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(54) **Title**: HYPOXIA-INDUCED EXPRESSION OF THERAPEUTIC PROTEINS IN MESENCHYMAL STEM CELLS

(57) **Abstract**: The invention relates to genetically modified mesenchymal stem cells (MSCs) comprising one or more exogenous nucleic acid molecules, wherein said exogenous nucleic acid molecules comprise a therapeutic protein-encoding region operably linked to a hypoxia-induced promoter. The invention further relates to their use in the treatment of medical conditions associated with hypoxia or hypoxic tissue, such as tumour diseases, lung diseases, gastrointestinal diseases, ischemic diseases and infectious diseases.

HYPOXIA-INDUCED EXPRESSION OF THERAPEUTIC PROTEINS IN MESENCHYMAL STEM CELLS

DESCRIPTION

The invention relates to genetically modified mesenchymal stem cells (MSCs) comprising one or more exogenous nucleic acid molecules, wherein said exogenous nucleic acid molecules comprise a therapeutic protein-encoding region operably linked to a hypoxia-induced promoter. The invention further relates to the use of said cells in the treatment of medical conditions associated with hypoxia or hypoxic tissue, such as tumour diseases, lung diseases, gastrointestinal diseases, ischemic diseases and infectious diseases, with the aim to direct the expression of the therapeutic protein to the site of hypoxia or hypoxic tissue.

In preferred embodiments the therapeutic transgene may be potentially harmful or toxic when administered or expressed systemically, such that site-directed expression under hypoxic conditions leads to the desired location of therapeutic effect, reduced off-target effects and corresponding reduction in unwanted side effects. In particular, immune-modulating agents are intended as therapeutic proteins, especially immune-modulating agents that exhibit unwanted systemic effects but show useful therapeutic properties when administered locally.

In preferred embodiments the therapeutic transgene may be potentially harmful or toxic to the producing cell, therefore the timely restriction of expression during the manufacturing of the cells is necessary to allow generation of the cell product in culture.

BACKGROUND OF THE INVENTION

Mesenchymal stem cells (MSCs) are cells of non-haematopoietic origin that reside in the bone marrow and other tissues. MSCs are commonly considered to be multipotent adult progenitor cells that have the ability to differentiate into a limited number of cell lineages, such as osteoblasts, chondrocytes, and adipocytes. Studies have been conducted on the use of MSCs as a therapeutic entity based on this capacity to differentiate directly into these end-stage phenotypes, including the use of MSCs to promote or augment bone repair and for the repair of cartilage defects (Vilquin and Rosset, Regenerative Medicine 2006: 1, 4, p 589, and Veronesi et al, Stem Cells and Development 2013; 22, p 181). The isolation and cultivation of MSCs for a number of therapeutic indications has been described and represents a promising approach towards treating inflammation-associated disorders (for example WO 2010/119039).

MSCs are known to exhibit immune evasive properties after administration to a patient. MSCs have been shown to exhibit a beneficial immune modulatory effect in cases of transplantation of allogeneic donor material (Le Blanc et al, Lancet 2004: 363, p 1439), thereby reducing a potentially pathogenic alloreactivity and rejection. Furthermore, MSCs are known to exhibit anti-tumourigenic effects, for example against Kaposi's sarcoma (Khakoo et al, J Exp Med 2006: 203, p 1235). MSCs treatment can also play a therapeutic role in wound healing. The therapeutic delivery of MSCs can be performed via systemic injection, followed by MSC homing to and

engraftment within sites of injury (Kidd et al, Stem Cells 2009: 27, p 2614). Although it is clear that MSCs have a regenerative effect on injured tissue, their use as a delivery vehicle for therapeutic proteins of interest has not yet been fully explored.

A number of medical conditions, especially tumours, are characterised by the presence of hypoxia or hypoxic regions in tissues affected by the disease. Until the present time, only limited means have been provided for delivery of therapeutic agents specifically to hypoxic tissue. Hypoxic tissue often exhibits limited blood flow, such that passive mechanisms of drug delivery, or delivery of gene expression constructs encoding therapeutic proteins without appropriate active targeting to hypoxic regions, have led to unsatisfactory results.

Hypoxia-inducible factor (HIF) is a DNA-binding transcription factor that associates with specific nuclear cofactors under hypoxic conditions to activate a number of genes involved in the adaptive response to compromised oxygen tension. Hypoxic signals are therefore local (tissue specific) and capable of inducing specific patterns of gene expression that may counter or protect the body from the hypoxic conditions.

Previous attempts have been made at treating cardiac disease with MSCs, for example by administering MSCs to differentiate into cardiac muscle cells and provide therapeutic gene function under control of hypoxia-induced promoters. For example, WO 2004/044142 A2 discloses genetically modified MSCs comprising a tissue protective polypeptide encoding gene under control of a hypoxia-induced (HI) promoter and treatment of cardiovascular disease. Phillips et al (Advanced Drug Delivery Reviews, 60:2, p160) discloses the potential application of a HIF responsive promoter in genetically modified stem cells with respect to treatment of heart failure. No disclosure is apparent of using hypoxia-induced expression of therapeutic genes, in particular immune-stimulatory agents, in alternative therapeutic settings.

EP 2 657 339 A2 and Binley et al (Gene Therapy, 6:10, p1721) disclose viral gene delivery systems that are capable of induction under hypoxic conditions. EP 2 657 339 A2 discloses for example a combination of HRP, E2F and TERT in order to provide a hypoxia-induced gene expression regulatory sequence. Neither approach describes using modified cellular vectors to introduce the hypoxia-induced element in vivo.

Earlier attempts have been conducted using MSCs as therapeutic vehicles for beneficial transgenes. Zischek et al (Annals of Surgery, 250:5, p747) and US 2012/087901 disclose genetically modified MSCs comprising a therapeutic transgene under control of the RANTES promoter. No mention is made of hypoxia-induced gene expression.

Additional experiments are known in the art using genetically modified stem cells to introduce therapeutic proteins of interest. Matsumo et al (Arteriosclerosis, Thrombosis and Vascular Biol., 25:6, p1168) discloses genetically modified MSCs expressing VEGF as a transgene and their use in the treatment of acute myocardial infarction. Ma et al (Am. J. Physiol., 287:3, pR600) discloses that thyroid hormones lead to increased HIF-1 alpha protein accumulation and thereby increased expression of hypoxia induced genes, such as erythropoietin. Dzau et al (J. Am. College of Cardiol., 46:7, p1351) discloses genetically modified MSCs comprising an HMO-1 or an AKT gene and their use in the treatment of heart disease. US 2008/095749, Jorgensen et al (Gene Therapy, 10:10, p928) and Jacobsen et al (Gene Therapy, Jan 2005, p1494) disclose MSCs and

their use in the treatment of various medical disorders, for example lung disease, autoimmune and inflammatory disease. No suggestion is evident of using hypoxia-induced expression of therapeutic proteins from an MSC cellular vehicle.

In light of the prior art, novel means are required that elicit specific and local therapeutic effects in hypoxic tissue without the unwanted side effects that often occur via systemic administration of pharmaceutical products.

SUMMARY OF THE INVENTION

In light of the prior art the technical problem underlying the present invention is to provide alternative and/or improved means for local induction of therapeutic protein expression in regions of or in proximity to hypoxic tissue with the aim to restrict expression of a therapeutic gene to a hypoxic target site, thereby preventing unwanted side effects.

This problem is solved by the features of the independent claims. Preferred embodiments of the present invention are provided by the dependent claims.

The invention therefore relates to genetically modified mesenchymal stem cells (MSCs) comprising one or more exogenous nucleic acid molecules, wherein said exogenous nucleic acid molecules comprise a therapeutic protein-encoding region operably linked to a hypoxia-induced promoter (HI promoter).

In a preferred embodiment the invention is characterised in that the therapeutic protein is potentially harmful or toxic when administered or expressed systemically. In particular in the context of tumour diseases, hypoxic regions exist within the tumour that may be infiltrated by genetically modified MSCs and subsequently induce expression of a potentially systemically harmful but locally therapeutic protein.

In one embodiment, the therapeutic transgene is characterised in that the therapeutic protein be potentially harmful or toxic to the producing cell itself, therefore the timely restriction of expression during the manufacturing of the cells is necessary to allow generation of the cell product in culture.

Proteins that may be characterised as potentially harmful or toxic when administered systemically may – in preferred embodiments – be immune-stimulating molecules, in particular such as the immune-stimulating cytokines, chemokines and antibodies described herein.

Proteins that enable an anti-tumour response, such as cytotoxic proteins, or immune-modulating agents, such as those capable of inducing or enhancing an anti-tumour immune response, may therefore be expressed specifically in the area of a tumour that exhibits hypoxia and therefore maintain reduced levels of off-target expression of said therapeutic agent. Various cytotoxic or immune-modulating agents are known to a skilled person that may be expressed with the system of the present invention for which unwanted expression (or systemic administration) is unwanted. A number of non-limiting examples are provided below.

The application of interferons or interleukins to treat cancer indications is associated with severe toxicity as very high doses have to be applied systemically to reach therapeutic meaningful concentrations locally at the site of the tumor. Using inducible promoters allows the restriction of the expression to the site where efficacy is needed.

Interferon alpha treatment is successfully applied to Melanoma patients (J Clin Oncol. 2002 Sep 1;20(17):3703-18. Mechanisms and management of toxicities associated with high-dose interferon alfa-2b therapy. Kirkwood JM1, Bender C, Agarwala S, Tarhini A, Shipe-Spotloe J, Smelko B, Donnelly S, Stover L.) Unfortunately, the systemic toxicity of this treatment may lead in some cases to the premature halting of the treatment (Pharm World Sci. 2005 Dec;27(6):423-31, Side effects of interferon-alpha therapy. Sleijfer S1, Bannink M, Van Gool AR, Kruit WH, Stoter G).

Similar observations have been made for interferon gamma (Oncol Rep. 1999 Jan-Feb;6(1):173-7. Toxicity and antitumor activity of interferon gamma alone and in combinations with TNFalpha and melphalan in isolated limb perfusion in the BN175 sarcoma tumor model in rats. Manusama ER1, De Wilt JH, Ten Hagen TL, Marquet RL, Eggermont AM.). Interferon gamma is effective in a sarcoma model in rats, but treatment is associated with significant toxicities.

High-dose interleukin-2 (IL-2, Proleukin), a highly toxic agent used in the treatment of renal cell carcinoma and melanoma, has resulted in treatment-related mortality. Although the incidence and severity of toxicities have decreased as clinicians have gained experience with this agent and implemented toxicity prevention and management strategies, high-dose IL-2 is still associated with significant morbidity (Oncology. 2002 Nov;16 (11 Suppl 13):11-20. Managing toxicities of high-dose interleukin-2. Schwartz RN1, Stover L, Dutcher J.).

Due to their ability to migrate to areas of inflammation and/or hypoxia, MSCs represent an optimal tool for the delivery of therapeutic agents. Without being bound by theory, the MSCs of the present invention represent a drug delivery tool or vehicle for effective delivery of a therapeutic agent to the site of disease.

It was particularly surprising that the hypoxia-induced promoters described herein would lead to effective transgene expression from a genetically modified MSC. Although HIF-inducible vectors are as such known in the art, it was not to be expected that MSCs would show sufficient homing, integration and/or engraftment into hypoxic tissue and sufficient expression levels of the transgene to enable a therapeutic response. The MSCs also possess the additional advantage of greater specificity than naked viral vectors with respect to their homing to tumors, and other areas of inflammation, thereby providing more precise expression after systemic administration of the MSCs than what is possible with a simple viral vector.

In a preferred embodiment of the invention the promoter or promoter/enhancer combination comprises one or more hypoxia-response elements (HREs). HREs comprise or consist of nucleic acid target sequences that physically interact with or bind to one or more transcription factors that are up-regulated (or interact with other components of a transcription factor complex) in hypoxic conditions.

The term “transcription factors that are up-regulated in hypoxic conditions” relates to transcription factors that are expressed, activated and/or stabilized, or show increased activity in any other manner, to a greater extent in hypoxic conditions compared to non-hypoxic conditions. Such up-regulation may relate to enhanced interaction with other members of a transcription factor complex in hypoxic conditions, thereby leading to enhanced activity of said transcription factor.

The result of the interaction between HI promoters and the transcription factor is transcription initiation from said promoter, in other words promoter induction, thereby driving expression of the operably linked gene. In the context of the present invention such transcription initiation leads to transcription of the nucleic acid sequence encoding the therapeutic protein selectively in hypoxic conditions, as described herein.

The term “promoter” of the present invention relates to a nucleic acid sequence capable of regulating transcription, preferably inducing transcription and/or providing a binding site for transcription inducing factors, of an operably linked nucleic acid sequence, preferably a protein coding sequence. In one embodiment the HI promoter may relate to a promoter/enhancer combination.

The term “hypoxia-induced promoter” (HI promoter) relates to a promoter that enables increased levels of transcription of an operably linked nucleic acid sequence under conditions of low oxygen (hypoxia) compared to conditions of normal oxygen saturation (normoxia), for example when oxygen saturation is reduced in vitro in cell culture to 5% or below. In a preferred embodiment the increase in transcription relates to more than 2-fold, more preferably more than 2.5-fold, increase in transcript levels in hypoxic vs normoxic conditions, as determined by microarray analysis.

In one embodiment the MSCs of the present invention are characterised in that the HI promoter is induced when a cell comprising said promoter or promoter/enhancer combination is cultured *in vitro* in conditions of equal to or less than 5% oxygen saturation, preferably less than 3%, more preferably 2%, 1% or 0.5% oxygen saturation.

In a preferred embodiment a hypoxia induced promoter is a promoter induced in vitro in cell culture at 0.5% oxygen saturation, 5% CO_2, at 37 degrees C, at 1×10^5 cells/cm^2 cell confluency in complete culture medium for 24 hours, in comparison to normoxic conditions. As a comparative value, normoxic conditions relate to 21% oxygen saturation, 5% CO_2, at 37 degrees C, at 1×10^5 cells/cm^2 cell confluency in complete culture medium for 24 hours.

These values provide an objective means of assessing the oxygen saturation reduction required for expression of a promoter of the present invention.

Hypoxia may also be simulated in vitro via chemical treatment, for example using $CoCl_2$, for example at concentrations of 50 µM, 100 µM, 300 µM or 500 µM in cell culture.

In a preferred embodiment of the invention the promoter or promoter/enhancer combination comprises one or more HREs bound by a hypoxia inducible factor (HIF), preferably hypoxia inducible factor 1 (HIF-1), more preferably HIF-1 alpha.

Hypoxia-inducible factors (HIFs) are transcription factors that respond to changes in available oxygen in the cellular environment, to be specific, to decreases in oxygen, or hypoxia. HIF-1

belongs to the PER-ARNT-SIM (PAS) subfamily of the basic helix-loop-helix (bHLH) family of transcription factors. The alpha and beta subunit are similar in structure and both contain the following domains: N-terminus (bHLH domain for DNA binding), central region (Per-ARNT-Sim (PAS) domain, which facilitates heterodimerization) and a C-terminus (recruits transcriptional coregulatory proteins).

HIFs include but are not limited to HIF-1α, HIF-1β, HIF-2α, HIF-2β, HIF-3α and HIF-3β.

Under normal oxygen tension, HIF alpha is subject to oxygen-dependent prolyl hydroxylation, which allows for substrate recognition and ubiquitylation by pVHL and its associated ubiquitin–ligase complex. Polyubiquitylated HIF alpha is degraded by the 26S proteasome. HIFs are typically expressed under normal oxygen conditions, but inactive due to their low stability.

Under low oxygen tension HIF alpha escapes prolyl hydroxylation and associates with nuclear HIF beta. The heterodimer binds to a core consensus sequence at the promoters of HIF-responsive genes, and upon binding to the coactivators p300/CBP and PKM2, initiates transcription. Stabilized HIF alpha dimerizes with HIF beta before binding to hypoxia-response elements (HREs) in the promoters of hypoxia induced genes.

In a preferred embodiment of the invention a hypoxia-response element (HREs) comprises a nucleic acid core 50-[A/G]CGTG-30 consensus sequence, but may comprise variable flanking sequences in the promoters of hypoxia induced genes.

In further preferred embodiments the HI promoters relate to those identified in Hu et al. (BMC Genomics 2014, 15:303).

Hu et al subjected human MSCs to hypoxic conditions (in cell culture at 0.5% oxygen saturation, 5% CO_2, at 37 degrees C, at 1×10^5 cells/cm^2 cell confluency in complete culture medium for 24 hours) and subsequently analyzed global gene expression by microarray and RT-qPCR analysis. 84 genes were found to be up-regulated more than 2-fold in hypoxic conditions (Table 1).

Table 1. Up-regulated genes in human MSCs under hypoxic conditions (source: Hu et al, BMC Genomics).

Gene description	Gene symbol	Fold change HX/NX
Leptin	LEP	74,30
Early Growth Response 2	EGR2	6,78
Inhibin, beta B	INHBB	6,25
Interleukin 11	IL11	5,50
Growth differentiation factor 6	GDF6	5,29
Hemopoietic cell kinase	HCK	4,82
Podoplanin	PDPN	3,65
Ephrin-A3	EFNA3	3,65
Semaphorin A7	SEMA7	2,70
Activin A receptor type IIA	ACVR2A	2,70
Matrix metalloproteinase 11	MMP11	2,52

Aggrecan	ACAN	2,50
Sparc/osteonectin (testican)	SPOCK1	2,20
Frizzled homologue-8	FZD8	3,20
Secreted frizzled related protein 4	SFRP4	2,30
Cadherin 11, type 2 (osteoblast)	CDH11	2,00
Transient receptor potential cation channel	TRPM7	6,63
Interleukin 11	IL11	5,50
Metallothionein 3	MT3	4,80
Stanniocalcin 1	STC1	4,80
Regucalcin	RGN	4,04
Vascular endothelial growth factor A	VEGFA	3,56
RAS p21 protein activator 1	RASA1	2,92
Immediate Early Response 3	IER3	2,52
Insulin-like growth factor binding protein 1	IGFBP1	8,93
Insulin-like growth factor binding protein 3	IGFBP3	3,10
Insulin-like growth factor binding protein 5	IGFBP5	3,10
Interleukin 11	IL11	5,50
Placental Growth Factor	PGF	3,28
Transcription elongation factor A3	TCEA3	2,82
Hepatocyte growth factor receptor	MET	2,70
Heparin-binding EGF-like GF	HBEGF	2,64
Jun B oncogene	JUNB	3,07
c-Jun oncogene	JUN	2,66
Inhibin, beta B	INHBB	6,25
Growth differentiation factor 6	GDF6	5,29
Inhibitor of growth family member 3	ING3	3,53
Early growth response 2	EGR2	6,78
Interleukin 11	IL11	5,50
Activated leukocyte cell adhesion molecule	ALCAM	2,89
Junctional adhesion molecule 2	JAM2	3,10
Semaphorin 5A	SEMA5A	4,02
Connective tissue growth factor	CTGF	2,40
Protein tyrosine phosphatase, receptor type, F	PTPRF	2,66
Signal-induced proliferation-associated 1 like 1	SIPA1L1	4,00
Intergrins alpha- 1, 3, 5, 6, 7	ITGA	>2.2
Integrin beta-1	ITGB	2,10
Periplakin	PPL	7,88
Desmoplakin	DSP	3,86
Keratin 16	KRT16	6,39

Keratin 14	KRT14	4,04
Keratin 20	KRT20	4,40
Keratin 19	KRT19	3,50
Keratin 15	KRT15	3,35
Keratin 13	KRT13	3,37
Keratin 24	KRT24	3,09
Keratin 17	KRT17	2,70
Keratin 12	KRT12	2,60
Mucin 1	MUC1	2,23
KIAA1199	KIAA1199	5,20
RAS p21 protein activator	RASA1	2,92
Microtubule-associated protein 1B	MAO1B	2,70
Microtubule-actin cross-linking factor 1	MACF1	2,40
Lysyl oxidase-like 4	LOX4	6,50
Lysyl oxidase-like 2	LOX2	2,50
Leptin	LEP	74,30
Stanniocalcin 1	STC1	4,70
Vascular endothelial growth factor A	VEGFA	4,61
Placental growth factor	PGF	3,28
Hepatocyte growth factor receptor	MET	2,70
PDGF receptor B	PDGFRB	2,10
PDGF receptor A	PDGFRA	2,00
Angiopoietin-like 4	ANGPTL4	2,80
Serpin peptidase inhibitor member 1	SERPINE1	4,92
Serpin peptidase inhibitor member 2	SERPINE2	2,40
Tissue plasminogen activator	PLAT	2,30
Endothelial tyrosine kinase, Ang 1 receptor	TEK	5,16
Lysyl oxidase-like 4	LOX4	6,50
Desmoplakin	DSP	3,86
Noggin	NOG	3,91
Glucose transporter member 1	SLC2A1	2,01
Glucose transporter member 3	SLC2A3	2,11
Glucose/fructose transporter member 5	SLC2A5	2,00
Glucose transporter, member 14	SLC2A14	2,88

According to Hu et al, genes were identified from the gene expression array using Genespring software. All values are significant ($p < 0.05$, $n = 3$).

Of these up-regulated genes, a number were confirmed to be HIF-1-responsive, as shown in Table 2.

Table 2. HIF-1 regulated transcripts upregulated in MSCs under hypoxic conditions (source: Hu et al, BMC Genomics).

Gene description	Gene symbol	Fold change HX/NX
Leptin	LEP	74,30
Insulin-like growth factor binding protein 1	IGFBP1	8,93
Phopshoglycerate kinase	PGK	7.10*
Lysyl oxidase-like 4	LOXL4	6,50
Carbonic anhydrase IX	CA9	5,55
Metallothionein 3	MT3	4,78
Vascular Endothelial Growth Factor A	VEGFA	4,61
Carbonic anhydrase XII	CA12	4,62
Lysyl Oxidase	LOX	3,99
Basic helix-loop-helix family member 40	BHLHE40	3,97
Adrenomedullin	ADM	3,39
Placental Growth Factor	PGF	3,28
Phosphofructokinase	PFK	3.20*
Insulin-like growth factor binding protein 3	IGFBP3	3,10
Angiopoietin-like 4	ANGPTL4	2,88
BCL2/adenovirus E1B 19 kDa interacting protein 3	Bnip3	2,17
Lactate Dehydrogenase-A	LDH-A	2,10
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH	1.48*
PhopshoGlycerate Mutase	PGAM	0.70*
Solute carrier family 2 (facilitated glucose transporter), member 1	SLC2A1 (GLUT 1)	2,01
Solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A1 (GLUT 3)	2,11

According to Hu, these genes were identified from the gene expression array and all values are significant ($p < 0.05$, $n = 3$). For genes with (*), transcripts were quantified by QPCR using 3 different preparations of hMSCs run in duplicate; (PGK $p < 0.001$; PFK, $p < 0.01$; GAPDH, $p = 0.21$; PGAM, $p = 0.02$; all $n = 7$).

In preferred embodiments the invention therefore relates to the use of the promoters of the genes listed in Tables 1 and 2 as HI promoters as described herein. A skilled person is capable of identifying the particular sequence of a promoter of any given gene using knowledge common in the art.

In further embodiments the HI promoter relates to a promoter module, wherein said promoter module comprises one or more HRE nucleic acid sequences in proximity to and/or interacting with further sequences that enable binding of transcription factors or subunits thereof, thereby enabling transcription in a hypoxia-dependent manner.

The combination of one or more HREs with one or more other regulatory promoter sequences is termed a “hypoxia-induced promoter module”.

Said one or more other regulatory promoter sequences may in some embodiments interact with other transcription factors or components of a transcription factor complex. In some embodiments these transcription factors may “partner” with, or physically interact with HIF in order to induce transcription from a HI promoter under hypoxic conditions. The transcription factors that "partner" with HIF may be constitutively expressed or induced in response to the low oxygen conditions.

The kind, location and relative distance between the HREs and other regulatory promoter sequences within the promoter may vary and is not intended to represent a limiting factor of the present invention.

One example of a HI promoter module is a combination of one or more HREs with one or more SMAD elements, thereby enabling TGF-Beta-induction of the promoter. Such promoter modules may therefore in preferred embodiments of the invention show transcription initiation under a combination of hypoxia and TGF-Beta induction/up-regulation/expression. The location and relative distance between the SMAD elements and HREs within the promoter may vary and is not intended to represent a limiting factor of the present invention.

The binding specificity of SMAD proteins was originally defined as 5′-GTCTAGAC-3′ and also shown to be 5′-GTCT-3′, or its complement 5′-AGAC-3′, called the Smad-binding element (SBE). Many Smad-responsive promoter regions contain one or more SBEs, which in some instances contain an extra base, as 5′-CAGAC-3′. The crystal structure of the MH1–SBE complex shows that Smads recognize the 5′-GTCT-3′ sequence through the β-hairpin in the MH1 domain (for further information on SMAD elements refer Massague et al, Genes & Dev 2005, 19:2783).

Further examples of an HI promoter module relate to combinations of one or more HREs with one or more binding sites for E-twenty six family transcription factors (Ets factors), one or more glucocorticoid response elements (GRE), one or more AP-1 binding sites and/or one or more GATA-2 binding sites. The combination between HIF-bound HREs and the above mentioned promoter elements lead to promoters that are induced under hypoxic conditions.

The ETS (E26 transformation-specific or E-twenty-six) family of transcription factors is unique to metazoans. All ETS family members are identified through a highly conserved DNA binding domain, the ETS domain, which is a winged helix-turn-helix structure that binds to DNA sites with a central GGA(A/T) DNA sequence.

Glucocorticoid response elements are short sequences of DNA within a gene promoter region that are able to bind specific transcription factors and regulate transcription of genes. For example under conditions of stress, a transcription activator protein binds to the response element and stimulates transcription. The glucocorticoid response element, to which the GR can bind, typically comprises the following consensus sequence: GTTACAnnnTGTTCT (SEQ ID No 8).

The activator protein 1 (AP-1) is a transcription factor which is a heterodimeric protein composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. It regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. AP-1 upregulates transcription of genes containing the TPA DNA response element (TRE; 5'-TGAG/C(N)TCA-3').

GATA2 is a transcription factor typically expressed in hematopoietic progenitors, including early erythroid cells, mast cells, and megakaryocytes, and also in nonhematopoietic embryonic stem cells. GATA-1, -2, and -3 have been shown to recognize a consensus sequence derived from regulatory elements in erythroid cell-specific genes, WGATAR (in which W indicates A/T and R indicates A/G). The consensus DNA sequence, AGATCTTA, is also recognized by GATA2. FUrther information on GATA2 binding sites are available from Ko et al (Mol Cell Biol 1993 13(7):4011).

Examples of HI promoter modules are provided below in Table 3. In further embodiments the promoter of the present invention relates to the promoter modules as described in Table 3.

The promoter module name of Table 3 relates to the combination of identified binding sites within the promoter module. “HIF” or “Ebox” relates to a recognized HRE site. Eboxes are also commonly referred to as enhancer boxes, in the present invention such Eboxes refer to enhancers comprising sequences for HIF transcription factor binding.

Table 3. Examples of HI promoter modules

Promoter Module Name	Function	Target Genes
HIF HASF	Both the HBS and HAS are present and involved as cis-elements for the activation of VEGF and EPO.	EPO, VEGFA
HIF SMAD 1	TGF-beta and hypoxia collaborate at the transcriptional level on the Epo 3’ enhancer via HIF-1 and Smad3/4 binding sites.	EPO
HIF SMAD 2	TGF-beta-dependent transcription factor SMAD4 cooperates with HIF-1 alpha to activate 4E-BP1 gene transcription under hypoxia	EIF4EBP1
HIF ETSF	Interaction between HIF-2alpha and endothelial Ets factors is involved in transcriptional activation of Flk-1 in endothelial cells	Kdr
HIF HIF GREF	Two Eboxes and one GRE are the regulatory elements responsible for Mup2 expression	Mup2
HIF HIF 2	HIF-1 binding sites HRE1 and HRE2 are required for hypoxia-induced COX4-2 promoter activity	Cox4-2
HIF HIF 1	Both CLOCK/BMAL1 and CLOCK/BMAL2 heterodimers activate the PAI-1 promoter through proximal and distal E-Box enhancers	SERPINE1
GREF HIF	Androgen-dependent gene expression of prostate-specific antigen (PSA) is enhanced synergistically by hypoxia in human prostate cancer cells. HIF-1 interacts with the AR an the human PSA gene promoter	KLK3
SMAD HIF	HIF-1 alpha cooperates with Smad3/Smad4 in activating the human VEGF prompter	VEGFA
GATA HIF AP1F	AP1 and GATA-2 play roles in stabilizing the binding of HIF-1 to the endothelin-1 hypoxia response element	EDN1
HIF SMAD NR2F	An interplay among the transcription factors mediating hypoxia (HIF-1), TGF-beta (Smads) and tissue-specific factors (HNF-4) occurs in the 3’ EPO enhancer through direct protein interaction	EPO

SF1F HIF	CLOCK-BMAL1, LRH-1 and SHP regulate the mSHP gene to generate the circadian oscillation	Nr0b2
ETSF HIF	Interaction between HIF-2alpha and endothelial Ets factors is required for the transcriptional activation of Flk-1 in endothelial cells	Kdr

Further examples of specific HI promoter modules are provided below in Table 4. In further embodiments the promoter of the present invention relates to the promoter modules as described in Table 4.

Table 4. Specific examples of HI promoter modules

Promoter Module Name	Gene	Sequence	Accession No.	SEQ ID No
ETSF HIF	Mouse vascular endothelial growth factor receptor-2 (Flk-1, Kdr).	GCCGCCCGGCACAGTTCCGGGG TAGTGGGGGAGTGGGCGTGGGA AACCGGG	AF153057	1
HIF SMAD 2	Human eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1).	GCGGGGGATCCCACGTGGAAGC AGCGTCCTGGAGCTGGGTGGGG CTGCGGCGCGGACTACAAATCCC AGGGGCGTGGGGCGGGAGAGGC GGAAGGGGCGTCCTGGGGCGGG GCGGCACGGGGCGAGGCGGAGC GAGGCTGGAGGCGCGGGAGGGC AGCGAGAGGTTCGCGGGTGCAG CGCACAGGAGACCATGTCCGGGG GCAGCAGCTGCAGCCAGACCCCA AGCC	AP006545	2
HIF SMAD 1	Human eritropoietin (EPO).	GGCTGGGCCCTACGTGCTGTCTC ACACAGCCTGTCTGACCTCTCG	M11319	3
HIF ETSF	Mouse vascular endothelial growth factor receptor-2 (Flk-1, Kdr).	ATCCAGTGGGGGGCGTGGCCGG ACGCAGGGAGTCCCCA	AF153057	4
SMAD HIF	Human vascular endothelial growth factor gene (VEGF)	GGTTTTGCCAGACTCCACAGTGC ATACGTGGGCTCCAACA	AF095785	5
HIF HIF	Human plasminogen activator inhibitor 1 (PAI-1,SERPINE1)	GAGAGTCTGGACACGTGGGGGA GTCAGCCGTGTATCATCGGAGGC GGCCGGGCACATGGCAGGGATG AGGGAAAGACCAAGAGTCCTCTG TTGGGCCCAAGTCCTAGACAGAC AAAACCTAGACAATCACGTGGCT GGCTGCA	X13323	6

HIF HIF 2	Human cytochrome c oxidase subunit M isoform 2 (COX4-2)	GCGCTCCCACGCCCTGCCGAAGC AGGACGTTCCCACGCTGGGGCG GCCCCGAGAAAGGCTGTGGGGC GGGCCGGGGGCGGGCGCCCGCA CTCAGGTCCCTCCGCAGCGGGTT CTCAGTTGCTCGCTGGGCAGACC CAGGTCGCGCTCCCACTGCCGAG CCCGCGAGGTGAGTGAGTTGGG GGAGGACCCCGCCACCCAAGCC CGCGCGGGTCAGGGGCGCGGCT CGGTCTCCTGCCTTCGCCGCTGG GTCACGCCCCAGTGGTCCCTGTC TTAGAGGGTTGGGGGGCTTCTTG TAGGTTAGAGTTGAAGACTCCTG GGGAGTCTGCATATGTGTGCCAT CTCTTTCCCCAAATATAGATTACA TCCTCCCTCTCTTGATATGTGGAG CTCTCCCACCCACTAATCATAATC ACTGGCCGGAGCCCCTCTTTTCC AGAAGTGTGGGCCGCCCCCTCCC ACTTATCACCTGTAGTGTGCCCCC TCCCTCACTCTATTTGTTGGGTGC CCCTCTCTCTCCCTAGATGTGGG ACTCCCCTTCTTTTCCCCAGATGT GTGTCTCCCTCCCTCTGACCCAG TTAAGTCCCCATCCTCTAAAGCCC CTCCCCACCACCATGGATGCCAA ACCCTGAGTCTTCCCTGGGGGTG GGAGACTGGGCTCAGCCAGACTG GCTAAGCCCAGTCCCCACCCCAA CCAGATCCAGCAGAGCAGGCCTG TGTGCACGTAGGCAGCCAATT	NG_012180	7

In one embodiment the MSCs of the present invention are characterised in that the promoter of the exogenous nucleic acid driving expression of the therapeutic gene comprises or consists of one or more endogenous hypoxia-induced promoter sequences, preferably a single endogenous hypoxia-induced promoter.

As preferred embodiments the invention encompasses the use of the promoter of the following human genes: EPO, VEGFA, 4E-BP1, Flk-1, Kdr, Mup2, Cox4-2, SERPINE1, KLK3 or EDN1. Further preferred embodiments relate to the promoters of the human genes VEGF, IL-6, SDF-1, FGF-beta und SLC2A1.

Previous attempts at using HREs in artificial promoters have led to potentially disadvantageous promoter constructs, whereby arrays of multiple HREs have been placed in adjacent positions in the promoter regions of the genes to be expressed. Such artificial HI promoters lead to potentially high levels of expression and potentially unwanted enhanced sensitivity to hypoxic conditions. This enhanced activity and/or sensitivity is desired in some embodiments of the invention, for example if the hypoxic conditions intended as a gene expression induction signal (associated with a particular disease-associated hypoxic region) are known to be relatively mildly hypoxic. Such enhanced activity and/or sensitivity is undesired in some embodiments of the invention, for example if the hypoxic conditions intended as a gene expression induction signal (associated with a particular disease-associated hypoxic region) are known to be relatively strongly hypoxic, so

that strong overexpression of the protein would be undesirable. The specific promoter to be applied can be selected by a skilled person, with respect to promoter strength, sensitivity to hypoxia, presence or absence of arrays, size, clonability, or other factors.

Arrays of multiple repeated sequences such as in an HRE array in a promoter may also exhibit increased frequencies of recombination between the sequences, or perhaps other similar sequences, thereby representing an additional risk factor with respect to HRE-array promoters.

Endogenous HI promoters are preferred in the present invention.

In some cases embodiments endogenous promoters may be modified, thereby creating a synthetic promoter, defined herein as a “modified endogenous promoter”, without introducing an array of HREs in adjacent positions.

According to further embodiments of the invention promoters comprising three or more HREs, or four or more HREs, such as three or four or more HREs in adjacent positions, are not encompassed by the present invention. Such arrangements relate to HRE arrays and are in some embodiments of the invention disadvantageous due to the reasons provided herein.

According to one embodiment of the invention a modified endogenous promoter relates to a modified HI promoter or promoter module comprising three or fewer HREs, preferably 1 or 2 HREs.

In one embodiment the MSCs of the present invention are characterised in that said mesenchymal stem cell comprises no additional exogenous nucleic acid molecules. Earlier experimental approaches towards using MSCs as a medicament involved the application of immortalized MSCs, in which additional genetic modifications were carried out in order to maintain enhanced replication of the MSCs in cell culture.

In one embodiment the MSCs of the present invention are characterised in that said mesenchymal stem cell is a patient-derived cell, a cell in primary cell culture or a cell in sub-culture after passaging from primary culture, wherein fewer than 5 passages from primary culture have occurred before introduction of the exogenous nucleic acid into the cells.

The introduction of exogenous nucleic acids comprising hypoxia-induced promoters poised for expression of a therapeutic gene into patient derived cells has not been described previously for all the embodiments described herein. The cells as described herein exhibit beneficial properties in cell culture, with respect to stability of the genetic modification and ease of expanding sufficient quantities of the cells, and in therapy. The introduction of such a genetic construct, without compromising the beneficial properties of the cells, represents an advantageous and unexpected development over the art.

The modified MSCs of the present invention avoid and/or minimize potential side effects due to systemic administration of therapeutic protein or therapeutic protein-encoding nucleic acid vectors. The use of MSCs as vehicles for administration of therapeutic proteins provides local production of the protein of interest in diseased regions of the body due to the homing capabilities of MSCs towards hypoxic and/or inflamed tissue.

Methods for the genetic modification of MSCs are known to those skilled in the art. Examples of suitable methods for genetic modification of MSCs are disclosed in WO 2010/119039 and WO 2008/150368.

In one embodiment the genetically modified mesenchymal stem cell as described herein is characterized in that the exogenous nucleic acid comprises a viral vector, for example in the form of a viral expression construct, more preferably a retroviral vector.

In one embodiment the genetically modified mesenchymal stem cell as described herein is characterized in that the exogenous nucleic acid is or comprises a non-viral expression construct.

In a further aspect the invention relates to the genetically modified mesenchymal stem cell as described herein for use as a medicament.

One embodiment of the invention is directed to the MSCs as described herein for use as a medicament in the treatment of a medical condition associated with hypoxia and/or hypoxic tissue. Medical conditions characterised by hypoxia and/or hypoxic tissue are described in more detail below.

In a further embodiment the invention relates to the use of the MSCs as described herein as a medicament in the treatment of a medical condition associated with hypoxia and/or hypoxic tissue, wherein said cells migrate towards and/or integrate into hypoxic tissue or in proximity to hypoxic tissue. It was a surprising finding that MSCs migrate towards hypoxic signals in vivo. This finding represents a novel and advantageous aspect of the invention that has been suggested in the prior art. The presence of the hypoxia-induced promoter in MSCs therefore provides a synergistic effect, whereby the combination of the hypoxia-induced MSC-migratory properties with the hypoxia-induced expression of the therapeutic gene provides precise local disease-specific expression of the therapeutic agent. This combination leads to reduced “off-target” effects that may be induced by unwanted production of the therapeutic protein outside the diseased tissue.

In a further embodiment the invention relates to the use of the MSCs as described herein as a medicament in the treatment of a medical condition associated with hypoxia and/or hypoxic tissue and inflammation associated with said hypoxia and/or hypoxic tissue.

A number of hypoxia-related medical conditions have been identified that exhibit regions of combined hypoxia and inflammation, for example tumour diseases, inflammatory bowel disease, lung diseases, such as lung conditions involving reduced air-flow and therefore oxygen-deprivation, ischemic disease and infectious diseases.

As described by Eltzschig et al (N Engl J Med. 2011; 364(7): 656–665) and Bartels et al (PNAS, vol. 110 no. 46, 18351–18352), Hypoxia and inflammation share an interdependent relationship. Hypoxia-elicited inflammation has been identified in various clinical settings, or inflammation during hypoxic conditions in the outcomes of a wide array of human diseases. Inflammatory disease states are frequently characterized by tissue hypoxia, or stabilization of hypoxia-dependent transcription factors, such as hypoxia-inducible factor (HIF).

For example, intestinal inflammation, such as occurs during inflammatory bowel disease, is characterized by the occurrence of severe hypoxia of the mucosal surface, and concomitant stabilization of HIF. Stabilization of HIF1 alpha during intestinal inflammation is caused by alterations in metabolic supply and demand ratios, particularly for oxygen, leading to "inflammatory hypoxia".

Similarly, lung inflammation, such as occurs during acute lung injury, is associated with metabolic alterations leading to the stabilization of HIF1 alpha. On the other hand, disease conditions that are primarily caused by a lack of oxygen are characterized by secondary inflammatory changes. For example, ischemia and reperfusion injury is characterized by inflammatory responses that lead to subsequent organ dysfunction.

Concentrations of oxygen in solid tumours, as compared with those in normal tissues, are frequently reduced. Solid tumours contain increased levels of HIF-1α and HIF-2α, and these elevated levels correlate with cancer-related death. Elevated levels of HIF-1α and HIF-2α in biopsy specimens of prostate tumours have also been detected and are associated with an adverse clinical course. Hypoxia in a solid tumour stabilizes HIF through hypoxia-dependent inhibition of PHDs.

Stabilization of HIF and induction of HIF-dependent genes occur during infections with pathogens. For example, infection with Bartonella henselae — the causative agent of bacillary angiomatosis — is associated with stabilization of HIF-1α and the transcription of genes that typically become transcribed in hypoxic conditions. In infected cells, changes in oxygen consumption, as well as cellular hypoxia and decreased ATP levels, correlate with HIF stabilization and the release of angiogenic factors during bacillary angiomatosis. Stabilization of HIF during infections can also be oxygen-independent. For example, under normoxic conditions, iron uptake by bacteria attenuates PHD activity, stabilizes HIF-1α, and induces the expression of genes targeted by HIF. Stabilization of HIF-1α has been found in liver-biopsy specimens obtained from patients with chronic hepatitis C and in skin-biopsy specimens obtained from patients with cutaneous infections caused by Staphylococcus aureus, varicella–zoster virus, human herpesvirus, or Candida albicans.

Pathogens may highjack the host's HIF pathway for their own advantage. Pseudomonas aeruginosa rapidly inactivates the adenosine that host cells produce in an HIF-dependent manner, thus depriving the host epithelium of the actions of extracellular adenosine signalling that promote intestinal barrier function during inflammation and hypoxia. During infection with group A streptococcus or P. aeruginosa, HIF-1α in immune cells induces inflammation that helps to eliminate the pathogen. In mice lacking HIF-1α, bactericidal activity is decreased in myeloid cells, and the systemic spread of infection cannot be contained.

A further aspect of the invention relates to the use of the MSCs as described herein as a medicament, wherein one or more thyroid hormones, preferably T3 and/or T4, are co-administered with said stem cells. As described in the examples below, the administration of T3 and T4 thyroid hormones leads to enhanced stability of HIF-1 alpha transcription factor under hypoxic conditions, in addition to enhanced hypoxic-driven migratory properties of the MSCs as described herein. This combined administration provide synergistic effects with respect to the

properties of both the MSCs and the TFs required for expression of the inducible promoter, providing unexpected benefits in expression of the protein of the present invention.

It was surprising, in light of the prior art, that the expression of the inducible promoters mentioned herein led to sufficient expression of the therapeutic protein upon appropriate stimulus at the site of hypoxia. The promoters provided herein show suitable inducible properties for rapid and sufficiently high expression of therapeutic protein upon entering into proximity with hypoxic tissue.

One aspect of the invention therefore relates to the MSCs as described herein for use as a medicament in the treatment of a tumour disease.

In one embodiment the MSCs of the present invention are characterised in that the therapeutic protein is a cytotoxic protein. Cytotoxic proteins are preferably selected from the group consisting of Herpes simplex viral thymidine kinase (HSV-tk) and cytosine deaminase (CD). Such embodiments relate preferably to co-administration of the MSCs with gangciclovir (for HSV-tk) or 5-fluorocytosine (for CD), according to known principles regarding the activation of the pro-drugs. The invention therefore relates to a local induced expression of the cytotoxic protein after HI promoter activation, thereby leading to activation of the gangciclovir or 5-fluorocytosine prodrug specifically in hypoxic tissue.

In one embodiment the MSCs of the present invention are characterised in that the therapeutic protein is a more immune response-stimulating or immune response-modulating cytokine and/or chemokine. This embodiment is particularly relevant in the treatment of tumour diseases.

In further embodiments of the invention the immune response-stimulating or immune response-modulating cytokine is selected from the group consisting of IL-2, IL-7, IL-12, IL-15, IL-21, IFN gamma and IFN beta. It is known that the various cytokines are potent anti-tumour agents. Rakhmilevich et al. have shown that IL-12 can be used to treat melanoma and mastocytoma in an in vivo model (Rakhmilevich AL, Timmins JG, Janssen K, Pohlmann EL, Sheehy MJ, Yang NS, J Immunother. 1999; 22 (2): 135-44). Furthermore, Ehtesham et al. have shown that interferone can be successfully used to treat glioma in an animal model. (Ehtesham M, Samoto K,Kabos P, Acosta FL, Gutierrez MAR, Black KL and Yu JS, Cancer Gene Therapy (2002) 9, 925–934 Treatment of intracranial glioma with in situ interferon-gamma and tumor necrosis factor-alpha gene transfer).

However, despite recent advances in cytokine-induced enhancement of anti-tumour immune responses (either innate, or instigated via additional immune therapies), unwanted side effects can occur via the systemic administration of immune modulating agents, in particular immune stimulating cytokines.

In one embodiment the invention encompasses the use of MSCs described herein as a medicament in the treatment of a tumour and/or tumour disease, for example by modulating the tumour microenvironment in order to attract immune effector cells and facilitate their activation and/or adoption of a memory phenotype.

In one embodiment the invention relates to a genetically modified mesenchymal stem cell for use as a medicament in the treatment of a tumour, wherein said MSCs comprise one or more exogenous nucleic acid molecule(s), wherein said exogenous nucleic acid molecule(s) comprise

a region encoding one or more immune response-stimulating cytokine(s) operably linked to a hypoxia-induced promoter (HI promoter).

One aspect of the invention relates to the use of the MSCs described herein in an anti-tumour treatment comprising the combined administration of said mesenchymal stem cells with an immunotherapy. One example of an immunotherapy is the administration of checkpoint inhibitors, including for example antibodies against CTLA-4, PD-1, PD-L1 and other immune co-stimulatory molecules, immune cells, for example T cells, such as T cells with artificial T cell receptors, for example a chimeric antigen receptor (CAR-Ts) or exogenous T-Cell Receptor (TCR) transduced cells, NK cells or macrophages/monocytes, or active immunotherapeutic drugs, for example, tumour antigens, patient-derived tumour material and other therapeutic drugs aiming to activate and/or direct the immune response against a tumour, or features of a tumour.

The present invention enables the stimulation of cells involved in an anti-tumour immune response and thereby the local activation, support and/or strengthening of an anti-tumour immune response. The MSCs as described herein migrate to tumour tissue due to either their natural or an engineered capacity for homing to areas of inflammation in vivo. The homing to and/or engraftment into tumour tissue (tumour stroma) leads to local expression of immune stimulating cytokines, thereby creating increased amounts and/or activities of immune cells in the local tumour environment, thereby enabling the immune system of the patient to attack tumour cells and also providing support for a combined immunotherapy.

A crucial limitation in the successful development and clinical use of immunotherapies is the ability of tumours to evade and suppress the natural immune response against the tumour cells, by establishing an immunosuppressive tumour microenvironment, This phenomenon is known as tumour-mediated immunosuppression and is mediated to a large extent by the secretion of anti-inflammatory cytokines by immune cells present in the tumour that display a regulatory phenotype (for example, Regulatory T-cells; TRegs and Monocyte-Derived Suppressor Cells; MDSCs). The invention therefore provides means to modify the tumour microenvironment, making it pro-inflammatory, promoting the activation of immune cells present in the tumour and recruitment and activation of external immune cells and thereby facilitating the broad activation of the immune system against the tumour and/or enhance the efficacy of anti-tumour immunotherapeutic treatments.

Unfortunately, systemic application of these recombinant cytokines leads in many cases to significant toxicity which prevents their application in clinical routine. In the present invention, it was surprising to find that use of a hypoxia-inducible promoters in MSCs allow expression of therapeutic meaningful cytokine amounts at the site of tumour, while at the same time systemic concentrations of the cytokines do not reach toxic levels.

In further embodiments of the invention the immune response-stimulating or immune response-modulating cytokine is selected from the group consisting of chemokine (C-C motif) ligand 2, 1, 17, 22 (CCL2, CCL1, CCL17, CCL22, stromal cell-derived factor 1 (SDF-1; CXCL12), chemokine (C-C motif) ligand 23 (CCL23; Macrophage inflammatory protein 3 (MIP-3)), chemokine (C-C motif) ligand 19 (CCL19; EBI1; ELC or macrophage inflammatory protein-3-beta (MIP-3-beta)) and chemokine (C-C motif) ligand 4 (CCL4; Macrophage inflammatory protein-1β (MIP-1β)).

Surprisingly, the MSCs modified with one or more immune response stimulating cytokine(s) as described herein show unexpectedly good expression and secretion of said cytokines both in vitro and in vivo. A skilled person would not expect that these particular cytokines could be expressed in sufficient quantities and exported from the cells in sufficient quantities to induce or enhance the desired local immune response, based on either the innate response or and immunotherapy. The present invention, with respect to using immune response-stimulating or immune response-modulating cytokines and/or chemokines as a therapeutic protein, enables the stimulation of cells involved in an anti-tumour immune response and thereby the local activation, support and/or strengthening of an anti-tumour immune response. The MSCs as described herein migrate to tumour tissue due to either their natural or an engineered capacity for homing to areas of inflammation in vivo. The homing to and/or engraftment into tumour tissue (tumour stroma) leads to local expression of immune stimulating cytokines, thereby creating increased amounts and/or activities of immune cells in the local tumour environment.

The present invention therefore makes use of MSCs as a cellular vehicle for the delivery of immunomodulatory effectors for simulating an immune response, thereby utilising the unique homing abilities of MSC to target regions of inflammation, in particular tumours, and thereby exert local therapeutic effects based on activation of an appropriate immune response, wherein the immune response relates preferably to the natural immune response of a host (subject), and thereby enhance the efficacy and therapeutic effect of immunotherapeutics, such as bi-specific antibodies, adoptive immunotherapies, anti-tumour vaccines and/or checkpoint inhibitors.

The invention also encompasses the administration of a combination of immune activating cytokine and chemokines via the MSC-based approach described herein, with the aim to attract immune effector cells, induce immune activation, promote the maturation of memory immune cells and/or suppress the emergence of suppressive and/or regulatory immune cells.

The invention also encompasses the expression of a combination of immune activating cytokine and/or chemokines in tumours via the MSC-based approach described herein, with the aim to attract immune effector and helper cells, induce immune activation, promote the maturation of memory immune cells and/or suppress the emergence and persistence of suppressive and/or regulatory immune cells.

In one embodiment, a combination of cytokines is used under control of a hypoxia-induced promoter, in order to promote the activation of different arms of the immune response, including the innate and adaptive immune response, effector, helper and/or antigen presenting cells.

It is envisioned that cytokines such as TNF-alpha will activate multiple aspects of the immune system and that this effect may however lack the necessary specificity for an anti-tumoral response.

On the other hand, IL-2, IL-7, IL-15 and IL-21 specifically activate cytotoxic lymphocytes such as T-cells and NK cells that mount a specific response against tumour cells. Likewise, IL-12 will activate cytotoxic lymphocytes, but also monocytes and helper cells.

The combination of IL-12, for example, with IL-2, IL-7, IL-15, and/or IL-21, will have the effect of activating (i) tumour-directed cytotoxic cells, (ii) helper cells that enhance the activation of

cytotoxic cells and/or (iii) monocytes that can develop an additive immunological response against the tumour. A combination of cytokines therefore yields synergistic effects, as is seen in the natural immune response, and in the present invention greatly increases the therapeutic efficacy. It was a surprising result that the natural immune response could, in effect, be mirrored, or analogously applied, in an enhanced manner using a MSC-based transgenic approach in which the cytokines were expressed under control of a hypoxia-induced promoter.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the exogenous nucleic acid comprises a region encoding two or more immune response-stimulating cytokines operably linked to one or more hypoxia induced promoters, wherein the cytokines are selected from the group consisting of IL-2, IL-7, IL-12, IL-15, IL21, IFN gamma and IFN beta.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the two or more immune response-stimulating cytokines comprise at least IL-12, and one or more of IL-2, IL-7, IL-15, and/or IL-21.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the two or more immune response-stimulating cytokines comprise least IL-7 and IL-21.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the two or more immune response-stimulating cytokines comprise at least one chemokine and at least one immune response-stimulating cytokine is selected from the group consisting of IL-2, IL-7, IL-12, IL-15, IL21, IFN gamma and IFN beta.

The present invention encompasses in some embodiments the combination of cytokine transgenes in the cells as described herein, in particular any given specific combination of individual cytokines or chemokines disclosed herein, preferably any given specific combination of two or more of -2, IL-7, IL-12, IL-15, IL21, IFN gamma, IFN beta, CD28, chemokine (C-C motif) ligand 1, 2, 4, 17, 19, 22, 23 (CCL1, CCL2, CCL4, CCL17, CCL19, CCL22, CCL23), stromal cell-derived factor 1 (SDF-1).

The MSCs defined by two or more immune response-stimulating cytokines and the method of tumour treatment comprising administration of MSCs defined by one or more immune response-stimulating cytokines in combination with other anti-tumour immunotherapies are bound by the surprising and beneficial concept of local immune system stimulation in an anti-tumour immune response, either by the innate immune system or by combined immunotherapies. It was unexpected that MSCs encoding transgenic immune-stimulating cytokines under control of a hypoxia-induced promoter may be used as an effective anti-tumour adjuvant in stimulating an anti-tumour response when expressed conditionally under hypoxic conditions.

The related prior art teaches only the use of MSC vehicles for the local administration of (transgenic) cytotoxic agents, with a direct effect. However, the use of MSCs encoding (potentially multiple) transgenic immune-stimulatory cytokines, or the combination of such MSCs with anti-tumour immunotherapies, to boost the local anti-tumour immune response, represent special technical features of the invention. The invention provides suitable means for local

stimulation of an immune response directed against hypoxic tumour tissue in a subject. This includes the natural immune response of the patient and immunotherapeutic treatments aiming to direct the immune response against the tumour (e.g. checkpoint inhibitors, CARTs against tumour antigens and other tumour immunotherapies). Such support or induction of the immune response may in various clinical settings be beneficial in order to initiate and maintain the immune response and evade the tumour-mediated immunosuppression that often blocks this activation.

In one embodiment the MSCs of the present invention are characterised in that the therapeutic protein is the sodium iodide symporter (NIS). As demonstrated in the experimental examples below, the NIS has been used as a model system for assessing induction of a HI promoter under hypoxic conditions.

The NIS represents a therapeutic protein for the treatment of tumour diseases. Endogenous NIS is expressed at the basolateral membrane of thyroid follicular cells and transports iodide actively into the thyroid. In combination with radioiodine treatment, the treatment of a patient with MSCs comprising NIS as the therapeutic gene enables MSC recruitment to tumours, HIF1-dependent promoter activation in tumours due to the local hypoxic conditions, local expression of NIS and selective uptake of radioiodine in tumours, thereby providing therapeutic efficacy with reduced side effects due to the local expression of the NIS.

In one embodiment the MSCs of the present invention are characterised in that the therapeutic protein is not the sodium iodide symporter (NIS).

In one embodiment the invention is directed towards the expression of a therapeutic antibody from the genetically modified MSCs described herein under the control of a hypoxic-induced promoter, as described herein. Antibodies represent effective therapeutic agents, in particular with respect to the treatment of cancer cells in a tumour environment. Despite showing antigen-specificity, therapeutic antibodies may bind their target in areas of the body where their action is unwanted, thereby leading to unwanted side effects. Monoclonal antibodies (mAbs) are established as targeted therapies for malignancies, transplant rejection, autoimmune and infectious diseases, as well as a range of new indications. However, administration of mAbs carries the risk of immune reactions such as acute anaphylaxis, serum sickness and the generation of antibodies (Nature Reviews Drug Discovery 9, 325-338, April 2010).

Additional means are therefore required to enable targeted expression of antibody-based therapeutics in regions of interest, such as in hypoxic tissue. The present invention therefore enables the hypoxia-specific induction of expression of an antibody-encoding transgene from the MSCs of the invention as described herein. Various therapeutic antibodies are known to a skilled person and may be selected by a skilled person without undue effort. For example, those antibodies that bind to cancer antigens may be used to treat cancer in the context of the modified MSCs described herein. Cell surface receptors are common targets for antibody therapies and include, for example, CD20, CD274, and CD279. Once bound to a cancer antigen, antibodies can induce antibody-dependent cell-mediated cytotoxicity, activate the complement system, or prevent a receptor from interacting with its ligand, all of which can lead to cell death. Multiple antibodies are approved to treat cancer, including Alemtuzumab, Ipilimumab, Nivolumab, Ofatumumab, and Rituximab

In one embodiment of the invention the tumour to be treated is metastatic.

In one embodiment of the invention the tumour to be treated is a solid tumour.

In one embodiment of the invention the tumour to be treated comprises a hypoxic region.

The tumour to be treated is preferably selected from the group consisting of a prostate tumour, a breast tumour, a pancreatic tumour, a squamous cell carcinoma, a breast tumour, a melanoma, a basal cell carcinoma, a hepatocellular carcinoma, testicular cancer, a neuroblastoma, a glioma or a malignant astrocytic tumour such as glioblastma multiforme, a colorectal tumour, an endometrial carcinoma, a lung carcinoma, an ovarian tumour, a cervical tumour, an osteosarcoma, a rhabdo/leiomyosarcoma, a synovial sarcoma, an angiosarcoma, an Ewing sarcoma/PNET and a malignant lymphoma.

In one embodiment of the invention the tumour to be treated is not hepatocellular carcinoma.

One aspect of the invention therefore relates to the MSCs as described herein for use as a medicament in the treatment of an ischemic disease.

Ischemic diseases are preferably selected from the group consisting of circulatory disorders, cardiovascular disease, artery or blood vessel conditions and ischemic obstructive or occlusive diseases or conditions, such as stroke, myocardial infarction or peripheral occlusive disease.

In particular with respect to the treatment of ischemic disease, the invention encompasses embodiments in which the therapeutic gene is VEGF, Erythropoietin or PDGF.

Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. The primary role of erythropoietin is an essential hormone for red cell production. Erythropoietin, also known as EPO, is a glycoprotein hormone that controls erythropoiesis, or red blood cell production. It is a cytokine for erythrocyte precursors in the bone marrow. The primary role of erythropoietin is an essential hormone for red blood cell production. Platelet-derived growth factor (PDGF) is a growth factor that regulates cell growth and division. In particular, it plays a significant role in blood vessel formation (angiogenesis), the growth of blood vessels from already-existing blood vessel tissue.

One aspect of the invention therefore relates to the MSCs as described herein for use as a medicament in the treatment of a lung disease. The lung disease is preferably an inflammatory disease of the lung, an acute lung injury, chronic obstructive pulmonary disease including chronic bronchitis, emphysema, bronchiectasis and bronchiolitis, acute respiratory distress syndrome, asthma, sarcoidosis, hypersensitivity pneumonitis and/or pulmonary fibrosis.

Genetically modified mesenchymal stem cell for use as a medicament in the treatment of a lung disease according to the preceding claim, wherein the therapeutic gene is Alpha-1 antitrypsin (AAT) or HGF.

Alpha-1 antitrypsin (also known as A1AT, AAT, PI, SERPINA1) is a ~52kDa glycoprotein, first described in 1906, that is one of the most abundant endogenous serine protease inhibitors (SERPIN superfamily). AAT is considered to be an acute phase protein, whereby AAT

concentration can rise many fold upon acute inflammation. AAT is capable of protecting tissues from enzymes of inflammatory cells and can reduce the production of inflammatory cytokines. Due to its anti-inflammatory properties AAT may be employed in the treatment of a number of inflammatory diseases. Hepatocyte growth factor/scatter factor (HGF/SF) is a paracrine cellular growth, motility and morphogenic factor. It is secreted by mesenchymal cells and targets and acts primarily upon epithelial cells and endothelial cells, but also acts on haemopoietic progenitor cells. It has been shown to have a major role in embryonic organ development, specifically in myogenesis, in adult organ regeneration and in wound healing.

One aspect of the invention therefore relates to the MSCs as described herein for use as a medicament in the treatment of an inflammatory bowel disease. The inflammatory bowel disease is preferably selected from the group consisting of Crohn's disease (CD), ulcerative colitis (UC), collagenous colitis, lymphocytic colitis, diversion colitis and Behçet's disease.

Genetically modified mesenchymal stem cell for use as a medicament in the treatment of inflammatory bowel disease according to the preceding claim, wherein the therapeutic gene is an anti-inflammatory cytokine, a protein that enhances endothelial cell growth, preferably vascular endothelial growth factor, a protein that modulates the immune function defensines, IL1RA or TLR antagonists.

The interleukin-1 receptor antagonist (IL-1RA) is a protein that in humans is encoded by the IL1RN gene. IL-1RA is a member of the interleukin 1 cytokine family. IL1Ra is secreted by various types of cells including immune cells, epithelial cells, and adipocytes, and is a natural inhibitor of the pro-inflammatory effect of IL1β. This protein inhibits the activities of interleukin 1, alpha (IL1A) and interleukin 1, beta (IL1B), and modulates a variety of interleukin 1 related immune and inflammatory responses. Toll-Like Receptors (TLRs) share common structures and signalling that leads to NF-κB activation. A number of substances are known to function as TLR antagonists, such as ligands that bind to the TLRs and block the signalling cascade. Such molecules are known for TLR4 and TLR9, for example neutralizing antibodies directed against extracellular TLRs.

One aspect of the invention therefore relates to the MSCs as described herein for use as a medicament in the treatment of an infectious disease, wherein the therapeutic gene is an anti-infective protein. The infectious disease is preferably selected from the group consisting of a bacterial, viral and/or fungal infection of a subject.

According to the present invention the anti-infective protein is selected from the group consisting of a defensin, bacteriocin, lactacin, Pokeweed Antiviral Protein (PAP), Zinc-Finger Antiviral Protein (ZAP) and/or IFITM3.

Defensins are small cysteine-rich cationic proteins found in both vertebrates and invertebrates. They have also been reported in plants. Defensins are generally considered to be host defense peptides. They are active against bacteria, fungi and many enveloped and non-enveloped viruses. They consist of 18-45 amino acids including six (in vertebrates) to eight conserved cysteine residues. Cells of the immune system contain these peptides to assist in killing phagocytosed bacteria, for example in neutrophil granulocytes and almost all epithelial cells. Most defensins function by binding to the microbial cell membrane, and, once embedded, forming

pore-like membrane defects that allow efflux of essential ions and nutrients. For example, defensin sequences may be obtained from the Defensins knowledgebase, a manually curated database and information source focused on the defensins family of antimicrobial peptides (refer Seebah et al, Nucleic Acids Res. Jan 2007; 35(Database issue): D265–D268).

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of bacteria. They are typically considered to be narrow spectrum antibiotics. For example, bacteriocin sequences may be obtained from BACTIBASE, a web-accessible database for bacteriocin characterization (Hammami et al, BMC Microbiology 2007, 7:89).

The pokeweed plant is a common plant that is often used as a dye; it is also eaten when the plant is young and tender. Studies and experiments have shown that a specific protein, the Pokeweed Antiviral Protein (PAP), may be used as a therapeutic protein in T-cell leukemia, lymphoma, Hodgkin's lymphoma, and AIDS. Suitable PAP sequences are disclosed, for example, in Kataoka et al, Plant Mol Biol. 1992 Dec;20(5):879-86. The Zinc-Finger Antiviral Protein (ZAP) is a specific antiviral protein that aids in the destruction of virus particles, specifically the Moloney murine leukemia virus (MLV) and the Sindbis virus (SIN). Suitable examples of ZAP are disclosed, for example, in Lee et al, Proc Natl Acad Sci U S A. 2013 Jul 23;110(30):12379-84. IFITM3 is an antiviral protein that can slow and prevent viruses from infecting and spreading. These proteins are involved in the immune system in humans, some of the proteins also represent interferons. Anti-HIV activity has been demonstrated, as has an effect against influenza (including influenza A virus subtype H1N1), West Nile virus and dengue virus.

The MSCs of the present invention, particularly with respect to the use of anti-infective therapeutic proteins, are suitable for treatment of infections in bradytrophic tissue, for example tissue for which poor blood circulation is available, or of infections not easily accessed by systemic administration of an antibiotic. For example, osteomyelitis in the bone marrow of a patient may be treated using the MSC described herein. The treatment of granulomatous infections and inflammation is also encompassed by the present invention, such as tuberculosis. Granuloma is an inflammation found in many infectious diseases. Granulomas typically form when the immune system attempts to wall off substances that it perceives as foreign but is unable to eliminate. Such substances include infectious organisms such as bacteria and fungi.

In the meaning of the invention an anti-infective protein may also be considered a protein that indirectly stimulated an immune response against any given infectious agent, for example an anti-infective protein may, in some embodiments, relate to any of the immune response-stimulating cytokines, chemokines or other immune modulating agents described herein. An anti-infective protein may also relate to a protein that exhibits a direct effect against an infectious agent, such as an anti-bacterial or anti-fungal and/or anti-viral protein.

In one embodiment the MSCs of the present invention are characterised in that a therapeutically effective number of the stem cells is introduced into the subject's bloodstream.

In one embodiment the MSCs of the present invention are characterised in that the therapeutically effective number of genetically modified mesenchymal stem cells is from about 1 x 10^5 to about 1 x 10^9, preferably about 1 x 10^6 to about 1 x 10^8 or more preferably about 5 x 10^6 to about 2 x 10^7 cells/kg body weight.

In one embodiment the MSCs of the present invention are characterised in that the therapeutic protein-encoding region operably linked to the hypoxia-induced promoter is integrated into the stem cell genome.

In one embodiment the MSCs of the present invention are characterised in that said MSCs comprise a selection marker gene operably linked to a constitutive promoter or promoter/enhancer combination, wherein the selection marker gene preferably comprises an antibiotic resistance gene or a gene encoding a surface marker protein, wherein said selection marker gene is preferably integrated into the stem cell genome.

In one embodiment the MSCs of the present invention are characterised in that said MSCs comprise an insulator sequence located between the cytotoxic protein-encoding region and the selection marker gene.

In one embodiment the MSCs of the present invention are characterised in that said MSCs comprise a proviral sequence integrated into the stem cell genome, wherein the cytotoxic protein-encoding region operably linked to the hypoxia-induced promoter and the selection marker gene operably linked to the constitutive promoter or promoter/enhancer combination are a part of the proviral sequence. In one embodiment the MSCs of the present invention are characterised in that the proviral sequence is a lentiviral, alpha-retroviral or gamma-retroviral sequence.

In one embodiment the MSCs as described herein are characterised in that said cells are obtained from bone marrow, umbilical cord, adipose tissue, or amniotic fluid.

In one embodiment the MSCs as described herein are characterised in that said cells are CD34 negative.

In one embodiment the MSCs as described herein are characterised in that said cells are human cells.

In one embodiment the MSCs as described herein are characterised in that said cells are CD38+ after stimulation with inflammatory mediators, preferably TNF alpha and/or IFN gamma.

In one embodiment the MSCs for use as a medicament as described herein are characterised in that the subject is human.

In one embodiment the MSCs for use as a medicament as described herein are characterised in that said genetically modified cells are allogeneic with respect to the subject.

In one embodiment the MSCs for use as a medicament as described herein are characterised in that said genetically modified cells are autologous with respect to the subject.

DETAILED DESCRIPTION OF THE INVENTION

All cited documents of the patent and non-patent literature are hereby incorporated by reference in their entirety.

The "mesenchymal cells" disclosed herein (also referred to in some embodiments as "mesenchymal stem cells" or “MSCs”) can give rise to connective tissue, bone, cartilage, and cells in the circulatory and lymphatic systems. Mesenchymal stem cells are found in the

mesenchyme, the part of the embryonic mesoderm that consists of loosely packed, fusiform or stellate unspecialized cells. As used herein, mesenchymal stem cells include, without limitation, CD34-negative stem cells.

In one embodiment of the invention, the mesenchymal cells are fibroblast-like plastic adherent cells, defined in some embodiments as multipotent mesenchymal stromal cells and also include CD34-negative cells.

For the avoidance of any doubt, the term mesenchymal cell encompasses multipotent mesenchymal stromal cells that also includes a subpopulation of mesenchymal cells, MSCs and their precursors, which subpopulation is made up of multipotent or pluripotent self-renewing cells capable of differentiation into multiple cell types in vivo.

As used herein, CD34-ngeative cell shall mean a cell lacking CD34, or expressing only negligible levels of CD34, on its surface. CD34-negative cells, and methods for isolating such cells, are described, for example, in Lange C. et al., “Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine”. J. Cell Physiol. 2007, Apr. 25.

Mesenchymal cells can be differentiated from hematopoietic stem cells (HSCs) by a number of indicators. For example, HSCs are known to float in culture and to not adhere to plastic surfaces. In contrast, mesenchymal cells adhere to plastic surfaces. The CD34-negative mesenchymal cells of the present invention are adherent in culture.

The genetically modified cell(s) described herein may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostaticaly, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctival, intravesicularlly, mucosally, intrapericardially, intraumbilically, intraocularally, orally, topically, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (e.g., liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

As used herein, "introducing" cells "into the subject's bloodstream" shall include, without limitation, introducing such cells into one of the subject's veins or arteries via injection. Such administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods. A single injection is preferred, but repeated injections over time (e.g., quarterly, half-yearly or yearly) may be necessary in some instances. Such administering is also preferably performed using an admixture of CD34-negative cells and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known to those skilled in the art and include, but are not limited to, 0.01-0.1 M and preferably 0.05 M phosphate buffer or 0.8% saline.

Additionally, such pharmaceutically acceptable carriers can be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions and suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as Ringer's dextrose, those based on Ringer's dextrose, and the like. Fluids used commonly for i.v. administration are found, for example, in Remington: The Science and Practice of Pharmacy, 20th Ed., p. 808, Lippincott Williams S- Wilkins (2000). Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like.

As used herein, “potentially harmful therapeutic protein” shall include all proteins or transgene encoded proteins which allow to elicit a therapeutic effect at a specific target site when a certain local concentration is reached, whereby the therapeutic protein is toxic if the same or higher concentration is reached systemically.

As used herein, a "therapeutically effective number of cells" includes, without limitation, the following amounts and ranges of amounts: (i) from about 1 x 10^2 to about 1 x 10^8 cells/kg body weight; (ii) from about 1 x 10^3 to about 1 x 10^7 cells/kg body weight; (iii) from about 1 x 10^4 to about 1 x 10^6 cells/kg body weight; (iv) from about 1 x 10^4 to about 1 x 10^5 cells/kg body weight; (v) from about 1 x 10^5 to about 1 x 10^6 cells/kg body weight; (vi) from about 5 x 10^4 to about 0.5 x 10^5 cells/kg body weight; (vii) about 1 x 10^3 cells/kg body weight; (viii) about 1 x 10^4 cells/kg body weight; (ix) about 5 x 10^4 cells/kg body weight; (x) about 1 x 10^5 cells/kg body weight; (xi) about 5 x 10^5 cells/kg body weight; (xii) about 1 x 10^6 cells/kg body weight; and (xiii) about 1 x 10^7 cells/kg body weight. Human body weights envisioned include, without limitation, about 5 kg, 10 kg, 15 kg, 30 kg, 50 kg, about 60 kg; about 70 kg; about 80 kg, about 90 kg; about 100 kg, about 120 kg and about 150 kg. These numbers are based on pre-clinical animal experiments and human trials and standard protocols from the transplantation of CD34+ hematopoietic stem cells. Mononuclear cells (including CD34+ cells) usually contain between 1:23000 to 1:300000 CD34-negative cells.

As used herein, "treating" a subject afflicted with a disorder shall mean slowing, stopping or reversing the disorder's progression. In the preferred embodiment, treating a subject afflicted with a disorder means reversing the disorder's progression, ideally to the point of eliminating the disorder itself. As used herein, ameliorating a disorder and treating a disorder are equivalent. The treatment of the present invention may also, or alternatively, relate to a prophylactic administration of said cells. Such a prophylactic administration may relate to the prevention of any given medical disorder, such as the prevention of inflammation, or the prevention of development of said disorder, whereby prevention or prophylaxis is not to be construed narrowly under all conditions as absolute prevention. Prevention or prophylaxis may also relate to a reduction of the risk of a subject developing any given medical condition, preferably in a subject at risk of said condition.

Typically, the term "inflammation" as used in its art-recognized sense relates to a localized or systemic protective response elicited by injury, infection or destruction of tissues which serves to

protect the subject from an injurious agent and the injured tissue. Inflammation is preferably characterized by fenestration of the microvasculature, leakage of the elements of blood into the interstitial spaces, and migration of leukocytes into the inflamed tissue, which may lead to an uncontrolled sequence of pain, heat, redness, swelling, and loss of function.

Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

In some embodiments of the invention the MSCs as described herein migrate towards physiological niches affected by a disease condition, such as areas of inflammation, in order to impart their therapeutic effect, for example in a local manner.

As used herein “cell migration” is intended to mean movement of a cell towards a particular chemical or physical signal. Cells often migrate in response to specific external signals, including chemical signals and mechanical signals. Chemotaxis is one example of cell migration regarding response to a chemical stimulus. In vitro chemotaxis assays such as Boyden chamber assays may be used to determine whether cell migration occurs in any given cell. For example, the cells of interest may be purified and analysed. Chemotaxis assays (for example according to Falk et al., 1980 J. Immuno. Methods 33:239-247) can be performed using plates where a particular chemical signal is positioned with respect to the cells of interest and the transmigrated cells then collected and analysed. For example, Boyden chamber assays entail the use of chambers isolated by filters, used as tools for accurate determination of chemotactic behaviour. The pioneer type of these chambers was constructed by Boyden (Boyden (1962) "The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes". J Exp Med 115 (3): 453). The motile cells are placed into the upper chamber, while fluid containing the test substance is filled into the lower one. The size of the motile cells to be investigated determines the pore size of the filter; it is essential to choose a diameter which allows active transmigration. For modelling in vivo conditions, several protocols prefer coverage of filter with molecules of extracellular matrix (collagen, elastin etc.) Efficiency of the measurements can be increased by development of multiwell chambers (e.g. NeuroProbe), where 24, 96, 384 samples are evaluated in parallel. Advantage of this variant is that several parallels are assayed in identical conditions.

Alternatively, tissue samples may be obtained from subjects (for example rodent models) after cell transplantation and assayed for the presence of the cells of interest in particular tissue types. Such assays may be of molecular nature, identifying cells based on nucleic acid sequence, or of histological nature, assessing cells on the basis of fluorescent markings after antibody labelling. Such assays are also particularly useful for assessing engraftment of transplanted cells. Assays for engraftment may also provide information on cell migration, as to some extent the engraftment is dependent on cell localization prior to engraftment.

In some embodiments of the invention the MSCs as described herein engraft in physiological niches affected by a disease condition, such as areas of hypoxia and/or inflammation, in order to impart their therapeutic effect, for example in a local manner.

As used herein “engraftment” relates to the process of incorporation of grafted or transplanted tissue or cells into the body of the host. Engraftment may also relate to the integration of transplanted cells into host tissue and their survival and under some conditions differentiation into non-stem cell states.

Techniques for assessing engraftment, and thereby to some extent both migration and the bio-distribution of MSCs, can encompass either in vivo or ex vivo methods. Examples of in vivo methods include bioluminescence, whereby cells are transduced to express luciferase and can then be imaged through their metabolism of luciferin resulting in light emission; fluorescence, whereby cells are either loaded with a fluorescent dye or transduced to express a fluorescent reporter which can then be imaged; radionuclide labelling, where cells are loaded with radionuclides and localized with scintigraphy, positron emission tomography (PET) or single photon emission computed tomography (SPECT); and magnetic resonance imaging (MRI), wherein cells loaded with paramagnetic compounds (e.g., iron oxide nanoparticles) are traced with an MRI scanner. Ex vivo methods to assess biodistribution include quantitative PCR, flow cytometry, and histological methods. Histological methods include tracking fluorescently labelled cells; in situ hybridization, for example, for Y-chromosomes and for human-specific ALU sequences; and histochemical staining for species-specific or genetically introduced proteins such as bacterial β-galactosidase. These immunohistochemical methods are useful for discerning engraftment location but necessitate the excision of tissue. For further review of these methods and their application see Kean et al., MSCs: Delivery Routes and Engraftment, Cell-Targeting Strategies, and Immune Modulation, Stem Cells International, Volume 2013 (2013).

Progenitor or multipotent cells, such as the mesenchymal cells of the present invention, may therefore be described as protein delivery vehicles, essentially enabling the localization and expression of therapeutic gene products in particular tissues or regions of the subject's body. Such therapeutic cells offer the potential to provide cellular therapies for diseases that are refractory to other treatments. For each type of therapeutic cell the ultimate goal is the same: the cell should express a specific repertoire of genes, preferably exogenous nucleic acids that code for therapeutic gene products, thereby modifying cell identity to express said gene product and provide a therapeutic effect, such as an anti-inflammatory effect. The cells of the invention, when expanded in vitro, represent heterogeneous populations that include multiple generations of mesenchymal (stromal) cell progeny, which lack the expression of most differentiation markers like CD34. These populations may have retained a limited proliferation potential and responsiveness for terminal differentiation and maturation along mesenchymal and non-mesenchymal lineages.

As used herein "inducible expression" or “conditional expression” relates to a state, multiple states or system of gene expression, wherein the gene of interest is preferably not expressed, or expressed at negligible levels, unless there is the presence of some molecule (an inducer) or other physiological conditions, such as low oxygen tension, that allows for gene expression. The

molecule or conditions are said to "induce expression". The invention may also relate to “up-regulation” of a gene under inflammatory and/or hypoxic conditions.

As used herein, in "proximity with" a tissue includes, for example, within 5 mm, within 1 mm of the tissue, within 0.5 mm of the tissue and within 0.25 mm of the tissue.

Given that stem cells can show a selective migration to different tissue microenvironments in normal as well as diseased settings, the use of additional tissue-specific promoters linked to the differentiation pathway initiated in the recruited stem cell is encompassed in the present invention and could be used to drive the selective expression of therapeutic genes only within a defined biologic context. Stem cells that are recruited to other tissue niches, but do not undergo the same program of differentiation, should not express the therapeutic gene. This approach allows a significant degree of potential control for the selective expression of the therapeutic gene within a defined microenvironment and has been successfully applied to regulate therapeutic gene expression during neovascularization. Potential approaches to such gene modifications are disclosed in WO 2008/150368 and WO 2010/119039, which are hereby incorporated in their entirety.

The main response of the immune system to tumours is to destroy the abnormal cells using killer T cells, sometimes with the assistance of helper T cells. Tumour antigens are presented on MHC class I molecules in a similar way to viral antigens. This allows killer T cells to recognize the tumour cell as abnormal. NK cells also kill tumorous cells in a similar way, especially if the tumour cells have fewer MHC class I molecules on their surface than normal; this is a common phenomenon with tumours. Some tumour cells also release products that inhibit the immune response; for example by secreting the cytokine TGF-β, which suppresses the activity of macrophages and lymphocytes.

The present invention therefore provides means for supporting an anti-tumour immune reaction by the expression of an immune-stimulating cytokine under control of a hypoxia-induced promoter from the genetically modified MSCs described herein.

Immunotherapy is to be understood in the context of the present invention to encompass any therapeutic agent that uses the immune system to treat cancer. Immunotherapy exploits the fact that cancer cells have subtly different molecules on their surface that can be detected by the immune system. These molecules, known as cancer antigens, are most commonly proteins, but also include molecules such as carbohydrates. Immunotherapy provokes or enhances the immune system in attacking the tumour cells by using these antigens as targets. Immunotherapy encompasses, without limitation, cellular and antibody therapy.

Cellular therapies typically involve the administration of immune cells isolated from the blood or from a tumour of the patient. Immune cells directed towards the tumour to be treated are activated, cultured and returned to the patient where the immune cells attack the cancer. Cell types that can be used in this way are, without limitation, natural killer cells, lymphokine-activated killer cells, cytotoxic T cells and dendritic cells. Dendritic cell therapy provokes anti-tumour responses by causing dendritic cells to present tumour antigens. Dendritic cells present antigens to lymphocytes, which activates them, priming them to kill other cells that present the antigen.

Antibodies are proteins produced by the immune system that bind to a target antigen on the cell surface. Those that bind to cancer antigens may be used to treat cancer. Cell surface receptors are common targets for antibody therapies and include for example CD20, CD274, and CD279. Once bound to a cancer antigen, antibodies can induce antibody-dependent cell-mediated cytotoxicity, activate the complement system, or prevent a receptor from interacting with its ligand, all of which can lead to cell death. Multiple antibodies are approved to treat cancer, including Alemtuzumab, Ipilimumab, Nivolumab, Ofatumumab, and Rituximab.

Tumour-associated antigens, or Tumour-specific antigens, may be targeted by the preferably cellular or antibody-based anti-tumour immunotherapy and include, without limitation, those antigens known to a skilled person or identifiable by a skilled person that are expressed solely or predominantly by tumour cells and may be targeted by immune therapy. As non-limiting examples, tumour associated or tumour specific antigens encompass proteins produced in tumour cells that have an abnormal structure due to mutation, such as proto-oncogenes, abnormal products of ras and p53 genes, or other proteins associated with tumour cells, such as tissue differentiation antigens, cluster of differentiation (often abbreviated as CD) cell surface molecules, mutant protein antigens, oncogenic viral antigens, cancer-testis antigens and vascular or stromal specific antigens. Glycoproteins, glycolipids, carbohydrates or growth factor receptors may also be considered tumour associated or tumour specific antigens as targets of anti-tumour immunotherapy.

The MSCs of the present invention are capable of supporting and/or enhancing the immunotherapies described herein through their unique properties derived from a combination of immune-response stimulating transgene cytokines and the MSCs inherent anti-inflammatory properties.

The antibodies described herein may be expressed from a genetically modified MSCs under control of a hypoxia-induced promoter, or may be administered separately (or in combination) as an immune therapy with MSCs of the present invention that encode immune-stimulating cytokines, as described herein.

As known in the art, an immune response-stimulating cytokine is to be understood as any cytokine that leads to or produces either directly or indirectly the induction, activation and/or enhancement of an immune response, preferably directed against an antigen, for example a tumour antigen. In particular, the immune response-stimulating cytokines of the invention are preferably considered as cytokines that leads to the induction, activation and/or enhancement of an immune response beneficial for the treatment of a tumour disease.

As used herein, the terms immune response-modulating cytokine or immune response-associated cytokine may be used interchangeably and relate to molecules that participate, modulate, control and/or regulate the immune response and/or inflammatory reactions including anti-tumour activity due to the differentiation, maturation and activation of immune cells.

Cytokines are a diverse group of non-antibody proteins that act as mediators between cells. Cytokines are currently being clinically used as biological response modifiers for the treatment of various disorders. The term cytokine is a general term used to describe a large group of proteins. Particular kinds of cytokines may include monokines, namely cytokines produced by mononuclear

phagocytic cells, Lymphokines, namely cytokines produced by activated lymphocytes, especially Th cells, Interleukins, namely cytokines that act as mediators between leukocytes and Chemokines, namely small cytokines primarily responsible for leucocyte migration. Cytokine signaling is flexible and can induce both protective and damaging responses. They can produce cascades, or enhance or suppress production of other cytokines. Despite the various roles of cytokines, a skilled person is aware of which cytokines may be considered as immune response-stimulating and therefore applied in the treatment of a tumour disease as described herein.

Cytokines have the ability to modulate immune responses and are often utilised by a tumour to allow it to grow and manipulate the immune response. These immune-modulating effects allow them to be used as drugs to provoke an immune response against the tumour.

The following cytokines may be referred to as immune-response stimulatory or immune response-modulatory cytokines.

Two commonly used groups of cytokines in anti-tumour therapy are the interferons and interleukins.

Interferons are cytokines produced by the immune system usually involved in an anti-viral response, but also show effectiveness in the treatment of cancer. There are three groups of interferons (IFNs): type I (IFN alpha and IFN beta), type 2 (IFN gamma) and the relatively newly discovered type III (IFN lambda). IFN alpha has been applied in the treatment of hairy-cell leukaemia, AIDS-related Kaposi's sarcoma, follicular lymphoma, chronic myeloid leukaemia and melanoma. Type I and II IFNs have been researched extensively and although both types promote the anti-tumour effects of the immune system, only type I IFNs have been shown to be clinically effective in cancer treatment so far. IFN lambda has been tested for its anti-tumour effects in animal models, and shows promise. Expression of one or more interferon under control of a hypoxia-induced promoter represents one preferred embodiment of the invention.

According to some embodiments of the present invention, the immune-response stimulatory or immune response-modulatory cytokines are preferably those involved in T cell regulation or with effector function for T cells (T cell regulatory cytokines). These cytokines exhibit desired properties with respect to inducing a pro-inflammatory microenvironment and thereby facilitating the activation of the immune system against the tumour and/or enhance the efficacy of anti-tumour immunotherapeutic treatments. Such cytokines may be able to attract immune effector cells, such as T cells, and promote the maturation of memory immune cells. Examples of these cytokines are IFN gamma, IL-2, IL-12, IL-23, IL-15 and IL-21 (refer Kelley's Textbook of Rheumatology; Firestein et al, 8th ed. (ISBN 978-1-4160-3285-4), p367 "Cytokines"). Expression of one or more interleukin under control of a hypoxia-induced promoter represents one preferred embodiment of the invention.

The specific molecules mentioned herein relate preferably to mammalian molecules, preferably human molecules, for reasons of suitability for administration in human subjects. Molecules disclosed comprising mouse nomenclature, for example mouse sequences, are included in the invention, as are the human counterparts of such mouse molecules (with analogous sequence or function). For example, the cytokines and/or chemokines mentioned herein relate preferably to the human sequences, as can be obtained by a skilled person without undue effort, for example

from a sequence database such as those maintained by the National Center for Biotechnology Information (NCBI). The sequence of any given gene, and the promoter of any gene, can be identified using databases known to a skilled person, such as the NCBI database.

Protein sequences or protein-coding nucleic acid sequences may be modified in comparison to commonly known sequences, for example by making e. g. conservative amino acid substitutions in a protein sequence, or by using the degeneracy of the genetic code in order to change the coding sequence without changing the encoded protein sequence. As a skilled person is aware, the sequences of biological molecules can be changed (mutated) via standard techniques, their properties thereby being improved or maintained in comparison to the known original sequence. Any modified cytokine sequence that maintains the basic properties of, or is functionally analogous to, the known sequence is therefore encompassed in the scope of the present invention. The invention therefore encompasses variant amino acid sequences of the specific transgenes disclosed herein, preferably the human genes, that display a sequence identity of at least 70%, 75%, 80%, 85%, 90% or preferably at least 95% to the known human protein sequence, and preferably exhibit functional analogy. Functional analogy is to be understood as a protein that fulfills essentially the same function as the known human protein sequence, although comprises a variant amino acid sequence.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the immune response-stimulating cytokine under control of the hypoxia-induced promoter is IL-2. Interleukin-2 is an example of a cytokine that can enhance the anti-tumour activity of the immune system and thus can be used as a treatment in cancer. Interleukin-2 has been used for the treatment of malignant melanoma (trade name, Proleukin®) and renal cell carcinoma. In normal physiology it promotes both effector T cells (cells that produce the immune response) and T-regulatory cells (cells that repress the immune response), but its exact mechanism in the treatment of cancer is unknown. Recent work indicates a beneficial effect of IL-2 expression in cancer treatment. IL-2 has been used in conjunction with adoptive immunotherapies, such as CART therapies, in order to promote T-cell activation. However, systemic administration of IL-2 carries the risk of broad immune activation which contributes to the toxicities associated with CART therapies.

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the immune response-stimulating cytokine under control of the hypoxia-induced promoter is IL-7. Interleukin 7 (IL-7) is a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus. IL-7 stimulates the differentiation of multipotent (pluripotent) hematopoietic stem cells into lymphoid progenitor cells. IL-7 as an immunotherapy agent has been examined in pre-clinical animal studies and more recently in human clinical trials for various malignancies and during HIV infection (Fry TJ, Mackall CL (June 2002). "Interleukin-7: from bench to clinic". Blood 99 (11): 3892–904).

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the immune response-stimulating cytokine under control of the hypoxia-induced promoter is IL-15. Interleukin 15 (IL-15) is a cytokine with structural similarity to IL-2. Like IL-2, IL-15 binds to and signals through a complex composed of IL-2/IL-15 receptor beta chain (CD122) and the common gamma chain (gamma-C, CD132). IL-15 induces cell proliferation of

natural killer cells; cells of the innate immune system whose principal role is to kill virally infected or cancerous cells. IL-15 has been shown to enhance the anti-tumour immunity of CD8+ T cells in pre-clinical models (Klebanoff CA, et al. Proc. Natl. Acad. Sci. U.S.A. 101 (7): 1969–74).

In a preferred embodiment the genetically modified mesenchymal stem cell as described herein is characterised in that the immune response-stimulating cytokine under control of the hypoxia-induced promoter is IL-21. Interleukin-21 (IL-21) is a cytokine that has potent regulatory effects on cells of the immune system, including natural killer (NK) cells and cytotoxic T cells that can destroy virally infected or cancerous cells. IL-21 has been approved for Phase 1 and 2 clinical trials in metastatic melanoma (MM) and renal cell carcinoma (RCC) patients (Søndergaard H, Skak K, Tissue Antigens 74 (6): 467–79). It has been shown to be safe for administration with flu-like symptoms as side effects. Dose-limiting toxicities included low lymphocyte, neutrophil, and thrombocyte count as well as hepatotoxicity.

Interleukin 12 (IL-12) is an interleukin that is naturally produced by dendritic cells, macrophages and human B-lymphoblastoid cells (NC-37) in response to antigenic stimulation. IL-12 is involved in the differentiation of naive T cells into Th1 cells. It is known as a T cell-stimulating factor, which can stimulate the growth and function of T cells, thereby falling under the concept of the present invention. IL-12 is known to stimulate the production of interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ. IL-12 also has a known anti-angiogenic activity. IL-12 functions by increasing production of IFN gamma, which in turn increases the production of the chemokine CXCL10. However, until the present time, excessive clinical toxicity and modest clinical response has been observed in clinical trials, thereby necessitating novel approaches and administration regimes that minimize toxicity without affecting the anti-tumour effect of IL-12. IL-12 has not been shown to have substantial activity in the tumours tested to this date via systemic administration in doses that are non-toxic to the subject.

The present invention therefore relates to the MSCs as described herein and medical use thereof, wherein the exogenous nucleic acid encodes IL-12 under control of the hypoxia-induced promoter. The MSC-based approach towards site-specific expression of IL-12 represents a novel and advantageous approach towards avoiding the toxicity inherent in IL-12 systemic administration.

The present invention enables a surprising and advantageous anti-tumour effect via the expression of an immune stimulatory cytokine in the MSCs as described herein. The expression of a stimulatory cytokine as described herein by the MSCs supports an anti-tumour immune response and leads to reduction in tumour size and/or growth, and shows a distinct reduction in and/or avoidance of the side effects produced by systemic administration of such cytokines known in the art. Side effects such as nausea and vomiting, sores in the mouth or on the lips, diarrhoea, drowsiness, allergic reactions, fever or chills, hives, itching, headache, coughing, shortness of breath, or swelling of the face, tongue, or throat, may be avoided by the MSC-based therapy described herein.

The present invention therefore provides means for reducing the side effects of cytokine therapy, and the concomitant use of cytokines with immunotherapies, by enabling local (or locally

confined) tumour-specific effects, achieved preferably by systemic administration of the cells, but exerted in a tissue specific manner via cell therapy using MSCs that comprise and express said cytokines under the appropriate tissue-specific conditions.

Chemokines refer to a sub-group of cytokines (signalling proteins) secreted by cells. Cytokines have the ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. Proteins are classified as chemokines according to shared structural characteristics such as small size (typically approximately 8-10 kilodaltons in size), and the presence of four cysteine residues in conserved locations that are key to forming their 3-dimensional shape. Cytokines may be known under alternative definitions, such as the SIS family of cytokines, SIG family of cytokines, SCY family of cytokines, Platelet factor-4 superfamily or intercrines. Chemokines have been classified into four main subfamilies: CXC, CC, CX3C and XC. All of these proteins exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors, which are selectively found on the surfaces of their target cells.

The invention therefore relates to the mesenchymal stem cells described herein, wherein the exogenous nucleic acid encodes an inflammatory chemokine under control of the hypoxia-induced promoter. Such chemokines are known to a skilled person. Examples of inflammatory chemokines relate to CXCL-8, CCL2, CCL3, CCL4, CCL5, CCL11 and CXCL10, CXCL1, CXCL2, CXCL13, CX3CL1, CXCL12, CCL23, CCL19. The chemokines described herein are capable of exhibiting T cell recruiting properties that are beneficial in an immune response against a tumour disease. The use of such chemokine molecules in the context of MSC-based expression and delivery of the molecules enables surprising benefits with respect to site-specific T cell recruitment and reduction in side effects associated non-target effects caused by systemic administration of such cytokines/chemokines.

Checkpoint inhibitors (also known as immune checkpoint modulators, or CPMs) are designed to lessen the effectiveness of checkpoint proteins. They may have a variety of mechanisms of action, but if effective, they enable the immune system to recognize other molecules on the surface of the cancer cells. In one embodiment the medical use of the genetically modified mesenchymal stem cell as described herein is characterised by the combined administration of a checkpoint inhibitor, preferably a PD-L1 and/or PD-1 inhibitor, with said MSCs. Examples include Nivolumab (BMS-936558, MDX-1106, ONO-4538), a fully human Immunoglobulin G4 (IgG4) monoclonal PD-1 antibody, Lambrolizumab (MK-3475), a humanized monoclonal IgG4 PD-1 antibody, and BMS-936559, a fully human IgG4 PD-L1 antibody. In one embodiment the medical use of the genetically modified mesenchymal stem cell as described herein is characterised by the combined administration of a checkpoint inhibitor, preferably a CTLA-4 inhibitor, with said MSCs. Examples include Tremelimumab (Pfizer, NY, USA) and ipilimumab, two fully human monoclonal antibodies against CTLA-4.

The combined administration of the MSCs expressing an immune response-stimulating cytokine and/or an immune stimulatory molecule that induces T-cell proliferation and/or differentiation under control of the hypoxia-induced promoter together with a checkpoint inhibitor leads to a synergistic effect with respect to the desired anti-cancer effect. The cytokine or other immune stimulator provides local enhancement of the T cell response against the cancer tissue, whilst the

checkpoint inhibitor also enables the T cells to more effectively attack and destroy cancerous tissue. The effects of these two agents are combined in a synergistic manner, resulting in a technical effect greater than the sum of these two aspects when considered alone.

The present invention therefore provides novel means for local and hypoxia-specific cytokine production for the stimulation of an anti-tumour immune response, mediated preferably by cytotoxic T cells and/or NK cells. Artificial T cell receptors (also known as chimeric T cell receptors, chimeric immunoreceptors, chimeric antigen receptors (CARs)) are engineered receptors, which graft an arbitrary specificity onto an immune effector cell. Typically, these receptors are used to graft the specificity of a monoclonal antibody onto a T cell; with transfer of their coding sequence facilitated by retroviral vectors.

Combined administration encompasses simultaneous treatment, co-treatment or joint treatment, and includes the administration of separate formulations of MSCs with immunotherapies, such as checkpoint inhibitors and/or immune cells, whereby treatment may occur within minutes of each other, in the same hour, on the same day, in the same week or in the same month as one another. Sequential administration of any given combination of combined agents (for example MSCs, immune cells and/or checkpoint inhibitors) is also encompassed by the term “combined administration”. A combination medicament, comprising one or more of said MSCs with another immunotherapeutic, such as checkpoint inhibitors and/or immune cells, may also be used in order to co-administer the various components in a single administration or dosage.

A combined immunotherapy may precede or follow treatment with genetically modified stem cells by intervals ranging from minutes to weeks. In embodiments where the other immunotherapeutic agent and genetically modified stem cells are administered separately to the site of interest, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the agent and the genetically modified stem cell would still be able to exert an advantageously combined effect on a treatment site. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

As used herein, "nucleic acid" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids or modified variants thereof. An "exogenous nucleic acid" or "exogenous genetic element" relates to any nucleic acid introduced into the cell, which is not a component of the cells “original” or "natural" genome. Exogenous nucleic acids may be integrated or non-integrated, or relate to stably transfected nucleic acids.

Any given gene delivery method is encompassed by the invention and preferably relates to viral or non-viral vectors, as well as biological or chemical methods of transfection. The methods can yield either stable or transient gene expression in the system used.

Genetically modified viruses have been widely applied for the delivery of genes into stem cells. Preferred viral vectors for genetic modification of the MSCs described herein relate to retroviral vectors, in particular to gamma retroviral vectors. The gamma retrovirus (sometimes referred to

as mammalian type C retroviruses) is a sister genus to the lentivirus clade, and is a member of the Orthoretrovirinae subfamily of the retrovirus family. The Murine leukemia virus (MLV or MuLV), the Feline leukemia virus (FeLV), the Xenotropic murine leukemia virus-related virus (XMRV) and the Gibbon ape leukemia virus (GALV) are members of the gamma retrovirus genus. A skilled person is aware of the techniques required for utilization of gamma retroviruses in genetic modification of MSCs. For example, the vectors described Maetzig et al (Gammaretroviral vectors: biology, technology and application, 2001, Viruses Jun;3(6):677-713) or similar vectors may be employed. For example, the Murine Leukemia Virus (MLV), a simple gammaretrovirus, can be converted into an efficient vehicle of genetic therapeutics in the context of creating gamma retrovirus-modified MSCs and expression of a therapeutic transgene from said MSCs after delivery to a subject.

Adenoviruses may be applied, or RNA viruses such as Lentiviruses, or other retroviruses. Adenoviruses have been used to generate a series of vectors for gene transfer cellular engineering. The initial generation of adenovirus vectors were produced by deleting the El gene (required for viral replication) generating a vector with a 4kb cloning capacity. An additional deletion of E3 (responsible for host immune response) allowed an 8kb cloning capacity. Further generations have been produced encompassing E2 and/or E4 deletions.

Lentiviruses are members of Retroviridae family of viruses (M. Scherr et al., Gene transfer into hematopoietic stem cells using lentiviral vectors. Curr Gene Ther. 2002 Feb; 2(1):45-55). Lentivirus vectors are generated by deletion of the entire viral sequence with the exception of the LTRs and cis acting packaging signals. The resultant vectors have a cloning capacity of about 8 kb. One distinguishing feature of these vectors from retroviral vectors is their ability to transduce dividing and non-dividing cells as well as terminally differentiated cells.

Non-viral methods may also be employed, such as alternative strategies that include conventional plasmid transfer and the application of targeted gene integration through the use of integrase or transposase technologies. These represent approaches for vector transformation that have the advantage of being both efficient, and often site-specific in their integration. Physical methods to introduce vectors into cells are known to a skilled person. One example relates to electroporation, which relies on the use of brief, high voltage electric pulses which create transient pores in the membrane by overcoming its capacitance. One advantage of this method is that it can be utilized for both stable and transient gene expression in most cell types. Alternative methods relate to the use of liposomes or protein transduction domains. Appropriate methods are known to a skilled person and are not intended as limiting embodiments of the present invention.

FIGURES

The invention is further described by the following figures. These are not intended to limit the scope of the invention.

Fig. 1: Tumour homing of NIS-transfected MSCs (HIF-NIS-MSC) via Hypoxia.

Fig. 2: NIS-expressing MSCs show significant iodide uptake *in vitro* after hypoxia simulation

Fig. 3: Hypoxia induces NIS expression in NIS transfected MSCs under the control of the HIF-responding promoter

Fig. 4: Induction of iodide accumulation after systemic MSC administration in an HCC tumour model

Fig. 5: HCC tumours show perchlorate-sensitive iodide uptake activity after systemic HIF-NIS-MSC injection

Fig. 6: Immunofluorescent microscopy imaging of a human hepatocellular carcinoma experimental tumour spheroid treated with genetically modified MSCs of the present invention.

Fig. 7: MSC migration in response to tumour derived factors

Fig. 8: Effect of T3, T4 on MSC migration to tumour derived signals

EXAMPLES

The invention is further described by the following examples. These are not intended to limit the scope of the invention.

EXAMPLE 1:

Preparation human mesenchymal stem cells:

The MSC were prepared according to processes established previously. The cells were isolated from bone marrow from healthy human donors by plastic adherence and are cultured in growth medium. For example the process comprised using the cell culture medium disclosed in US8557578 B2, which is hereby incorporated in its entirety by reference.

Human MSCs are isolated from bone marrow by plastic adherence and are cultured in growth medium e.g. FBS containing DMEM as described by Pittinger, M.F. (2008) Mesenchymal stem cells from adult bone marrow, In D.J. Prockop, D.G. Phinney, B.A. Bunnell, Methods in Molecular Biology 449, Mesenchymal stem cells, Totowa: Humana Press), or in the culture medium as described in US8557578 B2.

Generation of vectors for the expression of potentially toxic cytokines under the HI-promoter:

The transgene expression cassettes comprising a HIF promoter (hypoxia inducible promoter) and a gene (e.g. cDNA) for toxic cytokine like Interferon alpha, beta or interleukine-2, were produced as described in Julia Lodge, Peter Lund, Steve Minchin (2007) Gene Cloning, New York: Tylor and Francis Group.

The transgene is inserted into a suitable vector system (e.g. lentiviral or gamma-retroviral vector) by standard cloning techniques. A suitable vector is for example described by Baum (EP 1757703 A2). The vector may or may not include a second transgene cassette consisting of a promoter and a selectable marker gene (cell surface marker or resistance gene, for example the pac gene to confer puromycin resistance) to allow enrichment of genetically modified cells later in the process (David P. Clark, Nanette J. Pazdernik, 2009, Biotechnology: Applying the Genetic Revolution, London: Elsevier).

Genetic modification of mesenchymal stem cell:

The transduction is performed with modifications as described by Murray et al., 1999 Human Gene Therapy. 10(11): 1743-1752 and Davis et al., 2004 Biophysical Journal Volume 86 1234–1242. In detail:

6-well cell culture plates (e.g. Corning) are coated with Poly-L-Lysine (PLL) (e.g. Sigma-Aldrich, P4707-50ML): The PLL solution (0.01%) is diluted to final concentration between 0.0001% and 0.001% with PBS. 2ml of the diluted PLL are used for each well. The plate is incubated at least for 2h at room temperature. After incubation, the plates are washed carefully with PBS.

Viral vector supernatant in a final volume of 2ml is added to each PLL-coated well. The number of particles should between 2x10e3 and 1x10e6 per well, which will result in multiplicity of infection of 0.25 and 10. The loaded plate is centrifuged for 2000x g, 30min, 4°C. Afterwards the supernatant is discarded and 1x10e5 mesenchymal stem cells are seeded per well. The plates are incubated at 37° with 5% CO2 for further use.

Regulation of expression in vectors carrying the HI-promoter by hypoxic conditions:

MSc are transduced with retroviral vector encoding interferon alpha (IFN a). The transgene is either under control of the HIF-1 alpha promoter or under control of a constitutive promoter (CMV) as control. Transduced MSC are seeded into 2 different 6-well plates in 2ml Medium. One plate is kept under hypoxic conditions (<1% O2) in an hypoxia incubator for 24h. The second plate is kept under norm-oxic conditions (20% O2). After 24h the supernatant from plates is collected and amount of IFN alpha in the supernatant is determined by ELISA.

It was found that MSC which harbor the HIF-1 alpha promoter express IFN a only under hypoxic conditions and not under norm-oxid conditions. The CMV driven version of the vectors express IFN a under both conditions.

EXAMPLE 2:

The experimental examples presented below relate to the establishment of NIS-transfected MSCs with high levels of functional NIS expression under the control of a HIF1-responsive promoter. Using NIS as reporter gene, the inventors demonstrated active MSC recruitment and HIF1-responding promoter activation in liver cancer xenografts after systemic injection as shown by tumour-selective radioiodine accumulation (Figures 1 to 5). Immunostaining analyses confirmed active and tumour-selective recruitment of HIF-NIS-MSCs in liver cancer xenografts.

Figure 1 demonstrates schematically the experimental approach used to assess NIS-transfected MSCs. The coding sequence for hNIS is positioned behind a HIF-responsive promoter in an expression construct, which is subsequently introduced into MSCs for in vitro or in vivo assessment.

Figure 2 demonstrates the iodine uptake in vitro in MSCs with or without the expression construct described above, with or without CoCl2 treatment to simulate hypoxia. Iodine uptake in the modified MSCs is dependent on CoCl2 treatment, indicating the functional effect of hypoxia-induced NIS expression.

Figure 3 shows a western blot against hNIS with and without CoCl2 treatment, thereby demonstrating the up-regulation of NIS under hypoxic conditions.

Figure 4 shows iodide accumulation after systemic MSC administration in an HCC tumour model. Radionuclide biodistribution studies in vivo are shown in the figure. Gamma-camera imaging was carried out of mice harbouring Huh7 tumours after mesenchymal stem cell (MSC)-mediated sodium iodide symporter (NIS) gene delivery 3 hr following 123I or 188Re administration.

Figure 5 shows perchlorate-sensitive iodide uptake activity after systemic HIF-NIS-MSC injection. Evaluation of radionuclide biodistribution was carried out ex vivo 4 hours following injection of 18.5MBq 123I or 111MBq 188Re. Tumours in MSC-injected mice showed high perchlorate sensitive radionuclide uptake activity, while no significant radionuclide accumulation was measured in non-target organs. Results are reported as percent of injected dose per organ +/– SD.

Figure 6 shows immunofluorescent microscopic images of a human hepatocellular carcinoma experimental tumour spheroid treated with genetically modified MSCs of the present invention. In Fig. 6A HIF-1 alpha is stained within the spheroid. For Fig. 6B and 6C a hMSC line was stably transfected with the HIF-responsive promoter operably linked to a reporter transgene (Cherry). The MSCs invade the center of the experimental tumour spheroid, and subsequently induce expression of the reporter transgene (Cherry). Tumour tissue is marked using DAPI staining. The location of HIF or Cherry expression is indicated by the ring.

The data presented in the examples demonstrate tumour hypoxia-targeted NIS-mediated radionuclide therapy of extrathyroidal tumours after MSC-mediated gene delivery, in addition to invasion of MSCs into a hepatocellular carcinoma experimental tumour spheroid and subsequent expression of a hypoxia-induced reporter protein. The examples show the functionality of a HIF-responsive promoter used to express a transgene under hypoxic conditions from a MSC vehicle as an agent for delivery. The NIS or Cherry as a transgene is not limiting for the present invention and is used herein as an example of HI promoter function in MSCs.

The examples provided herein also demonstrate that T3 and T4 can stimulate the ability of MSCs to migrate towards tumour-derived signals (Figures 7 and 8). Experiments using the µ-Slide Chemotaxis3D model system enable visualization of MSC migration. MSCs are seeded in the observation channel of a µ-Slide Chemotaxis3D. Chambers are filled either with HCC-conditioned serum-free medium (-) or unconditioned serum-free medium (+). Chemotaxis is monitored by time-lapse microscopy over a 24 hour period. Single cells are tracked and analysed for their migration in relation to the gradient. FMI (forward migration index), directionality, velocity and distance travelled by the cells is measured. A reproducible effect of T3 and T4 was found, enhancing the ability of MSC to migrate towards tumour-derived signals.

CLAIMS:

1. Genetically modified mesenchymal stem cells (MSCs) comprising one or more exogenous nucleic acid molecules, wherein said exogenous nucleic acid molecules comprise a therapeutic protein-encoding region operably linked to a hypoxia-induced promoter.

2. Genetically modified mesenchymal stem cell according to the preceding claim, wherein the promoter comprises one or more hypoxia-response elements (HREs).

3. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein said HREs are bound by a hypoxia inducible factor (HIF) selected from HIF-1 or HIF-2.

4. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein said HREs are bound by HIF-1 alpha.

5. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein the promoter comprises or consists of one or more endogenous or modified endogenous promoter sequences, said modified endogenous promoter comprising three or fewer HREs.

6. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein the promoter comprises a hypoxia-induced promoter module, said module comprising one or more hypoxia-response elements (HREs) and one or more additional regulatory promoter sequence selected from a SMAD element, a binding site for an E-twenty six family transcription factor (Ets factors), a glucocorticoid response element (GRE), an AP-1 binding site and/or a GATA-2 binding site.

7. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein the promoter or promoter module is induced when a cell comprising said promoter or promoter module is cultured *in vitro* in conditions of equal to or less than 5% oxygen saturation, preferably 2%, more preferably 0.5% oxygen saturation.

8. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein said mesenchymal stem cell comprises no additional exogenous nucleic acid molecules.

9. Genetically modified mesenchymal stem cell according to any one of the preceding claims, wherein said mesenchymal stem cell is a human patient-derived cell, a cell in primary cell culture or a cell in sub-culture after passaging from primary culture.

10. Genetically modified mesenchymal stem cell according to the preceding claim, wherein said mesenchymal stem cell has undergone fewer than 5 passages from primary culture before introduction of the exogenous nucleic acid.

11. Genetically modified mesenchymal stem cell according to any one of the preceding claims for use as a medicament in the treatment of a medical condition associated with hypoxia and/or hypoxic tissue.

12. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein one or more thyroid hormones, preferably T3 and/or T4, are co-administered with said stem cells.

13. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the medical condition is a tumour disease.

14. Genetically modified mesenchymal stem cell for use as a medicament according to the preceding claim, wherein the therapeutic protein is a cytotoxic protein selected from Herpes simplex viral thymidine kinase or cytosine deaminase.

15. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the therapeutic protein is an immune response-stimulating or immune response-modulating cytokine.

16. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the therapeutic protein is an immune response-stimulating or immune response-modulating chemokine.

17. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the therapeutic protein is an antibody.

18. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims for the treatment of a tumour disease, wherein said stem cell comprises one or more immune response-stimulating or immune response-modulating cytokines and/or chemokines, and wherein said treatment comprises the combined administration of said mesenchymal stem cells with an anti-tumour immunotherapy.

19. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the medical condition is an ischemic disease, preferably selected from the group consisting of circulatory disorders, cardiovascular disease, artery or blood vessel conditions and ischemic obstructive or occlusive diseases or conditions, such as stroke, myocardial infarction or peripheral occlusive disease, wherein the therapeutic gene is preferably VEGF, Erythropoietin or PDGF.

20. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the medical condition is a lung disease, wherein the lung disease is preferably an inflammatory disease of the lung, an acute lung injury, chronic obstructive pulmonary disease including chronic bronchitis, emphysema, bronchiectasis and bronchiolitis, acute respiratory distress syndrome, asthma, sarcoidosis, hypersensitivity pneumonitis and/or pulmonary fibrosis, wherein the therapeutic gene is preferably Alpha-1 antitrypsin (AAT) or HGF.

21. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the medical condition is an inflammatory bowel disease, wherein the inflammatory bowel disease is preferably selected from

the group consisting of Crohn's disease (CD), ulcerative colitis (UC), collagenous colitis, lymphocytic colitis, diversion colitis and Behçet's disease, wherein the therapeutic gene is preferably an anti-inflammatory cytokine, a protein that enhances endothelial cell growth, preferably vascular endothelial growth factor, a protein that modulates the immune function of defensines, IL1RA or a TLR antagonist.

22. Genetically modified mesenchymal stem cell for use as a medicament according to any one of the preceding claims, wherein the medical condition is an infectious disease selected from the group consisting of a bacterial, viral and/or fungal infection of a subject, wherein the therapeutic gene is an anti-infective protein.

Fig. 1

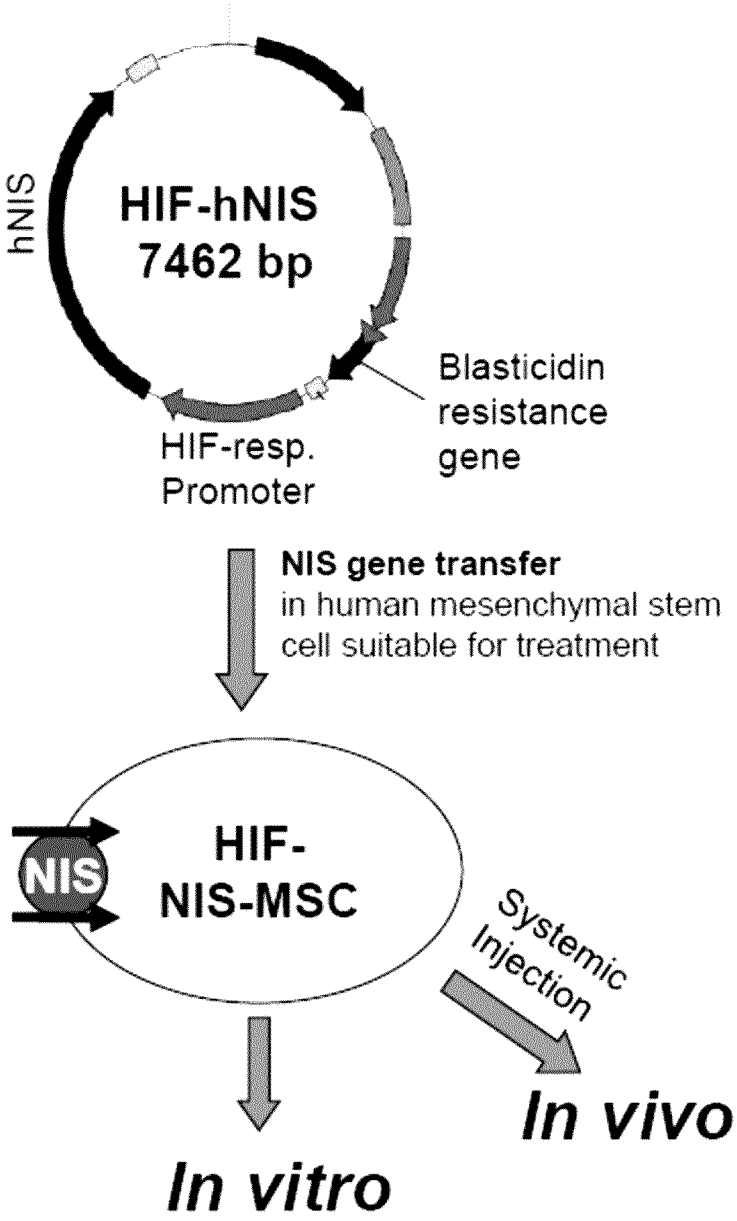
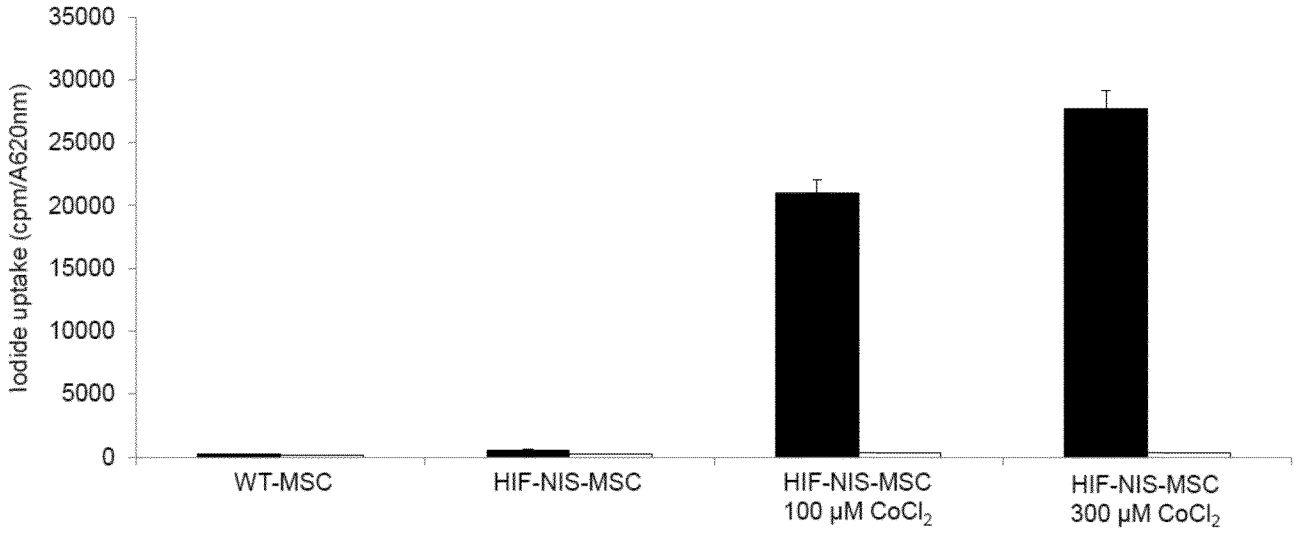

hNIS
HIF-hNIS
7462 bp
Blasticidin
resistance
gene
HIF-resp.
Promoter
NIS gene transfer
in human mesenchymal stem
cell suitable for treatment
NIS
HIF-
NIS-MSC
Systemic
Injection
In vivo
In vitro

Fig. 2


35000
30000
25000
20000
15000
10000
5000
0
Iodide uptake (cpm/A620nm)
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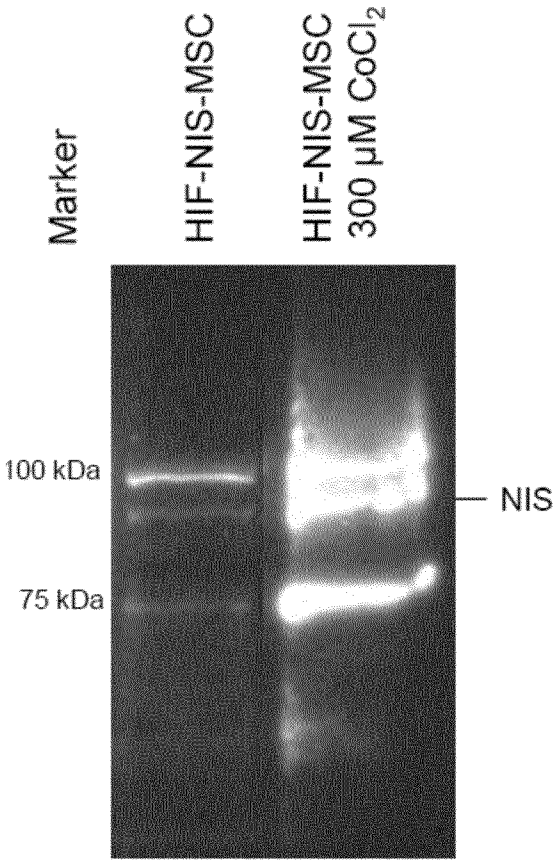
HIF-NIS-MSC
HIF-NIS-MSC
100 µM CoCl2
HIF-NIS-MSC
300 µM CoCl2

Fig. 3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HIF-NIS-MSC
300 µM CoCl2
100 kDa
75 kDa
NIS

Fig. 4

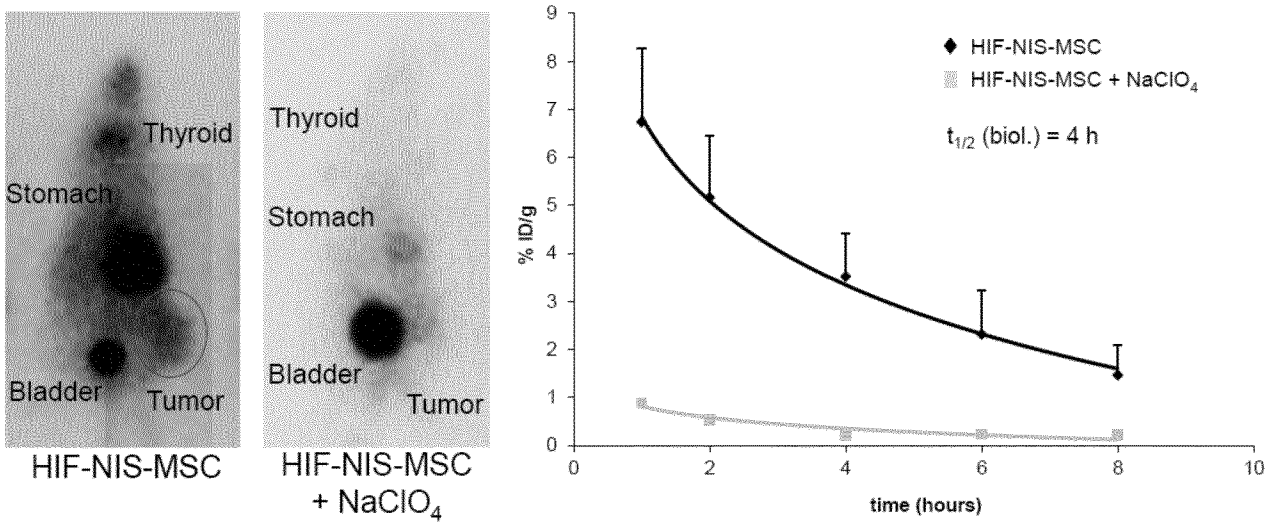
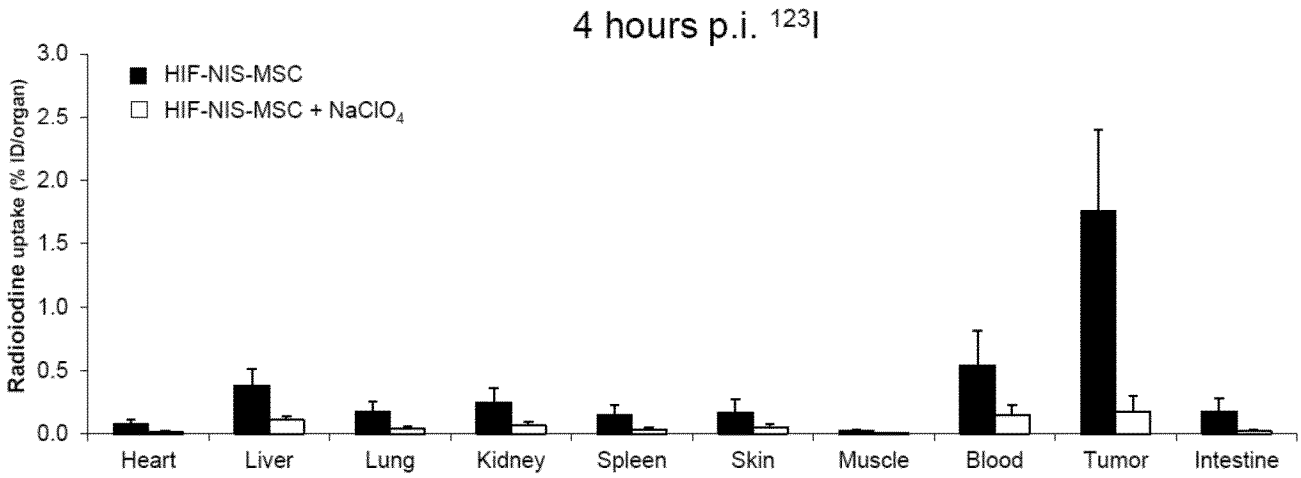

Thyroid
Stomach
Bladder
Tumor
HIF-NIS-MSC
Thyroid
Stomach
Bladder
Tumor
HIF-NIS-MSC + NaClO4
HIF-NIS-MSC
HIF-NIS-MSC + NaClO4
t1/2 (biol.) = 4 h
% ID/g
time (hours)
0
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10

Fig. 5


4 hours p.i. 123I
HIF-NIS-MSC
HIF-NIS-MSC + NaClO4
Radioiodine uptake (% ID/organ)
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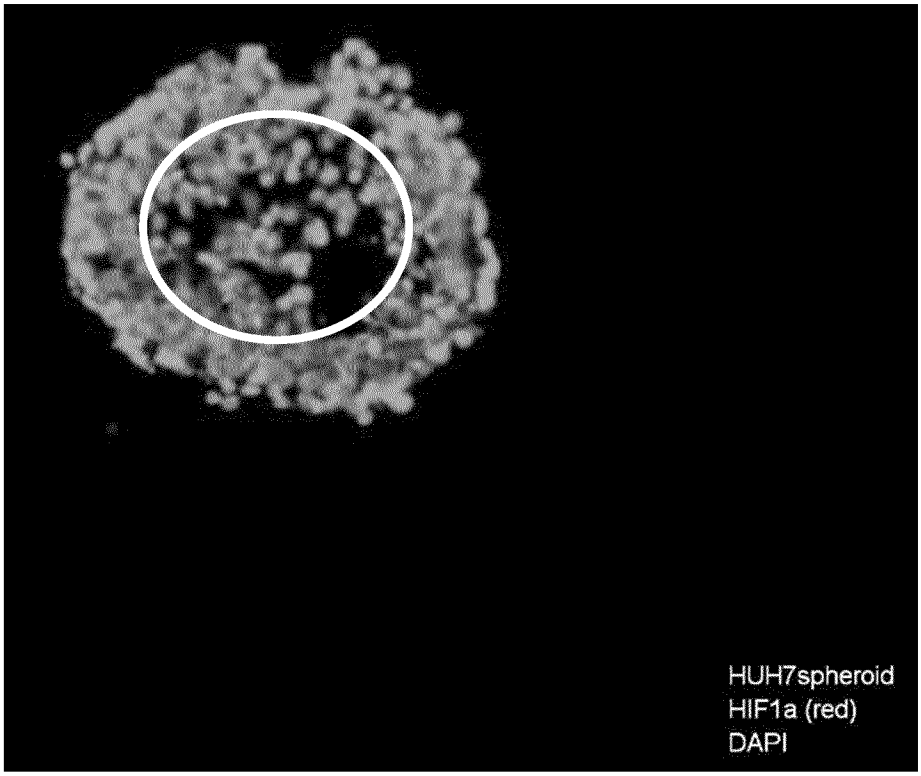
2.5
2.0
1.5
1.0
0.5
0.0
Heart
Liver
Lung
Kidney
Spleen
Skin
Muscle
Blood
Tumor
Intestine

Fig. 6

A


HUH7spheroid
HIF1a (red)
DAPI

B

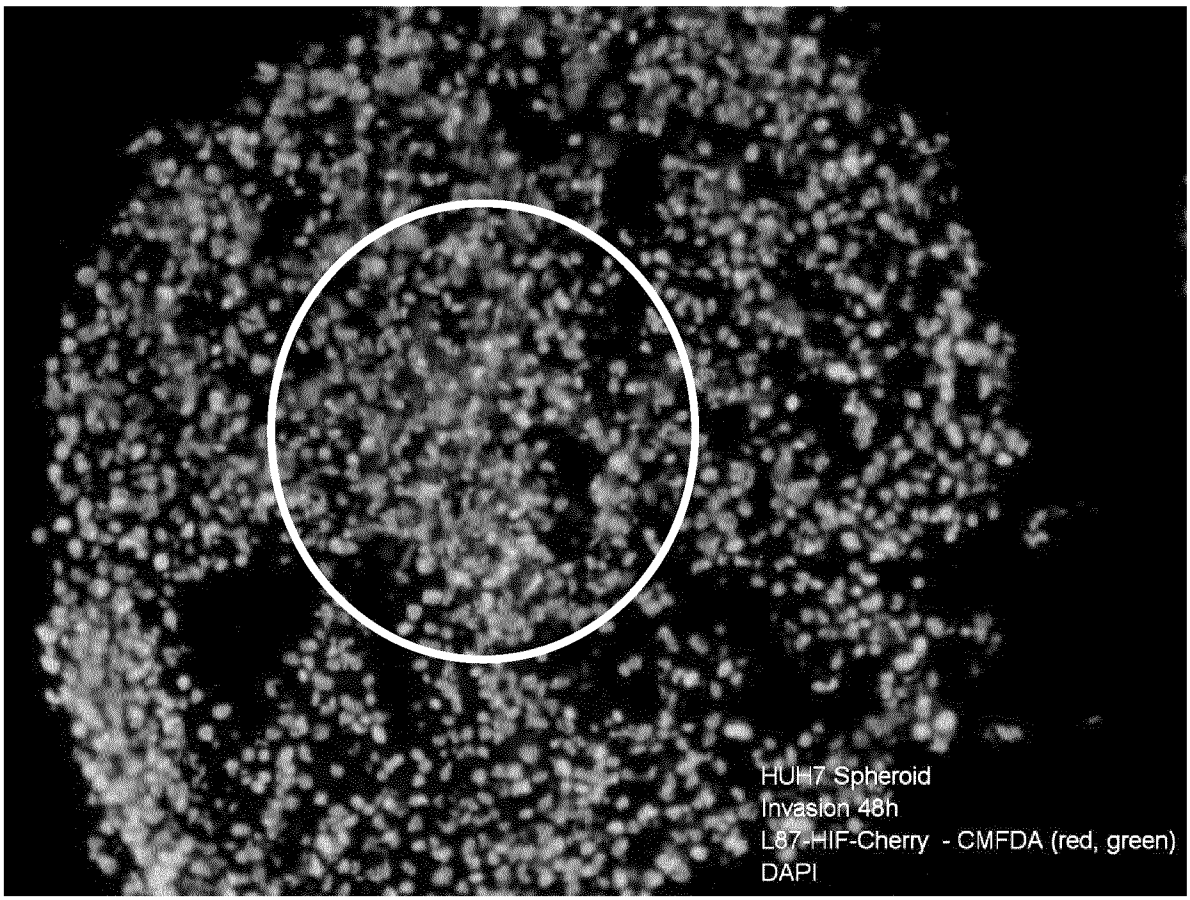

HUH7 Spheroid
Invasion 48h
L87-HIF-Cherry - CMFDA (red, green)
DAPI

Fig. 6 (cont.)

C

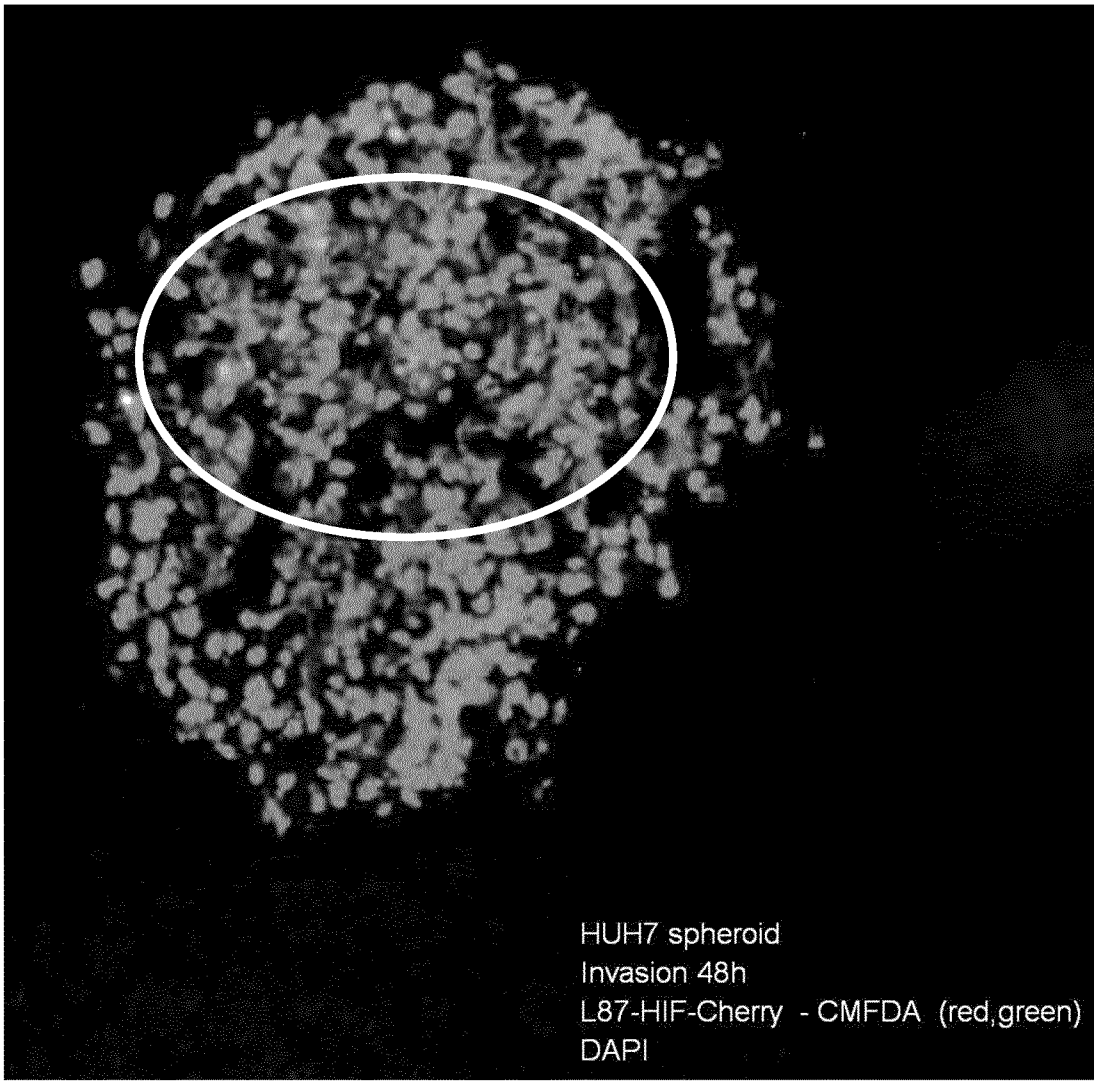
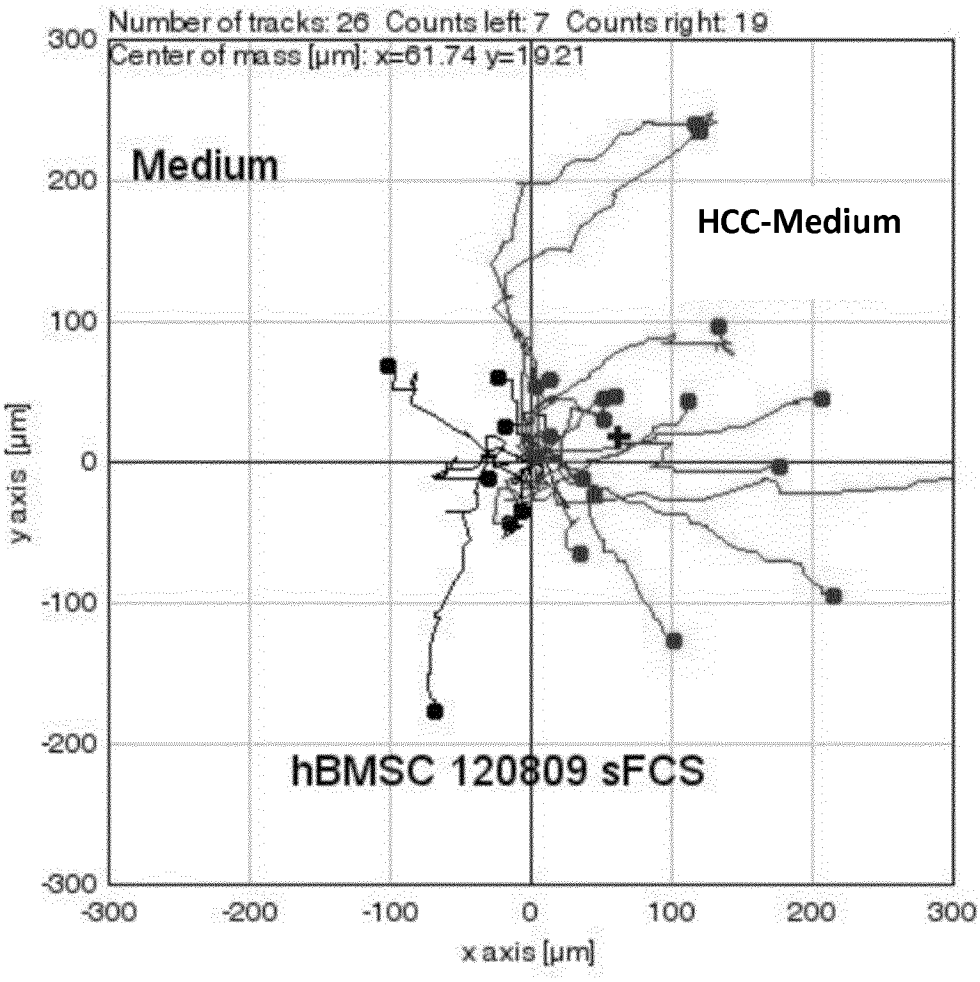

HUH7 spheroid
Invasion 48h
L87-HIF-Cherry - CMFDA (red,green)
DAPI

Fig. 7

Fig. 8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Center of mass [µm]: x=61.74 y=19.21
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HCC-Medium
hBMSC 120809 sFCS
300
200
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-100
-200
-300
y axis [µm]
-300
-200
-100
0
100
200
300
x axis [µm]

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/055979

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

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

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

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

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

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

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

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055979

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0775
ADD. C12N5/10 A61K35/28

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/044142 A2 (BRIGHAM & WOMENS HOSPITAL [US]; DZAU VICTOR J [US]; MANGI ABEEL [US];) 27 May 2004 (2004-05-27)	1-11
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
10 May 2016	20/05/2016

Name and mailing address of the ISA/	Authorized officer
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