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(54) **Title**: NEW COMPOSITIONS AND CELL THERAPY METHODS FOR THE TREATMENT OF CIRRHOSIS

(57) **Abstract**: The present invention provides an adaptor for transducing cells with adenovirus in an efficient and specific manner. The embodiment of the invention make use of an adaptor polypeptide that comprises a domain of coxsackievirus and adenovirus receptor (CAR) capable of binding to an adenoviral fiber protein or a functional variant thereof and a VEGF receptor (VEGFR) ligand.

NEW COMPOSITIONS AND CELL THERAPY METHODS FOR THE TREATMENT OF CIRRHOSIS

FIELD OF THE INVENTION

The invention relates to the field of cell therapy and, more particularly, to methods for the treatment of cirrhosis by specifically directing bone marrow derived EPC engineered to overexpress IGF-I by using an adaptor molecule which facilitates adenoviral transduction of said EPC.

BACKGROUND OF THE INVENTION

Cirrhosis is a consequence of chronic liver disease characterized by replacement of liver tissue by fibrosis, scar tissue and regenerative nodules (lumps that occur as a result of a process in which damaged tissue is regenerated), leading to loss of liver function. Cirrhosis is most commonly caused by alcoholism, hepatitis B and C, and fatty liver disease but has many other possible causes. Some cases are idiopathic, i.e., of unknown cause. Cirrhosis may develop to hepatic-insufficiency and hepatocellular carcinoma. Other complications can be bruising and bleeding due to decreased production of coagulation factors, hepatic encephalopathy, ascites etc.

At present, treatment of liver cirrhosis is limited to the management of complications and to effecting organ replacement in advanced disease. Liver transplantation, however, can be applied to only a small proportion of potential candidates. Recent progress in cell and gene therapy has opened new avenues to treat patients with hepatic disorders. The development of biological therapies capable of inducing fibrosis regression and improvement of hepatocellular function in cirrhotic livers is an urgent medical need. To this end, a strategy is the use of bone marrow (BM)-derived endothelial precursor cells (EPC).

These cells can be obtained by culturing BM mononuclear cells in specific differentiation medium. EPC share phenotypic markers with progenitor cells,

endothelial cells and hematopoietic cells, and produce a diversity of cytoprotective growth factors. Transfection of EPC with therapeutic or reporter genes has been widely described. These genetically modified and *ex vivo* expanded progenitor cells may become new promising agents that will be able to appropriately rescue impaired neovascularisation process under diseases conditions (Miller−Kasprzak and Jagodziński, *Arch. Immunol Ther Exp* 2007; 55:247–259 and Melo *et al. Arterioscler Thromb Vasc Biol* 2004; 24:1761-1774).

The idea that EPC therapy could be also an option for patients with liver cirrhosis stems from recent evidence showing that infusion of bone marrow-derived EPC to cirrhotic rats was able to improve liver fibrosis and liver function (Liu *et al., Liver Transplantation* 2009;15:1092-1100). Since EPC can be isolated and manipulated *in vitro*, it is reasonable to hypothesize that their transduction with vectors encoding specific hepatoprotective factors might enhance their capability to promote cirrhosis regression. In this context, it has been shown that direct transduction of liver parenchyma with gene therapy vectors encoding insulin-like growth factor 1 (IGF-I) leads to cirrhosis reversion in rats (Sobrevals *et al., Hepatology* 2010; 51(3):912-921).

IGF-I is a potent cytoprotective and anabolic hormone that is mainly produced in the liver, its levels being markedly decreased in liver cirrhosis as result of hepatocellular insufficiency. Intrahepatic expression of IGF-I has been shown to decrease fibrogenesis, to stimulate fibrolysis, to reduce inflammation and to exert hepatoprotective activities resulting in amelioration of liver function (Sobrevals *et al.*, *Hepatology* 2010;51(3):912-921). In fact, in a recent double blind pilot clinical trial using recombinant IGF-I the inventors found a significant elevation of serum albumin and improvement of Child-Pugh score in patients with alcoholic cirrhosis who received the therapy (Conchillo et al., *J Hepatol* 2005. 34:630-636).

Two distinct subpopulations exist in a heterogeneous parent EPC culture, one of which is amenable to transduction with adenovirus and one that is not. It has been described that EPC can be transfected with recombinant adenovirus, and that the transduction efficiency can be increased by the incorporation of a centrifugation step in the standard

protocol (Kealy *et al., Tissue engineering* 2009;15(2):223-231). Nevertheless, this method uses very high MOI.

However, the efficiency of transfection is still very low and the viability of the EPC cells is decreased because of the high sensitivity of the cells to the adenovirus. Thus, there is the need in the art of methods and reagents to facilitate the adenoviral transduction of EPC while maintaining the viability of the EPC.

DESCRIPTION OF THE FIGURES

Figura 1. Infection of murine EPC with an adenoviral vector encoding GFP.
Murine EPC were transfected after 5 days of differentiation with an adenovirus encoding for GFP at a MOI (multiplicity of infection) of 500 pfu/cell. Levels of GFP+ cells (mEPC.GFP+) were determined using flow-cytometry. Non transfected EPC were used as negative control.

Figure 2. Schematic representation of CAR-Fc-VEGF$_{121}$ adaptor.
A. Human CAR ectodomain and mouse or human VEGF isoform 121 are linked by the Fc portion of a human IgG1 (comprising hinge and CH2 and CH3 domains). B. Adaptor molecules should dimerize and bring together adenovirus and EPC by simultaneous binding via the CAR domain and through the VEGF receptor.

Figure 3. Characterization of rat Bone Marrow-derived EPC.
A. Adherent cells at day 5 of differentiation showed spindle-like morphology typical of endothelial lineage (200x, upper panel). Cells plated over Matrigel matrix initiated tube formation (100x, lower panel). B. FACS analysis was used to quantify VEGFR-1 and VEGFR-2 endothelial markers, homing receptor CXCR4, endothelial von Willebrand Factor (vWF) and to determine DiL-acLDL uptake and cell binding to lectin Ulex Europaeus-FITC. In all cases differentiated marked cells (DC) were compared to isotype cells (IC). Results shown are representative of 5 independent experiments.

Figure 4. Analysis of EPC transduction by adenovirus using CAR-Fc-mVEGF$_{121}$ adaptor.

A. EPC were mock transduced or transduced with AdIGFI-GFP at a MOI of 500 or 50 in the absence or presence of 25 ng of the CAR-Fc-mVEGF$_{121}$ adaptor for 48 h and GFP expression were visualized microscopically (40x, upper pictures). Arrows point at cell aggregates and detached cells. GFP expression was quantified by FACS analysis (lower graphs). Mock transduced cells (EPC) and AdIGFI-GFP transduced cells (EPC.IGFI) are shown. **B**. Bar-graph representing GFP expressing cells (% of GFP+ cells) after transduction. **C**. IGF-I expression (ng/mL 10^6 cells). IGF-I was quantified by ELISA of the supernatant of cells treated as described in A. *P <0.05, ***P <0.001.

Figure 5. Analysis of IGF-I and VEGF expression in EPC.

IGF-I (A) and VEGF (B) expression levels were quantified by ELISA in the supernatant of mock transduced cells (EPC) or EPC transduced for 48 hours with AdIGFI-GFP (EPC.IGF-I) at MOI 50 in the presence of 25 ng of the CAR-Fc-mVEGF$_{121}$ adaptor.

Figure 6. Biodistribution analysis of engineered EPC in cirrhotic rats (TAA model).

A. Six million EPC transduced with targeted AdIGFI-GFP for 48h as described in Figure 4, were labelled with DiL (red) and administered into cirrhotic rats by the tail vein. Cryosections from lung, spleen and liver were collected six days after cell transfer. DiL positive cells were quantified in 20 fields from 3 different animals. *P <0.05, ***P<0.001. B. GFP expression (arrows) was detected by immunohistochemistry of paraffin-embedded liver tissues.

Figure 7. IGF-I expression analysis in cirrhotic rats treated with engineered EPC or controls (TAA model).

Cirrhotic animals were treated with saline or six million MNC, EPC or EPC treated with targeted AdIGFI-GFP (EPC.IGF-I). Healthy animals were used as controls. 4 days after cell transfusion serum samples were collected to evaluate IGF-I by ELISA (A). 6 days after cell administration liver extracts were obtained and used to evaluate IGF-I by Western-blot analysis (B, lower picture). GAPDH was also evaluated as a loading control. Quantification of the Western-blot is also shown (B, upper graph). *P<0.05.

Figure 8. Analysis of liver fibrosis in cirrhotic rats treated with engineered EPC or controls.

Cirrhotic animals (TAA model in left panels and CCl_4 model in right panels), treated as described, were sacrificed 6 days after cell transfusion. A. Liver sections were stained with Masson trichromic and the fibrotic area was quantified. B. Liver extracts were used to evaluate Collagen I (Col. I) mRNA expression by quantitative RT-PCR. *P<0.05; **P<0.01.

Figure 9. Immunhistochemical analysis of α-SMA in cirrhotic animals treated with EPC or EPC transduced with Ad-IGFI-GFP using the protein adaptor CAR-Fc-mVEGF$_{121}$.

The number of cells positive for the Hepatic stellate cells (HSC) activation marker α-SMA was analyzed in histological hepatic sections of treated animals in at least 15 fields, with a 20X objective and using the ImageJ software. Data of marked area in treated animals relative to the positive area in healthy animals are shown in graphs (A) and representative photographs (B). The treatment of cirrhotic rats with EPC engineered to overexpress IGF-I reduced significantly the number of activated HSC in fibrotic septum.

Figure 10. Analysis of HSC apoptosis in cirrhotic rats (TAA model) treated with engineered EPC or controls.

Liver sections from animals were stained by immunofluorescence for caspase 3 and α-SMA. Cell nuclei were labelled with DAPI. Double positives for caspase 3 and α-SMA were quantified. Doble stainnig of α-SMA (specific antigen of activated HSC) and active caspase-3 was found in liver sections. The treatment of cirrhotic animals with EPC engineered to overexpress IGF-I significantly increased the apoptosis in HSC compared to saline group and to non genetically modified rat EPC, suggesting that the combination between therapeutic gene IGF-I and EPC activated the cell death program of activated HSC, responsible of collagen production in cirrhotic livers. *P<0.05, ***P<0.001.

Figure 11. Analysis of cell proliferation in cirrhotic rats treated with engineered EPC or controls.

Cell proliferation analysis in liver sections from healthy or cirrhotic animals treated with MNC, EPC or EPC.IGF-I, that were immunohistochemically stained for Ki67. Cell proliferation is represented as Ki67 positive cells per field. Results showed that treatment with EPC engineered to over-expressed IGF-I significantly increased the hepatic proliferation compared to non-treated animals. A. TAA model. B. CCl_4 model. *P<0.05.

DETAILED DESCRIPTION OF THE INVENTION

Polypeptide of the invention

The authors of the present invention have observed that a recombinant protein comprising a portion or domain of the coxsackievirus and adenovirus receptor (CAR) capable of binding to an adenoviral fiber protein and a vascular endothelial growth factor receptor (VEGFR) ligand can function as an adaptor protein and facilitate the transduction with adenovirus of cells that express VEGFR in the membrane by means of the binding of the CAR region of the adaptor protein to the adenoviral fiber protein and by the binding of the VEGFR ligand to the VEGFR on the surface of the cell.

Thus, in a first aspect, the invention relates to a polypeptide (hereinafter polypeptide of the invention, adaptor polypeptide of the invention, or adaptor of the invention) comprising:

(a) a domain of coxsackievirus and adenovirus receptor (CAR) capable of binding to an adenoviral fiber protein or a functional variant thereof and

(b) a VEGF receptor (VEGFR) ligand.

As used herein, the term "coxsackievirus and adenovirus receptor" or "CAR" (hereinafter also referred to as component (a) of the polypeptide of the invention) relates to a 46 kDa transmembrane protein that is a member of the immunoglobulin superfamily which acts as primary receptor for Ad subgroups A (e.g. Ad12), C (e.g.

Ad2 and Ad5), D (e.g. Ad8, Ad9, Ad10, Ad13, Ad15, Ad17, Ad19, Ad20, Ad22, Ad30, Ad32, Ad33, Ad36-39 and 42-49), E and F (Ad40 and Ad41) as well as for Coxsackie B viruses.

CAR proteins for use in the present invention include, without limitation, human CAR, rat CAR and mouse CAR.

Human CAR (CXAR_HUMAN Coxsackievirus and adenovirus receptor from homo sapiens, UniProt Accession number P78310 or SEQ ID NO:1) is a 365 amino acids polypeptide wherein amino acids 1-19 form a signal sequence and amino acids 20-365 form the mature CAR protein. The soluble region of CAR ectodomain is formed by amino acids 20-237, wherein amino acids 20-134 form the domain Ig-like C2-type 1 and amino acids 141-228 form the domain Ig-like C2-type 2.

SEQ ID NO:1

MALLLCFVLLCGVVDFARSLSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLIS
PADNQKVDQVIILYSGDKIYDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTYQC
KVKKAPGVANKKIHLVVLVKPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQKLSD
SQKMPTSWLAEMTSSVISVKNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKAGLIA
GAIIGTLLALALIGLIIFCCRKKRREEKYEKEVHHDIREDVPPPKSRTSTARSYIGSNHS
SLGSMSPSNMEGYSKTQYNQVPSEDFERTPQSPTLPPAKVAAPNLSRMGAIPVMIPAQSK
DGSIV

Rat CAR (CXAR_RAT Coxsackievirus and adenovirus receptor homolog, Isoform I, Rattus norvegicus, UniProt Accession number Q9R066 or SEQ ID NO:2) is a 365 amino acids polypeptide wherein amino acids 1-19 form a signal sequence and amino acids 20-365 form the mature CAR protein. The soluble region of CAR ectodomain is formed by amino acids 20-238, wherein amino acids 20-136 form the domain Ig-like C2-type 1 and amino acids 141-228 form the domain Ig-like C2-type 2.

SEQ ID NO:2

MALLLCFVLLCGVADFTSSLSITTPEQRIEKAKGETAYLPCKFTLEPEDQGPLDIEWLIS
PSDNQKVDQVIILYSGDKIYDNYYPDLKGRVHFTSNDVKSGDASINVTNLQLSDIGTYQC
KVKKAPGVANRKFLLTVLVKPSGTRCFVDGSGEIGNDFKLKCEPKEGSLPLQYEWQKLSD
SQKMPTPWLAEMTSPVISVKNASSEYSGTYSCTVQNRVGSDQCMLRLDVVPPSNRAGTIA
GAVIGTLLALVLIGAILFCCHKKRREEKYEKEVHHDIREDVPPPKSRTSTARSYIGSNHS
SLGSMSPSNMEGYSKTQYNQVPSEDFERAPQSPTLAPAKVAAPNLSRMGAVPVMIPAQSK
DGSIV

Mouse CAR (CXAR_MOUSE Coxsackievirus and adenovirus receptor homolog, Isoform I, Mus musculus, UniProt Accession number P97792 or SEQ ID NO:3) is a 365 amino acids polypeptide wherein amino acids 1-19 form a signal sequence and amino acids 20-365 form the mature CAR protein. The soluble region of CAR ectodomain is formed by amino acids 20-237, wherein amino acids 20-136 form the domain Ig-like C2-type 1 and amino acids 141-228 form the domain Ig-like C2-type 2.

SEQ ID NO:3

MARLLCFVLLCGIADFTSGLSITTPEQRIEKAKGETAYLPCKFTLSPEDQGPLDIEWLIS
PSDNQIVDQVIILYSGDKIYDNYYPDLKGRVHFTSNDVKSGDASINVTNLQLSDIGTYQC
KVKKAPGVANKKFLLTVLVKPSGTRCFVDGSEEIGNDFKLKCEPKEGSLPLQFEWQKLSD
SQTMPTPWLAEMTSPVISVKNASSEYSGTYSCTVQNRVGSDQCMLRLDVVPPSNRAGTIA
GAVIGTLLALVLIGAILFCCHRKRREEKYEKEVHHDIREDVPPPKSRTSTARSYIGSNHS
SLGSMSPSNMEGYSKTQYNQVPSEDFERAPQSPTLAPAKVAAPNLSRMGAVPVMIPAQSK
DGSIV

The term “domain of coxsackievirus and adenovirus receptor (CAR) capable of binding to an adenoviral fiber protein” refers to any region from the extracellular domain of CAR (also identified as ectodomain) which maintains substantially the same capacity than the full-length CAR for binding to the adenoviral fibre protein. Typically, the domain of coxsackievirus and adenovirus receptor, when expressed in a target cell under conditions which allows the domain to be attached to the cell membrane facing the extracellular space. allows infection of said cell by an adenovirus. The domain may comprise the complete extracellular region (amino acids 20-237 of the human CAR, amino acids 20-238 of the rat CAR or amino acids 20-237 of the mouse CAR), the

domain Ig-like C2-type 1 (amino acids 20-134 of the human CAR, amino acids 20-136 of the rat CAR or amino acids 20-134 of the mouse CAR), the domain Ig-like C2-type 2 (amino acids 141-228 of the human CAR, amino acids 141-228 of the rat CAR and amino acids 141-228 of the mouse CAR), a region comprising both the domains Ig-like C2-type 1 and the Ig-like C2-type 2 domains or any region which is capable of binding to the adenoviral fiber protein with sufficient specificity so as to ensure efficient infection of cells expressing said receptor. Methods for determining the capacity of a CAR domain for binding to the adenoviral fiber protein include, without limitation, surface plasmon resonance as described in Kirby et al. (*J.Virol.*, 2000, 74:2804-2813). Suitable domains for use in the adaptor molecules of the present invention include those having a binding constant of less than 10^{-7} M, preferably less than 10^{-8} M, more preferably of less than $9x10^{-9}$ M, less than $8x10^{-9}$ M, less than $7x10^{-9}$ M, less than $6x10^{-9}$ M, less than $5x10^{-9}$ M, less than $4x10^{-9}$ M, less than $3x10^{-9}$ M, less than $2x10^{-9}$ M, less than 10^{-9} M, less than $9x10^{-10}$ M, less than $8x10^{-10}$ M, less than $7x10^{-10}$ M, less than $6x10^{-10}$ M, less than $5x10^{-10}$ M, less than $4x10^{-10}$ M, less than $3x10^{-10}$ M, less than $2x10^{-10}$ M, less than 10^{-10} M.

The term "functionally equivalent variant" or "functional variant" as used herein, relates to any polypeptide derived from CAR by insertion, deletion or substitution of one or more residues and which maintains substantially the ability to interact with the adenoviral fiber protein as determined above. Suitable functional variants are those showing a degree of identity with respect to the CAR domain of about greater than 25% amino acid sequence identity, such as 25% 40%, 60%, 70%, 80%, 90% or 95%. The degree of identity between two polypeptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. The identity between two amino acid sequences is preferably determined by using the BLASTP algorithm [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)]. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query

sequence, which either match or satisfy some positive- valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul et al, *supra*). These initial neighbourhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always 0) and N (penalty score for mismatching residues; always 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. Suitable values of the BLASTP parameters are, without limitation, the default values of a word length of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sd. USA, 1989, 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

In an embodiment, the CAR domain comprises SEQ ID NO: 4, which is the ectodomain of the human CAR (P78310 | amino acids 20-236). In another embodiment, the CAR domain is the SEQ ID NO: 4.

SEQ ID NO: 4.

LSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLISPADNQKVDQVIILYSGDKI
YDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTYQCKVKKAPGVANKKIHLVVLV
KPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQKLSDSQKMPTSWLAEMTSSVISV
KNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKA

The second component of the polypeptide of the invention is a "VEGF receptor (VEGFR) ligand" (hereinafter also referred to as component (b) of the polypeptide of the invention). As used herein, the term "VEGF receptor ligand" refers to any molecule which is capable of specifically binding to VEGFR.

The term "vascular endothelial growth factor receptor" (VEGFR) is understood as any member of the VEGFR family of kinase receptors of the cell surface. VEGFR dimerize and become activated through transphosphorylation when the one of the VEGF family members binds to the VEGFR. In humans the VEGFR comprises the VEGFR-1 (Flt-1), VEGF-R2 (KDR/Flk-1) o VEGFR3 (Flt-4).

Typically, the ligand of the VEGFR is a member of the VEGF subfamily of growth factors, specifically the platelet-derived growth factor family of cystine-knot growth factors (Olsson et al., *Nature Reviews Molecular Cell Biology* 2006;7:359-371).

All naturally occurring VEGF polypeptides that bind to the VEGFR can be used to manufacture the polypeptide of the invention, and numerous such sequences are known through Genbank, scientific literature, and patent literature. VEGF family members include those of avian and mammalian species and of viral species that infect avian and mammalian species. Exemplary mammalian species are primates, rodents, bovine, equine, canine, porcine and feline species. Exemplary sequences include (1) all such VEGF family member sequences listed in the publicly accessible database GENBANK; (2) all VEGF sequences that hybridize to specific human sequences identified herein under moderate or high stringency conditions; and (3) all VEGF sequences that can be amplified from a genomic or cDNA library using PCR and related techniques and using primers designed from highly conserved regions of the sequences provided herein. It is possible to use human VEGF sequences and viral sequences, such as those described in the following paragraphs.

In an embodiment, the VEGFR ligand is VEGF-A (or VEGF). VEGF-A was originally purified from several sources on the basis of its mitogenic activity toward endothelial cells, and also by its ability to induce microvascular permeability, hence it is also called vascular permeability factor (VPF). (Ferrara, *J. Mol. Med.* 1999;77: 527-543 ; Neufeld, et al., *FASEB J* 1999;13:9-22; Zachary, Intl. *J. Biochem. Cell. Bio.* 1998;30:1169-74). Human VEGF-A is a secreted, disulfide-linked homodimeric glycoprotein composed of 23 kD subunits. VEGF-A is a ligand for VEGFR-1/Flt-1 (fms-like tyrosine kinase-1)

and VEGFR-2/Kdr/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1). Typically, any VEGF-A can be used in the adaptor proteins of the invention including, without limitation, VEGF-A isoform a (NP_001020537.2), VEGF-A isoform b (NP_003367.4), VEGF-A isoform c (NP_001020538.2), VEGF-A isoform d (NP_001020539.2), VEGF-A isoform g (NP_001028928.1), VEGF-A isoform e (NP_001020540.2) and VEGF-A isoform f (NP_001020541.2).

Other suitable VEGFR ligands include without limitation:

- VEGF-B (NP_003368.1), which is described in detail in International Patent Publication No. WO 96/26736 and U.S. Pat. Nos. 5,840,693 and 5,607,918. VEGF-B shares approximately 44% amino acid identity with VEGF-A. VEGF-B is expressed as two isoforms of 167 and 186 amino acid residues generated by alternative splicing. Both VEGF-B_{167} (AAL79000.1) and VEGF-B_{186} (AAC50721.1) can form disulfide-linked homodimers or heterodimers with VEGF-A.
- VEGF-C (NP_005420.1), the isolation and characteristics of which are described in detail in Joukov et al, (EMBO J. 15 290-298, 1996) and U.S. Pat. Nos. 6,221,839; 6,235,713; 6,361,946; 6,403,088; and 6,645,933 and International Patent Publ. Nos. WO 97/05250, WO 98/07832, and WO 98/01973.
- VEGF-D (or FIGF) (NP_004460.1), which is initially expressed as a prepro-peptide that undergoes removal of a signal peptide (residues 1-21 of NP_004460.1), N-terminal (residues 22-92 of NP_004460.1) and C-terminal (residues 202-354 of NP_004460.1) proteolytic processing, and forms non-covalently linked dimers. Isolation of a biologically active fragment of VEGF-D designated VEGF-DΔNΔC, is described in International Patent Publication No. WO 98/07832 (PCT/US97/14696), incorporated herein by reference. VEGF-DΔNΔC consists of amino acid residues 93 to 201 of VEGF-D and binds VEGFR-2 and VEGFR-3. Partially processed forms of VEGF-D bind to VEGFR-3. In addition, VEGF-D is described in great detail in International Patent Publication No. WO 98107832 and U.S. Pat. No. 6,235,713, and describes VEGF-D polypeptides and variants thereof that are useful in producing the chimeras of the present invention. VEGF-D related molecules also

are described in International Patent Publication Nos. WO 98102543 and WO 97112972, and U.S. Pat. No. 6,689,580, and U.S. patent application Ser. Nos. 09/1219,345 and 09/1847,524.

Fragments of VEGF-C or -D for use in the adaptor molecules of the invention may be continuous fragments that bind one or more of the VEGF receptors. However, it has been demonstrated that VEGFR binding can be achieved with molecules that incorporate discrete, discontinuous fragments of VEGF-C, fused, e.g., to fragments of VEGF-A or other amino acid sequences. Such chimeric VEGFR ligands are described in U.S. patent application Ser. No. 09/795,006, and International Patent Publication No. WO 01/62942.

Four additional members of the VEGF subfamily collectively referred to as VEGF-E factors have been identified in poxviruses, which infect humans, sheep and goats. The orf virus-encoded VEGF-E and NZ2 VEGF are potent mitogens and permeability enhancing factors. Both show approximately 25% amino acid identity to mammalian VEGF-A, and are expressed as disulfide-linked homodimers. Another variant of orf virus VEGF-E like protein from strain NZ10 is described in WO 00/25805. VEGF-like proteins have also been identified from two additional strains of the orf virus, D1701 (GenBank Acc. No. AF106020; described in Meyer et al., *EMBO J.* 1999; 18:363-374) and NZ10 (described in International Patent Application WO 00/25805). These viral VEGF-like proteins have been shown to bind VEGFR-2 present on host endothelium, and this binding is important for development of infection and viral induction of angiogenesis (Meyer et al., *EMBO J.* 1999; 18:363-374; International Patent Application WO 00/25805).

Moreover, smaller fragments of most or all of the VEGF family also may bind to their respective receptors and the identity of such smaller fragments is determined by RTK binding assays such as those described in US2007142282 AA or in the literature.

As used herein, "VEGF receptor (VEGFR) ligand" is understood as a protein that specifically binds to VEGFR and includes all the VEGF family members described

above as well as functional variants thereof and fragments of said VEGF family members that are able to bind to VEGFR. The term "functional variant", as used herein, relates to any polypeptide derived from a VEGFR ligand by insertion, deletion or substitution of one or more residues and which maintains substantially the ability to interact with the corresponding VEGFR as determined above. Suitable functional variants are those showing a degree of identity with respect to the VEGF of about greater than 25% amino acid sequence identity, such as 25% 40%, 60%, 70%, 80%, 90% or 95%. The degree of identity between two polypeptides is determined using computer algorithms and methods that are widely known for the persons skilled in the art. The identity between two amino acid sequences is preferably determined by using the BLASTP algorithm (explained above) [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990)].

Different assays are available in the state of the art to determine the binding capacity of a functional variant of a VEGFR ligand to the corresponding VEGFR. Examples of the different assays that can be used are standard receptor-binding assay, such as the RTK binding assays described on US2007142282 AA, or in Zheng Y. et al.(*Arteriosclerosis, Thrombosis, and Vascular Biology* 2006;26:2019) or radio-ligand binding assay as described in Ito N. et al. (*Angiogenesis* 1999;3:159).

Basically the different assays that can be used are based in the detection of the auto-phosphorylation capacities of the VEGFR when it is activated by a ligand (Meyer, M. et al. *EMBO J* 1999;18:363-74., Dougher-Vermazen, M. et al. (*Biochem Biophys Res Commun* 1994;205:728-38). Further methods for the detection of the binding capacity of a ligand to bind to a VEGFR are those based on the detection of the phosphorylation of a VEGFR substrate. Basically the assay consists in the detection of the phosphorylation of a known VEGFR substrate that is detected using, for example an antibody that binds to the phosphorylated substrate peptide. Moreover, different cell-based assays based on the different effects observed for the different VEGF family members can be used. For example, it has been described that VEGF-A has a mitogenic activity toward endothelial cells (Ferrara, *J. Mol. Med.* 1999; 77:527-543; Neufeld, et al., *FASEB. J.* 1999;13:9-22; Zachary, Intl. *J. Biochem. Cell. Bio.* 1998; 30:1169-74).

Thus, for determine the binding capacities of a functional variant of a VEGFR-A, can be measured the ability of said variant for inducing mitogenesis in endothelial cells.

In an embodiment, the VEGFR ligand is selected from the VEGF isoforms $VEGF_{121}$, $VEGF_{121b}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{189}$ and $VEGF_{206}$. In another embodiment the VEGFR ligand is human or mouse $VEGF_{121}$.

In a particular embodiment, the VEGFR ligand is a $VEGF_{121}$ isoform having the sequence of SEQ ID NO:5, which is encoded by polynucleotide sequence SEQ ID NO:6.

SEQ ID NO:5

APMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGG
CCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEKCDK
PRR

SEQ ID NO:6

GCACCCATGGCAGAAGGAGGAGGGCAGAATCATCACGAAGTGGTGAAGTTCATGGATGT
CTATCAGCGCAGCTACTGCCATCCAATCGAGACCCTGGTGGACATCTTCCAGGAGTACC
CTGATGAGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCCTGATGCGATGCGGGGGC
TGCTGCAATGACGAGGGCCTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCA
GATTATGCGGATCAAACCTCACCAAGGCCAGCACATAGGAGAGATGAGCTTCCTACAGC
ACAACAAATGTGAATGCAGACCAAAGAAAGATAGAGCAAGACAAGAAAAATGTGACAAG
CCGAGGCGG

In another embodiment, the VEGF R ligand is a $VEGF_{121}$ isoform having the sequence SEQ ID NO:7, which is encoded by polynucleotide sequence SEQ ID NO:8.

SEQ ID NO:7

APTTEGEQKSHEVIKFMDVYQRSYCRPIETLVDIFQEYPDEIEYIFKPSCVPLMRCAGC
CNDEALECVPTSESNITMQIMRIKPHQSQHIGEMSFLQHSRCECRPKKDRTKPEK

SEQ ID NO:8

GCACCCACGACAGAAGGAGAGCAGAAGTCCCATGAAGTGATCAAGTTCATGGATGTCTA
CCAGCGAAGCTACTGCCGTCCGATTGAGACCCTGGTGGACATCTTCCAGGAGTACCCCG
ACGAGATAGAGTACATCTTCAAGCCGTCCTGTGTGCCGCTGATGCGCTGTGCAGGCTGC
TGTAACGATGAAGCCCTGGAGTGCGTGCCCACGTCAGAGAGCAACATCACCATGCAGAT
CATGCGGATCAAACCTCACCAAAGCCAGCACATAGGAGAGATGAGCTTCCTACAGCACA
GCAGATGTGAATGCAGACCAAAGAAAGACAGAACAAAGCCAGAAAAA

In a particular embodiment, the polypeptide of the invention further comprises a dimerization domain (hereinafter also referred to as component (c) of the polypeptide of the invention) which is found between the domain of CAR and the VEGFR ligand.

As used herein, the term "dimerization domain" refers to a protein binding domain (of either immunological or non-immunological origin) that has the ability to bind to another protein binding domain with sufficient strength and specificity such as to form a dimer. The dimerization domain can associate with other dimerization domain of identical amino acid sequence (a homodimer), or with a dimerization domain of different amino acid sequence (a heterodimer). Such an interaction may be caused by covalent bonds between the components of the dimerizing domains as well as by hydrogen bond forces, hydrophobic forces, van der Waals forces and salt bridges.

Preferred dimerization domains contain at least one cysteine that is capable of forming an intermolecular disulfide bond with a cysteine on the partner fusion protein. The dimerization domain can contain one or more cysteine residues such that disulfide bond(s) can form between the partner fusion proteins. In one embodiment, dimerization domains contain one, two or three to about ten cysteine residues. In an embodiment, the dimerization domain comprises the hinge region of an immunoglobulin. In another embodiment, the dimerization domain comprises the Fc fragment of an immunoglobulin.

The term "Fc fragment" as used herein, relates to the tail region of an immunoglobulin which is capable of specifically interacting with cell surface receptors called Fc

receptors and some proteins of the complement system. In IgG, IgA and IgD antibody isotypes, the Fc fragment is composed of two identical protein fragments, derived from the second and third constant domains of the antibody's two heavy chains; IgM and IgE Fc fragments contain three heavy chain constant domains (CH domains 2–4) in each polypeptide chain. The Fc fragments of IgGs bear a highly conserved N-glycosylation site. The Fc fragment also comprises the hinge region that permit dimerization of both identical protein fragments.

In an embodiment, the Fc fragment is a Fc fragment of a IgG. In an embodiment, the immunoglobulin is IgG1.

In a another embodiment, the Fc fragment comprises or consist of the amino acid sequence SEQ ID NO:9.

SEQ ID NO:9

KSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKV

Further suitable dimerization domains are, without limitation, leucine zippers, helix-loop helix domains or ankyrin and the dimerization component derived from SHBG (EP2072527A). The dimerization domain may be also an inducible dimerization domain as described on WO09124285A.

The skilled person will appreciate that the different elements of the polypeptide of the invention may be arranged in any order as long as the tridimensional structure of the VEGFR ligand and of the CAR region are preserved and maintain their respective functions of interaction with VEGFR or interaction with the adenoviral fiber proteins. Thus, suitable arrangements of the adaptor polypeptide of the invention depicted schematically in N- to C-terminal direction include:

- CAR ectodomain - VEGFR ligand
- VEGFR ligand – CAR ectodomain
- CAR ectodomain - dimerization domain -VEGFR ligand
- VEGFR ligand - dimerization domain- CAR ectodomain

In an embodiment, the adaptor polypeptide comprises, in order from the N-terminus to the C-terminus, the CAR ectodomain, a dimerization motif and the VEGFR ligand.

The different elements of the polypeptide of the invention may be directly attached, i.e. the C-terminus of an element is linked directly to the N-terminal region of the following element. However, it is also possible that the elements are contacted via a linker region. The linker can be located between any two components of the polypeptide. Thus, in another embodiment, the polypeptide of the invention further comprises one or more peptide linkers between the components a, b and c.

According to the invention, said linker amino acid sequence acts as a hinge region between said domains, allowing them to move independently from one another while maintaining the three-dimensional form of the individual domains. In this sense, a possible intermediate amino acid sequence according to the invention would be a hinge region characterized by a structural ductility allowing this movement. In a particular embodiment, said intermediate amino acid sequence is a flexible linker. In an embodiment, said flexible linker is a flexible linker peptide with a length of 20 amino acids or less.

The effect of the linker region is to provide space between the CAR ectodomain, VEGFR ligand and the dimerization domain. It is thus assured that the secondary structure of the CAR domain is not affected by the presence of component VEGFR ligand or the dimerization domain and vice versa. The spacer is preferably of a polypeptide nature. The linker peptide preferably comprises at least 2 amino acids, at least 3 amino acids, at least 5 amino acids, at least 10 amino acids, at least 15 amino acids, at least 20 amino acids, at least 30 amino acids, at least 40 amino acids, at least 50 amino acids, at least 60 amino acids, at least 70 amino acids, at least 80 amino acids,

at least 90 amino acids or approximately 100 amino acids.

In an embodiment, the linker peptide comprises 2 or more amino acids selected from the group consisting of glycine, serine, alanine and threonine. In an embodiment of the invention, said flexible linker is a polyglycine linker. The possible examples of linker/spacer sequences include SGGTSGSTSGTGST (SEQ ID NO: 10), AGSSTGSSTGPGSTT (SEQ ID NO:11) or GGSGGAP (SEQ ID NO:12). These sequences have been used for binding designed coiled coils to other protein domains (Muller, K.M., Arndt, K.M. and Alber, T., Meth. Enzimology, 2000, 328: 261-281). Preferably, said linker comprises or consists of amino acid sequence GGGVEGGG (SEQ ID NO:13).

The linker can include tetranectin residues 53-56, which in tetranectin forms a β-sheet, and residues 57-59 forming a turn in tetranectin (Nielsen, B.B. *et al.*, FEBS Lett. 412: 388-396, 1997).

Alternatively, a suitable linker peptide can be based on the sequence of 10 amino acid residues of the upper hinge region of murine IgG3. This peptide (PKPSTPPGSS, SEQ ID NO:14) has been used for the production of dimerized antibodies by means of a coiled coil (Pack P. and Pluckthun, A., 1992, Biochemistry 31:1579-1584) and can be useful as a spacer peptide according to the present invention. Even more preferably, it can be a corresponding sequence of the upper hinge region of human IgG3. The sequences of human IgG3 are not expected to be immunogenic in human beings. Additional linker peptides that can be used in the adaptor polypeptide of the invention include the peptide of sequence APAETKAEPMT (SEQ ID NO:15), the peptide of sequence GAP, the peptide of sequence AAA and the peptide of sequence AAALE.

In an embodiment, the linker is selected form AAQPAEP (SEQ ID NO:16), AAA, and GGPGS and is at the C-terminus of the CAR domain and/or C-terminus of the dimerization domain. In another embodiment, the polypeptide of the invention comprises a first linker region having the sequence AAQPAEP between the CAR domain and the dimerization domain and a second linker region having the AAA

sequence between the dimerization domain and the VEGF ligand. In another embodiment, the polypeptide of the invention comprises a CAR domain and a dimerization domain which are directly linked and a linker region having a GGPGS sequence located between the dimerization domain and the VEGFR ligand.

For the purpose of facilitating the isolation and purification of the adaptor polypeptide of the invention, said polypeptide can contain, if desired, an additional peptide which can be used for the purposes of isolating or purifying the fusion protein, such as a tag peptide. Said tag peptide can be located in any position of the adaptor polypeptide which does not alter the functionality of any of components or domains of the polypeptide of the invention (a), (b) and (c). By way of a non-limiting illustration, said tag peptide can be located in the N-terminal position of the polypeptide of the invention such that the C-terminal end of the tag peptide is bound to the N-terminal end of the adaptor polypeptide of the invention. Alternatively, the tag peptide can be located in the C-terminal position of the polypeptide of the invention such that the N-terminal end of the tag peptide is bound to the C-terminal end of the polypeptide of the invention. Virtually any peptide or peptide sequence allowing the isolation or purification of the adaptor polypeptide can be used, for example, polyhistidine sequences, peptide sequences which can be recognized by antibodies which can serve to purify the resulting polypeptide by immunoaffinity chromatography, such as tag peptides, for example, influenza virus hemagglutinin (HA)-derived epitopes (Field et al., 1988, Mol. Cell. Biol., 8: 2159-2165), C-myc and the antibodies 8F9, 3C7, 6E10, G4, B7 and 9E10 against it (Evan et al., 1985, Molecular and Cellular Biology, 5:3610-3616); the Herpes Simplex virus D (gD) tag protein and the antibodies thereof (Paborsky et al., 1990, Protein Engineering, 3:547-553). Other tag peptides include the Flag peptide (Hopp et al., 1988, BioTechnology, 6:1204-1210) and the KT3 epitope (Martin et al., 1993, Science, 255: 192-194). The tag peptide is generally arranged at the amino- or carboxy-terminal end. In another embodiment, the tag peptide is a His tag, more preferably a hexahistidine tag.

In another embodiment, the polypeptide of the invention is the polypeptide referred to herein as CAR-Fc-mVEGF$_{121}$ (SEQ ID NO: 17) and having the sequence:

LSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLISPADNQKVDQVIILYSGDK
IYDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTYQCKVKKAPGVANKKIHLVV
LVKPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQKLSDSQKMPTSWLAEMTSSV
ISVKNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKA**KSSDKTHTCPPCPAPELLG**
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKVggpgsAPTTEGEQKSHEVIKFMDV
YQRSYCRPIETLVDIFQEYPDEIEYIFKPSCVPLMRCAGCCNDEALECVPTSESNITMQ
IMRIKPHQSQHIGEMSFLQHSRCECRPKKDRTKPEK

wherein the sequence of the CAR ectodomain is underlined, the sequence of the dimerization domain is bolded, the sequence of the GGPGS linker region appears as in lower case and the sequence of the VEGFR ligand appears as regular text.

In another embodiment, the polypeptide of the invention is the polypeptide referred to herein as CAR-Fc-hVEGF$_{121}$ (SEQ ID NO: 18) and having the sequence:

LSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLISPADNQKVDQVIILYSGDK
IYDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTYQCKVKKAPGVANKKIHLVV
LVKPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQKLSDSQKMPTSWLAEMTSSV
ISVKNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKAaaqpaep**KSSDKTHTCPPC**
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK
TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQ
VYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL
YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKVaaaAPMAEGGGQNHHEV
VKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEE
SNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEKCDKPRR

wherein the sequence of the CAR ectodomain is shown underlined, the sequence of the AAQPAEP and AAA linker regions are shown in regular lower case text, the sequence of the IgG Fc is shown in bold and the sequence of the VEGF$_{121}$ as regular text.

Polynucleotides, gene constructs and vectors of the invention.

In another aspect, the invention relates to a polynucleotide encoding a polypeptide of the invention, hereinafter polynucleotide of the invention. A person skilled in the art will understand that the polynucleotide of the invention will encode the adaptor molecule regardless of the relative orientation and regardless of the fact that the components of the adaptor molecule are directly connected or separated by a spacer region.

In an embodiment the polynucleotide comprises or consists of SEQ ID NO: 19, which encodes a polypeptide sequence SEQ ID NO:20, that comprises the signal sequence of human CAR linked to CAR-Fc-mVEGF$_{121}$ (SEQ ID NO:17).

SEQ ID NO: 19

atgGCGCTCCTGCTGTGCTTCGTGCTCCTGTGCGGAGTAGTGGATTTCGCCAGAAGTTT
GAGTATCACTACTCCTGAAGAGATGATTGAAAAAGCCAAAGGGGAAACTGCCTATCTGC
CGTGCAAATTTACGCTTAGTCCCGAAGACCAGGGACCGCTGGACATCGAGTGGCTGATA
TCACCAGCTGATAATCAGAAGGTGGATCAAGTGATTATTTTATATTCTGGAGACAAAAT
TTATGATGACTACTATCCAGATCTGAAAGGCCGAGTACATTTTACGAGTAATGATCTCA
AATCTGGTGATGCATCAATAAATGTAACGAATTTACAACTGTCAGATATTGGCACATAT
CAGTGCAAAGTGAAAAAAGCTCCTGGTGTTGCAAATAAGAAGATTCATCTGGTAGTTCT
TGTTAAGCCTTCAGGTGCGAGATGTTACGTTGATGGATCTGAAGAAATTGGAAGTGACT
TTAAGATAAAATGTGAACCAAAAGAAGGTTCACTTCCATTACAGTATGAGTGGCAAAAA
TTGTCTGACTCACAGAAAATGCCCACTTCATGGTTAGCAGAAATGACTTCATCTGTTAT
ATCTGTAAAAAATGCCTCTTCTGAGTACTCTGGGACATACAGCTGTACAGTCAGAAACA
GAGTGGGCTCTGATCAGTGCCTGTTGCGTCTAAACGTTGTCCCTCCTTCAAATAAAGCC
AAATCTTCTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGG
ACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCC
CTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAAC
TGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTA
CAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATG
GCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACC

ATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCG
GGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCA
GCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACG
CCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAA
GAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACA
ACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAAGTCggtggccccgggtcg
GCACCCACGACAGAAGGAGAGCAGAAGTCCCATGAAGTGATCAAGTTCATGGATGTCTA
CCAGCGAAGCTACTGCCGTCCGATTGAGACCCTGGTGGACATCTTCCAGGAGTACCCCG
ACGAGATAGAGTACATCTTCAAGCCGTCCTGTGTGCCGCTGATGCGCTGTGCAGGCTGC
TGTAACGATGAAGCCCTGGAGTGCGTGCCCACGTCAGAGAGCAACATCACCATGCAGAT
CATGCGGATCAAACCTCACCAAAGCCAGCACATAGGAGAGATGAGCTTCCTACAGCACA
GCAGATGTGAATGCAGACCAAAGAAAGACAGAACAAAGCCAGAAAAAtga

wherein the sequence encoding the CAR domain (signal sequence and ectodomain) is shown underlined, the sequence encoding the dimerization domain is shown in bold, the sequence encoding the linker region is shown in lower case and the sequence encoding the VEGFR ligand is shown in regular upper case text.

SEQ ID NO: 20

MALLLCFVLLCGVVDFARSLSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLI
SPADNQKVDQVIILYSGDKIYDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTY
QCKVKKAPGVANKKIHLVVLVKPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQK
LSDSQKMPTSWLAEMTSSVISVKNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKA
KSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKT
ISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK*Vggpgs*
APTTEGEQKSHEVIKFMDVYQRSYCRPIETLVDIFQEYPDEIEYIFKPSCVPLMRCAGC
CNDEALECVPTSESNITMQIMRIKPHQSQHIGEMSFLQHSRCECRPKKDRTKPEK

In another embodiment, the polynucleotide comprises or consists of SEQ ID NO: 21, which encodes a polypeptide sequence SEQ ID NO: 22, that comprises the signal sequence of human CAR linked to CAR-Fc-hVEGF$_{121}$ (SEQ ID NO:18).

SEQ ID NO: 21

ATGGCGCTCCTGCTGTGCTTCGTGCTCCTGTGCGGAGTAGTGGATTTCGCCAGAAGTTT
GAGTATCACTACTCCTGAAGAGATGATTGAAAAAGCCAAAGGGGAAACTGCCTATCTGC
CGTGCAAATTTACGCTTAGTCCCGAAGACCAGGGACCGCTGGACATCGAGTGGCTGATA
TCACCAGCTGATAATCAGAAGGTGGATCAAGTGATTATTTTATATTCTGGAGACAAAAT
TTATGATGACTACTATCCAGATCTGAAAGGCCGAGTACATTTTACGAGTAATGATCTCA
AATCTGGTGATGCATCAATAAATGTAACGAATTTACAACTGTCAGATATTGGCACATAT
CAGTGCAAAGTGAAAAAAGCTCCTGGTGTTGCAAATAAGAAGATTCATCTGGTAGTTCT
TGTTAAGCCTTCAGGTGCGAGATGTTACGTTGATGGATCTGAAGAAATTGGAAGTGACT
TTAAGATAAAATGTGAACCAAAAGAAGGTTCACTTCCATTACAGTATGAGTGGCAAAAA
TTGTCTGACTCACAGAAAATGCCCACTTCATGGTTAGCAGAAATGACTTCATCTGTTAT
ATCTGTAAAAAATGCCTCTTCTGAGTACTCTGGGACATACAGCTGTACAGTCAGAAACA
GAGTGGGCTCTGATCAGTGCCTGTTGCGTCTAAACGTTGTCCCTCCTTCAAATAAAGCC
gcggcccagccggccgagccc**AAATCTTCTGACAAAACTCACACATGCCCACCGTGCCC**
AGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACA
CCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAA
GACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGAC
AAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCC
TGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTC
CCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGT
GTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCC
TGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG
GAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTA
CAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCG
TGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGT
AAAGTCgcggccgctGCACCCATGGCAGAAGGAGGAGGGCAGAATCATCACGAAGTGGT
GAAGTTCATGGATGTCTATCAGCGCAGCTACTGCCATCCAATCGAGACCCTGGTGGACA
TCTTCCAGGAGTACCCTGATGAGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCCTG
ATGCGATGCGGGGGCTGCTGCAATGACGAGGGCCTGGAGTGTGTGCCCACTGAGGAGTC
CAACATCACCATGCAGATTATGCGGATCAAACCTCACCAAGGCCAGCACATAGGAGAGA
TGAGCTTCCTACAGCACAACAAATGTGAATGCAGACCAAAGAAAGATAGAGCAAGACAA
GAAAAATGTGACAAGCCGAGGCGGtga

wherein the sequence encoding the CAR domain (signal sequence and ectodomain) is shown underlined, the sequence encoding the dimerization domain is shown in bold, the sequences encoding the linker region are shown in lower case and the sequence encoding the VEGFR ligand is shown in regular upper case text.

SEQ ID NO: 22

MALLLCFVLLCGVVDFARSLSITTPEEMIEKAKGETAYLPCKFTLSPEDQGPLDIEWLI
SPADNQKVDQVIILYSGDKIYDDYYPDLKGRVHFTSNDLKSGDASINVTNLQLSDIGTY
QCKVKKAPGVANKKIHLVVLVKPSGARCYVDGSEEIGSDFKIKCEPKEGSLPLQYEWQK
LSDSQKMPTSWLAEMTSSVISVKNASSEYSGTYSCTVRNRVGSDQCLLRLNVVPPSNKA
aaqpaep**KSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE**
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
KVaaaAPMAEGGGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDEIEYIFKPSCVPL
MRCGGCCNDEGLECVPTEESNITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQ
EKCDKPRR

The polynucleotide of the invention may be isolated or may form part of a gene construct. The construct preferably comprises the polynucleotide of the invention located under the operative control of sequences regulating the expression of the polynucleotide of the invention. A person skilled in the art will understand that the polynucleotide of the invention must access the nucleus of a target tissue and there be transcribed and translated to give rise to the biologically active fusion protein.

In principle, any promoter can be used for the gene constructs of the present invention provided that said promoter is compatible with the cells in which the polynucleotide is to be expressed. Thus, promoters suitable for the embodiment of the present invention include, without being necessarily limited to, constitutive promoters such as the derivatives of the genomes of eukaryotic viruses such as the polyoma virus, adenovirus, SV40, CMV, avian sarcoma virus, hepatitis B virus, the promoter of the metallothionein

gene, the promoter of the herpes simplex virus thymidine kinase gene, retrovirus LTR regions, the promoter of the immunoglobulin gene, the promoter of the actin gene, the promoter of the EF-1alpha gene as well as inducible promoters in which the expression of the protein depends on the addition of a molecule or an exogenous signal, such as the tetracycline system, the NFκB/UV light system, the Cre/Lox system and the promoter of heat shock genes, the regulatable promoters of RNA polymerase II described in WO/2006/135436 as well as tissue-specific promoters.

The polynucleotides of the invention or the gene constructs forming them can form part of a vector. Thus, in another aspect, the invention relates to a vector comprising a polynucleotide or a gene construct of the invention. A person skilled in the art will understand that there is no limitation as regards the type of vector which can be used because said vector can be a cloning vector suitable for propagation and for obtaining the polynucleotides or suitable gene constructs or expression vectors in different heterologous organisms suitable for purifying the adaptor polypeptides. Thus, suitable vectors according to the present invention include expression vectors in prokaryotes such as pUC18, pUC19, Bluescript and their derivatives, mp18, mp19, pBR322, pMB9, CoIEl, pCRl, RP4, phages and shuttle vectors such as pSA3 and pAT28, expression vectors in yeasts such as vectors of the type of 2 micron plasmids, integration plasmids, YEP vectors, centromeric plasmids and the like, expression vectors in insect cells such as the pAC series and pVL series vectors, expression vectors in plants such as vectors of expression in plants such as pIBI, pEarleyGate, pAVA, pCAMBIA, pGSA, pGWB, pMDC, pMY, pORE series vectors and the like and expression vectors in superior eukaryotic cells based on viral vectors (adenoviruses, viruses associated to adenoviruses as well as retroviruses and lentiviruses) as well as non-viral vectors such as pSilencer 4.1-CMV (Ambion), pcDNA3, pcDNA3.1/hyg pHCMV/Zeo, pCR3.1, pEFl/His, pIND/GS, pRc/HCMV2, pSV40/Zeo2, pTRACER-HCMV, pUB6/V5-His, pVAXl, pZeoSV2, pCI, pSVL and pKSV-10, pBPV-1, pML2d and pTDTl.

The vector of the invention can be used to transform, transfect or infect cells which can be transformed, transfected or infected by said vector. Said cells can be prokaryotic or eukaryotic. By way of example, the vector wherein said DNA sequence is introduced

can be a plasmid or a vector which, when it is introduced in a host cell, is integrated in the genome of said cell and replicates together with the chromosome (or chromosomes) in which it has been integrated. Said vector can be obtained by conventional methods known by the persons skilled in the art (Sambrook et al., 2001, *supra*.).

Therefore, in another aspect, the invention relates to a cell comprising a polynucleotide, a gene construct or a vector of the invention. The cell is typically obtained by transformation, transfection or infection with a construct or vector provided by this invention. The transformed, transfected or infected cells can be obtained by conventional methods known by persons skilled in the art (Sambrook et al., 2001, *supra*.). In a particular embodiment, said host cell is an animal cell transfected or infected with a suitable vector.

Host cells suitable for the expression of the adaptor polypeptides of the invention include, without being limited, mammal, plant, insect, fungal and bacterial cells. Bacterial cells include, without being limited to, Gram-positive bacterial cells such as species of the Bacillus, Streptomyces and Staphylococcus genus and Gram-negative bacterial cells such as cells of the Escherichia and Pseudomonas genus. Fungal cells preferably include cells of yeasts such as Saccharomyces, Pichia pastoris and Hansenula polymorpha. Insect cells include, without being limited to, Drosophila cells and Sf9 cells. Plant cells include, among others, cells of crop plants such as cereals, medicinal, ornamental or bulbous plants.

In an embodiment, the cell comprising the polypeptide, polynucleotide of the invention, the gene construct of the invention or the vector of the invention is a human cell. Suitable human cells in the present invention include epithelial cell lines, osteosarcoma cell lines, neuroblastoma cell lines (human, etc.), epithelial carcinomas (human, etc.), glial cells (murine, etc.), hepatic cell lines (from monkey, etc.), COS cells, BHK cells, HeLa cells, 911, AT1080, A549, 293 or PER.C6, NTERA-2 human ECC cells, D3 cells of the mESC line, human stem cells such as HS293 and BGV01, SHEF1, SHEF2 and HS181, NIH3T3 cells, 293T, REH and MCF-7 and hMSC cells.

The adaptor polypeptide of the invention may be obtained by recombinant expression in a suitable host. For this purpose, a polynucleotide of the invention is introduced in a vector suitable for its expression in a heterologous organism together with transcription and, optionally, translation control elements. The transcription and, optionally, translation control elements present in the expression cassette of the invention include promoters, which direct the transcription of the nucleotide sequence to which they are operatively linked and other sequences which are necessary or suitable for the transcription and its suitable regulation in time and place, for example, initiation and termination signals, cleavage sites, polyadenylation signal, replication origin, transcriptional enhancers, transcriptional silencers, etc. Said elements, as well as the vectors used for constructing the expression cassettes and the recombinant vectors according to the invention are generally chosen according to the host cells to be used.

Thus, in another aspect, the invention relates to a method of obtaining a polypeptide of the invention, the method comprising:

a) culturing a host cell of the invention under conditions that allow expression of the polypeptide; and
b) isolating the polypeptide from said culture of host cells.

In an embodiment, the host cell wherein expression is carried out is a human cell. Suitable human cells for producing the polypeptide of the invention include, without limitation, any of the cell lines defined above in connection with the cells of the invention. Different methods for isolating the peptide are known by the person skilled in the art.

Compositions of the invention.

The adaptor polypeptide of the invention may be contacted with an adenovirus thus resulting in the formation of a binary complex by way of the interaction between the CAR region in the adaptor polypeptide and the adenoviral fiber protein. Thus, in another aspect, the invention relates to a composition or complex comprising:

(a) an adaptor polypeptide of the invention and

(b) an adenovirus comprising a transgene nucleotide sequence encoding a product of interest.

The term "composition", as used herein, relates to any composition of matter comprising the components of the invention, i.e., the adaptor polypeptide of the invention and the adenovirus encoding a therapeutic protein or a reporter protein. It will be understood that the composition may be formulated as a single component or, alternatively, it can be provided as separated formulations which may then combined for their joint administration. The compositions of the invention may also be provided as a kit-of-parts wherein each of the components is separately formulated but packaged in a single container. The molar ratio of the components forming the compositions of the invention may vary but preferably includes ratios of the two components being between 50:1 and 1:50, more in particular between 20:1 and 1:20, between 1:10 and 10:1, o between 5:1 and 1:5.

The term "complex", as used herein, relates to compositions of matter wherein one or more adenoviral particles encoding a product of interest are bound by one or more molecules of the adaptor molecules of the invention via the specific interaction between the CAR domain in the adaptor molecule and the adenoviral fiber protein. It will be understood that the stoichiometry of the complex will depend on the number of fiber proteins available on the adenoviral capsid which may simultaneously bind the dimeric adaptor protein. The adenoviral capsid is an assembly of seven polypeptides, organized into an ~900 Å-diameter icosahedral shell.

Twelve trimers of hexon, the major capsid component, are arranged onto each of 20 interlocking triangular facets, with penton capsomeres and their protruding fibers occupying each of the 12 vertex positions. Thus, since the adenovirus comprises 12 fibers, the complex of the invention may comprise at the most 12 adaptor molecules bound simultaneously to each adenoviral particle. Thus, preferably, the stoichiometry of the complex of the invention is 12 adaptor molecules per adenoviral particles, although stoichiometries of 11:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1 and 1:1 are also possible and contemplated by the present invention.

The domains (a, b and c) of the polypeptide of the invention have been described in detail in the context of the polypeptide of the invention.

The second component of the composition or complex of the invention is an adenovirus encoding a product of interest. As used herein, the term "adenovirus" or "adenoviral particle" is used to include any virus that can be categorized as an adenovirus, including any adenovirus that infects a human or an animal, including all groups, subgroups, and serotypes and that uses CAR as receptor for infection of target cells. There are at least 51 serotypes of Adenovirus that classified into several subgroups. For example, subgroup A includes adenovirus serotypes 12, 18, and 31. Subgroup C includes adenovirus serotypes 1, 2, 5, and 6. Subgroup D includes adenovirus serotype 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, and 42-49. Subgroup E includes adenovirus serotype 4. Subgroup F includes adenovirus serotypes 40 and 41. These latter two serotypes have a long and a short fiber protein. Thus, as used herein an adenovirus or adenovirus particle is a packaged vector or genome. Moreover, the term "adenovirus" and "adenovirus particle" also refer to derivatives thereof containing one or more modifications with respect to the wild-type. Such modifications include, but are not limited to, modifications to the adenovirus genome that is packaged in the particle in order to make an infectious virus. Exemplary modifications include deletions known in the art, such as deletions in one or more of the E1a, E1b, E2a, E2b, E3, or E4 coding regions. Other exemplary modifications include deletions of all of the coding regions of the adenoviral genome. Such adenoviruses are known as "gutless" adenoviruses. The terms also include replication-conditional adenoviruses, which are viruses that preferentially replicate in certain types of cells or tissues but to a lesser degree or not at all in other types. For example, among the adenoviral particles provided herein, are adenoviral particles that replicate in abnormally proliferating tissue, such as solid tumors and other neoplasms. These include the viruses disclosed in U.S. Pat. No. 5,998,205 and U.S. Pat. No. 5,801,029. Such viruses are sometimes referred to as "cytolytic" or "cytopathic" viruses (or vectors), and, if they have such an effect on neoplastic cells, are referred to as "oncolytic" viruses (or vectors).

The adenoviruses forming part of the compositions or complex of the invention comprise a polynucleotide sequence encoding a product of interest.

The "product of interest" can be virtually any protein or peptide of interest, e.g., a reporter protein or peptide (such as β-gal, a fluorescent protein such as GFP, YFP, etc.); or a peptide, a protein, or an antibody (or a functional fragment thereof) with therapeutic or diagnostic applications; or any recombinant protein or peptide of interest. As used herein, the term "product of interest" further includes "recombinant", i.e., it does not appear naturally.

In an embodiment, the product of interest is a hepatoprotective secretable factor. The term "hepatoprotective secretable factor" refers to any factor that is secretable (i.e. that after being synthesized in a cell is released from this cell to the extracellular space) and has a protective effect of the liver cells (e.g. hepatocytes, cholangiocytes, progenitor cells, Kupffer cells, endothelial cells, etc), affecting positively the survival and function of said cells. The hepatoprotective factor may be secretable either because it corresponds to a polypeptide which is naturally secreted or because it corresponds to a polypeptide that has been modified so as to incorporate a signal sequence which is capable of mediating the transfer of the nascent polypeptide chain into the secretory pathway and its subsequent secretion into the medium.

Suitable signal sequences which can be used for promoting the secretion of a given polypeptide include, without limitation, the signal sequence of a secreted protein such as insulin, GLP-1, a cytokine, a clotting factor, an immunoglobulin, a secretory enzyme or a hormone (including the pituitary adenylate cyclase activating polypeptide (PACAP)/glucagon superfamily) and a serum protein such as the tissue-type plasminogen activator.

Products of interest and, in particular, hepatoprotective secretable factors include, without limitation, erythropoietin (EPO), leptins, adrenocorticotropin-releasing hormone (CRH), somatotropic hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), prolactin-releasing

hormone (PRH), melatonin-releasing hormone (MRH), prolactin-inhibiting hormone (PIH), somatostatin, adrenocorticotropin hormone (ACTH), somatotropic hormone or growth hormone (GH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyrotropin (TSH or thyroid-stimulating hormone), prolactin, oxytocin, antidiuretic hormone (ADH or vasopressin), melatonin, Müllerian inhibiting factor, calcitonin, parathyroid hormone, gastrin, cholecystokinin (CCK), Arg-vasopressin, thyroid hormones, azoxymethane, triiodothyronine, LIF, amphiregulin, soluble thrombomodulin, SCF, osteogenic protein 1, BMPs, MGF, MGSA, heregulins, melanotropin, secretin, insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), atrial natriuretic peptide (ANP), human chorionic gonadotropin (hCG), insulin, glucagon, somatostatin, pancreatic polypeptide (PP), leptin, neuropeptide Y, renin, angiotensin I, angiotensin II, factor VIII, factor IX, tissue factor, factor VII, factor X, thrombin, factor V, factor XI, factor XIII, interleukin 1 (IL-1), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 6 (IL-6), interleukin 7 (IL-7), interleukin 8 (IL-8), interleukin 9 (IL-9), interleukin 10 (IL-10), interleukin 11 (IL-11), interleukin 12 (IL-12), interleukin 13 (IL-13), interleukin 14 (IL-14), interleukin 15 (IL-15) interleukin 16 (IL-16), interleukin 24 (IL-24), tumor necrosis factor alpha (TNF-α), interferons alpha, beta, gamma, CD3, CD134, CD137, ICAM-1, LFA-1, LFA-3, chemokines including RANTES 1α, MIP-1α, MIP-1β, nerve growth factor (NGF), WT1 protein encoded by the Wilms' tumor suppressor gene, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-beta), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGF and KGF), epidermal growth factor (EGF and related factors), vascular endothelial growth factor (VEGF), granulocyte colony-stimulating factor (GM-CSF), glial growth factor, keratinocyte growth factor, endothelial growth factor, glial-cell line-derived, neurotrophic factor (GDNF), alpha 1-antitrypsin, tumor necrosis factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), cardiotrophin-1 (CT-1), oncostatin M (OSM), serpin (A1, A2, A3, A4, A5, A6, A7, A8, A9, A10, A11, A12, A13, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, B11, B12, B13, C1, D1, E1, E2, F1, F2, G1, H1, I1 and I2), cyclosporine, fibrinogen, the EDA domain of fibronectin, lactoferrin, tissue-type plasminogen activator (tPA), chymotrypsin, immunoglobins, hirudin, superoxide dismutase, imiglucerase, β-Glucocerebrosidase, alglucosidase-α, α-L-iduronidase, iduronate-2-

sulfatase, galsulfase, human α-galactosidase A, α-1 proteinase inhibitor, lactase, pancreatic enzymes (lipase, amylase, protease), adenosine deaminase, immunoglobulins, albumin, Botulinum toxins type A and B, collagenase, human deoxyribonuclease I, hyaluronidase, papain, L-asparaginase, lepirudin, streptokinase, porphobilinogen deaminase (PBGD), cell transforming factor beta (TGF-β) inhibitor peptides, IL10 inhibitors, FoxP3 inhibitors, TNFα inhibitors, VEGF inhibitors, PD-1 inhibitors and CD152 inhibitors, cardiotrophin-1 (CT1), oncostatin M (OSM), interferon alpha (e.g., IFNa5), endothelial cell protein C/activated protein C receptor (EPCR). Moreover, the product of interest can be an antibody (or functional fragments thereof) of interest or of therapeutic or diagnostic application, etc. Therefore, the polynucleotide comprising the nucleotide sequence encoding the product of interest present in the viral vector of the invention comprises the sequence encoding the product of interest.

In an embodiment the product of interest is IGF-I or a functional variant thereof. Insulin-like growth factor 1 (IGF-I) also known as somatomedin C or mechano growth factor, as used in the present invention, refers to any of the isoforms which are encoded by the human IGF-I gene including, the isoform 4 preprotein (NP_000609.1), the isoform 1 preprotein (NP_001104753.1), the isoform 2 preprotein (NP_001104754.1), the isoform 3 preprotein (NP_001104755.1) as well to orthologues thereof from other species like chimpanzee (XP_001156521.1, XP_001156403.1, XP_001156345.1, XP_001156459.1) dog (XP_853117.1, XP_866935.1, XP_866946.1), mouse (NP_034642.1, NP_908941.1), rat (NP_849197.1) and the like. However, in an embodiment, the product of interest is the human IGF-I.

The term "functional variant thereof" as used herein, relates to any polypeptide derived from IGF-I by insertion, deletion or substitution of one or more residues and which maintains substantially the ability to interact with and signal by the IGF1R. Suitable functional variants are those showing a degree of identity with respect to the IGF-I of about greater than 25% amino acid sequence identity, such as 25% 40%, 60%, 70%, 80%, 90% or 95%. The degree of identity between two polypeptides is determined using computer algorithms and methods that are widely known for the persons skilled in

the art. The identity between two amino acid sequences is preferably determined by using the BLASTP algorithm as described previously [BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894, Altschul, S., et al., *J. Mol. Biol.* 1990;215: 403-410].

Different assays are available in the art for determining whether a given polypeptide can be seen as functionally equivalent variant of IGF-I. By way of an example, it is possible to test whether a polypeptide is capable of specifically binding to the IGF-I receptor using standard receptor-binding assay or radioligand binding assay (Ballard J. et al. *Biochemical and Biophysical Research Communications* 1987;149(2):398-404). It is also possible to test a putative functionally equivalent variant of IGF-I by measuring the phosphorylation of the different IGF1R substrates after the stimulation with the suspected functionally equivalent variant of IGF-I. Suitable substrates that can be assayed include Shc (R. Gregg Richards, The *Journal of Biological Chemistry* 1998; 273:11962-11969), detection of phosphorylated Shc can be carried out using standard methods, including the use of antibodies specific towards the phosphorylated peptide. Moreover, it is possible to determine whether a polypeptide is a functionally equivalent variant of IGF-I by detecting the effect of said polypeptide in a cell based system. Those assays are based on the different effects induced by the binding of IGF-I to IGF1R in different cells. For instance, it is possible to detect IGF-I – mediated stimulation of proteoglycan synthesis in articular cartilage as described by Starkman B. G. et al. (*Biochem. J.* 2005; 389:723-729) or the IGF-I-mediated PTEN suppression and enhanced cell invasion and proliferation via activation of the IGF-I/PI3K/Akt signaling pathway in pancreatic cancer cells (Ma J. et al. *Journal of Surgical Research* 2010; 160:90-101).

The polynucleotide encoding the product of interest may be operatively coupled to a transcriptional regulatory element. In an embodiment, said transcriptional regulatory element is a promoter specific for EPC. Suitable EPC-specific promoters include, without limitation, the IdI promoter, the VEGFR2 promoter or the VE-cadherin promoter.

Method for obtaining a cell expressing a product of interest

The authors of the present invention have observed that the adaptor polypeptide of the invention allows the efficient adenoviral transduction of EPC cells by adenovirus with a lower MOI and higher survival cell rate than when the transduction is carried out in the absence of the adaptor polypeptide (see example 1 of the present invention).

Thus, in another aspect, the invention relates to a method of obtaining a cell expressing a product of interest (hereinafter first method of the invention), comprising the steps of

(i) contacting a cell with a polypeptide of the invention, and an adenovirus encoding a product of interest (comprising a polynucleotide sequence encoding the product of interest), wherein said contacting can be carried out by separately adding the polypeptide of the invention and the adenovirus encoding a product of interest or by adding a preformed polypeptide–adenovirus complex,

(ii) maintaining the mixture obtained in step (i) under conditions adequate for the formation of a ternary complex between said polypeptide, said adenovirus and said cell and

(iii) maintaining the cells under conditions adequate for the expression of the therapeutic protein.

In the first step of the method of the invention, a cell is contacted with a polypeptide of the invention and an adenovirus encoding a product of interest. The cell used in the first method of the invention can be any eukaryotic cell. In an embodiment, the cell is a human cell. In another embodiment, the cell is an endothelial progenitor cell (EPC).

The term “endothelial progenitor cell (EPC)”, as used herein, shall be construed in its broadest context to mean a cell that, when functional or normal, is capable of proliferating to form blood vessels e.g., in the formation of a vascular network or in vascular repair, such as by angiogenesis and/or neovascularisation. EPCs may be functional, nonfunctional, or have impaired function (for example with respect to this proliferative ability and developmental capability), and non-functional EPCs or EPCs

having impaired function may be identified readily (for example by their aberrant morphology and impaired proliferative ability). These cells are typically circulating, bone marrow-derived cell population and appear to participate in both vasculogenesis and vascular homeostasis. This progenitor (stem) cell population were first described as CD34+/CD133+ cells in the bone marrow by Asahara et al. in 1997 (Science Vol. 275(30):964-967), but can be isolated from the peripheral blood mononuclear cell (PBMC). Recently, several subpopulations of EPCs have been identified (Shantsila et al. J Am Coll Cardiol, 2008;51:669-671) by means of the cell surface antigen expression such as CD14, CD45, KDR and CD34. There are two subpopulations of EPCs: the so-called "early" and "late" EPCs according to the timing of their emergence during culture. The early EPCs form the more conventional CFU-ECs (cells grow endothelial cell colony-forming units), and late EPCs are thought to represent those "outgrowth" endothelial cells (OECs) as described by Sieveking et al. (Sieveking DP et al. *J. Am. Coll. Cardiol.* 2008;51:660-668)

EPC may be obtained from human mononuclear cells obtained from peripheral blood or bone marrow of the patient before treatment. EPC may also be obtained from heterologous or autologous umbilical cord blood. Peripheral blood is preferred due to convenience. The leukocyte fraction of peripheral blood is most preferred. Several approaches have been used for the isolation of EPC, including density centrifugation and magnetic bead selection (Methods in Molecular Medicine vol. 139 2008. Human Press). Other methods for isolating EPC are based in the combined use of antibodies that recognize EPC specific antigens on immature human hematopoietic progenitor cells (HSCs) and FACS techniques. For example, CD34 is commonly shared by EPC and HSCs. CD34 is expressed by all HSCs but is lost by hematopoietic cells as they differentiate. For example, the “early” EPCs populations are CD14+/CD34–/CD45/CD133/KDR low expression and the “late” EPCs are CD34+/KDR+.

The isolation of EPC can be done using methodology present in the art such as the isolation of EPC from human peripheral blood by magnetic bead selection on the basis of said cell surface antigen expression (Asahara et al. *Science* 1997; 275 (5302):964-966). Other population called “Activated EPCs” can be isolated as described on

WO2009075565.

To obtain the EPC progenitors from peripheral blood about 5 ml to about 500 ml of blood is taken from the patient. Preferably, about 50 ml to about 200 ml of blood is taken. EPC can be expanded *in vivo* by administration of recruitment growth factors, e.g., GM-CSF and IL-3, to the patient prior to removing the progenitor cells. Optionally, the cells may be expanded *ex vivo* using, for example, the method disclosed by U.S. Pat. No. 5,541,103.

The process of contacting said cell with the polypeptide of the invention and the adenovirus can be carried out by separately adding the polypeptide of the invention and the adenovirus encoding a product of interest or by adding a preformed polypeptide–adenovirus complex.

In a second step, the mixture obtained in step (i) is maintained under conditions adequate for the formation of a ternary complex between said polypeptide, said adenovirus and said cell.

The "adequate conditions" are those known by the person skilled in the art that allows the formation of a ternary complex between the peptide of the invention, the adenovirus comprising a transgene nucleotide sequence encoding a product of interest and the cell such as temperature, pH, etc.

In a further step, the cells are maintained under conditions adequate for the expression of the product of interest. In an embodiment, the product of interest is a hepatoprotective secretable factor. In another embodiment, the product of interest is IGF-I or a functionally equivalent variant thereof.

In a particular embodiment, the first method of the invention further comprises the isolation of the product expressing cell. The cells can be isolated using standard procedures based on the presence of cell surface markers specific for EPC cells as it was described previously.

Thus, in another aspect, the invention relates to a cell expressing a product of interest (hereinafter "first cell of the invention") obtained by this first method of the invention.

In an embodiment, the product of interest is a hepatoprotective secretable factor. In another embodiment, the hepatoprotective secretable factor is IGF-I or a functionally equivalent variant thereof.

The terms "hepatoprotective secretable factor", "IGF-I" and "functionally equivalent variant of IGF-I" have been described in detail above and are used with the same meaning in the method of obtaining a cell expressing a product of interest.

Nevertheless, as will be understood by the person skilled in the art, the first cells of the invention can be substantially purified or can be forming part of a composition that has, preferably more than 20% of first cells of the invention, more preferably more that 30%, 40%, 50%, 60%, 70%, 80%, 90%, and more preferably more than 99% of first cells of the invention. The first cells of the invention can be present in the composition together with other cell types, cells that do not express the product of interest, etc.

Modified EPCs of the invention

The authors of the present invention have also been able to obtain endothelial precursor cells (EPCs) expressing IGF-1. While the cells have been obtained using the adaptor polypeptide of the invention, the contribution of the inventors is not limited to cells obtained using the adaptor polypeptide but comprises cells obtained by any method known in the art for the insertion of exogenous DNA in a cell of interest.

Thus, in another aspect, the invention relates to an EPC expressing IGF-1 or a functional variant thereof, hereinafter called "EPCs of the invention" or "second cell of the invention".

The term "EPC" has been described in detail above and applies equally to the modified

EPCs of the invention. The EPC of the invention has been modified so as to incorporate an exogenous nucleic acid encoding IGF-I or a functionally equivalent variant thereof.

The term "exogenous nucleic acid encoding IGF-I" or "exogenous polynucleotide encoding IGF-I" in the present invention refers to any sequence that encodes IGF-I that is not the sequence naturally present in the EPC genome.

Any method known in the art for introducing the IGF-I coding sequence in the EPC can be used to produce the cells of the invention. Examples of said methods are, without limitation, chemical-based methods (highly branched organic compounds, so-called dendrimers, liposomes, cationic polymers such as DEAE-dextran or polyethylenimine, etc), non chemical methods (such as electroporation, Optical transfection, etc), particle-based methods (such as gene gun, Magnetofection or Magnet assisted transfection, etc) or viral methods (viral transduction such as the one described in the examples of this invention). In an embodiment, the EPCs cells of the invention which express IGF-I are obtained by transduction with adenoviral vectors encoding IGF-I using an adaptor protein comprising a CAR domain and a VEGFR ligand as defined above.

As will be understood by the person skilled in the art, the second cells of the invention can be substantially purified or can be forming part of a composition that has, preferably more than 20% of first cells of the invention, more preferably more that 30%, 40%, 50%, 60%, 70%, 80%, 90%, and more preferably more than 99% of first cells of the invention. The second cells of the invention can be present in the composition together with other cell types, cells that do not express the product of interest, etc.

<u>Pharmaceutical compositions comprising the composition and complex of the invention and therapeutic methods of the compositions and complexes of the invention</u>

The compositions or complexes of the invention comprising an adaptor polypeptide of the invention and an adenovirus encoding a product of interest can be used as pharmaceutical compositions wherein a disease is to be treated or prevented using the product of interest. Thus, in another aspect, the invention relates to a pharmaceutical

composition (hereinafter pharmaceutical composition of the invention) comprising a composition or complex of the invention and a pharmaceutically acceptable carrier. In yet another aspect, the invention relates to a polypeptide of the invention, or a composition or complex of the invention for use in medicine.

The terms "composition" and, "complex" have been described in detail above.

As used herein, the term "pharmaceutically acceptable carrier" means a non- toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Remington's Pharmaceutical Sciences. Ed. by Gennaro, Mack Publishing, Easton, Pa., 1995 discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as lactose, glucose, and sucrose; starches such as com starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as TWEEN™ 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. If filtration or other terminal sterilization methods are not feasible, the formulations can be manufactured under aseptic conditions.

The carriers and the auxiliary substances necessary to manufacture the desired pharmaceutical dosage form of the pharmaceutical composition of the invention will depend, among others factors, on the pharmaceutical dosage form chosen. Said pharmaceutical dosage forms of the pharmaceutical composition will be manufactured

according to conventional methods known by the person skilled in the art. A review of different administration methods for active ingredients, excipients which are to be used and processes for producing them can be found in "Tratado de Farmacia Galénica", C. Faulí i Trillo, Luzán 5, S.A. de Ediciones, 1993. Examples of pharmaceutical compositions include any solid (tablets, pills, capsules, granulates, etc.) or liquid (solutions, suspensions or emulsions) composition for oral, topical or parenteral administration. Furthermore, the pharmaceutical composition can contain, as appropriate, stabilizers, suspensions, preservatives, surfactants and the like.

Under certain conditions, it may be preferable to provide the complex or composition of the invention as a controlled release formulation. The term "controlled release" (and variants of that term) as used herein (e.g., in the context of "controlled-release system") is generally meant to encompass release of a substance (e.g., a drug or a protein) at a selected site or otherwise controllable in rate, interval, and/or amount. Controlled release encompasses, but is not necessarily limited to, substantially continuous delivery, patterned delivery (e.g., intermittent delivery over a period of time that is interrupted by regular or irregular time intervals), and delivery of a bolus of a selected substance (e.g., as a predetermined, discrete amount if a substance over a relatively short period of time (e.g., a few seconds or minutes).

The complexes of the invention can be used for direct administration to a subject in need thereof whereby the subject's own EPC will be transduced *in vivo* by the adenoviral particles.

In another aspect, the invention relates to a composition or complex of the invention, wherein the product of interest of the adenovirus of the composition or complex is IGF-I or a functionally equivalent variant thereof for use in the prevention or the treatment of a fibrosis-associated disease.

In another aspect, the invention relates to the use of a composition or complex of the invention, wherein the product of interest of the adenovirus of the composition or complex is IGF-I or a functionally equivalent variant thereof for the manufacture of a

medicament for the prevention or the treatment of a fibrosis-associated disease.

In another aspect, the invention relates to a method of treatment of a fibrosis-associated disease in a subject which comprises the administration to said subject of a composition or complex of the invention wherein the product of interest of the adenovirus of the composition or complex is IGF-I or a functionally equivalent variant thereof.

The term “Fibrosis-associated disease”, as used herein, includes, without limitation any disease caused by fibrosis in any organ due to pathological conditions or diseases, fibrosis due to physical trauma ('traumatic fibrosis'), fibrosis due to radiation damage, and fibrosis due to exposure to chemotherapeutics. Fibrosis is defined as a pathological lesion made of scaring tissue including fibrillar proteins or glycoproteins (collagens, proteoglycans and the like...). Typically, fibrosis includes (i) fibrosis associated to organ or tissue function loss, e.g., pulmonary fibrosis, liver fibrosis (cirrhosis), renal fibrosis, corneal fibrosis, etc.; and (ii) surgical and/or aesthetic complications, e.g., fibrosis associated to skin and peritoneal surgery, fibrosis associated with burns, osteoarticular fibrosis, keloids, etc.

As used herein, the term "organ fibrosis" includes but is not limited to liver fibrosis, fibrosis of the kidneys, fibrosis of lung, and fibrosis of the intestine As used herein, "liver fibrosis" includes liver fibrosis due to any cause, including but not limited to virally-induced liver fibrosis such as that due to hepatitis B or C virus; exposure to alcohol (alcoholic liver disease), certain pharmaceutical compounds including but not limited to methotrexate, some chemotherapeutic agents, and chronic ingestion of arsenicals or vitamin A in megadoses, oxidative stress, cancer radiation therapy or certain industrial chemicals including but not limited to carbon tetrachloride and dimethylnitrosamine; and diseases such as primary biliary cirrhosis, primary sclerosing colangitis, fatty liver, obesity, non-alcoholic steatohepatitis, cystic fibrosis, hemochromatosis, auto-immune hepatitis, and steatohepatitis. In an embodiment, the fibrosis-associated disease is cirrhosis.

Cirrhosis is a consequence of chronic liver disease characterized by replacement of liver

tissue by fibrosis, scar tissue and regenerative nodules (lumps that occur as a result of a process in which damaged tissue is regenerated), leading to loss of liver function. Cirrhosis is most commonly caused by alcoholism, hepatitis B and C, and fatty liver disease but has many other possible causes. Some cases are idiopathic, i.e., of unknown cause.

The composition or complex of the invention can be administered to a patient by any means known in the art including oral and parenteral routes. According to such embodiments, inventive compositions may be administered by injection (e.g., intravenous, subcutaneous or intramuscular, intraperitoneal injection), rectally, vaginally, topically (as by powders, creams, ointments, or drops), or by inhalation (as by sprays).

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. In one embodiment, the inventive conjugate is suspended in a carrier fluid comprising 1 % (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) TWEEN™ 80. The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

The composition or complex of the invention may be encapsulated within biodegradable polymeric microspheres or liposomes. Examples of natural and synthetic polymers useful in the preparation of biodegradable microspheres include carbohydrates such as

alginate, cellulose, polyhydroxyalkanoates, polyamides, polyphosphazenes, polypropylfumarates, polyethers, polyacetals, polycyanoacry lates, biodegradable polyurethanes, polycarbonates, polyanhydrides, polyhydroxyacids, poly(ortho esters), and other biodegradable polyesters. Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides.

Pharmaceutical compositions for oral administration can be liquid or solid. Liquid dosage forms suitable for oral administration of inventive compositions include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to an encapsulated or unencapsulated composition or complex of the invention, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Pharmaceutical compositions comprising the cells of the invention and therapeutic methods using the cells of the invention

As mentioned before, the first and second cells of the invention modified according to the present invention can exert a therapeutic effect resulting from the expression of the product of interest when administered to a subject in need thereof. In particular, the authors of the present invention have obtained modified EPCs by *ex vivo* transduction with adenoviral vectors using the adaptor protein of the invention (see example 1). Said example shows that the adaptor of the invention allows efficient EPC transduction at low MOI with excellent cell viability, thus permitting the use of engineered cells for therapeutic purposes. Moreover, the authors of the present invention have observed that EPCs modified so that they express a product of interest (IGF-I) can be administered to

a subject resulting in a therapeutic effect derived from the expression of the product of interest which is higher than that observed when unmodified EPC are administered (see the example of the present invention).

In this way, the invention provides therapeutic compositions which combine the effects of cell therapy (resulting from the effect of the EPC on the liver cells) and gene therapy (by the effect of IGF-1 produced by the EPC). Thus, in another aspect, the invention relates to a first cell of the invention wherein the product of interest encoded by exogenous polynucleotide sequence of the cell is IGF-I or a functionally equivalent variant thereof or to a second cell of the invention for use in medicine. Moreover, the invention relates to a first cell of the invention wherein the product of interest encoded by exogenous polynucleotide sequence of the cell is IGF-I or a functionally equivalent variant thereof or to a second cell of the invention for use in the prevention or the treatment of a fibrosis-associated disease.

In another aspect, the invention relates to the use of a first cell of the invention, wherein the product of interest encoded by exogenous polynucleotide sequence of the cell is IGF-I or a functionally equivalent variant thereof or to the use of a second cell of the invention for the manufacture of a medicament for the prevention or the treatment of a fibrosis-associated disease.

In another aspect, the invention relates to a method of treatment of a fibrosis-associated disease in a subject which comprises the administration to said subject of a first cell of the invention wherein the product of interest encoded by exogenous polynucleotide sequence of the cell is IGF-I or a functionally equivalent variant thereof or of a second cell of the invention.

The term “fibrosis-associated disease” has been described in detail above in the context of the pharmaceutical compositions comprising the compositions and complexes of the invention and are to be used equally in the present case. In an embodiment, the fibrosis associated disease is cirrhosis.

The cells used in the cell therapy can be autologous or heterologous. As used herein, the term "autologous" means that the cells are from the same individual which is then treated. As used herein, the term "heterologous" means that the cells derive from an individual which is different to the individual which is then treated. When the modified cells are to be administered to a patient, they are preferably isolated or derived from precursor cells from that patient (i.e., the EPC are administered to an autologous patient). However, the cells may be infused into HLA-matched allogeneic, or HLA-mismatched allogenic patients. In the latter case, immunosuppressive drugs may be administered to the recipient.

The cells may be administered in any suitable manner, preferably with a pharmaceutically acceptable carrier (e.g., saline). Usually administration will be intravenous, but intra-articular, intramuscular, intradermal, intraperitoneal, and subcutaneous routes are also acceptable. Administration may be repeated at time intervals. Infusions of first or second cells of the invention may be combined with administration of growth factors that act to maintain cells number and activity. The cell may also be administrated included in microparticles, microcapsules or microspheres.

The term "microcapsules" refers to vesicular systems in which the first or second cells of the invention are confined in a cavity surrounded by a single (usually polymeric) membrane. The term "microspheres" refers to matrix systems in which the cells are dispersed over the entire particle.

The term "microparticle" relates to a spherical or non-spherical particle, inside which there are microcapsules or microspheres. In the present invention, "microparticle" is understood as that particle comprising a diameter less than 1 mm, preferably between 1 and 0.9, between 0.9 and 0.8, between 0.8 and 0.7, between 0.7 and 0.6, between 0.6 and 0.5, between 0.5 and 0.4, between 0.4 and 0.3, between 0.3 and 0.2, between 0.2 and 0.1 or less than 0.1 mm in diameter. In a particular embodiment, the microparticle of the invention has a diameter between 0.380 and 0.404 mm, preferably, 0.392 mm.

Nevertheless, as will be understood by the person skilled in the art, the average size of

the microparticle is affected by different technological factors of the process for producing said microparticle, such as the concentration of the different components of the microparticle, stirring speed, etc.

The microcapsules, microspheres and microparticles can be formed by any biocompatible polymeric material allowing the continuous secretion of the therapeutic products and acting as a support of the first and second cell of the invention. Thus, said biocompatible polymeric material can be, for example, thermoplastic polymers or hydrogel polymers.

The thermoplastic polymers include acrylic acid, acrylamide, 2-aminoethyl methacrylate, poly(tetrafluoroethylene-co-hexafluoropropylene), methacrylic acid-(7-coumaroxy)ethyl ester, N-isopropylacrylamide, polyacrylic acid, polyacrylamide, polyamidoamine, poly(amino)-p-xylylene, poly(chloroethyl vinyl ether), polycaprolactone, poly(caprolactone-co-trimethylene carbonate), poly(carbonate-urea)urethane, poly(carbonate)urethane, polyethylene, polyethylene and acrylamide copolymer, polyethylene glycol, polyethylene glycol methacrylate, poly(ethylene terephthalate), poly(4-hydroxybutyl acrylate), poly(hydroxyethyl methacrylate), poly(N-2-hydroxypropyl methacrylate), poly(lactic acid-glycolic acid), poly(L-lactic acid), poly(gamma-methyl, L-glutamate), poly(methylmethacrylate), poly(propylene fumarate), poly(propylene oxide), polypyrrole, polystyrene, poly(tetrafluoroethylene), polyurethane, polyvinyl alcohol, ultra high molecular weight polyethylene, 6-(p-vinylbenzamido)-hexanoic acid and N-p-vinylbenzyl-D-maltonamide and copolymers containing more than one of said polymers.

The hydrogel type polymers include natural materials of the type of alginate, agarose, collagen, starch, hyaluronic acid, bovine serum albumin, cellulose and derivatives thereof, pectin, chondroitin sulfate, fibrin and fibroin, as well as synthetic hydrogels such as sepharose and sephadex.

In principle, the number of cells which must form part of the microparticle is not essential for the invention provided that there are a number of cells sufficient so that a

beneficial therapeutic response in the patient over time can be measured. Thus, the amount of cells for each mL of polymer solution is between 1 and 10 $x10^6$, preferably between 2 and 9 $x10^6$, more preferably between 3 and 8 $x10^6$, still more preferably between 4 and 7 $x10^6$ and still more preferably between 5 and 6 x 10^6. The number of cells in the initial mixture is preferably 5; 3.75; 2.5 or 1.25 $x10^6$ for each mL of polymer solution.

The dose administered to a patient should be sufficient to reach a sufficiently high concentration of cells in the target region of the body of the patient and sufficient to have a generation of the product of interest so that a beneficial therapeutic response in the patient over time can be measured. For example, in the case that the cells are EPC, the product of interest if IGF-I and the disease is cirrhosis, then suitable cell dosages are typically, between $0,625x10^6$ EPC/Kg and $1,25x10^6$ EPC/Kg or more are administered. The cells can be administered one or more times to a patient to impart beneficial results. One skilled in the art will be able to determine the appropriate timing for administering the cells. The timing of the first and/or subsequent dose(s) of the cells can depend on a variety of factors, including, but not limited to a patient's health, stability, age, and weight. The cells can be administered at any appropriate time interval; for example, including but not limited to, once per week, once every two weeks, once every three weeks, once per month. Appropriate dosages of the cells also depend on a variety of factors, including, but not limited to, a patient's health, stability, age, and weight. Once a sufficient level of expression of the product of interest has been achieved to achieve clinical benefit, maintenance administrations may be required, but can generally be given on a less frequent basis (e.g., monthly or semi-annually).

The cells, especially the EPC used for cell therapy are preferably formulated so that they can be used as an off-the-shelf pharmaceutical. In this case, there may be a histocompatibility mismatch between the cells in the preparation and the patient being treated which may dampen the effect of the cell therapy by promoting premature elimination of the cells (especially after multiple administration). In this context, it may be advantageous to use a cell preparation in which at least some of the HLA Class I alleles on the cells (especially at the A locus and, more in particular, the A2 allele) are

shared with the patient. In this way, at least some of the tumor target antigen will be presented in autologous Class I molecules, enhancing the anti-tumor response and diminishing the allo response.

Partial match can be achieved simply by providing a cell therapy made of a mixture of cells bearing two or more of the common HLA-A allotypes (HLA-A2, A1, A19, A3, A9, and A24). Complete match for most patients can be achieved by providing the clinician with a battery of different cells from which to select, each possibly bearing only a single allotype at the HLA-A locus. Treatment would involve identifying one or more HLA allotype(s) in the patient by standard tissue typing, and then treating the patient with cells having HLA allotype(s) that match those of the patient. For example, a patient that was HLA-A2 and A19 could be treated with either HLA-A2 or HLA-A19 homozygous cells, or with a mixture of both.

In an embodiment, the cell used in the cell therapy is an EPC. In another embodiment, the cell is autologous to the subject to be treated. In an embodiment, the fibrosis-associated disease is cirrhosis. In another embodiment, the product of interest is a hepatoprotective secretable factor. In another embodiment, the hepatoprotective secretable factor is IGF-I or a variant thereof.

EXAMPLES

MATERIALS AND METHODS

Animals and experimental design.

Cirrhosis was induced in male Sprague-Dawley rats (Harlan Laboratories) by intraperitoneal administration of thioacetamide (TAA) (200 mg/kg) (Sigma Aldrich, St. Louis, MO, USA) in saline three times a week for 7 weeks or by intragastric administration of carbon tetrachloride (CCl_4) along with addition of phenobarbital (Kern Pharma) (35 mg/dl), following the protocol described by Runyon *et al.*, (*Gastroenterology* 1991; 100: 489-93), for 8 weeks. Control rats received vehicle alone.

At the end of the cirrhosis induction period, animals were divided into 4 groups (n=6) which received respectively saline, non-differentiated bone marrow mononuclear cells (MNC), EPC and EPC engineered to overexpress IGF-I (EPC-IGF). Each rat received 6 x 10^6 cells in 300 µL of saline via the tail vein one week after the last TAA or CCl_4 dose. One group of normal rats was used as healthy controls. The rats were given standard laboratory chow and free access to food and water. Animals were killed 6 days after cell transplantation, and liver and serum were collected.

All animals received human care, and study protocols complied with the institution's guidelines. An additional group of cirrhotic rats received DiL labeled EPC to analyze EPC recruitment to the damaged liver.

Cell lines.

HEK-293 (Human Embryo Kidney, ATCC-CRL1573) were cultures in DMEM supplemented with 10% con FBS, 2mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL, CA, US). 293 cells are stable transduced with the E1 region of the adenovirus and thus allows the replication of recombinant virus.

Adenoviral vector

Rat or murine IGF-I and GFP cDNAs were cloned into pTrack-CMV to construct pTrack-CMV-IGFICMV-GFP. The latter plasmid was linearized by *PmeI* and cotransformed into BJ5183 competent cells by electroporation with pAdEasy-1. The resultant recombinant plasmid was transfected into 293 cells with lipofectamine 2000 (Invitrogen Corporation, CA, USA) to generate adenoviral vector AdIGFI-GFP. Vectors were propagated in 293 cells and purified by centrifugation on cesium chloride by standard procedures (Graham and Van der Eb, *Virology* 1973;54:536-9). Titers of adenoviruses were determined by immunoassay with anti-hexon antibody to the 293 cells.

Production, purification and sequence of the adaptor proteins

A polynucleotide construct (SEQ ID NO: 19) encoding an adaptor polypeptide CAR-Fc-mVEGF$_{121}$ was constructed. The polynucleotide construct was composed of hCAR

signal sequence and ectodomain and mVEGF isoform 121 linked by the human IgG Fc [consisting of the hinge, CH2 and CH3]. A GGPGS region was added between Fc and $mVEGF_{121}$.

Another polynucleotide (SEQ ID NO: 21) encoding an adaptor polypeptide CAR-Fc-$hVEGF_{121}$ was also constructed. The polynucleotide construct was composed of hCAR signal sequence and ectodomain and hVEGF isoform 121 linked by the human IgG Fc [consisting of the hinge, CH2 and CH3] was constructed for future studies. An AAQPAEP region (SEQ ID NO: 16) was added between CAR domain and Fc fraction. Another AAA sequence was added between Fc and $hVEGF_{121}$.

Adaptor protein was produced in HEK-293 cells transduced with lentiviral vector carrying the cDNA of the protein. Purification was performed using chromatography with protein A column (GE Healthcare, NY, USA). Functional adaptor protein forms homodimers and interacts with the cellular receptors of VEGF.

EPC isolation, culture and characterization.

Bone marrow derived mononuclear cells (MNC) were isolated by ficoll-gradient (Lympholyte CL5045, Cederlane Laboratories, Burlington, NC, USA) from tibiae and femora of healthy Sprague-Dawley donor rats (200-250 g weight) or C57BL/6 mice and cultured on fibronectin coated-plates at 10^6 cells/cm^2 for 4 days as described (Griese et al., *Cardiovasc Res* 2003;58:469-77). EPC medium consisted of EBM-2 (Clonetics, Walkersville, MD, USA) supplemented with 5% FBS, 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), rrVEGF 10 ng/ml, bFGF 1 ng/ml, rmIGF-I 10 ng/ml (R&D, Mineapollis, MN, USA), rHuEGF 10 ng/ml (Promokine, Amiens Cedex, France) and hydrocortisone 1 µg/ml (Sigma Aldrich), following Griese *et al.* (*Cardiovasc Res* 2003;58:469-77). At day 4 of differentiation, non adherent cells were removed and fresh medium was substituted. EPC at day 5 of culture were identified by the spindle-shape morphology and by flow cytometry labeling cells with monoclonal antibodies as rabbit anti-von Willebrand factor (vWF) (Dako Cytomation, Glostrop, Denmark) and mouse anti-CXCR4 (Sigma Aldrich), VEGFR2 and VEGFR1 (RnD). Samples were analyzed by BD FACSCalibur flow cytometer (Beckton Dickinson,

Franklin Lakes, NJ, USA) and Cell Quest software (Beckton Dickinson). EPC functional capacities were also studied by binding of FITC–Ulex europaeus agglutinin-1 at 50 μg/ml (Vector Laboratories, Burlingame CA, USA), acLDL uptake at 10 μg/ml (Molecular Probes, OR, USA) and EPC tube formation as described (Wang *et al. J Cell Biochem* 2008;103:21-9).

EPC transduction and tracking

Transduction of EPC by AdIGFI-GFP was facilitated using the adaptor protein CAR-Fc-mVEGF$_{121}$. Prior to infection, AdIGFI-GFP (moi50) was incubated for 30 min at 37°C with the adaptor at 25 ng/10^6 cells to be infected. The infective mix was then added to EPC for 48h. IGF-I production by EPC was analyzed in cell supernatant by ELISA (R&D) and EPC transduction was confirmed by the analysis of GFP+ cells by flow cytometry and fluorescent microscope (Leica, IL, USA). Adenoviral transduction of EPC without the adaptor follows exactly the same protocol described, except that the adenoviral vector (moi50 and moi500) is directly added to the cell culture medium.

To analyze EPC-IGF recruitment to the cirrhotic liver cells were labelled with red fluorescent CM-DiL cell Tracker (Molecular Probes) (Zhou et al., *J Lipid Res* 2007;48:518-27) 48 h after EPC transduction with AdIGFI-GFP. 6x10^6 of labeled EPC were administered by tail vein into cirrhotic rats (TAA model) and cryosections from lung, spleen and liver were collected 6 days following transplantation (n=3). DiL+ cells were detected by fluorescent microscope (Leica, Wetzlar, Germany). ImageJ software was used for image analysis (Softonic, CA, US).

Fibrosis assessment, biochemical tests and ELISA

Liver fibrosis grade was evaluated by staining paraffin-embedded liver tissue with Masson´s trichrome and was scored by a pathologist who was blinded in order to evaluate the percentage of fibrotic area using morphometric analysis and ImageJ1.40G analysis software (Wyne Rasband, National Institutes of Health, USA). Serum AST, ALT, ALP, and bilirubin were determined by Cobas Integra 400plus analyzer (Roche, Basel, Switzerland). IGF-I and VEGF levels were determined using IGF-I and VEGF ELISA DuoSet Economy Pack (R&D).

Immunohistochemistry and immunofluorescence.

Immunohistochemistry for GFP was performed using specific antibody (1:200) (Abcam, Cambridge, Ma, USA). Hepatic Stellate Cells (HSC) activation and hepatocyte proliferation were detected in paraffin-embedded liver sections by immunohistochemistry against α-SMA (α-smooth muscle actin) (DAKO) and Ki67 (Neomarkers, CA, USA) respectively. EnVision+ System- HRP and DAB+ Chromogen were used (Dako, Glostrup, Denmark). Liver tissue was counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Double immunofluorescence staining of caspase-3 and α-SMA was developed using the following primary antibodies: caspase-3 (Cell Signaling, Danvers, MA, USA) and anti α-SMA (Dako, Glostrup, Denmark) diluted 1:100. Secondary antibodies used were anti-rabbit FITC and anti-mouse Cy3 (Jackson Immunoresearch, Suffolk, UK). Nuclei were stained with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) and images were uptaken with Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Munich, Germany) and analyzed by MetaMorph 7.6 software (Downingtown, PA, USA).

Real time PCR and Western blot.

Collagen I gene expression analysis was performed by RNA isolation, retrotranscription and real-time PCR methods using iQ5cycler and iQ SYBR Green Supermix (Bio-Rad, CA, US) (Berasain *et al. J Biol Chem* 2005;280:19012-20; Chomczynski and Sacchi. *Anal Biochem* 1987;162:156-9). RNA isolation from laser dissected histology sections was perfomed by RNA Nanoprep Kit (Stratagene La Jolla, CA, USA). Primer sequences and temperature of detection were as previously described (Møller S *et al., J Hepatol* 1993;17:315–320).

Western blot analysis was performed (Berasain *et al. J Biol Chem* 2005;280:19012-20) using antibodies specific for rat IGF-I (Santa Cruz Biotechnology, CA, USA) and GAPDH (Abcam, MA, US) (Sobrevals *et al., Hepatology* 2010;51(3):912-921). The densitometry of bands was quantified by ImageQuantECL&TL software (GE Healthcare).

Gene	Forward primers	Reverse primers
GADPDH	CTTCCACGATGCCAAAGTTG (SEQ ID NO: 23)	GATGGTGAAGGTCGGTGTG (SEQ ID NO:24)
Collagen I	CAAGAATGGCGACCGTGGTGA (SEQ ID NO:.25)	GGTGTGACTCGTGCAGCCATC (SEQ ID NO:26)

Statistical Analysis

Differences between groups were examined for statistical significance using the non parametric Mann–Whitney U tests. Data are reported as mean ± standard deviation (SD). A p value of <0.05 was considered significant.

EXAMPLE 1

Mice EPC infection with a GFP coding adenoviral vector.

Although *ex vivo* genetic engineering of EPC employing adenoviral vectors is an appealing therapeutic strategy, the results showed that EPC could only be efficiently transduced when using a high moi of adenovirus. As shown in Figure 1, the amount of adenovirus (Ad-CMV-GFP) required to efficiently infect mouse EPC (57%), maintaining proper cell viability, is moi 500 pfu/cell.

This represents a considerable obstacle for the implementation of combined cell and gene therapy, since at high moi, cell viability is compromised and only a small proportion of the transduced EPC remain alive and useful for infusion into the subject.

Structure and function of the adaptor protein CAR-Fc-mVEGF$_{121}$.

In order to increase the number of transduced cells, an adaptor protein CAR-Fc-mVEGF121 composed of the human CAR ectodomain and the murine VEGF isoform 121 linked by the CH2 and CH3 regions of the human IgG Fc was designed (Fig. 2A) as described in materials and methods. This bivalent protein is able to interact with the know fiber of the adenovirus through the CAR domain and on the other hand is able to

interact with the VEGFR through murine isoform 121 of VEGF thus creating a molecular bridge between the vector and the target cell (Figure 2B).

Characterization of EPC and EPC engineering.

On the fifth day of culture in the differentiation medium, rat BM mononuclear cells acquired the spindle-like morphology typical of endothelial lineage and formed capillary-like structures within 12 hours when plated on Matrigel (Fig 3A). Flow cytometric analysis showed that the cells were able to bind Ulex-FITC and to internalize DiLacLDL (Fig. 3B). They also exhibited positivity for CXCR4, vWF, VEGFR-1 (FLT-1) and VEGFR-2 (FLK-1) (Fig. 3B). EPC engineering was performed by their *ex vivo* transduction with adenoviral vectors. Cell transduction with the adenoviral vector using a moi of 500 achieved high transduction efficiency but at the expense of compromised cell viability. As shown in Fig. 4A and 4B, a large proportion of detached dead GFP+ cells was observed when using moi of 500, while the percentage of transduced living cells was very low (15.6 ± 4.8 %). With moi of 50, cell viability was preserved but transduction efficiency was poor (29.7± 4.3 %).

By preincubating AdIGFI-GFP with the CAR-Fc-mVEGF$_{121}$ adaptor, EPC transduction was increased to 41.0±4.6 % using moi 50 pfu/cell and excellent cell viability was maintained (Fig. 4A and 4B). This enhancement of transduction mediated by CAR-Fc-mVEGF121 was reflected by a significant increase in IGF-I synthesis with respect to cells transduced in the absence of the adaptor (Fig. 4C) without compromising cell viability.

An interesting observation was that EPC-IGF surpassed EPC not only in the production of IGF-I but also in the synthesis of VEGF, another relevant hepatoprotective factor (Fig 5). This finding suggests that EPC-IGF could exert therapeutic activities more potent than those of non-engineered EPC.

The adaptor of the invention allowed efficient EPC transduction at low MOI with excellent cell viability, thus permitting the use of IGF-I engineered cells for therapeutic

purposes. Interestingly, IGF-I engineered cells overexpress not only IGF-I but also VEGF, two factors that have been shown to exert potent hepatoprotective activities. VEGF, on the other hand, is known to stimulate neovascularization and microvasculature reconstruction, effects that could potentially reduce areas of ischemia that could be present in cirrhotic livers. The data reveal that by engineering EPC with IGF-I, this cells can be converted in a source of cytoprotective molecules of utility for the treatment of liver cirrhosis.

Distribution of transplanted EPC and transgene expression in the cirrhotic liver (TAA model).

Homing axes involved in the recruitment of cells to injured tissues are SDF-1, CXCR4, VEGF, VEGFR and HGF- cmet receptor. As we used an adaptor molecule composed of VEGF-Fc-CAR, we are using the VEGF receptors to engineer EPC by adenoviral vectors, so it could be possible that as we are blocking this homing axe (VEGF-VEGFR), engineered EPC recruitment by injured liver could be affected. In order to solve this question, IGF-1-GFP engineered EPC were also labeled with DiL cell traker, which allow us to follow the recruitment of EPC into liver tissue following their IV administration. Figure 6, shows the DiL+ EPC found in the liver parenchyma, as well as GFP+ EPC were also detected by immunohistochemistry.

In order to determine whether the engineered EPC were indeed recruited to the damaged liver the cells were labelled with the red fluorescent CM-DiL tracker 48h after their transduction with AdIGFI-GFP. On day 6 after EPC infusion, it was found that DiL+ cells were present in significantly higher numbers in the cirrhotic liver than in lungs or spleen (Fig. 6A). EPC engraftment in hepatic tissue was also analyzed by GFP immunostaining of liver samples. GFP+ cells were detected mainly in fibrous septa surrounding regenerative nodules but also occasionally inside liver nodules, as shown in Fig 6B. These findings also indicate that the transduced cells were able to efficiently express the transgene in the injured organ. In accord with this observation, western blot analysis of liver extracts demonstrated greater IGF-I abundance in EPC.IGFI treated rats than in those which received EPC or saline (Fig. 7B). Similarly, serum levels of

IGF-I were significantly higher in EPC.IGF group than in animals given saline or EPC on day 4 after cell administration (Fig. 7A).

In the present study, the infused EPC were demonstrated to be recruited to the liver, mainly to portal tracts, where they effectively express the transgene. The presence of IGF expressing EPC in the fibrous septa would lead to the release of antifibrogenic and cytoprotective factors within the inflamed tissue. This would enhance the effectiveness of the combined cell and gene therapy which appears to mediate significant beneficial effects with relatively low doses of engineered cells.

Effect of therapy on liver biochemistry.

In rats subjected to thioacetamide poisoning that were treated with EPC or EPC.IGFI, but not in those given MNC, an improvement of serum biochemistry was found with respect to saline-treated controls. Serum alkaline phosphatase was reduced in both EPC and EPC.IGF groups as compared to controls, the values being significantly lower in EPC.IGF than in EPC animals. Serum bilirubin was reduced in EPC and EPC-IGF rats with respect to saline-treated controls but differences only reached statistical significance in the EPC.IGF group (Table I).

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirrubin (mg/dL)
Healthy	84.86 ± 8.2	80.44 ± 19.8	63.0 ± 6.6	0.037 ± 0.005
Saline	116.5 ± 12.0	108.5 ± 42.5	160.1 ± 45.2	0.107 ± 0.067
MNC	117.5 ± 12.8	127.25 ± 51.1	125.4 ± 60.9	0.100 ± 0.097
EPC	79.75 ± 27.1	91.0 ± 30.9	92.7 ± 36.9*	0.054 ± 0.026
EPC-IGF	88.4 ±19.6	82.8 ± 6.3	63.7 ± 35.2 #, ***	0.042 ± 0.027 *

Table 1. Analysis of liver functionality in cirrhotic rats treated with engineered EPC or controls. ALT, AST, ALP and total bilirubin were measured in serum samples obtained from animals treated as described in methods. *P<0.05 vs. Saline; ***P<0.001 vs. Saline; # P<0.05 vs. EPC group.

In rats subjected to CCl_4 poisoning that were treated with EPC.IGFI an improvement of serum ALT, AST and ALP levels was found with respect to saline-treated controls but differences only reached statistical significance for ALT and AST (Table II).

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirrubin (mg/dL)
Healthy	84.86 ± 8.2	80.44 ± 19.8	15±4	0.05 ± 0.01
Saline	133 ± 47	156± 111	123±63	0.14 ± 0.02
MNC	127 ± 21	111 ± 34	126 ± 44	0.19 ± 0.07
EPC	117± 32	148±49	156 ±104	0.17 ± 0.09
EPC.IGF-1	88± 21*#	94±48#	101±55	0.16 ± 0.09

Table2. Analysis of liver functionality in cirrhotic rats treated with engineered EPC or controls. ALT, AST, ALP and total bilirubin were measured in serum samples obtained from animals treated as described in methods. *P<0.05 vs. Saline; # P<0.05 vs. EPC group.

Effect of therapy on liver histology.

After 7 weeks of TAA administration or 8 weeks of CCl_4 administration, rats given saline showed well-established cirrhosis with thick fibrotic septa. Notably, a reduction of fibrotic areas was observed in all groups of animals that received infusions of BM-derived cells. The decrease in fibrosis was, however, more manifest in rats treated with EPC or EPC.IGF, and was significantly more marked in the latter group (Fig 8A). In keeping with these findings, Col I gene expression was reduced in rats that received EPC or EPC.IGF as compared to controls given saline (Fig. 8B). Importantly, in TAA model, Col I mRNA levels were significantly lower in EPC.IGF group than in animals treated with EPC (Fig. 8B, left panel). In the CCl4 model, no differences were observed in the mRNA colagen I levels in the EPC group vs EPC.IGF1 group. However, the comparison between the EPC vs EPC.IGF1 is significant ($p<0.01$). This indicates an improvement in the EPC.IGF1 treated animals (Fig. 8B, right panel).

In order to quantify the number of activated hepatic stellate cells (HSC) we developed an immunohistochemistry of the marker alfa-SMA, which detect activated HSC, responsible of collagen synthesis in cirrhosis (Sobrevals, Hepatology 2009). We counted the number of activated HSC in liver tissue in two models of cirrhosis induced by TAA or CCl_4. Results show that the treatment of cihrrotic animals with IGF-1

engineered EPC induced a significant decrease of the number of HSC compared to non treated animals (TAA model) (Figure 9A, upper panel). In CCl_4 model, we observed that IGF-1 genetically modified EPC reduced significantly the number of alfa-SMA positive cells in liver septum compared to the animals which received non modified EPC (Figure 9B, lower panel). These results correspond with expression level of collagen type I found in EPC.IGF1 treated animals.

Since it has also been shown that regression of liver cirrhosis is associated with apoptosis of HSC in fibrous septa (Iredale *et al., J Clin Invest* 1998;102:538–549), immunohistochemical studies were performed to detect the presence of double positive cells for caspase-3 and α-SMA in liver samples from the three groups of TAA treated animals. It was found that TAA-treated animals which received EPC.IGF therapy had a significant increase in the number of caspase-3^+/α-SMA^+ cells compared to EPC and saline groups (Fig. 10). This finding suggests that an increase in the rate of apoptosis of collagen-producing cells may contribute to the antifibrogenic effect of EPC.IGF therapy.

Data from transgenic mice overproducing IGF-I in the liver when challenged with hepatotoxicants indicate that IGF-I may stimulate hepatocellular regeneration upon injury (Sanz *et al. Gut* 2005;54:134–141). To analyze the impact of combined cell and gene therapy on liver regeneration in the liver cirrhosis, Ki67 immunostaining was performed in liver samples obtained 6 days after therapy. It was found in the TAA model (Fig. 11A) that the percentage of Ki67 positive nuclei was significantly increased in EPC.IGF group as compared to controls. Although there was a tendency for increased regeneration in the EPC group, the differences with respect to controls did not reach statistical significance in comparison to saline (Fig. 11A). There were no differences in Ki67+ nuclei in MNC group as compared to the control group treated with saline (Fig. 11A).

Although no differences in the number of cells ki67 positive are observed in the cirrhosis model CCL4 (chronic intoxication), a tendency of increase can be observe in

the EPC IGF1 treated group (20.1 ± 16.8 saline vs 29.3± 6.8 EPC.IGF group) (Fig. 11B).

These results show that the IGF-I gene transfer to EPC has a boosting effect on the antifibrogenic and hepatoprotective properties of these cells in two different models of liver cirrhosis. Indeed, EPC-IGF treated rats exhibited less fibrosis and Col I mRNA in the liver than those which received non-modified EPC. Double immunohistochemical staining for caspase 3 and α-SMA in liver samples suggested that the ability of EPC-IGF therapy to induce cirrhosis regression might be mediated in part by induction of apoptosis of hepatic stellate cells (HSCs), the main producers of collagen in the liver. Although IGF-I behaves *in vitro* as an antiapoptotic molecule for HSC, it has been shown that its *in vivo* effects on activated HSCs in chronically inflamed livers are the opposite. In this situation IGF-I activates a biological program, which leads to resolution of inflammation and promotion of tissue repair by reducing scar formation and stimulating liver regeneration. According to this notion we also found a significant increase of hepatocellular regeneration in livers from rats with IGF expressing EPC, as compared to saline treated controls while the differences did not reach statistical significance in the group that received EPC.

Final conclusion: All together the data provided in the present invention indicate that combined gene and cell therapy is capable of improving liver cirrhosis both histologically and functionally with greater efficacy than when using unmodified EPC.

CLAIMS

1. A polypeptide comprising:
   (a) a domain of coxsackievirus and adenovirus receptor (CAR) capable of binding to an adenoviral fiber protein or a functionally equivalent variant thereof and
   (b) a VEGF receptor (VEGFR) ligand.

2. A polypeptide according to claim 1 further comprising
   (c) a dimerization domain which connects the domain of CAR and the VEGFR ligand.

3. A polypeptide according to claims 1 or 2 wherein the domain of CAR is the sequence SEQ ID NO:4.

4. A polypeptide according to any of the previous claims, wherein the VEGFR ligand is the VEGF isoform 121 (VEGF121).

5. A polypeptide according to any of the previous claims, wherein the VEGF isoform 121 is SEQ ID NO:5 or SEQ ID NO:7.

6. A polypeptide according to any of claims 2 to 5, wherein the dimerization domain is an immunoglobulin Fc fragment.

7. A polypeptide as defined in claim 6 wherein the immunoglobulin Fc fragment is an IgG1 Fc fragment.

8. A polypeptide according to claim 7, wherein the Fc fragment of IgG1 is SEQ ID NO: 9.

9. A polypeptide according to any of the previous claims, further comprising one or more peptide linkers between the components a, b and c.

10. A polypeptide according to claim 9, wherein the peptide linker is at the C-terminus of the CAR domain and/or at the C-terminus of the dimerization domain.

11. A polypeptide according to claim 10, wherein the peptide linker is selected from the group of AAQPAEP (SEQ ID NO:16), AAA and GGPGS.

12. A polypeptide according to claims 10 or 11, wherein the polypeptide comprises SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 20, or SEQ ID NO: 22.

13. A polynucleotide encoding a polypeptide as defined in any of claims 1 to 12.

14. A polynucleotide according to claim 13, wherein the sequence is SEQ ID NO: 19 or SEQ ID NO: 21.

15. A gene construct or a vector comprising a polynucleotide as defined in claims 13 or 14.

16. A cell comprising a polynucleotide as defined in claim 13 or 14, or a gene construct or a vector as defined in claim 15.

17. A method of making a polypeptide as defined in any of claims 1 to 12 comprising the steps of:
    (a) culturing a host cell as defined in claim 16 under conditions that allow production of the polypeptide; and
    (b) isolating the polypeptide from said culture of host cells.

18. A composition or complex comprising
    (a) a polypeptide as defined in any of claims 1 to 12, and
    (b) an adenovirus comprising a nucleotide sequence encoding a product of interest.

19. The composition or complex according to claim 18, wherein the product of interest is a hepatoprotective secretable factor.

20. The composition or complex according to claim 19, wherein the hepatoprotective secretable factor is IGF-I or a functionally equivalent variant thereof.

21. A method of obtaining a cell expressing a product of interest, comprising the steps of

(i) contacting a cell with a polypeptide as defined in any of claims 1 to 12 and with an adenovirus encoding a product of interest, or with a composition or complex according to claim 18 or 20, wherein said contacting can be carried out by separately adding the polypeptide and the adenovirus or by adding a preformed polypeptide–adenovirus complex,

(ii) maintaining the mixture obtained in step (i) under conditions adequate for the formation of a ternary complex between said polypeptide, said adenovirus and said cell, and

(iii) maintaining the cells under conditions adequate for the expression of the product of interest.

22. The method of claim 21, further comprising isolating the cell expressing the product of interest.

23. The method according to claims 21 or 22, wherein the cell is an endothelial progenitor cell (EPC).

24. The method according to any of claims 21 to 23, wherein the product of interest is a hepatoprotective secretable factor.

25. The method according to any of claims 21 to 24, wherein the hepatoprotective secretable factor is IGF-I or a functionally equivalent variant thereof.

26. A cell expressing a product of interest obtained by the method of any of claims 21 to 25.

27. EPC that comprises an exogenous polynucleotide encoding IGF-I or a functionally equivalent variant thereof.

28. A pharmaceutical composition comprising a composition or complex as defined in any of claims 18 to 20, or a cell as defined in any of claims 26 or 27 and a pharmaceutically acceptable carrier.

29. The pharmaceutical composition according to claim 28, which comprises a composition or complex or a cell as defined in any of claims 26, wherein the product of interest is IGF-I or a functionally equivalent variant thereof.

30. A polypeptide as defined in any of claims 1 to 12, a composition or complex as defined in any of claims 18 to 20, or a cell as defined in claims 26 or 27 for use in medicine

31. A composition or complex as defined in any of claims 18 to 20, wherein the product of interest is IGF-I or a functionally equivalent variant thereof; a cell as defined in claim 26 wherein the product of interest is IGF-I or a functionally equivalent variant thereof or a cell as defined in claim 27 for use in the prevention or the treatment of a fibrosis-associated disease.

32. Use of a composition or complex as defined in any of claims 18 to 20, wherein the product of interest is IGF-I or a functionally equivalent variant thereof; of a cell as defined in claims 26 wherein the product of interest is IGF-I or a functionally equivalent variant thereof or of a cell according to claim 27 in the manufacture of a medicament for the prevention or the treatment of a fibrosis-associated disease.

33. A polypeptide, a composition or a cell for use according to claim 31, or a use according to claim 32 wherein the fibrosis-associated disease is cirrhosis.

34. A cell for use according to any of claims 31 or 33 or a use according to claim 32 wherein the cell is autologous to the subject to be treated.

FIGURE 1

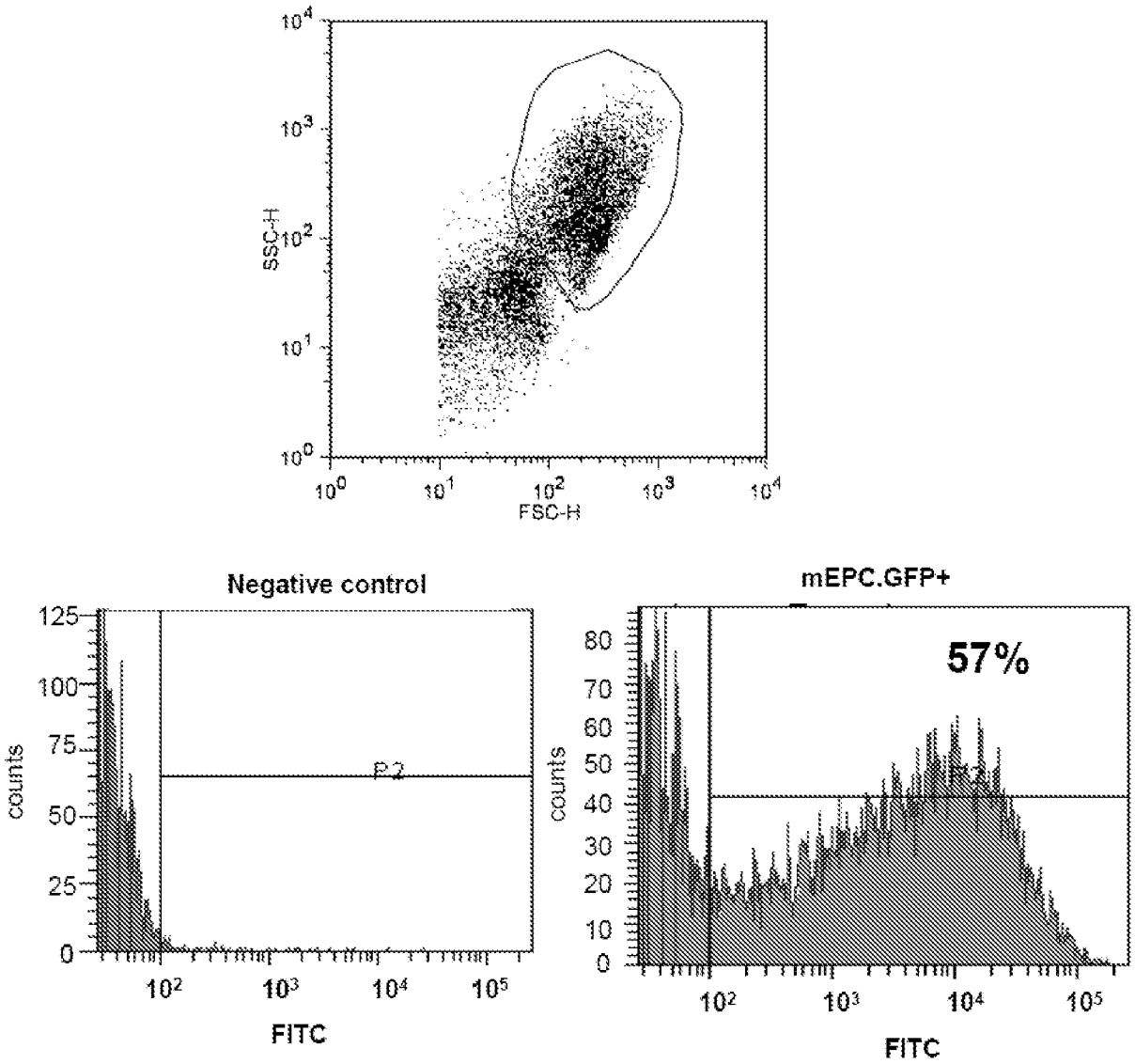

SSC-H
FSC-H
Negative control
counts
P2
FITC
mEPC.GFP+
57%
counts
FITC

FIGURE 2

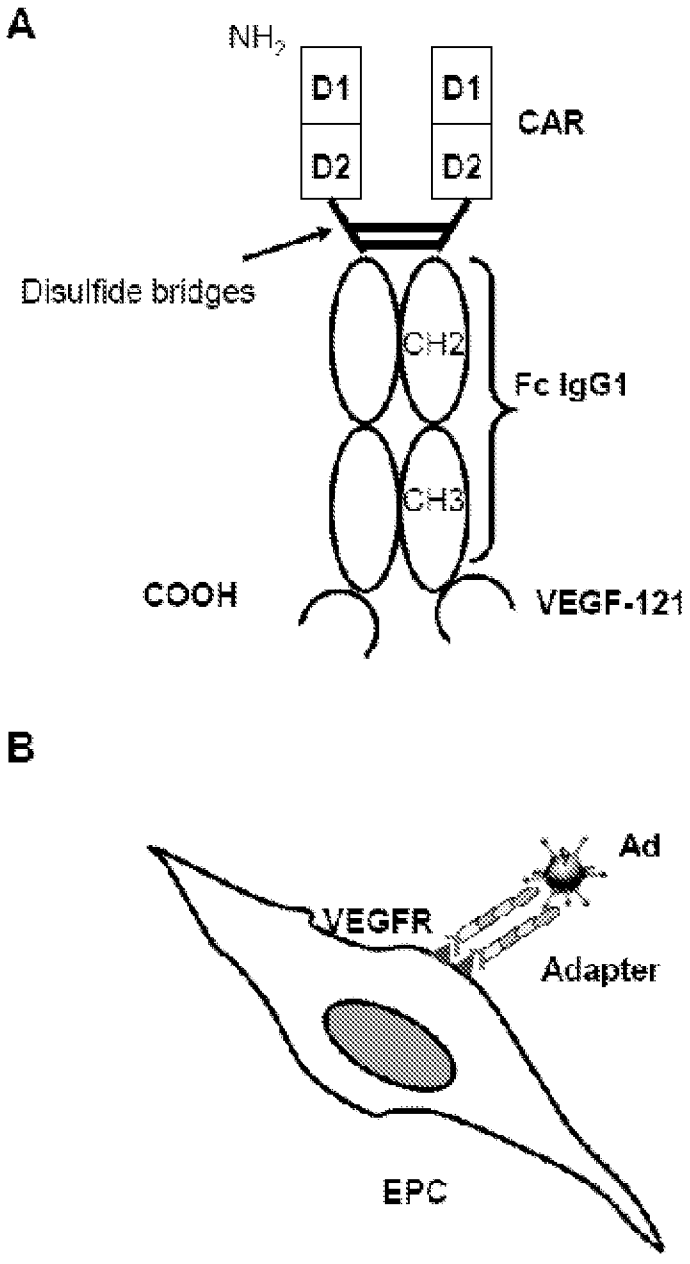
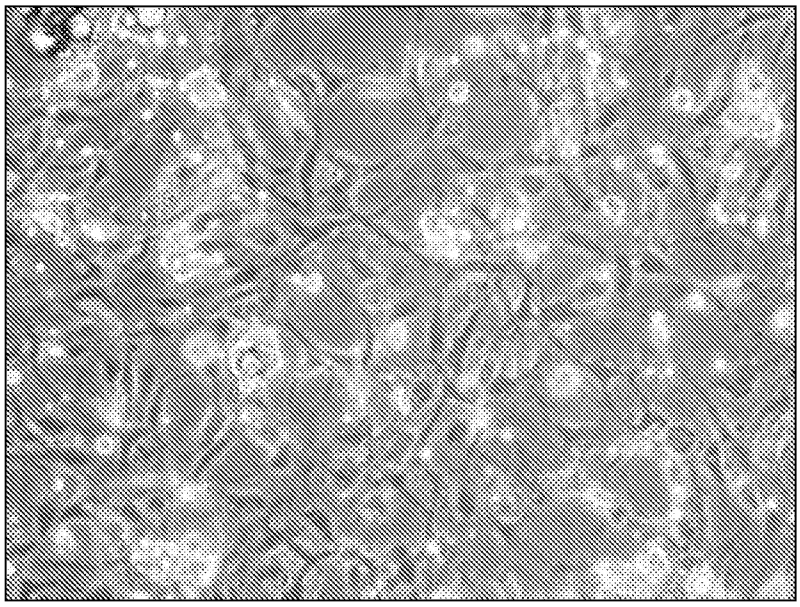

A
NH2
D1
D2
D1
D2
CAR
Disulfide bridges
CH2
Fc IgG1
CH3
COOH
VEGF-121
B
Ad
VEGFR
Adapter
EPC

FIGURE 3A

Spindle-like morphology



Tube formation

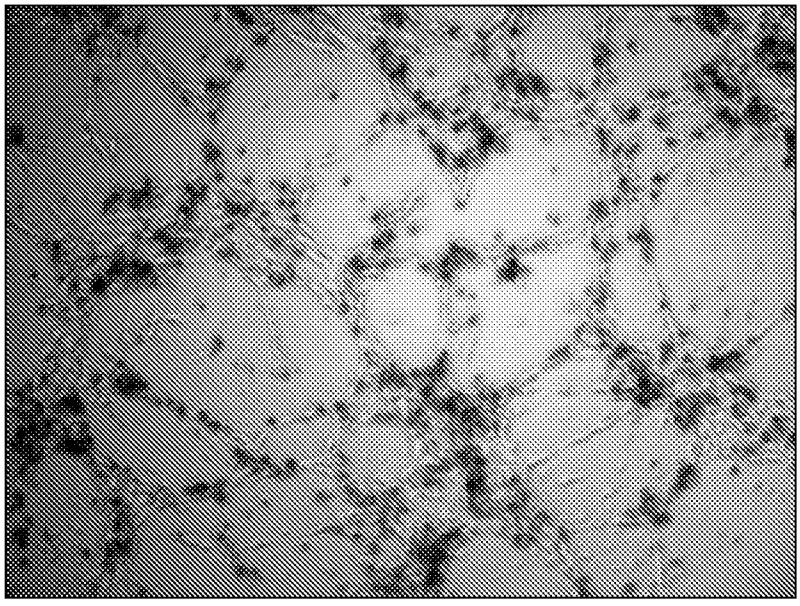


FIGURE 3B

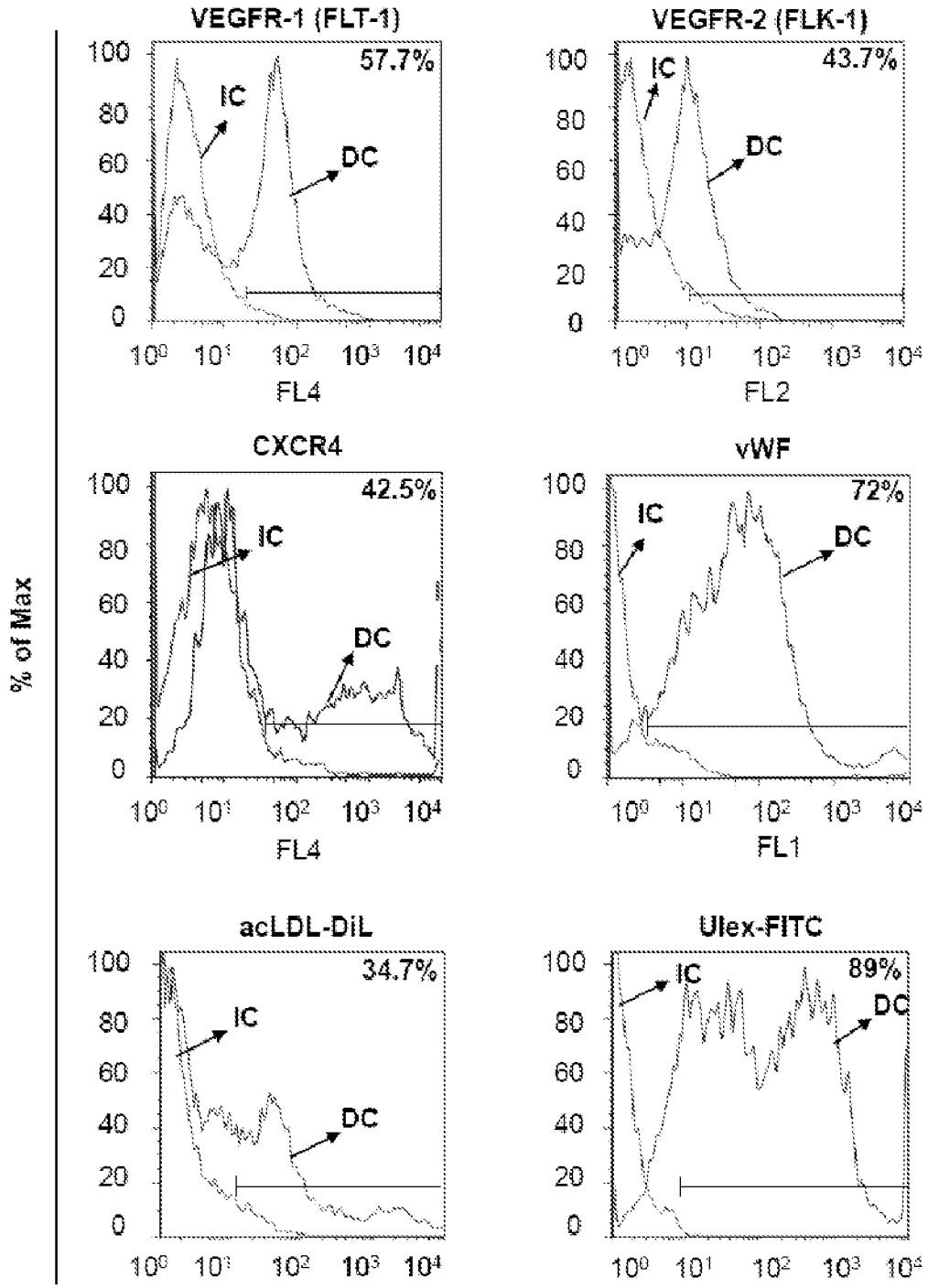

VEGFR-1 (FLT-1)
57.7%
IC
DC
FL4
VEGFR-2 (FLK-1)
43.7%
IC
DC
FL2
CXCR4
42.5%
IC
DC
FL4
vWF
72%
IC
DC
FL1
acLDL-DiL
34.7%
IC
DC
Ulex-FITC
89%
IC
DC
% of Max
100
80
60
40
20
0
10^0 10^1 10^2 10^3 10^4

FIGURE 4

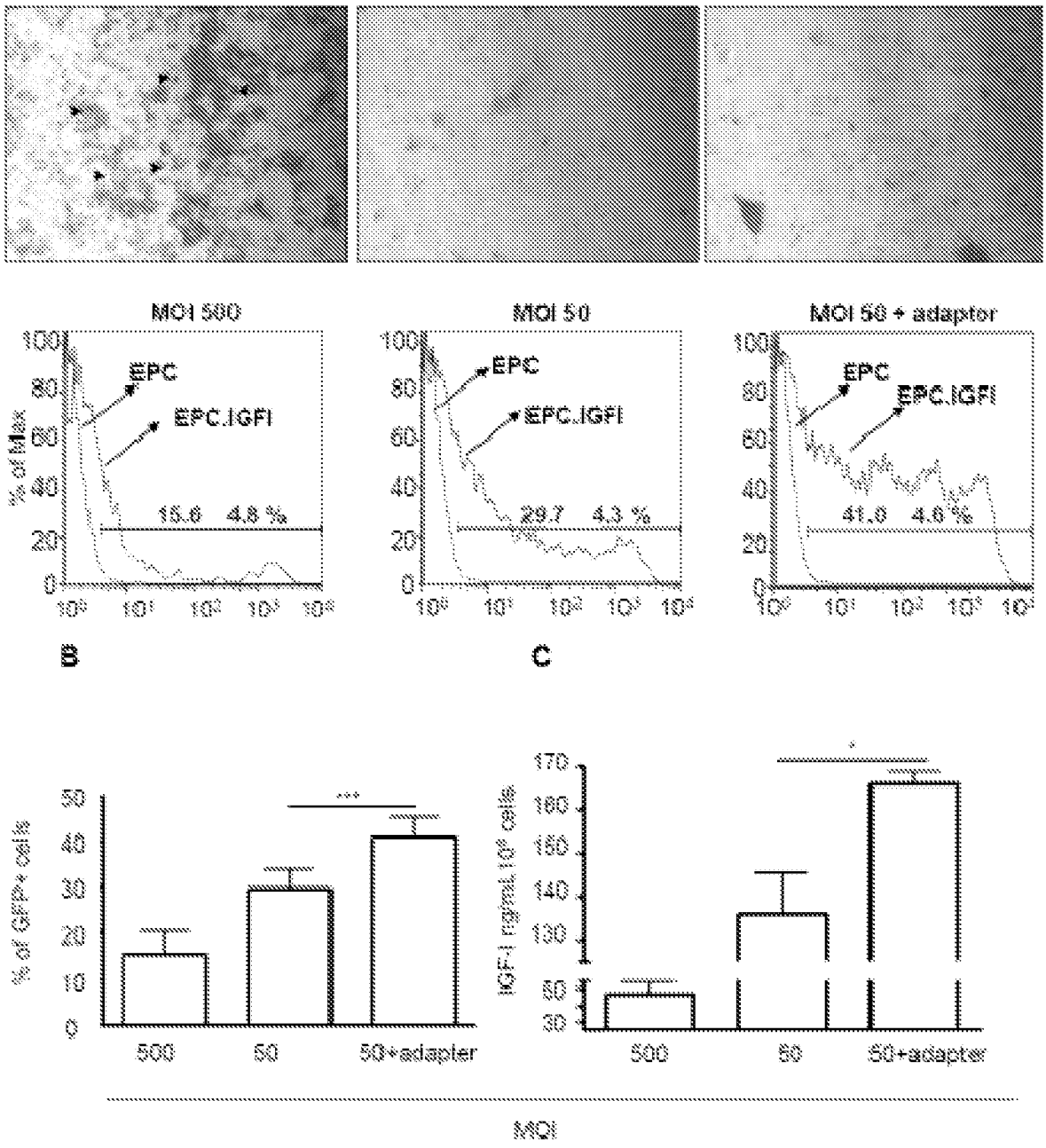

MOI 500
MOI 50
MOI 50 + adaptor
EPC
EPC.IGFI
% of Max
15.6 4.8 %
29.7 4.3 %
41.0 4.6 %
B
C
% of GFP+ cells
IGFI ng/mL/10^6 cells
500
50
50+adapter
MOI

FIGURE 5

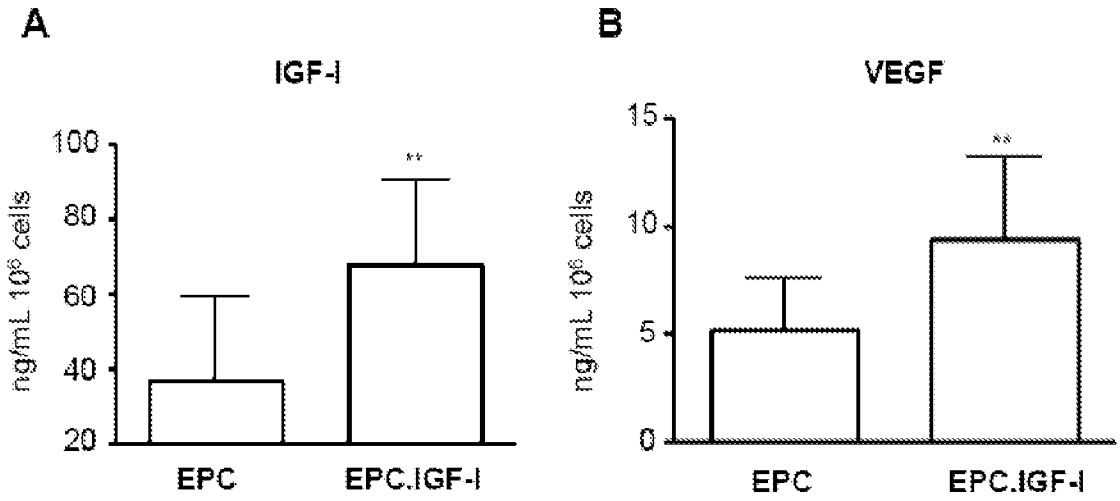

A
IGF-I
ng/mL 10⁶ cells
100
80
60
40
20
**
EPC
EPC.IGF-I
B
VEGF
ng/mL 10⁶ cells
15
10
5
0
**
EPC
EPC.IGF-I

FIGURE 6

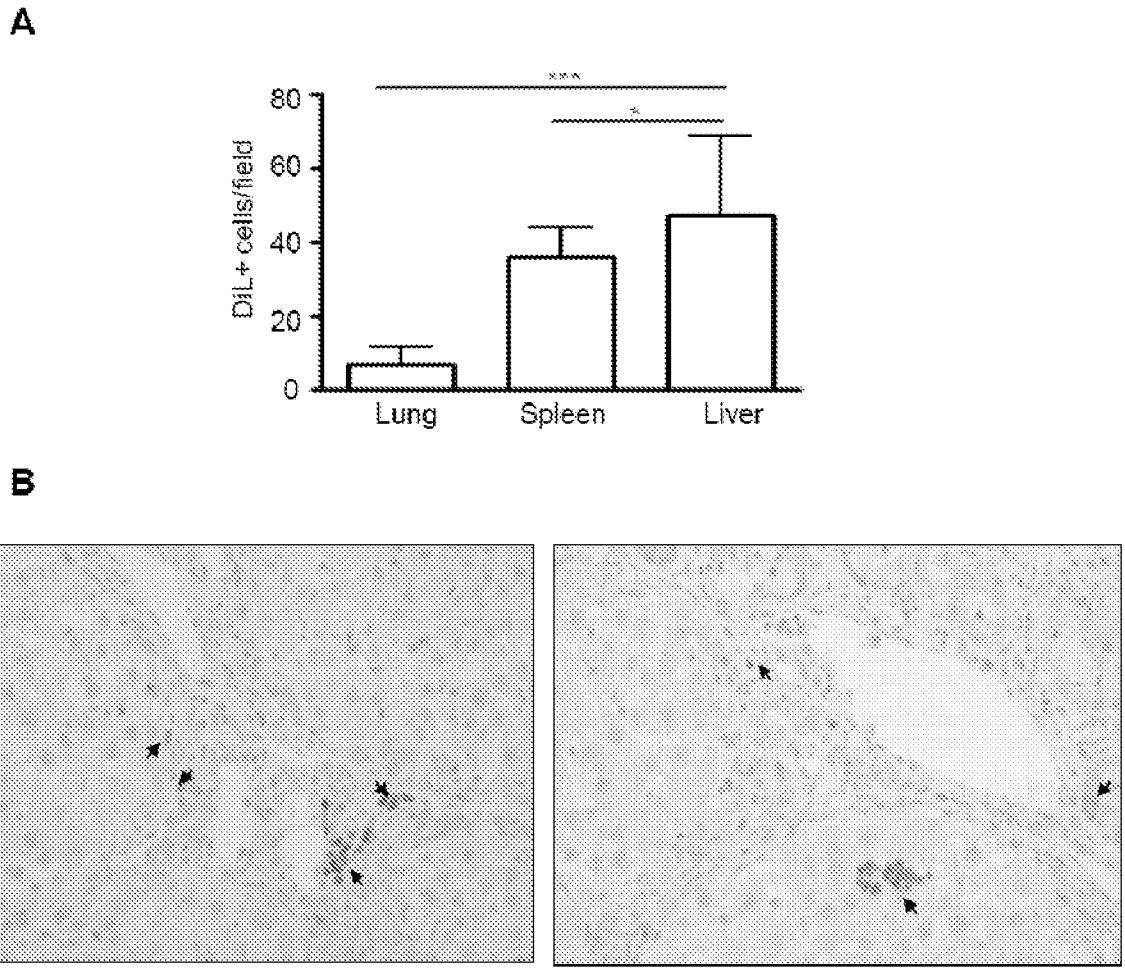


FIGURE 7

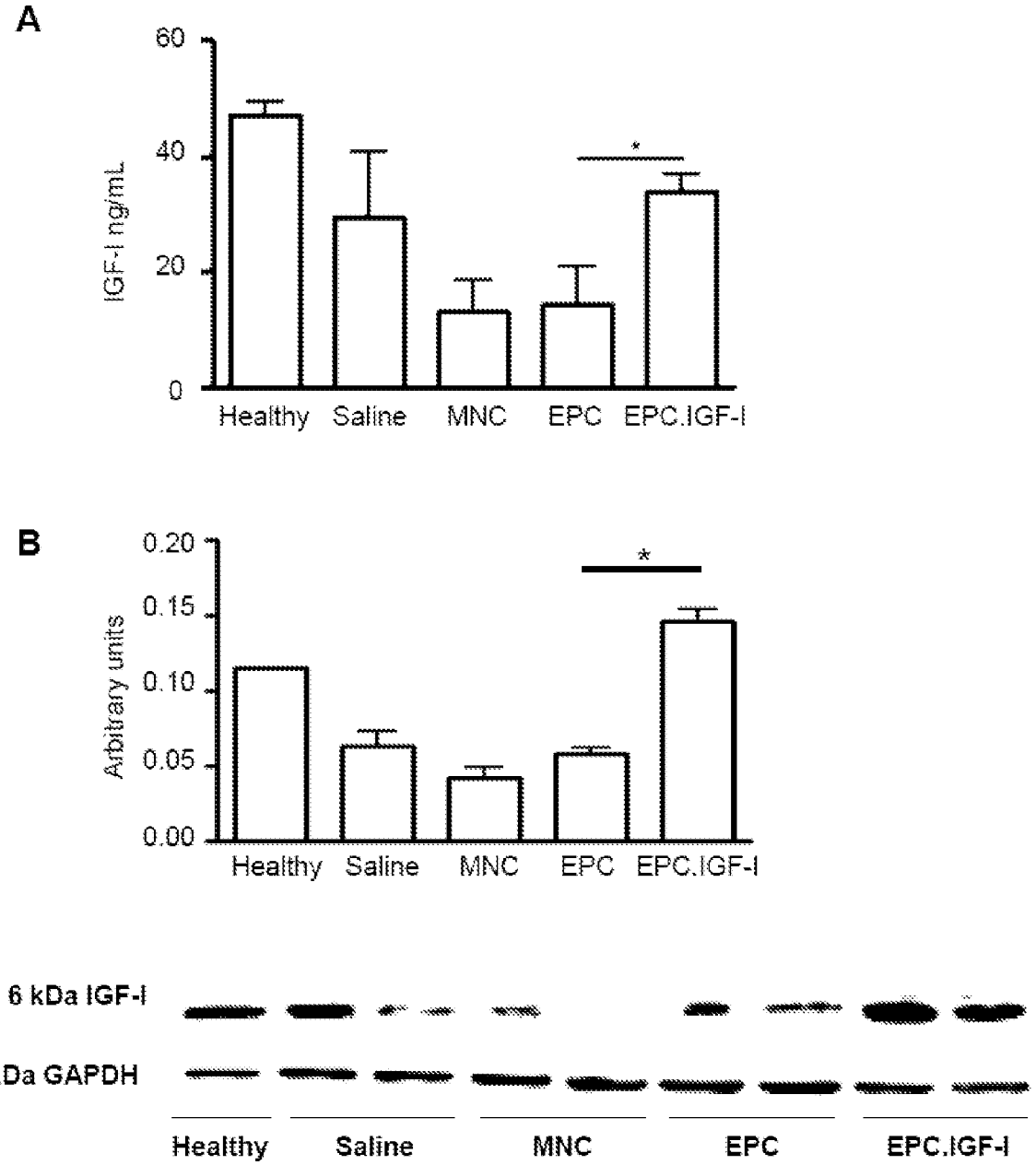

A
60
40
20
0
IGF-I ng/mL
*
Healthy
Saline
MNC
EPC
EPC.IGF-I
B
0.20
0.15
0.10
0.05
0.00
Arbitrary units
*
Healthy
Saline
MNC
EPC
EPC.IGF-I
6 kDa IGF-I
38 kDa GAPDH
Healthy
Saline
MNC
EPC
EPC.IGF-I

FIGURE 8

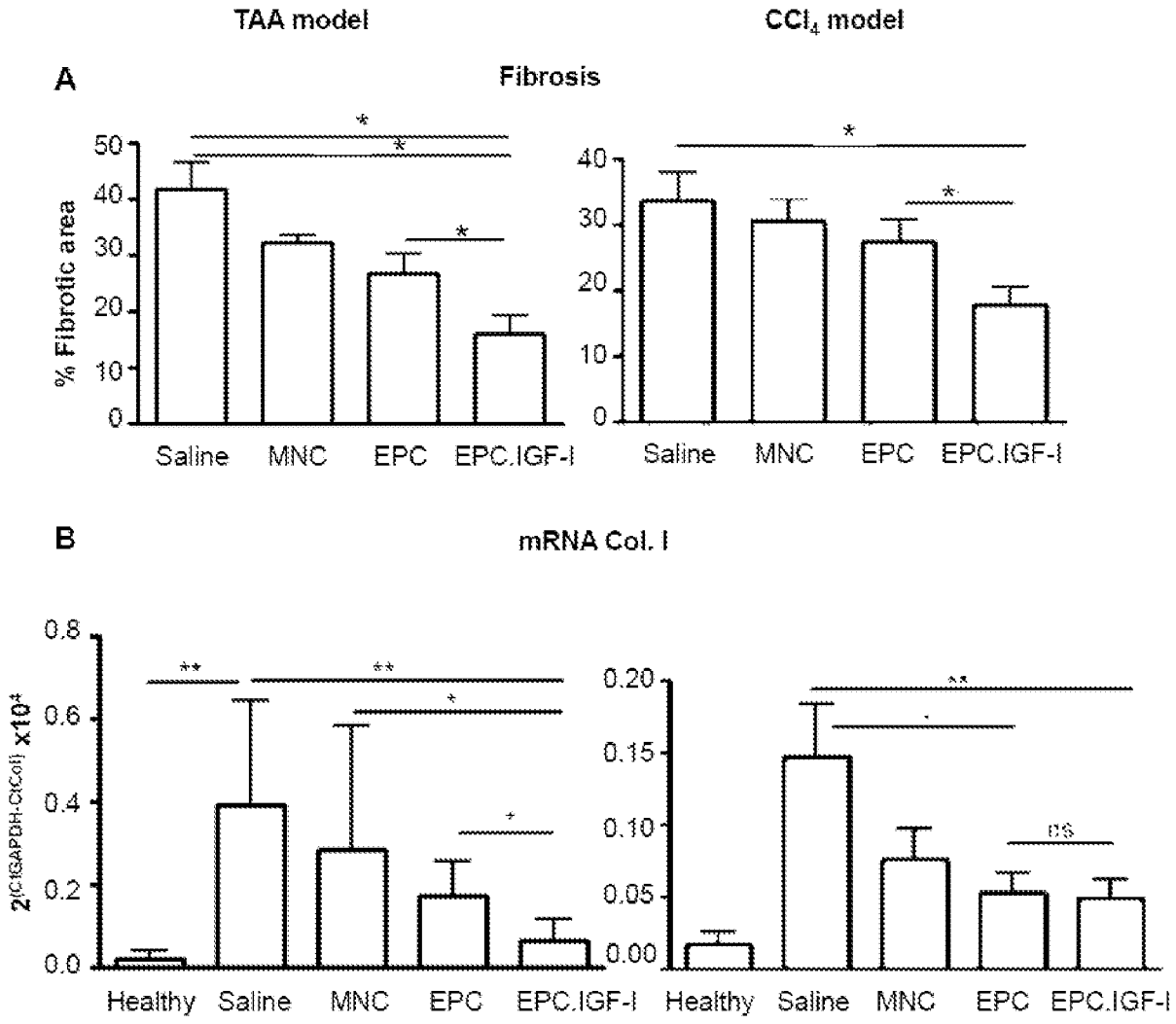

TAA model
CCl4 model
A
Fibrosis
% Fibrotic area
50
40
30
20
10
0
Saline
MNC
EPC
EPC.IGF-I
B
mRNA Col. I
0.8
0.6
0.4
0.2
0.0
0.20
0.15
0.10
0.05
0.00
Healthy
Saline
MNC
EPC
EPC.IGF-I
NS

FIGURE 9A

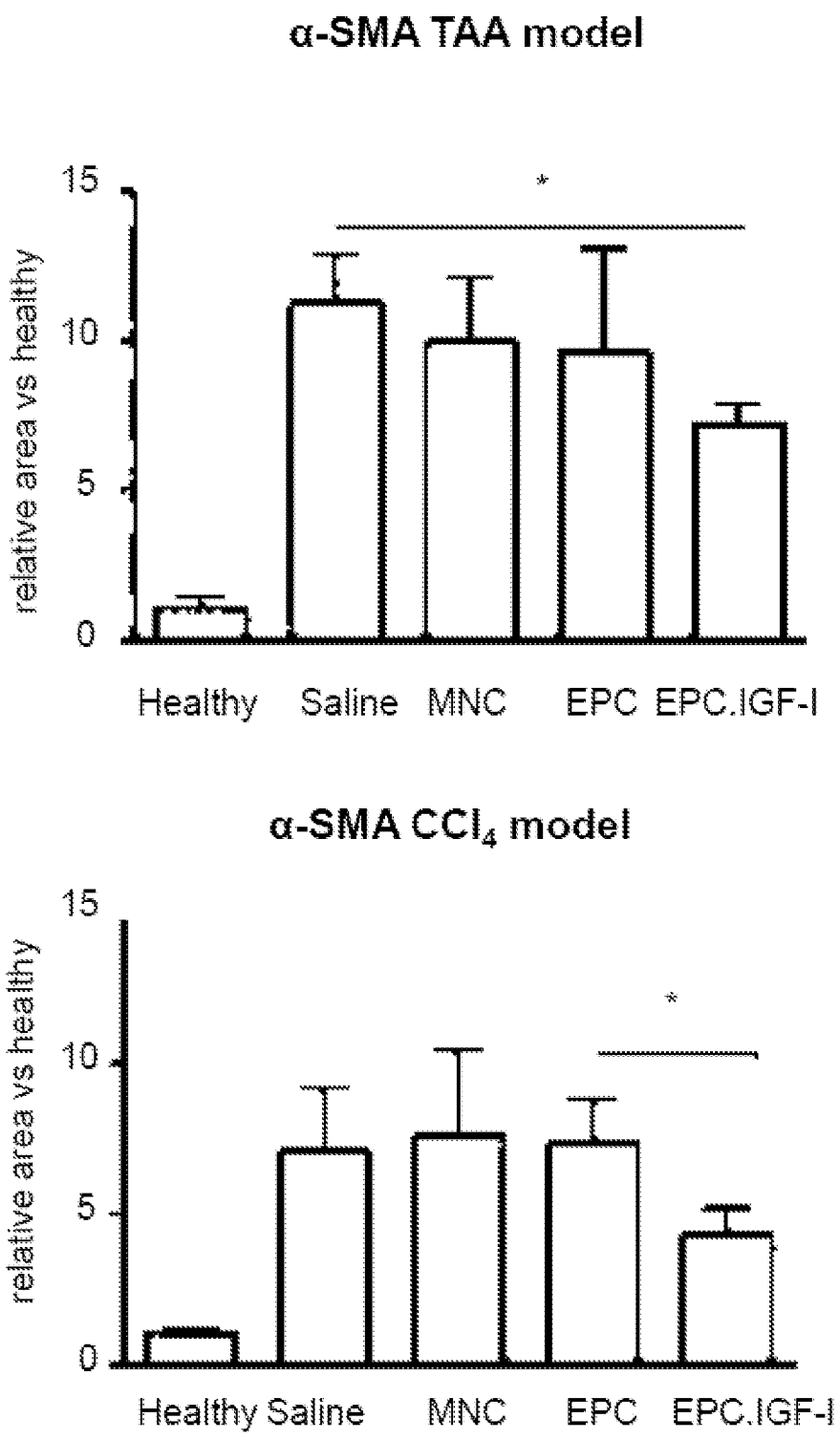

α-SMA TAA model
relative area vs healthy
15
10
5
0
*
Healthy
Saline
MNC
EPC
EPC.IGF-I
α-SMA CCl4 model
relative area vs healthy
15
10
5
0
*
Healthy
Saline
MNC
EPC
EPC.IGF-I

FIGURE 9B

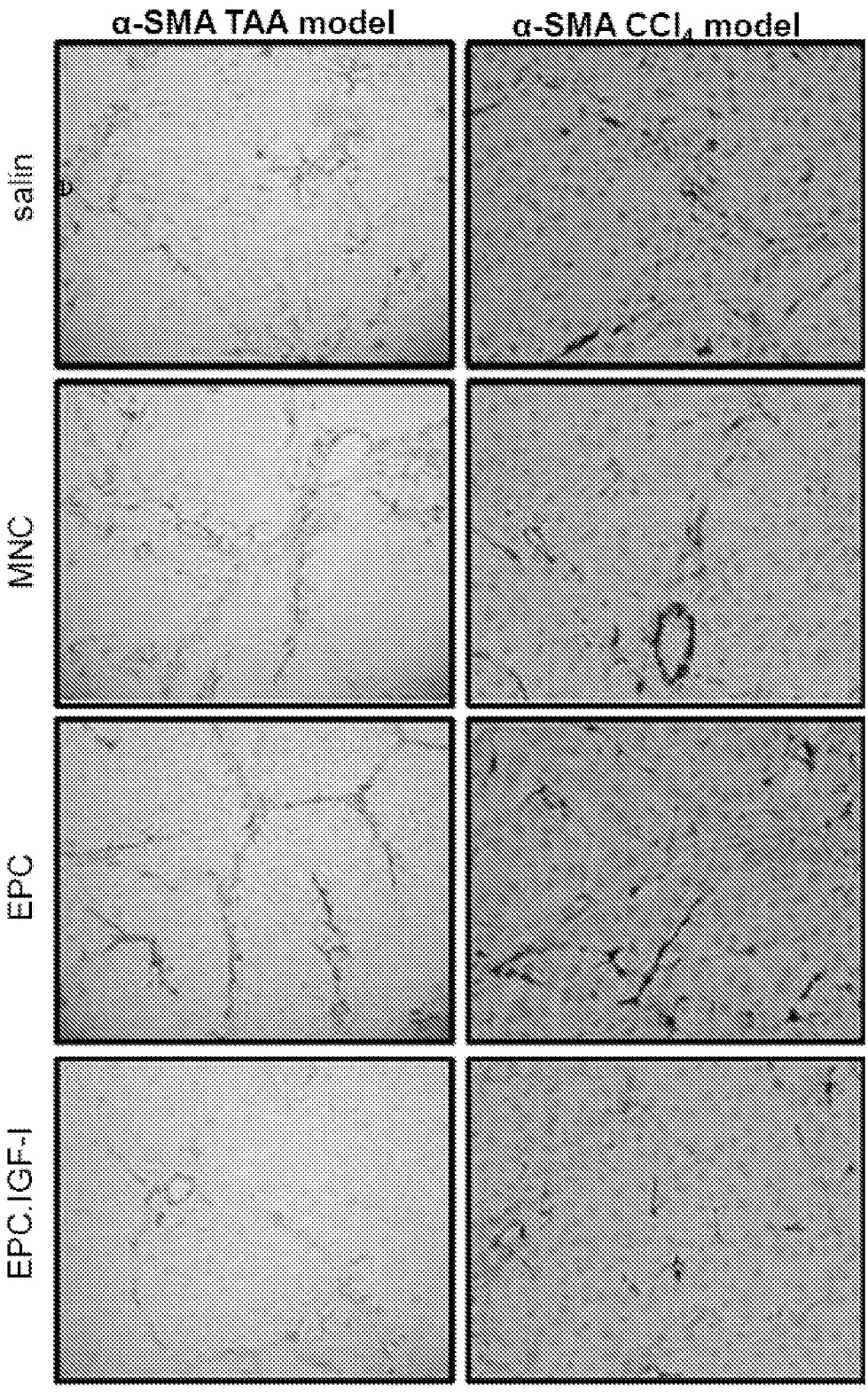


FIGURE 10

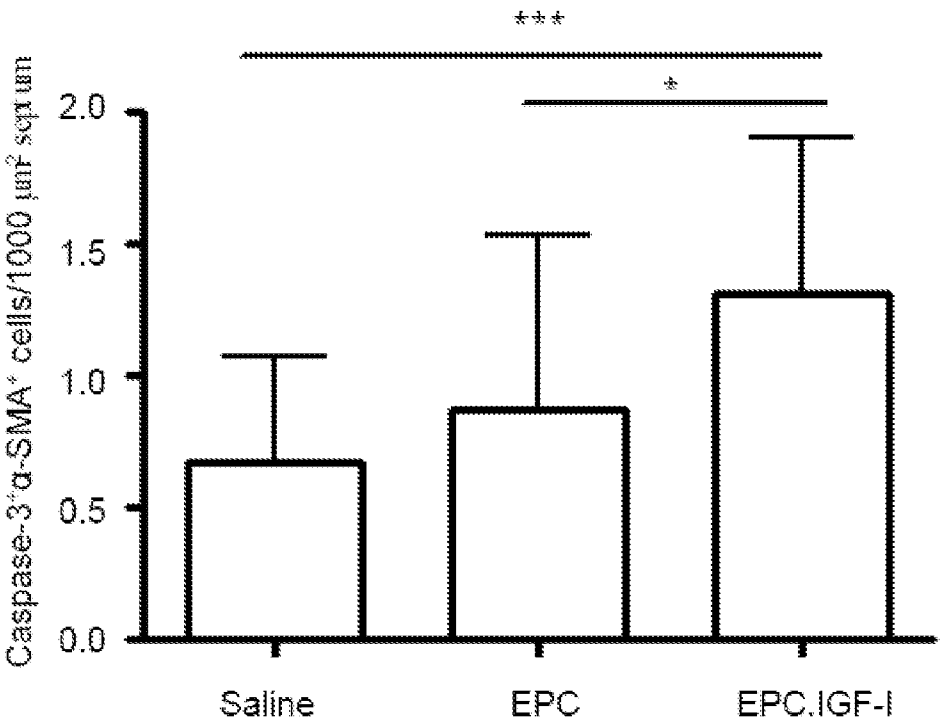
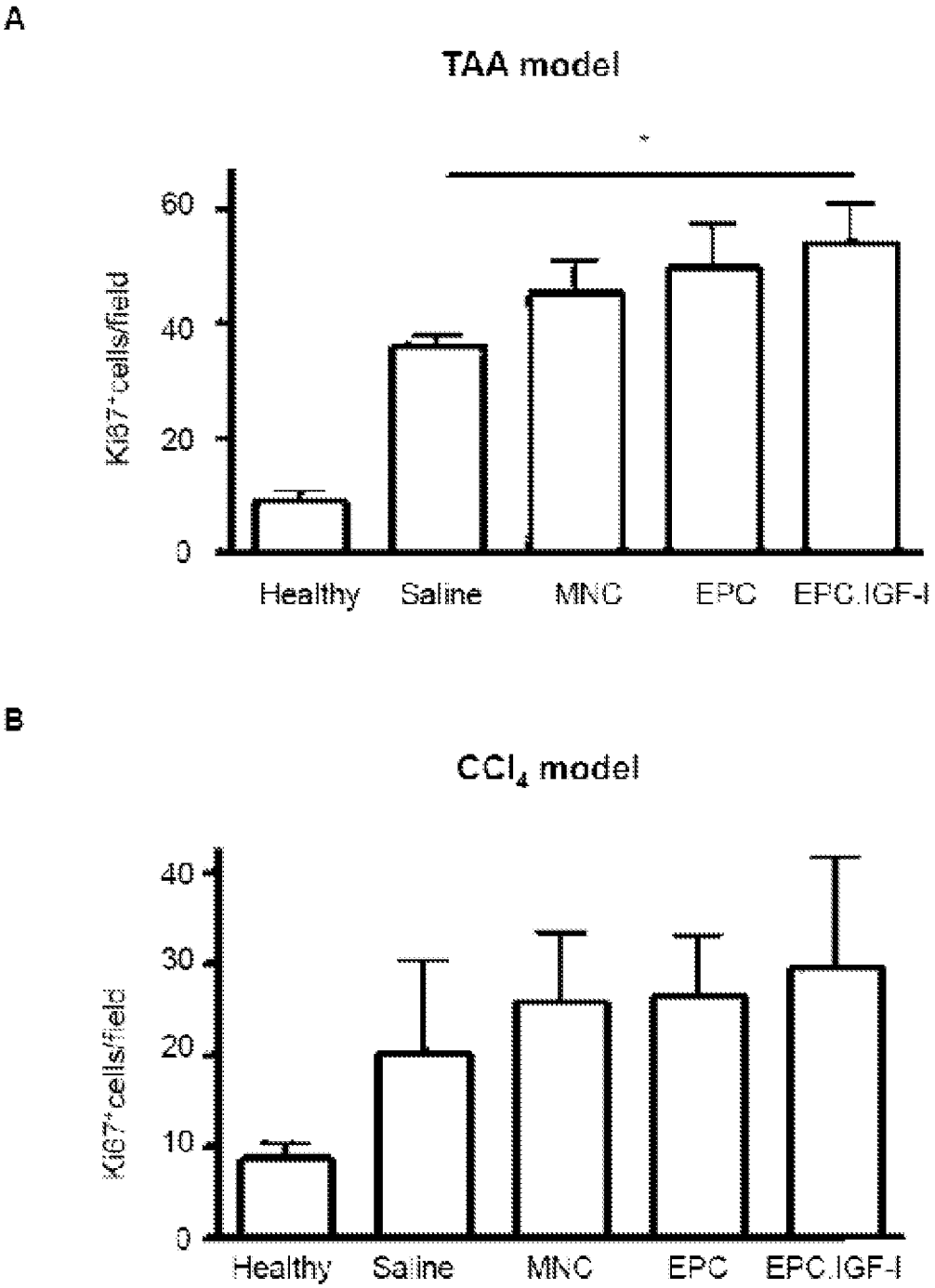

***
*
Caspase-3+α-SMA+ cells/1000 μm² sqn um
2.0
1.5
1.0
0.5
0.0
Saline
EPC
EPC.IGF-I

FIGURE 11


A
TAA model
Ki67+cells/field
60
40
20
0
Healthy
Saline
MNC
EPC
EPC.IGF-I
B
CCl4 model
Ki67+cells/field
40
30
20
10
0
Healthy
Saline
MNC
EPC
EPC.IGF-I

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2011/059137

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/861 A61K48/00 C07K14/52 C07K14/65 A61K38/30
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)
C12N A61K C07K

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 practical, 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.
X	VERA M ET AL: "Liver transduction with a simian virus 40 vector encoding insulin-like growth factor I reduces hepatic damage and the development of liver cirrhosis", GENE THERAPY, MACMILLAN PRESS LTD., BASINGSTOKE, GB, vol. 14, no. 3, 1 February 2007 (2007-02-01), pages 203-210, XP002589972, ISSN: 0969-7128, DOI: DOI:10.1038/SJ.GT.3302858 [retrieved on 2006-10-05]	26
Y	the whole document	1-34

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[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

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"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
5 August 2011	16/08/2011

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	Renggli-Zulliger, N

1

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.
X	ZARATIEGUI M ET AL: "IGF1 gene transfer into skeletal muscle using recombinant adeno-associated virus in a rat model of liver cirrhosis", JOURNAL OF PHYSIOLOGY AND BIOCHEMISTRY, PAMPLONA, vol. 58, no. 3, 1 September 2002 (2002-09-01), pages 169-176, XP008123888, ISSN: 1138-7548	26
Y	the whole document	1-34

X	WO 2009/141335 A1 (PROYECTO BIOMEDICINA CIMA SL [ES]; UAB RESEARCH FOUNDATION [US]; PEREB) 26 November 2009 (2009-11-26)	26
Y	the whole document	1-34

X	PEREBOEV ALEXANDER V ET AL: "Enhanced gene transfer to mouse dendritic cells using adenoviral vectors coated with a novel adapter molecule", MOLECULAR THERAPY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 9, no. 5, 1 May 2004 (2004-05-01), pages 712-720, XP009120711, ISSN: 1525-0016, DOI: 10.1016/J.YMTHE.2004.02.006 [retrieved on 2004-04-30] the whole document	26

Y	TANIGUCHI ET AL: "Endothelial Progenitor Cell Transplantation Improves the Survival Following Liver Injury in Mice", GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, vol. 130, no. 2, 1 February 2006 (2006-02-01), pages 521-531, XP005284552, ISSN: 0016-5085, DOI: DOI:10.1053/J.GASTRO.2005.10.050 the whole document	1-34

Y	NAKAMURA ET AL: "Significance and Therapeutic Potential of Endothelial Progenitor Cell Transplantation in a Cirrhotic Liver Rat Model", GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, vol. 133, no. 1, 10 July 2007 (2007-07-10) , pages 91-107.E1, XP022135959, ISSN: 0016-5085, DOI: DOI:10.1053/J.GASTRO.2007.03.110 the whole document	1-34

	-/--	

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HUR JIN ET AL: "Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis", ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY, HIGHWIRE PRESS, PHILADELPHIA, PA, US, vol. 24, no. 2, 1 February 2004 (2004-02-01), pages 288-293, XP009107673, ISSN: 1524-4636, DOI: DOI:10.1161/01.ATV.0000114236.77009.06 the whole document	1-34
Y,P	WO 2010/109053 A1 (PROYETO DE BIOMEDICINA CIMA S [ES]; FORTES ALONSO MARIA PURIFICACION []) 30 September 2010 (2010-09-30) the whole document	1-34
Y,P	VERONICA FERNANDEZ-RUIZ ET AL: "Treatment of murine fulminant hepatitis with genetically engineered endothelial progenitor cells", JOURNAL OF HEPATOLOGY, 1 February 2011 (2011-02-01), XP55004138, ISSN: 0168-8278, DOI: 10.1016/j.jhep.2011.01.036 the whole document	1-34

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2011/059137

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009141335 A1	26-11-2009	AU 2009249682 A1	26-11-2009
		CA 2725162 A1	26-11-2009
		CN 102131931 A	20-07-2011
		EP 2304034 A1	06-04-2011
		US 2011076304 A1	31-03-2011
WO 2010109053 A1	30-09-2010	NONE	