Title: ISOLATION OF A NOVEL SENESECE–FACTOR GENE, p23

Abstract

Provided are an isolated senescence–related nucleic acid molecule encoding a 23 kilodalton polypeptide (p23), methods for expressing the 23 kilodalton polypeptide in cultured cells, recombinant p23 polypeptides, expression vectors and host cells expressing p23, and antibodies against p23. Also provided are methods for using p23 to modulate senescence, and for determining p23 expression in biological samples.
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ISOLATION OF A NOVEL SENESCENCE-FACTOR GENE, P23

Field of the Invention

This invention relates to nucleic acid sequences from a gene, referred to herein as p23, that is involved in the senescence of human epithelial cells, the recombinant polypeptides encoded by the sequences, expression vectors and host cells containing the sequences, antibodies that specifically bind p23 polypeptides, and methods of modulating senescence and determining the amounts of the polypeptides in biological samples.

Background of the Invention

Replicative senescence, i.e., the inability of a cell to divide in response to mitogens, was first described for cultured normal human fibroblasts (Hayflick, Exptl. Cell Res. 37: 614-635, 1965). Since then, a variety of other human cell types have been observed to become senescent after repeated passages in culture (Smith and Pereira-Smith, Science 273: 63-67, 1996). Senescent cells are arrested in their growth with a G1 DNA content and do not enter S phase, although they remain metabolically active, and resist death by apoptosis for long periods of time in culture.

Although senescent cells in culture can be identified by their inability to divide in response to mitogens, until recently it was not possible to distinguish senescent cells in vivo from cells that retained the ability to divide. However, a biological marker, an enzyme with β-galactosidase activity, has been described recently that apparently identifies senescent human cells in culture (Dimri et al., Proc. Natl. Acad. Sci. USA 92: 9363-9367, 1995). These studies demonstrated in cultured cells an inverse relationship between the ability to incorporate 3H-thymidine into newly synthesized DNA and the expression of the β-galactosidase. This
enzyme's presence can be detected easily by providing cells with a substrate that yields a blue-colored product upon enzymatic cleavage. In addition, these investigators observed an age-dependent rise in this senescence-associated β-galactosidase in human skin, suggesting that the accumulation of the enzyme provides a marker for senescence in fibroblasts and keratinocytes in the skin and perhaps other epithelial tissues.

The senescent phenotype appears to be controlled by more than one gene. In one study, cell fusion experiments were performed with 40 different immortal human cell lines to determine whether the senescent phenotype could thus be restored. Based on the results, the cell lines were assigned to four different complementation groups, indicating that at least four genes or genetic pathways contribute to senescence (Smith and Pereira-Smith, *Science* 273:63-67, 1996). Other experiments have indicated that genes located on human chromosomes 1, 4, 6, 7, 11, 18, and X are involved in senescence (*ibid.)*. Recently, a specific gene (mac25) that is overexpressed in senescent epithelial cells was isolated and mapped to the long arm of chromosome 4 (Swisshelm et al., *Proc. Natl. Acad. Sci. USA* 92:4472-4476, 1995).

Because senescent cells appear to be blocked in G₁ phase, one approach to identifying senescence-related genes has been to compare the transcripts expressed during G₁ in young quiescent cells and in senescent cells after serum starvation. Using this approach, a number of differences have been documented in gene expression between young and senescent cells (Smith and Pereira-Smith, 1996; WO 96/13601, 1996). Senescent fibroblasts have been observed also to express reduced levels of transcription factor binding activities (*ibid.*). However, there have been no reports of inducing senescent cells to enter the cell cycle by introducing single gene products that normally are down-regulated in such cells.

Some lines of experimentation have suggested that senescence may have evolved as a mechanism for tumor suppression, and that aging is an indirect effect of this circumstance. Because constraints on growth control are absent from tumor cells, such cells most likely have switched off the expression of genes whose products promote or maintain the senescent state. For example, *in vitro* studies have indicated that senescence can be partially circumvented by the inactivation of tumor suppressor proteins such as the retinoblastoma tumor suppressor gene RB₁ (Weinberg, *Cell* 81:323-330, 1995). This suggests the possibility that the loss of
functional tumor suppressor genes in vivo could permit cells to gain a replicative advantage and eventually to undergo immortalization.

It has been observed also that the decline in immune response associated with increasing age stems from a decreased proliferative response of T-lymphocytes that have been exposed to antigen (Smith and Pereira-Smith, 1996). This decreased responsiveness of T cells to antigens is reminiscent of the decreased responsiveness to mitogens seen in senescent cultured cells, thus suggesting that the decreased immune response of old age may result from the same or similar mechanisms. Thus, if the genes that cause senescence were known, their relationship to the loss of immune response with age could be elucidated, and it may become feasible to manipulate these genes therapeutically to boost the immune system.

To directly examine the role of senescence in tumor suppression, experiments were conducted in which immortalized and non-immortalized human fibroblasts were infected with either a plasmid expressing the SV40 T antigen, a Ki-ras-bearing RNA tumor virus, or both. Only the immortalized fibroblasts were rendered tumorigenic by this means, suggesting that distinct molecular mechanisms must govern immortalization and tumorigenesis, and indicating further that the former may be a prerequisite for the latter (Sager, R., Environ. Health Perspect. 93:59-62, 1991). Various studies have indicated that escape from senescence, i.e., immortalization, results from the alteration in expression or loss of one or more senescence genes. If these genes could be identified and isolated, their role in cancer could be further elucidated, and it may become possible to manipulate their expression to restore controlled growth to malignant tissues.

Summary of the Invention

A novel gene has been identified that is expressed at high levels in senescent cells. A cDNA corresponding to the novel gene has been isolated and sequenced and found to contain an open reading frame encoding a protein having a deduced molecular weight of 23 kilodaltons (kDa) (SEQ ID NO:1). Hence, this gene has been named "p23." Messenger RNA transcribed from p23 is reproducibly detectable at higher levels in senescent than in proliferating cultured normal human mammary epithelial cells. The function of p23 is not known, but analysis of its deduced amino acid sequence (SEQ ID NO:2) suggests that it belongs to a family of transmembrane proteins known as the "PMP 22" family or "epithelial membrane protein" (EMP) family (e.g., see Taylor et al., J. Biol. Chem. 270:28824-28833, 1995; Lobsiger et al., Genomics 36:379-387, 1996; Taylor and Suter, Gene 175:115-120, 1996).
p23 is expressed in several human tissues, including adult and fetal liver, pancreas, placenta, adrenals, prostate, and ovary, all of which are composed primarily of epithelial cells having endocrine or secretory function. It was noted further that p23 RNA is markedly decreased or absent from a number of human breast cancer cell lines, thus suggesting that the p23 polypeptide may play a role in suppressing the malignant phenotype in normal breast tissue. p23-positive normal human mammary epithelial cells (HMECs) express reduced levels of p23 when cultured with retinoic acid, thus indicating that the p23 gene is transcriptionally regulated through a retinoic acid receptor pathway.

In one aspect, the present invention thus provides isolated p23 nucleic acid molecules that are involved in the senescence of human epithelial cells, as well as recombinant p23 polypeptides encoded by the nucleic acid molecules. In other aspects, the invention provides vectors and host cells comprising the nucleic acid molecules, antibodies specific to the polypeptides, and methods of modulating senescence and of measuring the levels of p23 in a biological sample.

**Brief Description of the Drawings**

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same becomes better understood by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 shows a comparison of the p23 polypeptide whose deduced amino acid sequence is shown in SEQ ID NO:2 with the coding sequence of the apoptosis-related rat ventral prostate gene 1 (RVP1) shown in SEQ ID NO:3.

FIGURE 2 shows structural features of the p23 polypeptide whose deduced amino acid sequence is shown in SEQ ID NO:2. These features were determined by analysis with the Motifs subroutine of the Genetics Computer Group (GCG) computer program for analyzing nucleotide and amino acid sequences. Triangles indicate hydrophobic regions, while ovals indicate hydrophilic regions. The "O" shown below the line between residues 50 and 100 indicates the position of a putative glycosylation site at amino acid residue 72.

**Detailed Description of the Preferred Embodiment**

The identification of genes involved in inducing and maintaining the senescent state furthers the goal of regulating disease states such as cancer, persistent inflammation, and various proliferative and degenerative disorders. This invention provides a nucleic acid molecule encoding a polypeptide having the amino acid sequence of p23.
sequence shown in SEQ ID NO:2, which is a representative example of a p23 polypeptide. A representative example of a nucleic acid molecule that encodes a p23 polypeptide comprises the nucleotide sequence shown in SEQ ID NO:1, which corresponds to a cDNA encoding the polypeptide whose amino acid sequence is shown in SEQ ID NO:2. The open reading frame in SEQ ID NO:1 is located between nucleotides 221 and 853, thus the majority of this nucleotide sequence is untranslated. A representative example of a p23 polypeptide is provided by the amino acid sequence shown in SEQ ID NO:2.

Many of the uses of the nucleic acid shown in SEQ ID NO:1 depend on the ability of complementary nucleic acid strands to form duplexes, i.e., to hybridize with one another. "Stringent hybridization conditions" means generally that the nucleic acid duplexes that form under these conditions are perfectly matched or nearly perfectly matched (Sambrook et al., Molecular Cloning [2d ed.], 1989, which is hereby incorporated by reference). Thus, under stringent conditions, complementary nucleic acid molecules derived from different allelic forms of a gene are expected to form stable hybrids, as allelic forms of a gene typically differ at very few nucleotide positions. Similarly, probes derived from a specific cDNA are expected to form stable hybrids under stringent conditions with cDNAs or genes corresponding to allelic forms or mutant forms of that same gene.

Stringent hybridization conditions for polynucleotide molecules >200 nucleotides in length typically involve hybridizing at a temperature 15°-25°C below the melting temperature (Tm) of the expected duplex, most preferably at 25°C below the Tm, and for oligonucleotide probes (<30 nucleotides), by hybridizing at 5°-10°C below the Tm (e.g., Sambrook et al., 1989; see Section 11.45). The Tm of a nucleic acid duplex can be calculated using a formula based on the % G+C contained in the nucleic acids, and that takes chain length into account, such as the formula \[ Tm = 81.5 - 16.6 \left( \log [Na^+] \right) + 0.41 \left( \% G+C \right) - (600/N), \] where N = chain length (Sambrook et al., 1989; see Section 11.46). It is apparent from this formula that the effect of chain length on Tm is significant only when rather short nucleic acids are hybridized, and also that the length effect is negligible for nucleic acids longer than a few hundred bases. Thus, one skilled in the art can derive suitable p23 probes from virtually any portion or segment of SEQ ID NO:1. So long as the selected probe molecule exceeds about 15 nucleotides in length, conditions for stringent hybridization can be calculated by using the above formula or using some similar formula. For any given probe, the Tm can be confirmed empirically by hybridizing.
the probe with a cloned p23 nucleic acid molecule, such as the one in SEQ ID NO:1, then incrementally increasing the temperature until the duplexes are melted. The optimal hybridization temperature for a given probe likewise can be confirmed empirically by testing the rate of hybrid duplex formation at different temperatures. Moreover, probes that are at least 15 nucleotides in length are expected to hybridize specifically because sequences exceeding this length are extremely unlikely to be represented more than once in a mammalian genome (Sambrook et al., 1989, at Section 11.7).

The choice of hybridization conditions will be evident to one skilled in the art and will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of desired relatedness between the sequences. As discussed above, methods for hybridization and representative buffer formulations for high and low stringency hybridization are well established and are provided in the published literature (e.g., Sambrook et al., 1989; see also Hames and Higgins, eds., *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington DC, 1985; Berger and Kimmel, eds., *Methods in Enzymology, Vol. 52, Guide to Molecular Cloning Techniques*, Academic Press Inc., New York, NY, 1987; and Bothwell, Yancopoulos and Alt, eds., *Methods for Cloning and Analysis of Eukaryotic Genes*, Jones and Bartlett Publishers, Boston, MA 1990; which are incorporated by reference herein in their entirety).

One of ordinary skill in the art realizes that the stability of nucleic acid duplexes will decrease with an increased number of mismatched bases. Thus, the stringency of hybridization may be manipulated to maximize or minimize the stability of such duplexes. Hybridization stringency can be altered by: adjusting the temperature of hybridization; adjusting the percentage of helix-destabilizing agents, such as formamide, in the hybridization mix; and adjusting the temperature and/or salt concentration of the wash solutions. In general, the stringency of hybridization is adjusted during the post-hybridization washes by varying the salt concentration and/or the temperature. Stringency of hybridization may be lowered by reducing the percentage of formamide in the hybridization solution or by decreasing the temperature of the wash solution. High stringency conditions may involve high temperature hybridization (e.g., 65-68°C in aqueous solution containing 4-6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), or 42°C in 50% formamide) combined with washes at high temperature (e.g., 5-25°C below the Tm) in a solution having a low salt concentration (e.g., 0.1 X SSC). Low stringency conditions may
involve lower hybridization temperatures (e.g., 35-42°C in 20-50% formamide) with
washes conducted at an intermediate temperature (e.g., 35-60°C) and in a wash
solution having a relatively high salt concentration (e.g., 2-6 X SSC). Moderate
stringency conditions, which may involve hybridization in 0.2-0.3 M NaCl at a
temperature between 50°C and 65°C and washes in 0.1 X SSC, 0.1% SDS at between
50°C and 55°C, may be used in conjunction with the disclosed polynucleotide
molecules as probes to identify genomic or cDNA clones encoding related proteins,
e.g., other members of the EMP family.

A nucleic acid molecule comprising the sequence shown in SEQ ID NO:1
provides a tool that can be used to identify and isolate the entire gene encoding p23,
as well as variants of the p23 gene, such as allelic variants or mutant forms of the
gene. By hybridizing p23 probe, i.e., probes derived from all or subparts of SEQ ID
NO:1, under stringent conditions with a phage or cosmid library of the human
genome, DNA molecules corresponding to all or part of the p23 gene can be
identified.

This invention also includes variant forms such as allelic variants and
mutated forms of the p23 protein, gene, and cDNA. Genes and cDNAs encoding
variants of p23 are easily identified and subsequently isolated by using probes based
on SEQ ID NO:1 as a tool for screening cDNA or genomic libraries made from cells
of interest, i.e., cells that may contain variant forms of the p23 gene or mRNA. To
maximize specificity of the screening, hybridizations are conducted under stringent
conditions. Confirmation that clones thus isolated are p23 variants is accomplished
by determining the nucleotide sequence of the cloned DNA and comparing the
sequence, particularly the coding regions, with the p23 sequence shown in SEQ ID
NO:1. Variants of p23 are expected to share at least 90-95% of their nucleotide
sequences.

In some instances, cells may express a non-functional p23 protein or may
contain no p23 protein due to genetic mutation or somatic mutations. Such cells,
which may include genetically deficient cells or cancer cells, may thus escape the
senescent state. For cancer cells having defects in p23, the cancer cells may be
treated in a manner to cause the over-expression of wild-type p23 to force the cells to
stabilize in G1. Thus, the subject invention provides methods of inducing a senescent
phenotype in a cell by introducing into the cell a nucleic acid molecule that encodes
p23, such as, for example, the representative p23 sequence shown in SEQ ID NO:1.
Such methods for inducing senescence in a cell may involve introducing into non-senescent cells *in vivo* or *in vitro* a nucleic acid molecule that encodes the protein whose amino acid sequence is shown in SEQ ID NOS:1 and 2, or that encodes an allelic form of the protein having essentially the same biological activity. Moreover, the untranslated regions of the p23 cDNA shown in SEQ ID NO:1 may provide important regulatory functions that affect mRNA stability or processing, or other aspects of mRNA function.

Included in the subject invention are recombinant expression vectors for expressing p23 in eukaryotic cells, including yeast cells (e.g., retroviruses, Herpes simplex viruses, plasmids, vaccinia viruses, adenoviruses, defective paroviruses, CMV, and the like), and plasmid or cosmid vectors for expressing p23 in prokaryotic cells. Recombinant expression vectors of the invention are constructed, for example, by operably linking a nucleic acid molecule capable of encoding the p23 protein of SEQ ID NO:2 to suitable control sequences. Nucleotides 221-853 of SEQ ID NO:1 provide a representative nucleotide sequence having the requisite coding capacity. "Operably linking" is used herein to mean ligating a p23 nucleic acid molecule to an expression vector nucleic acid in a manner that correctly positions the regulatory elements necessary for transcription and translation of p23, preferably under the predetermined positive (or negative) regulatory control exerted by control sequences in the expression vector (i.e., regulatory sequences capable of driving expression, over-expression, or constitutive expression of the p23 gene, e.g., promoter, enhancer, operator sequences, and the like). The vector may contain an inducible promoter, for example, one that directs transcription only in the presence of a particular hormone (e.g., zinc), growth-factor, co-factor, or metabolic substrate. Selectable markers may also be present in the expression vector. Representative examples of such selectable markers include enzymes, antigens, drug resistance markers, or markers satisfying the growth requirements of the cell. Regulatory elements may be present that exert control either in eukaryotic cells or in prokaryotic cells, or both types of regulatory elements may be present in a single vector.

The subject expression vectors are useful for transfecting or transducing cells to express transgenic p23 polypeptides, mutant p23 polypeptides, and antisense nucleic acids capable of forming duplexes with endogenous p23 mRNA. Cells induced to express exogenous p23 are called "transgenic cells." Thus, the invention provides cell lines transformed by vectors that direct the expression of transgenic p23 polypeptide in the transformed cells. The transgenic cells of the subject invention
can be used to produce the p23 polypeptide in large quantities. To facilitate harvesting the p23 polypeptide from transgenic cells, the transgene, i.e., the DNA fragment encoding p23, can be linked in-frame to coding regions for amino acids that provide signals that direct the secretion of the transgenic polypeptide into the culture medium. Transgenic cells expressing p23 may be either eukaryotic or prokaryotic.

Another embodiment of the invention provides methods of inducing a senescent phenotype in a eukaryotic cell. For this method, a p23 expression vector that constitutively, conditionally, or transiently over-expresses p23 is introduced into the cell. As a result of the subsequent p23 expression in the transduced cell, the cell proliferates at a rate slower than its parent cell, or ceases proliferation altogether, i.e., the cell attains a senescent phenotype. p23-transduced human diploid cells, for example, will become arrested in G₁. Cultured cells or cells taken from a live host may be the target cells for the p23 expression vector. Thus, when cultured cells are the target, the invention provides cell lines capable of expressing transgenic p23 polypeptide. If cells taken from a live host are transduced, these can be returned to the host or further studied in culture.

Moreover, skilled artisans will understand the advantages in gene therapy of removing cells from a patient, transfecting or transducing the cells with an expression vector expressing p23, or conversely with a vector expressing antisense RNA capable of suppressing endogenous expression of p23, and thereafter returning the cells to the patient (i.e., ex vivo genetic manipulation). It will also be understood that transgenic animals (e.g., experimental and domestic animals) may be constructed that express p23 under the control of tissue-specific or inducible promoters, or that express antisense RNA for suppressing endogenous p23 expression.

In addition, nucleotide sequences encoding p23 may be used to obtain transient expression of p23 in cells by introducing cloned p23 nucleic acids into cells by such methods as electroporation, calcium phosphate precipitation, or in liposomes. Transient expression results when mRNA is transcribed from the initially introduced vector DNA prior to vector integration.

Antisense p23 nucleotide sequences, that is, nucleotide sequences complementary to the transcribed or the non-transcribed strand of a p23 gene, may be used to block normal or mutant p23 expression in cancer cells or other proliferating cells. The use of antisense oligonucleotides and their applications have been reviewed in the literature (see, for example, Mol and Van der Krul, eds., Antisense Nucleic Acids and Proteins Fundamentals and Applications, New York, NY, 1992;
which is incorporated by reference herein in its entirety). Suitable antisense oligonucleotides are at least 11 nucleotides in length and may include untranslated (upstream or intron) and associated coding sequences. As will be evident to one skilled in the art, the optimal length of an antisense oligonucleotide depends on the strength of the interaction between the antisense oligonucleotide and its complementary target sequence, the temperature and ionic environment in which translation takes place, the base sequence of the antisense oligonucleotide, and the presence of secondary and tertiary structure in the target mRNA and/or in the antisense oligonucleotide. Suitable target sequences for antisense oligonucleotides include intron-exon junctions (to prevent proper splicing), regions in which DNA/RNA hybrids will prevent transport of mRNA from the nucleus to the cytoplasm, initiation factor binding sites, ribosome binding sites, and sites that interfere with ribosome progression. A particularly preferred target region for antisense oligonucleotide is the 5' untranslated (promoter/enhancer) region of the gene of interest. Antisense oligonucleotides may be prepared by the insertion of a DNA molecule containing the target DNA sequence into a suitable expression vector such that the DNA molecule is inserted downstream of a promoter in a reverse orientation as compared to the gene itself. The expression vector may then be transduced, transformed or transfected into a suitable cell resulting in the expression of antisense oligonucleotides. Alternatively, antisense oligonucleotides may be synthesized using standard manual or automated synthesis techniques. Synthesized oligonucleotides may be introduced into suitable cells by electroporation, calcium phosphate precipitation, liposomes, microinjection, or other means. The stability of antisense oligonucleotide-mRNA hybrids may be increased, for example, by the addition of stabilizing agents to the oligonucleotide, such as intercalating agents covalently attached to one end of the oligonucleotide, or by incorporating phosphodiester, phosphonates, phosphorothioates, phosphoroselenoates, phosphoramidates, or phosphorodithioates into the phosphodiester backbone.

Protein harvested from transgenic cells expressing p23 can be used for a number of purposes, for example, for raising antiserum against p23. Thus, the invention provides immunologic binding partners for p23 polypeptides such as polyclonal and monoclonal antibody molecules, and various antigen-binding fragments thereof, that are capable of specifically binding a p23 polypeptide such as the polypeptide whose amino acid sequence is shown in SEQ ID NO:2. Antibodies may be raised against whole p23, or against fragments of the polypeptide. The p23
used as an antigen may be denatured or in its native form prior to injection. Antibodies against denatured proteins are often able to react with either native or denatured protein, and are often useful for Western blotting. The antibodies can be used also for the identification of senescent cells in culture or in tissue biopsies using standard immunostaining protocols.

Immunospecific reagents capable of specifically binding p23 may be produced by hybridoma or by repeated injection of the purified protein or selected peptides derived from p23 in combination with an appropriate adjuvant (e.g., Freund's, ISCOMs, or the like) into a suitable animal such as a rabbit, sheep, or goat. Antibodies against p23 find utility in therapeutic, purification, and diagnostic applications. Therapeutic applications include binding partners that inhibit the binding of p23 to ligands that normally bind to it, thus promoting cell proliferation in the treated cell. Representative examples of purification applications include immunological methods and immunoaffinity chromatography wherein the antibody is used as an affinity reagent to purify p23 from tissues in which it occurs naturally, or from cultured cells expressing transgenic p23. Representative examples of diagnostic applications include enzyme-linked and radioisotopic immunoassays (i.e., ELISA and RIA), immunofluorescence, time-resolved fluorescence immunoassay and the like, to determine levels of p23 protein in tissue samples, such as tumor cells.

In addition, antibody against p23 could be used to selectively kill the senescent cells in a cell population. As p23 appears to be a transmembrane protein, antibody against p23 can be used to selectively lyse cells in whose membranes the protein is present. Thus, an aging culture of cells could be rejuvenated by exposure to the antibody under conditions that permit the antibody to lyse cells expressing the protein, or by using anchored anti-p23 to cull p23-expressing cells from a cell suspension. In addition, these same procedures could be used to cull or enrich for senescent cells from tissue samples removed from patients. Young cells from such culled cell populations could be returned to the body, or if indicated, the senescent cells instead could be returned to the body.

The p23-specific immunologic binding partners further find general utility in diagnostic assays for detecting and quantitating levels (e.g., protein or antigen) of p23 in a cell such as a cultured cell, to provide an indicator of the remaining number of cell divisions that can be expected for that cell line. For this type of assay, the measured level of p23 in the cell line is compared with the levels previously
measured in early, intermediate, and late passage cultures of the same cell type. For example, the life expectancy of cultured epithelial cells can be predicted by comparison of p23 levels in the test culture with standard p23 levels measured after various numbers of passages in a representative epithelial cell line, e.g., HMECs. Alternatively, one can estimate the number of remaining passages that could be expected as a function of the proportion of cells in the culture that are expressing p23 as detected by immunostaining, in situ, or Northern blot hybridization to detect mRNA, or by any other convenient method. In such an assay, if greater than 90% of the cells are expressing high levels of p23 as compared with levels expressed during early passage cells, it can be assumed that the culture is senescent and will not substantially expand if replated.

Skilled artisans will further understand that the disclosure herein of recombinant p23 nucleic acids, cells expressing exogenous p23, and in vitro assays provide opportunities to screen for compounds that modulate, or completely alter, the functional activity of a p23 protein or p23 nucleic acid in a cell. In this context "modulate" is intended to mean that the subject compound increases or decreases one or more functional activities of a p23 protein or nucleic acid, while "alter" is intended to mean that the subject compound completely changes the p23 protein or nucleic acid functional activity to a different functional activity. In this context, an example of a compound that "modulates" the activity of a p23 protein is an inhibitor capable of decreasing the level of p23 expression following the administration of the compound to a cell expressing p23. As cells in which p23 is suppressed are expected to become receptive to mitogen stimulation, the functional activity of the p23 added to the cells can be assayed by measuring the recipient cell's restored responsiveness to mitogens. Retinoic acid is an example of a compound that reduces p23 expression.

Included among the compounds that may modulate p23 activity are artificial p23 polypeptides, organic chemical mimetics, and the like. Such compounds find broad utility as selective inhibitors of p23. By providing competitive inhibition of p23, such inhibitors could be used to restore the ability of a cell to proliferate in response to growth factors, mitogens, cytokines, and like agents. "Artificial p23 polypeptides" is understood to include fragments of the p23 polypeptide, which can be produced from full length p23 by physical or enzymatic fragmentation or by use of recombinant DNA technology to express subportions of the p23 polypeptide.
The subject p23 polypeptides encompass normal p23 polypeptides (i.e., found in normal cells), mutant p23 polypeptide (e.g., resulting from mutagenesis, or found in tumor cells), and chemically modified p23 polypeptides (e.g., having one or more chemically altered amino acids, in which case a designated amino acid can be converted into another amino acid, or chemically substituted or derivatized and the like). Functional sites in the p23 polypeptides are identified by constructing mutants of the p23 nucleic acid, e.g., and testing the constructs for expression products having altered functional properties such as failure to induce senescence when introduced into actively proliferating cultured cells.

It is further understood that mutant p23 nucleotide sequences may be constructed from the nucleotide sequence shown in SEQ ID NO:1. Skilled artisans will recognize a variety of methods by which the sequence in SEQ ID NO:1 may be mutated (e.g., with chemical agents or radiation or using recombinant DNA technology), and by which clones of cells containing the mutated p23 nucleotide sequences may be identified and/or selected. The subject mutant p23 nucleotide sequences are useful for modulating or altering the activity of p23 in a cell. The subject mutant p23 nucleotide sequences may be introduced into cells using vectors such as retroviral vectors, adenovirus vectors, or bacteriophage or plasmid vectors.

The subject invention includes assays for: a) detecting the absolute levels and activities of p23 expression in nonsynchronized cell populations (e.g., in tissue samples such as tumor biopsy specimens); b) comparing the levels and activities of p23 polypeptide or mRNA in synchronized or non-synchronized cell populations after various numbers of passages in culture; and c) determining the levels and activities of p23 expression products in biological fluids (i.e., blood, serum, plasma, mucus secretions, CNS fluid, cell extracts, and the like). The absolute levels and activities of p23 expressed in malignant biological fluids (e.g., tumor cell extracts, serum from cancer patients, and the like), as well as the levels and activities expressed in cell extracts prepared after various numbers of passages of a tumor cell in culture may provide information on the aggressiveness of a tumor or may shed light on the likelihood that the tumor cells can be arrested in G1 by restoring p23. In this regard the assayed levels and activities of p23 may serve as diagnostic markers for:

a) staging tumors, since at least some types of malignant cells capable of metastasizing express little or no p23;
b) determining prognosis, i.e., predicting patient survivability and time to
recurrence of tumor, because rapidly growing malignant cells capable of metastasis
may generally express less p23 than differentiated cells; and/or

5 c) predicting therapeutic success, i.e., of a particular therapeutic regimen,
because more slowly growing cells may express higher levels (or activities) of p23
(i.e., than rapidly growing metastatic cells) and also be more responsive to less
drastic and more prolonged therapeutic regimens.

Those skilled in the art will recognize that the subject diagnostic assays may
provide results that are useful to a physician in deciding how to stage a tumor, how to
10 select an appropriate therapeutic regimen, how to evaluate the success of therapy, and
how to evaluate patient risk or survivability.

The invention further provides methods for measuring the level of p23
expression in a biological sample. The sample may be a cultured cell, a biological
15 fluid, a patient tissue specimen, a tumor biopsy, or other sample. The expression
level can be measured, for example, by hybridizing RNA from the biological sample
with a nucleic acid probe corresponding to an at least 15 nucleotide region of the
nucleotide sequence of SEQ ID NO:1, and comparing the results with RNA standards
from young and senescent cultured epithelial cells. Probes are generally labeled, for
example, with ^32P or biotin, using enzymes such as polynucleotide kinase, Klenow,
or whole DNA polymerase, and using routine protocols (see, e.g., Sambrook et al.,
1989). In one commonly used method of detecting p23 expression in the sample, i.e.,
Northern blotting, extracted RNA is immobilized on a membrane filter, hybridized
with the denatured labeled probe, and hybrids detected by autoradiography or
chromogenic methods. Comparison with RNA standards, e.g., RNA from young
(i.e., low passage number) and senescent cultured epithelial cells, provides a basis for
determining whether the amount of p23 RNA in a test sample is "low" or "high," i.e.,
the level in young standard cells from the selected standard cell line is defined as
"low," while the level in senescent standard cells is defined as "high." Alternatively,
25 p23 expression levels can be determined by using antibody against p23 to measure
the amount of p23 polypeptide. Again, amounts of p23 polypeptide in young and
senescent epithelial cells provide a standard for comparison. Thus, assays for p23
levels provide a valuable tool in managing use of scarce or valuable cell lines, such
as cell lines established from unique tissue samples, or for maximizing the efficient
use of non-immortalized cell lines whose passage history is not known. Moreover,
such assays could be used to characterize biopsy samples from normal or diseased tissue, e.g., tumor biopsies or tissue biopsies from degenerating tissues.

In other embodiments, the invention provides assays for detecting chromosomal rearrangements in chromosome 3 in a human cell. The chromosomal location of p23 has been mapped by computerized analysis (Unigene program; Boguski et al., *Nature Genet.* 10:369-371, 1995) to the distal long arm of chromosome 3, between bands q28 and q29. Thus, the cloned p23 cDNA sequences provide a hybridization probe that can be used for *in situ* hybridization to visualize the p23 gene in metaphase chromosomes, thus enabling one to detect translocations involving this region of chromosome 3. Translocation of the p23 gene, i.e., from its normal location to a different chromosome, may contribute to a phenotype of uncontrolled cell growth by removing p23 from regulatory control elements that ensure its expression and subsequent cell senescence. Thus, rearrangement of a p23 gene in a cell may have dramatic results. If a rearrangement induces under-expression, the cell may acquire a malignant (i.e., uncontrolled) growth phenotype, and if a rearrangement induces over-expression, the cell may undergo premature senescence. Screening cellular samples from individuals for chromosomal rearrangements involving p23 may provide information related to that patient's relative risk of developing specific types of cancer or other disease conditions, such as autosomal dominant optic atrophy (see below). Moreover, rearrangements in the long arm of chromosome 3 involving band q28 have been associated with at least one type of tumor, i.e., liposarcomas (*Nature Genetics Special Issue*, April, 1997, page 433).

The location on chromosome 3 of the p23 gene is the same as that determined for a OPA1, an autosomal dominant genetic disease that is manifest by retinal ganglion cell or optic nerve degeneration. (Lunkes, A., *Am. J. Hum. Genet.*, Oct., 1995; or Eiberg et al., *Human Mol. Genetics* 3:977-980, 1994). Both p23 and the OPA1 trait map to the long arm of chromosome 3 between q28-q29, suggesting the possibility that optic atrophy could result from a mutation in p23 itself. Given its association with cell senescence, a mutation in p23 could well trigger premature or excessive expression of the gene, and the consequent premature entry of the affected cells into a senescent or aberrant state, thus manifesting as nerve cell degeneration. As this interband region of chromosome 3 is large enough to accommodate several genes, it remains possible that p23 is not directly responsible for OPA1, but rather is
closely linked to the responsible gene, thus providing a genetic marker for the disease locus due to its proximity to the actual OPA1 gene.

If rearrangements of the p23 gene result in a loss of growth control, e.g., cancer, or to inappropriate atrophy, e.g., OPA1, normal growth may be restored by providing the missing control elements to the translocated gene, thus reversing the malignant phenotype, or by suppressing the inappropriate overexpression of p23, e.g., in treating OPA1. Thus, the p23 gene and its regulatory elements may serve as targets for gene therapy vectors that are designed to reactivate or to inactivate the rearranged gene, e.g., using in situ-directed recombination/mutagenesis or targeted integration to disrupt the translocated gene.

EXAMPLE 1

Cloning of a Gene that Is Up-Regulated in Senescent Breast Epithelial Cells

The technique of differential display (DD) of mRNA (Liang and Pardee. Science 257:967-971, 1992; Liang et al., Nucl. Ac. Res. 22:5763-5764, 1994) has been used to identify genes whose level of expression correlates with cellular senescence. In this technique, two populations of messenger RNAs are compared by creating partial cDNA sequences from subsets of the messenger RNA populations using reverse transcription and then amplifying the cDNA using the polymerase chain reaction (PCR). Different primers can be used for the initial reverse transcription. The primer used to transcribe the first DNA strand always hybridizes to a portion of the poly(A) tail of the mRNA template as well as to one or two non-(A) residues at the 3' end of the mRNA at the poly(A) junction. For second strand synthesis, primers are used that have a random sequence that is intended to be complementary to an internal sequence somewhere upstream (i.e., in a 5' direction) from the first primer. By varying the identity of the base or bases complementary to non-(A) residues for the first primer, different subsets of mRNA are targeted. Using whole cell RNA as a template to synthesize cDNA that is subsequently amplified by PCR, each primer pair will typically generate about 50-100 bands that range in size from 50-500 base pairs. After being amplified by PCR in the presence of 35S-labeled nucleotides, these mixtures of short cDNA sequences are displayed for comparison on a polyacrylamide sequencing gel. By comparing the products obtained using the same primers with messenger RNA from two different types of cells, those bands present in one cell type but absent from the other can thus be identified. The cDNAs that differ between the two populations can be recovered from the dried gel, reamplified with PCR, and subsequently cloned and further characterized. This
method has been used successfully to identify a large number of senescence-related ESTs from fibroblasts (WO 96/13610).

Sources of cells and culture conditions used for these experiments were as follows. Normal human mammary epithelial cells (HMECs), strains AG11132 and AG11134, were obtained from the Coriell Institute (National Institutes of Aging Cell Repository, Camden, New York). HMECs were maintained in serum-free mammary essential basal medium (MEBM; Clonetics, San Diego, CA) supplemented with 0.4% bovine pituitary extract (Clonetics), 10 mM HEPES (Sigma), 10 ng/ml human recombinant epidermal growth factor (EGF) (Upstate Biotechnology, Lake Placid, NY), 5 μg/ml human recombinant insulin (UBI), 0.5 μg/ml hydrocortisone (Sigma, H4001), and 10^{-5} M isoproterenol (Sigma, 15627). Breast tumor cells were obtained from the American Type Culture Collection, Rockville, MD and maintained in alph-MEM (BRL/Gibco) supplemented with 5% fetal bovine serum (HyClone), 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 1 x non-essential amino acids (Sigma), 12.5 ng/ml EGF (Sigma), 1 μg/ml insulin (Sigma) and 1 μg/ml hydrocortisone (Sigma).

Differential display was performed comparing cDNAs from young and senescent AG11134 cells. AG11134 is a line of normal HMECs that already had been serially passaged 6-8 at the time it was obtained from the Coriell Institute. The cells were passaged and expanded weekly by 1:4 to 1:2 dilutions, and cells were harvested for RNA preparation after 18 and 26 passages (p18 and p26). Total cellular RNA was purified as previously described (Swisshelm et al., *Cell Growth Differentiation* 5:133-141, 1994). At p18, the cells had doubled about 60-65 times, and still proliferated rapidly, but by p26, corresponding to about 85 doublings, 80-90% of the cells failed to replicate when replated, thus had become senescent. The senescent phenotype was verified by assaying for the presence of the pH-dependent β-galactosidase that is differentially expressed in senescent cells (Dimre et al., 1995). In the proliferating population (p18), about 12% of the cells stained positive for β-galactosidase, while in the p26 population, 99.4% of the cells stained positive for this enzyme.

RNA was extracted from the young and senescent AG11134 cells, and differential display was performed to compare transcription in p18 and p26 HMECs. Differential display was conducted in accord with published procedures (Liang and Pardee, 1992; Liang et al., *Methods Enzymol.* 254:304-321, 1995; Swisshelm et al.,...

Primers for the hybridization with the poly(A) end of the mRNA ("anchor" primers) included three different primers having a 5' Hind III site to facilitate cloning of the amplified fragments (Liang et al, 1994; GenHunter Corp., Brookline, MA). These three "H-T11" primers had the following sequences:

5' AAGCTTTTTTTTTTAG 3' (SEQ ID NO:4)
5' AAGCTTTTTTTTTTTA 3' (SEQ ID NO:5)
5' AAGCTTTTTTTTTTTC 3' (SEQ ID NO:6)

In addition to the H-T11 primers, the following T12 anchor primers (obtained from Operon Technology, Alameda, CA) were used:

5' TTTTTTTTTTTTTAA 3' (SEQ ID NO:7)
5' TTTTTTTTTTTTGA 3' (SEQ ID NO:8)
5' TTTTTTTTTTTTCA 3' (SEQ ID NO:9)
5' TTTTTTTTTTTTAG 3' (SEQ ID NO:10)
5' TTTTTTTTTTTTGG 3' (SEQ ID NO:11)
5' TTTTTTTTTTTTCG 3' (SEQ ID NO:12)
5' TTTTTTTTTTTTAC 3' (SEQ ID NO:13)
5' TTTTTTTTTTTTGC 3' (SEQ ID NO:14)
5' TTTTTTTTTTTTC 3' (SEQ ID NO:15)
5' TTTTTTTTTTTAT 3' (SEQ ID NO:16)
5' TTTTTTTTTTTGT 3' (SEQ ID NO:17)
5' TTTTTTTTTTTTCT 3' (SEQ ID NO:18)

The above primers were coupled in PCR reactions with each of 30 different random-sequence primers obtained either from Operon Technology or GenHunter, each having 60-70% G+C and no self-complementary ends.

PCR reactions were conducted as follows: denaturation at 94°C, 30 seconds; annealing at 40°C, 2 minutes; extension at 72°C, 30 seconds. These steps were repeated for a total of 40 cycles, which were terminated with a 5-minute extension step. For individual PCR reactions, each anchor primer was paired in a separate reaction tube with each of the random primers, except that the T12 primers ending with the same base were pooled together in a single reaction.

The cDNA band patterns corresponding to early passage and senescent cells were displayed and compared on DD gels, and a number of bands were excised from the dried gels that appeared to be either more abundant or less abundant in the
senescent cells as compared with the young cells. Initially, about fifty candidate cDNA
fragments were extracted from the gels and reamplified by PCR. Each of
these amplified cDNA fragments was labeled with $^{32}$P and used as a hybridization
probe to analyze RNA from young and senescent AG11134 cells on Northern blots
containing 5-10 µg/lane of whole cell RNA from each type of cell. The Northern
blots were hybridized at 37°C in buffer containing 0.25 M NaPO$_4$, 0.25 M NaCl, 7%
SDS, 1m M EDTA, 5% dextran sulfate, 100 mg/ml salmon sperm DNA, and 50%
formamide. The Northern blots contained whole cell RNA from each of the cell
cultures. Filters were washed at 37°C in a buffer containing 2 X SSC and 0.1% SDS.

A probe corresponding to one of the excised DNA fragments, which was
named "DD19," was found to hybridize with a mRNA of approximately 4 kb in size
that was present at much higher levels in senescent than in rapidly dividing cells.
The primer pair flanking this particular cDNA consisted of 5′ GGAGGGGTGTT 3′
(SEQ ID NO:19) (random primer OPB15, from Operon, Kit B) and
5′ AAGCTTTTTTTTTC 3′ (SEQ ID NO:6) (i.e., anchor primer H-T$_{11}$C). The
DD19 probe was labeled with $^{32}$P-dCTP using a random primer kit (Boehringer -
Mannheim).

To confirm that DD19 corresponded to a mRNA elevated in senescent cells,
the Northern blot analysis was repeated using whole cell RNA from AG11132 as
well as from AG11134 cells, the former also being a line of normal HMECs. Results
of this experiment indicated that transcripts hybridizing with the DD19 probe were
present at higher levels in senescent than in young cells for both strains of HMEC
cells. The most prominent band that hybridized with the probe had a size of about
4.0 kb, but a less abundant transcript with a size of about 3.0 kb was present also. It
is possible that the 3.0 kb mRNA results from differential splicing of the primary p23
transcript.

The amplified DD19 DNA fragment, which had a size of 326 bp, was cloned
into the plasmid vector pCR (Invitrogen, Carlsbad, CA), using the TA cloning kit.
The insert from the cloned DD19 was sequenced both manually using SEQUENASE
(USB) and by the fluorescence method using an ABI 377 automated sequencer
(Murdock Laboratories, University of Montana). Sequencing was conducted using
primers defined by the vector, i.e., T7 and SP6.

DD19 DNA cloned in the pCR vector was labeled and hybridized as
described above with a panel of RNAs to confirm the initial observation that the 4.0
kb mRNA is elevated in senescent cells. Sources of RNA for this panel were young,
senescent, and quiescent AG11134 cells, as well as young, senescent, and quiescent AG11132 cells. Quiescent cells are cells that have stopped dividing, but that retain the capacity to divide if placed under favorable conditions, e.g., if exposed to a mitogen, or if diluted and replated. Quiescent cells were prepared from early passage cells by allowing cells to become confluent, then maintaining them in culture with occasional feeding for an additional two weeks without further passage. RNA was isolated from quiescent cells about 48 hours after the final addition of fresh medium. As an internal control, p23 was stripped from the filters, and the filters were rehybridized with labeled probe made from a cloned cDNA that corresponds to 36B4, which is a phosphoprotein present in ribosomes, and whose corresponding mRNA, which has a size of 1.5 Kb, is present at relatively constant levels in a wide variety of cell types (Masiakowski et al., Nucl. Ac. Res. 10:7895-7903, 1982; Rio et al., Proc. Natl. Acad. Sci. USA 84:9243-9247, 1987; Laborda, J., Nucl. Acids Res. 19:3998, 1991).

When Northern blots containing whole cell RNA from young, senescent, and quiescent cells were analyzed by hybridization as described above, the results confirmed that the cloned DD19 DNA corresponded to transcripts expressed at elevated levels in senescent cells for both of the HMEC cell lines. As measured by densitometric analysis, the expression level for the 4.0 kb transcript in AG11132 cells was about 7-fold higher in senescent than in young cells. DD19-related transcripts were not elevated in quiescent cells for either strain of HMEC cells.

Further hybridization experiments were conducted to ascertain the levels of expression of the 4.0 kb transcript in a panel of breast tumor cell lines. These were Hs578T, MCF7A, MDA-MD-435, MDA-MB-231, SKBR3, and T47D cells. These hybridizations were conducted using two different probes, one of which was the cloned DD19 DNA fragment, and the other of which was DD19.5 DNA, a cDNA clone corresponding to most or all of the 4.0 kb transcript (described below). Hybridization conditions were as described above, except that filters hybridized with DD19.5 probe were washed at 50°C instead of 37°C. Identical results were obtained with both probes. No transcripts hybridizing with labeled DD19 were observed in any of these cells except T47D, in which levels comparable to senescent cells were observed. Interestingly, the 3.0 kb RNA was not detected in any of the six breast cancer cell lines, including T47D. This circumstance suggests that the absence of the 3 kb mRNA may provide a marker for breast cancer cells.
Using cloned DD19 DNA as a probe, a cDNA clone corresponding to most or all of the 4.0 transcript was obtained as follows. The cloned DD19 DNA fragment was labeled and used as a hybridization probe to screen a cDNA library that previously had been prepared in the lambda Zap II vector using RNA from senescent 76N cells as template for reverse transcription of long cDNA (Swisshelm et al., 1994). These cells are a strain of normal human mammary epithelial cells. Hybridization buffer contained 50% formamide, 5 X SSC, 100 mg/ml carrier DNA, 0.1% SDS, 0.1% BSA, 0.1% polyvinylpyrrolidone, and 0.1% ficoll. Hybridizations were conducted at 37°C, and the filters were washed at 37°C in 2 X SSC and 0.1% SDS. Approximately 1.25 x 10⁶ plaques were screened. Three positive clones were selected for further characterization.

Inserts from the three positive clones were sequenced both manually and with the ABI automated sequencer. The longest of the three cDNA clones, which was named "DD19.5," encompassed the inserts in the other two selected clones. The cDNA cloned in DD19.5 was sequenced in its entirety using walking primers, and the cloned insert proved to be 3443 nucleotides in length. Cloned DD19.5 was deposited on August 4, 1997, in accord with the terms of the Budapest Treaty at the American Type Culture Collection, located at 12301 Parklawn Drive, Rockville, MD, 20852, U.S.A., and was assigned the accession number ____. The nucleotide sequence of DD19.5 was analyzed using the Wisconsin Package Version 9.0 (Genetics Computer Group, University Research Park, Madison, WI), hereafter referred to as the "GCG" package or program.

Analysis of the DD19.5 nucleotide sequence indicated that it contained an open reading frame (ORF) capable of encoding a protein of 211 amino acid residues, having a predicted molecular weight of 23 kDa. Hence, this gene was named "p23." The length of this open reading frame indicated that a large proportion of the 4.0 kb mRNA was untranslated. This long untranslated region is in accord with the assumption that p23 belongs to the EMP family of transmembrane proteins, as this family often has mRNAs with large untranslated regions (e.g., Chen et al., Genomics 41:40-48, 1997; Lobsiger et al., Genomics 36:379-387, 1996).

Using FASTA homology search, it was determined that p23 is similar or identical to several anonymous partial cDNAs (i.e., expressed sequence tags or "ESTs"), including an anonymous cDNA from a pancreatic islet cDNA library (GenBank accession number W51940), and at least six other ESTs. These latter are GenBank accession number AC000088, with 45.3% identity in a 214 amino acid
overlap; accession number AC000005, with 46.5% identity in a 198 amino acid overlap; accession number U19582, with 34.6% identity in a 188 amino acid overlap; accession number X94770, with a 24.3% identity in a 136 amino acid overlap; accession number X15436, with a 52.0% identity in a 25 amino acid overlap; and, accession number M97881, with a 43.2% identity in a 37 amino acid overlap. No significant homology was detected between p23 and the senescence-related ESTs disclosed in WO 96/13610.

Computer analysis indicated also that p23 is related to a gene known as "RVP1" that was cloned from a rat ventral prostate-androgen withdrawal cDNA library (Genbank accession No. A39484; Briehl and Miesfeld, Molec. Endocrinol. 5:1381-1388, 1991). When aligned to maximize their similarities, p23 is identical at 48% of its amino acids to RVP1, and similar at 69% of its amino acids, i.e., the amino acids either are the same or represent conservative substitutions 69% of the time. The comparison of these two protein sequences is shown in FIGURE 1. Sizes of the most abundant transcripts for these two genes are quite different, with that of the rat gene being approximately 1.2 kb, and that of the p23 gene being 4.0 kb. However, the putative protein products of the rat gene and p23 gene are more similar in size than their transcripts, the RVP1 protein having 280 amino acids, and the p23 protein having 211 amino acids. The degree of homology observed between p23 and the rat protein suggests that these two proteins are distantly related, although RVP1 is elevated not in senescent cells, but in apoptotic cells.

Functional motifs in the open reading frame from p23 were identified based on the amino acid sequence and consensus sequence domain, using the Motifs tool, which is a subroutine in the GCG computer program package for sequence analysis. A single putative asparagine N-glycosylation site was identified at residue 72 within the consensus sequence "NLSS." A cAMP/cGMP phosphorylation site was noted at residue 192 embedded within the consensus sequence "RKTTS." Two potential protein kinase C substrates, a threonine and serine residue, were identified at amino acids 193 and 206, respectively. Various features of the secondary structure predicted for the p23 protein are shown in FIGURE 2, in which the hydrophobic regions are indicated by triangles and the hydrophilic regions by ovals. Shown also is the O-glycosylation site at residue 72. In addition, the analysis indicated further that p23 has an isoelectric point of pH 8.02.

Based on Engelman et al. (Ann. Rev. Biophys. Biochem. 15:321-353, 1986), and Kyte-Doolittle hydrophobicity plots (Kyte and Doolittle, J. Mol. Biol. 157:105-
132, 1982), two and possibly four domains in the p23 amino acid sequence of SEQ ID NO:2 appear to contain integral transmembrane regions. This is notable because several of the EMP family of proteins, a family to which p23 has been tentatively assigned, are characterized by containing four putative transmembrane regions (see, e.g., Schiemann et al., Anticancer Res. 17:13-20, 1997). EMP proteins also are associated with cell growth arrest and degeneration, although it has been proposed that they play a dual role in development and differentiation. For example, PMP22, the prototype gene for this family, may be involved in both growth arrest and in differentiation in Schwann cells (Taylor et al., 1995; Taylor and Sutor, Gene 175:115-120, 1996). The putative transmembrane regions identified in p23 are located at amino acid residues 82-98 (76-108), 119-135 (115-141), 8-24 (3-28), and 170-186 (165-187) (the numbers in parentheses represent alternative overlapping possibilities). This shared feature with EMP proteins supports the proposal that p23, like the EMP proteins it resembles, functions to suppress cell division.

In addition to RVPI, two other proteins have been identified that appear to be related to the p23 polypeptide. One of these is the product of the "TMVCF" gene, a gene associated with human autosomal dominant genetic disorders involving multiple physical abnormalities. The TMVCF gene encodes a 219 amino acid protein that by Kyle-Doolittle analysis has four putative transmembrane regions (Sirotkin et al., Genomics 42:245-251, 1997). The other p23-related protein was isolated from monkey cells and encodes a receptor for the toxin produced by Clostridium perfringins, and is called the "CPE-R" gene (Katahina et al., J. Cell Biol. 136:1239-1247, 1997). The CPE-R protein encodes a 209 amino acid polypeptide, and also is predicted to contain four transmembrane regions. When compared with p23 using the GCG program, the TMVCF protein had about a 46% identity and 55% similarity with p23, while the CPE-R protein had a 46% identity and a 57% similarity. The CPE-R and TMVCF genes give rise, respectively, to mRNAs of 1.8 and 1.4 kb. As no transcripts of these sizes were detected on Northern blots with p23 probes, it appears that the coding sequences of CPE-R and TMVCF have diverged from the p23 coding sequences to a degree such that they do not support cross-hybridization with p23 probes under the hybridization conditions that were used in the Northern blots described above.

The results of preliminary Southern blots have indicated that the human genome contains only a single gene capable of hybridizing with a probe corresponding to p23. For these analyses, genomic DNA from five types of cells
were digested with Bam H1, an enzyme having a single cut site in the p23 cDNA sequence. As expected, two fragments were observed when Southern blot analysis was performed on the cleaved DNA and the blots probed with labeled DD19.5. These blots were hybridized in 2 x SSC, 0.1% SDS, at 50°C.

The position of the p23 gene in the human chromosomes was mapped by computerized analysis using the Unigene program (Boguski et al., *Nature Genet*. 10:369-371, 1995). The gene was found thus to be located on the distal long arm of chromosome 3, between bands q28 and q29. This location coincides with a chromosomal location that is strongly associated by pedigree analysis with the disease OPA1. This common map site suggests the possibility that mutations in the p23 gene could be the underlying cause of OPA1, although this interband region is large enough to accommodate several genes. It may be of significance that a breakpoint at this chromosomal location has been associated with at least one type of cancer, i.e., liposarcoma (Mitelman et al., *Nature Genetics* 15(suppl.):417-474, 1997).

**EXAMPLE 2**

**Expression of p23 in Various Tissue Types**

Expression of p23 was further investigated by analyzing several different premade Northern blots (ClonTech, Palo Alto, CA) containing various panels of poly(A)+ mRNA. The hybridization probe used in these analyses was the cloned 326 bp DD19 fragment. Results of these hybridizations revealed that the gene is expressed in a wide variety of tissues at different levels. p23 expression was observed in heart, placenta, liver, fetal liver, lung, skeletal muscle, kidney, spleen, thymus, prostate, ovary, and small intestine. A human endocrine tissue panel Northern blot showed abundant expression in the pancreas, and also in the adrenal gland, with somewhat lower levels in the thyroid, testis and thymus. A human brain panel Northern blot showed expression from the occipital pole, lower levels of expression in the medulla, and very little expression elsewhere in the brain. A human immune system Northern blot showed expression in spleen, lymph node, thymus, and appendix. Of all the tissues analyzed, the highest levels observed were in liver, pancreas, and fetal liver. Levels expressed in the other organs were about 10-50% lower than those seen in liver and pancreas.

Direct comparisons of p23 RNA levels observed in the ClonTech Northern blot panels with p23 transcript levels in senescent cells was not possible, as the ClonTech blots did not include senescent cell RNA. Meaningful comparisons were
further obviated by the fact that the senescent cell RNA analyses described in Example 1 used total RNA, whereas the ClonTech blots contained polyadenylated RNA.

Also analyzed for p23 transcripts was a ClonTech human cancer cell line panel, which included HL-60 (promyelocytic leukemia), HeLa S3 (cervical carcinoma), K-562 (chronic myelogenous leukemia), MOLT-4 (lymphoblastic leukemia), Raji (Burkitt's lymphoma), SW480 (colorectal adenocarcinoma), A549 (lung carcinoma), and G361 (melanoma) cells. Northern blot results indicated that p23 was expressed at low levels in the SW480 and A549 cells, but none was detected in the other cell lines in this panel. It may be significant that SW480 and A549 are epithelial cells. These results support the hypothesis that low levels of p23 expression are associated with uncontrolled cell growth.

EXAMPLE 3

p23 Expression in Presence of Retinoic Acid

The response of p23 expression to retinoic acid was tested in senescent and early passage AG11132 cells, early passage AG11134 cells, MCF7 tumor cells (which express no detectable p23), and T47D tumor cells (which express levels of p23 comparable to senescent epithelial cells). Cells were cultured for 48 hours as described in Example 1, with the addition of 1μM retinoic acid to the culture medium. Control cultures received no retinoic acid. Whole cell RNA was extracted from exposed and control cells, and was subjected to Northern blot analysis. The probe used was cloned DD19, and the hybridization conditions were as described in Example 1 for initial Northern blot testing with RNA from young and senescent AG11134 cells. Signals on the resulting autoradiograms were quantified by densitometry, using an AGFA flatbed scanner and the program NIH Image. Results indicated a 25-50% reduction in the amount of p23 mRNA in all of the cells exposed to retinoic acid.

EXAMPLE 4

Suppression of the Transformed Phenotype in Cultured Breast Cancer Cells

For these experiments, MDA-MD-231, Hs578T, MDA-MD-431, SKBR3, and MCF7 cells are cultured as described in Example 1. The cloned p23 gene is introduced into the cells as follows. The coding region of the p23 cDNA is ligated into the LXS N retroviral vector as described in Seewaldt et al., Cell Growth and Differentiation 6:1077-1088, 1995, which is hereby incorporated by reference.
This vector harbors a gene that confers resistance to the drug G418, thus providing a basis for selection of cultured cells that are effectively transduced. For G418 selection, G418 (GIBCO) is added to the culture medium at 1 mg/ml, a concentration that is toxic to non-transduced cells. Dividing cells are transduced by adding the p23-encoding vector at a multiplicity of infection of 1:1 in the presence of 4 mg/ml POLYBRENE (Sigma). Cell selection is as described previously (Seewaldt et al., 1995). As controls, some of the cell cultures are transduced with the "empty" vector, i.e., vector not containing p23 coding sequences.

Expression of p23 in transduced G418-resistant cells is verified by Northern blotting. Total cell RNA is extracted with guanidinium hydrochloride, and analyzed after formaldehyde denaturation by electrophoresis in agarose gels, transferred to nylon membranes, and hybridized with probe made by labeling DD19 or DD19.5. Alternatively, suitable synthetic probes are based on the nucleotide sequence of SEQ ID NO:1, and specificity of the synthetic probes is verified by demonstrating that the probe hybridizes under stringent conditions to a 4.0 kb and a 3.0 kb mRNA expressed at higher levels in senescent than in young HMECs, and not with other mRNAs in those same cells.

Breast cancer cells transduced with a p23 expression vector are expected to divide less rapidly than control cells, and to enter a senescent state wherein they are arrested in G1. To determine whether cells are actively dividing, cells are re-plated, and 3H-thymidine is added to the medium at 1-10 μCi/ml. After 1-6 hours, cells are harvested, and DNA is extracted and assessed for the amount of 3H that was incorporated into DNA. Senescent cells do not incorporate 3H-thymidine into their DNA, as they are arrested in G1.

The effects of transduced p23 will be further assessed by evaluating cell doubling times in p23-transduced and mock-transduced (i.e., cells infected with the empty vector) breast cancer cells. Cells are plated at 5 x 10^4 cells per 35 mm tissue culture Petri dish and grown in standard medium containing 1 mg/ml G418. Individual plates are trypsinized at 24 to 48 hour intervals, and harvested cells are counted in duplicate. Doubling times are obtained by plotting cell number on a log scale against time on a linear scale.

Cells expressing transduced p23 and exhibiting increased doubling times are further assessed to determine whether they have become less tumorigenic than prior to transduction. Transgenic and mock-infected cells are injected subdermally into nude mice to assess tumorigenicity using 10^6 cells per injection intradermally, intra-
peritoneally, or into the mammary fat pad, depending on the type of cells being injected. For example, transduced breast tumor cells are injected into the mammary fat pad. Tumor mean diameter is measured at weekly intervals following the inoculation. Reduced rate of tumor growth with p23-transduced cells as compared with mock-transduced cells will indicate that induction of p23 expression can provide a therapeutic treatment for breast cancer or other types of cancer that involve epithelial cells.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.
The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated nucleic acid molecule that encodes a polypeptide comprising the amino acid sequence shown in SEQ ID NO:2.

2. A nucleic acid molecule of Claim 1 which comprises the nucleotide sequence shown in SEQ ID NO:1.

3. An isolated polypeptide comprising the amino acid sequence shown in SEQ ID NO:2.

4. A method of inducing a senescent phenotype in a cell comprising introducing into the cell a nucleic acid molecule of Claim 1.

5. A recombinant expression vector comprising a nucleic acid molecule of Claim 1 operably linked to regulatory elements in the vector.

6. A recombinant expression vector of Claim 5, wherein the regulatory elements provide for expression in a eukaryotic cell.

7. A method of inducing a senescent phenotype in a eukaryotic cell, comprising introducing into the cell a vector of Claim 6.

8. A cell line transformed by an expression vector of Claim 5, said cell line being capable of expressing transgenic p23 polypeptide.

9. The cell line of Claim 8, wherein the transgenic p23 polypeptide is secreted.

10. An immunospecific reagent capable of specifically binding a polypeptide comprising the amino acid sequence of SEQ ID NO:2.

11. A reagent of Claim 10 which comprises a monoclonal antibody, or specific binding fragment thereof.

12. A reagent of Claim 10 which comprises a polyclonal antibody, or specific binding fragment thereof.
13. A method of measuring the level of p23 expression in a biological sample comprising the steps:

hybridizing RNA from the cell under stringent hybridization conditions with a nucleic acid probe corresponding to an at least 15 nucleotide region of the nucleotide sequence of SEQ ID NO:1;

determining that the level of p23 in the cell is high or low by comparison with standards comprising RNA from young and senescent cultured epithelial cells.
Fig. 1.
PLOTSTRUCTURE of: DD19.5.pep ck: 2066
TRANSLATE of: dd19.5.seq check: 4921 from: 253 to: 886

CHOU–FASMAN PREDICTION

Fig. 2.
SEQUENCE LISTING

(1) GENERAL INFORMATION:
   (i) APPLICANT: Swisshelm, Karen 
       Hosier, Suzanne
       Kubbies, Manfred
   (ii) TITLE OF INVENTION: A Novel Senescence-Related Gene
   (iii) NUMBER OF SEQUENCES: 19
   (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Christensen O'Connor Johnson & Kindness
        (B) STREET: 1420 5th Ave., Suite 2800
        (C) CITY: Seattle
        (D) STATE: WA
        (E) COUNTRY: US
        (F) ZIP: 98101-2347
   (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: MS WINDOWS 95
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: PCT
        (B) FILING DATE: 05-August-1998
   (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: 08/908,873
        (B) FILING DATE: 08-August-1997
   (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Sheiness, Diana K.
        (B) REGISTRATION NUMBER: 35,356
        (C) REFERENCE/DOCKET NUMBER: UOFW-1-12608
   (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (206) 682-8100; (206) 224-0735, direct
        (B) TELEFAX: (206) 224-0779

(2) INFORMATION FOR SEQ ID NO:1:
   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3443 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA to mRNA
   (iii) HYPOTHETICAL: NO
   (iv) ANTI-SENSE: NO
   (v) ORIGINAL SOURCE:
        (A) ORGANISM: Homo sapiens
   (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 221..853
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGACCCAGAG CTTCCCGAG GCGGCGCGAG CGAGCAGGGG TCCCCGCCTT AACTTCCTCC

GCGGCGGAGA GCCACCTTCG GAGATCCGGG TTGCCACCTT GCAAACTCTC CGCCCTTGC

ACCTGCGCACCC TCTGAGCCAG CGCGGCGGCC CGAGCGAGTC ATG GCC AAC GCG GGG

Met Ala Asn Ala Gly

CTG CAG CTG TTG GCC TTC ATT CTC GCC TTC CTG GGA TGG ATT GGC GCC
Leu Gln Leu Leu Gly Phe Ile Leu Ala Phe Leu Gly Trp Ile Gly Ala

10 15 20

ATC GTC AGC ACT GCC CTG CCC CAG TGG AGG ATT TAC TCC TAT GCC GGC
Ile Val Ser Thr Ala Leu Pro Gln Trp Arg Ile Tyr Ser Tyr Ala Gly

283 331
(2) INFORMATION FOR SEQ ID NO:2:
   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 211 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: protein
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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     1  5  10  15
Gly Trp Ile Gly Ala Ile Val Ser Thr Ala Leu Pro Gln Trp Arg Ile  
     20  25  30
Tyr Ser Tyr Ala Gly Asp Asn Ile Val Thr Ala Gln Ala Met Tyr Glu  
     35  40  45
Gly Leu Trp Met Ser Cys Val Ser Gln Ser Thr Gly Gin Ile Gln Cys  
     50  55  60
Lys Val Phe Asp Ser Leu Leu Asn Leu Ser Ser Thr Leu Gln Ala Thr  
     65  70  75  80
Arg Ala Leu Met Val Val Gly Ile Leu Leu Gly Val Ile Ala Ile Phe  
     85  90  95
Val Ala Thr Val Gly Met Lys Cys Met Lys Cys Leu Glu Asp Asp Glu  
    100 105 110
Val Gln Lys Met Arg Met Ala Val Ile Gly Gly Ala Ile Phe Leu Leu  
    115 120 125
Ala Gly Leu Ala Ile Leu Val Ala Thr Ala Trp Tyr Gly Asn Arg Ile  
    130 135 140
Val Gln Glu Phe Tyr Asp Pro Met Thr Pro Val Asn Ala Arg Tyr Glu  
    145 150 155 160
Phe Gly Gln Ala Leu Phe Thr Gly Trp Ala Ala Ala Ser Leu Cys Leu  
    165 170 175
Leu Gly Gly Ala Leu Leu Cys Cys Ser Cys Pro Arg Lys Thr Thr Ser  
    180 185 190
Tyr Pro Thr Pro Arg Pro Tyr Pro Lys Pro Ala Pro Ser Ser Gly Lys  
    195 200 205
Asp Tyr Val  
    210

(2) INFORMATION FOR SEQ ID NO:3:
   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 247 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: peptide
   (iii) HYPOTHETICAL: YES
   (iv) ANTI-SENSE: NO
   (vi) ORIGINAL SOURCE:
       (A) ORGANISM: Rattus norvegicus
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Met Ser Met Ser Leu Glu Ile Thr Gly Thr Ser Leu Ala Val Leu Gly  

SUBSTITUTE SHEET (RULE 26)
(2) INFORMATION FOR SEQ ID NO:4:
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      (A) LENGTH: 16 nucleotides
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (x) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (x) SEQUENCE DESCRIPTION: SEQ ID NO:5:

   SUBSTITuted SHEET (RULE 26)
(2) INFORMATION FOR SEQ ID NO:6:
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       (A) LENGTH: 16 nucleotides
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
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(2) INFORMATION FOR SEQ ID NO:8:
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       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
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       (B) TYPE: nucleic acid
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(2) INFORMATION FOR SEQ ID NO:10:
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       (B) TYPE: nucleic acid
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       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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(2) INFORMATION FOR SEQ ID NO:11:
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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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(2) INFORMATION FOR SEQ ID NO:16:
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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
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       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
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   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
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(2) INFORMATION FOR SEQ ID NO:18:
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      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTTTTTTTTT TTCT

(2) INFORMATION FOR SEQ ID NO:19:
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      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: oligonucleotide
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGAGGGTGT
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12Q 1/68; C12P 21/00; C12N 15/63; C07H 21/04; C07K 16/00
US CL : 435/6, 70.1, 320.1, 325; 536/23.5; 530/287.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 70.1, 320.1, 325; 536/23.5; 530/287.1

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>A</td>
<td>US 5,580,726 A (VILLEPONTEAU et al) 03 December 1996, see entire document.</td>
<td>1-13</td>
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</tbody>
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[ ] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

Date of the actual completion of the international search 14 SEPTMBER 1998
Date of mailing of the international search report 22 DEC 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer DEBRA SHOEMAKER
Telephone No. (703) 308-1096

Form PCT/ISA/210 (second sheet) (July 1992)
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>ORKIN et al. Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy. 07 December 1995, pages 1-38, see page 1, 5, 9, and 20-22.</td>
<td>4, 7</td>
</tr>
</tbody>
</table>
B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Searched inventors and keywords: senescence and epithelial and gene or marker in APS, CAPLUS, MEDLINE, SCISEARCH, CANCERLIT, LIFESCI, BIOSIS, EMBASE. Searched sequences from priority document in commercial databases.