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(54) Title: MODULATION OF CELL PROLIFERATION, METHODS AND REAGENTS

(57) Abstract

The present invention relates to polypeptides capable of bypassing a cell cycle checkpoint. Specific applications for inducing cell growth using such polypeptides (e.g., a macrophage migration inhibitory factor) are described.

Modulation of Cell Proliferation, Methods and Reagents

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Background of the Invention

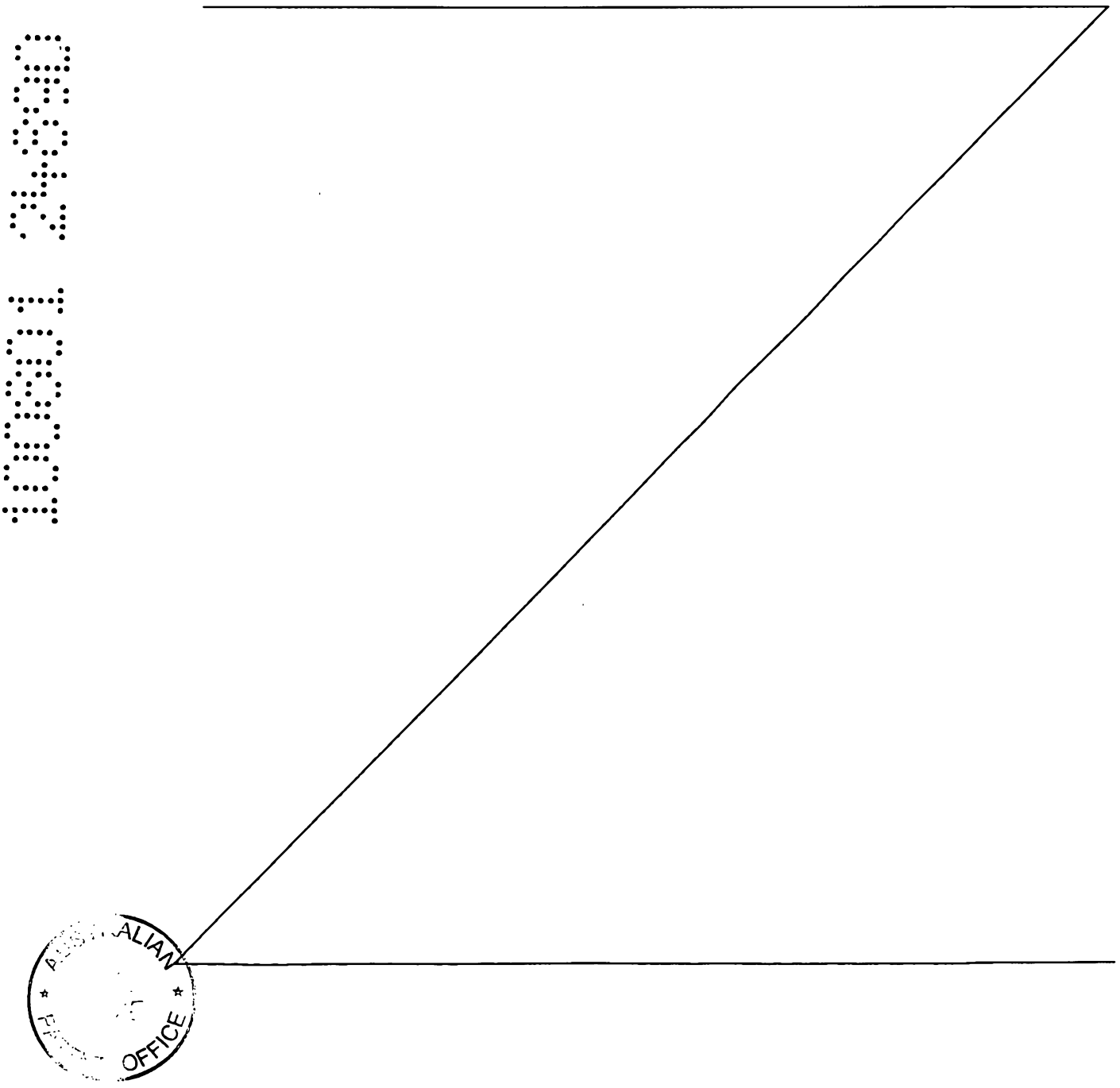
Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

Entry of cells into mitosis characteristically involves coordinated and simultaneous events, which include, for example, cytoskeletal rearrangements, disassembly of the nuclear envelope and of the nucleoli, and condensation of chromatin into chromosomes. The progression of a proliferating eukaryotic cell through the cell-cycle checkpoints is controlled by an array of regulatory proteins that guarantee that mitosis occurs at the appropriate time. These regulatory proteins can provide exquisitely sensitive feedback-controlled circuits that can, for example, prevent exit of the cell from S phase when a fraction of a percent of genomic DNA remains unreplicated (Dasso et al. (1990) *Cell* 61:811-823) and can block advance into anaphase in mitosis until all chromosomes are aligned on the metaphase plate (Rieder et al. (1990) *J. Cell Biol.* 110:81-95).

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells. Recent advances have revealed signal transduction pathways that transmit checkpoint signals in

response to DNA damage, replication blocks, and spindle damage. Checkpoint pathways have components shared among all eukaryotes, underscoring the conservation of cell cycle regulatory machinery.

For instance, negative controls on cell cycle progression are exerted during development, differentiation, senescence, and cell death. These negative controls may play an important role in preventing tumorigenesis. In many cases, arrest of cell proliferation takes place under circumstances in which the integrity of the genome has been compromised, and failure to arrest proliferation would release cells with highly unstable genomes that could evolve into cancer cells. Such circumstances might include, for example (i) senescence, in which telomeres are lost or become short, and unstable



dicentric chromosomes are formed; (ii) "programmed cell death" or apoptosis, in which DNA-degrading nucleases are unleashed; and (iii) immune cell development, in which requisite immunoglobulin and T cell receptor gene rearrangements require double-strand DNA breaks. These are all programmed events that may include an arrest of proliferation.

Cells also have the capacity to arrest cell cycle progression when damage is induced by unprogrammed extrinsic events, such as exposure to inhibitors of DNA replication or spindle assembly or to agents that physically damage DNA. These perturbations result in arrest of cell cycle progression at a specific stage. For example, when DNA replication is inhibited by hydroxyurea, the cell arrests in early S phase and does not undergo mitosis. This dependence of mitosis on prior completion of DNA replication is due to the action of specific gene products. Inactivation of these genes by mutation relieves the cell of this dependence, and such mutants will enter mitosis with incompletely replicated DNA. The genes that establish dependence in the cell cycle constitute checkpoints.

Cell death occurs by a variety of processes including, for example, programmed cell death and necrosis. The term " apoptosis" describes the morphological features of cells undergoing the process of programmed cell death, which is responsible for maintaining a steady-state level of cells in a self-renewing tissues. Under normal conditions, apoptosis assures that the number of dying cells in a tissue is roughly equivalent to the number of newly produced cells. However, in various disease states or as a result of an insult to a tissue, dysregulation of the process of apoptosis can occur. Similarly, various diseases states are associated with increased levels of cell death due to processes other than apoptosis.

In Alzheimer's disease, Parkinson's disease, Huntington's chorea, epilepsy, amyotrophic lateral sclerosis, stroke, ischemic heart disease, spinal cord injury and many viral infections, for example, abnormally high levels of cell death occur. In at least some of these diseases, there is evidence that the excessive cell death occurs through mechanisms consistent with apoptosis. Among these are 1) spinal cord injury, where the severing of axons deprives neurons of neurotrophic factors necessary to sustain cellular viability; 2) stroke, where after an initial phase of necrotic cell death due to ischemia, the rupture of dead cells releases excitatory neurotransmitters such as glutamate and oxygen free radicals that stimulate apoptosis in neighboring healthy neurons; and 3) Human Immunodeficiency Virus (HIV) infection, which induces apoptosis of T-lymphocytes.

In contrast, the level of apoptosis is decreased in cancer cells, which allows the cancer cells to survive longer than their normal cell counterparts. As a result of the increased number of surviving cancer cells, the mass of a tumor can increase even if the

doubling time of the cancer cells does not increase. Furthermore, the high level of expression in a cancer cell of the bcl-2 gene, which is involved in regulating apoptosis and, in some cases, necrotic cell death, renders the cancer cell relatively resistant to chemotherapeutic agents and to radiation therapy.

The p53 tumor suppressor protein (p53), e.g., a checkpoint gene, is an example of a protein that is involved in the process of apoptosis. The wild-type p53 protein induces apoptosis in a cell, whereas mutant p53 proteins do not induce apoptosis. Many cancers have mutations in the genes encoding p53 and, therefore, either do not express any p53 protein or express a mutant p53 protein. Thus, the absence of a wild-type p53 tumor suppressor in a cancer cell also can contribute to the low level of apoptosis that occurs in cancer cells.

The ability to manipulate the mechanism by which the genes involved in cell death are regulated would provide physicians with a potential target for therapies aimed at ameliorating the effects of diseases that are characterized by abnormal levels of cell death and also would allow for the development of methods to identify agents that can effectively regulate, for example, apoptosis in a cell. However, the mechanisms by which these genes are regulated in a cell have not yet been described. Thus, there exists a need to identify methods to manipulate the regulatory elements for genes involved in apoptosis.

Normal mammalian diploid cells placed in culture have a finite proliferative life-span and enter a nondividing state termed senescence, which is characterized by altered gene expression (Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol. Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64). Replicative senescence is dependent upon cumulative cell divisions and not chronologic or metabolic time, indicating that proliferation is limited by a "mitotic clock" (Dell'Orco et al. (1973) Exp. Cell Res. 77:356; Hadey et al. (1978) J. Cell. Physiol. 97:509). The reduction in proliferative capacity of cells from old donors and patients with premature aging syndromes (Martin et al. (1970) Lab. Invest 23:86; Schneider et al. (1976) PNAS 73:3584; Schneider et al. (1972) Proc. Soc. Exp. Biol. Med. 141:1092; Elmore et al. (1976) Cell Physiol. 87:229), and the accumulation in vivo of senescent cells with altered patterns of gene expression (Stanulis-Praeger et al. (1987) Mech. Ageing Dev. 38:1; and Dimri et al. (1995) PNAS 92:9363), implicate cellular senescence in aging and age-related pathologies ((Hayflick et al. (1961) Exp. Cell Res. 25:585; Wright et al. (1989) Mol. Cell. Biol. 9:3088; Goldstein, (1990) Science 249:112; Campisi, (1996) Cell 84:497; Campisi (1997) Eur. J. Cancer 33:703; Faragher et al. (1997) Drug Discovery Today 2:64).

Cellular senescence is believed to contribute to multiple conditions in the elderly that could in principle be remedied by cell life-span extension in situ. Examples include atrophy of the skin through loss of extracellular matrix homeostasis in dermal fibroblasts; age-related macular degeneration caused by accumulation of lipofuscin and downregulation of a neuronal survival factor in RPE cells; and atherosclerosis caused by loss of proliferative capacity and overexpression of hypertensive and thrombotic factors in endothelial cells.

Extended life-span cells also have potential applications ex vivo. Cloned normal diploid cells could replace established tumor cell lines in studies of biochemical and physiological aspects of growth and differentiation; long-lived normal human cells could be used for the production of normal or engineered biotechnology products; and expanded populations of normal or genetically engineered rejuvenated cells could be used for autologous or allogeneic cell and gene therapy. Thus the ability to extend cellular life-span, while maintaining the diploid status, growth characteristics, and gene expression pattern typical of young normal cells, has important implications for biological research, the pharmaceutical industry, and medicine.

Summary of the Invention

The present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell.

The present invention also relates to the treatment of proliferative disorders by inhibiting MIF-induced bypass of the p53 checkpoint.

A first aspect of the present invention provides a method for identifying a polypeptide which is capable of by-passing a cell-cycle checkpoint comprising,

(a) producing a cell which overexpresses a cell-cycle checkpoint gene so as to cause growth arrest of the cell;

(b) ectopically expressing a nucleic acid encoding a polypeptide product; and

(c) determining if the polypeptide product of the nucleic acid bypasses the checkpoint-mediated growth arrest and leads to cell proliferation.

The method can be practised using a cell wherein the cell-cycle checkpoint is selected from the group consisting of p53 and p53-like tumor suppressors, Ink4 gene products, and CIP/KIP gene products.

A second aspect of the invention provides a method for inducing cell growth comprising contacting a cell, with an amount of the polypeptide formulated according to method of claim 3 sufficient to induce cell proliferation

A third aspect of the present invention provides a method of inducing cell growth comprising contacting the cell with an MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof in an amount sufficient to induce cell proliferation.

A fourth aspect of the present invention provides a method for inducing cell growth in a cell whose growth is arrested by a tumor suppressor, comprising contacting the cell with an amount of a macrophage migration inhibitory factor (MIF), in an amount sufficient to induce cell proliferation.

A fifth aspect of the present invention provides a method for inducing cell growth in a cell whose growth is arrested by a tumor suppressor, comprising contacting the cell, with a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof in an amount sufficient to induce cell proliferation.

A sixth aspect of the invention provides a method for increasing the number of mitotic divisions a cell can undergo, comprising contacting the cell, with a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof, in an amount sufficient to increase the replicative capacity of the cell.

A seventh aspect of the present invention provides a method for improving the chemosensitivity of a cancer cell to a chemotherapeutic agent, comprising contacting the cell with an MIF polypeptide in an amount sufficient and under conditions wherein the cell undergoes p53-dependent cell death.

In preferred emdodiments of the above methods which utilize an MIF polypeptide are carried out with an MIF polypeptide that is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

The subject methods can be used, for example, to increase the proliferative capacity of a stem cell or progenitor cells. Such cells can be selected from the group consisting of neuronal, hematopoietic, pancreatic, and hepatic stem and progenitor cells.

In a preferred embodiment, the subject method can be used as part of a treatment of skin or other epithelial cells.

In another preferred embodiment, the subject method can be used as part of a treatment of mesenchymal cells.

In still another preferred embodiment, the subject method can be used as part of a treatment of chondrocyte or osteocytes.

As described herein, the cells which are treated can be treated in culture or in an implant, or by administration to an animal, e.g., *in vivo*.

An eighth aspect of the present invention provides method for inhibiting MIF-dependent proliferation of cells, comprising contacting the cell with an agent that inhibits MIF-mediated bypass of p53 wherein said agent competitively inhibits formation of MIF complexes or interaction of MIF polypeptide with its receptor.

A ninth aspect of the present invention provides a pharmaceutical preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth, and a pharmaceutically acceptable excipient.

A tenth aspect of the present invention provides a cosmetic preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth, in an amount suitable to promote proliferation of cells of a dermal layer when applied topically, and a pharmaceutically acceptable excipient for topical application.

The subject method can be carried out wherein MIF polypeptide is applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

An eleventh aspect of the present invention provides use of an MIF polypeptide in the manufacture of a medicament for promoting the healing of a wound, which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

A twelfth aspect of the present invention provides a kit for conjoint administration comprising, (a) the preparation of the ninth or tenth aspects, and (b) a trophic factor.

A thirteenth aspect of the present invention provides a kit for conjoint administration comprising, (a) the preparation of the ninth or tenth aspects, and (b) a chemotherapeutic agent.

A fourteenth aspect of the present invention provides use of a macrophage migration inhibitory factor (MIF) for the manufacture of a medicament for inducing cell growth in a cell whose growth is arrested by a tumor suppressor.

A fifteenth aspect of the present invention provides use of a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof for the manufacture of a medicament for inducing cell growth in a cell whose growth is arrested by a tumor suppressor.

A sixteenth aspect of the present invention provides use of a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof, for the manufacture of a medicament for increasing the number of mitotic divisions a cell can undergo.

A seventeenth aspect of the present invention provides use of an MIF polypeptide for the manufacture of a medicament for improving the chemosensitivity of a cancer cell to a chemotherapeutic agent wherein the cell undergoes p53-dependent cell death.

An eighteenth aspect of the present invention provides use of an agent that inhibits MIF-mediated bypass of p53 in the manufacture of a medicament for inhibiting MIF-dependent proliferation of cells, which agent competitively inhibits formation of MIF complexes or interaction of MIF polypeptide with its receptor.

A nineteenth aspect of the present invention provides method for promoting the healing of a wound comprising contacting the wound, tissue or cell with MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

A twentieth aspect of the present invention provides method for enhancing tissue regeneration process comprising contacting the tissue prophylactically with MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill

of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

Brief Description of the Drawings

Figure 1. Sequence alignment of human (SEQ ID No. 2), mouse, rat and cow MIF, with consensus sequence (SEQ ID No. 3).

Figure 2. Is a schematic diagram of an assay for identifying genes which are capable of bypassing p53-induced cell cycle arrest.

Figure 3. Is a photograph of culture plates.

Detailed Description of the Invention

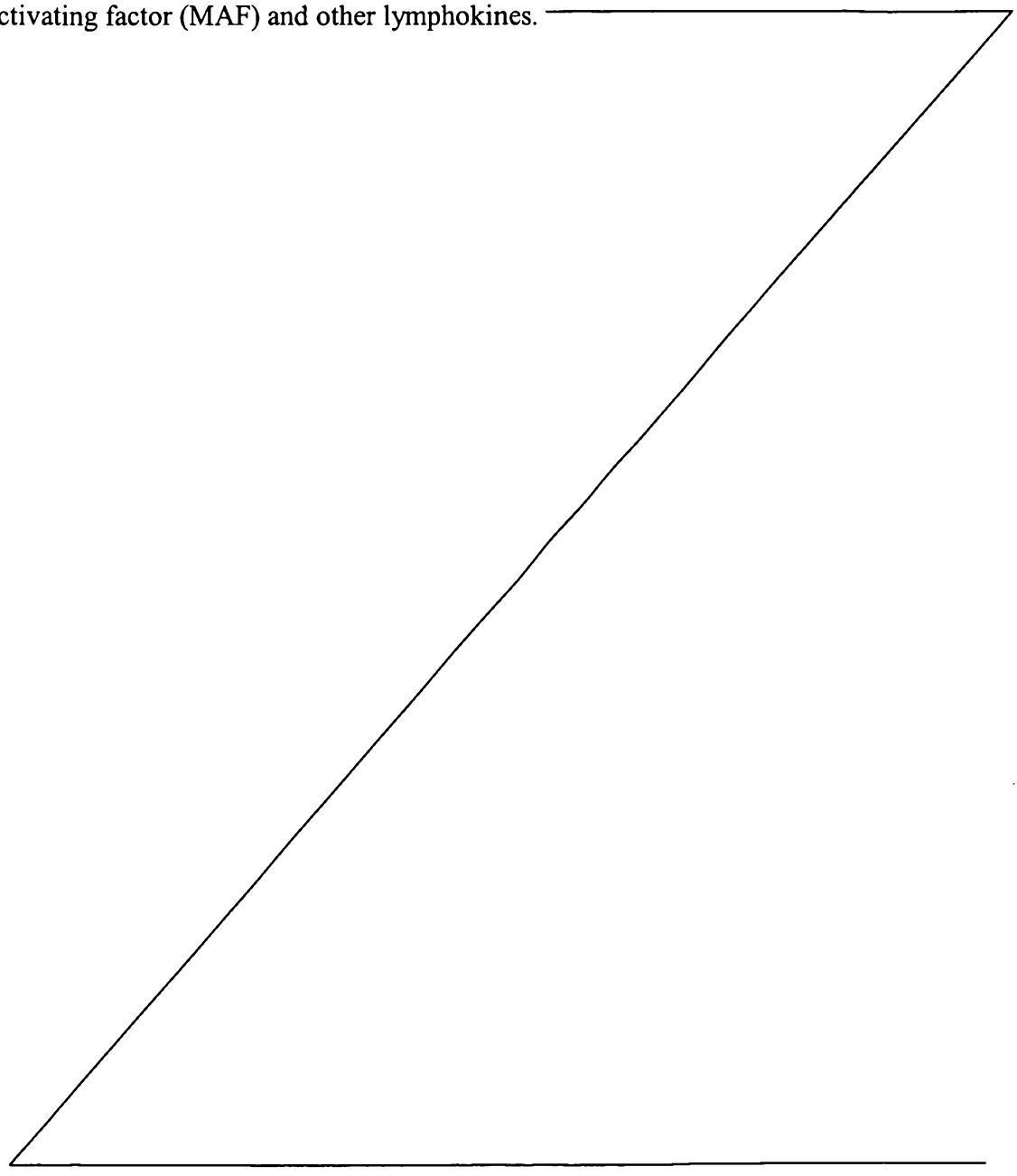
(i) Overview

The p53 protein has a key role in the negative regulation of cell proliferation, in the maintenance of genomic stability and in the suppression of transformation and tumorogenesis. The p53 gene product acts to prevent the accumulation of deleterious mutations by inducing cell cycle arrest or apoptosis following DNA damage. The importance of p53 in tumor suppression is underscored by the observation that mutations in p53 are the most common genetic alteration in human tumors. p53 can also be functionally inactivated by mutation-independent mechanisms. In a proportion of tumors, wild-type p53 is inactivated by abnormal subcellular localization or decreased stability.

In order to identify regulators of p53 activity, we undertook a screen to identify genes that, when expressed, were capable of bypassing p53 mediated growth arrest. See Figure 2 and US Patent No. 6,255,071. Briefly, mouse embryo fibroblasts (MEF) which lack endogenous p53 genes (from p53 knock-out mice) were engineered to conditionally express a fluorescently tagged p53 protein (GFP-p53). When activated the fluorescent p53 is localized to the nucleus and enforces cell cycle arrest. We used this cell line in a phenotype-based screen to identify negative regulators of p53 activity encoded by cDNA libraries. Cells that were no longer inhibited by p53 induction gave raise to colonies. CDNAs which were able to bypass the p53 arrest were isolated and sequenced. A recurring clone was the macrophage migration inhibitory factor (MIF). See SEQ ID No. 1 (nucleic acid) and SEQ ID No. 2 (protein).

A large number of publications have reported the isolation and identification of putative MIF molecules. For example, MIF-1 was purified to homogeneity from the serum-free culture supernatant of a human T cell hybridoma clone called F5 (Oki et al. (1991) Lymphokine Cytokine Res. 10:273-80). Also, an MIF-2, which is more hydrophobic than MIF-1, was purified to homogeneity from the same clone, (Hirose et al.(1991) Microbiol. Immunol. 35:235-45). A lymphocyte migration inhibitory factor (LyMIF) has also been identified (Masayuma et al. (1984) Cell Immunol 85:154-67). MIF belongs to the group of so-called lymphokines which comprises biologically active, soluble polypeptides that are secreted by lymphocytes and monocytes or macrophages when these are stimulated by antigens, mitogens or the like. According to the known state of the art, human MIF consists of a group of polypeptides that inhibit the migration

ability of macrophages. Human MIF is secreted not only by activated lymphocytes, T- and B-cells, but also by non-lymphoid cells, for example by growing fibroblasts and certain tumour cells. MIF can be clearly differentiated from γ-interferon, macrophage-activating factor (MAF) and other lymphokines.



Human MIF plays a decisive role in the early phase of an inflammation reaction ("delayed type hypersensitivity reaction"). It induces the differentiation of monocytes and quiescent tissue macrophages to mature inflammatory macrophages. Human MIF and related proteins are therefore important markers for inflammatory conditions.

Since MIF was originally identified as a cytokine, we tested for its ability to function when added *in trans*. The particular assay that was used was a classic one of p53 function, the ability to extend the life span of primary fibroblasts. As a source of MIF, we constructed a maltose binding protein -MIF fusion (MBP-MIF), expressed it in *E. Coli*, affinity purified the fusion protein and cleaved it to release the MIF polypeptide chain. Cultures of late passage (just presenescent) mouse fibroblasts were split into new culture plates in the presence or absence of 200ng/ml recombinant MIF, and allowed to grow from 15 days. The tissue culture media was replaced every three days with fresh media (containing MIF where appropriate). Numerous colonies were formed on plates where MIF was contained in the media, whereas none were observed for the cells growing in the absence of MIF. See Figure 3.

The dose response for the extension of life span of the primary MEFS was assayed in an experiment using 0, 25, 50, 75, 100, 150, 200, 300, 400, 600 ng MIF/ml media. Under these conditions, 150 ng/ml was found to be the optimal concentration.

Applicants understand that this is the first example of a natural protein capable of functionally inactivating the growth arrest phenotype of a tumor suppressor *in trans*. In addition, it is, as far as the Applicants are aware, the first example of a molecule capable of extending the life span of primary cells, *in trans*. In addition, since MIF has been identified as having a proinflammatory role, both systemicallt and locally, this observation provides a link between the fields of inflammation and tumor biology.

One aspect of the present invention relates to methods and reagents for extending the life-span, e.g., the number of mitotic divisions, of a cell. In general, the subject method relies on the ectopic expression or application of a MIF protein, or a bioactive fragment or peptidomimetic thereof, or other "MIF therapeutic" as defined below. By "ectopic expression", it is meant that a cell is caused to express, e.g., by expression of a heterologous or endogenous gene, a higher than normal level of MIF than the cell normally would for the particular starting phenotype. The subject method is useful both *in vivo*, *ex vivo* and *in situ*. Exemplary uses include, merely to illustrate, the extension of stem cell or progenitor cell cultures or implants, the extension of skin or other epithelial cell cultures or grafts, the expansion of mesenchymal cell cultures or grafts, and the expansion of chondrocyte or osteocyte cultures or grafts. Exemplary stem and progenitor cells which can be extended by the subject method include neuronal, hematopoietic,

pancreatic, and hepatic stem and progenitor cells. The subject methods and compositions of MIF can be used cosemetically as well.

The subject MIF treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

(ii) Definitions

For convenience, certain terms used herein as defined below.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid comprising an open reading frame encoding a polypeptide, including both exonic and (optionally) intronic sequences. An exemplary recombinant gene encoding a subject MIF protein is represented by SEQ. ID No: 1.

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein with respect to transfected nucleic acid, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a MIF polypeptide of the present invention.

"Expression vector" refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) genetic element(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a DNA sequence encoding a desired protein (in this case, a MIF protein of the present invention) which is transcribed into mRNA and translated into protein, and (3) appropriate transcription and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are

used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated. Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of the MIF gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of one of the naturally-occurring forms of an MIF protein.

As used herein, the term "tissue-specific promoter" means a DNA sequence that serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a urogenital origin, e.g. renal cells, or cells of a neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

The terms “MIF proteins” and “MIF polypeptides” refer to a family of related polypeptides having such bioactivities as described herein. Exemplary MIF proteins are encoded by the nucleic acid of SEQ ID No:1, or by a nucleic acid which hybridizes thereto. Thus, the MIF proteins useful in the subject method can be at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or even at least 95% identical to the human MIF of SEQ ID No:2, or a fragment thereof which reconsitutes a telomerase elongation enzyme in a host cell (such as a human cell). A variety of different techniques are available in the art for

assessing the activity of a particular MIF polypeptide, e.g., which may vary in sequence and/or length relative to SEQ ID No: 1.

As used herein, the term "fusion protein" is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

The term "MIF therapeutic agent" refers to any agent, such as a protein, peptide, small organic molecule, nucleic acid, etc, which can mimic or potentiate MIF-induced bypass of the p53 checkpoint (an agonist) or inhibit MIF MIF-induced bypass of the p53 checkpoint (an antagonist).

The term "progenitor cell" refers to an undifferentiated cell which is capable of proliferation and giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable daughter cells. As used herein, the term "progenitor cell" is also intended to encompass a cell which is sometimes referred to in the art as a "stem cell". In a preferred embodiment, the term "progenitor cell" refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues.

As used herein the term "substantially pure", with respect to progenitor cells, refers to a population of progenitor cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to progenitor cells making up a total cell population. Recast, the term "substantially pure" refers to a population of progenitor cell of the present invention that contain fewer than about 20%, more preferably fewer than about 10%, most preferably fewer than about 5%, of lineage committed cells in the original unamplified and isolated population prior to subsequent culturing and amplification.

The term "cosmetic preparation" refers to a form of a pharmaceutical preparation which is formulated for topical administration.

As used herein, the term "cellular composition" refers to a preparation of cells, which preparation may include, in addition to the cells, non-cellular components such as cell culture media, e.g. proteins, amino acids, nucleic acids, nucleotides, co-enzyme, anti-oxidants, metals and the like. Furthermore, the cellular composition can have components which do not affect the growth or viability of the cellular component, but which are used

to provide the cells in a particular format, e.g., as polymeric matrix for encapsulation or a pharmaceutical preparation.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophegeal, epidermal, and hair follicle epithelial cells. Other exemplary epithlelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, which that characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g. tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow; and "hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

A "wound to eye tissue" refers to severe dry eye syndrome, corneal ulcers and abrasions and ophthalmic surgical wounds.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of cutaneous tissue. These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic

dermatitis. For example, epidermodysplasia is a form of faulty development of the epidermis.. Another example is "epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and bullae either spontaneously or at the site of trauma.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma", which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; "epidermoid carcinoma", which refers to cancerous cells which tend to differentiate in the same way as those of the epidermis; i.e., they tend to form prickle cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of tubular cells in varying arrangements. Another carcinomatous epithelial growth is "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder which alters the skin's regulatory mechanisms. In particular, lesions are formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be refered to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an AR sequence of the present invention.

(iii) Exemplary Uses

The present method can be used to increase the proliferative capacity and/or lifespan of cells *in vivo*, *in vitro* and as part of an ex vivo protocol. While the method of the invention is applicable to any normal cell type, the method is preferably practiced using normal cells that have encountered, or will encounter, an environmental cue which activates a p53 checkpoint or other checkpoint. For purposes of the present invention, the term "normal" refers to cells other than tumor cells, cancer calls, or transformed cells. An

exemplary cell is an embryonic stem cells, such as disclosed in Thomson et al. (1998) Science 282:1145 and Shamblott et al. (1998) PNAS 95:13726. Especially preferred cells for use in the present method include embryonic, fetal, neonatal, and adult stem cells of any organ, and adult pluripotent hematopoietic stem cells.

In one embodiment, the cells are stem and/or progenitor cells. These include hematopoietic stem cells, e.g., which are derived from bone marrow, mobilized peripheral blood cells, or cord blood. In other embodiments, the cells are progenitor cells for pancreatic or hepatic tissue, or other tissue deriving from the primative gut. In still other embodiments, the stem is a neuronal stem cell, such as neural crest which can be used to form neurons or smooth muscle cells.

In other embodiments, the cells are not stem or progenitor cells, e.g., they are committed cells, such as pancreatic β cells, smooth muscle cells (or other myocytic cells), fibroblasts, lymphocytic cells, e.g., B or T cells, osteocytes or chondrocytes, to name but a few.

While the subject method can be used either *in vivo* or *in vitro*, the invention has particular application to the cultivation of cells *ex vivo*, and provides especially important benefits to therapeutic methods in which cells are cultured *ex vivo* and then reintroduced to a host. For example, the subject method can be used to extend the proliferative capacity of cells which are harvested, or otherwise isolated in culture, which are to be transplanted to a patient.

Such protocols can find use in bone marrow transplants wherein bone marrow, or isolated hematopoietic progenitor cells are treated according to the present invention, with the by-pass of the checkpoint being reversed to the wild-type phenotype before, or shortly after, transplantation.

The subject method can also be used to extend T cell life in HIV and Down's patients.

It also has application in protocols for the formation of artificial tissues such as prosthetic devices, e.g., deriving from stem or committed cells. Exemplary tissues include pancreatic, hepatic, neural, myocytic, cartilaginous and osseous tissue.

In one embodiment, the subject method can be used to extend the proliferative capacity of hematopoietic cells and hematopoietic stem/progenitor cells. The term "hematopoietic cells" herein refers to fully differentiated myeloid cells such as erythrocytes or red blood cells, megakaryocytes, monocytes, granulocytes, and eosinophils, as well as fully differentiated lymphoid cells such as B lymphocytes and T lymphocytes. Thus, a hematopoietic stem/progenitor cell includes the various

hematopoietic precursor cells from which these differentiated cells develop, such as BFU-E (burst-forming units-erythroid), CFU-E (colony forming unit-erythroid), CFU-Meg (colony forming unit-megakaryocyte), CFU-GM (colony forming unit-granulocyte-monocyte), CFU-Eo (colony forming unit-eosinophil), and CFU-GEMM (colony forming unit-granulocyte-erythrocyte-megakaryocyte-monocyte).

In another embodiment, the subject method can be use to extend the proliferative capacity pancreatic cells and pancreatic stem/progenitor cells. The term "pancreatic progenitor cell" refers to a cell which can differentiate into a cell of pancreatic lineage, e.g. a cell which can produce a hormone or enzyme normally produced by a pancreatic cell. For instance, a pancreatic progenitor cell may be caused to differentiate, at least partially, into α, β, δ, or ϕ islet cell, or a cell of exocrine fate. The pancreatic progenitor cells of the invention can also be cultured prior to administration to a subject under conditions which promote cell proliferation and differentiation. These conditions include culturing the cells to allow proliferation and confluence *in vitro* at which time the cells can be made to form pseudo islet-like aggregates or clusters and secrete insulin, glucagon, and somatostatin.

The endocrine portion of the pancreas is composed of the islets of Langerhans. The islets of Langerhans appear as rounded clusters of cells embedded within the exocrine pancreas. Four different types of cells- α, β, δ, and ϕ-have been identified in the islets. The α cells constitute about 20% of the cells found in pancreatic islets and produce the hormone glucagon. Glucagon acts on several tissues to make energy available in the intervals between feeding. In the liver, glucagon causes breakdown of glycogen and promotes gluconeogenesis from amino acid precursors. The δ cells produce somatostatin which acts in the pancreas to inhibit glucagon release and to decrease pancreatic exocrine secretion. The hormone pancreatic polypeptide is produced in the ϕ cells. This hormone inhibits pancreatic exocrine secretion of bicarbonate and enzymes, causes relaxation of the gallbladder, and decreases bile secretion. The most abundant cell in the islets, constituting 60-80% of the cells, is the β cell, which produces insulin. Insulin is known to cause the storage of excess nutrients arising during and shortly after feeding. The major target organs for insulin are the liver, muscle, and fat-organs specialized for storage of energy.

In an exemplary embodiment, the subject agents can be used to extend the proliferative capacity of implanted pancreatic tissue, e.g., implanted β-islet cells. Recently, tissue-engineering approaches to treatment have focused on transplanting pancreatic islets, usually encapsulated in a membrane to avoid immune rejection. Many methods for encapsulating cells are known in the art. For example, a source of β islet cells producing insulin is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the β islet cells (Aebischer et al.

U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) *Expt. Neurobiol.* 110:39-44; Jaeger et al. (1990) *Prog. Brain Res.* 82:41-46; and Aebischer et al. (1991) *J. Biomech. Eng.* 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the β islet cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) *Trans. Am. Artif. Intern. Organs* 35:791-799; Sefton et al. (1987) *Biotehnol. Bioeng.* 29:1135-1143; and Aebischer et al. (1991) *Biomaterials* 12:50-55).

In any of the above-embodiments, the pancreatic cells can be treated by the subject method ex vivo, and/or treated by the subject method by subsequent delivery of, e.g., a MIF therapeutic, to an animal in which the device is implanted. Such cells can be used for treatment of diabetes because they have the ability to differentiate into cells of pancreatic lineage, e.g., β islet cells. The pancreatic cells of the invention can be cultured *in vitro* under conditions which can further induce these cells to differentiate into mature pancreatic cells, or they can undergo differentiation in vivo once introduced into a subject.

Moreover, in addition to providing a source of implantable cells, either in the form of the progenitor cell population of the differentiated progeny thereof, the subject method can be used to extend the proliferative capacity of normal pancreatic cells used to produce cultures for the production and purification of secreted factors. For instance, cultured cells can be provided as a source of insulin. Likewise, exocrine cultures can be provided as a source for pancreatin.

In still another embodiment, the subject method can be used to extend the proliferative capacity of hepatic cells and hepatic stem cells. The term "hepatic progenitor cell" as used herein refers to a cell which can differentiate in a cell of hepatic lineage, such a liver parenchymal cell, e.g., a hepatocyte. Hepatocytes are some of the most versatile cells in the body. Hepatocytes have both endocrine and exocrine functions, and synthesize and accumulate certain substance, detoxify others, and secrete others to perform enzymatic, transport, or hormonal activities. The main activities of liver cells include bile secretion, regulation of carbohydrate, lipid, and protein metabolism, storage of substances important in metabolism, degradation and secretion of hormones, and transformation and excretion of drugs and toxins. The subject method can be used to facilitate the long term culture of hepatic cells and hepatic progenitor cells either in vitro or subsequent to implantation.

In still another embodiment, the subject method can be used to extend the proliferative capacity of "feeder" cell layers for cell co-cultures.

In another embodiment, the subject method can be used to enhance large-scale cloning, e.g., of non-human animals, by enhancing the presence of actively dividing fetal fibroblasts for nuclear transfer.

Prior research in nuclear transplantation has shown that the cell cycle stage of the donor cell affects the extent of development of the embryo after nuclear transfer. When the donor cell is fused to the recipient oocyte, which is arrested in the second metaphase in meiosis, the nuclear envelope breaks down and the chromosomes condense until the oocyte is activated. This condensation phase has been shown to cause chromosomal defects in donor cells that are undergoing DNA synthesis. Donor cells in the G_1 phase of the cell cycle (before DNA synthesis), however, condense normally and support a high rate of early development.

Our rationale in selecting an optimal donor cell for nuclear transplantation was that the cell should not have ceased dividing (which is the case in G_0) but be actively dividing, as an indication of a relatively undifferentiated state and for compatibility with the rapid cell divisions that occur during early embryo development. The cells should also be in G_1, either by artificially arresting the cell cycle or by choosing a cell type that has an inherently long G1 phase.

The subject methods are also applicable to general cell culture techniques. For example, the method can be used to increase the replicative capacity of hybrids between immortal and mortal human cells, such as hybrids between human B-lymphocytes and myeloma cells, e.g., to increase the replicative capacity of antibody producing human hybridomas.

More generally, the subject method can be used to increase the replicative capacity of cells in culture which have been engineered to produce recombinant proteins. Indeed, the subject method can permit the use of "normal" cells as the recombinant cell, so that problems which may occur with the use of immortal cells (such as differences in post-translation modifications) can be avoided, particularly for producing secreted proteins.

In still another embodiment, the subject method can be used to enhance the life of "feeder" cell layers for cell co-cultures.

In another aspect, the present invention provides pharmaceutical preparations and methods for controlling the proliferation of epithelially-derived tissue utilizing, as an active ingredient, a MIF therapeutic agent. The invention also relates to methods of controlling proliferation of epithelial-derived tissue by use of the pharmaceutical preparations of the invention. To illustrate, an MIF therapeutic agent of the present invention may be used as part of regimens in the treatment of disorders of, or surgical or

cosmetic repair of, such epithelial tissues as skin and skin organs; corneal, lens and other ocular tissue; mucosal membranes; and periodontal epithelium. The methods and compositions disclosed herein provide for the treatment or prevention of a variety of damaged epithelial and mucosal tissues. For instance, the subject method can be used to control wound healing processes, as for example may be desirable in connection with any surgery involving epithelial tissue, such as from dermatological or periodontal surgeries. Exemplary surgical repair for which use of an MIF therapeutic agent is a candidate treatment include severe burn and skin regeneration, skin grafts, pressure sores, dermal ulcers, fissures, post surgery scar reduction, and ulcerative colitis.

The subject method has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of contacting a cell, in vitro or in vivo, with an amount of an MIF therapeutic agent sufficient to alter the life span of the treated epithelial tissue. For in vivo use, the mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues.

A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment. "Promotion of wound healing" can also mean that the method causes the extends the proliferative and growth phase of, *inter alia,* keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g. the take rates of skin grafts,) when used together with the method of the present invention.

Complications are a constant risk with wounds that have not fully healed and remain open. Although most wounds heal quickly without treatment, some types of wounds resist healing. Wounds which cover large surface areas also remain open for extended periods of time. In one embodiment of the present invention, the subject method can be used to enhance and/or otherwise accelerate the healing of wounds involving epithelial tissues, such as resulting from surgery, burns, inflammation or irritation. The MIF therapeutic agents of the present invention can also be applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

Full and partial thickness burns are an example of a wound type which often covers large surface areas and therefore requires prolonged periods of time to heal. As a result,

life-threatening complications such as infection and loss of bodily fluids often arise. In addition, healing in burns is often disorderly, resulting in scarring and disfigurement. In some cases wound contraction due to excessive collagen deposition results in reduced mobility of muscles in the vicinity of the wound. The compositions and method of the present invention can be used to enhance the healing of burns and to promote healing processes that result in more desirable cosmetic outcomes and less wound contraction and scarring.

Severe burns which cover large areas are often treated by skin autografts taken from undamaged areas of the patient's body. The subject method can also be used in conjunction with skin grafts to impove the grafts performance and life span in culture, as well as improve the "take" rates of the graft by accelerating growth of both the grafted skin and the patient's skin that is proximal to the graft.

Dermal ulcers are yet another example of wounds that are amenable to treatment by the subject method, e.g., to cause healing of the ulcer and/or to prevent the ulcer from becoming a chronic wound. For example, one in seven individuals with diabetes develop dermal ulcers on their extremities, which are susceptible to infection. Individuals with infected diabetic ulcers often require hospitalization, intensive services, expensive antibiotics, and, in some cases, amputation. Dermal ulcers, such as those resulting from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) and arterial ulcers also resist healing. The prior art treatments are generally limited to keeping the wound protected, free of infection and, in some cases, to restore blood flow by vascular surgery. According to the present method, the afflicted area of skin can be treated by a therapy which includes a MIF therapeutic agent which promotes epithelization of the wound, e.g., accelerates the rate of the healing of the skin ulcers.

In another exemplary embodiment, the subject method is provided for treating or preventing gastrointestinal diseases. Briefly, a wide variety of diseases are associated with disruption of the gastrointestinal epithelium or villi, including chemotherapy- and radiation-therapy-induced enteritis (i.e. gut toxicity) and mucositis, peptic ulcer disease, gastroenteritis and colitis, villus atrophic disorders, and the like. For example, chemotherapeutic agents and radiation therapy used in bone marrow transplantation and cancer therapy affect rapidly proliferating cells in both the hematopoietic tissues and small intestine, leading to severe and often dose-limiting toxicities. Damage to the small intestine mucosal barrier results in serious complications of bleeding and sepsis. The subject method can be used to promote proliferation of gastrointenstinal epithelium and thereby increase the tolerated doses for radiation and chemotherapy agents. Effective

treatment of gastrointestinal diseases may be determined by several criteria, including an enteritis score, other tests well known in the art.

With age, the epidermis thins and the skin appendages atrophy. Hair becomes sparse and sebaceous secretions decrease, with consequent susceptibility to dryness, chapping, and fissuring. The dermis diminishes with loss of elastic and collagen fibers. Moreover, keratinocyte proliferation (which is indicative of skin thickness and skin proliferative capacity) decreases with age. An increase, or prolinged rate of keratinocyte proliferation is believed to conteract skin aging, i.e., wrinkles, thickness, elasticity and repair. According to the present invention, an MIF therapeutic agent can be used either therapeutically or cosmetically to counteract, at least for a time, the effects of aging on skin.

The subject method can also be used in treatment of a wound to eye tissue. Generally, damage to corneal tissue, whether by disease, surgery or injury, may affect epithelial and/or endothelial cells, depending on the nature of the wound. Corneal epithelial cells are the non-keratinized epithelial cells lining the external surface of the cornea and provide a protective barrier against the external environment. Corneal wound healing has been of concern to both clinicians and researchers. Opthomologists are frequently confronted with corneal dystrophies and problematic injuries that result in persistent and recurrent epithelial erosion, often leading to permanent endothelial loss. The use of MIF therapeutic agents can be used in these instances to promote epithelialization of the affected corneal tissue. To further illustrate, specific disorders typically associated with epithelial cell damage in the eye, and for which the subject method can provide beneficial treatment, include persistent corneal epithelial defects, recurrent erosions, neurotrophic corneal ulcers, keratoconjunctivitis sicca, microbial corneal ulcers, viral cornea ulcers, and the like. Moreover, superficial wounds such as scrapes, surface erosion, inflammation, etc. can cause lose of epithelial cells. According to the present invention, the corneal epithelium is contacted with an amount of an MIF therapeutic agent effective to enhance proliferation of the corneal epithelial cells to appropriately heal the wound.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, more than 40,000 corneal transplants were performed in the United States in 1996. Human epidermal cells can be grown in vitro and used to populate burn sites and chronic skin ulcers and other dermal wounds. The subject method can be used to enhance the life span of epithelial tissue *in vitro*, as well as to enhance the grafting of the cultured epithelial tissue to an animal host

The present method can be used for improving the "take rate" of a skin graft. Grafts of epidermal tissue can, if the take rate of the graft is to long, blister and shear, decreasing the likelihood that the autograft will "take", i.e. adhere to the wound and form a basement membrane with the underlying granulation tissue. Take rates can be increased by the subject method by enhancing the proliferation of the keratinocytes. The method of increasing take rates comprises contacting the skin autograft with an effective wound healing amount of an MIF therapeutic agent described in the method of promoting wound healing and in the method of promoting the growth and proliferation of keratinocytes, as described above.

Skin equivalents have many uses not only as a replacement for human or animal skin for skin grafting, but also as test skin for determining the effects of pharmaceutical substances and cosmetics on skin. A major difficulty in pharmacological, chemical and cosmetic testing is the difficulties in determining the efficacy and safety of the products on skin. One advantage of the skin equivalents of the invention is their use as an indicator of the effects produced by such substances through in vitro testing on test skin.

Thus, in one embodiment of the subject method can be used as part of a protocol for skin grafting of, e.g., denuded areas, granulating wounds and burns. The use of MIF therapeutic agents can enhance such grafting techniques as split thickness autografts and epidermal autografts (cultured autogenic keratinocytes) and epidermal allografts (cultured allogenic keratinocytes). In the instance of the allograft, the use of the subject method to enhance the formation of skin equivalents in culture helps to provide/maintain a ready supply of such grafts (e.g., in tissue banks) so that the patients might be covered in a single procedure with a material which allows permanent healing to occur.

In this regard, the present invention also concerns composite living skin equivalents comprising an epidermal layer of cultured keratinocyte cells which have been expanded in the presence of an MIF therapeutic agent. The subject method can be used as part of a process for the preparation of composite living skin equivalents. In an illustrative embodiment, such a method comprises obtaining a skin sample, treating the skin sample enzymically to separate the epidermis from the dermis, treating the epidermis enzymically to release the keratinocyte cells, culturing, in the presence of an MIF therapeutic agent, the epidermal keratinocytes until confluence, in parallel, or separately, treating the dermis enzymatically to release the fibroblast cells, culturing the fibroblasts cells until sub-confluence, inoculating a porous, cross-linked collagen sponge membrane with the cultured fibroblast cells, incubating the inoculated collagen sponge on its surface to allow the growth of the fibroblast cells throughout the collagen sponge, and then inoculating it

with cultured keratinocyte cells, and further incubating the composite skin equivalent complex in the presence of an MIF therapeutic agent to enhance the life span of the cells.

In other embodiments, skin sheets containing both epithelial and mesenchymal layers can be isolated in culture and expanded with culture media supplemented with an MIF therapeutic agent.

Any skin sample amenable to cell culture techniques can be used in accordance with the present invention. The skin samples may be autogenic or allogenic.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which control of epithelial cell proliferation in and around periodontal tissue is desired. In one embodiment, proliferative forms of the MIF therapeutics can be used to enhance reepithelialization around natural and prosthetic teeth, e.g., to promote formation of gum tissue.

In yet another aspect, the subject method can be used to help control guided tissue regeneration, such as when used in conjunction with bioresorptable materials. For example, incorporation of periodontal implants, such as prosthetic teeth, can be facilitated by the instant method. Reattachment of a tooth involves both formation of connective tissue fibers and re-epithelization of the tooth pocket. The subject methodtreatment can be used to enhance tissue reattachment by controlling the mitotic capacity of basal epithelial cells in the wound healing process.

In another aspect of the present invention, MIF therapeutic preparations can be used to effect the growth of hair, as for example in the treatment of alopecia whereby hair growth is potentiated, or for example in cosemetic removal of hair (depilation) whereby hair growth is inhibited.

In certain embodiments, the subject compositions can be used to inhibit, rather than promote, growth of epithelial-derived tissue. For instance, certain of the compositions disclosed herein may be applied to the treatment or prevention of a variety hyperplastic or neoplastic conditions. The method can find application for the treatment or prophylaxis of, e.g., psoriasis; keratosis; acne; comedogenic lesions; folliculitis and pseudofolliculitis; keratoacanthoma; callosities; Darier's disease; ichthyosis; lichen planus; molluscous contagiosum; melasma; Fordyce disease; and keloids or hypertrophic scars. Certain of the formulations of the present invention may also be used as part of treatment regimens in auto-immune diseases for affecting healing of proliferative manifestations of the disorder, as for example, part of a treatment for aphthous ulcers, pemphigus such as pemphigus vulgaris, pemphigus foliaceus, pemphigus vegetans or pemphigus erythematous, epidermolysis, lupus lesions or desquamative lesions.

In yet another embodiment of the present invention, a MIF therapeutic can be used to modulate spermatogenesis, e.g., either inhibit (as a contraceptive) or potentiate (as male fertility drug).

It is now apparent from the observation herein that MIF may play a role in tumorogenesis, e.g., in the steps of immortalization and/or the maintenance of a tumor. Accordingly, inhibitors of MIF-induced bypass of p53 can be used a chemotherapeutics, e.g., preferably in the treatment of proliferative disorders with a p53+ phenotype.

Moreover, apoptosis is a major determinant of the effectiveness of antitumor chemotherapy since most drugs used in cancer treatments provoke cell death by this process. In many instances, the mechanism is by p53-mediated apoptosis (e.g., in p53+ tumors). For instance, chemosensitivity to genistein is apparently dependent on cellular p53 content. Likewise, induction of $p21^{waf1}$ in cells (a transcriptional target of p53) increases the cells' sensitivity to doxorubicin, tumudex and methotrexate as compared to uninduced cells; this condition is associated with apoptosis. Accordingly, inhibitors of native MIF-induced p53 bypass, e.g., as may result from MIF produced by macrophages infiltrating a tumor, can help to improve the chemosensitivity of cancer cells for chemotherapeutic agents, e.g., at least ones which are potentiated by p53-mediated apoptosis.

Thus, MIF antagonists can be delivered conjointly with chemotherapeutic agents such as alkylating agents, including: alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodepa, carboquone, meturedepa and uredepa; ethylenimines and methylmelamines such as altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, chclophosphamide,estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide and uracil mustard; nitrosoureas such as carmustine, chlorozotocin, foremustine, lomustine, nimustine and ranimustine; and others such as dacarbazine, mannomustine, mitobronitol, mitolactol and pipobroman.

In one embodiment, the cells to be treated are hyperproliferative cells of adipocytic lineage, e.g., arising from adipose or adipose precursor cells. For instance, the instant method can be carried out to prevent the proliferation of an adipose cell tumor. The adipose tumor cells can be of a liposarcoma. The term "liposarcoma" is recognized by those skilled in the art and refers to a malignant tumor characterized by large anaplastic lipoblasts, sometimes with foci of normal fat cells. Exemplary liposarcoma types which are can be treated by the present invention include, but are not limited to, well

differentiated/dedifferentiated, myxoid/round cell and pleiomorphic (reviewed in Sreekantaiah, C. et al., (1994) *supra*).

Another adipose cell tumor which may be treated by the present method include lipomas, e.g., benign fatty tumors usually composed of mature fat cells. Likewise, the method of the present invention can be used in the treatment and/or prophylaxis of lipochondromas, lipofibromas and lipogranulomas. Lipochondroma are tumors composed of mature lipomatous and cartilaginous elements; lipofibromas are lipomas containing areas of fibrosis; and lipogranuloma are characterized by nodules of lipoid material associated with granulomatous inflammation.

The subject method may also be used to inhibit the proliferation of hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. For instance, the present invention contemplates the treatment of various myeloid disorders including, but not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) *Crit Rev. in Oncol./Hemotol.* 11:267-97). Lymphoid malignancies which may be treated by the subject method include, but are not limited to acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas contemplated by the treatment method of the present invention include, but are not limited to, non-Hodgkin's lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease.

In another embodiment, the subject method can be used in the treatment of various carcinomas. In a representative embodiment, a MIF inhibitor is used to treat certain breast cancers. In similar fashion, MIF antagonist can be used in therapeutic protocols for the treatment of malignant melanoma. In yet another embodiment, the subject method is used in the treatment of sarcomas, e.g. an osteosarcoma or Kaposi's sarcoma.

The subject method can be used in the treatment of hyperproliferative vascular disorders, e.g. smooth muscle hyperplasia (such as atherosclerosis) or restinosis, as well as other disorders characterized by fibrosis, e.g. rheumatoid arthritis, insulin dependent diabetes mellitus, glomerulonephritis, cirrhosis, and scleroderma. For example, in the case of restinosis, the MIF inhibitor can be delivered, for example, by percutaneous transluminal transfer (Mazur et al. (1994) *Tex Heart Inst J* 21:104-111).

(iv) Exemplary MIF Therapeutics and formulations

The MIF therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the MIF therapeutics are preferably derived from vertebrate MIF proteins, e.g., have sequences corresponding to naturally occurring MIF proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the MIF polypeptide can correspond to a MIF protein (or fragment thereof) which occurs in any metazoan organism.

The various naturally-occurring MIF proteins from which the subject therapeutics can be derived are well characterized in the art. See for example GenBank accession M25639, U62326, U20999, M95776, U88035 and SWISS-PROT accession P80177, P34884, S32394. The MIF proteins have no obvious secretion signal peptide, yet are generally understood to be secreted.

Moreover, mutagenesis can be used to create modified MIF polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*), or generating antagonists (e.g., dominant negative mutants). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified MIF polypeptides can also include those with aitered post-translational processing relative to a naturally occurring MIF protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the MIF therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a MIF coding sequence represented in SEQ ID No:1. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other MIF proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a MIF protein can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a

number of known techniques. The gene encoding a MIF protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a MIF polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence of SEQ ID No: 2. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID Nos:8-14 are also within the scope of the invention.

In addition to native MIF proteins, MIF polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by SEQ ID No: 2. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence of SEQ ID No: 2 are also within the scope of the invention.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a MIF polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant MIF gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native MIF protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring MIF polypeptides, e.g., mutational variants.

As is known in the art, MIF polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide MIF may be secreted and isolated from a mixture of cells and medium containing the recombinant MIF polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant MIF gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant MIF polypeptide can be isolated from cell culture

medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant MIF polypeptide is a fusion protein containing a domain which facilitates its purification, such as an MIF/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

Recombinant MIF genes can be produced by ligating nucleic acid encoding an MIF protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject MIF polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a MIF polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used. In an illustrative embodiment, an MIF polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of the human MIF genes represented in SEQ ID No:1.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A*

Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant MIF polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

When it is desirable to express only a portion of an MIF protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al. (1987) *PNAS 84*:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing MIF-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the MIF polypeptides of the present invention. For example, MIF polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the MIF polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the MIF protein (e.g.of the pro-form, in order to permit purification of the poly(His)-MIF protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide

sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

MIF polypeptides may also be chemically modified to create MIF derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of MIF proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

In a preferred embodiment, the MIF polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface associated proteins which may normally be associated with the MIF polypeptide, unless provided in the form of fusion protein with the MIF polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of MIF polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated MIF polypeptides can include all or a portion of the amino acid sequences represented SEQ ID No: 2, or a homologous sequence thereto. With respect to bioctive fragments of MIF polypeptide, preferred MIF therapeutics include at least 50 (contiguous) amino acid residues of a MIF

polypeptide, more preferably at least 100 (contiguous), and even more preferably at least 150 (contiguous) residues.

Isolated peptidyl portions of MIF proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a MIF polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") MIF protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant MIF polypeptides of the present invention also include homologs of the authentic MIF proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. MIF homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein.

Modification of the structure of the subject MIF polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the MIF polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine.

Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur -containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional MIF homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring MIF proteins. In one embodiment, the invention contemplates using MIF polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are mimic or antagonize native MIF proteins. To illustrate, MIF homologs can be engineered by the present method to provide interfere with the formation of oligomeric complexes of native MIF proteins, e.g., to antagonize the function of the wild-type protein. MIF is understood to form trimeric complexes which may be important to at least a portion of its bioactivity. Dominant negative MIF homologs can be used to inhibit the p53-bypass activity of wild-type MIF. In other embodiments, such mutational analysis can be used to disect the various activities of the protein. For instance, the p53-bypass activity of the protein may be isolated from other activities of the protein, such as its tautomerase activity. Moreover, manipulation of certain domains of MIF by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679

illustrate specific techniques which one skilled in the art could utilize to generate libraries of MIF variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the MIF polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of MIF proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illsutrate, the amino acid sequences for a population of MIF homologs or other related proteins are aligned, preferably to promote the highest homology possible. See for example Figure 1. Such a population of variants can include, for example, MIF homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of MIF variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential MIF sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display) containing the set of MIF sequences therein.

In an illustrative embodiment, alignment of various mammalian MIF proteins can be used to produce a degenerate set of MIF polypeptides represented by the general formula:

MPMFXVNTNVPRASVPXGFLSELTQQLAQATGKXPQYIAVHVVPDQLMXFX
GXXXPCALCSLHSIGKIGGAQNRXYSKLLCGLLXXRLXISPDRXYINXXDM
NAAXVGWNXSTFA

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, rat or cow MIF clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. In an even more expansive library, each X can be selected from any amino acid.

There are many ways by which the library of potential MIF homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential MIF sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of MIF homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate MIF sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is used to transfect a eukaryotic cell that can be co-cultured with GFP-p53 cells as described above. A functional MIF protein secreted by the cells expressing the combinatorial library will diffuse to neighboring MEF cells and induce proliferation. The pattern of detection of proliferation will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing MIF homologs active as proliferative agents with respect to epithelial cells. Likewise, MIF antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type MIF added to the culture media.

To illustrate, target FP-p53 engineered cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial MIF gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant MIF homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a MIF protein to produce a measurable response in the target cells, such as proliferation, the inserts are removed and the effect of the variant MIF proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the MIF protein to generate mimetics, e.g. peptide or non-peptide agents, which mimic or inhibit wild-type MIF. Thus, such mutagenic techniques as described above are also useful to map the determinants of the MIF proteins which are critical to bioactivity. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject MIF proteins which are involved in activity, peptidomimetic compounds can be generated which mimic or inhibit MIF activity. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden,

Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the MIF proteins can be produced using, e.g, expression vectors containing a nucleic acid encoding a MIF polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a MIF polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding MIF polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of MIF polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a MIF polypeptide. Thus, another aspect of the invention features expression vectors for *in*

vivo transfection of a MIF polypeptide in particular cell types so as cause ectopic expression of a MIF polypeptide in a target tissue.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the MIF coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of MIF expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for introduction of nucleic acid encoding one an MIF polypeptide into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) Blood 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by nucleic acid encoding a MIF polypeptide, rendering

the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψCre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, in vitro and/or in vivo (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

In choosing retroviral vectors as a gene delivery system for the subject MIF proteins, it is important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the recombinant gene, is that the target cells must be dividing. In general, this requirement will not be a hindrance to use of retroviral vectors to deliver the subject MIF gene constructs. In fact, such limitation on infection can be beneficial in circumstances where the tissue (e.g. nontransformed cells) surrounding the target cells does not undergo extensive cell division and is therefore refractory to infection with retroviral vectors.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral env protein (Roux et al. (1989) PNAS 86:9079-9083; Julan et al. (1992) J. Gen Virol 73:3251-3255; and Goud et al. (1983) Virology 163:251-254); or coupling cell surface ligands to the viral env proteins (Neda et

al. (1991) J Biol Chem 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the env protein to an asialoglycoprotein), as well as by generating MIF proteins (e.g. single-chain antibody/env MIF proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the MIF gene of the retroviral vector.

Another viral gene delivery system useful in the present invention utilitizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al. (1988) BioTechniques 6:616; Rosenfeld et al. (1991) Science 252:431-434; and Rosenfeld et al. (1992) Cell 68:143-155). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including endothelial cells (Lemarchand et al. (1992) Proc. Natl. Acad. Sci. USA 89:6482-6486), and smooth muscle cells (Quantin et al. (1992) Proc. Natl. Acad. Sci. USA 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham (1986) J. Virol. 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) Cell 16:683; Berkner et al., supra; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted MIF gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject MIF polypeptides is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. Curr. Topics in Micro. and Immunol. (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) Am. J. Respir. Cell. Mol. Biol. 7:349-356; Samulski et al. (1989) J. Virol. 63:3822-3828; and McLaughlin et al. (1989) J. Virol. 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) Proc. Natl. Acad. Sci. USA 81:6466-6470; Tratschin et al. (1985) Mol. Cell. Biol. 4:2072-2081; Wondisford et al. (1988) Mol. Endocrinol. 2:32-39; Tratschin et al. (1984) J. Virol. 51:611-619; and Flotte et al. (1993) J. Biol. Chem. 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistent expression of the subject MIF proteins in cells of the central nervous system, such as neuronal stem cells, and ocular tissue (Pepose et al. (1994) Invest Ophthalmol Vis Sci 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a the subject proteins in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding one of the subject proteins can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) No Shinkei Geka 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with

monoclonal antibodies against glioma-associated antigen (Mizuno et al. (1992) Neurol. Med. Chir. 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, WO92/19749, and WO92/06180). For example, the subject gene construct can be used to transfect hepatocytic cells in vivo using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via receptor-mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al. (1993) Science 260-926; Wagner et al. (1992) PNAS 89:7934; and Christiano et al. (1993) PNAS 90:2122).

In clinical settings, the subject gene delivery systems can be introduced into a cell or tissue culture, or a patient as appropriate, by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) PNAS 91: 3054-3057) or by topical application of the gene therapy vector.

Moreover, the pharmaceutical preparation can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced in tact from recombinant cells, e.g. retroviral packages, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system. In the case of the latter, methods of introducing the viral packaging cells may be provided by, for example, rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested in vivo in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals, and can be

adapted for release of viral particles through the manipulation of the polymer composition and form. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of an the viral particles by cells implanted at a particular target site. Such embodiments of the present invention can be used for the delivery of an exogenously purified virus, which has been incorporated in the polymeric device, or for the delivery of viral particles produced by a cell encapsulated in the polymeric device.

By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, Concise Encyclopedia of Medical & Dental Materials, ed. by David Williams (MIT Press: Cambridge, MA, 1990); and the Sabel et al. U.S. Patent No. 4,883,666. In another embodiment of an implant, a source of cells producing the recombinant virus is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the viral source (Aebischer et al. U.S. Patent No. 4,892,538; Aebischer et al. U.S. Patent No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng. 113:178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the viral packaging cells (Lim U.S. Patent No. 4,391,909; Sefton U.S. Patent No. 4,353,888; Sugamori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotechnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55). Again, manipulation of the polymer can be carried out to provide for optimal release of viral particles.

In yet another embodiment, ectopic expression of MIF can be by way of a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous MIF gene. For instance, the gene activation construct can replace the endogenous promoter of a MIF gene with a heterologous promoter, e.g., one which causes consitutive expression of the MIF gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. A vareity of different formats for the gene activation constructs are available. See, for example, the Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous MIF gene

(exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic MIF gene upon recombination of the gene activation construct. For use in generating cultures of MIF producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native MIF gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous MIF gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regualtory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp. Med.*, 169:13), the human β-actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-

797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native MIF gene, e.g., to activate expression.

The reversibility of the checkpoint by-pass, such as by MIF, can be generated by use of an expression system which is inducible because of the presence of an inducible transcriptional regulatory sequence controlling the expression of the coding sequence of the by-pass agent, e.g., the coding sequence for a MIF polypeptide.. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. Where the cells are to be transplanted into a patient, the inducible promoter is preferably one which is regulated by a small molecule or other factor which is not endogenous to the host animal.

Exemplary regulatable promoters include the tetracycline responsive promoters, such as described in, for example, Gossen et al. (1992) PNAS 89:5547-5551; and Pescini et al., (1994) Biochem. Biophys. Res. Comm. 202:1664-1667. In another another embodiment, the subject method utilizes the multimerization technology first pioneered by Schreiber and Crabtree. This technique permits the regulation of expression of an endogenous or heterologous gene, in this case a coding sequence for TRT or a telomerase activator, by use of chimeric transcription factors which are dependent on small molecules "dimerizers" to assemble transcriptionally active complexes. See, for example, PCT publications WO 9612796; WO 9505389; WO 9502684; WO 9418317; WO 9606097; and WO 9606110. Moreover, a number of techniques have been deveolped more recently which permit the recruitment of endogenous DNA binding and activation domains to the transcriptional regulatory sequences by use of artificial dimerization molecules. See, for example, PCT publication WO 9613613.

In another embodiment, the reversibility of the system is generated by the use of such vectors as described in PCT application WO 98/12339. These vectors include excision elements, such as recombinase sites, which flank the coding sequence for the MIF polypeptide. Upon contacting the cell with the appropriate recombinase, the vector sequence between the recombinase sites is excised. In preferred embodiments, the vector is a retroviral vector and the recombinase sites are located in the LTRs such that excision of a proviral sequence occurs, e.g., the viral vector is completely, or nearly completely excised from the chromosomal DNA of the host cell.

In other embodiments, the reversibility of MIF expression can be accomplished by use of conditionally active (or conditionally inactivable) forms of MIF. For instance, temperature-sensitive mutants can be employed in the subject method. In embodiments wherein the cells are to be transplanted into an animal, the ts mutant can be inactive at body temperature (the non-permissive temperature) and active at a lower or higher cell culture temperature.

To illustrate, one strategy for producing temperature-sensitive mutants, that does not require a search for a ts mutation in a gene of interest, is based on a portable, heat-inducible N-degron. The N-degron is an intracellular degradation signal whose essential determinant is a "destabilizing" N-terminal residue of a protein. A set of N-degrons containing different destabilizing residues is manifested as the N-end rule, which relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-degron consists of at least two determinants: a destabilizing N-terminal residue and a specific internal Lys residue (or residues) of a substrate. The Lys residue is the site of attachment of a multiubiquitin chain. Ubiquitin is a protein whose covalent conjugation to other proteins plays a role in a number of cellular processes, primarily through routes that involve protein degradation. For a description of exemplary heat-inducible N-degron modules which can be adapted for generating conditional mutants of MIF or other checkpoint bypass agents, see US Patents 5,705,387 and 5,538,862, and Dohmen et al. (1994) Science 263:1273-6.

In embodiments wherein the cells are treated in culture, RNA encoding MIF or other checkpoint bypass agents can be introduced directly into the cell, e.g., from RNA generated by *in vitro* transciption.

In yet another embodiment, membrane permeable drugs (e.g., preferably small organic molecules) can be identified which activate the expression of an endogenous MIF gene. In light of the availability of the genomic MIF gene, it will be possible to produce reporter constructs in which a reporter gene is operably linked to the transcriptional regulatory seqence of the MIF gene. When transfected into cells which possess the appropriate intracellular machinery for activation of the reporter construct through the MIF regulatory sequence, the resulting cells can be used in a cell-based approach for identifying such compounds.

In yet another embodiment, to the extent it is relevant, the intracellular level of MIF can be upregulated by inhibiting its natural turnover rate. For example, inhibitors of ubiquitin-dependent or independent degradation of MIF can be used to cause ectopic expression of MIF in the sense that the concentration of the protein in the cell can be artificially elevated.

In still another embodiment, non-peptide inhibitors of MIF activity can be used in those embodiments of the subject methods which rely on antagonism of MIF activity. The availability of purified and recombinant MIF polypeptides, as well as the p53-bypass assay described herein, facilitates the generation of assay systems which can be used to screen for drugs, such as small organic molecules, which are either agonists or antagonists of the normal p53-bypass function of a MIF protein, particularly their role in the pathogenesis of cell proliferation and/or differentiation. Thus, in certain embodiments, the MIF therapeutic can be, e.g., a peptide, a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

To further illustrate, the subunit structure of human MIF (Sun et al. (1996) Protein Eng 9: 631-5) and suggests that the major form of the protein in solution is a trimer. The subunit arrangement of MIF is similar to that of tumor necrosis factor and suggests that signal transduction might require trimerization of receptor subunits. Simple competitve assays can be carried out to identify agents which disrupt the formation of MIF complexes and which, as MIF therapeutics, would presumably be antagonists of wild-type MIF. Likewise, functional evidence suuprts the notion that the C-terminus of MIF is important for trimer formation (Mischke et al. (1997) FEBS Lett 414:226-32). Accordingly, fragments of MIF including oligomerization domains may, in complexes with the full-length protein, reduce the biological activity of the wild-type protein (e.g., as dominant negative inhibitors or weak agonsits).

Another aspect of the invention provides a conjoint therapy wherein one or more other therapeutic agents are administered with the MIF therapeutic agent. Such conjoint treatment may be achieved by way of the simultaneous, sequential or separate dosing of the individual components of the treatment. For example, the MIF therapeutic can be administered conjointly with a growth factors and other mitogenic agents. Mitogenic agent, as used herein, refers to any compound or composition, including peptides, proteins, and glycoproteins, which is capable of stimulating proliferation of a target cell population. For example, the MIF therapeutic agent can be conjointly administered with a T-cell mitogenic agent such as lectins, e.g., concanavalin A or phytohemagglutinin. Other exemplary mitogenic agents include insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and certain of the transforming growth factors (TGFs).

The source of the MIF therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically

synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of MIF polypeptides. Recombinant sources of MIF polypeptides are also available as described above.

Those of skill in treating humans and other animals can determine the effective amount of an MIF therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The MIF therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular MIF therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositons suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a

suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeuitcs, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellent such as nitrogen carbon dioxide, a freon, or without a propellent such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discreate units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications whcih can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the MIF therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol,

stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrocloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the MIF therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the MIF therapeutic is formulated in liposome-containing compositions.

Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a MIF polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of MIF therapeutic, such as an organic mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of MIF therapeutics is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated MIF therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum, hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatydylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phospha-tidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such a benzoic acid, methyl paraben and propyl paraben may also be added.

Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a MIF therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

EDITORIAL NOTE

24890/99

The following pages are numbered 1 to 2 followed by page 55.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 523 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 51..395

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CTCGAGCTGC AGAGCTGCCT CTGCGCGGGT CTCCTGGTCC TTCTGCCATC ATG CCG          56
                                                        Met Pro
                                                          1

ATG TTC ATC GTA AAC ACC AAC GTG CCC CGC GCC TCC GTG CCG GAC GGG        104
Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val Pro Asp Gly
          5                  10                  15

TTC CTC TCC GAG CTC ACC CAG CAG CTG GCG CAG GCC ACC GGC AAG CCC        152
Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala Thr Gly Lys Pro
     20                  25                  30

CCC CAG TAC ATC GCG GTG CAC GTG GTC CCG GAC CAG CTC ATG GCC TTC        200
Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met Ala Phe
 35                  40                  45                  50

GGC GGC TCC AGC GAG CCG TGC GCG CTC TGC AGC CTG CAC AGC ATC GGC        248
Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser Ile Gly
                 55                  60                  65

AAG ATC GGC GGC GCG CAG AAC CGC TCC TAC AGC AAG CTG CTG TGC GGC        296
Lys Ile Gly Gly Ala Gln Asn Arg Ser Tyr Ser Lys Leu Leu Cys Gly
             70                  75                  80

CTG CTG GCC GAG CGC CTG CGC ATC AGC CCG GAC AGG GTC TAC ATC AAC        344
Leu Leu Ala Glu Arg Leu Arg Ile Ser Pro Asp Arg Val Tyr Ile Asn
         85                  90                  95

TAT TAC GAC ATG AAC GCG GCC AGT GTG GGC TGG AAC AAC TCC ACC TTC        392
Tyr Tyr Asp Met Asn Ala Ala Ser Val Gly Trp Asn Asn Ser Thr Phe
    100                 105                 110

GCC TAAGAGCCGC AGGGACCCAC GCTGTCTGCG CTGGCTCCAC CCGGGAACCC             445
Ala
115

GCCGCACGCT GTGTTCTAGG CCCGCCCACC CCAACCTTCT GGTGGGGAGA AATAAACGGT      505

TTAGAGACAG CTCTGCAG                                                    523
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 115 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Pro Met Phe Ile Val Asn Thr Asn Val Pro Arg Ala Ser Val Pro
 1               5                  10                  15

Asp Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala Thr Gly
            20                  25                  30

Lys Pro Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
        35                  40                  45

Ala Phe Gly Gly Ser Ser Glu Pro Cys Ala Leu Cys Ser Leu His Ser
    50                  55                  60

Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Ser Tyr Ser Lys Leu Leu
65                  70                  75                  80

Cys Gly Leu Leu Ala Glu Arg Leu Arg Ile Ser Pro Asp Arg Val Tyr
                85                  90                  95

Ile Asn Tyr Tyr Asp Met Asn Ala Ala Ser Val Gly Trp Asn Asn Ser
            100                 105                 110

Thr Phe Ala
        115
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 115 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Pro Met Phe Xaa Val Asn Thr Asn Val Pro Arg Ala Ser Val Pro
1               5                  10                  15

Xaa Gly Phe Leu Ser Glu Leu Thr Gln Gln Leu Ala Gln Ala Thr Gly
            20                  25                  30

Lys Xaa Pro Gln Tyr Ile Ala Val His Val Val Pro Asp Gln Leu Met
        35                  40                  45

Xaa Phe Xaa Gly Xaa Xaa Xaa Pro Cys Ala Leu Cys Ser Leu His Ser
    50                  55                  60

Ile Gly Lys Ile Gly Gly Ala Gln Asn Arg Xaa Tyr Ser Lys Leu Leu
65                  70                  75                  80

Cys Gly Leu Leu Xaa Xaa Arg Leu Xaa Ile Ser Pro Asp Arg Xaa Tyr
                85                  90                  95

Ile Asn Xaa Xaa Asp Met Asn Ala Ala Xaa Val Gly Trp Asn Xaa Ser
            100                 105                 110

Thr Phe Ala
        115
```

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method for identifying a polypeptide which is capable of by-passing a cell-cycle checkpoint comprising,

(a) producing a cell which overexpresses a cell-cycle checkpoint gene so as to cause growth arrest of the cell;

(b) ectopically expressing a nucleic acid encoding a polypeptide product; and

(c) determining if the polypeptide product of the nucleic acid bypasses the checkpoint-mediated growth arrest and leads to cell proliferation.

2. The method of claim 1, wherein the cell-cycle checkpoint is selected from the group consisting of p53 and p53-like tumor suppressors, Ink4 gene products, and CIP/KIP gene products.

3. The method of claim 1 or claim 2, comprising a further step of formulating, as a pharmaceutical preparation, a polypeptide identified in step (c) by the ability to by-pass the checkpoint-mediated growth arrest.

4. The method of claim 3, wherein the polypeptide is a soluble extracellular polypeptide.

5. The method of claim 1 or claim 2, comprising a further step of formulating, as a pharmaceutical preparation, a nucleic acid encoding a polypeptide identified in step (c) by the ability to by-pass the checkpoint-mediated growth arrest.

6. A method for inducing cell growth comprising contacting a cell, with an amount of the polypeptide formulated according to method of claim 3 sufficient to induce cell proliferation.

7. A method of inducing cell growth comprising contacting the cell with an MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof in an amount sufficient to induce cell proliferation.

8. A method for inducing cell growth in a cell whose growth is arrested by a tumor suppressor, comprising contacting the cell, with an amount of a macrophage migration inhibitory factor (MIF), in an amount sufficient to induce cell proliferation.

9. A method for inducing cell growth in a cell whose growth is arrested by a tumor suppressor, comprising contacting the cell, with a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof in an amount sufficient to induce cell proliferation.

10. The method of claim 8 or claim 9, wherein the MIF polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

11. The method of claim 8 or claim 9, wherein said cell is a primary cell, a stem cell or a progenitor cell.

12. The method of claim 11, wherein the cell is a neuronal, hematopoietic, pancreatic, or hepatic stem cell, or a progenitor cell.

13. The method of claim 8 or claim 9, wherein said cell is a skin cell or other epithelial cells.

14. The method of claim 8 or claim 9, wherein said cell is a mesenchymal cell.

15. The method of claim 8 or claim 9, wherein said cell is a chondrocyte or an osteocyte.

16. A method for increasing the number of mitotic divisions a cell can undergo, comprising contacting the cell, with a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof, in an amount sufficient to increase the replicative capacity of the cell.

17. The method of claim 16, for overcoming tumor suppressor-mediated growth arrest.

18. The method of any one of claims 8 to 17, wherein the tumor suppressor is p53.

19. A method for improving the chemosensitivity of a cancer cell to a chemotherapeutic agent, comprising contacting the cell with an MIF polypeptide in an amount sufficient and under conditions wherein the cell undergoes p53-dependent cell death.

20. The method of any one of claims 16 to 19, wherein the MIF polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

21. The method of any one of claims 16 to 20, wherein said cell is a primary cell, a stem cell or a progenitor cell.

22. The method of claim 21, wherein the cell is a neuronal, hematopoietic, pancreatic, or hepatic stem cell, or a progenitor cell.

23. The method of any one of claims 16 to 20, wherein said cell is a skin cell or other epithelial cells.

24. The method of any one of claims 16 to 20, wherein said cell is a mesenchymal cell.

25. The method of any one of claims 16 to 20, wherein said cell is a chondrocyte or an osteocyte.

26. The method of any one of claims 22 to 25, wherein said cell grows in culture or in an implant.

27. The method of any one of claims 22 to 25, wherein said cell grows *in vivo*.

28. Method for inhibiting MIF-dependent proliferation of cells, comprising contacting the cell with an agent that inhibits MIF-mediated bypass of p53 wherein said agent competitively inhibits formation of MIF complexes or interaction of MIF polypeptide with its receptor.

29. A pharmaceutical preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth, and a pharmaceutically acceptable excipient.

30. A cosmetic preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth, in an amount suitable to promote proliferation of cells of a dermal layer when applied topically, and a pharmaceutically acceptable excipient for topical application.

31. Use of an MIF polypeptide in the manufacture of a medicament for promoting the healing of a wound, which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

32. The use of claim 31, for promoting healing of epithelial tissues.

33. The use of claim 31, for promoting healing of a wound resulting from surgery, burns, inflammation or irritation.

34. The use of claim 31, wherein the medicament is for prophylactic application, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes.

35. The use of claim 31, for promoting healing of a dermal ulcer.

36. The use of claim 35, wherein the dermal ulcers is a result from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) or arterial ulcers.

37. A kit for conjoint administration comprising, (a) the preparation of claim 29 or 30, and (b) a trophic factor.

38. A kit for conjoint administration comprising, (a) the preparation of claim 29 or 30, and (b) a tropic factor.

39. A kit for conjoint administration comprising, (a) the preparation of claim 29 or 30, and (b) a chemotherapeutic agent.

40. The method of any one of claims 18 to 26 or a kit of claim 37, wherein the chemotherapeutic agent is selected from the group consisting of: alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodepa, carboquone, meturedepa and uredepa; ethylenimines and methylmelamines such as altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, chclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide and uracil mustard; nitrosoureas such as carmustine, chlorozotocin, foremustine, lomustine, nimustine and ranimustine; dacarbazine; mannomustine; mitobronitol; mitolactol; and pipobroman.

41. Use of a macrophage migration inhibitory factor (MIF) for the manufacture of a medicament for inducing cell growth in a cell whose growth is arrested by a tumor suppressor.

42. Use of a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof for the manufacture of a medicament for inducing cell growth in a cell whose growth is arrested by a tumor suppressor.

43. Use of claim 41 or claim 42, wherein the MIF polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

44. Use of claim 41 or claim 42, wherein said cell is a primary cell, a stem cell or a progenitor cell.

45. Use of claim 44, wherein the cell is a neuronal, hematopoietic, pancreatic, or hepatic stem cell, or a progenitor cell.

46. Use of claim 41 or claim 42, wherein said cell is a skin cell or other epithelial cells.

47. Use of claim 41 or claim 42, wherein said cell is a mesenchymal cell.

48. Use of claim 41 or claim 42, wherein said cell is a chondrocyte or an osteocyte.

49. Use of a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof, for the manufacture of a medicament for increasing the number of mitotic divisions a cell can undergo.

50. Use of claim 49, for overcoming the tumor suppressor-mediated growth arrest.

51. Use of any one of claims 41 to 50, wherein the tumor suppressor is p53.

52. Use of an MIF polypeptide for the manufacture of a medicament for improving the chemosensitivity of a cancer cell to a chemotherapeutic agent wherein the cell undergoes p53-dependent cell death.

53. Use of claim 52, wherein the MIF polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to SEQ ID No. 1.

54. Use of claim 52 or claim 53, wherein said cell is a primary cell, a stem cell or a progenitor cell.

55. Use of claim 54, wherein the cell is a neuronal, hematopoietic, pancreatic, or hepatic stem cell, or a progenitor cell.

56. Use of claim 52 or claim 53, wherein said cell is a skin cell or other epithelial cells.

57. Use of claim 52 or claim 53, wherein said cell is a mesenchymal cell.

58. Use of claim 52 or claim 53, wherein said cell is a chondrocyte or an osteocyte.

59. Use of any one of claims 55 to 58, wherein said cell grows in culture or in an implant.

60. Use of any one of claims 55 to 58, wherein said cell grows *in vivo*.

61. Use of any one of claims 19 to 27 or a kit of claim 39, wherein the chemotherapeutic agent is selected from the group consisting of: alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodepa, carboquone, meturedepa and uredepa; ethylenimines and methylmelamines such as altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, chclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide and uracil mustard; nitrosoureas such as carmustine, chlorozotocin, foremustine, lomustine, nimustine and ranimustine; dacarbazine; mannomustine; mitobronitol; mitolactol; and pipobroman.

62. Use of an agent that inhibits MIF-mediated bypass of p53 in the manufacture of a medicament for inhibiting MIF-dependent proliferation of cells, which agent competitively inhibits formation of MIF complexes or interaction of MIF polypeptide with its receptor.

63. Method for promoting the healing of a wound comprising contacting the wound, tissue or cell with MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

64. Method of claim 63, for promoting healing of epithelial tissues.

65. Method of claim 63, for promoting healing of a wound resulting from surgery, burns, inflammation or irritation.

66. Method of claim 63, for promoting healing of a dermal ulcer.

67. Method of claim 66, wherein the dermal ulcers is a result from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) or arterial ulcers.

68. Method for enhancing tissue regeneration process comprising contacting the tissue prophylactically with MIF polypeptide which MIF polypeptide has an amino acid sequence at least 90% identical to SEQ ID No. 2, or an active portion thereof that can induce cell growth.

69. Method of claim 68, wherein the MIF polypeptide is in the form of a cosmetic preparation.

70. A method for identifying a polypeptide which is capable of by-passing a cell-cycle checkpoint, substantially as herein described with reference to any one of the Examples.

71. A method for inducing cell growth, substantially as herein described with reference to any one of the Examples.

72. A method for inducing cell growth in a cell whose growth is arrested by a tumor suppressor, substantially as herein described with reference to any one of the Examples.

73. A method for increasing the number of mitotic divisions a cell can undergo, substantially as herein described with reference to any one of the Examples.

74. A method for improving the chemosensitivity of a cancer cell to a chemotherapeutic agent, substantially as herein described with reference to any one of the Examples.

75. Method for inhibiting MIF-dependent proliferation of cells, substantially as herein described with reference to any one of the Examples.

76. A pharmaceutical preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, substantially as herein described with reference to any one of the Examples.

77. A cosmetic preparation comprising, as an active component, a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2, substantially as herein described with reference to any one of the Examples.

78. Use of an MIF polypeptide, substantially as herein described with reference to any one of the Examples.

79. A kit for conjoint administration, substantially as herein described with reference to any one of the Examples.

80. Use of a polypeptide including an MIF amino acid sequence at least 90% identical to SEQ ID No. 2 or an active portion thereof, substantially as herein described with reference to any one of the Examples.

81 Use of an agent that inhibits MIF-mediated bypass of p53, substantially as herein described with reference to any one of the Examples.

82 Method for promoting the healing of a wound, substantially as herein described with reference to any one of the Examples.

83. Method for enhancing tissue regeneration process, substantially as herein described with reference to any one of the Examples.

DATED this 10th day of January 2003

COLD SPRING HARBOR LABORATORY

Attorney: IVAN A. RAJKOVIC
Fellow Institute of Patent and Trade Mark Attorneys of Australia
of BALDWIN SHELSTON WATERS

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concensus   MPMFXVNTNVPRASVPXGFLSELTQQLAQATGKXPQYIAVHVVPDQLMXFXGX
human       MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGS
cow         MPMFVVNTNVPRASVPDGLLSELTQQLAQATGKPAQYIAVHVVPDQLMTFGGS
rat         MPMFIVNTNVPRASVPEGFLSELTQQLAQATGKPAQYIAVHVVPDQLMTFSGT
MOUSE       MPMFIVNTNVPRASVPEGFLSELTQQLAQATGKPAQYIAVHVVPDQLMTFSGT

consensus   XXPCALCSLHSIGKIGGAQNRXYSKLLCGLLXXRLXISPDRXYINXXDMNAAX
            SEPCALCSLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAAS
            SEPCALCSLHSIGKIGGAQNRSYSKLLCGLLTERLRISPDRIYINFCDMNAAN
            SDPCALCSLHSIGKIGGAQNRNYSKLLCGLLSDRLHISPDRVYINYYDMNAAN
            NDPCALCSLHSIGKIGGAQNRNYSKLLCGLLSDRLHISPDRVYINYYDMNAAN

consensus   VGWNXSTFA
            VGWNNSTFA
            VGWNGSTFA
            VGWNGSTFA
            VGWNGSTFA
```

Fig. 1

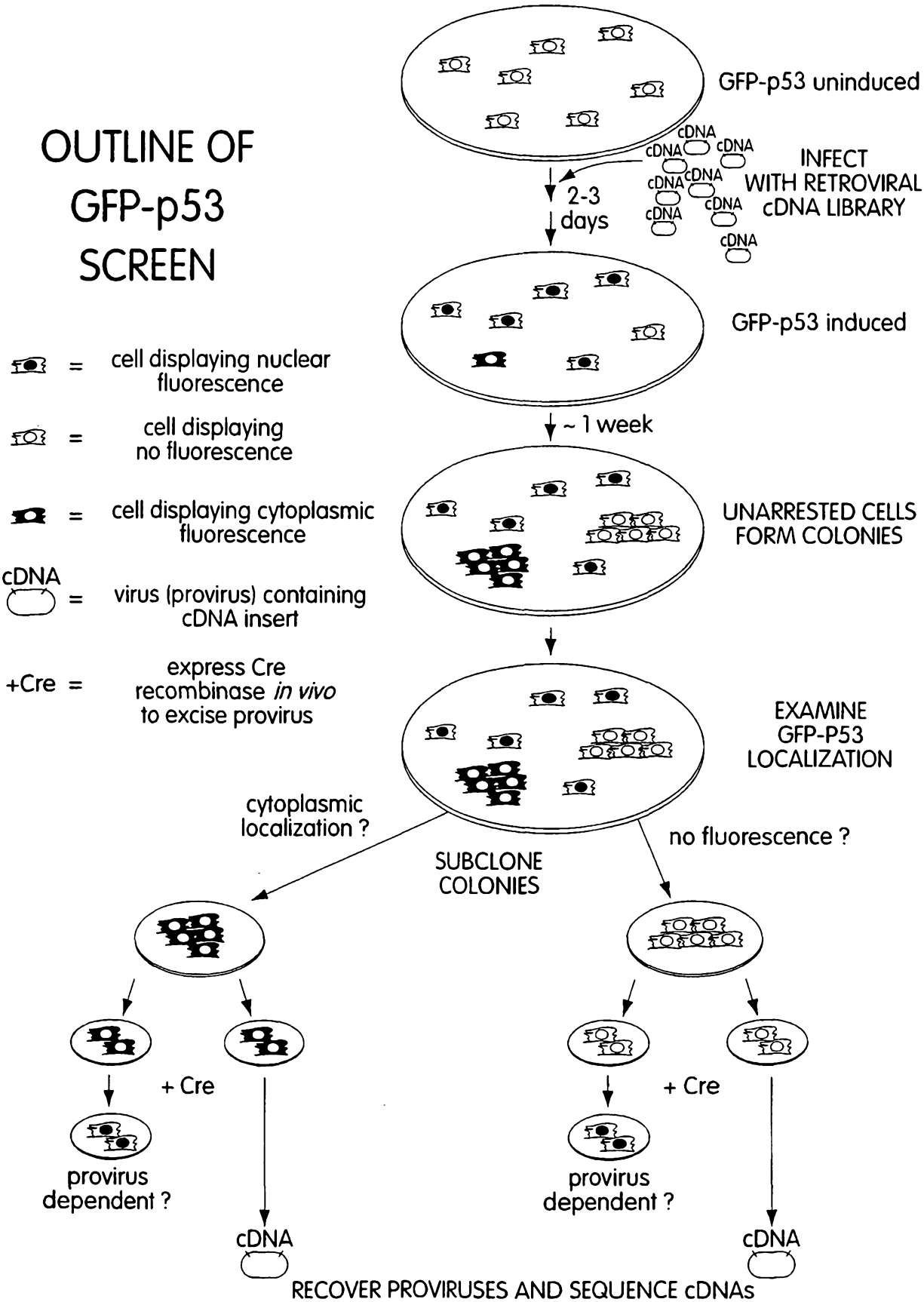
OUTLINE OF GFP-p53 SCREEN
= cell displaying nuclear fluorescence
= cell displaying no fluorescence
= cell displaying cytoplasmic fluorescence
cDNA = virus (provirus) containing cDNA insert
+Cre = express Cre recombinase *in vivo* to excise provirus
GFP-p53 uninduced
cDNA
INFECT WITH RETROVIRAL cDNA LIBRARY
2-3 days
GFP-p53 induced
~ 1 week
UNARRESTED CELLS FORM COLONIES
EXAMINE GFP-P53 LOCALIZATION
cytoplasmic localization ?
no fluorescence ?
SUBCLONE COLONIES
+ Cre
provirus dependent ?
cDNA
RECOVER PROVIRUSES AND SEQUENCE cDNAs

Fig. 2

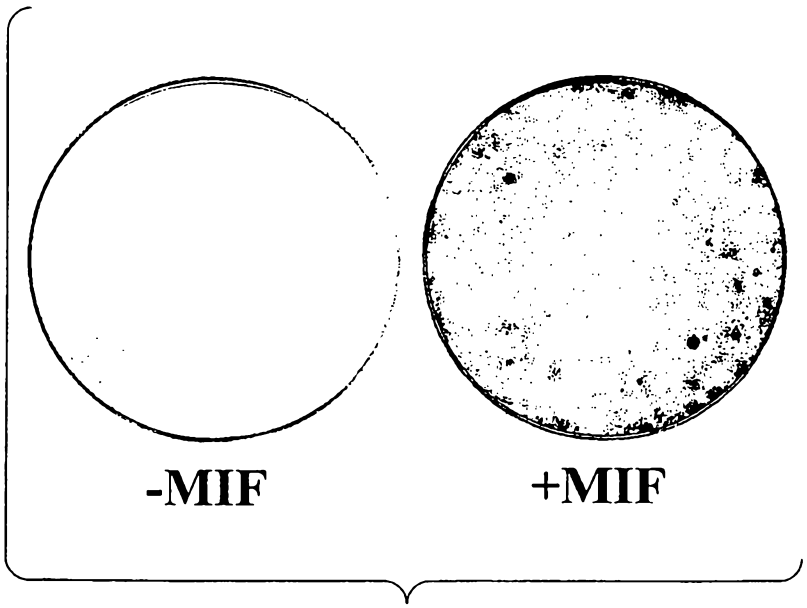

Fig. 3