiment, the stellate cell population of the second part of the kit of the invention is an activated stellate cell population. In another embodiment, the stellate cell population of the second part of the kit of the invention is a quiescent stellate cell population. In another embodiment, the stellate cell population of the second part of the kit of the invention is a reversed stellated cell population.

In one embodiment, the second part of the kit of the invention comprises a stellate cell population, wherein the stellate cells population is in the form of a frozen stock solution. In one embodiment, the stellate cells population is stocked in a composition according to the invention.

A further aspect of the present invention is a method for determining the pro-fibrotic activity of a tested agent, comprising the steps of:

a) *in vitro* incubating stellate cells in a culture medium of the invention or in a composition according to the invention,
b) adding the tested agent in the culture medium, and
c) determining the phenotype of said stellate cells, preferably the activated or quiescent phenotype of said stellate cells.

In one embodiment, "determining the phenotype of said stellate cells" corresponds to determining the expression profile of at least one gene marker of activated stellate cells in said stellate cells, and comparing this expression profile with a reference expression profile.

Examples of gene markers of activated stellate cells include, but are not limited to, ACTA2, COL1A1, LOX, LOXL1, LOXL2, LOXL3, PDGFRB, COL4A1, COL4A2, COL5A1, ADAM12, ADAMTS2, ACTG2, NOTCH3 and CRYAB, preferably ACTA2, COL1A1 and LOX.

As used herein, the term "expression" may refer alternatively to the transcription of a gene marker of activated stellate cells (i.e. expression of the RNA) or to the translation (i.e. expression of the protein) of a gene marker of activated stellate cells.

Methods for determining the expression profile are well-known from the skilled artisan, and include, without limitation, determining the transcriptome (in an embodiment wherein expression relates to transcription of a gene marker of activated stellate cells) or proteome (in an embodiment wherein expression relates to translation of a gene marker of activated stellate cells) of a stellate cell population cultured in presence of the tested agent.

In one embodiment of the invention, the expression of the at least one gene marker of activated stellate cells is assessed at the RNA level. Methods for assessing the transcription level of a gene marker are well known in the prior art. Examples of such methods include, but are not limited to, RT-PCR, RT-qPCR, Northern Blot, hybridization techniques such as, for example, use of microarrays, and combination thereof including but not limited to, hybridization of amplicons obtained by RT-PCR, sequencing such as, for example, next-generation DNA sequencing (NGS) or RNA-seq (also known as "Whole Transcriptome Shotgun Sequencing") and the like.

In one embodiment of the invention, the expression of the at least one gene marker of activated stellate cells is assessed at the protein level. Methods for determining a protein level in a sample are well-known in the art. Examples of such methods include, but are not limited to, immunohistochemistry, Multiplex methods (Luminex), western blot, enzyme-linked immunosorbent assay (ELISA), sandwich ELISA, fluorescent-linked immunosorbent assay (FLISA), enzyme immunoassay (EIA), radioimmunoassay (RIA) and the like.

In one embodiment of the invention, the reference expression profile is the expression profile of the at least one gene marker of activated stellate cells in stellate cells incubated in a culture medium according to the invention without the tested agent.

In one embodiment, a tested agent has a pro-fibrotic activity if the expression of the at least one gene marker of activated stellate cells is higher than the reference expression

profile. In one embodiment, the term "higher" means at least 10% higher, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% higher or more than the reference expression profile.

In another embodiment, "determining the phenotype of said stellate cells" corresponds to evaluating the presence or the number of lipid containing droplets in said stellate cells.

In one embodiment, a tested agent has a pro-fibrotic activity if lipid containing droplets are absent of stellate cells incubated with said tested agent.

In another embodiment, a tested agent as a pro-fibrotic activity if the number of lipid containing droplets is lower in stellate cells incubated with said tested agent than in stellate cells incubated in the absence of the tested agent. In one embodiment, the term "lower" means at least 10% lower, preferably at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% lower or more than the number of lipid containing droplets in stellate cells incubated in the absence of the tested agent.

Methods for evaluating the presence of lipid containing droplets are well-known from the skilled artisan, and include, without limitation, Nile Red, BODIPY, LD450 and Oil Red O.

In another embodiment, the step c) of determining the phenotype of said stellate cells comprises the step of:

- determining the expression profile of at least one gene marker of activated stellate cells in said stellate cells, and comparing this expression profile with a reference expression profile, and
- evaluating the presence of lipid containing droplets in said stellate cells.

In a preferred embodiment, the method for determining the pro-fibrotic activity of a test agent, comprising the steps of:

a) *in vitro* incubating stellate cells in a culture medium of the invention or a composition used as a culture medium according to the invention;
b) adding the tested agent in the culture medium;
c) determining the phenotype of said stellate cells by:

- determining the expression profile of at least one gene marker of activated stellate cells in said stellate cells, and comparing this expression profile with a reference expression profile, and
- evaluating the presence of lipid containing droplets in said stellate cells.

Another aspect of the present invention is a method for determining the anti-fibrotic activity of a tested agent, comprising the steps of:

a) *in vitro* incubating stellate cells in a culture medium which is not a culture medium or a composition used as a culture medium according to the invention;
b) adding the tested agent in the culture medium;
c) determining the expression profile of at least one gene marker of activated stellate cells in said stellate cells; and
d) comparing the expression profile obtained in step c) with a reference expression profile.

In one embodiment of the invention, the reference expression profile is the expression profile of the at least one gene marker of activated stellate cells in stellate cells incubated in a culture medium according to the invention without the tested agent.

In one embodiment, a tested agent has an anti-fibrotic activity if the expression of the at least one gene marker of activated stellate cells is similar to the reference expression level. In one embodiment, the term "similar" means that the difference between the expression levels are of less than 20%, preferably less than 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1% or less.

Another object of the invention is a pharmaceutical composition comprising EGF and/or FGF2 as described hereinabove, in combination with at least one pharmaceutically acceptable excipient.

Suitable excipients include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol®, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, such as, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like.

In one embodiment, the pharmaceutical composition comprises EGF in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition comprises FGF2 in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition comprises EGF and FGF2 in combination with at least one pharmaceutically acceptable excipient.

In one embodiment, the composition may comprise a pharmaceutically acceptable salt of EGF and/or FGF2.

Examples of pharmaceutically acceptable salts include salts with inorganic bases, salts with organic bases, salts with inorganic acids, salts with organic acids, salts with basic or acidic amino acids and the like. Examples of salts with an inorganic base include alkali metal salts, such as a sodium salt and a potassium salt; an alkaline earth metal salt such as a calcium salt and a magnesium salt; an aluminum salt; and an ammonium salt. Examples of salts with an organic base include salts with trimethylamine, triethylamine, pyridine, picoline, 2,6-lutidine, ethanolamine, diethanolamine, triethanolamine, cyclohexylamine, dicyclohexylamine and N,N'-dibenzylethylenediamine. Examples of salts with an inorganic acid include salts with hydrochloric acid, boric acid, nitric acid, sulfuric acid and phosphoric acid. Examples of salts with an organic acid include salts with formic acid, acetic acid, trifluoroacetic acid, phthalic acid, fumaric acid, oxalic acid, tartaric acid, maleic acid, citric acid, succinic acid, malic acid, methanesulfonic acid, benzenesulfonic acid and p-toluenesulfonic acid. Examples of salts with a basic amino acid include salts with arginine, lysine and ornithine. Examples of salts with an acidic amino acid include salts with aspartic acid and glutamic acid. A list of suitable salts is disclosed in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., p 1418, 1985, the entire disclosure of which is incorporated herein by reference.

In one embodiment, the pharmaceutical composition according to the invention further comprises at least one dietary fatty acid.

In one embodiment, the at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, arachidonic acid, myristic acid, retinoic acid (or retinol) or a mixture thereof. In a particular embodiment, the at least one dietary acid is selected from the group comprising oleic acid, palmitic acid and retinol, or a mixture thereof. In one preferred embodiment, the pharmaceutical composition of the invention further comprises oleic acid, palmitic acid and retinol.

In one embodiment, the pharmaceutical composition of the invention comprises EGF and at least one dietary fatty acid in combination with at least one pharmaceutically acceptable excipient. In one embodiment, the pharmaceutical composition of the invention comprises EGF and oleic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, oleic acid and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, oleic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, oleic acid, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient.

In one embodiment, the pharmaceutical composition of the invention comprises FGF2 and at least one dietary fatty acid in combination with at least one pharmaceutically acceptable excipient. In one embodiment, the pharmaceutical composition of the invention comprises FGF2 and oleic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical

composition of the invention comprises FGF2 and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises FGF2 and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises FGF2, oleic acid and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises FGF2, oleic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises FGF2, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises FGF2, oleic acid, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient.

In one embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2 and at least one dietary fatty acid in combination with at least one pharmaceutically acceptable excipient. In one embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2 and oleic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2 and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2 and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2, oleic acid and palmitic acid in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2, oleic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient. In another embodiment, the pharmaceutical composition of the invention comprises EGF, FGF2,

oleic acid, palmitic acid and retinol in combination with at least one pharmaceutically acceptable excipient.

Another object of the invention is a medicament comprising EGF and/or FGF2 or a pharmaceutically acceptable salt or solvate thereof, as described hereinabove.

In one embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof. In another embodiment, the medicament of the invention comprises EGF and FGF2 or a pharmaceutically acceptable salt or solvate thereof.

In one embodiment, the medicament further comprises at least one dietary fatty acid. In one embodiment, the at least one dietary fatty acid is selected from oleic acid, palmitic acid, retinol and mixture thereof.

In one embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and at least one dietary fatty acid. In one embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and oleic acid. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and palmitic acid. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and retinol. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and oleic acid and palmitic acid. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof and oleic acid and retinol. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof, palmitic acid and retinol. In another embodiment, the medicament of the invention comprises EGF or a pharmaceutically acceptable salt or solvate thereof, oleic acid, palmitic acid and retinol.

In one embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof and at least one dietary fatty acid. In

one embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof and oleic acid. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof and palmitic acid. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof and retinol. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof and oleic acid and palmitic acid. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof, oleic acid and retinol. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof, palmitic acid and retinol. In another embodiment, the medicament of the invention comprises FGF2 or a pharmaceutically acceptable salt or solvate thereof, oleic acid, palmitic acid and retinol.

In one embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, and at least one dietary fatty acid. In one embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, and oleic acid. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, and palmitic acid. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, and retinol. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, oleic acid and palmitic acid. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, oleic acid and retinol. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, palmitic acid and retinol. In another embodiment, the medicament of the invention comprises EGF, FGF2, or a pharmaceutically acceptable salt or solvate thereof, oleic acid, palmitic acid and retinol.

Another object of the invention is a composition, a pharmaceutical composition or a medicament as described here above for treating fibrosis or for use in treating fibrosis.

In one embodiment, fibrosis is selected in the group comprising liver fibrosis (such as for example alcoholic hepatitis, cirrhosis, or acute-on-chronic hepatitis), pulmonary fibrosis (such as for example idiopathic pulmonary fibrosis or cystic fibrosis), heart fibrosis, Crohn's Disease, progressive kidney disease, pancreatic fibrosis, scleroderma/systemic sclerosis, post-surgery or peritoneal adhesions, retroperitoneal fibrosis, pleural fibrosis, pericardial fibrosis, uterine fibroid, and graft fibrosis.

In one embodiment, the subject is affected with a liver disease, preferably selected from the list comprising significant fibrosis, cirrhosis, the fibrosis being from alcoholic or non-alcoholic origin and/or the subject is a patient affected with a chronic disease, preferably said chronic disease is selected from the group comprising chronic viral hepatitis C, chronic viral hepatitis B, chronic viral hepatitis D, chronic viral hepatitis E, non-alcoholic fatty liver disease (NAFLD), alcoholic chronic liver disease, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis and Wilson disease.

The invention also relates to a method of treating fibrosis comprising administering to a subject in need thereof a therapeutically effective amount of a composition, pharmaceutical composition or medicament of the present invention.

In one embodiment of the invention, the subject is an animal, preferably a mammal, such as for example, a rat or a pet, such as, for example, a cat or a dog. According to a preferred embodiment, the subject is a human. In one embodiment of the invention, the human is a male, a female or a child. According to an embodiment, the subject, including a human, is at risk of presenting fibrosis in an organ or a tissue; or presents fibrosis in an organ or a tissue. In one embodiment, the subject is at risk of being affected by hepatitis. In another embodiment, the subject is an excessive alcohol drinker. In another embodiment, the subject has a history of smoking and/or breathing secondhand smoke. In another embodiment, the subject is overweight. In another embodiment, the subject is genetically predisposed to develop fibrosis.

In one embodiment, the composition, pharmaceutical composition or medicament of the invention is in a form adapted for oral administration.

Examples of forms adapted for oral administration include, but are not limited to, tablets, orodispersing/orally disintegrating tablets, effervescent tablets, powders, granules, pills (including sugarcoated pills), dragees, capsules (including soft gelatin capsules), syrups, liquids, gels or other drinkable solutions, suspensions, slurries, liposomal forms and the like.

In one embodiment, the composition, pharmaceutical composition or medicament of the invention is in a form adapted for injection, such as, for example, for intramuscular, subcutaneous, intradermal, transdermal or intravenous injection or infusion and the like.

Examples of forms adapted for injection include, but are not limited to, solutions, such as, for example, sterile aqueous solutions, dispersions, emulsions, suspensions, solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to use, such as, for example, powder, liposomal forms and the like.

Administration of the composition, pharmaceutical composition or medicament of the invention may be accomplished by any acceptable method which allows the therapeutically components to reach its target. Any acceptable method known to one of ordinary skill in the art may be used to administer a composition, pharmaceutical composition or medicament to the subject. The administration may be localized (i.e., to a particular region, physiological system, tissue, organ, or cell type) or systemic, depending on the condition being or to be treated. In one embodiment, the targeted tissue comprises stellate cells, preferably HSCs.

In one embodiment, a therapeutically effective amount of a composition, pharmaceutical composition or medicament of the present invention can be delivered via a nanoparticle-based drug delivery system. In another embodiment, the composition, pharmaceutical composition or medicament can be perfused directly through the targeted tissue, such as the liver. In another embodiment, the composition, pharmaceutical composition or medicament can be delivered using a bioerodible implant by way of diffusion or by degradation of a polymer matrix.

Other suitable delivery systems include, but are not limited to, time-release, delayed release, sustained release, or controlled release delivery systems. Many types of release delivery system are available and known to those of ordinary skill in the art. They include, for example, polymer-based systems or non-polymer systems such as for example liposome-based systems or hydrogel release systems. In one embodiment, the composition, pharmaceutical composition or medicament can be delivered by vitamin A-coupled liposomes.

It will be understood that the total daily usage of the compound of the invention, composition, pharmaceutical composition and medicament of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific composition employed; the duration of the treatment; drugs used in combination or coincidental with the specific composition employed; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of a compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the composition may be varied over a wide range from about 10 to about 10000 mg of active components per adult per day, preferably 100 to about 5000, more preferably from about 200 to about 2000 mg per adult per day. Preferably, the compositions contain 10, 50, 100, 250, 500, 1000 and 2,000 mg of the active ingredient for the symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 10 to about 10000 mg of the active ingredient, preferably 100 to about 5000, more preferably from about 200 to about 2000 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.1 mg/kg to about 100 mg/kg of body weight per day, preferably from about 1 mg/kg to 40 mg/kg of body weight per day, more preferably from about 2 mg/kg to 20 mg/kg of body weight per day.

In another embodiment of the invention, the composition, pharmaceutical composition or medicament of the invention may further comprise at least one anti-fibrotic agent.

In another embodiment of the invention, the composition, pharmaceutical composition or medicament of the invention may be used in combination with at least one anti-fibrotic agent.

Examples of anti-fibrotic agents include, but are not limited to, corticosteroids, mucolytics, anti-inflammatory agents, antiviral agents (such as for example an anti-HCV or anti-HBV agent), angiotensin II receptor antagonists, and immunosuppressant drugs.

The use of the anti-fibrotic agents described above is generally well characterized in the fibrosis therapy arts, and their use herein falls under the same considerations for monitoring tolerance and effectiveness and for controlling administration routes and dosages, with some adjustments. Typical dosages of an effective anti-fibrotic agent can be in the ranges recommended by the manufacturer, and where indicated by *in vitro* responses or responses in animal models, can be reduced by up to about one order of magnitude concentration or amount. Thus, the actual dosage will depend upon the judgment of the physician, the condition of the patient, and the effectiveness of the therapeutic method based on the *in vitro* responsiveness of cells or tissue samples, or the responses observed in the appropriate animal models.

Another object of the invention is an *in vivo* process for reverting stellate cells, preferably HSCs, thereby treating fibrosis. In one embodiment, the *in vivo* process of the invention comprises administering a composition, pharmaceutical composition or medicament according to the invention to a subject in need thereof.

Another object of the present invention is a method for inhibiting gene markers of activated stellate cells, preferably HSCs, comprising administering a composition, pharmaceutical composition or medicament according to the invention to a subject in need thereof.

Examples of gene markers of activated stellate cells include, but are not limited to, ACTA2, COL1A1, LOX, LOXL1, LOXL2, LOXL3, PDGFRB, COL4A1, COL4A2,

COL5A1, ADAM12, ADAMTS2, ACTG2, NOTCH3 and CRYAB, preferably ACTA2, COL1A1 and LOX.

The invention also relates to a method for inducing inactivated stellate cells, preferably HSCs, specific markers, comprising administering a composition, pharmaceutical composition or medicament according to the invention to a subject in need thereof.

Examples of inactivated stellate cells specific markers include, but are not limited to, CXCL1, CXCL2, XCL10, CTSS, LY86, CAPN6, RND1, KRT20, CX3CR1 and GPC3.

The invention also relates to a method for inducing vitamin A storage in cytoplasmic lipid droplets of stellate cells, preferably HSCs, comprising administering a composition, pharmaceutical composition or medicament according to the invention to a subject in need thereof.

The invention also relates to a method for reducing stellate cells, preferably HSCs, proliferation rate, comprising administering a composition, pharmaceutical composition or medicament according to the invention to a subject in need thereof.

This invention will be better understood from the Experimental Details that follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter, and are not to be considered in any way as limited thereto.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows light microscopic and α-SMA immunocytochemistry images of the freshly isolated, qHSC-enriched cell population and fully culture activated HSCs (passage 4).

Figure 2 is a set of 2 graphs showing log2 mRNA expression levels of COL1A1 and LOX in freshly isolated, non-plated qHSCs and aHSCs from 5 different donors. In the graphs, **p<0.01.

Figure 3 is a photograph showing protein levels for PDGFRB and GAPDH in aHSCs (passage 4).

Figure 4 is a histogram showing mRNA expression levels of ACTA2, COL1A1 and LOX in human aHSCs incubated with EGF, FGF2 or a combination of both, presented as relative fold change to untreated control cells (gray line). In the graph, ns = not significant $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 5 is a histogram showing mRNA expression levels of ACTA2, COL1A1 and LOX in human aHSCs incubated with a combination of oleic acid (OA), palmitic acid (PA) and 5μM retinol (RE), or a combination of OA+PA+R+EGF+FGF2 (further called RM), presented as relative fold change to untreated control cells (gray line). In the graph, ns = not significant $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 6 is a set of 3 histograms showing mRNA expression levels of ACTA2, COL1A1 and LOX in non-cultured quiescent HSCs (qHSCs), culture aHSCs and *in vitro* aHSCs reverted to quiescence-like by RM (rHSCs) from 3 different donors. The expression levels are presented as relative fold change to aHSCs. In the graphs, ns = not significant $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 7 shows light microscopic (10x magnification) and Oil Red O staining images (63x magnification) of aHSCs and rHSCs.

Figure 8 is two graphs showing FACS-detection and quantification of the intrinsic fluorescence (at wavelength of ~328 nm) of all-trans retinyl esters in UV-excited aHSCs and rHSCs.

Figure 9 is a set of 3 histograms showing relative expression of ACTA2, COL1A1 and LOX in rHSCs after a recovery of 2 days in presence or absence of recombinant TGFβ1 (10ng/mL) in FBS free medium, from 5 different donors. The expression levels are presented as relative fold change to rHSCs. In the graphs, ns = not significant $p \geq 0.05$, *$p < 0.05$, **$p < 0.01$.

Figure 10 is a set of 3 histograms showing relative mRNA expression of Acta1, Col1a1 and Lox in freshly isolated, uncultured qHSCs, aHSCs (day 6 of culture) and rHSCs (day 6 of culture in RM).

Figure 11 is a set of 3 histograms showing ratio of EdU-positive nuclei over DAPI-positive nuclei in aHSCs and rHSCs cultured for 6 hours in the presence of EdU and stained with DAPI (A), percentage of degraded gelatin (stained area) over the total analyzed area after aHSCs or rHSCs culture for 5 days on quenched-gelatin coated coverslips under control conditions or in RM (B) and the number of migrated aHSCs and rHSCs seeded in collagen-coated boyden-chamber and stimulated with PDGFbb or with its solvens as a control in the lower compartment, in a trans-well migration assay (C). In the graphs, ns = not significant $p \geq 0.05$, *$p < 0.05$.

Figure 12 is a set of 6 photographs showing Sirius Red staining of liver slices from Group 1 (A), Group 2 (B), Group 3 (C), and Group 4 (D) mice.

EXAMPLES

The present invention is further illustrated by the following examples.

Patient samples

The protocol and experiments were approved by the ethical committees of the St-Luc Hospital and faculty of Medicine of Université Catholique de Louvain. An agreement from the Belgian Ministry of Health was obtained for the Hepatocytes and Hepatic Stem Cells Bank. Five livers were used for the following experiments, for which the clinical characteristics are summarized in Table 1.

Table 1: Clinical characteristics of livers

Donor number	Health status	Age	Gender	Ischemia time
L4	Healthy	12 years	Female	16h30
L8	Healthy	1 day	Male	4h40
L10	Healthy	7 months	Female	5h20
L11	Healthy	7 days	Male	4h25
L12	FH*	13 years	Male	1h30

*FH=Familial Hypercholesterolemia

Isolation of high purity human quiescent HSCs

The human liver parenchymal and non-parenchymal cell fractions were separated from each other by sequential perfusion of liver pieces with pre-warmed EGTA-containing EBSS medium (Lonza, Verviers, Belgium) and a digestion enzyme solution (EBSS supplemented with 0.9 mg/mL collagenase P and 0.03 mg/mL soybean trypsin inhibitor (Roche)) for 9 to 12 minutes. Collagenase digestion was stopped with ice-cold M199 wash medium (Lonza) containing 0.03 mg/mL of soybean trypsin inhibitor and 100 mL/L of human plasma (Najimi et al, Cell Transplant. 2007, 16(7):717-28). After filtration, the non-parenchymal cells were separated from the parenchymal cells by subsequent low-speed centrifugation steps (50g) and submitted to an additional centrifugation step (700g for 8 minutes). Non parenchymal cell pellets were then resuspended and cryopreserved in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 20% fetal bovine serum (FBS) (Biochrom GmbH, Berlin, Germany) and 5% dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). The isolation of high purity human qHSCs was performed according to the following method (adapted from Guimaraes et al., Journal of Hepatology. 2010, 52(3):389-397): dissociated and washed single non-parenchymal cells were suspended in a 5% FBS, 2mM EDTA (Sigma) buffer (10^6cells/100µL) and incubated for 30 minutes at 4°C with 500 ng/10^6 cells anti-CD32 (Abcam, Cambridge, United Kingdom) and 1 µg/10^6 cells anti-CD45 (BD Biosciences, San Jose, CA). 7-aminoactinomycin (7-AAD) (eBioscience, San Diego, CA) was used for the exclusion of non-viable cells. Pure populations of qHSCs were sorted as CD32-CD45-UV+ cells, using a FACSAria (BD Biosciences). RNA from freshly isolated qHSCs was obtained using RNEasy MicroKit (Qiagen). RNA samples were amplified using Ovation Pico WTA system V2 (NuGEN) producing microgram quantities of cDNA.

Isolation and *in vitro* reversion of HSCs

Human aHSCs were obtained by plating the qHSC-enriched population obtained after Nycodenz (Myegaard, Oslo, Norway) gradient centrifugation of the non-parenchymal cell fraction (Berardis et al. PLoS ONE. 2014, 9:e86137). Homogeneous populations of aHSCs were obtained after 3 passages in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere with 5% CO2. Prior to each passage, cells were washed with

phosphate buffered saline (PBS) and lifted using 0.05 % Trypsin (Lonza). For reversion of the activated phenotype, human aHSCs were seeded at a density of 10.000 cells/cm2 (unless stated differently) and 24 hours later the cells were washed and incubated with DMEM supplemented with 1% FBS, 20 ng/mL epidermal growth factor (EGF) (Peprotech, London, UK), 10 ng/mL fibroblast growth factor 2 (FGF2) (Peprotech), 100 µM oleic acid (Sigma), 100 µM palmitic acid (Sigma) and 5 µM retinol (Sigma). The medium was refreshed every two days for 5 days (day 1, 3 and 5) and the cells were harvested for further analysis on day 6. The effect of this mixture of growth factors and dietary components (further referred to as human HSC reverting medium (RM)) on the activation status of the cells was assessed by relative comparison to control cells cultured in DMEM with 1% FBS for the same period of time.

Mouse HSCs were isolated from male BalbC mice (age 20-25 weeks) (Charles River Laboratories, L'arbresle, France) and cultured in DMEM with 10% FBS, as described previously (Mannaerts et al. Hepatology. 2010, 51: 603-614). For treatment experiments with mouse HSC reverting medium (mRM) (DMEM supplemented with 10% FBS, 40 ng/mL human EGF, 20 ng/mL human FGF2, 100 µM oleic acid, 100 µM palmitic acid and 5 µM retinol), freshly isolated mouse qHSCs were seeded at a density of 7.500 cells/cm². The inhibition of activation was assessed by relative comparison to control cells cultured in DMEM supplemented with 10% FBS for the same period of time. All procedures on animals were carried out in accordance with University's guidelines for the care and use of laboratory animals in research. The performed experiments were approved by the ethical committee of the Vrije Universiteit Brussel in project 12-212-1.

Gene expression profiling and analysis

Double-stranded cDNA was synthesized from total RNA originating from cultured (activated and reverted HSCs; n=3 from corresponding donors) and uncultured (FACS-sorted quiescent HSCs; n=3 from two corresponding donors) human HSCs. cDNA was labeled and fragmented using Encore Biotin Module (NuGEN) and hybridized to the Affymetrix HG-U219 genechip (Affymetrix, Santa Clara, California, USA). Data normalization and analysis was performed using GeneSpring GX12 (Agilent, Santa Clara, CA) as described previously (Mannaerts et al. PLoS ONE. 2013, 8:e84071). Briefly,

Affymetrix gene expression data were normalized using the robust multi-array algorithm (Irizarry et al. Nucleic Acids Research. 2003, 31:e15). For the detection of differentially expressed genes, a p-value cut-off of 0.05 was used in combination with a fold-change cut-off of 2.0. Functional analysis of gene expression data (gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)-pathways) was conducted using an open access, high-level cross-platform microarray dataset analysis tool (InCroMAP) (http://www.ra.cs.uni-tuebingen.de/software/InCroMAP/).

Immunocytochemistry

Nycodenz-isolated HSCs cultured for 1 day or for 3 passages were washed with PBS and fixed for 10 minutes with 4% buffered formaldehyde (Merck, Darmstadt, Germany). Following permeabilization with 0.1% Triton-X 100 (in PBS containing 1% bovine serum albumin), cells were incubated overnight with anti-αSMA (1/1000) (Sigma). Primary antibody binding was visualized using an Alexa488-labeled secondary antibody (1/200) (Invitrogen, Eurgene, OR). Images were taken with an AxioCam MRc5 digital camera (Carl Zeiss).

Lipid staining

The cells were washed with PBS and fixed in 10% formalin solution. The cells were then washed with deionized water, incubated with isopropanol for 5 minutes and stained with diluted (3/2 in water) and filtered Oil Red O (Sigma-Aldrich) at 0.3% (w/v) in 99% isopropanol for 20 minutes. The red stained lipid droplets were visualized with light microscopy (Carl Zeiss).

Western blot

Cells were washed with ice-cold PBS and scraped with ice-cold lysis buffer (170 mM NaCl, 10 mM EDTA, 50 mM Tris pH 7.4, 50 mM NaF, 0,2 mM dithiothreitol and 0.5% NP-40) supplemented with protease (Roche Diagnostics, Mannheim, Germany) and phosphatase (Roche Diagnostics) inhibitors. Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co, Rockford, IL). Thirty microgram of protein was separated on a 8% tris-glycine SDS-polyacrylamide gel and electroblotted

onto polyvinylidene difluoride membranes (Amersham Biosciences, Little Chalfront, UK) using a wet blotting apparatus (Mini Trans-Blot Cell, BioRad, Nazareth, Belgium). Blots were blocked with 5% milk powder in tris buffered saline (TBS) with 0.2% tween (Sigma) and subsequently incubated overnight with primary anti-PDGFRB (diluted 1/1000 in blocking buffer) (Abcam) or anti-GAPDH (diluted 1/30.000) (Abcam). The membranes were washed and incubated with a horseradish peroxidase conjugated secondary antibody (1/20000) (Dako, Glostrup, Denmark) for 1 hour and the antigen was visualized by enhanced chemiluminescence using ECL substrate (Pierce Chemical co.).

Proliferation assay

Cell proliferation of human HSCs was assessed with the Click-iT EdU Cell Proliferation Assay Kit (Invitrogen, Eugene, OR). HSCs were cultured under control conditions or in RM for 6 days. On day 6, the cells were labeled with 10 µM EdU for 6 hours and subsequently formalin fixed and mounted with Prolong Gold antifade reagent with DAPI (Invitrogen). EdU incorporation was visualized according to the manufacturer's instructions. The percentage proliferation was calculated as the ratio of EdU positive cells to DAPI positive cells. The quantification was performed on at least 500 cells per donor and is presented as the mean percentage measured in 3 different donors.

***In situ* Zymography**

The gelatinase activity of human HSCs was assessed using the highly quenched, fluorescein-labeled pig skin gelatin (DQ gelatin, Invitrogen). Upon proteolytic digestion, its green fluorescence is revealed and can be used to measure enzymatic activity. A 1 mg/mL stock solution of DQ-gelatin was prepared using deionized water and stored at 4°C. Prior to cell seeding (20.000 cells/cm²), glass coverlips (12 mm diameter) were coated with 50µg DQ-gelatin for 1 hour. 24 Hours post-seeding, the cells were washed with serum-free DMEM and cultured for 6 days under control conditions or RM. On day 6, the cells were formalin fixed and mounted with Prolong Gold antifade reagent with DAPI. The percentage green stained area was calculated using ImageJ (http://imagej.nih.gov/ij/index.html) at the adjusted threshold of 34-255. The

quantification was performed on 10 images per donor and is represented as the mean percentage measured in 3 different donors.

Migration assay

Both aHSCs and rHSCs were seeded in collagen-coated Boyden chambers (Millipore, 40.000 cells/chamber) in serum-free DMEM. After 60 minutes, the chambers were transferred to wells with 20 ng/mL platelet derived growth factor (PDGFbb) (R&D systems, Minneapolis, MN) or its solvent as a control. After 16 hours, non-migrated cells were cleared and migrated cells were fixed with ice-cold 100% methanol and mounted with Prolong Gold antifade reagent with DAPI. The quantification was performed by manually counting the totality of migrated cells on images covering the entire membrane and is represented as the mean of 3 different donors.

RNA purification and RTq-PCR

RNA was extracted and purified from cultured and uncultured cells using the Reliaprep RNA cell Miniprep system (Promega, Madison, WI). Total RNA was converted to cDNA by reverse transcription using the Revert Aid Kit (ThermoFisher Scientific, St. Leon-Rot, Germany). Quantitative real-time polymerase chain reaction was performed using the GoTaq qPCR Master Mix with BRYTE green (Promega). A 7500 real time PCR system was used and data was analyzed using System SDS software v2.0.6 (Applied biosystems). Fold change differences between samples were determined using the comparative Ct method ($\Delta\Delta$Ct). The expression level of different target genes, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the calibrator, was given by 2-$\Delta\Delta$Ct. Gene specific primers were produced by Integrated DNA technologies (Leuven, Belgium).

Statistical analysis

GraphPad Prism v4.0.0 (GraphPad Software, La Jolla, CA) was used for statistical analysis. Data in the figures are expressed as means ± SEM. Differences among groups were tested for statistical significance by Student t-test or analysis of variance (ANOVA) followed by Tukey's test, depending on the number of groups. ns= not significant $p \leq 0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Example 1: EGF and FGF2 revert the culture activated phenotype of human HSCs

Human HSCs were isolated by density gradient centrifugation from the non-parenchymal liver fraction, allowed to activate by seeding on plastic culture dishes and expanded for at least 3 passages. The activated status of the cells was assessed by the typical myofibroblastic phenotype (Fig. 1), the strong increase in alpha smooth muscle actin (ACTA2) protein by immunocytochemistry and collagen type 1 alpha 1 (COL1A1) and lysyl oxidase (LOX) expression by RT-qPCR (Fig. 2), and the high protein level of platelet-derived growth factor receptor beta (PDGFRB), a membrane receptor associated with HSCs (Fig. 3). These features have been demonstrated on all the HSC isolated from different donor livers.

Thereafter, different culture conditions have been tested for their ability to revert the activated phenotype of culture expanded human primary HSCs. These conditions included pharmacological agents previously shown to inhibit rodent HSC activation, i.e. TWS119, valproic acid, Trichostatin A, valinomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, bafilomycin, hydroxychloroquine, 3-methyladenine; an adipogenic differentiation mixture and a diverse pool of growth factors and cytokines (data not shown). Among these agents, results show that FGF2 and EGF significantly down-regulate the expression of ACTA2, COL1A1 and LOX, alone or in combination (Fig.4).

Example 2: Oleic acid, palmitic acid and retinol revert the culture activated phenotype of human HSCs

The same experiment has been realized with a medium containing dietary fatty acids. Results show that oleic acid (OA), palmitic acid (PA) and retinol (R) also down-regulate the expression of ACTA2, COL1A1 and LOX (Fig. 5, "OA+PA+R" columns).

Moreover, aHSCs cultured in medium containing the mixture of dietary fatty acids displayed a quiescent-like phenotype, characterized by a thinner cell body and the presence of intra-cytoplasmic lipid droplets (data not shown).

Example 3: EGF and FGF2 act synergistically with dietary fatty acids and retinol to revert the culture activated phenotype of human HSCs

By combining the tested agents in the culture medium, it was observed that the effect of EGF and FGF2 on the expression of ACTA2, COL1A1 and LOX expression was strongly potentiated by the mixture of dietary fatty acids (OA, PA and R) to reach levels of expression that are similar to those measured in freshly isolated, non-cultured qHSCs (Fig. 5 and 6). HSCs reverted by this mix of growth factors and dietary components (further referred to as RM) present a thinner cell body and intra-cytoplasmic lipid droplets (Fig. 7).

An important functional hallmark of qHSCs is their ability to store vitamin A in their cytoplasmic lipid droplets. In order to assess whether the *in vitro* reverted HSCs (rHSCs) had the molecular machinery both to metabolize and store vitamin A, the retinyl ester auto-fluorescence at 328 nm was measured by fluorescence-activated cell sorting (FACS). Results show that around 75% of the aHSCs cultured in RM became UV-positive. Analysis of the rHSCs under UV-light shows that the auto-fluorescent signal co-localizes with the intra-cytoplasmic lipid droplets, indicating that vitamin A is indeed metabolized and stored inside the lipid droplets (Fig. 8).

Example 4: Analysis of the reversibility of the quiescent-like state of HSCs

To investigate whether this induced quiescent-like state is reversible, the cells were allowed to recover for 2 days in the absence or presence of transforming growth factor beta (TGFβ), a profibrogenic molecule, in serum-free medium. In the absence of TGFβ, the cells maintained low expression levels of profibrogenic genes. However, in the presence of TGFβ, the cells again upregulated the expression of ACTA2, COL1A1 and LOX (Fig. 9). To investigate whether the described mix of growth factors and dietary components can prevent the activation process and maintain a quiescent phenotype, freshly isolated mouse qHSCs were seeded in normal control conditions or RM optimized for mouse HSC cultures (mRM) until day 6. It was observed that cells grown under mRM conditions maintained low expression levels of Acta2, ColA1 and Lox, similar to

uncultured D0 mouse qHSCs (Fig. 10) and have an intermediary phenotype with a large intra-cytoplasmic lipid droplet content (data not shown).

Example 5: *In vitro* reverted human HSCs have a reduced proliferation rate

It is known that aHSCs functionally differ from qHSCs by their increased proliferation rate, unbalanced ECM homeostasis and higher migratory potential (Friedman, Physiological Reviews. 2008, 88: 125-172). Therefore, it was further investigated whether human *in vitro* rHSCs functionally differed from their activated counterparts by comparing their proliferation (EdU-incorporation), ECM degradation (*in situ* zymography) and PDGFbb-induced migration (transwell assay). Results show that rHSCs (obtained with RM) displayed a 75% reduction in proliferation compared to aHSC (Fig. 11A). Although rHSCs express slightly higher levels of TIMP1 and show no difference in MMP2 and MMP9 expression (data not shown), no difference in overall gelatinase activity between aHSCs and rHSCs is observed (Fig. 11B). As this assay is dependent on cell density and that we observed a difference in proliferation between both conditions, only areas with a similar number of cells were used for quantification. In concordance with the *in situ* zymography results, we measured no difference in PDGFbb-induced migration (Fig. 11C).

Example 6: The overall gene expression profile of *in vitro* rHSCs is distinct from that of aHSCs and qHSCs

To gain more insight into the gene expression changes elicited by this transient reversion into a quiescent-like state, the global gene expression profile of rHSCs was assessed and compared to that of freshly isolated, non-cultured qHSCs and culture aHSCs using the human genome U219 arrays. Results show that the global gene expression profile of rHSCs resembles more closely to that of aHSCs than qHSCs, with 2277 (rHSC vs aHSC) against 9122 (rHSC vs qHSC) genes significantly differentially regulated (student t-test, $p \leq 0,05$). Many of the top upregulated genes in rHSCs are inflammation-related, i.e. IL-8, IL-33, IL-1β, CXCL1, and CXCL6 (Table 2). Over 60% of the ≥2-fold deregulated genes in rHSCs compared to aHSCs are downregulated and include genes such as TNNT2, SULT1E1, ACTG2, and SYNPO2L (Table 2).

Table 2: The fold-change of the top 15 upregulated and top 15 downregulated genes in rHSCs, compared to the aHSCs

Max upregulated after reversion	Fold	Max downregulated after reversion	Fold
IL8	23	TNNT2	35
IL33	19	SULT1E1	11
IL1B	19	ACTG2	10
CXCL6	18	SYNPO2L	9
CST1	14	DES	9
CXCL1	13	KRT7	8
RARRES1	11	CNN1	8
AADAC	10	AQP1	7
PTGS2	10	OXTR	6
NPTX1	9	KIAA1199	6
GNG2	9	LMCD1	6
MMP1	8	FAM46B	6
TFPI2	8	SLC24A3	6
TFPI2	8	ADAMTS5	5
RARB	7	MEOX2	5

Example 7: *In vitro* reverted human HSCs upregulate the expression of *in vivo* inactivated HSC specific markers

To determine whether human HSCs reverted to quiescence-like by RM share characteristics with *in vivo* inactivated HSCs (iaHSCs) found in the mouse liver after recovery from experimentally induced fibrosis, the expression of a panel of genes (n=17) identified as highly specific for *in vivo* iaHSCs was measured and compared to aHSCs and qHSCs (Kisseleva et al. PNAS. 2012; Troeger et al. Gastroenterology. 2012, 143: 1073-1083.e1022; Friedman and Arthur, Science & Medicine. 2002, 8(4):194; Xiao Liu et al. Curr Pathobiol. 2013, Rep 1: 209-214) in aHSCs and rHSCs from 3 liver donors. Although there was some variation between HSCs from different donors, 10 genes of the 17 genes tested are consistently upregulated in rHSCs of each donor, i.e. CXCL1, CXCL2, CXCL10, CTSS, LY86, CAPN6, RND1, KRT20, CX3CR1 and GPC3 (Table 3).

Table 3: Overview of the fold-difference in expression of *in vivo* iaHSC signature genes in rHSCs and aHSCs

Gene	Accession	Donor L4	Donor L8	Donor L12
CXCL1	NM_001511	52	30.7	26
CXCL2	NM_002089	39.1	7.3	223.6
CXCL10	NM_001565	3.7	32.7	nd
CTSS (tv2)	NM_001199739	27.6	20.5	10.1
LY86	NM_004271	1.8	26.4	6.2
CAPN6	NM_014289	2.6	5.2	14.1
RND1	NM_014470	1.9	11.7	6.6
KRT20	NM_019010	6	nd	3.9
CX3CR1 (tv1)	NM_001171174	3.2	nd	5.4
GPC3 (tv4)	NM_001164619	1.5	1.8	1.2

tv: transcript variant; nd: not detected

Example 8: *In vivo* evaluation of EGF and FGF2 impact on liver fibrosis

Material and methods

Mouse model

The proposed mouse model is well-documented and recapitulates the important cellular and molecular events characterizing the development of liver fibrosis in patients. This model is used routinely by the researchers to study the activation process of HSCs. It is known that a 4-week treatment induces remarkable fibrosis that is well-suited to study the impact of potential anti-fibrotic agents (Mannaerts et al. 2010; Mannaerts et al., 2015). Therefore, we opt for a 4-week treatment of CCl_4 with or without a co-treatment of the EGF/FGF2 solution in the first or last two weeks of treatment.

Mice were male BALB/c (Charles River) of 6-7 weeks.

Treatment Administrations

CCl_4 and corn oil injections

The scheme of treatment administrations is detailed in Table 4. Each animal received 100 µl of CCl_4 solution (a freshly-prepared mix of volume of 15 µL CCl_4 (Sigma Fluka,

87031) and 85 µL of corn oil) or same volume of corn oil via the IP routes according to QA/PROD34.

The injection was done 2 times a week: one IP on the right side of the mouse and one on the left side of the mouse.

Table 4: Groups of Animals, Doses and Administration Routes

Groups	Animal number	Treatment 2 times/week	Treatment route	EGF and FGF2 pump	Length of experiment
GP1	8	Corn oil	IP	No	4 weeks, no recovery
GP2	8	CCl_4	IP	No	4 weeks, no recovery
GP3	8	CCl_4	IP	2 first weeks of treatment	4 weeks, no recovery
GP4	8	CCl_4	IP	2 last weeks of treatment	4 weeks, no recovery

IP: intraperitoneal

EGF and FGF2 pump placement

EGF/FGF2 solution preparation

The EGF/FGF2 solution containing EGF (Peprotech London, M315-09) at 20 ng/ml and FGF2 (Peprotech London, 450-33) at 10 ng/ml was prepared with PBS for all the Alzet pumps. The solution was aliquoted and stored at -20°C. The ALZET pump model 2002 (volume of 200 µl for 2 weeks with a 0.5 µl/h rate) was used.

Analgesia

Five (5) mg/kg BW of carprofen - Rimadyl® was injected subcutaneously at the base of the neck before surgery.

Surgery

Surgery was done under general anaesthesia (isoflurane gaz anaesthesia, B132 equipment) according to SOP QA/PROD31. Animals were left in a recovering chamber till they recover from the anaesthesia.

The mice of GP2 were subjected to anaesthesia without pump placement at Day +14. This anaesthesia was lasted the same time then the anaesthesia of the animals with pump placement. Animals were left on a heating pad till they recover from the anaesthesia.

Sampling of organs and histological analysis

All mice were euthanized by cervical dislocation according to SOP QA/PROD31 and necropsied. Two control mice of GP1 to GP4 were euthanized after 2 weeks of treatment at Day+14.

One day after the last injection for GP1 to GP4 (Day+26), the mice were euthanized and the organ sampling was done.

Livers were harvested under Ketamine/xylasine anesthesia. Liver tissue was fixed and processed for paraffin sections. Formalin fixed and paraffin imbedded 5 µm liver sections were deparaffinized and rehydrated in graded alcohol bathes. To evaluate liver fibrosis, liver slices were stained using Sirius red and counterstained with fast green and light microscopic images were captured.

Sampling of blood and RT-qPCR analysis

At necropsy, the blood was collected by retroorbital punction. The blood serum was aliquoted (100 µl/tube) and stored at -20°C until the end of the study.

Total RNA was extracted from the liver samples using Tripure isolation Reagent (Roche, Belgium) and Fastprep lysing matric (ceramic beads). DNAse I treated samples, were thereafter processed for First-strand cDNA synthesis using high capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems).

Samples were then and subsequently diluted with nuclease-free water (Invitrogen) to 10 ng/mL cDNA.

PCR amplification mixtures (25 µL) contained 25 ng template cDNA, Master Mix buffer (12.5 mL; Applied Biosystems), and the corresponding Taqman assay were run in duplicate and performed on a StepOnePlus Real-time PCR (Applied Biosystems). The cycling conditions comprised 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min. Relative quantification was normalized against the house keeping gene β-2-microglobulin. The Applied Biosystems assays used for the current study are listed in Table 5.

Table 5: Genes studied by RT-qPCR

Gene symbol	Gene name	Gene ID	Ref Applied Biosystems
β2m	beta-2-microglobulin	12010	Mm00437762_m1
ACTA2	actin, alpha 2, smooth muscle, aorta	11475	Mm01546133_m1
COL1α1	collagen, type I, alpha 1	12842	Mm00801666_g1

Results

Histological analysis

To examine whether the EGF/FGF2 solution of the invention is able to modulate liver fibrosis, CCl_4 was administrated to mice for 4 weeks and the effect of these two growth factors was investigated both at early phase of liver fibrosis induction (Group 3) and late phase of liver fibrosis induction (Group 4).

Subsequently to CCl_4 administration to Balb/c male mice, liver fibrosis was induced in CCl_4-treated mice, as demonstrated by Sirius Red staining commonly used to visualize collagen fibers (Group 2) as compared to sham mice (Group 1) (Figures 12 A and B respectively).

On the contrary, Figures 12 C and D show that mice treated with EGF/FGF2 solution presented a reduction in liver fibrosis, in both groups 3 and 4 respectively.

Therefore, the EGF/FGF2 solution of the invention is able to reduce liver fibrosis.

RT-qPCR analysis

Recovered livers of the studied mice were processed for mRNA extraction and analysis of genes related to fibrosis. RT-qPCR using Taqman probes and dCT analysis have revealed that CCl_4 treatment leads to an upregulation of the expression of alpha smooth muscle actin (ACTA1) and collagen type I alpha 1 (COL1α1) mRNAs (Table 6, GP1 compared to GP2), two widely used markers reflecting an *in situ* activation of hepatic stellate cells.

In mice treated with EGF and FGF2, results presented in Table 6 (GP3 and GP4 compared to GP2) show a downregulation of both mRNAs expression.

Table 6: RT-qPCR values

Group	CT values			dCT	
	β2m	ACTA2	COL1α1	ACTA2	COL1α1
GP1	24.0	33.8	31.4	9.8	7.3
GP2	24.5	28.9	27.3	4.4	2.8
GP3	25.5	32.5	29.4	6.5	3.9
GP4	27.5	34.0	32.7	6.5	5.2

Therefore, treatment with a composition comprising EGF and FGF2 leads to an inhibition of the CCl_4 induced fibrosis.

CLAIMS

1. A composition for treating fibrosis comprising EGF and FGF2.

2. The composition for treating fibrosis according to claim **1**, wherein said composition further comprises at least one dietary fatty acid.

3. The composition for treating fibrosis according to claim **2**, wherein said at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, arachidonic acid, myristic acid, and retinol, preferably oleic acid, palmitic acid or retinol, or a mixture thereof.

4. The composition for treating fibrosis according to claims **1** to **3**, wherein said fibrosis is selected from the group comprising liver fibrosis, pulmonary fibrosis, heart fibrosis, Crohn's Disease, progressive kidney disease, pancreatic fibrosis, scleroderma/systemic sclerosis, post-surgery adhesions, peritoneal adhesions, retroperitoneal fibrosis, pleural fibrosis, pericardial fibrosis, uterine fibroid, and graft fibrosis.

5. The composition for treating fibrosis according to claim **4**, wherein said liver fibrosis is cirrhosis.

6. The composition for treating fibrosis according to claim **4**, wherein said pulmonary fibrosis is idiopathic pulmonary fibrosis or cystic fibrosis.

7. *In vitro* process for reverting stellate cells comprising a step of incubating stellate cells into a composition comprising EGF and/or FGF2, and optionally at least one dietary fatty acid.

8. The *in vitro* process according to claim **7** wherein said at least one dietary fatty acid is selected from the group comprising oleic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, arachidonic acid, myristic acid, and retinol, preferably oleic acid, palmitic acid or retinol, or a mixture thereof.

9. The *in vitro* process according to anyone of claims **7** or **8** wherein said stellate cells are selected from the group comprising hepatic stellate cells (HSCs), pancreatic stellate cells (PaSCs), lung stellate cells, intestinal stellate cells, kidney stellate cells, spleen stellate cells, adrenal gland stellate cells, ductus deferens stellate cells and vocal cords stellate cells, preferably HSCs, PaSCs, lung stellate cells and intestinal stellate cells, more preferably HSCs.

10. An isolated reversed stellate cells population obtained by the *in vitro* process according to anyone of claims **7** to **9**.

11. A composition comprising EGF, FGF2, oleic acid, palmitic acid and retinol, wherein

- the concentration of EGF ranges from 0.1 to 100 ng/mL,
- the concentration of FGF2 ranges from 0.05 to 80 ng/mL,
- the concentration of oleic acid ranges from 1 to 1000 nmol/mL,
- the concentration of palmitic acid ranges from 1 to 1000 nmol/mL, and
- the concentration of retinol ranges from 0.01 to 100 nmol/mL.

12. A culture medium comprising the composition according to claim **11**.

13. An *in vitro* method for determining the pro-fibrotic activity of a tested agent comprising the steps of:

a) *in vitro* incubating stellate cells in a culture medium according to claim **12**,

b) adding the tested agent in the culture medium,

c) determining the phenotype of said stellate cells, preferably the activated or quiescent phenotype of said stellate cells.

14. A kit of part comprising two parts, wherein the first part comprises a composition according to claim **11** or a culture medium according to claim **12**, and the second part comprises a stellate cells population.

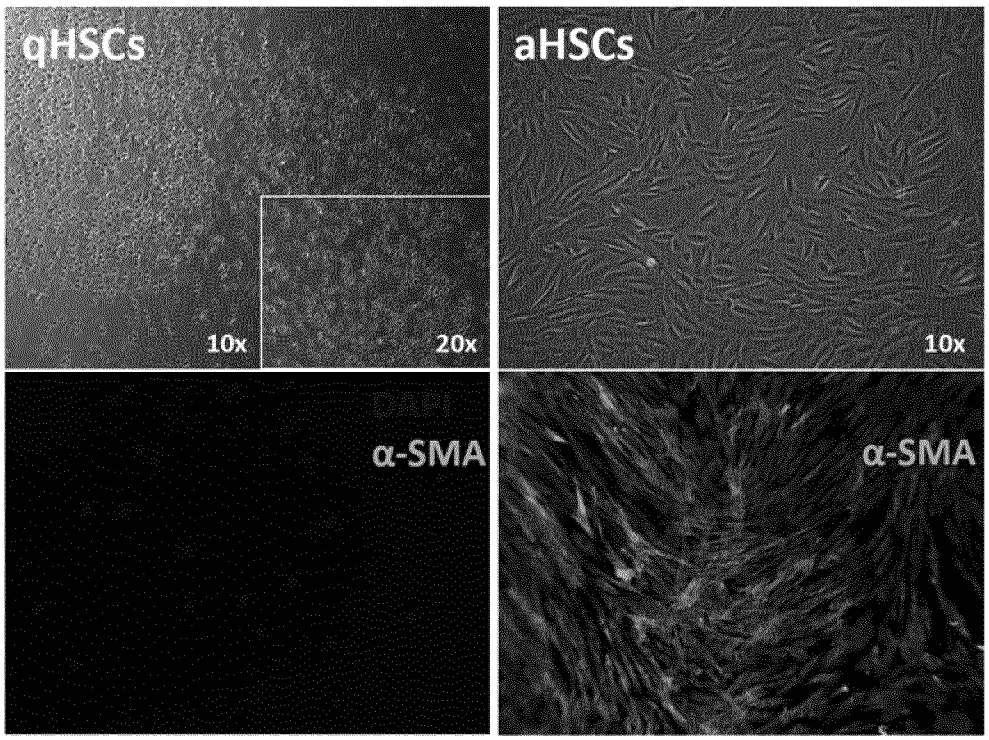

qHSCs
10x
20x
aHSCs
10x
α-SMA
α-SMA

FIG. 1

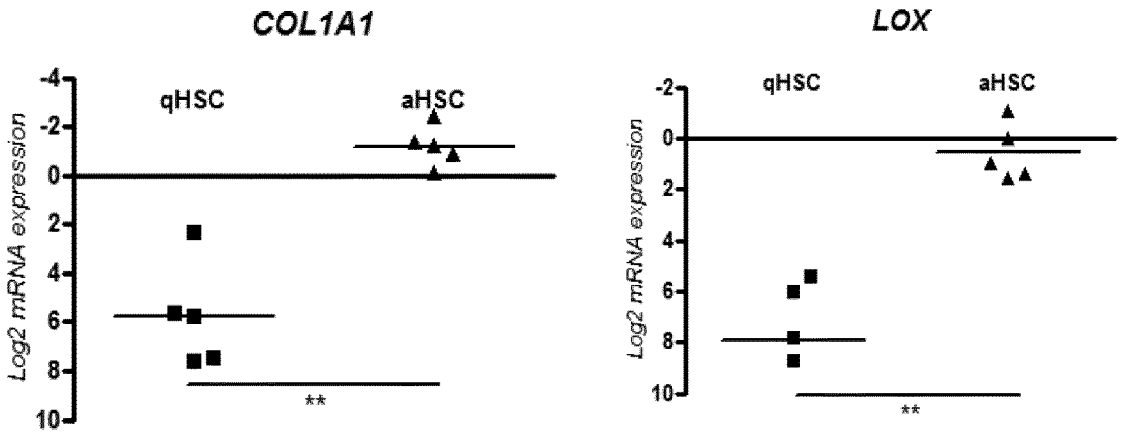
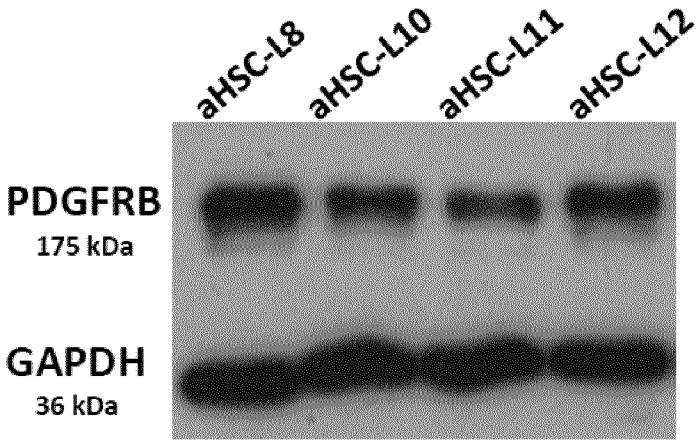

COL1A1
qHSC
aHSC
Log2 mRNA expression
-4
-2
0
2
4
6
8
10
**
LOX
qHSC
aHSC
Log2 mRNA expression
-2
0
2
4
6
8
10
**

FIG. 2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aHSC-L10
aHSC-L11
aHSC-L12
PDGFRB
175 kDa
GAPDH
36 kDa

FIG. 3

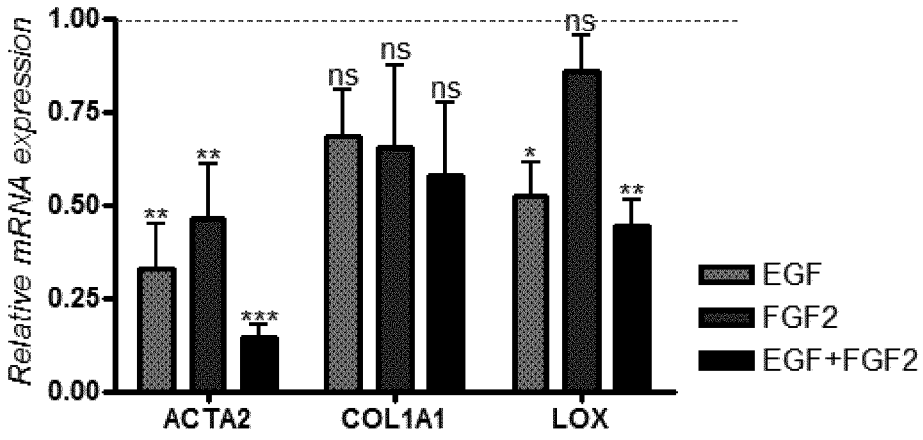

Relative mRNA expression
1.00
0.75
0.50
0.25
0.00
**
**
***
ns
ns
ns
*
ns
**
ACTA2
COL1A1
LOX
EGF
FGF2
EGF+FGF2

FIG. 4

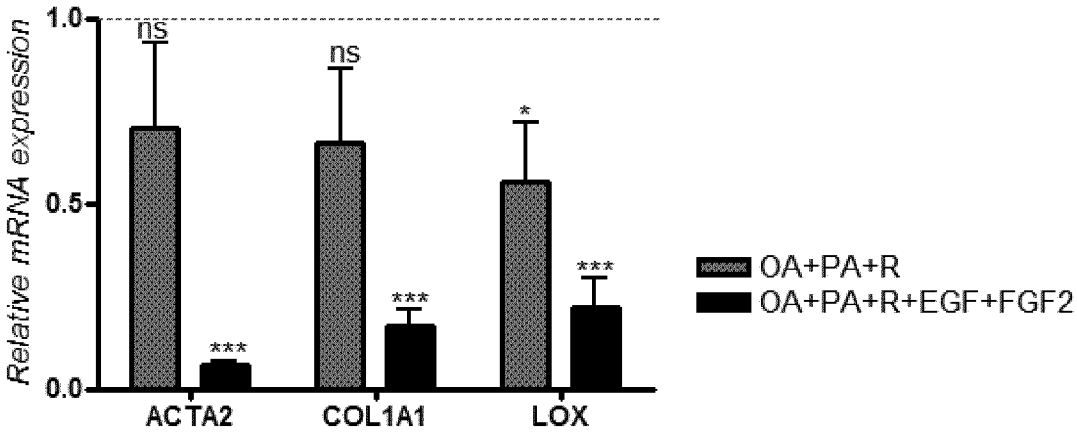

Relative mRNA expression
1.0
0.5
0.0
ns
ns
*
***
***
***
ACTA2
COL1A1
LOX
OA+PA+R
OA+PA+R+EGF+FGF2

FIG. 5

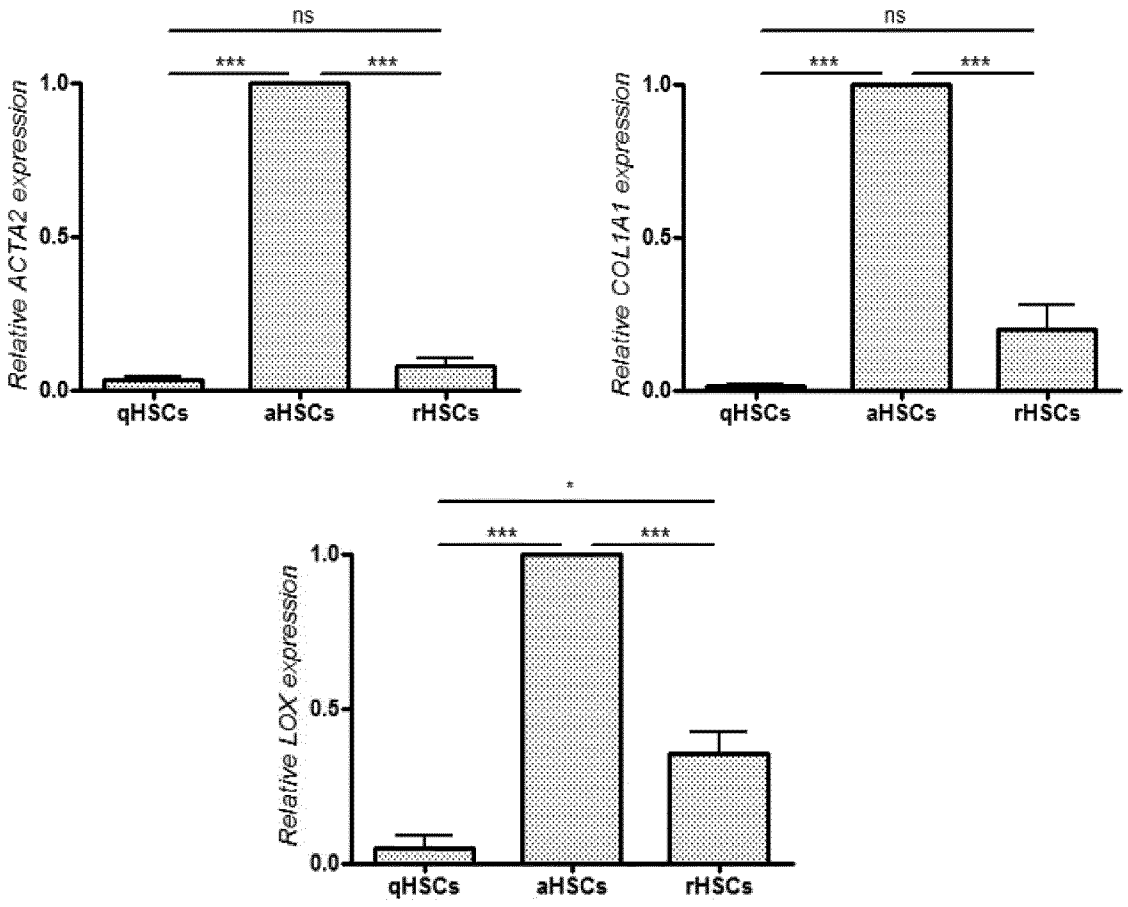
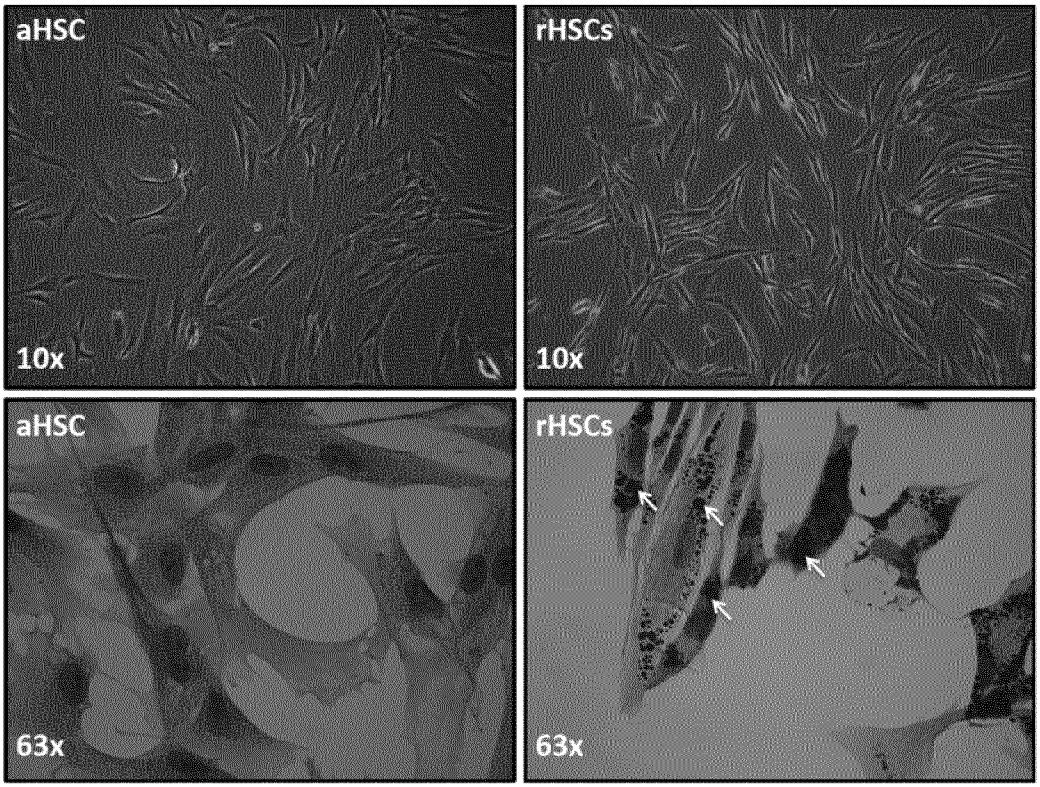

Relative ACTA2 expression
Relative COL1A1 expression
Relative LOX expression
1.0
0.5
0.0
ns
*
***
***
qHSCs
aHSCs
rHSCs

FIG. 6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rHSCs
10x
aHSC
63x
rHSCs
63x

FIG. 7

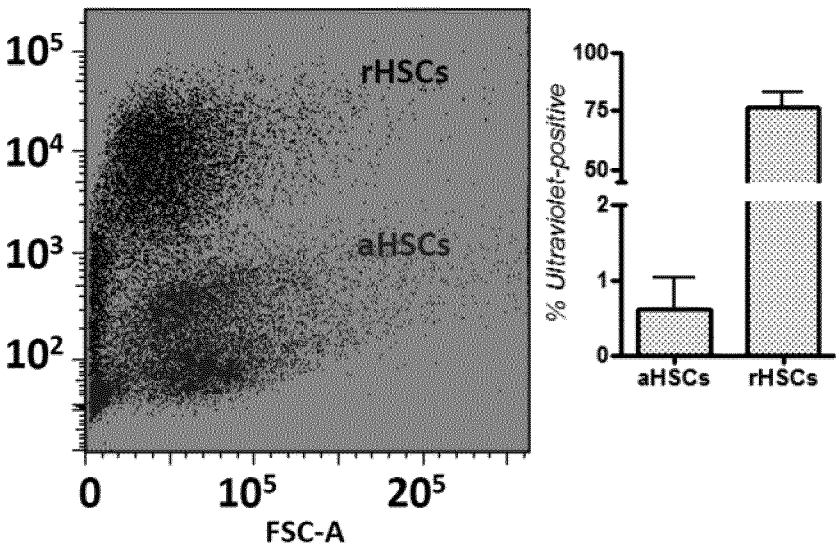
10^5
10^4
10^3
10^2
rHSCs
aHSCs
0
10^5
2^05
FSC-A
% Ultraviolet-positive
100
75
50
2
1
0
aHSCs
rHSCs

FIG. 8

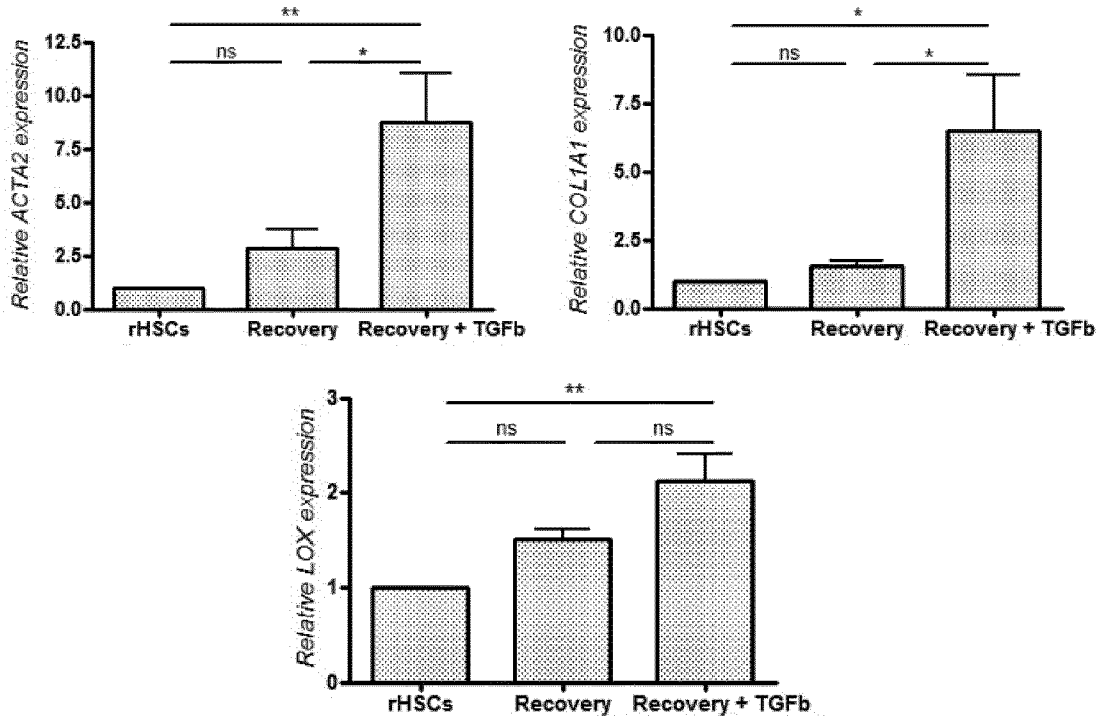
Relative ACTA2 expression
12.5
10.0
7.5
5.0
2.5
0.0
**
ns
*
rHSCs
Recovery
Recovery + TGFb
Relative COL1A1 expression
10.0
7.5
5.0
2.5
0.0
*
ns
*
rHSCs
Recovery
Recovery + TGFb
Relative LOX expression
3
2
1
0
**
ns
ns
rHSCs
Recovery
Recovery + TGFb

FIG. 9

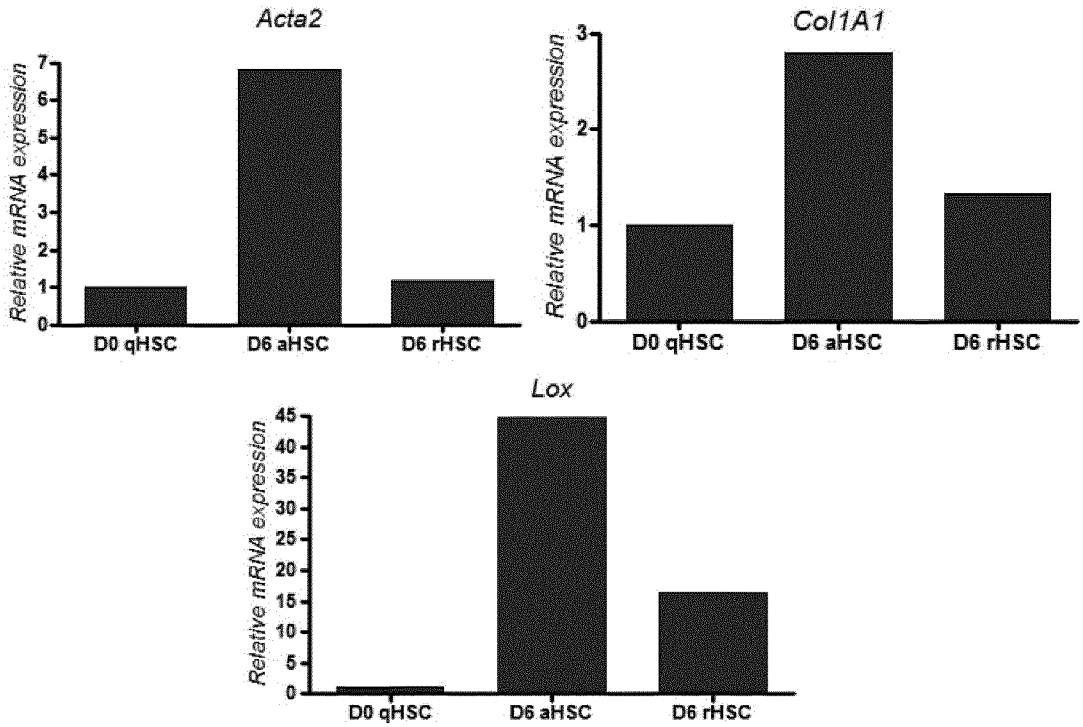
Acta2
Relative mRNA expression
7
6
5
4
3
2
1
0
D0 qHSC
D6 aHSC
D6 rHSC
Col1A1
Relative mRNA expression
3
2
1
0
D0 qHSC
D6 aHSC
D6 rHSC
Lox
Relative mRNA expression
45
40
35
30
25
20
15
10
5
0
D0 qHSC
D6 aHSC
D6 rHSC

FIG. 10

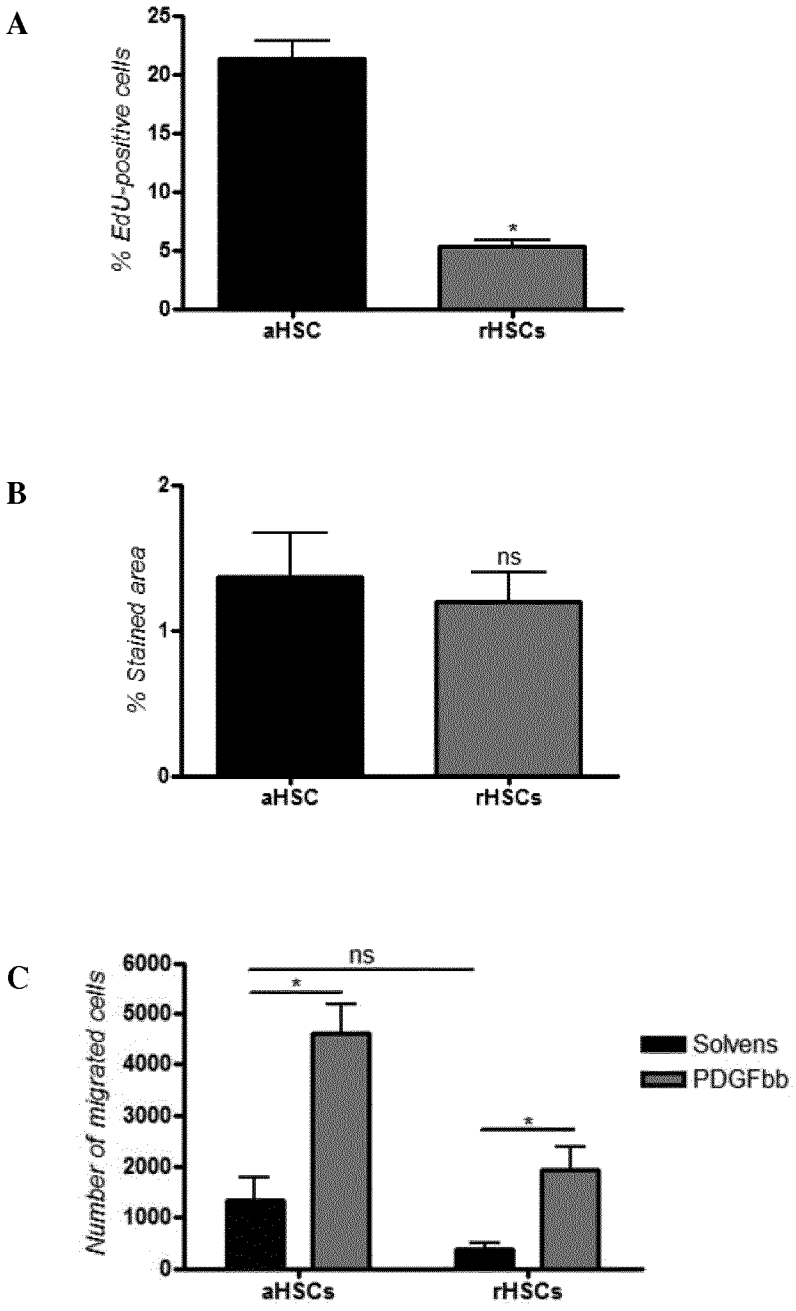
A
% EdU-positive cells
25
20
15
10
5
0
*
aHSC
rHSCs
B
% Stained area
2
1
0
ns
aHSC
rHSCs
C
Number of migrated cells
6000
5000
4000
3000
2000
1000
0
ns
*
*
aHSCs
rHSCs
Solvens
PDGFbb

FIG. 11

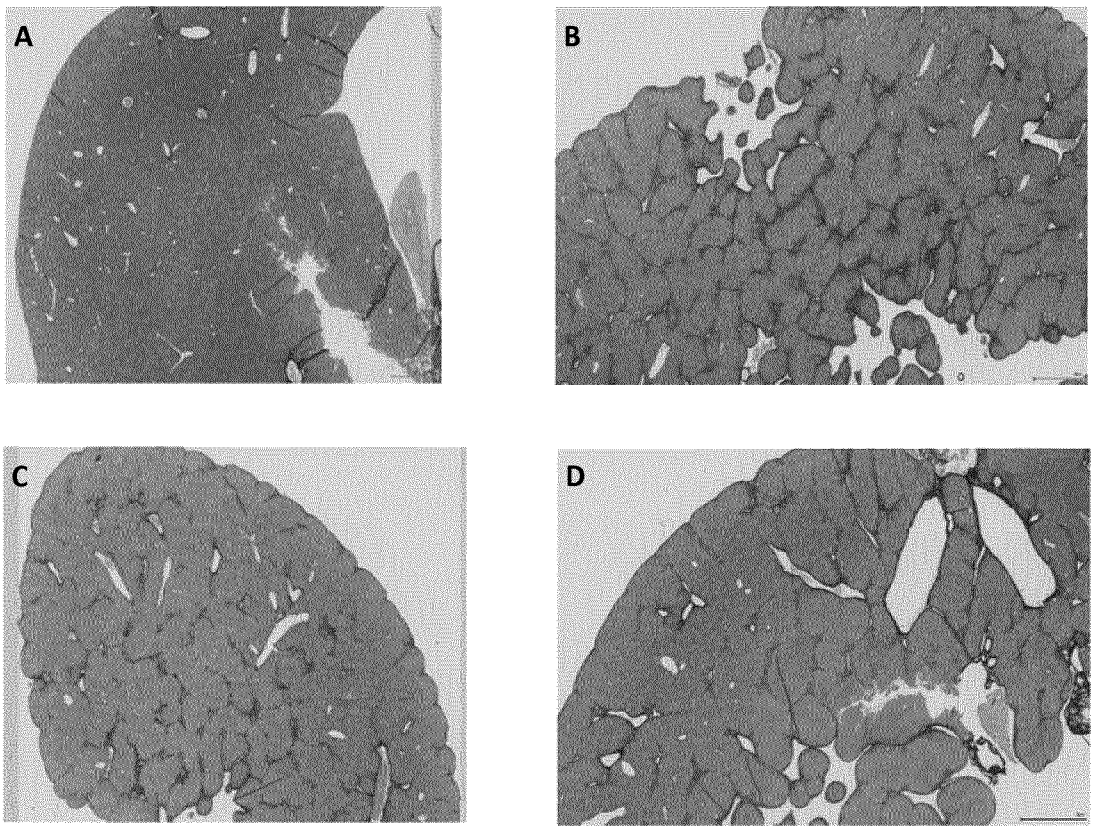

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/066213

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/18 A61P1/16
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ATUL SINHA ET AL: "Epidermal Growth Factor Enemas with Oral Mesalamine for Mild-to-Moderate Left-Sided Ulcerative Colitis or Proctitis", NEW ENGLAND JOURNAL OF MEDICINE, vol. 349, no. 4, 24 July 2003 (2003-07-24) , pages 350-357, XP055225301, US ISSN: 0028-4793, DOI: 10.1056/NEJMoa013136 page 353 ----- -/--	1-5

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 2016	23/11/2016

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

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KUMAR KRISHNAN ET AL: "Intestinal growth factors: Potential use in the treatment of inflammatory bowel disease and their role in mucosal healing", INFLAMMATORY BOWEL DISEASES, vol. 17, no. 1, 1 January 2011 (2011-01-01), pages 410-422, XP055225334, Raven Press, HAGERSTOWN, MD; US ISSN: 1078-0998, DOI: 10.1002/ibd.21316 page 415	1-5
A	LI-PING WANG ET AL: "Original Article BMP-7 attenuates liver fibrosis via regulation of epidermal growth factor receptor", INTERNATIONAL JOURNAL OF CLINICAL AND EXPERIMENTAL PATHOLOGY, vol. 7, no. 7, 15 June 2014 (2014-06-15), pages 3537-3547, XP055225341, US ISSN: 1936-2625 page 358	1-5
A	BRYAN C. FUCHS ET AL: "Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma", HEPATOLOGY, vol. 59, no. 4, 28 April 2014 (2014-04-28) , pages 1577-1590, XP055225363, USA ISSN: 0270-9139, DOI: 10.1002/hep.26898 page 1578	1-5
A	IKUO NAKAMURA ET AL: "Brivanib Attenuates Hepatic Fibrosis In Vivo and Stellate Cell Activation In Vitro by Inhibition of FGF, VEGF and PDGF Signaling", PLOS ONE, vol. 9, no. 4, 7 April 2014 (2014-04-07), page e92273, XP055223650, DOI: 10.1371/journal.pone.0092273 page 1	1-5

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SILVIA BERARDIS ET AL: "Gene Expression Profiling and Secretome Analysis Differentiate Adult-Derived Human Liver Stem/Progenitor Cells and Human Hepatic Stellate Cells", PLOS ONE, vol. 9, no. 1, 21 January 2014 (2014-01-21), page e86137, XP055225466, DOI: 10.1371/journal.pone.0086137 cited in the application page 3	1-5

A	L. G. KOMUVES ET AL: "Expression of Epidermal Growth Factor and Its Receptor in Cirrhotic Liver Disease", JOURNAL OF HISTOCHEMISTRY AND CYTOCHEMISTRY, vol. 48, no. 6, 1 June 2000 (2000-06-01), pages 821-830, XP055225539, US ISSN: 0022-1554, DOI: 10.1177/002215540004800610 page 822	1-5

A,P	ADIL EL TAGHDOUINI ET AL: "In vitro reversion of activated primary human hepatic stellate cells", FIBROGENESIS & TISSUE REPAIR, vol. 31, no. 4, 6 August 2015 (2015-08-06) , pages 1-15, XP055299364, DOI: 10.1186/s13069-015-0031-z page 2	1-5

A	KATI RIES ET AL: "ELEVATED EXPRESSION OF HORMONE-REGULATED RAT HEPATOCYTE FUNCTIONS IN A NEW SERUM-FREE HEPATOCYTE-STROMAL CELL COCULTURE MODEL", IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY - ANIMAL, vol. 36, no. 8, 1 January 2000 (2000-01-01), pages 502-512, XP055299382, New York ISSN: 1543-706X, DOI: 10.1290/1071-2690(2000)036<0502:EEOHRR>2.0 .CO;2 page 503	1-5

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	David F Burke ET AL: "A Recommended Numbering Scheme for Influenza A HA Subtypes", PLoS One vol 9 (11), 12 November 2014 (2014-11-12), pages 1-6, XP055267693, DOI: 10.1371/journal.pone Retrieved from the Internet: URL:http://journals.plos.org/plosone/article/asset?id=10.1371/journal.pone.0112302.PDF [retrieved on 2016-04-21] page 1	1-5
A	KEISUKE OKABE ET AL: "Wound Treatment Using Growth Factors", MODERN PLASTIC SURGERY, vol. 03, no. 03, 1 July 2013 (2013-07-01), pages 108-112, XP055299446, ISSN: 2164-5213, DOI: 10.4236/mps.2013.33022 page 108	1-5
A	US 2007/110731 A1 (RILEY PATRICIA A [US]) 17 May 2007 (2007-05-17) paragraph [0037] - paragraph [0038]	1-5
A	SZABO SANDOR ET AL: "Angiogenic and Anti-Angiogenic Therapy for Gastrointestinal Ulcers: New Challenges for Rational Therapeutic Predictions and Drug Design", CURRENT PHARMACEUTICAL DESIGN, vol. 17, no. 16, June 2011 (2011-06), pages 1633-1642, XP009191523, page 1634	1-5
A	WO 2010/138002 A1 (AUCKLAND UNISERVICES LTD [NZ]; GREEN COLIN RICHARD [NZ]; SHERWIN TREVO) 2 December 2010 (2010-12-02) page 30 - page 31	1-5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/066213

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.:
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:

see additional 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 an 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.:

5(completely); 1-4(partially)

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.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5(completely); 1-4(partially)

A composition for treating fibrosis comprising EGF and FGF2, wherein the fibrosis is liver fibrosis.

2-12. claims: 6(completely); 1-4(partially)

A composition for treating fibrosis comprising EGF and FGF2, wherein said fibrosis is (invention number in brackets): pulmonary fibrosis (2), heart fibrosis or pericardial fibrosis (3), Crohn's Disease (4), progressive kidney disease (5), pancreatic fibrosis (6), scleroderma/systemic sclerosis (7), post-surgery adhesions (8), peritoneal adhesions or retroperitoneal fibrosis (9), pleural fibrosis (10), uterine fibroid (11) or graft fibrosis (12).

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13. claims: 7-10, 13, 14(all partially)

In vitro process comprising a step of incubating stellate cells into a composition comprising EGF and optionally FGF2, wherein said stellate cells are hepatic stellate cells, and the isolated cells obtainable thereby, as well as the method of claim 13 (wherein both EGF and FGF2 are present) employing hepatic stellate cells, and the kit of claim 14 (wherein both EGF and FGF2 are present) comprising hepatic stellate cells.

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14. claims: 7-10(partially)

In vitro process comprising a step of incubating stellate cells into a composition comprising FGF2, wherein said stellate cells are hepatic stellate cells, and the isolated cells obtainable thereby.

15-22. claims: 7-10, 13, 14(all partially)

In vitro process comprising a step of incubating stellate cells into a composition comprising EGF and/or FGF2, and the isolated cells obtainable thereby, as well as the method of claim 13 (wherein both EGF and FGF2 are present) and the kit of claim 14 (wherein both EGF and FGF2 are present), wherein said stellate cells are (invention number in brackets): pancreatic stellate cells (15), lung stellate cells (16), intestinal stellate cells (17), kidney stellate cells (18), spleen stellate cells (19), adrenal gland stellate cells (20), ductus deferens stellate cells (21) or vocal cords stellate cells (22).

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23. claims: 11, 12

A composition comprising EGF, FGF2, oleic acid, palmitic acid and retinol, wherein- the concentration of EGF ranges from 0.1 to 100 ng/mL,- the concentration of FGF2 ranges from 0.05 to 80 ng/mL,- the concentration of oleic acid ranges from 1 to 1000 nmol/mL,- the concentration of palmitic acid ranges from 1 to 1000 nmol/mL, and- the concentration of retinol ranges from 0.01 to 100 nmol/mL,anda culture medium comprising the composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/066213

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007110731 A1	17-05-2007	NONE	
WO 2010138002 A1	02-12-2010	NONE	