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(54) Title: REAGENTS AND METHODS FOR IDENTIFYING AND MODULATING EXPRESSION OF GENES REGULATED BY CDK INHIBITORS

(57) Abstract: This invention provides methods and reagents for identifying compounds that inhibit the induction of genes involved in viral infection, cancer and age-related diseases, such genes being induced by cyclin-dependent kinase inhibitors.

REAGENTS AND METHODS FOR IDENTIFYING AND MODULATING EXPRESSION OF GENES REGULATED BY CDK INHIBITORS

BACKGROUND OF THE INVENTION

This application claims priority to U.S. Provisional Application Serial No.: 60/315,791, filed August 29, 2001.

This application was supported by a grant from the National Institutes of Health, Nos. R01 CA89636 and R01 AG17921. The government may have certain rights in this invention.

1. Field Of The Invention

This invention is related to cellular senescence and stress response and changes in cellular gene expression that accompany senescence and stress response. In particular, the invention is related to the identification of genes the expression of which is modulated by a class of cellular gene products termed cyclin dependent kinase (CDK) inhibitors, induced in cells at the onset of senescence and in response to different forms of stress. More specifically, the invention provides markers of cellular senescence and stress response that are genes whose expression is induced by such CDK inhibitors. The invention provides methods for identifying compounds that inhibit pathological consequences of cellular senescence and stress response by detecting inhibition of induction of these marker genes by CDK inhibitors in the presence of such compounds. Also provided are reagents that are recombinant mammalian cells containing recombinant expression constructs encoding different cellular CDK inhibitors, such as p21, p16 or p27 that are experimentally-inducible, and recombinant mammalian cells containing a recombinant expression construct that expresses a reporter gene under the transcriptional control of a promoter for a gene whose expression is induced by endogenous or exogenous, experimentally-inducible, CDK inhibitors.

2. Summary Of The Related Art

Cell cycle progression is regulated to a large extent by a set of serine/threonine kinases, known as cyclin-dependent kinases (CDKs). A special group of proteins, known as CDK inhibitors, interact with and inhibit CDKs, thus causing cell cycle arrest in a variety of physiological situations (*see* Sielecki *et al.*, 2000, *J. Med. Chem.* 43: 1-18 *and references therein*). There are two families of CDK inhibitors. The first one, known as Cip/Kip, includes $p21^{Waf1/Cip1/Sdi1}$, $p27^{Kip1}$, and $p57^{Kip2}$. The second family, Ink4, includes $p16^{Ink4A}$, $p15^{Ink4b}$, $p18^{Ink4c}$, and $p19^{Ink4d}$. Expression of specific CDK inhibitors is activated by different factors. For example, contact inhibition induces p27 and p16 expression (Dietrich *et al.*, 1997, *Oncogene* 15: 2743-2747), extracellular anti-mitogenic factors such as TGFα induce p15 expression (Reynisdottir *et al.*, 1995, *Genes Dev.* 9: 1831-1845), serum starvation induces p27 expression (Polyak *et al.*, 1994, *Genes Dev.* 8: 9-22), and UV radiation induces p16 expression (Wang *et al.*, 1996, *Cancer Res.* 56: 2510-2514). In addition, all of the above treatments, as well as different forms of DNA damage induce expression of p21, the most pleiotropic of the known CDK inhibitors (Dotto, 2000, *BBA Rev. Cancer* 1471: M43-M56).

Of special importance to the field of this invention, two of the CDK inhibitors, p21 and p16, have been intimately associated with the process of senescence in mammalian cells. At the onset of replicative senescence (Alcorta *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93: 13742-13747) and damage-induced accelerated senescence (Robles & Adami, 1998, *Oncogene* 16: 1113-1123), p21 induction results in cell growth arrest. This surge of p21 expression is transient, however, and is followed by stable activation of p16, which is believed to be responsible for the maintenance of growth arrest in senescent cells. The knockout of p21 (Brown *et al.*, 1997, *Science* 277: 831-

834) or p16 (Serrano *et al.*, 1996, *Cell* 85: 27-37) delays or prevents the onset of senescence. Furthermore, ectopic overexpression of either p21 or p16 induces growth arrest accompanied by phenotypic markers of senescence in both normal and tumor cells (Vogt *et al.*, 1998, *Cell Growth Differ.* 9: 139-146; McConnell *et al.*, 1998, *Curr. Biol.* 8: 351-354; Fang *et al.*, 1999, *Oncogene* 18: 2789-2797).

p21 has been independently identified in the art as a protein that binds and inhibits CDKs (Harper *et al.*, 1993, *Cell* 75: 805-816), as a gene upregulated by wild-type p53 (el-Deiry *et al.*, 1993, *Cancer Res.* 55: 2910-2919), and as a growth-inhibitory gene overexpressed in senescent fibroblasts (Noda *et al.*, 1994, *Exp. Cell. Res.* 211: 90-98). Because of its pivotal role in p53-regulated growth arrest, p21 is usually regarded as a tumor suppressor. Nevertheless, p21 mutations in human cancer are rare (Hall & Peters, 1996, *Adv. Cancer Res. 68*: 67-108), and p21 knockout mice develop normally and do not show an increased rate of tumorigenesis (Deng *et al.*, 1995, *Cell* 82: 675-684).

Cellular levels of p21 are increased in response to a variety of stimuli, including DNA-damaging and differentiating agents. Some of these responses are mediated through transcriptional activation of the p21 gene by p53, but p21 is also regulated by a variety of p53-independent factors (reviewed in Gartel & Tyner, 1999, *Exp. Cell Res.* 227: 171-181).

Transient induction of p21 mediates different forms of damage-induced growth arrest, including transient arrest that allows cells to repair DNA damage, as well as permanent growth arrest (also termed "accelerated senescence"), which is induced in normal fibroblasts (DiLeonardo *et al.*, 1994, *Genes Develop.* 8: 2540-2551; Robles & Adami, 1998, *Oncogene* 16: 1113-1123) and tumor cells (Chang *et al.*, 1999, *Cancer Res.* 59: 3761-3767) by DNA damage or introduction of oncogenic RAS (Serrano *et al.*, 1997, *Cell* 88: 593-602). A surge of p21 expression also coincides with the onset of

terminal growth arrest during replicative senescence of aging fibroblasts (Noda *et al.*, 1994, *ibid.*; Alcorta *et al.*, 1996, *Proc. Natl. Acad. Sci USA* 93:13742-13747; Stein *et al.*, 1999, *Mol. Cell. Biol.* 19: 2109-2117) and terminal differentiation of postmitotic cells (El-Deiry *et al.*, 1995, *ibid.*; Gartel *et al.*, 1996, *Exp. Cell Res.* 246: 280-289).

While p21 is not a transcription factor *per se*, it has indirect effects on cellular gene expression that may play a role in its cellular functions (Dotto, 2000, *BBA Rev. Cancer* 1471:M43-M56 *and references therein*). One of the consequences of CDK inhibition by p21 is dephosphorylation of Rb, which in turn inhibits E2F transcription factors that regulate many genes involved in DNA replication and cell cycle progression (Nevins, 1998, *Cell Growth Differ.* 9: 585-593). A comparison of p21-expressing cells (p21 +/+) and p21-nonexpressing cells (p21 -/-) has implicated p21 in radiation-induced inhibition of several genes involved in cell cycle progression (de Toledo *et al.*, 1998, *Cell Growth Differ.* 9: 887-896). Another effect of p21 is stimulation of the transcription cofactor histone acetyltransferase p300, that enhances many inducible transcription factors including NFκB (Perkins *et al.*, 1988, *Science* 275: 523-527). Activation of p300 may have a pleiotropic effect on gene expression (Snowden & Perkins, 1988, *Biochem. Pharmacol.* 55: 1947-1954). p21 may also affect gene expression through its interactions with many transcriptional regulators and coregulators other than CDK, such as JNK kinases, apoptosis signal-regulating kinase 1, Myc and others (Dotto, 2000, *BBA Rev. Cancer* 1471:M43-M56). These interactions may affect the expression of genes regulated by the corresponding pathways.

Another CDK inhibitor of particular relevance to the present invention is $p16^{INK4A}$; the human protein has been described by Serrano *et al.* (1993, *Nature* 366: 704-707). As mentioned above, p16 is an essential regulator of senescence in mammalian cells. It is also a *bona fide* tumor suppressor and one of the most commonly mutated genes in human cancers (Hall & Peters, 1996, *Adv. Cancer Res.* 68: 67-108).

p16 is known to directly inhibit CDK4 and CDK6, and may indirectly inhibit CDK2 as well (McConnell *et al.*, 1999, *Molec. Cell. Biol.* 19: 1981-1989).

Still another CDK inhibitor of particular relevance to the present invention is $p27^{Kip1}$. p27 was initially identified as an inhibitor of CDK2 in cells that had been growth arrested by contact inhibition, TGF-β or lovastatin (Hengst *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91: 5291-5295; Polyak *et al.*, 1994, *Cell* 78: 59-66). p27 also mediates cell growth arrest in response to differentiation, serum starvation, growth in suspension and other factors. Levels of p27 expression are frequently altered (both reduced and increased) in human cancers relative to normal tissues (*reviewed in* Philipp-Staheli *et al.*, 2001, *Exp. Cell Res.* 264: 148-161). p27 has also been proposed to cooperate with tumor suppressor PTEN in one of the pathways leading to senescence (Bringold and Serrano, 2000, *Exp. Gerontol.* 35: 317-329).

There remains a need in this art to identify genes whose expression is modulated by induction of CDK inhibitor genes such as p21, p16 or p27. There is also a need in this art to develop targets for assessing the effects of compounds on cellular senescence, carcinogenesis, viral diseases and age-related diseases.

SUMMARY OF THE INVENTION

This invention provides reagents and methods for identifying genes whose expression is modulated by induction of CDK inhibitor gene expression. The invention also provides reagents and methods for identifying compounds that inhibit the effects of CDK inhibitors such as p21, p27 and p16 on cellular gene expression, as a first step in rational drug design for preventing pathogenic consequences of cellular senescence and stress response, such as carcinogenesis, viral diseases and age-related diseases.

In a first aspect, the invention provides a mammalian cell containing an inducible CDK inhibitor gene. In preferred embodiments, the CDK inhibitor gene encodes p21,

p16 or p27. In preferred embodiments, the mammalian cell is a recombinant mammalian cell comprising a recombinant expression construct encoding an inducible p21 gene or an inducible p16 gene or an inducible p27 gene. More preferably, the construct comprises a nucleotide sequence encoding p21, most preferably human p21, under the transcriptional control of an inducible promoter. In alternative embodiments, the construct comprises a nucleotide sequence encoding the amino-terminal portion of p21 comprising the CDK binding domain, more preferably comprising amino acids 1 through 78 of the p21 amino acid sequence. In additional embodiments, the construct comprises a nucleotide sequence encoding p16, most preferably human p16, under the transcriptional control of an inducible promoter. In additional embodiments, the construct comprises a nucleotide sequence encoding p27, preferably human p27 or mouse p27, under the transcriptional control of an inducible promoter. In preferred embodiments, the inducible promoter in each such construct can be induced by contacting the cells with an inducing agent, most preferably a physiologically-neutral inducing agent, that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such promoter. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. In a particularly preferred embodiment are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof.

In another embodiment of the first aspect of the invention are provided recombinant mammalian cells comprising a recombinant expression construct in which a reporter gene is under the transcriptional control of a promoter derived from a cellular gene whose expression is modulated by a CDK inhibitor, most preferably p21, p16 or p27. In a preferred embodiment, the promoter is derived from a cellular gene whose expression induced by a CDK inhibitor such as p21, p16 or p27. In these embodiments,

the promoter is most preferably derived from a gene identified in Table II and Table V; however, those with skill in the art will recognize that a promoter from any gene whose expression is induced by CDK inhibitor gene expression can be advantageously used in such constructs. Most preferably, the promoter is derived from serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19), tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes comprising the recombinant expression constructs of the invention include firefly luciferase, Renilla luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase.

In additional preferred embodiments, the invention provides a mammalian cell comprising a first recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter for a mammalian gene whose expression is modulated by a CDK inhibitor, most preferably p21, p16 or p27, and a second recombinant expression construct encoding a mammalian CDK inhibitor gene, wherein expression of the CDK inhibitor is experimentally-induced in the mammalian cell thereby. In preferred embodiments, the CDK inhibitor gene is p21, p16 or p27. In

preferred embodiments, the recombinant expression construct encoding a mammalian CDK inhibitor gene is under the transcriptional control of an inducible heterologous promoter, wherein expression of the CDK inhibitor from the recombinant expression construct is mediated by contacting the recombinant cell with an inducing agent that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such promoter. Preferably, the construct comprises a nucleotide sequence encoding p21, most preferably human p21. In other embodiments, the construct comprises a nucleotide sequence encoding the amino-terminal portion of p21 comprising the CDK binding domain, more preferably comprising amino acids 1 through 78 of the p21 amino acid sequence. In alternative preferred embodiments, the construct comprises a nucleotide sequence encoding p16, most preferably human p16. In alternative preferred embodiments, the construct comprises a nucleotide sequence encoding p27, preferably human p27 or mouse p27. In a preferred embodiment of the second recombinant expression construct encoding a reporter gene, the promoter is derived from a cellular gene whose expression is induced by a CDK inhibitor such as p21, p16 or p27. In these embodiments, the promoter is most preferably derived from a gene identified in Table II or Table V, or a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (*for example*, a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes comprising the second recombinant expression constructs of the invention include firefly luciferase, Renilla luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase. In a particularly preferred embodiment are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably human HT1080 fibrosarcoma cell line and derivatives thereof. The product of the

reporter gene or an endogenous gene that is induced by the CDK inhibitor is preferably detected using an immunological reagent, by assaying for an activity of the gene product, or by hybridization to a complementary nucleic acid.

In a second aspect, the invention provides a screening method for identifying compounds that inhibit CDK inhibitor-induced expression of mitogenic or anti-apoptotic factors in mammalian cells. In preferred embodiments, the method comprises the steps of inducing the expression of a CDK inhibitor, most preferably p21, p16 or p27, in the cells in the presence or absence of a compound, and comparing expression of a mitogen or anti-apoptotic compound, or a plurality thereof, in the conditioned media. Inhibitors of CDK inhibitor effects are identified by having a lesser amount of the mitogen or anti-apoptotic compound, or a plurality thereof, in the conditioned media in the presence of the compound than in the absence of the compound. In the methods provided in this aspect of the invention, any CDK inhibitor-expressing cell is useful, most preferably cells expressing p21, p16 or p27, and p21, p16 or p27 expression in such cells can be achieved by inducing endogenous p21, p16 or p27, or by using cells containing an inducible expression construct encoding p21, p16 or p27 according to the invention. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. In a particularly preferred embodiment are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably human HT1080 fibrosarcoma cell line and derivatives thereof. Mitogen or anti-apoptosis compound expression is detected using an immunological reagent, by assaying for an activity of the gene product, or by hybridization to a complementary nucleic acid.

In alternative embodiments, the invention provides methods for identifying compounds that inhibit CDK inhibitor-induced expression of mitogenic or anti-apoptotic factors in mammalian cells, wherein the cells comprise a recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter of a

cellular gene encoding a mitogenic or anti-apoptotic factor that is induced by a CDK inhibitor such as p21, p16 or p27. In preferred embodiments, promoters include the promoters for connective tissue growth factor (CTGF; SEQ ID NO: 3), activin A (SEQ ID NO: 5), epithelin/granulin (SEQ ID NO: 11), galectin-3 (SEQ ID NO: 9), prosaposin (SEQ ID NO: 7), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor –C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase, β-galactosidase, alkaline phosphatase and green fluorescent protein. In these embodiments, inhibition of CDK inhibitor-mediated induction of reporter gene expression is used to identify compounds that inhibit induction of mitogens or anti-apoptotic factors in CDK inhibitor-expressing cells.

In this aspect, the invention also provides a method for inhibiting production of mitogenic or anti-apoptotic factors or compounds in a mammalian cell, the method comprising the steps of contacting the cell with a compound that inhibits production of mitogenic or anti-apoptotic factors, wherein said compound is identified by the aforesaid methods of this aspect of the invention. In preferred embodiments, the mammalian cells contacted with the inhibitory compounds in which production of mitogenic or anti-apoptotic factors is inhibited are fibroblasts, most preferably stromal fibroblasts. In preferred embodiments, the compounds are inhibitors of nuclear factor kappa-B (NFκB) activity or expression.

In a third aspect, the invention provides methods for identifying compounds that inhibit CDK inhibitor-mediated induction of cellular or viral gene expression. These

methods comprise the steps of inducing or otherwise producing expression of a CDK inhibitor gene in a mammalian cell; assaying the cell in the presence of the compound for changes in expression of cellular genes whose expression is induced by the CDK inhibitor; and identifying compounds that inhibit CDK inhibitor-mediated induction of cellular gene expression if expression of the cellular genes is changed to a lesser extent in the presence of the compound than in the absence of the compound. In preferred embodiments, the CDK inhibitor is p21, p16 or p27. In preferred embodiments, the cellular genes are induced by a CDK inhibitor, and compounds that inhibit this induction of cellular gene expression are detected by detecting expression of the genes at levels less than those detected when the CDK inhibitor is expressed in the absence of the compound. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p16 or p27. In preferred embodiments, the genes are identified in Table II. In further alternative embodiments, the method is performed using a recombinant mammalian cell comprising a reporter gene under the transcriptional control of a promoter derived from a gene whose expression is induced by a CDK inhibitor. When using constructs comprising promoters derived from genes induced by a CDK inhibitor, the reporter gene product is produced at lesser levels in the presence than the absence of the compound when the compound inhibits or otherwise interferes with CDK inhibitor-mediated gene expression modulation. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p16 or p27. In these embodiments, the promoter is most preferably derived from a gene identified in Table II and Table V. Most preferably, the promoter is derived from serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10),

granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes comprising the recombinant expression constructs of the invention include firefly luciferase, Renilla luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase. In other preferred embodiments, the cell comprises a first recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter for a mammalian gene whose expression is induced by a CDK inhibitor, and a second recombinant expression construct encoding a mammalian CDK inhibitor gene, wherein expression of the CDK inhibitor is experimentally-induced in the mammalian cell thereby. The product of the reporter gene or the endogenous gene that is induced by the CDK inhibitor is preferably detected using an immunological reagent, by assaying for an activity of the gene product, or by hybridization to a complementary nucleic acid.

In a fourth aspect, the invention provides methods for identifying compounds that inhibit pathogenic consequences of senescence in a mammalian cell, wherein such pathogenic consequences are mediated at least in part by expression of genes induced by CDK inhibitors. These methods comprise the steps of treating the mammalian cell in the presence of the compound with an agent or culturing the mammalian cell under conditions that induce CDK inhibitor gene expression ; assaying the mammalian cell for induction of genes that are induced by CDK inhibitors; and identifying the compound as

an inhibitor of senescence or pathogenic consequences of senescence if expression of genes that are induced by the CDK inhibitor are induced to a lesser extent in the presence of the compound than in the absence of the compound. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p16 or p27. In preferred embodiments, the genes are identified in Table II and Table V. In further alternative embodiments, the method is performed using a recombinant mammalian cell comprising a reporter gene under the transcriptional control of a promoter derived from a gene whose expression is modulated by a CDK inhibitor. In these embodiments, production of the product of the reporter gene at lesser levels in the presence than the absence of the compound using constructs comprising promoter derived from genes induced by the CDK inhibitor, is detected when the compound is an inhibitor of pathogenic consequences of cell senescence. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p16 or p27. The promoters are preferably derived from genes identified in Table II and Table V. The promoter most preferably is derived from serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an

SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). In other preferred embodiments, the cell comprises a first recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter for a mammalian gene whose expression is induced by a CDK inhibitor, and a second recombinant expression construct encoding a mammalian CDK inhibitor gene, wherein expression of the CDK inhibitor is experimentally-induced in the mammalian cell thereby. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p16 or p27. In a particularly preferred embodiment are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably human HT1080 fibrosarcoma cell line and derivatives thereof. The product of the reporter gene or an endogenous gene that is induced by the CDK inhibitor is preferably detected using an immunological reagent, by assaying for an activity of the gene product, or by hybridization to a complementary nucleic acid.

In a fifth aspect, the invention provides methods for inhibiting or preventing viral gene expression induction by CDK inhibitors. In preferred embodiments, the methods comprise the step of contacting a cell, preferably a virally-infected cell (either acutely or latently) or a cell at risk for viral infection with a compound identified by the inventive methods for identifying compounds that inhibit or prevent viral gene expression induction by CDK inhibitors. In preferred embodiments, effective amounts of the compounds are formulated into pharmaceutical compositions using pharmaceutically-acceptable carriers or other agents and administered to an animal, most preferably an animal suffering from a viral disease caused by CDK inhibitor-induced gene expression. In preferred embodiments, the disease is infection with cytomegalovirus (CMV), human immunodeficiency virus (HIV), and simian virus 40 (SV40).

In a sixth aspect, the invention provides antiviral compounds and methods for identifying antiviral compounds that inhibit p21-induced expression of viral genes. In

preferred embodiments, the antiviral compounds are effective against viruses including but not limited to cytomegalovirus (CMV), human immunodeficiency virus (HIV), and simian virus 40 (SV40).

In a seventh aspect, the invention provides methods for inhibiting pathogenic consequences of cellular senescence, such as carcinogenesis or age-related diseases, the method comprising the steps of contacting the cell with a compound that inhibits senescence or the pathogenic consequences of senescence as determined using the methods provided in the aforesaid aspects of the invention.

In an eighth aspect, the invention provides compounds that are identified using any of the methods of the invention as disclosed herein.

In ninth aspect, the invention provides methods for inhibiting or preventing gene expression induction by CDK inhibitors. In preferred embodiments, the methods comprise the step of contacting a cell with a compound identified by the inventive methods for identifying compounds that inhibit or prevent gene expression induction by CDK inhibitors. In preferred embodiments, effective amounts of the compounds are formulated into pharmaceutical compositions using pharmaceutically-acceptable carriers or other agents and administered to an animal, most preferably an animal suffering from a disease caused by CDK inhibitor-induced gene expression. In preferred embodiments, the disease is cancer, Alzheimer's disease, renal disease, arthritis or atherosclerosis. In preferred embodiments, the methods employ compounds that are NFκB inhibitors.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the IPTG-regulated retroviral vector

LNp21CO3 used to produce the human HT1080 fibrosarcoma cell line variant HT1080 p21-9.

Figure 2A is a graph of the time course of p21 induction after the addition of 50 μM IPTG, where p21 levels were determined by ELISA.

Figure 2B is a graph of the time course of p21 decay after removal of IPTG.

Figure 3A are photographs of gel electrophoresis patterns of RT-PCR experiments (left), northern blot analysis of cellular mRNA expression (middle) and immunoblotting assays for IPTG-induced changes in expression of the denoted genes (right); C: control untreated HT1080 p21-9 cells; I: cells treated for 3 days with 50 μM IPTG. ß2-microglobulin (ß2-M) was used as a normalization control for RT-PCR and S14 ribosomal protein gene for northern hybridization.

Figure 3B are photographs of gel electrophoresis of RT-PCR experiments (left) and immunoblotting analysis (right) showing the time course of changes in the expression of the denoted p21-inhibited genes upon IPTG addition and release.

Figure 3C are photographs of gel electrophoresis patterns of RT-PCR experiments (left) and northern hybridization analysis (right) of the time course of changes in the expression of the denoted p21-induced genes upon IPTG addition.

Figure 3D is a comparison of gene expression in untreated control HT1080 p21-9 cells (C), serum-starved quiescent cells (Q) and IPTG-treated senescent cells (I).

Figure 4 is a schematic diagram of the IPTG-regulated retroviral vector LNp16RO2 used to produce the human HT1080 fibrosarcoma cell line variant HT1080/ LNp16RO2.

Figures 5A and 5B are diagrams of changes in cell cycle distribution of HT1080 p16-5 (Figure 5A) or HT1080 p27-2 (Figure 5B) cells upon the addition of 50 μM IPTG.

Figures 6A and 6B are photographs of gel electrophoresis patterns of RT-PCR experiments for detecting IPTG-induced changes in expression of the denoted genes upon IPTG-induced expression of p16 in HT1080 p16-5 cells (Figure 6A) or p27 in HT1080 p27-2 cells (Figure 6B). -: control untreated cells; +: cells treated for 3 days with 50 μM IPTG. β-actin was used as a normalization control for RT-PCR.

Figure 7 illustrates the effects of p21 induction in HT1080 p21-9 cells on the expression of luciferase reporter genes driven by the promoters of the indicated p21-inducible genes. The assays were carried out following transient transfection, after two days (for prosaposin promoter) or three days of culture (for all the other promoters) in the presence or in the absence of 50 μM IPTG. The assays were carried out in triplicate (for prosaposin) or in quadruplicate (for all the other constructs).

Figures 8A and 8B are graphs showing IPTG dose dependence of luciferase expression in LuNK4p21 cell line after 24 hrs of IPTG treatment (Figure 8A) and the time course of luciferase expression upon the addition of 50 μM IPTG (Figure 8B).

Figures 9A through 9I illustrate the effects of p21 induction in HT1080 p21-9

cells on the expression of luciferase reporter genes driven by the NFκB-dependent promoter (Figures 9A through 9C) or by the promoters of the indicated p21-inducible genes (Figures 9D through 9I). In the experiments in Figures 9C through 9I, the promoter-reporter constructs were mixed at a molar ratio 1:2 with vectors expressing a dominant inhibitor of NFκB (IKK), C-truncated E1A mutant that inhibits p300/CBP (E1AΔCR2), or non-functional N- and C-truncated version of E1A (E1AΔN/ΔCR2). Luciferase levels were measured after 3 days in the presence or absence of IPTG, used at the indicated concentrations in Figures 9A and 9B or at 50 μM in all the other figures, and normalized either by the levels of Renilla luciferase expressed from the co-transfected pRL-CMV plasmid in the absence of IPTG or (in Fig. 9E) by the level of cellular protein. The experiments were carried out in triplicates.

Figure 10 is a bar graph of luciferase activity in LuNK4p21 cells in the presence and absence of IPTG and incubated with different amounts of NSAIDs.

Figure 11 is a photograph of gel electrophoresis patterns of RT-PCR experiments using LuNK4p21 for detecting inhibition of IPTG-induced changes in expression of the denoted genes by different amounts of sulindac; β-actin was used as a normalization control for RT-PCR.

Figures 12A through 12E illustrate the effects of p16 induction in HT1080 p16-5 cells, p21 induction in HT1080 p21-9 cells, and p27 induction in HT1080 p27-2 cells on the expression of luciferase reporter genes driven by the NFκB-dependent promoter (Figure 12A) or by the promoters of the indicated p21-inducible genes (Figures 12B through 12E), and the effects of a dominant inhibitor of NFκB (IKK) on such induction.

The presence or absence of IPTG or cotransfected IKK is indicated for each experiment. Luciferase levels were measured after 3 days in the presence or absence of IPTG and normalized by the levels of Renilla luciferase expressed from the co-transfected pRL-CMV plasmid. All the experiments were carried out in triplicates.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention provides reagents and methods for identifying genes involved in mediating CDK inhibitor-induced pathogenic consequences of senescence and stress response, and compounds capable of inhibiting pathogenic consequences of senescence and stress response in mammalian cells. Particularly provided are embodiments of such reagents and methods for identifying genes induced by CDK inhibitors p21, p27 or p16.

For the purposes of this invention, the term "CDK inhibitor" is intended to encompass members of a family of mammalian genes having the biochemical activity of cyclin-dependent kinase inhibition. Explicitly contained in this definition are the CDK inhibitors p15, p14, p18 and particularly p21, p16 or p27, the latter three of which are particularly preferred embodiments of the reagents and methods of this invention.

For the purposes of this invention, reference to "a cell" or "cells" is intended to be equivalent, and particularly encompasses *in vitro* cultures of mammalian cells grown and maintained as known in the art.

For the purposes of this invention, reference to "cellular genes" in the plural is intended to encompass a single gene as well as two or more genes. It will also be understood by those with skill in the art that effects of modulation of cellular gene expression, or reporter constructs under the transcriptional control of promoters derived from cellular genes, can be detected in a first gene and then the effect replicated by testing a second or any number of additional genes or reporter gene constructs.

Alternatively, expression of two or more genes or reporter gene constructs can be assayed simultaneously within the scope of this invention.

For the purposes of this invention, reference to "viral genes" in the plural is intended to encompass a single gene as well as two or more genes. It will also be understood by those with skill in the art that effects of modulation of viral gene expression, or reporter constructs under the transcriptional control of promoters derived from viral genes, can be detected in a first gene and then the effect replicated by testing a second or any number of additional genes or reporter gene constructs. Alternatively, expression of two or more genes or reporter gene constructs can be assayed simultaneously within the scope of this invention.

As used herein, the term "conditioned media" is intended to encompass cell culture media conditioned by growth of CDK inhibitor--expressing cells that contains mitogenic or anti-apoptotic factors. The conditioned media is produced in a preferred embodiment by culturing CDK inhibitor--expressing cells in a mammalian cell culture medium, most preferably a synthetic medium that does not contain serum additives. Any CDK inhibitor-expressing cell is useful for the production of said conditioned media, and CDK inhibitor expression in such cells can be achieved by inducing endogenous CDK inhibitors (such as by treatment with DNA damaging agents, ionizing or ultraviolet radiation, or contact inhibition) or by using cells containing an inducible CDK inhibitor expression construct according to the invention and culturing the cells in a physiologically-neutral inducing agent. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. A particularly preferred embodiment are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably human HT1080 fibrosarcoma cell line and derivatives thereof.

For the purposes of this invention, the term "senescence" will be understood to include permanent cessation of DNA replication and cell growth not reversible by growth factors, such as occurs at the end of the proliferative lifespan of normal cells or in normal or tumor cells in response to cytotoxic drugs, DNA damage or other cellular insult.

Senescence can be induced in a mammalian cell in a number of ways. The first is a natural consequence of normal cell growth, either *in vivo* or *in vitro*: there are a limited number of cell divisions, passages or generations that a normal cell can undergo before it becomes senescent. The precise number varies with cell type and species of origin (Hayflick & Moorhead, 1961, *Exp. Cell Res.* 25: 585-621). Another method for inducing senescence in any cell type is treatment with cytotoxic drugs such as most anticancer drugs, radiation, and cellular differentiating agents. *See,* Chang *et al.,* 1999, *Cancer Res.* 59: 3761-3767. Senescence also can be rapidly induced in any mammalian cell by transducing into that cell a tumor suppressor gene (such as p53, p21, p16 or Rb) and expressing the gene therein. *See,* Sugrue *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94: 9648-9653; Uhrbom *et al.*, 1997, *Oncogene* 15: 505-514; Xu *et al.*, 1997, *Oncogene* 15: 2589-2596; Vogt *et al.*, 1998, *Cell Growth Differ.* 9: 139-146

For the purposes of this invention, the term "pathological consequences of senescence" is intended to encompass diseases such as cancer, atherosclerosis, Alzheimer's disease, amyloidosis, renal disease and arthritis.

For the purposes of this invention, a "viral disease" is a disease caused by or associated with infection, replication, gene expression or production of a virus in a mammalian, most preferably a human, cell. In particular, the term is intended to encompass viruses having at least one gene the expression of which is responsive to and induced by p21. Most particularly, the term refers to DNA viruses, specifically double-

stranded DNA viruses, or viruses having a portion of their life cycle in double-stranded DNA form (including but not limited to retroviruses and lentiviruses, particularly HIV).

The reagents of the present invention include any mammalian cell, preferably a rodent or primate cell, more preferably a mouse cell and most preferably a human cell, that can induce expression of a CDK inhibitor gene, most preferably p21, p16 or p27, wherein such gene is either the endogenous gene or an exogenous gene introduced by genetic engineering. Although the Examples disclose recombinant mammalian cells comprising recombinant expression constructs encoding inducible p21, p27 and p16 genes, it will be understood that these embodiments are merely a matter of experimental design choice and convenience, and that the invention fully encompasses induction of endogenous CDK inhibitor genes such as p21, p27 and p16.

In preferred embodiments, the invention provides mammalian cells containing a recombinant expression construct encoding an inducible mammalian p21 gene. In preferred embodiments, the p21 gene is human p21 having nucleotide and amino acid sequences as set forth in U.S. Patent No: 5,424,400, incorporated by reference herein. In alternative embodiments, the p21 gene is an amino-terminal portion of the human p21 gene, preferably comprising amino acid residues 1 through 78 of the native human p21 protein (as disclosed in U.S. Patent No: 5,807,692, incorporated by reference) and more preferably comprising the CDK binding domain comprising amino acids 21-71 of the native human p21 protein (Nakanishi *et al.,* 1995, *EMBO J.* 14: 555-563). Preferred host cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof. A most preferred cell line is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p21-9, deposited on

April 6, 2000 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA 1664.

In alternative preferred embodiments, the invention provides mammalian cells containing a recombinant expression construct encoding an inducible mammalian p16 gene. In preferred embodiments, the p16 gene is human p16 having nucleotide and amino acid sequences as set forth in NCBI RefSeq NM_000077 and NP_000068. Preferred host cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof. A most preferred cell line is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p16-5, deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4020.

In alternative preferred embodiments, the invention provides mammalian cells containing a recombinant expression construct encoding an inducible mammalian p27 gene. In preferred embodiments, the p27 gene is human p27 having nucleotide and amino acid sequences as set forth in NCBI RefSeq NM_004064 and NP_004055 or mouse p16 having nucleotide and amino acid sequences as set forth in NCBI RefSeq NM_009875 and NP_034005. Preferred host cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof. A most preferred cell line is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p27-2, deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4021.

Recombinant expression constructs can be introduced into appropriate mammalian cells as understood by those with skill in the art. Preferred embodiments of said constructs are produced in transmissible vectors, more preferably viral vectors and most preferably retrovirus vectors, adenovirus vectors, adeno-associated virus vectors, and vaccinia virus vectors, as known in the art. See, generally, MOLECULAR VIROLOGY: A PRACTICAL APPROACH, (Davison & Elliott, ed.), Oxford University Press: New York, 1993.

In additionally preferred embodiments, the recombinant cells of the invention contain a construct encoding an inducible CDK inhibitor gene, wherein the gene is under the transcriptional control of an inducible promoter. In more preferred embodiments, the inducible promoter is responsive to a *trans*-acting factor whose effects can be modulated by an inducing agent. The inducing agent can be any factor that can be manipulated experimentally, including temperature and most preferably the presence or absence of an inducing agent. Preferably, the inducing agent is a chemical compound, most preferably a physiologically-neutral compound that is specific for the *trans*-acting factor. In the use of constructs comprising inducible promoters as disclosed herein, expression of CDK inhibitor from the recombinant expression construct is mediated by contacting the recombinant cell with an inducing agent that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such promoter. In preferred embodiments of this aspect of the inventive methods, the CDK inhibitor is p21, p27 or p16. A variety of inducible promoters and cognate *trans*-acting factors are known in the prior art, including heat shock promoters than can be activated by increasing the temperature of the cell culture, and more preferably promoter/factor pairs such as the *tet* promoter and its cognate *tet* repressor and fusions thereof with mammalian transcription factors (as are disclosed in U.S. Patent Nos. 5,654,168, 5,851,796, and 5,968,773), and the bacterial *lac* promoter of the lactose operon and its

cognate *lac*I repressor protein. In a preferred embodiment, the recombinant cell expresses the *lac*I repressor protein and a recombinant expression construct encoding human p21 under the control of a promoter comprising one or a multiplicity of *lac*-responsive elements, wherein expression of p21 can be induced by contacting the cells with the physiologically-neutral inducing agent, isopropylthio-β-galactoside. In this preferred embodiment, the *lac*I repressor is encoded by a recombinant expression construct identified as 3'SS (commercially available from Stratagene, LaJolla, CA). In alternative preferred embodiments, the recombinant cell expresses the *lac*I repressor protein and a recombinant expression construct encoding human p16 under the control of a promoter comprising one or a multiplicity of *lac*-responsive elements, wherein expression of p16 can be induced by contacting the cells with the physiologically-neutral inducing agent, isopropylthio-β-galactoside. In this preferred embodiment, the *lac*I repressor is encoded by the 3'SS recombinant expression construct (Stratagene). In alternative preferred embodiments, the recombinant cell expresses the *lac*I repressor protein and a recombinant expression construct encoding human p27 or mouse p27 under the control of a promoter comprising one or a multiplicity of *lac*-responsive elements, wherein expression of p27 can be induced by contacting the cells with the physiologically-neutral inducing agent, isopropylthio-β-galactoside. In this preferred embodiment, the *lac*I repressor is encoded by the 3'SS recombinant expression construct (Stratagene).

The invention also provides recombinant expression constructs wherein a reporter gene is under the transcriptional control of a promoter of a gene whose expression is modulated by a CDK inhibitor such as p21, p16 or p27. These include genes whose expression is induced by CDK inhibitors. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. In preferred embodiments, the promoters are derived from genes whose expression is induced or

otherwise increased by CDK inhibitor expression, and are identified in Table II or Table V. Most preferably, the promoter is derived from serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). These reporter genes are then used as sensitive and convenient indicators of the effects of CDK inhibitor gene expression, and enable compounds that inhibit the effects of CDK inhibitor expression in mammalian cells to be easily identified. Host cells for these constructs include any cell in which CDK inhibitor gene expression can be induced, and preferably include cells also containing recombinant expression constructs containing an inducible CDK inhibitor gene as described above. Reporter genes useful in the practice of this aspect of the invention include but are not limited to firefly luciferase, Renilla luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, and alkaline phosphatase. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof. A most preferred cell line is an HT 1080 fibrosarcoma cell line derivative identified as

HT1080/LUNK4p21, deposited on May 17, 2001 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-3381.

In preferred embodiments, cells according to the invention comprise both a first recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter for a mammalian gene whose expression is modulated by a CDK inhibitor, and a second recombinant expression construct encoding a mammalian CDK inhibitor gene, wherein CDK inhibitor expression is experimentally-inducible thereby in the mammalian cell. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. In alternative embodiments, the invention provides a mammalian cell comprising a recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter for a mammalian gene whose expression is induced by a CDK inhibitor, wherein the promoter is from the gene encoding connective tissue growth factor serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO:9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81) or a CMV early gene promoter (SEQ ID NO. 82).

In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27.

The invention also provides screening methods for identifying compounds that inhibit CDK inhibitor-induced expression of mitogenic or anti-apoptotic factors in mammalian cells. In preferred embodiments, CDK inhibitor expression is induced in a mammalian cell culture in the presence or absence of compounds to be identified as inhibitors of CDK inhibitor-induced expression of mitogenic or anti-apoptotic factors. Compounds are identified as inhibitors by inducing expression of CDK inhibitor in the cells, and comparing the extent of expression of a mitogenic or anti-apoptotic factor, or a plurality thereof, in the presence of the compound with expression in the absence of the compound, and inhibitors identified as compounds that have a reduced amount of expression of a mitogenic or anti-apoptotic factor, or a plurality thereof, in the presence of the compound. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Any CDK inhibitor-expressing cell is useful for the production of said conditioned media, and CDK inhibitor expression in such cells can be achieved by inducing endogenous CDK inhibitors (such as by treatment with DNA damaging agents and other cytotoxic compounds, and ionizing or ultraviolet radiation, or contact inhibition) or by using cells containing an inducible CDK inhibitor expression construct according to the invention and culturing the cells in a physiologically-neutral inducing agent. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably cells of the human HT1080 fibrosarcoma cell line and derivatives thereof. An exemplary cell line according to this particularly preferred embodiment of the invention is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p21-

9, deposited on April 6, 2000 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA 1664. An exemplary cell population is a human HT1080 fibrosarcoma derivative identified as HT1080/LNp16RO2, deposited on October 10, 2000 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-2580. Another exemplary cell line according to this particularly preferred embodiment of the invention is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p16-5, deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4020. Another exemplary cell line according to this particularly preferred embodiment of the invention is an HT 1080 fibrosarcoma cell line derivative identified as HT1080 p27-2, deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4021.

In alternative embodiments, the invention provides methods for identifying compounds that inhibit CDK inhibitor-induced expression of mitogenic or anti-apoptotic factors in mammalian cells, wherein the cells comprise a recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter of a cellular gene that is induced by a CDK inhibitor. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Preferred promoters include the promoters for connective tissue growth factor (CTGF; SEQ ID NO: 3), activin A (SEQ ID NO: 5), epithelin/granulin (SEQ ID NO: 11), galectin-3 (SEQ ID NO: 9), prosaposin (SEQ ID NO: 7), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID NO: 18), vascular endothelial growth factor –C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID

NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase, β-galactosidase, alkaline phosphatase and green fluorescent protein, all of which are commercially available. In these embodiments, CDK inhibitor expression is induced in the cells, and the extent of expression of the reporter gene is compared in the presence of the compound with expression in the absence of the compound. Inhibitors are identified as compounds that provide a reduced amount of expression of the reporter gene in the presence of the compound than in the absence of the compound. Any CDK inhibitor-expressing cell is useful in this aspect of the invention, and CDK inhibitor expression in such cells can be achieved by inducing the endogenous inhibitor gene (for example, by treatment with DNA damaging agents or other cytotoxic compounds, ionizing or ultraviolet radiation, or contact inhibition) or by using cells containing an inducible CDK inhibitor expression construct according to the invention and culturing the cells in a physiologically-neutral inducing agent. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Preferred cells include mammalian cells, preferably rodent or primate cells, and more preferably mouse or human cells. A particularly preferred embodiment is fibrosarcoma cells, more preferably human fibrosarcoma cells and most preferably human HT1080 fibrosarcoma cell line and derivatives thereof. A most preferred cell line is an HT1080 fibrosarcoma cell line derivative identified as HT1080/LUNK4p21, deposited on May 17, 2001 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-3381.

The invention provides methods for identifying compounds that inhibit pathogenic consequences of cell senescence, whereby the effects of the compound are assayed by determining whether the compounds inhibit induction of genes whose expression is induced by a CDK inhibitor. In the practice of the methods of the

invention, cultured mammalian cells in which a CDK inhibitor can be induced are treated to induce the inhibitor gene, for example, by ionizing or ultraviolet radiation, or contact inhibition treatment or treatment with cytotoxic drugs, or transduced with a transmissible vector encoding a CDK inhibitor. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. More preferably, HT1080 p21-9 cells are used in which p21 can be induced by contacting the cells with IPTG (deposited on April 6, 2000 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA 1664), or HT1080 p16-5 cells (deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4020) are used in which p16 can be induced with IPTG, or HT1080 p27-2 cells (deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-4021) are used in which p27 can be induced with IPTG. Typically, cells are grown in appropriate culture media (*e.g.*, DMEM supplemented with 10% fetal calf serum (FCS) for HT1080 derivatives). In HT1080 p21-9, HT1080 p16-5 or HT1080 p27-2 cells, CDK inhibitor gene expression is induced by adding IPTG to the culture media at a concentration of about 50μM. Typically, the CDK inhibitor is induced in these cells in the presence or absence of the compound to be tested according to the methods of the invention. mRNA is then isolated from cells in which the CDK inhibitor is induced, and expression of genes that are regulated by CDK inhibitors is analyzed. Expression is compared in cells in which the CDK inhibitor is induced in the presence of the compound with expression induced in the absence of the compound, and the differences used to identify compounds that affect cellular gene expression according to the methods set forth herein. In certain embodiments, cellular gene expression is analyzed using microarrays of oligonucleotides or cellular cDNAs such as are commercially available (for example, from Genome Systems, Inc., St. Louis, MO). In alternative embodiments, genes known to be induced by CDK inhibitors are

assayed. Gene expression can be assayed either by analyzing cellular mRNA or protein for one or a plurality of CDK inhibitor-modulated genes. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. Most preferably, the genes used in these assays are genes identified in Table II and Table V.

In alternative embodiments, such compounds are identified independently of CDK inhibitor-directed experimental manipulation. In such assays, cells are treated to induce senescence in any of the ways disclosed above, including but not limited to treatment with cytotoxic drugs, radiation or cellular differentiating agents, or introduction of a tumor suppressor gene. Expression of genes that are induced by CDK inhibitors is analyzed in the presence or absence of the test compound. Most preferably, the genes used in these assays are genes identified in Table II, using the types of mRNA and protein assays discussed above for gene expression analysis.

In alternative embodiments, the cells in which a CDK inhibitor is induced further comprise a recombinant expression construct encoding a reporter gene under the transcriptional control of a promoter of a cellular gene that is induced by a CDK inhibitor. In preferred embodiments of this aspect of the invention, the CDK inhibitor is p21, p16 or p27. In preferred embodiments, the cellular gene is a gene that is induced by the CDK inhibitor, and the promoter is derived from a gene identified in Table II and Table V. Examples of known promoters for such genes include serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (SEQ ID

NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one (SEQ ID No. 79) or a multiplicity of tandemly-repeated NFκB recognition sequences (a promoter comprising a 5-fold tandem repeat of NFκB recognition sequence is set forth as SEQ ID NO. 78), an SV40 early promoter (SEQ ID NO. 81), or a CMV early gene promoter (SEQ ID NO. 82). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase, β-galactosidase, alkaline phosphatase and green fluorescent protein, all of which are commercially available.

The invention provides methods for identifying compounds that inhibit viral infection, viral gene expression or the pathogenic consequences thereof, whereby the effects of the compound are assayed by determining whether the compounds inhibit induction of viral genes whose expression is induced by a CDK inhibitor.

The invention provides methods for identifying compounds that can inhibit induction of viral genes associated with the pathogenic consequences of viral infection. Such compounds would be expected to exhibit the capacity to prevent, retard or reverse viral diseases by their effects on CDK inhibitor-mediated induction of gene expression.

In one embodiment this invention provides methods for inhibiting gene expression induced by CDK inhibitors such as p21, p16 or p27. In preferred embodiments, such inhibiting is achieved by contacting cells with an effective amount of a compound that inhibits gene expression on promoters from DNA viruses, most preferably double-stranded DNA viruses that infect humans. In additional preferred embodiments, the compound inhibits lentivirus gene expression, most preferably HIV gene expression and HIV infectivity.

The invention thus provides methods for inhibiting viral infection of cells, most preferably human cells, by inhibiting CDK inhibitor induction of viral gene expression. Diseases that can be treated or prevented using the compounds identified by the methods

of the invention include but are not limited to the infections by HIV, cytomegalovirus, herpes simplex virus types 1 and 2, adenovirus, *varicella-zoster* virus, Epstein-Barr virus, human papillomavirus, hepatitis virus, human polyomavirus and diseases caused by or incident to infection with any of these viruses. In particular, the invention provides methods for inhibiting viral gene expression in latently-infected cells by said viruses especially HIV and herpes zoster virus.

The invention also provides methods for identifying genes associated with cellular senescence and pathogenic consequences of senescence or that mediate the effects of CDK inhibitor-induced cellular senescence. Induction of CDK inhibitors turns out to be an integral part of cell growth arrest associated with senescence, terminal differentiation and response to cellular damage. As described in the Examples below, cDNA array hybridization showed that these effects were due to p21-induced changes in gene expression. p21 selectively induced genes that have been associated with cellular senescence and aging or have been implicated in age-related diseases, including atherosclerosis, Alzheimer's disease, amyloidosis, renal disease and arthritis. These findings suggested that cumulative effects of p21 induction in an organism may contribute to the pathogenesis of cancer and age-related diseases. In addition, a number of p21-activated genes encode secreted proteins with potential paracrine effects on cell growth and apoptosis. In agreement with this observation, conditioned media from p21-induced cells showed mitogenic and anti-apoptotic activity.

In addition, the results presented in the Examples below demonstrated that induced expression of p16 or p27 mimicked the effects of p21 gene expression, and that the same genes whose expression was modulated by p21 gene expression were also modulated by p16 or p27 gene expression (*see* Figure 6). Thus, the methods of the invention have been extended to include cells in which p16 or p27 gene expression is

induced, either by induction of the endogenous p16 or p27 gene or in recombinant cells comprising an inducible expression construct encoding p16 or p27.

The observed effects of CDK inhibitor induction, particularly p21, p16 and p27 induction on gene expression show numerous correlations with the changes that have been associated with cell senescence and organism aging. Some of these correlations come from the analysis of genes that are inhibited by CDK inhibitors. Thus, senescent fibroblasts were reported to express lower levels of Rb (Stein *et al.*, 1999, *Mol. Cell. Biol.* 19: 2109-2117), as was observed upon p21 induction. It is also interesting that three genes that are inhibited by CDK inhibitors, CHL1, CDC21 and RAD54 encode members of the helicase family. A deficiency in another protein of the helicase group has been identified as the cause of Werner syndrome, a clinical condition associated with premature aging and, at the cellular level, accelerated senescence of cells in culture (Gray *et al.*, 1997, *Nature Genet.* 17: 100-103).

The strongest correlations with the senescent phenotype, however, come from identification of CDK inhibitor-induced genes, many of which are known to increase their levels during replicative senescence or organism aging. Overexpression of extracellular matrix (ECM) proteins is a known hallmark of replicative senescence, and two CDK inhibitor-induced genes in this group, fibronectin 1 and plasminogen activator inhibitor 1 (PAI-1), have been frequently associated with cellular senescence (reviewed in Crisofalo & Pignolo, 1996, *Exp. Gerontol.* 31: 111-123). Other CDK inhibitor-induced genes that were also reported to be overexpressed in senescent fibroblasts include tissue-type plasminogen activator (t-PA; West *et al.*, 1996, *Exp. Gerontol.* 31: 175-193), cathepsin B (diPaolo *et al.*, 1992, *Exp. Cell Res.* 201: 500-505), integrin β3 (Hashimoto *et al.*, 1997, *Biochem. Biophys. Res. Commun.* 240: 88-92) and APP (Adler *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* 88: 16-20). Expression of several CDK inhibitor-induced proteins was shown to correlate with organism aging, including t-PA

and PAI-1 (Hashimoto *et al.*, 1987, *Thromb. Res.* 46: 625-633), cathepsin B (Bernstein *et al.*, 1990, *Brain Res. Bull.* 24: 43-549) activin A (Loria *et al.*, 1998, *Eur. J. Endocrinol.* 139: 487-492), prosaposin (Mathur *et al.*, 1994, *Biochem. Mol. Biol. Int.* 34: 1063-1071), APP (Ogomori *et al.*, 1988, *J. Gerontol.* 43: B157-B162), SAA (Rosenthal & Franklin, 1975, *J. Clin. Invest.* 55: 746-753) and t-TGase (Singhal *et al.*, 1997, *J. Investig. Med.* 45: 567-575).

The most commonly used marker of cell senescence is the SA-β-gal activity (Dimri *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92: 9363-9367). This gene is strongly elevated in IPTG-treated HT1080 p21-9 cells (Chang *et al.*, 1999, *Oncogene* 18: 4808-4818). SA-β-gal was suggested to represent increased activity and altered localization of the lysosomal β-galactosidase (Dimri *et al.*, 1995, *ibid.*), and other studies have described elevated lysosome activities in senescent cells (Cristofalo & Kabakijan, 1975, *Mech. Aging Dev.* 4: 19-28). Five lysosomal enzymes appear in Table II, including N-acetylgalactosamine-6- sulfate sulfatase (GALNS), cathepsin B, acid α-glucosidase, acid lipase A and lysosomal pepstatin-insensitive protease. p21 also upregulated genes for mitochondrial proteins SOD2, metazin and 2, 4-dienoyl-CoA reductase, which correlates with reports of different mitochondrial genes overexpresssed in senescent cells (Doggett *et al.*, 1992, *Mech. Aging Dev.* 65: 239-255; Kodama *et al.*, 1995, *Exp. Cell Res.* 219: 82-86; Kumazaki *et al.*, 1998, *Mech. Aging Dev.* 101: 91-99).

Strikingly, products of many genes that we found to be induced by p21, p16 or p27 have been linked to age-related diseases, including Alzheimer's disease, amyloidosis, atherosclerosis and arthritis. Thus, APP gives rise to β-amyloid peptide, the main component of Alzheimer's amyloid plaques. Complement C3 (Veerhuis *et al.*, 1995, *Virchows Arch.* 426: 603-610) and AMP deaminase (Sims *et al.*, 1998, *Neurobiol. Aging* 19: 385-391) were also suggested to play a role in Alzheimer's disease. It is especially interesting that t-TGase, which is most rapidly induced by p21 and which has

been described as a pleiotropic mediator of cell differentiation, carcinogenesis, apoptosis and aging (Park *et al.*, 1999, *J. Gerontol. A Biol. Sci.* 54: B78-B83), is involved in the formation of plaques associated with both Alzheimer's disease and amyloidosis (Dudek & Johnson, 1994, *Brain Res.* 651: 129-133). The latter disease is due to the deposition of another CDK inhibitor-induced gene product, SAA, which has also been implicated in atherosclerosis, osteoarthritis and rheumatoid arthritis (Jensen & Whitehead, 1998, *Biochem. J.* 334: 489-503). Two other CDK inhibitor upregulated secreted proteins, CTGF and galectin 3 are involved in atherosclerosis (Oemar *et al.*, 1997, *Circulation* 95: 831-839; Nachtigal *et al.*, 1998, *Am. J. Pathol.* 152: 1199-1208). In addition, cathepsin B (Howie *et al.*, 1985, *J. Pathol.* 145: 307-314), PAI-1 (Cerinic *et al.*, 1998, *Life Sci.* 63: 441-453), fibronectin (Chevalier, 1993, *Semin. Arthritis Rheum.* 22: 307-318), GALNS and Mac-2 binding protein (Seki *et al.*, 1998, *Arthritis Rheum.* 41: 1356-1364) have been associated with osteoarthritis and/or rheumatoid arthritis. Furthermore, senescence-related changes in ECM proteins, such as increased PAI-1 expression, were proposed to result in age-specific deterioration in the structure of skin and other tissues (Campisi, 1998, *J. Investig. Dermatol. Symp. Proc.* 3: 1-5). Increased fibronectin production by aging cells was also suggested to increase the density of the fibronectin network in ECM, which may contribute to slower wound healing in aged individuals (Albini *et al.*, 1988, *Coll. Relat. Res.* 8: 23-37).

p21 and p21-inducible genes have also been implicated in diabetic nephropathy and chronic renal failure. Kuan *et al.* (1998, *J. Am. Soc. Nephrol.* 9: 986-993) found that p21 is induced under conditions of glucose-induced mesangial cell hypertrophy, an *in vitro* model of diabetic nephropathy. Megyesi *et al.* (1996, *Am. J. Physiol.* 271: F1211-1216) demonstrated that p21 is induced *in vivo* in several animal models of acute renal failure, and this p21 induction is independent of p53. The functional role of p21 in these pathogenic processes has been demonstrated by Al-Douahji *et al.* (1999, *Kidney Int.* 56:

1691-1699), who found that p21(-/-) mice do not develop glomerular hypertrophy under the conditions of experimental diabetes, and by Megyesi *et al.* (1999, *Proc Natl Acad Sci U S A.* 96:10830-10835), who showed that p21(-/-) mice do not develop chronic renal failure after partial renal ablation. Remarkably, Murphy *et al.* (1999, *J. Biol. Chem.* 274: 5830-5834), working with the same *in vitro* model used by Kuan *et al.* (1998, *J. Am. Soc. Nephrol.* 9: 986-993), reported that mesangial cell hypertrophy involves upregulation of several genes that are shown herein to be inducible by p21. These include CTGF, fibronectin and plasminogen activator inhibitor 1. The latter study also showed that CTGF plays a functional role in mesangial matrix accumulation in this model system (Murphy *et al.*, 1999, *J. Biol. Chem.* 274: 5830-5834). These results implicate p21 and p21-mediated induction of gene expression in the pathogenesis of renal failure.

Of special interest, p21 induced expression of $p66^{shc}$, a gene recently found to potentiate oxidative damage, with p66(-/-) mice showing increased stress resistance and significantly extended lifespan (Migliaccio *et al.*, 1999, *Nature* 402: 309-313). These observations suggest that the effects of p21 on gene expression may contribute to the pathogenesis of multiple diseases and overall restriction of the mammalian lifespan.

A major new class of anticancer drugs undergoing clinical trials is angiogenesis inhibitors. These agents target not the tumor cells, but rather the growth of stromal capillaries, stimulated by tumor-secreted angiogenic factors (*see* Kerbel, 2000, *Carcinogenesis* 21:505-515, *for a recent review*). The vasculature, however, is not the only stromal element required for tumor growth. It has been shown in multiple studies that stromal fibroblasts also support the growth of tumor cells *in vitro* and *in vivo*, and that normal and immortalized fibroblasts secrete paracrine factors that promote tumorigenicity and inhibit death of carcinoma cells (Gregoire and Lieubeau, 1995, *Cancer Metastasis Rev.* 14: 339-350; Camps *et al.*, 1990, *Proc. Natl. Acad. Sci. U. S. A.*

87: 75-79; Noel *et al.*, 1998, *Int. J. Cancer 76*: 267-273; Olumi *et al.*, 1998, *Cancer Res.* 58: 4525-4530). Such factors have been identified in fibroblast-conditioned media (Chung, 1991, *Cancer Metastasis Rev* 10: 263-74) and in coculture studies. In particular, Olumi *et al.* (1998, *Cancer Res.* 58: 4525-4530) showed that coculture of prostate carcinoma cells with normal prostate fibroblasts strongly decreases carcinoma cell death and promotes xenograft tumor formation. The paracrine effects of fibroblasts also have a tumor-promoting activity in carcinogenesis, as has been demonstrated for initiated prostate epithelial cells (Olumi *et al.*, 1999, *Cancer Res.* 59: 5002-5011). Despite these results, this paracrine carcinogenic and tumor-stimulating activity of tumor-associated fibroblasts has not yet been exploited as a target for pharmacological intervention. The present invention provides methods for detecting and identifying compounds capable of inhibiting mitogen production from such stromal fibroblasts, thus providing a way to inhibit tumor cell growth.

This paracrine tumor-promoting activity was recently shown to be selectively increased during replicative senescence of normal human fibroblasts (Krtolica *et al.*, 2000, Proc. Amer. Assoc. Can. Res. 41, Abs. 448), a process that involves induction of p21 and pl6. The tumor-promoting effect of stromal tissue was also shown in a mouse mammary carcinogenesis model to be induced by ionizing radiation (Barcellos-Hoff and Ravani, 2000, *Cancer Res.* 60: 1254-60), a treatment that produces high p21 levels in stromal fibroblasts (Meyer *et al.*, 1999, *Oncogene* 18: 5795-5805). These results indicate that the paracrine anti-apoptotic and mitogenic activities disclosed herein in conditioned media of p21-overexpressing cells most likely represent the same biological phenomenon.

The results disclosed herein indicate that CDK inhibitor induction affects cellular gene expression in a way that may increase the probability of the development of cancer or age-related diseases. A surge of CDK inhibitor expression occurs not only in normal

replicative senescence but also in response to cellular damage; in both cases, the undesirable effects of CDK inhibitor induction would be expected to accumulate in an age-dependent manner.

The results disclosed herein indicate that CDK inhibitor induction also increase the promoter function of different viral genes, thus indicating that transcriptional effects of CDK inhibitors also promote different types of viral infection and pathological consequences thereof.

Thus, the invention provides methods for identifying compounds that can inhibit induction of genes associated with the pathogenic consequences of cellular senescence, particularly genes that are induced during senescence, and particularly genes that are induced by CDK inhibitor expression. Such compounds would be expected to exhibit the capacity to prevent, retard or reverse age-related diseases by their effects on CDK inhibitor-mediated induction of gene expression. Such compounds would also be expected to prevent, treat, retard or cure viral diseases.

In one embodiment this invention provides methods for inhibiting gene expression induced by CDK inhibitors such as p21, p16 or p27. In preferred embodiments, such inhibiting is achieved by contacting cells with an effective amount of a compound that inhibits activity, expression or nuclear translocation of nuclear factor kappa-B (NFκB). It will be understood by those with skill in the art that NFκB activity can be inhibited in cells in at least three ways: first, down-regulating or inhibiting transcription, processing and/or translation of either of the genes making up the NFκB heterodimer; second, inhibiting translocation of NFκB from the cytoplasm to the nucleus, which can depend on inhibiting inactivation of IκB expression and/or activity in cells; and third, by inhibiting the activity of NFκB itself. This invention encompasses methods for inhibiting NFκB activity, and thereby inhibiting induction of genes by CDK inhibitors, in any and all of these ways. Examples of NFκB inhibitors known in the art

include N-heterocycle carboximide derivatives (as disclosed, *for example*, in International Application Publication NO: WO01/02359); anilide compounds (as disclosed, *for example*, in International Application Publication NO: WO00/15603); 4-pyrimidinoaminoindane derivatives (as disclosed, *for example*, in International Application Publication NO: WO00/05234); 4H-1-benzopyran-4-one derivatives (as disclosed, *for example*, in Japanese Application NO: JP11193231); xanthine derivatives (as disclosed, *for example*, in Japanese Application NO: JP9227561); carboxyalkenylkbenzoquinone and carboxyalkenylnaphthol derivatives (as disclosed, *for example*, in Japanese Application NO: JP7291860); disulfides and derivatives thereof (as disclosed, *for example*, in International Application Publication NO: WO99/40907); protease inhibitors (as disclosed, *for example*, in European Application Publication NO: EP652290); flurbiprofen, thalidomide, dexamethasone, pyrrolidine dithiocarbamate, dimethylfumarate, mesalizine, pimobendan, sulfasalazine, methyl chlorogenate, chloromethylketone, alpha-tocopherol succinate, tepoxaline, and certain non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, sodium salicylate and sulindac

The following Examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

EXAMPLE 1
Production of a Mammalian Cell comprising an Inducible p21 Gene

A recombinant derivative of human fibrosarcoma cell line HT1080 p21-9, was produced essentially according to Chang *et al.* (1999, *Oncogene* 18: 4808-4818, incorporated by reference herein). This cell line contained a p21 coding sequence under the transcriptional control of a promoter regulated by isopropyl-β-thiogalactoside (IPTG). Expression of p21 can be induced by culturing these cells in the presence of a sufficient amount of IPTG, thereby permitting the sequellae of p21 expression to be

studied in the absence of any additional effects that induction of the endogenous p21 gene might provoke. This cell line has been deposited on April 6, 2000 in the American Type Culture Collection (A.T.C.C.), Manassas, VA and given Accession Number PTA 1664.

Briefly, a subline of HT1080 expressing a murine ecotropic retrovirus receptor and a modified bacterial *lac*I repressor encoded by the plasmid 3'SS (Stratagene) (described in Chang & Roninson, 1996, *Gene* 33: 703-709, incorporated by reference) was infected with retroviral particles containing recombinant retrovirus LNp21CO3, the structure of which is shown in Figure 1. This retroviral vector contains the bacterial neomycin resistance gene (*neo*) under the transcriptional control of the retroviral long terminal repeat promoter. p21-encoding sequences are cloned in the opposite orientation to the transcriptional direction of the *neo* gene, and under the control of a modified human cytomegalovirus promoter. Specifically, the CMV promoter contains a three-fold repeat of bacterial *lac* operator sequences that make expression from the promoter sensitive to the *lac*I repressor expressed in the cell. LNp21CO3 was constructed by cloning a 492bp fragment of DNA comprising the p21 coding sequence into the *Not*I and *Bgl*II sites of the parent vector, LNXCO3 (disclosed in Chang & Roninson, *ibid.*).

After infection, cells infected with the LNp21CO3X vector were selected by culturing the cells in the presence of 400μg/mL G418 (obtained from BRL-GIBCO, Gaithersburg, MD). Clonal line HT1080 p21-9 was derived from LNp21CO3 transduced, G418-resistant cell lines by end-point dilution until a clonal cell line was obtained.

EXAMPLE 2
Cell Growth Assays

HT1080 p21-9 cells produced as described in Example 1 were used in cell

growth assays to determine what changes in cell growth occurred when p21 was expressed in the cell.

p21 expression from the LNp21CO3 vector in HT1080 p21-9 cells was induced by culturing the cells in DMEM medium containing 10% fetal calf serum (Hyclone, Logan, UT) and IPTG. Results of these assays are shown in Figures 2A and 2B. Figure 2A shows the time course of p21 protein production in cells cultured in the presence of 50μM IPTG. p21 gene expression increased between 6 and 12 hours after introduction of IPTG into the growth media, which expression peaked at about 24 hours post-induction. Upon removing the cells from IPTG-containing media, p21 expression fell about as rapidly as it had risen, returning to pre-induction levels at about 24 hours after IPTG was removed (Figure 2B).

Cell growth in the presence of IPTG was assayed in three ways: measuring ^{3}H-thymidine incorporation (termed the "labeling index"); observing the number of mitotic cells in the culture by microscopy (termed the "mitotic index") and determining the distribution of the culture cells in different portions of the cell cycle (termed the "cell cycle distribution").

^{3}H-thymidine incorporation assays were performed substantially as described by Dimri *et al.* (1995, *Proc. Natl. Acad. Sci. USA* 92: 9363-9367). Cells were cultured in the presence of ^{3}H-thymidine for 3h, and then analyzed by autoradiography. DNA replication was determined by autoradiography ceased entirely by 9 hours after addition of IPTG to the culture media. The mitotic index was determined by observing cells microscopically and calculating the number of cells in mitosis after staining with 5μg/mL 4,6-diamino-2-phenylindole (DAPI), and images were collected using a Leica DMIRB fluorescence microscope and Vaytek (Fairfield, Iowa) imaging system. Microscopically-detectable mitotic cells disappeared from these cultures by 14 hrs of IPTG treatment.

Cell cycle distribution was determined using FACS analysis of DNA content after staining with propidium iodide as described by Jordan *et al.* (1996, *Cancer Res.* 56: 816-825) using Becton Dickinson FACSort. Cell cycle distribution stabilized after 24 hrs of IPTG treatment. By this time, 42-43% of IPTG-treated cells were arrested in G1 and G2, respectively, and about 15% of the cells were arrested with S-phase DNA content. IPTG-treated HT1080 p21-9 cells also developed morphological senescence markers (enlarged and flattened morphology and increased granularity), as well as SA-β-gal activity (Chang *et al.*, 1999, *ibid.*). These results indicated that induced expression of p21 produces both cell cycle arrest and a variety of other changes that are characteristic of cell senescence.

EXAMPLE 3
Analysis of Gene Expression Modulated by p21 Gene Expression

The results disclosed in Example 2 suggested that the morphological and cell cycle consequences of p21 induction could reflect multiple changes in gene expression. The effects of p21 induction on cellular gene expression were examined as follows.

Poly(A)$^+$ RNA was isolated from untreated HT1080 p21-9 cells and from cells that were treated for 3 days with 50 μm IPTG. cDNA was prepared from the poly(A)$^+$ RNA and used as probes for differential hybridization with the Human UniGEM V cDNA microarray (as performed by Genome Systems, Inc., St. Louis, MO), which contains over 4,000 sequence-verified known human genes and 3,000 ESTs. More than 2,500 genes and ESTs showed measurable hybridization signals with probes from both untreated and IPTG-treated HT1080 p21-9 cells. Genes that were downregulated with balanced differential expression $\geq$2.5 or upregulated with balanced differential expression $\geq$2.0 are listed in Tables I and II, respectively.

Expression of 69 of these genes was individually tested by RT-PCR or northern

hybridization. RT-PCR analysis was carried out essentially as described by Noonan *et al.* (1990, *Proc. Natl. Acad. Sci. USA* 87: 7160-7164). Probes for northern hybridization were derived from inserts of the cDNA clones present in the microarray; these cDNAs were obtained from Genome Systems, Inc. In addition, changes in the expression of several p21-regulated gene products were analyzed by immunoblotting. The following primary antibodies were used for immunoblotting: mouse monoclonal antibodies against Cdc2 (Santa Cruz), cyclin A (NeoMarkers), Plk 1 (Zymed) and Rb (PharMingen); rabbit polyclonal antibodies against MAD2 (BadCo), p107 (Santa Cruz), CTGF (Fisp-12; a gift of Dr. L. Lau), Prc 1 (a gift of Drs. W. Jiang and T. Hunter), and topoisomerase IIα (Ab0284; a gift of Dr. W.T. Beck), and sheep polyclonal antibody against SOD2 (Calbiochem). Horse radish peroxidase (HRP)-conjugated secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG (Santa Cruz) and rabbit anti-sheep IgG (KPL). Protein concentrations in all samples were equalized after measurement with BioRad protein assay kit. Immunoblotting was carried out by standard procedures, and the signal was detected by chemiluminescence using LumiGlo (KPL).

These results are shown in Figures 3A through 3C. The changes in gene expression predicted by the microarray assays described above were confirmed for 38/39 downregulated and 27/30 upregulated genes. The observed signal differences in northern hybridization or RT-PCR for most of the tested genes (Figure 3A through 3C) appeared to be higher than the values of balanced differential expression determined from the cDNA array (Tables I and II), suggesting that cDNA array hybridization tends to underestimate the magnitude of p21 effects on gene expression. Changes in the expression of 6 downregulated and 4 upregulated genes were also tested at the protein level by immunoblotting (Figure 3B) or zymography (not shown) and were confirmed in all cases tested.

It was recognized that p21-mediated changes in gene expression were comprised

of near-term effects and longer-term effects that followed p21-induced cell growth arrest. For this purpose, the time course of changes in the RNA levels of a subset of p21-inhibited (Fig. 3B) and p21-induced genes (Fig. 3C) after the addition and removal of IPTG was determined. Immunoblotting was used to analyze the time course of p21-induced changes in Rb phosphorylation (as indicated by electrophoretic mobility) and in the cellular levels of Rb and several proteins that were inhibited by p21 according to the cDNA array; these results are shown in Figure 3B. Rb was found to become dephosphorylated as early as 6 hrs after the addition of IPTG. Furthermore, Rb protein levels decreased sharply between 12-24 hrs (shown in Figure 3B), but no significant changes were detected in RB mRNA levels (data not shown). A similar decrease was observed for a Rb-related protein p107 (shown in Fig. 3A).

1. Gene expression inhibited by p21

All the tested p21-inhibited genes showed a rapid response to p21 induction and release. Five of these genes (topoisomerase IIα, ORC1, PLK1, PRC1 and XRCC9) showed significant inhibition at both RNA and protein levels between 4 and 8 hrs after the addition of IPTG (Fig. 3B). This pattern has been termed an "immediate response," which parallels the kinetics of cell growth arrest and Rb dephosphorylation. Other p21-inhibited genes (such as CDC2 or DHFR) showed an "early response" pattern that lags slightly behind the cessation of DNA replication and mitosis, with a major decrease in mRNA levels detectable only 12 hrs after the addition of IPTG. All p21-inhibited genes, however, resumed their expression 12-16 hrs after the removal of IPTG, when the cells were still growth-arrested and before the resumption of DNA replication and mitosis (Fig. 3B). This analysis indicated that changes in the expression of p21-inhibited genes were near-term effects of p21 induction and release and were not a consequence of cell growth arrest and recovery.

In summary, 69 genes and 3 ESTs were identified by the cDNA microarray as downregulated in p21-induced cells, with balanced differential expression of 2.5-12.6 (Table IA); five additional genes that are associated with cell cycle progression and have been identified by our separate assays as downregulated in IPTG-treated cells are listed in Table IB. A strikingly high fraction of downregulated genes identified by the cDNA array (43 of 69) were associated with mitosis, DNA replication, segregation and repair and chromatin assembly, indicating a highly selective nature of p21-mediated inhibition of gene expression.

The largest group of p21-downregulated genes are that have been implicated in the signaling, execution and control of mitosis. Many p21-inhibited genes are involved in DNA replication and segregation, chromatin assembly and DNA repair. Some of these genes encode enzymes involved in nucleotide biosynthesis, other proteins are involved in DNA replication. Several p21-inhibited genes are associated with DNA repair. These results suggest opportunities for discovering components of the cellular program of p21-induced growth arrest that would be targets for therapeutic intervention.

2. Gene expression induced by p21

In addition to genes repressed by p21 expression, the assays described above detected genes induced by p21. The pattern of gene expression of p21-induced genes is shown in Figure 3C. In contrast to p21-inhibited genes, p21-upregulated genes increased their expression only 48 hrs after the addition of IPTG, *i.e.* after the onset of growth arrest in all cells. Only one tested gene, tissue transglutaminase (t-TGase), showed a detectable increase 12 hrs after the addition of IPTG, but its expression reached a maximum only by 48 hrs (as shown in Fig. 3C). Furthermore, elevated expression of all the tested genes (except for t-TGase) persisted for at least three days after release from IPTG, well after resumption of the cell cycle (not shown). This "late

response" kinetics indicated that p21 induction of such genes was a delayed effect relative to p21-mediated growth arrest.

48 known genes and 6 ESTs or genes with unknown functions were identified as upregulated in p21-induced cells, with balanced differential expression of 2.0-7.8 (Table II). A very high fraction (20/48) of identifiable genes in this group encode extracellular matrix (ECM) components (*e.g.* fibronectin 1, laminin α2, Mac-2 binding protein), other secreted proteins (*e.g.* activin A, connective tissue growth factor, serum amyloid A), or ECM receptors (such as integrin β3). Several of these secreted proteins, as well as a large group of p21-induced intracellular proteins (Table II), are known to be induced in different forms of stress response or to play a role in stress-associated signal transduction. Remarkably, many genes that we found to be induced by p21 are also upregulated in cellular senescence, organism aging, or different age-related diseases, indicating that suppression of p21-mediated gene induction may provide a way to prevent the development of such diseases. As disclosed in Example 5 below, several p21-induced genes encode secreted factors with paracrine anti-apoptotic and mitogenic activities, and conditioned media from p21-induced cells exhibits two biological effects predicted by the nature of p21-upregulated genes: stimulation of cell growth and suppression of apoptosis. This finding, suggests that "paracrine" effects of p21 may contribute to carcinogenesis through a tumor-promoting effect on neighboring cells. This raises the possibility that suppression of p21-mediated gene induction may also provide a way to achieve an anti-carcinogenic effect.

EXAMPLE 4
Identifying the Specificity of p21 Induction by Comparing IPTG-treated and Serum-Starved HT1080 p21-9 Cells

The identity of p21-induced changes in cellular gene expression that are likely to be a consequence of cell growth arrest was determined as follows.

Growth arrest (quiescence) was induced in HT1080 p21-9 cells by serum starvation produced by culturing the cells in serum-free media for 4 days. In serum-starved cells, unlike IPTG-treated HT1080 p21-9 cells, the cells did not develop a senescent morphology and showed only very weak SA-β-gal expression. p21 levels in serum-starved cells were increased only about 2-fold, as opposed to the 15-20 fold increase seen in IPTG-treated cells. Fig. 3D shows RT-PCR analysis performed as described above of the expression of a group of p21-inhibited and p21-induced genes in HT1080 p21-9 cells that were growth- arrested after 4 days in serum-free media or 3 days in the presence of 50 μM IPTG. Genes that were completely inhibited in HT1080 p21-9 cells when the culture media contained 50 μM IPTG were also inhibited in serum-starved cells, but most of these genes were inhibited to a lesser extent than in IPTG-treated cells.

Genes whose expression is induced by p21 showed three distinct patterns. The first group are genes whose expression is induced as strongly in quiescent cells as in senescent cells. These include galectin-3, superoxide dismutase 2, complement C3 and prosaposin, indicating that their induction was a consequence of cell growth arrest or that such genes were exquisitely sensitive to slightly elevated p21 levels. The second group are genes that were up-regulated in quiescent cells but not as strongly as in senescent cells. These genes include fibronectin-1, Mac2 binding protein and the Alzheimer precursor protein serum amyloid A. The third group are genes that are not detectably induced in quiescent cells but are strongly induced in senescent cells. These genes include CTGF, plasminogen activator inhibitor 1, tissue transglutaminase or natural killer cell marker protein NK4, integrin beta 3 and activin A.

The difference between the response of certain genes to induction of quiescence by serum starvation and cellular senescence through IPTG-induced overexpression of p21 identified these genes as diagnostic markers of senescence. Furthermore, novel

senescence markers can now be identified by comparing their expression between p21-expressing and quiescent cells.

EXAMPLE 5
Production of Conditioned Media containing Mitogenic Factors and Mitogenic Activity Assays

Several p21-upregulated genes (Table II) encode secreted proteins that act as growth factors, including CTGF (Bradham *et al.*, 1991, *J. Cell Biol.* 114: 1285-1294), activin A (Sakurai *et al.*, 1994, *J. Biol. Chem.* 269: 14118-14122), epithelin/granulin (Shoyab *et al.*, 1990, *Proc. Natl. Acad. Sci. USA* 87: 7912-7916) and galectin-3 (Inohara *et al.*, 1998, *Exp Cell Res.* 245: 294-302). In addition, galectin-3 (Akahani *et al.*, 1997, *Cancer Res.* 57: 5272-5276) and prosaposin (Hiraiwa *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94: 4778-4781) were shown to have anti-apoptotic activity. Paracrine anti-apoptotic or mitogenic activities have also been reported for several p21-inducible gene products that are not listed in Table II, since their balanced differential expression values in cDNA microarray hybridization were 1.8-1.9. This is below the arbitrarily chosen minimum value of 2.0 that we have used for inclusion into this Table or verification by RT-PCR. These proteins are clusterin (Koch-Brandt and Morgans, 1996, *Prog. Mol. Subcell. Biol.* 16: 130-149), prostacyclin-stimulating factor (PSF) (Yamauchi *et al.*, 1994, *Biochem. J.* 303: 591-598), vascular endothelial growth factor-C (VEGF-C) (Joukov *et al.*, 1996, *EMBO J.* 15: 290-298), gelsolin (Ohtsu *et al.*, 1996, *EMBO J* 16: 4650-4656) and tissue inhibitor of metalloproteinase-1 (TIMP-1) (Li *et al.*, 1999, *Cancer Res.* 59: 6267-6275).

To verify the induction of secreted mitogenic and anti-apoptotic factors by p21, conditioned media from IPTG-treated HT1080 p21-9 cells were tested to investigate whether they would have an effect on cell growth and apoptosis. In these experiments, conditioned media were prepared by plating 10^6 HT1080 p21-9 cells per 15cm plate in

the presence of DMEM/ 10% FCS. The next day, IPTG was added to a final concentration of 50μM, and this media was replaced three days later with DMEM supplemented with 0.5% FCS and 50μM IPTG. Two days later (days 3-5 of IPTG treatment), this conditioned media was collected and stored at 4°C up to 15 days before use. Control media were prepared by adding IPTG-free DMEM/ 0.5% FCS to untreated cells grown to the same density as IPTG-treated cells and collecting the media two days thereafter.

The slow-growing human fibrosarcoma cell line HS 15.T was used to detect mitogenic activity in these conditioned media. For mitogenic activity assays, both types of conditioned media, as well as fresh media and 1:1 mixtures of conditioned media and fresh media were used to test mitogenic activity. In these experiments, the conditioned media were supplemented with 1% or 2% FCS. Briefly, HS 15.T cells were plated in 12-well plates at 15,000 cells per well. Two days later, these cells were cultured in different types of media. The cells were grown in conditioned media for 60hr, and the ^{3}H-thymidine at a concentration of 3.13 μCi/mL was added and incubated for 24 hrs. Cells were then collected and their ^{3}H-thymidine incorporation determined as described by Mosca *et al.* (1992, *Mol. Cell. Biol.* 12: 4375-4383).

The addition of IPTG to fresh media had no effect in this assay. There was no significant difference between cell growth in fresh media and in conditioned media from untreated HT1080 p21-9 cells. In contrast, conditioned media from IPTG-treated cells increased ^{3}H-thymidine incorporation up to three-fold. Growth stimulation of HS 15.T by conditioned media from IPTG-treated cells was also detectable by methylene blue staining.

The effect of this conditioned media on apoptosis was also determined. These experiments used a mouse embryo fibroblast line C8, immortalized by E1A. This cell line is highly susceptible to apoptosis induced by different stimuli (Lowe *et al.*, 1994,

Science 266: 807-810; Nikiforov *et al.*, 1996, *Oncogene* 13: 1709-1719), including serum starvation (Lowe *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91: 2026-2030). Apoptosis was analyzed by plating 3 x 10^5 C8 cells per 6-cm plate, and replacing the media on the following day with fresh media supplemented with 0.4% serum or with conditioned media (no fresh serum added). DNA content analysis and DAPI staining were carried out after 24 hrs and 48 hrs, and relative cell numbers were measured by methylene blue staining (Perry *et al.*, 1992, *Mutat. Res.* 276: 189-197) after 48 hrs in low-serum media.

The addition of low-serum fresh media or conditioned media from IPTG-treated or untreated cells rapidly induced apoptosis in C8 cells, as evidenced by cell detachment and apoptotic morphology detectable in the majority of cells after DAPI staining (not shown). Conditioned media from IPTG-treated cells, however, strongly increased cell survival relative to fresh media and conditioned media from untreated cells, as measured by methylene blue staining of cells that remained attached after 48 hrs. The effect of the conditioned media from p21-induced cells was even more apparent in FACS analysis of cellular DNA content, which was carried out on combined attached and floating C8 cells 24 hrs and 48 hrs after media change. Unlike many other cell lines, apoptosis of C8 cells produces only a few cells with decreased (sub-G1) amount of DNA, and it is characterized by selective disappearance of cells with G2/M DNA content (Nikiforov *et al.*, 1996, *ibid.*). Serum-starved cells in conditioned media from IPTG-treated cells retained the G2/M fraction and showed cell cycle profiles that resembled control cells growing in serum-rich media. The addition of IPTG by itself had no effect on apoptosis in C8 cells. Thus, p21 induction in HT1080 cells results in the secretion of mitogenic and anti-apoptotic factors, as predicted by the nature of p21-unregulated genes.

EXAMPLE 6
Production of Mammalian Cell comprising Inducible $p16^{Ink4A}$ or $p27^{Kip1}$ Genes

Mammalian cell lines comprising inducible CDK inhibitors $p16^{Ink4A}$ (which preferentially inhibits CDK4/6; Serrano et al., Nature 16, 704-707, 1993) or $p27^{Kip1}$ (which preferentially inhibits CDK2; Blain *et al.*, 1997, *J. Biol. Chem.* 272: 25863-25872) were produced generally as described in Example 1 for production of an inducible p21 containing cell line. A recombinant derivative of human HT1080 fibrosarcoma cell line containing a recombinant expression construct encoding the bacterial lacI gene and expressing a murine ecotropic retrovirus receptor (HT1080 3'SS6; Chang & Roninson, 1996, *Gene* 183: 137-142) was used to make the inducible lines. For the inducible expression of p16, a DNA fragment containing a 471bp coding sequence of human p16 (as disclosed in U.S. Patent 5,889,169, incorporated by reference) was cloned into the IPTG-regulated retroviral vector LNXRO2 (Chang & Roninson, 1996, *Gene* 183: 137-142). This retroviral vector contains the bacterial neomycin resistance gene (*neo*) under the transcriptional control of the retroviral long terminal repeat promoter, permitting selection using G418 (BRL-GIBCO). The resulting construct, designated LNp16RO2, is depicted schematically in Figure 4. For the inducible expression of p27, a vector LNp27RO2, carrying murine p27 cDNA (NCBI RefSeq NM_009875) in the same LNXR02 vector has been developed and described by Kokontis *et al.*, 1998, *Mol. Endocrinol.* 12: 941-953, and provided to us by Dr. N. Hay, University of Illinois at Chicago).

The LNp16RO2 and LNp27RO2 constructs were introduced individually into HT1080 3'SS cells using conventional retroviral infection methods. The infected cells were selected by culturing the cells in the presence of 400μg/mL G418 (obtained from BRL-GIBCO). The G418-selected population of LNp16RO2 transduced cells was designated HT1080/LNp16RO2. This cell population has been deposited on October 10,

2000 in the American Type Culture Collection (A.T.C.C.), Manassas, VA and given Accession Number PTA-2580.

This cell population was subcloned, and 20 clonal cell lines were isolated and tested for IPTG-inducible growth inhibition. Cell line showing the strongest growth inhibition was designated HT1080 p16-5. This cell line has been deposited on January 31, 2002 in the American Type Culture Collection (A.T.C.C.), Manassas, VA and given Accession Number PTA-4020. Figure 5A shows changes in the cell cycle distribution of HT1080 p16-5 cells upon the addition of 50 μM IPTG. Fractions of cells in different phases of the cell cycle were determined using FACS analysis of DNA content after staining with propidium iodide as described by Jordan *et al.* (1996, *Cancer Res.* 56: 816-825) using Becton Dickinson FACSort. Cell cycle distribution stabilized after 24 hrs of IPTG treatment, by which time 93% of IPTG-treated cells were arrested in G1. Such G1 arrest is expected from the inhibition of CDK4/6 by p16.

Similarly, the G418-selected population of LNp27RO2 transduced cells was subcloned, and 38 clonal cell lines were isolated and tested for IPTG-inducible growth inhibition. Cell line showing the strongest growth inhibition was designated HT1080 p27-2. This cell line has been deposited on January 31, 2002 in the American Type Culture Collection (A.T.C.C.), Manassas, VA and given Accession Number PTA-4021. Figure 5B shows changes in the cell cycle distribution of HT1080 p27-2 cells upon the addition of 50 μM IPTG. Cell cycle distribution stabilized after 24 hrs of IPTG treatment, by which time 89% of IPTG-treated cells were arrested in G1. Such G1 arrest is expected from the inhibition of CDK4/6 by p16.

EXAMPLE 7

Effects of p16 and p27 on the Expression of p21-inducible Genes

The HT1080 derivatives HT1080 p16-5 and HT1080 p27-2, carrying p16 or p27

genes inducible with IPTG as described in Example 6 were used in gene expression assays as follows.

RNA was obtained from these cell lines, cultured in the presence or absence of 50μM IPTG for three days. These RNA samples were then used in RT-PCR assays performed essentially as described above in Example 3, except that β-actin rather than β_2-microglobulin was used for normalization. Eighteen genes shown above to be induced by p21 were analyzed for the effects of p16 or p27 gene expression induced by IPTG treatment of these cells. The tested genes included the genes involved in Alzheimer's disease, amyloidosis, arthritis, atherosclerosis and paracrine apoptotic and mitogenic effects as described above with regard to induced p21 expression. The results for p16 are shown in Figure 6A and for p27 in Figure 6B. All the tested p21-induced genes were also induced by IPTG-induced p16 expression, and almost all of the tested genes (except for t-PA and CTGF) were also induced by p27. The results shown in Figure 6 also illustrate that p16 or p27 expression has no detected effect on p21 expression.

EXAMPLE 8
Production of Recombinant Expression Constructs containing a Reporter Gene Expressed by a p21-responsive Promoter

Promoter-reporter constructs were prepared from promoters of several p21-inducible human genes, including NK4, SAA, Complement C3 (CC3), prosaposin, βAPP and t-TGase as follows. The promoter region of the CC3 gene was identified in the human genome sequence (NCBI Accession number M63423.1) as adjacent to the 5' end of CC3 cDNA (Vik *et al.*, 1991, *Biochemistry* 30: 1080-1085). The promoter region of the NK4 gene was identified in the human genome sequence (Accession number AJ003147) as adjacent to the 5' end of NK4 cDNA (Accession number M59807). The

previously described promoter of the SAA gene (Edbrooke *et al.*, 1989, *Mol. Cell. Biol.* 9: 1908-1916) was identified in the human genome sequence (Accession number M26698). The promoter region of the βAPP gene was identified in the human genome sequence (Accession number X12751) as adjacent to the 5' end of βAPP cDNA (Accession number XM009710). The promoter region of the t-TGase gene was identified in the human genome sequence (Accession number Z46905) as adjacent to the 5' end of t-TGase cDNA (Accession number M55153). Polymerase chain reaction (PCR) amplification of promoter-specific DNA was performed using genomic DNA from HT1080 p21-9 cells as the template. PCR was carried out using *PfuTurbo* DNA Polymerase (Stratagene) and primer sets listed in Table IIIa. The PCR conditions for each primer set are described in Table IIIb. Primer sets for amplifying promoter sequences from several genes induced by CDK inhibitors, including the gene promoters used as disclosed in this Examiner, are set forth in Table IIIc.

PCR products were obtained and cloned into the TOPO TA cloning vectors pCR2.1/TOPO (for SAA, CC3, βAPP and t-TGase) or pCRII/TOPO (for NK4). These constructs were verified by sequencing, and the *Kpn* I-*Xho* I fragments containing promoters in the correct orientation were then inserted into the *Kpn* I and *Xho* I sites in a firefly luciferase-reporter vector pGL2 basic (Promega, Madison, WI) using standard recombinant genetic techniques (Sambrook *et al.*, *ibid.*). The clone containing a 480 bp sequence of the prosaposin promoter, driving firefly luciferase expression has been described by Sun et al. (1999, Gene 218, 37-47) and provided by Dr. Grabowski (Children's Hospital Medical Center, Cincinnati, OH).

Plasmid clones for each promoter construct were tested for p21-regulation by a transient transfection assay. Transient transfection of HT1080 p21-9 cells was carried out by electroporation, essentially as described in the Bio-Rad protocols. For each

electroporation, HT1080 p21-9 cells were grown to 95% confluence in 15cm plates using DMEM supplemented with 10% FC2 serum and containing penicillin, streptomycin and glutamine. The cells were then trypsinized, resuspended in DMEM or Opti-MEM medium (GibcoBRL) and spun down at 1,000 rpm in an IEC HN-SII centrifuge for 10 minutes. Following centrifugation the media were aspirated and the cells were again resuspended in Opti-MEM at a concentration of 18-20 million cells per ml. 400 µl of cell suspension (approximately 7 to 8 million cells) was transferred to a 4 cm gap electroporation cuvette (Bio-Rad). 10-20 µg of the promoter-luciferase construct was added to the cells. In some experiments, a control plasmid pCMVbgal expressing bacterial β-galactosidase from the CMV promoter, was added to the mixture at a ratio of 1:10 for normalization. In other experiments, normalization was carried out by adding vector pRL-CMV expressing Renilla luciferase from the CMV promoter at a 1:20 molar ratio, and the firefly luciferase and Renilla luciferase activities were measured in the same samples using the Dual Luciferase Essay kit (Promega). Electroporations were performed using Bio-Rad Gene Pulser at 0.22 volts, with a capacitance extender set to 960µFD, providing a τ value of 27 to 30. In preliminary experiments, cell survival and attachment after electroporation was determined to be approximately 33%. Cells were plated in triplicate at an initial density of approximately 50,000 attached cells/well in 12-well plates. After letting the cells settle for a period of 3-6 hours, the media was aspirated and replaced with fresh media with or without 50 µM IPTG. 2 to 4 days later, cells were washed twice with phosphate-buffered saline and collected in 300 µL of 1x Passive Lysis Buffer or Reporter Lysis Buffer (Promega). The lysate was centrifuged briefly at 10,000 g to pellet debris, and 50 µL aliquots were transferred to fresh tubes for use in the Firefly Luciferase assay (Promega). Luciferase activity was measured using a Turner 20/20 luminometer at 52.1% sensitivity with a 5 second delay period and 10-15

second integration time.

Figure 7 shows the results of representative experiments. After 2-4 days of p21-induction in transfected cells, expression from promoter constructs of p21-induced genes was increased about 7.0-fold for NK4, 3.7-fold for SAA, 12.5-fold for CC3, 3.0-fold for prosaposin, 2.6-fold for βAPP, and 2.3-fold for t-TGase. These results indicated that p21 up-regulates expression of these genes by regulating their promoters, and that promoter constructs of such genes can be used to assay for p21-mediated regulation of gene expression. Such assays can be used to identify compounds that inhibit p21-mediated gene activation, as described below in Example 9.

Table IIIa. Primer sequences

Promoter	Sense primer (5'->3')	Antisense primer (5'->3')
CC3	GCTAAGAGGATATTGACATTAGA CAGG (SEQ ID NO: 21)	AGGGGGAGGTGGGTTAGTAG (SEQ ID NO: 22)
NK4	TGGAGCTAGAAGAGCCCGTAGG (SEQ ID NO: 23)	GCCAAAAGTTCAAGGAGCCAA (SEQ ID NO: 24)
SAA	CAGAGTTGCTGCTATGTCCACCA (SEQ ID NO: 25)	CACTCCTTGTGTGCTCCTCACC (SEQ ID NO: 26)
βAPP	TTGCTCCTTTGGTTCGTTCT (SEQ ID NO: 27)	GCTGCCGAGGAAACTGAC (SEQ ID NO: 28)
t-TGase	CCCAGGGAGAAATATCCACTGAA GCAAC (SEQ ID NO: 29)	TCGGGCGGGGGCGGTGGCTCCT TCCACT (SEQ ID NO: 30)

Table IIIb. PCR conditions

Promoter	Denaturation	Annealing	Extension	Cycles	Product size
CC3	95°, 1 min	63°, 1 min	72°, 1 min 40 sec	31	1018 bp
NK4	94°, 1 min	65°, 1 min	72°, 1 min 40 sec	32	877 bp
SAA	94°, 1 min	68°, 1 min	72°, 1 min 40 sec	32	1000 bp
βAPP	94°, 1 min	62.9°, 1 min	72°, 1 min 40 sec	30	623 bp
t-TGase	94°, 1 min	66.5°, 1 min	72°, 1 min 40 sec	33	1600 bp

Table IIIc. Primer Sequences

ID NO:	Gene Name	Upstream (Sense, 5'-3')	Downstream (Antisense, 5'-3')
1	SAA	cagagttgctgctatgtccacca (SEQ ID NO: 25)	cactccttgtgtgctcctcacc (SEQ ID NO: 26)
2	Compl. C3	cctaagaggatattgacattagacagg (SEQ ID NO: 21)	agggggaggtgggttagtag (SEQ ID NO: 22)
3	CTGF	gcctcttcagctacctacttcctaa (SEQ ID NO: 31)	cgaggaggaccacgaagg (SEQ ID NO: 32)
4	Integrin B3	Gattggtcttgccctcaacag (SEQ ID NO: 33)	ccagcacagtcgcccaga (SEQ ID NO: 34)
5	Activin	tgattccaatgtttttctaaaagg (SEQ ID NO: 35)	gaatgtctaaagagctcagaagt (SEQ ID NO: 36)
6	NK4	tggagctagaagagcccgtagg (SEQ ID NO: 23)	gccaaaagttcaaggagccaag (SEQ ID NO: 24)
7	Prosaposin	ggtttaagcaatttctggcctct (SEQ ID NO: 37)	cgtctgactctccgcagtctgcaat (SEQ ID NO: 38)
8	Mac2-BP	gtaaaactccctgatgattccttct (SEQ ID NO: 39)	ctctgcagactggtcctttgac (SEQ ID NO: 40)
9	GAL-3	tgtcttcacaaggtggaagtgg (SEQ ID NO: 41)	ctggagggcagagcacag (SEQ ID NO: 42)
10	MnSOD	taccaaccctaggggtaaaaataaa (SEQ ID NO: 43)	atgctgctagtgctggtgctac (SEQ ID NO: 44)
11	Granulin	gagactaggaagccacttctctttc (SEQ ID NO: 45)	ctggaatgctgtgttcttttctact (SEQ ID NO: 46)
12	p66shc	gtggcagacagggcactc (SEQ ID NO: 47)	ctcctgagctgcctcaatg (SEQ ID NO: 48)
14	Cathepsin B	Ctcccgagtagctgggatta (SEQ ID NO: 51)	ccacgtgaccaccgcgca (SEQ ID NO: 52)
15	βAPP	ttgctcctttggttcgttct (SEQ ID NO: 27)	gctgccgaggaaactgac (SEQ ID NO: 28)
16	t-Tgase	cccagggagaaatatccactgaagcaac (SEQ ID NO: 29)	tcgggcgggggcggtggctccttccact (SEQ ID NO: 30)
17	Clusterin	agccccttgacttctctcct (SEQ ID NO: 53)	ctcctggcgacgccgcgtt (SEQ ID NO: 54)
18	PSF	aaagtgctgggattagaggcgtga (SEQ ID NO: 55)	tatgtattgctaagggaagctattggag (SEQ ID NO: 56)
19	VEGF-C	gttcttggatcatcaggcaactt (SEQ ID NO: 57)	gtggaaggaccgggggtgg (SEQ ID NO: 58)
20	TIMP-1	agaaccggtacccatctcaga (SEQ ID NO: 59)	ctgtacctctggtgtctctct (SEQ ID NO: 60)

EXAMPLE 9
Production of Cells Stably Transfected with a p21-inducible Reporter Construct

To develop a stably transfected cell line with p21-regulated luciferase expression, the NK4 promoter-luciferase construct, described in Example 8 and termed pLuNK4, was introduced into HT1080 p21-9 cells, which carry IPTG-inducible p21, by cotransfection with pBabePuro carrying puromycin N-acetyltransferase as a selectable marker. Transfection was carried out using LIPOFECTAMINE 2000 (Life Technologies, Inc., Gaithersburg, MD), using a 10:1 ratio of pLuNK4 and pBabePuro. Stable transfectants were selected using 1 μg/mL puromycin for 5 days. 54 puromycin-resistant cell lines were isolated and tested for luciferase activity (using a Luciferase Assay System, Promega), in the presence and in the absence of 50 μM IPTG.

This assay was performed as follows. Cells were plated at a density of 40,000 cells/well in 12 well plates in 1 mL of media containing penicillin/streptomycin, glutamine and 10% fetal calf serum (FCS). After attachment, cells were treated with 50 μM IPTG or left untreated for different periods of time. Luciferase activity was then measured as described in Example 8 above. An additional aliquot was removed from the cell lysate to measure protein concentration using the Bio-Rad protein assay kit (Bradford assay). Luciferase activity for each sample was normalized to protein content and expressed as luciferase activity/ μg protein. All assays were carried out in triplicate and displayed as a mean and standard deviation.

21 of 54 tested cell lines showed measurable luciferase activity, but only one cell line, designated HT1080 LuNK4p21, showed higher luciferase expression in the presence than in the absence of IPTG. The results of assays carried out with p21LuNK4 cell line are shown in Figure 8A and 8B. Fig. 8A shows the IPTG dose dependence of luciferase expression after 24 hrs of IPTG treatment, and Fig. 8B shows the time course of luciferase expression upon the addition of 50 μM IPTG. This analysis shows that

most of the induction can be achieved using as little as 5 μM IPTG and a treatment period as short as 17 hrs.

These results demonstrated that the pLuNK4 reporter construct could be used to produce stably transfected cell lines that were responsive to p21 induction of reporter gene transcription. Such constructs and cells provide a basis for a screening assay for identifying compounds that inhibit p21-mediated gene activation. The relatively short time required for luciferase induction (about 17 hrs), together with the pronounced (approximately 3-fold) increase in luciferase levels in IPTG-treated cells, should make the LuNK4p21 cell line suitable for high-throughput screening of compounds that would inhibit the inducing effect of p21. Other cell lines with similar (and potentially better) inducibility can also be developed through the methods disclosed herein used to derive LuNK4p21. The results described in Example 8 demonstrate that the same type of screening can also be conducted using transient transfection assays with promoter constructs of p21-inducible genes rather than stably-transfected cell lines. The methods for high-throughput screening based on luciferase expression are well known in the art (see Storz *et al.*, 1999, *Analyt. Biochem.* 276: 97-104 for a recent example of a transient transfection-based assay and Roos *et al.*, 2000, *Virology* 273: 307-315 for an example of screening based on a stably transfected cell line). Compounds identified using these cells and assays are in turn useful for developing therapeutic agents that can inhibit or prevent p21-mediated induction of age-related genes.

EXAMPLE 10
Use of NFκB and p300/CBP Inhibitors to Inhibit p21-Mediated Induction in Transient Transfection Assays

Examination of promoter sequences of p21-inducible genes showed that many of these promoters, including NK4, contain known or potential NFκB binding sites.

Several p21-induced genes are known to be positively regulated by NFκB, including superoxide dismutase 2 (SOD2) (Jones *et al.*, 1997, *Mol. Cell. Biol.* 17: 6970-6981), t-TGase (Mirza *et al.*, 1997, *Amer. J. Physiol.* 272: G281-G288), Alzheimer's β-amyloid precursor protein (APP) (Grilli *et al.*, 1996, *J. Biol. Chem.* 271: 15002-15007) and the inflammatory protein serum amyloid A (SAA) (Jensen and Whitehead, 1998, *Biochem J.* 334: 489-503). p21 has been previously shown by transient co-transfection experiments to activate NFκB-dependent transcription (Perkins *et al.*, 1997, *Science* 275: 523-527) in human immunodeficiency virus, indicating that the HIV promoter residing in the viral long terminal repeat (SEQ ID NO. 85) is responsive to and regulated by NFκB. This effect of p21 was shown to be due to the stimulation of transcription cofactors p300 and CBP (Perkins *et al.*, 1997, *Science* 275: 523-527); it is possible that activation of p300/CBP or related transcription cofactors may be responsible for the effect of p21 on some of the upregulated genes. Thus, inhibitors of NFκB or p300/CBP may potentially prevent the induction of transcription by p21.

To determine if IPTG-inducible p21 expression in HT1080 p21-9 cells stimulates the transcriptional activity of NFκB, we have used transient transfection assays to investigate the effect of p21 induction on luciferase expression from the plasmid pNFkB-Luc, commercially available from Stratagene. This plasmid expresses firefly luciferase from an artificial promoter containing five tandemly repeated NFκB consensus sequences. We have also generated by PCR a version of the same promoter that contains only one NFκB consensus sequence using primers identified as SEQ ID Nos. 80 and 83. The sequences of these two promoters are identified as SEQ ID NOs. 78 and 79.

The ability of p21 to induce these promoters was tested by transient transfection assays, as described in Example 8, using different concentrations of IPTG that induce p21 to different levels. As shown in Fig. 9A and Fig. 9B, both promoters showed dose-

dependent induction by IPTG, but both the basal level of activity and the fold induction by IPTG were much higher in the promoter containing five NFκB sites. These results indicate that p21 induces NFκB-dependent transcription in our system, and that promoters containing one or more NFκB site can be used as an alternative to the promoters of p21-inducible cellular genes.

To evaluate the effects of genetic inhibitors of NFκB on luciferase expression from pNFkB-Luc, 20 μg of the latter plasmid were mixed (at a molar ratio 1:2) with a plasmid MAD3 (a.k.a. pRC/βactin-HA-IKKα) that expresses a dominant mutant of IκB kinase α that selectively inhibits NFκB (DiDonato *et al.*, 1996, *Mol. Cell. Biol.* 16: 1295-1304) (provided by Dr. M. Karin, University of California San Diego). This plasmid is referred to below as IKK. To determine the effect of p300/CBP inhibition on luciferase expression from pNFkB-Luc, the latter plasmid was similarly mixed in another assay with a vector expressing a truncated gene for adenoviral E1A protein with a C-terminal deletion {ΔCR2 (120-140)}. The C-truncated E1A (termed E1AΔCR2) is known to inhibit p300/CBP and related factors (such as PCAF) but it does not inhibit Rb, the target of the C-terminal domain of E1A (Chakravarti *et al.*, 1999, *Cell* 96: 393-403). As a negative control, pNFkB-Luc was mixed with a functionally inactive form of E1A with deletions at both the C-terminus and the N-terminus {ΔN(2-36)}, termed E1AΔN/ΔCR2. The E1AΔCR2 and E1AΔN/ΔCR2 constructs were provided by Dr. V. Ogryzko (NICHHD, NIH). The mixtures of pNFkB-Luc with IKK, E1AΔCR2 or E1AΔN/ΔCR2 were transfected into HT1080 p21-9 cells by electroporation, as described in Example 8 (with pRL-CMV plasmid further added for normalization). After electroporation, equal numbers of transfected cells were treated with 50 μM IPTG or untreated for three days (in triplicates). The firefly luciferase activity was measured and normalized to Renilla luciferase activity measured (in the absence of IPTG) in each

transfected sample.

The results of this analysis are shown in Figure 9C. pNFkB-Luc mixed with the negative control (E1AΔN/ΔCR2) showed up to 15-fold induction in the presence of IPTG, demonstrating an increase in NFκB transcriptional activity in HT1080 p21-9 cells. Mixing pNFkB-Luc with the IKK inhibitor almost completely abolished luciferase expression in IPTG-treated or untreated cells, demonstrating the efficacy of this inhibitor. E1AΔCR2 had a similar but weaker effect than IKK, suggesting the requirement of p300/CBP for NFκB activity in HT1080 p21-9 cells (Fig. 9 C).

The same analysis was carried out using promoter-luciferase constructs for six p21-inducible genes. The results for SAA are shown in Figure 9D, for prosaposin in Figure 9E, for βAPP in Figure 9F, for t-TGase in Figure 9G, for complement C3 in Figure 9H, and for NK4 in Figure 9I. Both IKK and E1AΔCR2 inhibited the induction of all the tested promoters in the presence of IPTG, indicating that the regulation of these promoters by p21 is mediated in part through p300/CBP and NFκB. Quantitatively, however, the effects of these inhibitors varied among the promoters. Both basal and IPTG-stimulated expression of most of the tested promoters of SAA (Fig. 9D) and NK4 (Fig. 9I) was inhibited by IKK and E1AΔCR2 almost as strongly as that of NFκB. In contrast, these inhibitors had little or no effect on the basal expression from the promoters of prosaposin (Fig. 9E), βAPP (Fig. 9F), t-TGase (Fig. 9G), or complement C3 (Fig. 9H), but interfered with the induction of these promoters in the presence of IPTG. The effect of IKK on the induction of the prosaposin promoter (Fig. 9E) by IPTG, however, was substantially weaker than for the other promoters.

These results indicate that p300/CBP and NFκB are involved in the induction of all the tested promoters by p21, although the relative contribution of these factors to the effect of p21 may vary among the promoters.

EXAMPLE 11
Use of Non-Steroidal Anti-Inflammatory Drugs to Inhibit p21-Mediated Gene Induction

The best-studied NFκB inhibitors in clinical use are certain non-steroidal anti-inflammatory drugs (NSAID), such as aspirin, sodium salicylate and sulindac (Kopp and Ghosh, 1994, *Science* 265: 956-959; Yin *et al.*, 1998, *Nature* 396: 77-80; Yamamoto *et al.*, 1999, *J. Biol. Chem.* 274: 27307-27314). The LuNK4p21 cell line described in Example 9 above was used to determine whether the induction of luciferase expression by p21 in this cell line can be inhibited by NSAID with NFκB -inhibitory activity.

Luciferase assays were performed substantially as described in Example 9. Luciferase activity was measured after 16 hrs of incubation with or without 50 μM IPTG, followed by an additional 20 hr treatment in the presence or in the absence of 20 mM sodium salicylate, 1 mM sulindac, or 10 mM aspirin. In addition, two NSAIDs were tested that do not inhibit NFκB: indomethacin and ibuprofen (at 25 μM each) (Yamamoto *et al.*, 1999, *ibid.*). NSAID concentrations were based on the pharmacologic concentrations of these agents in the serum of patients required for their anti-inflammatory properties (Yin *et al.*, 1998, *ibid.*).

The results of these assays are shown in Figure 10. IPTG increased luciferase expression approximately 3-4 fold in the absence of NSAID, but this induction was completely or almost completely abolished in the presence of salicylate, sulindac, or aspirin. In contrast, indomethacin and ibuprofen made no significant difference to the induction of luciferase by IPTG.

To determine whether NFκB -inhibiting NSAID inhibited not only the induction of transcription from the NK4 promoter but also RNA expression of the endogenous p21-inducible genes, LuNK4p21 cells were plated at 125,000 cells per well in 6-well plates and were either untreated or treated with 50 μM IPTG for 48 hrs (the period of

time required for maximal stimulation of p21-inducible genes; Chang *et al.*, 2000, *Proc. Natl. Acad. Sci. USA* 97: 4291-4296), in the presence or in the absence of sulindac, at 250 μM, 500 μM or 1 mM concentrations. After this incubation, RNA was extracted from the cells using Qiagen RNeasy Mini Kit, and relative RNA levels of several p21-inducible genes were determined by reverse transcription-PCR (RT-PCR), essentially as described by Noonan *et al.* (1990, *Proc. Natl. Acad. Sci. USA* 87: 7160-7164), except that β-actin rather than β_2-microglobulin was used for cDNA normalization. The sequences of the PCR primers for each of the tested genes are provided in Table IVa. The PCR cycles were as follows: for the 1st cycle, 3 min for denaturation, 2 min for annealing and 2 min for extension, and the rest of cycles, 30 sec for denaturation; 30 sec for annealing; and 1 min for extension. The temperature conditions of the PCR cycles and the sizes of the PCR products are provided in Table IVb.

The results of the RT-PCR analysis are shown in Fig. 11. For NK4 (the promoter of which was used to drive luciferase expression in LuNK4p21 cells), the addition of sulindac had very little effect on gene expression in the absence of IPTG, but all the concentrations of sulindac produced a dose-dependent decrease in NK4 RNA levels in the presence of IPTG. Very similar results were obtained with t-TGase RNA. With all the other tested genes, sulindac produced a dose-dependent increase in gene expression in the absence of IPTG. As a result of this effect, the highest tested dose of sulindac (1 mM) did not decrease gene expression in the presence of IPTG, but a noticeable decrease in the IPTG effects was observed at lower doses of sulindac. In particular, the effects of IPTG were diminished by 250 and 500 μM sulindac for the APP gene, but only by 250 μM sulindac for $p66^{Shc}$, CTGF and Mac2-binding protein (Mac2-BP) genes. None of the tested sulindac concentrations produced a significant decrease in IPTG-induced RNA levels of prosaposin or superoxide dismutase 2 (SOD2). The lack of sulindac effect on prosaposin is in agreement with a moderate effect of IKK inhibitor

on the prosaposin promoter (see Example 10 above). Hence, a moderate dose of sulindac (250 μM) inhibits the ability of p21 to induce transcription for most of the tested genes.

Table IVa. Primer sequences

GENE	SENSE PRIMER (5'->3')	ANTISENSE PRIMER (5'->3')
NK4	AGCACCAGGCCATAGAAAGA (SEQ ID NO: 13)	GGTGTCAGCTCCTCCTTGTC (SEQ ID NO: 49)
T-TGASE	ACTACAACTCGGCCCATGAC (SEQ ID NO: 50)	GCCAGTTTGTTCAGGTGGTT (SEQ ID NO: 61)
BAPP	CTCGTTCCTGACAAGTGCAA (SEQ ID NO: 62)	TGTTCAGAGCACACCTCTCG (SEQ ID NO: 63)
$P66^{SHC}$	GAGGGTGTGGTTCGGACTAA (SEQ ID NO: 64)	GCCCAGAGGTGTGATTTGTT (SEQ ID NO: 65)
CTGF	GGAGAGTCCTTCCAGAGCAG (SEQ ID NO: 66)	ATGTCTTCATGCTGGTGCAG (SEQ ID NO: 67)
MAC2-BP	ACCATGAGTGTGGATGCTGA (SEQ ID NO: 68)	ACAGGGACAGGTTGAACTGC (SEQ ID NO: 69)
GRANULIN	ACCACGGACCTCCTCACTAA (SEQ ID NO: 70)	ACACTGCCCCTCAGCTACAC (SEQ ID NO: 71)
PROSAPOSIN	CCAGAGCTGGACATGACTGA (SEQ ID NO: 72)	GTCACCTCCTTCACCAGGAA (SEQ ID NO: 73)
SOD2	CAAATTGCTGCTTGTCCAAA (SEQ ID NO: 74)	CATCCCTACAAGTCCCCAAA (SEQ ID NO: 75)
B-ACTIN	GGGAAATCGTGCGTGACATTAA G (SEQ ID NO: 76)	TGTGTTGGCGTACAGGTCTTTG (SEQ ID NO: 77)

Table IVb. PCR temperatures (in °C)

Gene	Denaturation	Annealing	Extension	Cycles	Product size
NK4	94	58	72	24	481
t-TGase	94	58	72	24	499
B-APP	94	58	72	20	500
$p66^{shc}$	94	58	72	22	514
CTGF	94	64	72	28	499
MAC2-BP	94	58	72	21	517
Granulin	94	64	72	25	446
Prosaposin	94	58	72	21	500
SOD2	94	58	72	23	505
β-actin	94	60	72	17	275

These results demonstrated that assays for interference with p21-mediated induction of reporter expression from the promoters of p21-inducible genes are capable of identifying agents that inhibit p21-mediated induction of genes associated with carcinogenesis and age-related diseases. In particular, an agent (sulindac) that was first identified as an effective inhibitor in a promoter-based assay using LuNK4p21 cell line was found to inhibit the induction of several aging-associated genes by p21. These results further demonstrated that NSAIDs that are active as NFκB inhibitors can prevent the induction of aging-associated genes by CDK inhibitors.

Agents that inhibit the induction of transcription by CDK inhibitors may be clinically useful for chemoprevention or slowing down the development of age-related diseases, including Alzheimer's disease, amyloidosis, atherosclerosis and arthritis. In addition, such compounds, through their effects on the expression of secreted growth factors (such as CTGF) may have value in cancer therapy or prevention. In fact, the available clinical data on NSAIDs with NFκB -inhibitory activity support these fields of use. Thus, several NSAID, including sulindac, aspirin and salicylate, were shown to have chemopreventive value in colorectal carcinomas and various other types of cancer and promoted the disappearance of colonic polyps (Lee *et al.*, 1997, "Use of aspirin and other nonsteroidal anti-inflammatory drugs and the risk of cancer development." *in* DeVita *et al.*, eds., CANCER. PRINCIPLES & PRACTICE OF ONCOLOGY, Lippincott-Raven: Philadelphia, pp. 599-607). The use of aspirin and other NSAIDs was also shown to decrease the risk of Alzheimer's disease (Stewart *et al.*, 1997, *Neurology* 48: 626-632). Long-term aspirin therapy was further reported to decrease the incidence of atherosclerosis (Sloop, 1998, *Angiology* 49 : 827-832). Finally, sulindac has been one of the most commonly used drugs with proven clinical efficacy in the treatment of arthritis (Brogden *et al.*, 1978, *Drugs* 16: 97-114). While some of these beneficial effects of NSAIDs have been attributed to their activity as cyclooxygenase 2 inhibitors (Pennisi,

1998, *Science* 280: 1191-1192), the results disclosed herein suggest that these clinical activities may also be due to the inhibition of p21-induced gene expression, presumably through the NFκB -inhibitory activity of these compounds. The assays and screening system provided by the instant invention enable one with ordinary skill in the art to test various NSAID derivatives for the improvement in this activity. Furthermore, these results provide the basis for using the general category of NFκB and p300/CBP inhibitors as agents for chemoprevention or treatment of cancer and age-related diseases.

EXAMPLE 12

Stimulation of the promoters of p21-inducible genes by p16 and p27.

As demonstrated in Example 7, expression of p21-inducible genes is also upregulated by other CDK inhibitors, $p16^{Ink4A}$ and $p27^{Kip1}$. To determine if the promoters of p21-inducible genes are stimulated by the latter CDK inhibitors, pNFkB-Luc and several of the promoter-luciferase constructs described in Example 8 (SAA, NK4, Complement C3 and prosaposin) were transfected into HT1080 derivatives with IPTG-inducible expression of p16 (HT1080 p16-5) or p27 (HT1080 p27-2), which are described in Example 6. For comparison, concurrent assays were carried out in HT1080 p21-9 cells with IPTG-inducible expression of p21. The effect of IPTG on the expression of these promoters was then analyzed as described for the p21-inducible line in Example 8. The specificity of the observed induction for NFκB was determined by cotransfection with the IKK inhibitor. As shown in Fig. 12, all the tested promoters were induced not only by p21 but also by p16 and p27. NFκB inhibitor IKK strongly inhibited the effects of all three CDK inhibitors on all the tested promoters, except for prosaposin (Fig. 12E), where IKK had only a weak effect with all three CDK inhibitors.

These findings indicate that p21-inducible promoters are activated not only by p21 but also by other CDK inhibitors, such as p16 and p27, and that the effects of the

CDK inhibitors are mediated to a large extent through NFκB.

EXAMPLE 13

p21 Induction of Viral Gene Promoters

The promoter of human immunodeficiency virus (HIV) was previously shown to be inducible by p21 in p300/CBP-dependent manner (Perkins *et al.*, 1997, *Science* 275: 523). To determine if p21 inducibility is a general property of different viral promoters, two other complete promoters of primate virus origin were tested for p21 inducibility and for the possible dependence of such inducibility on p300/CBP. These promoters, commonly used in mammalian expression vectors, are from CMV (enhancer and early promoter) and SV40 (early enhancer/promoter). These promoters were tested in pRL-CMV and pRL-SV40 constructs, which were obtained from Promega, Inc., and which express *Renilla* luciferase as the reporter gene.

pRL-CMV and pRL-SV40 were tested by transient transfection into HT1080 p21-9 cells (A.T.C.C. Accession No. PTA-1664) with isopropyl-β-thio-galactoside (IPTG)-inducible p21 expression, as described in Examples 1 and 2 above. To investigate the role of p300/CBP, vectors were used that expressed adenovirus protein E1A, which inhibits both p300/CBP and Rb. The reporter constructs were mixed at a molar ratio 1:2 with pcDNA3 vectors expressing the wild-type E1A, E1A mutant with a deletion of CR2 domain required for Rb inhibition, (E1AΔCR2), or a non-functional truncated form of E1A with deletions of the N-terminus (the portion of the E1A protein responsible for p300/CBP inhibition) and ΔCR2 (E1AΔN/ΔCR2) (as disclosed in Example 10 above).

After transfection (by electroporation as described in Example 3 above), cells were treated with 50 μM IPTG or untreated for three days (in triplicates). *Renilla*

luciferase activity was measured using Promega *Renilla* luciferase assay kit. The results of this analysis are shown in the following Table.

Table V

Reporter plasmid	Co-transfected with	50 μM IPTG (+ or -)	*Renilla* luciferase units (mean ± SD)	Fold induction by IPTG
pRL-CMV	E1A (w.t.)	-	671±3	
pRL-CMV	E1A (w.t.)	+	758±8	1.1
pRL-CMV	E1AΔCR2	-	654±26	
pRL-CMV	E1AΔCR2	+	927±9	1.4
pRL-CMV	E1AΔN/ΔCR2	-	915±7	
pRL-CMV	E1AΔN/ΔCR2	+	2970±100	3.2
pRL-SV40	E1A (w.t.)	-	326±3	
pRL-SV40	E1A (w.t.)	+	716±10	2.2
pRL-SV40	E1AΔCR2	-	318±9	
pRL-SV40	E1AΔCR2	+	1329±16	4.2
pRL-SV40	E1AΔN/ΔCR2	-	1308±8	
pRL-SV40	E1AΔN/ΔCR2	+	8433±109	6.4

The results of the above assays demonstrated that both CMV and SV40 promoters are inducible by p21 in mixtures with a non-functional control (E1AΔN/ΔCR2). Both wild-type E1A and E1AΔCR2, which inhibit p300/CBP, also inhibited induction of the CMV promoter by p21, but they did not have a major effect on the activity of this promoter when p21 was not induced. Wild-type E1A and E1AΔCR2 also strongly inhibit the SV40 promoter, with or without p21 induction. These results indicated that p21 induction of both CMV and SV40 promoters may require p300/CBP, although the role of other E1A-binding proteins cannot be excluded. The SV40 promoter but not CMV also requires p300/CBP or some other E1A-binding proteins for its basal expression.

Thus, all three tested promoters from different primate viruses (CMV, SV40 and HIV, tested by Perkins et al, 1997) have shown inducibility by p21. Interestingly, p21 induction, one of the most general stress responses in human cells, is also a frequent corollary of viral infection. For example, hepatitis C virus induces p21 expression in p53-independent manner (as disclosed in Majumder *et al.*, 2001, *J. Virol.* 75: 1401-7),

and Hepatitis B virus-X protein upregulates p21 expression (Park *et al.*, 2000, *Oncogene* 19: 3384). Overexpression of p21 is also observed in human T-cell lymphotropic virus type 1-infected cells (de La Fuente *et al.*, 2000, *J. Virol.* 74: 7270). CMV infection produces a transient increase in p21 expression, followed by p21 downregulation at a later stage of infection (Chen *et al.*, *J. Virol.* 75: 3613). Schmidt-Grimminger *et al.* (1998, *Am. J. Pathol.* 152: 1015) observed p21 expression in different papillomavirus-infected human tissues and proposed that p21 induction is a host response that inhibits viral DNA replication. Taking advantage of this host response to increase viral gene expression appears to be a reasonable and general evolutionary strategy for different viruses.

Based on these considerations, it is likely that compounds interfering with p21-mediated induction of gene expression will have therapeutic benefit in the treatment or prevention of different viral diseases.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

WE CLAIM:

1. A recombinant expression construct encoding a reporter gene operably linked to a promoter from a mammalian viral or cellular gene induced by a cyclin-dependent kinase inhibitor.

2. A recombinant expression construct according to Claim 1, wherein the reporter gene encodes firefly luciferase, Renilla luciferase, chloramphenicol acetyltransferase, beta-galactosidase, green fluorescent protein, or alkaline phosphatase.

3. A recombinant expression construct according to Claim 1, wherein the promoter is a promoter from a human viral or cellular gene induced by a CDK inhibitor.

4. A recombinant expression construct according to Claim 3, wherein the promoter is a promoter from a human gene identified in Table II or Table V.

5. A recombinant expression construct according to Claim 2, wherein the promoter is a promoter from a serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin β-3 (SEQ ID NO: 4), activin A (SEQ ID NO: 5), natural killer cell protein 4 (SEQ ID NO: 6), prosaposin (SEQ ID NO: 7), Mac2 binding protein (SEQ ID NO: 8), galectin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulin/epithelin (SEQ ID NO: 11), $p66^{shc}$ (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14), β-amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (EQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19), tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20), a promoter comprising one or a multiplicity of tandemly-repeated NFκB recognition

sequences, SV40 early promoter (SEQ ID NO. 81), human immunodeficiency virus promoter (SEQ ID NO. 85) or cytomegalovirus early promoter (SEQ ID NO.82).

6. A recombinant expression construct according to claim 4, wherein the promoter is a promoter from human natural killer cell protein 4 (SEQ ID NO: 6), serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), tissue transglutaminase (SEQ ID NO: 16), β-amyloid precursor protein (SEQ ID NO: 15), prosaposin (SEQ ID NO: 7), SV40 early promoter (SEQ ID NO. 81), human immunodeficiency virus promoter (SEQ ID NO. 85) or cytomegalovirus early promoter (SEQ ID NO.82).

7. A recombinant expression construct according to claim 4, wherein the recombinant expression construct is pLuNK4.

8. A mammalian cell comprising a recombinant expression construct according to claim 1, 2, 3, 4, 5, 6 or 7.

9. The mammalian cell of claim 8, identified by A.T.C.C. Accession No. PTA 3381 (HT1080 LuNK4p21).

10. A mammalian cell according to Claim 8 wherein expression of the recombinant expression construct is modulated by NFκB.

11. A mammalian cell according to claim 8, further comprising a second recombinant expression construct encoding a mammalian CDK inhibitor gene.

12. A mammalian cell according to claim 11, wherein expression of the CDK inhibitor is experimentally-induced in the mammalian cell.

13. The mammalian cell of claim 11, wherein the recombinant expression construct encoding a mammalian CDK inhibitor gene is under the transcriptional control of an inducible promoter, wherein expression of the CDK inhibitor from the

recombinant expression construct is mediated by contacting the recombinant cell with an inducing agent that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such a promoter.

14. The mammalian cell of claim 13, wherein the mammalian CDK inhibitor gene is a human p21 gene or CDK-binding fragment thereof.

15. The mammalian cell of claim 13, wherein the mammalian CDK inhibitor gene is a human p16 gene or CDK-binding fragment thereof.

16. The mammalian cell of claim 13, wherein the mammalian CDK inhibitor gene is a mouse or human p27 gene or CDK-binding fragment thereof.

17. A mammalian cell according to claim 13, further comprising a recombinant expression construct encoding a bacterial lactose repressor, wherein transcription thereof is controlled by a mammalian promoter, wherein the recombinant expression construct encoding a mammalian CDK inhibitor gene comprises a lactose repressor-responsive promoter element and wherein transcription of the CDK inhibitor gene is controlled by said lactose-repressor responsive promoter element, and wherein expression of the CDK inhibitor gene from the recombinant expression construct is mediated by contacting the recombinant cell with a lactose repressor-specific inducing agent.

18. The mammalian cell of claim 8, wherein the cell is a human HT1080 fibrosarcoma cell.

19. The mammalian cell of claim 11, wherein the cell is a human HT1080 fibrosarcoma cell.

20. The mammalian cell of claim 17, wherein the cell is a human HT1080

fibrosarcoma cell.

21. The mammalian cell of claim 11, wherein the second expression construct is LNp21CO3.

22. The mammalian cell of claim 21, identified by A.T.C.C. Accession No. PTA 1664 (HT1080 p21-9).

23. The mammalian cell of claim 11, wherein the second expression construct is LNp16RO2.

24. The mammalian cell of claim 23, identified by A.T.C.C. Accession No. PTA-4020 (HT1080 p16-5).

25. The mammalian cell of claim 11, wherein the second expression construct is LNp27RO2.

26. The mammalian cell of claim 25, identified by A.T.C.C. Accession No. PTA-4021 (HT1080 p27-2).

27. The mammalian cell of claim 17, wherein the lactose repressor-specific inducing agent is a β-galactoside.

28. A method for identifying a compound that inhibits induction of viral or cellular genes induced by a CDK inhibitor in a mammalian cell, the method comprising the steps of:

(a) culturing a recombinant mammalian cell according to claim 8 under conditions that induce expression of viral or cellular genes induced by a CDK inhibitor in mammalian cells in the presence and absence of a compound;

(b) comparing reporter gene expression in said cell in the presence of the

compound with reporter gene expression in said cell in the absence of the compound; and

(c) identifying the compound that inhibits induction of genes induced by a CDK inhibitor if reporter gene expression is lower in the presence of the compound than in the absence of the compound.

29. The method of Claim 28, wherein the cell is cultured under conditions that induce expression of a CDK inhibitor in said cell.

30. The method of Claim 29, wherein the CDK inhibitor is p21, p27 or p16 or CDK-binding fragments thereof.

31. The method of Claim 28, wherein the cell further comprises a second recombinant expression construct encoding a mammalian CDK inhibitor gene.

32. The method of claim 31, wherein the second recombinant expression construct comprises a mammalian CDK inhibitor gene under the transcriptional control of an inducible promoter, wherein expression of the CDK inhibitor from the recombinant expression construct is mediated by contacting the recombinant cell with an inducing agent that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such promoter.

33. The method of claim 32, wherein the mammalian CDK inhibitor gene is a human p21 gene or CDK-binding fragment thereof.

34. The method of claim 32, wherein the mammalian CDK inhibitor gene is a human p16 gene or CDK-binding fragment thereof.

35. The method of claim 32, wherein the mammalian CDK inhibitor gene is a human p27 gene or CDK-binding fragment thereof.

36. The method of claim 32, wherein the cell is a human HT1080 fibrosarcoma cell.

37. The method of claim 32, wherein the mammalian cell further comprises a recombinant expression construct encoding a bacterial lactose repressor, wherein transcription thereof is controlled by a mammalian promoter, wherein the recombinant expression construct encoding a mammalian CDK inhibitor gene comprises a lactose repressor-responsive promoter element and wherein transcription of the CDK inhibitor gene is controlled by said lactose-repressor responsive promoter element, and wherein expression of the CDK inhibitor gene from the recombinant expression construct is mediated by contacting the recombinant cell with a lactose repressor-specific inducing agent.

38. A method for identifying a compound that inhibits CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the steps of:

(a) producing expression of a CDK inhibitor in a mammalian cell;

(b) assaying the cell in the presence of the compound for changes in expression of cellular genes whose expression is modulated by the CDK inhibitor; and

(c) identifying the compound as an inhibitor of CDK inhibitor-mediated modulation of viral or cellular gene expression if expression of the cellular genes of subpart (b) is changed to a lesser extent in the presence of the compound.

39. The method of claim 38 wherein the CDK inhibitor is p16, p27 or p21.

40. The method of Claim 39, wherein the mammalian cell comprises a recombinant expression construct encoding a mammalian CDK inhibitor under the

transcriptional control of an inducible heterologous promoter, wherein expression of the CDK inhibitor from the recombinant expression construct is mediated by contacting the recombinant cell with an inducing agent that induces transcription from the inducible promoter or by removing an agent that inhibits transcription from such promoter.

41. The method of Claim 40, wherein the CDK inhibitor is p16.

42. The method of Claim 40, wherein the CDK inhibitor is p21.

43. The method of Claim 40, wherein the CDK inhibitor is p27.

44. The method of claim 38, wherein expression of the viral or cellular gene is induced by p21.

45. The method of claim 38, wherein expression of the viral or cellular gene is induced by p16.

46. The method of claim 38, wherein expression of the viral or cellular gene is induced by p27.

47. The method of claim 38, wherein the viral or cellular gene is identified in Table II or Table V.

48. The method of claim 40, wherein the viral or cellular gene is identified in Table II or Table V.

49. The method of claim 38, wherein expression of the viral or cellular gene is detected using an immunological reagent.

50. The method of claim 38, wherein expression of the viral or cellular gene is detected by assaying for an activity of the cellular gene product.

51. The method of claim 38, where expression of the viral or cellular gene is

detected by hybridization to a complementary nucleic acid.

52. A method for identifying a compound that inhibits CDK inhibitor-mediated induction of viral or cellular gene expression in a mammalian cell, the method comprising the steps of:

(a) treating the mammalian cell in the presence and absence of the compound with an agent or culturing the mammalian cell under conditions that induce senescence;

(b) assaying the mammalian cell for induction of viral or cellular genes that are induced by CDK inhibitor gene expression; and

(c) identifying the compound as an inhibitor of CDK inhibitor-mediated induction of viral or cellular gene expression if genes that are induced by the CDK inhibitor are induced to a lesser extent, in the presence of the compound than in the absence of the compound.

53. The method of claim 52, wherein the CDK inhibitor is p21, p16 or p27.

54. The method of claim 52, wherein the genes are identified in Table II or Table V.

55. The method of claim 52, wherein expression of the gene is detected using an immunological reagent.

56. The method of claim 52, wherein expression of the gene is detected by assaying for an activity of the gene product.

57. The method of claim 52, where expression of the gene is detected by hybridization to a complementary nucleic acid.

58. A method for identifying a compound that inhibits CDK inhibitor-

mediated induction of viral or cellular gene expression in a mammalian cell, the method comprising the steps of:

(a) contacting a mammalian cell in the presence or absence of the compound with an agent or culturing the mammalian cell under conditions that induce senescence, wherein the cell comprises a reporter gene under the transcriptional control of a promoter for a mammalian viral or cellular gene whose expression is modulated by a CDK inhibitor;

(b) assaying the cell for changes in expression of the reporter gene; and

(c) identifying the compound as an inhibitor of CDK inhibitor-mediated induction of viral or cellular gene expression if expression of the reporter gene is changed to a lesser degree in the presence of the compound than in the absence of the compound.

59. The method of claim 58, wherein the CDK inhibitor is p21, p16 or p27.

60. The method of claim 58, wherein the mammalian gene promoter is a promoter of a mammalian gene identified in Table II or Table V.

61. The method of claim 58, wherein expression of the cellular gene is detected using an immunological reagent.

62. The method of claim 58, wherein expression of the cellular gene is detected by assaying for an activity of the cellular gene product.

63. The method of claim 58, where expression of the cellular gene is detected by hybridization to a complementary nucleic acid.

64. A method for inhibiting CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the step of contacting the cell with a

compound produced according to the method of claim 28.

65. A method for inhibiting CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 38.

66. A method for inhibiting CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 52.

67. A method for inhibiting CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 58.

68. A method for inhibiting CDK inhibitor-mediated induction of viral or cellular gene expression, the method comprising the step of contacting the cell with an effective amount of a compound that inhibits NFκB activity.

69. A method for treating a disease in an animal accompanied by CDK inhibitor induced gene expression, the method comprising the step of administering to the animal an effective amount of a non-steroidal anti-inflammatory drug (NSAID) that inhibits NFκB activity.

70. A method according to Claim 69, wherein the disease is cancer other than colon cancer.

71. A method according to Claim 69, wherein the disease is renal failure.

72. A method according to Claim 69, wherein the disease is Alzheimer's disease and the NSAID is other than aspirin or salicylate.

73 A method according to Claim 69, wherein the disease is atherosclerosis

and the NSAID is other than aspirin.

74. A method according to Claim 69, wherein the disease is arthritis and the NSAID is other than aspirin, sulindac or salicylate.

75. A compound that inhibits viral or cellular genes associated with pathogenic consequences of senescence in a mammalian cell, wherein the compound is produced by a method having the steps of:

(a) treating the mammalian cell in the presence of the compound with an agent or culturing the mammalian cell under conditions that induce senescence;

(b) assaying the mammalian cell for induction of cellular genes that are induced by CDK inhibitor gene expression; and

(c) identifying the compound as an inhibitor of senescence if genes that are induced by the CDK inhibitor are induced to a lesser extent, in the presence of the compound.

76. A compound of claim 69, wherein the CDK inhibitor is p21, p16 or p27.

77. A compound that inhibits production of viral or cellular gene products induced by a CDK inhibitor in a mammalian cell, wherein the compound is produced by a method having the steps of:

(a) treating the mammalian cell in the presence of the compound with an agent or culturing the mammalian cell under conditions that induce expression of a CDK inhibitor;

(b) assaying the mammalian cell for induction of viral or cellular genes that are induced by CDK inhibitor gene expression; and

(c) identifying the compound as an inhibitor of CDK inhibitor induction if genes that are induced by the CDK inhibitor are induced to a lesser extent, in the presence of the compound.

78. A compound of claim 77, wherein the CDK inhibitor is p21, p27 or p16.

79. A method for inhibiting production of anti-apoptotic or mitogenic factors in a mammalian cell, the method comprising the steps of contacting the cell with a compound that inhibits induction of gene expression by a CDK inhibitor.

80. The method of claim 79, wherein the mammalian cell is a stromal fibroblast.

81. The method of claim 79, wherein the compound is an NFκB inhibitor or a p300/CPB inhibitor.

82. A method for treating an animal to prevent or ameliorate the effects of a disease accompanied by CDK inhibitor induced gene expression, the method comprising the steps of administering to an animal in need thereof a therapeutically-effective dose of a pharmaceutical composition of a compound identified according to the method of claims 28, 38, 52, or 58.

83. A method for inhibiting or preventing expression of a gene induced by a CDK inhibitor in a mammalian cell, the method comprising the step of contacting the mammalian cell with an amount of a compound identified according to the method of claims 28, 38, 52, or 58 effective to inhibit or prevent expression of the a gene induced by a CDK inhibitor.

84. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering an NFκB inhibitor to an animal in need

of such treatment.

85. A method of claim 84, wherein the NFκB inhibitor is a non-steroidal anti-inflammatory compound.

86. The method of claim 85, wherein the animal is a human.

87. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 28.

88. The method of claim 87, wherein the animal is a human.

89. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 38.

90. The method of claim 89, wherein the animal is a human.

91. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 52.

92. The method of claim 91, wherein the animal is a human.

93. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 58.

94. The method of claim 93, wherein the animal is a human.

95. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a

compound produced by the method of claim 75.

96. The method of claim 95, wherein the animal is a human.

97. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claims 28, 38, 52, or 58.

98. The method of claim 97, wherein the animal is a human.

99. A method for selectively inhibiting induction of viral or cellular genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound according to claim 77.

100. The method of claim 99, wherein the animal is a human.

101. A method for treating a viral infection in an animal, the method comprising administering to the animal a therapeutically effective amount of a compound according to claim 77.

102 The method of claim 100, wherein the animal is a human.

103. A method for treating a viral infection in an animal, the method comprising administering to the animal a therapeutically effective amount of a compound produced by the method of claims 28, 38, 52, or 58.

104. The method of claim 100, wherein the animal is a human.

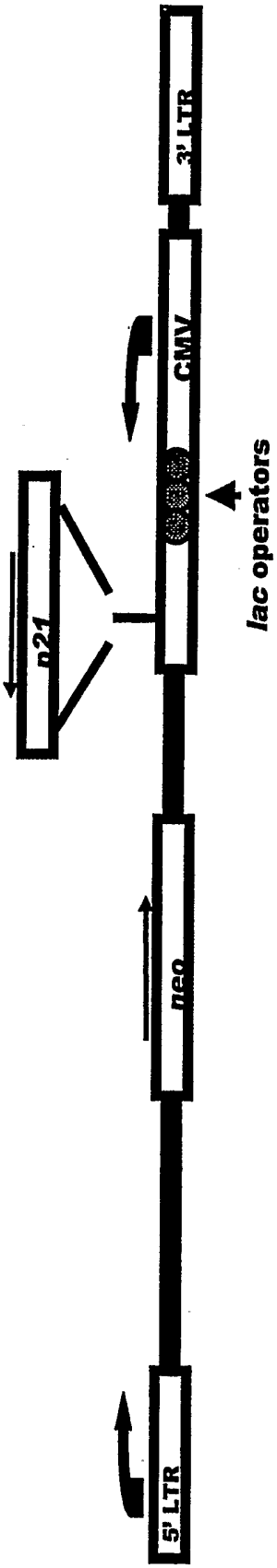


Figure 1. IPTG-regulated retroviral vector LNp21CO3

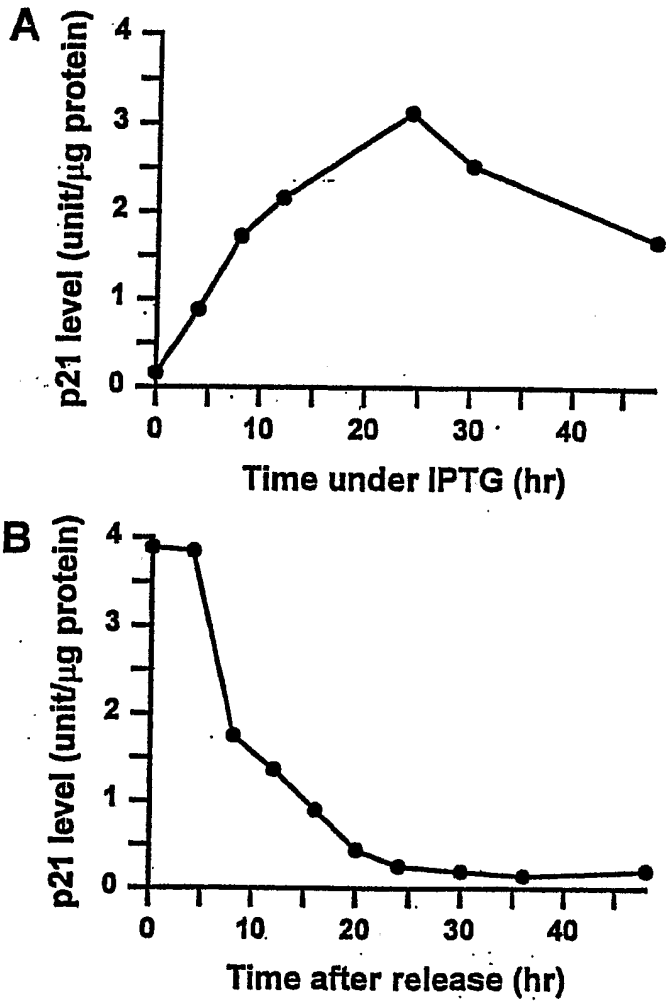

A
p21 level (unit/μg protein)
0
1
2
3
4
0
10
20
30
40
Time under IPTG (hr)
B
p21 level (unit/μg protein)
0
1
2
3
4
0
10
20
30
40
Time after release (hr)

Fig. 2

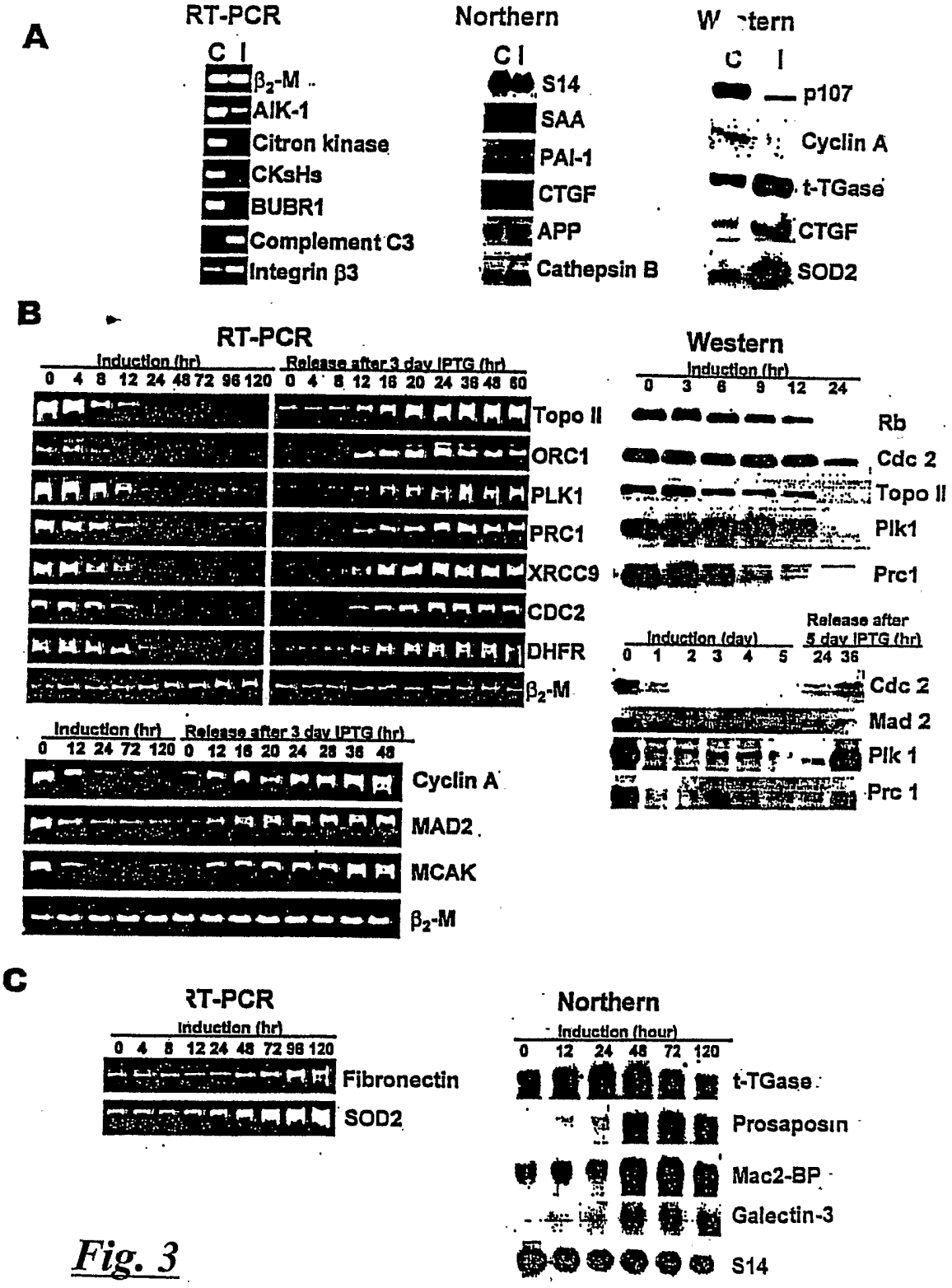
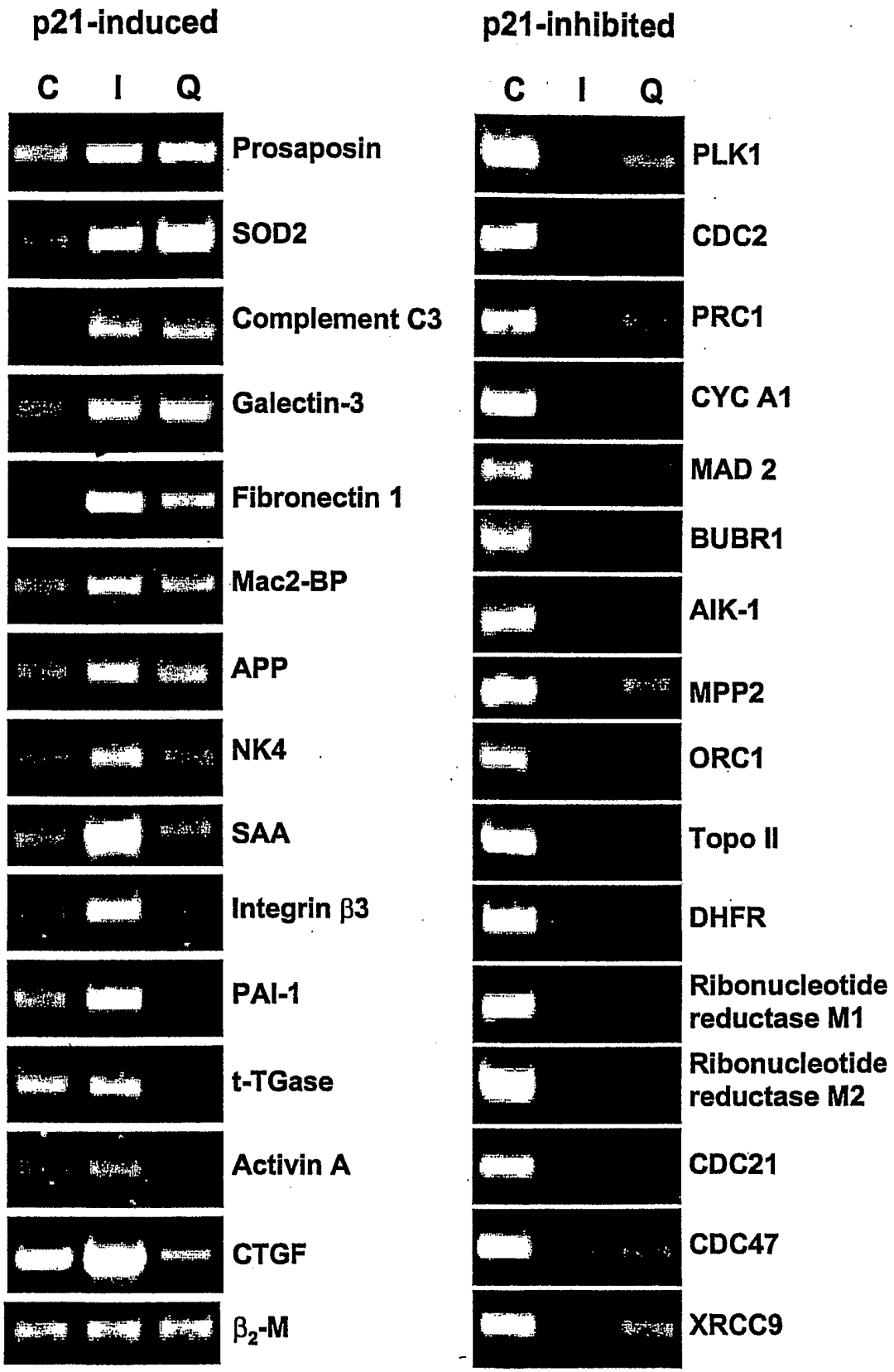

A
RT-PCR
C I
β2-M
AIK-1
Citron kinase
CKsHs
BUBR1
Complement C3
Integrin β3
Northern
C I
S14
SAA
PAI-1
CTGF
APP
Cathepsin B
W tern
C I
p107
Cyclin A
t-TGase
CTGF
SOD2
B
RT-PCR
Induction (hr)
0 4 8 12 24 48 72 96 120
Release after 3 day IPTG (hr)
0 4 8 12 16 20 24 36 48 60
Topo II
ORC1
PLK1
PRC1
XRCC9
CDC2
DHFR
β2-M
Western
Induction (hr)
0 3 6 9 12 24
Rb
Cdc 2
Topo II
Plk1
Prc1
Induction (day)
Release after 5 day IPTG (hr)
0 1 2 3 4 5 24 36
Cdc 2
Mad 2
Plk 1
Prc 1
Induction (hr)
Release after 3 day IPTG (hr)
0 12 24 72 120 0 12 16 20 24 28 36 48
Cyclin A
MAD2
MCAK
β2-M
C
RT-PCR
Induction (hr)
0 4 8 12 24 48 72 96 120
Fibronectin
SOD2
Northern
Induction (hour)
0 12 24 48 72 120
t-TGase
Prosaposin
Mac2-BP
Galectin-3
S14

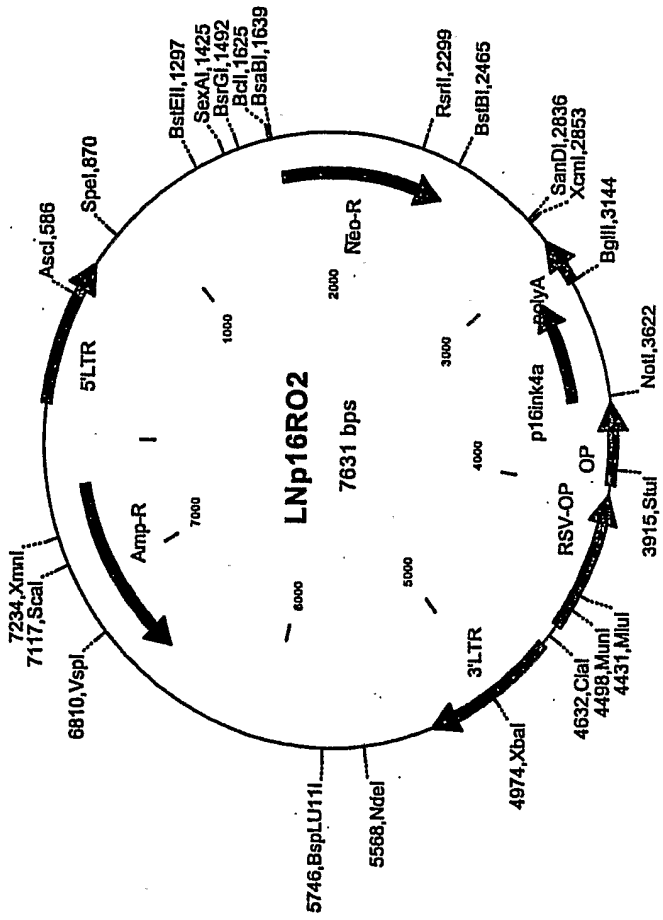
Fig. 3



C, control untreated cells; I, IPTG-treated,
Q, serum-starved (quiescent) cells

Fig. 3D

Figure 4


LNp16RO2
7631 bps
Neo-R
Amp-R
5'LTR
3LTR
RSV-OP
OP
p16ink4a
polyA
1000
2000
3000
4000
5000
6000
7000
AscI,586
SpeI,870
BstEII,1297
SexAI,1425
BsrGI,1492
BclI,1625
BsaBI,1639
RsrII,2299
BstBI,2465
SanDI,2836
XcmI,2853
BglII,3144
NotI,3622
3915,StuI
4431,MluI
4498,MunI
4632,ClaI
4974,XbaI
5568,NdeI
5746,BspLU11I
6810,VspI
7117,ScaI
7234,XmnI

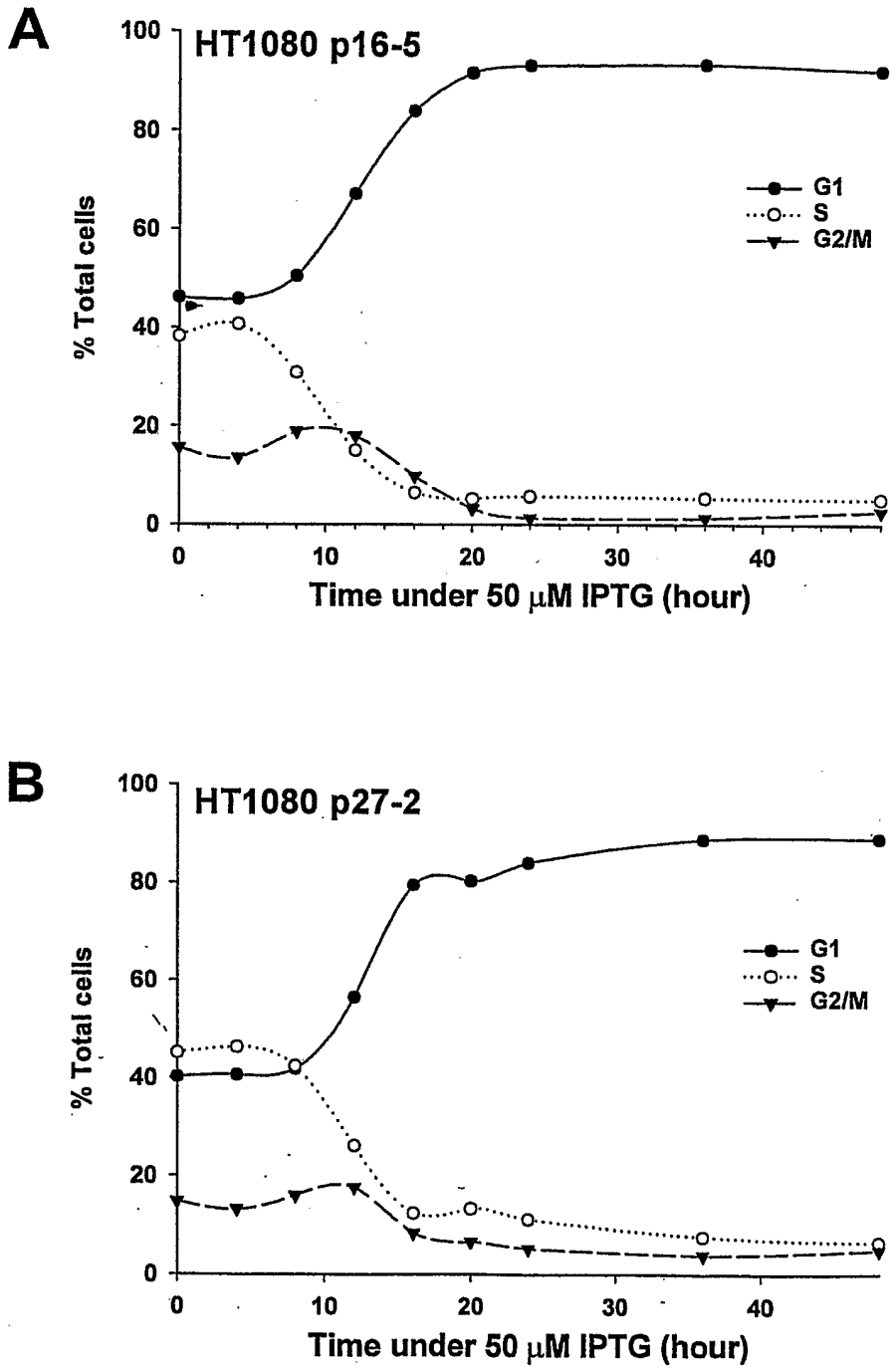

A
HT1080 p16-5
% Total cells
100
80
60
40
20
0
0
10
20
30
40
Time under 50 μM IPTG (hour)
G1
S
G2/M
B
HT1080 p27-2
% Total cells
100
80
60
40
20
0
0
10
20
30
40
Time under 50 μM IPTG (hour)
G1
S
G2/M

Fig. 5

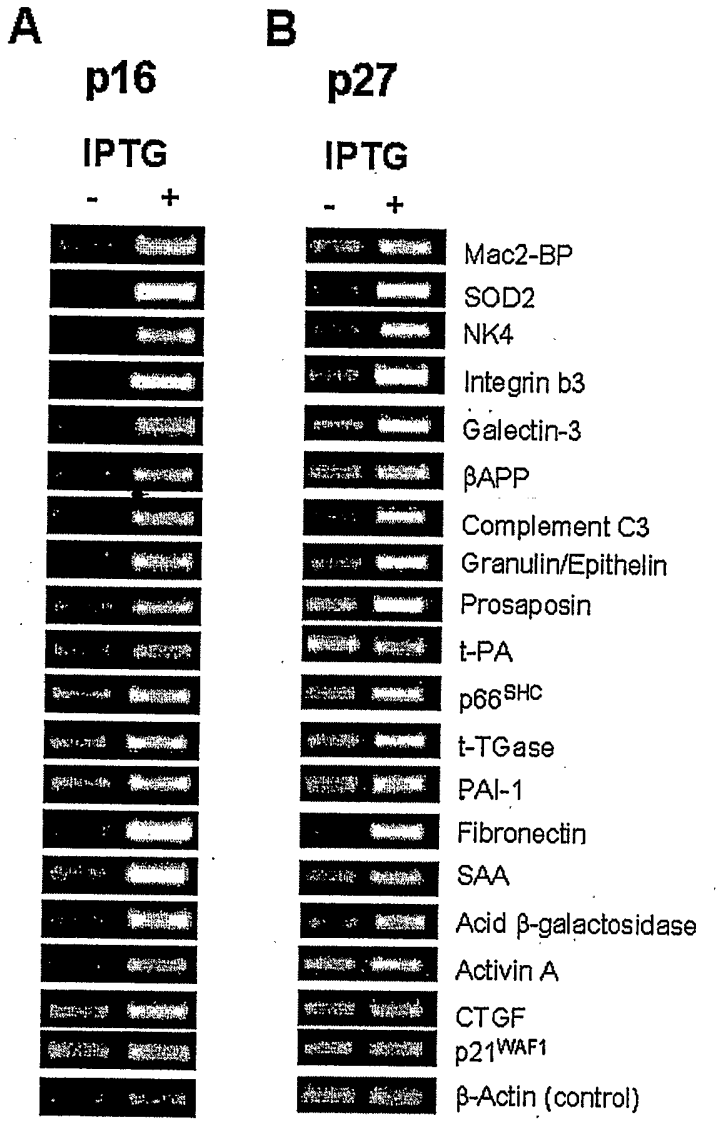

A
p16
IPTG
- +
B
p27
IPTG
- +
Mac2-BP
SOD2
NK4
Integrin b3
Galectin-3
βAPP
Complement C3
Granulin/Epithelin
Prosaposin
t-PA
p66SHC
t-TGase
PAI-1
Fibronectin
SAA
Acid β-galactosidase
Activin A
CTGF
p21WAF1
β-Actin (control)

Fig. 6

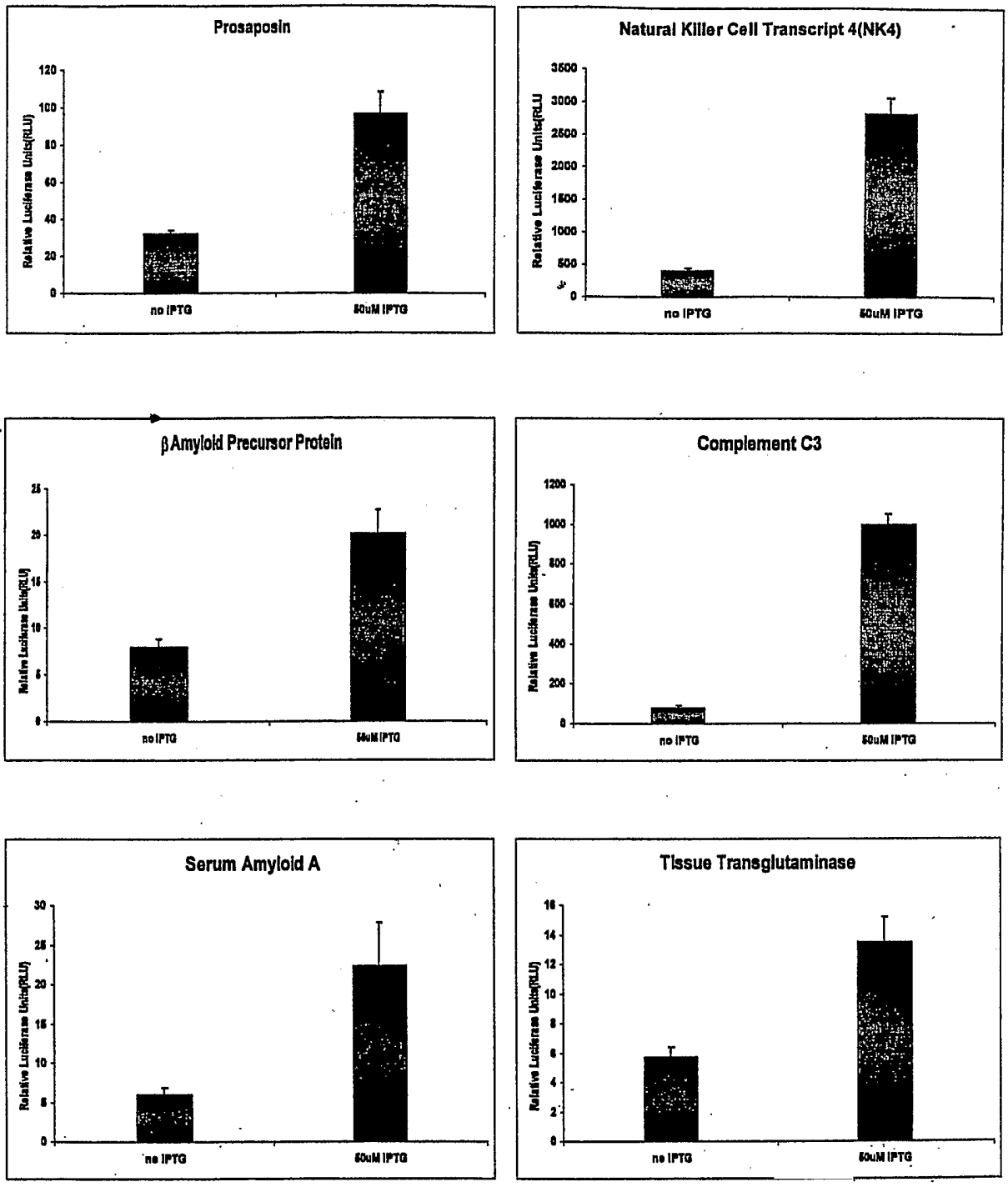

Prosaposin
Relative Luciferase Units(RLU)
120
100
80
60
40
20
0
no IPTG
50uM IPTG
Natural Killer Cell Transcript 4(NK4)
Relative Luciferase Units(RLU)
3500
3000
2500
2000
1500
1000
500
0
no IPTG
50uM IPTG
β Amyloid Precursor Protein
Relative Luciferase Units(RLU)
no IPTG
50uM IPTG
Complement C3
Relative Luciferase Units(RLU)
1200
1000
800
600
400
200
0
no IPTG
50uM IPTG
Serum Amyloid A
Relative Luciferase Units(RLU)
30
25
20
15
10
5
0
no IPTG
50uM IPTG
Tissue Transglutaminase
Relative Luciferase Units(RLU)
16
14
12
10
8
6
4
2
0
no IPTG
50uM IPTG

Figure 7

Figure 8

IPTG dose-dependence (24 hrs treatment) (A) and time course (50 μM IPTG) (B) of luciferase expression in HT1080 LuNK4p21 cells

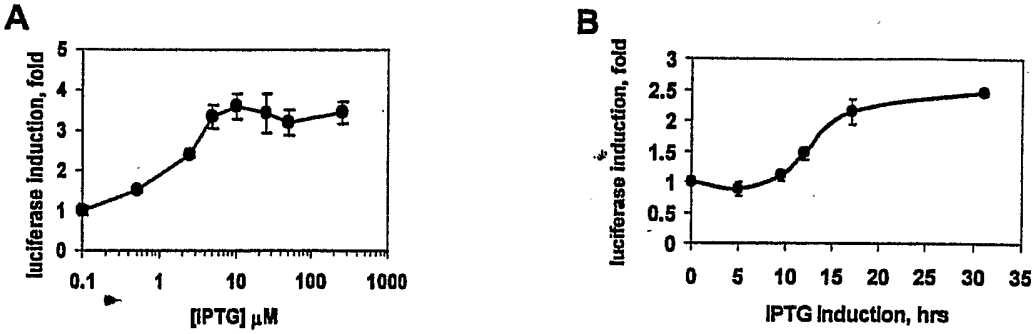
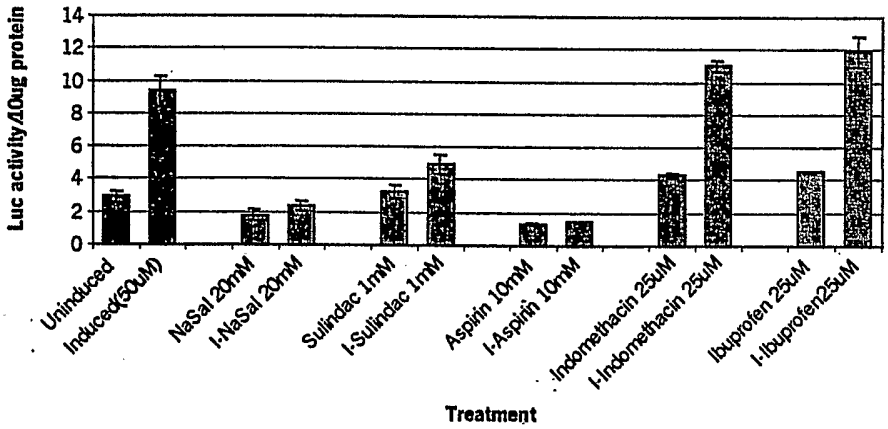
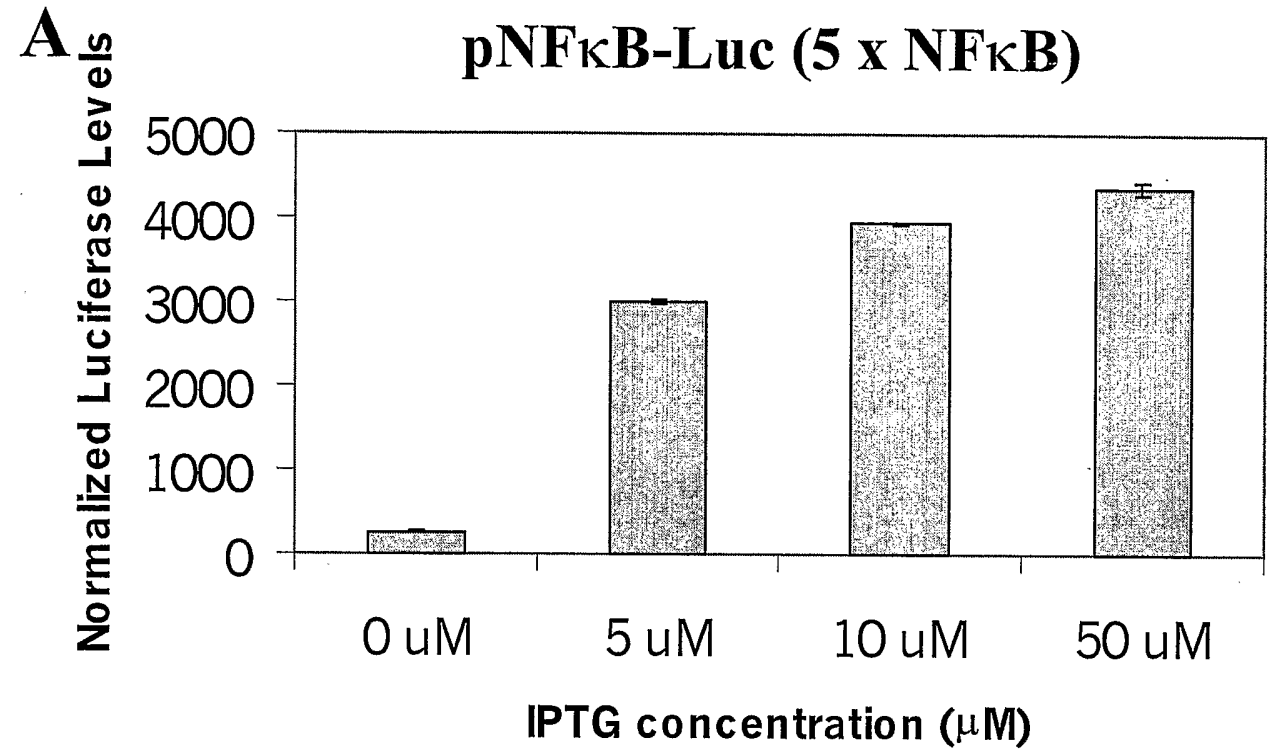


Figure 10

Effects of the indicated NSAID on luciferase expression in HT1080 LuNK4p21 cells, untreated or treated (I) with 50 μM IPTG




A
pNFκB-Luc (5 x NFκB)
Normalized Luciferase Levels
5000
4000
3000
2000
1000
0
0 uM
5 uM
10 uM
50 uM
IPTG concentration (μM)

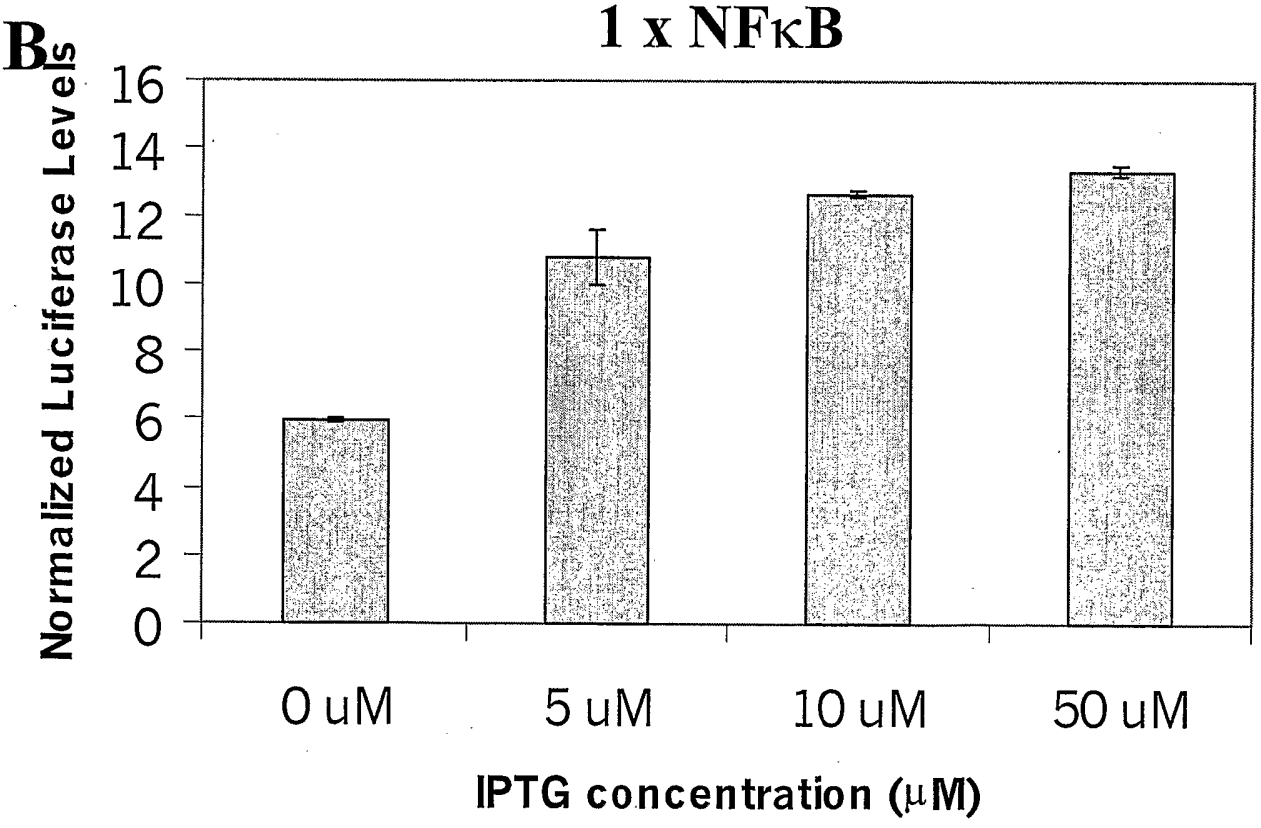
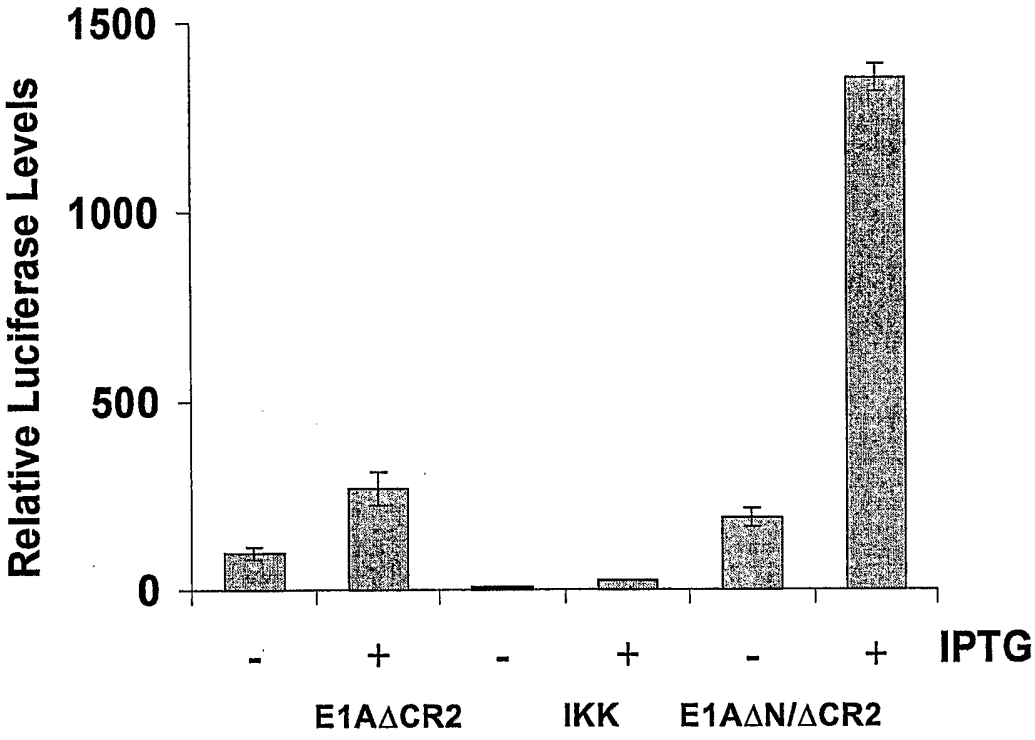

B
1 x NFκB
Normalized Luciferase Levels
16
14
12
10
8
6
4
2
0
0 uM
5 uM
10 uM
50 uM
IPTG concentration (μM)

FIGURE 9

C Nuclear Factor-kB



D Serum Amyloid A

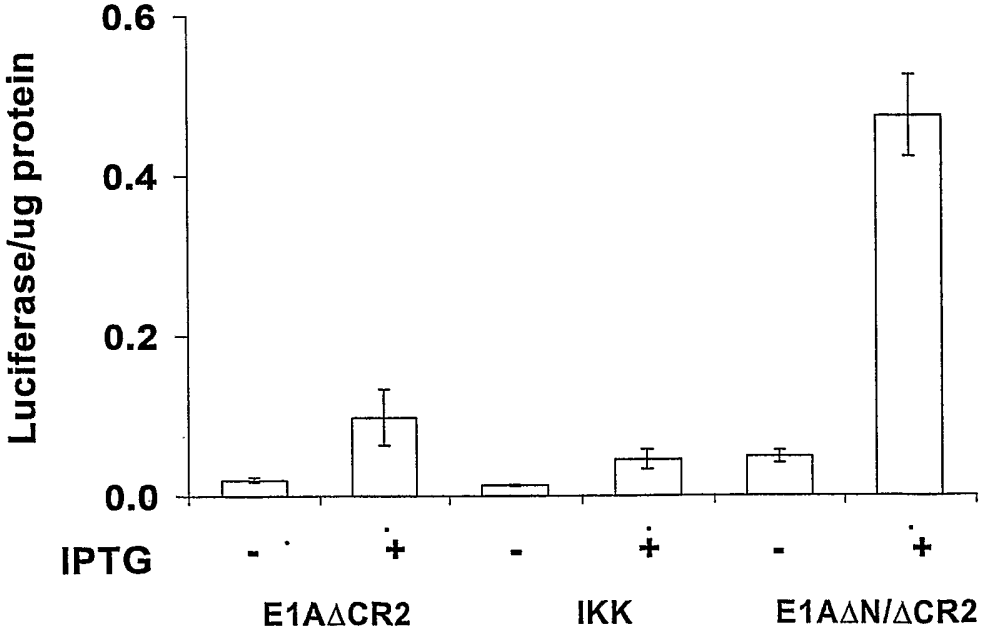
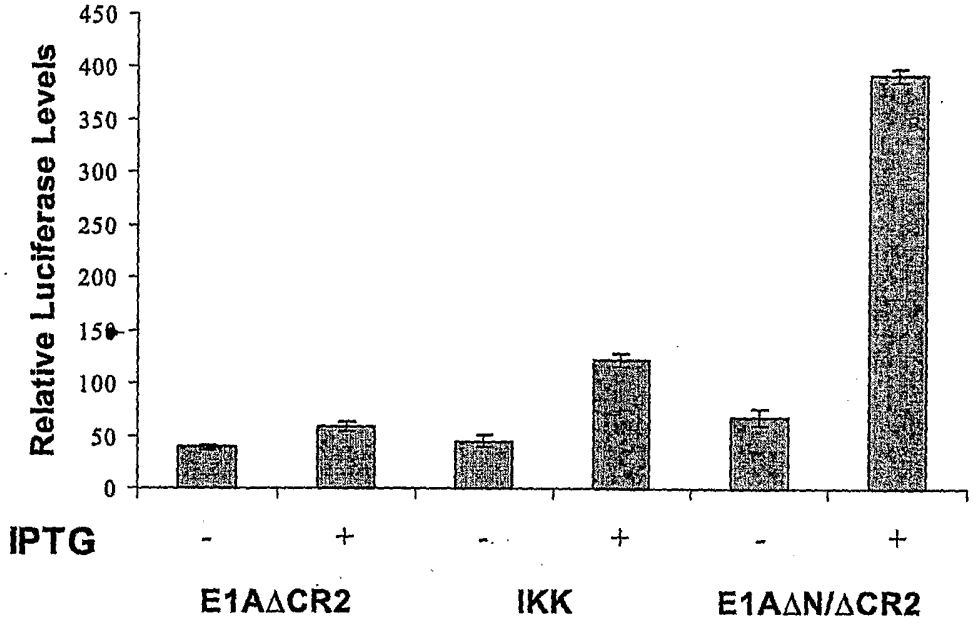


Fig. 9

E Prosaposin



E β-Amyloid Precursor Protein

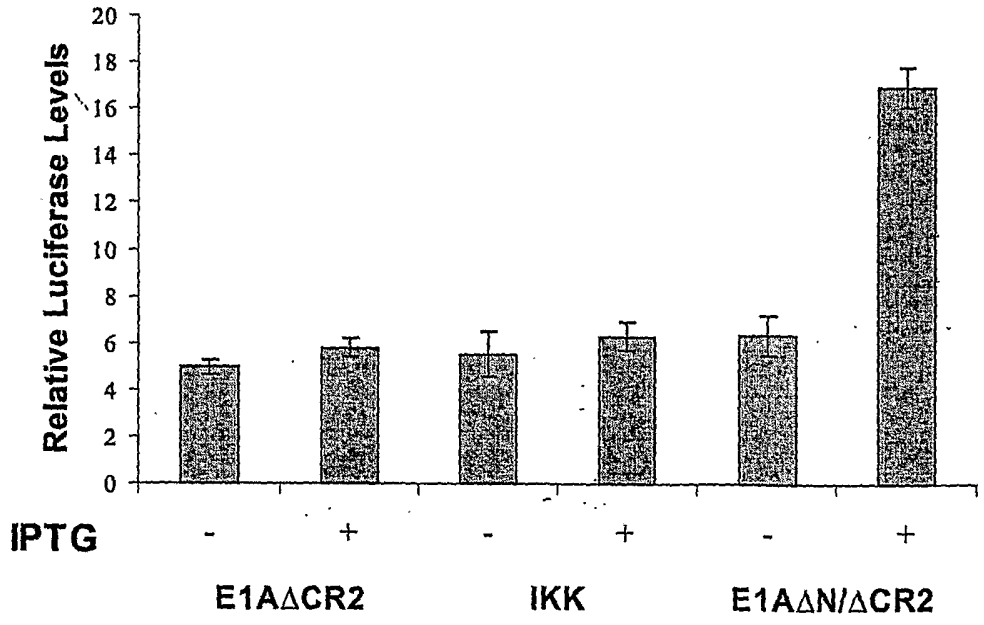
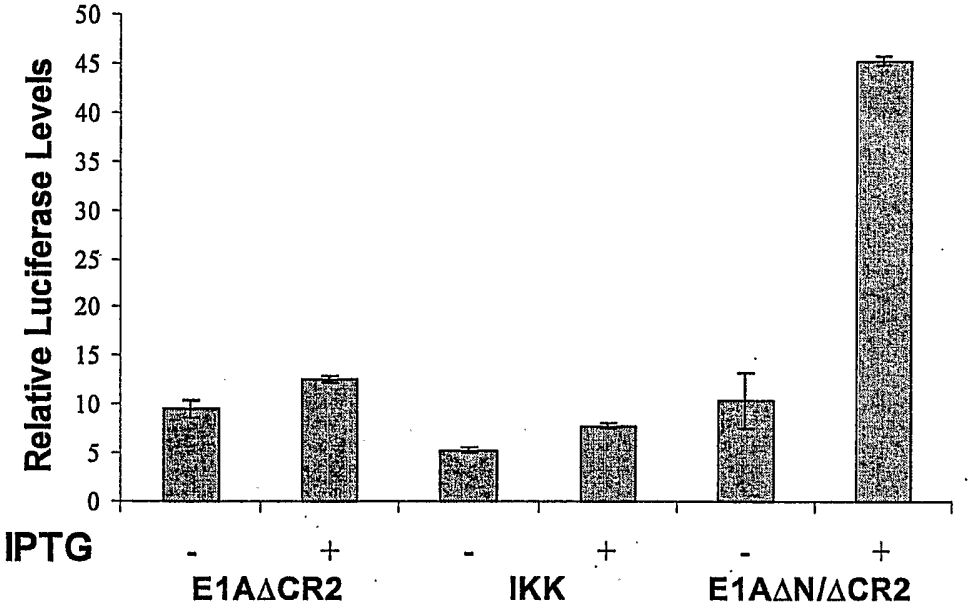


Fig. 9.

G Tissue Transglutaminase



H Complement C3

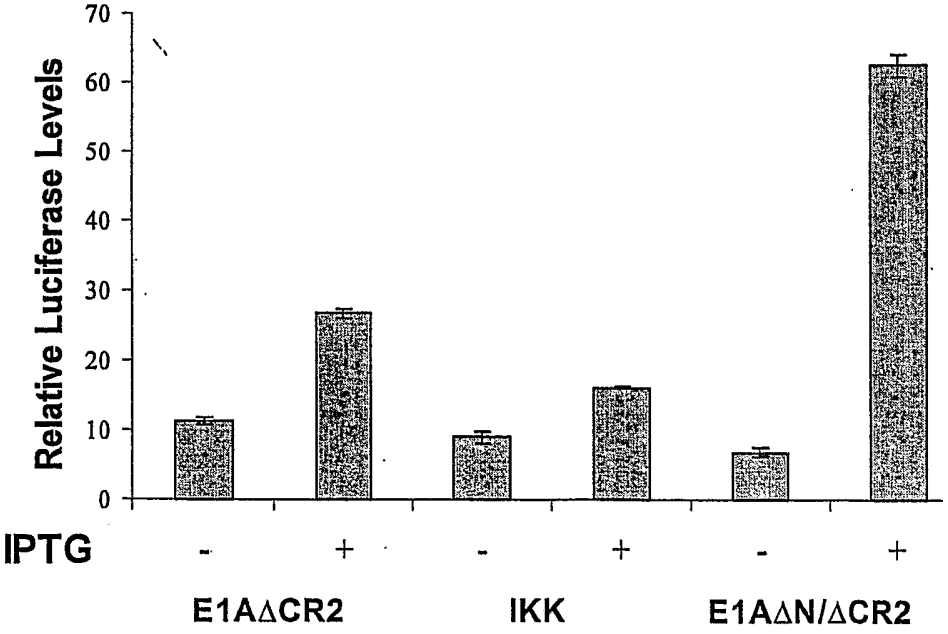


Fig. 9

I NK Cell Transcript 4 (NK4)

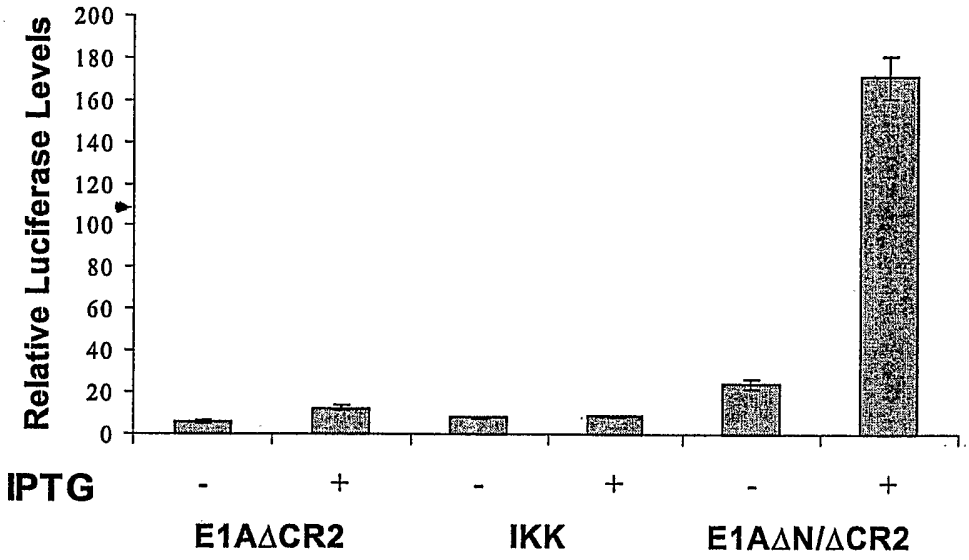
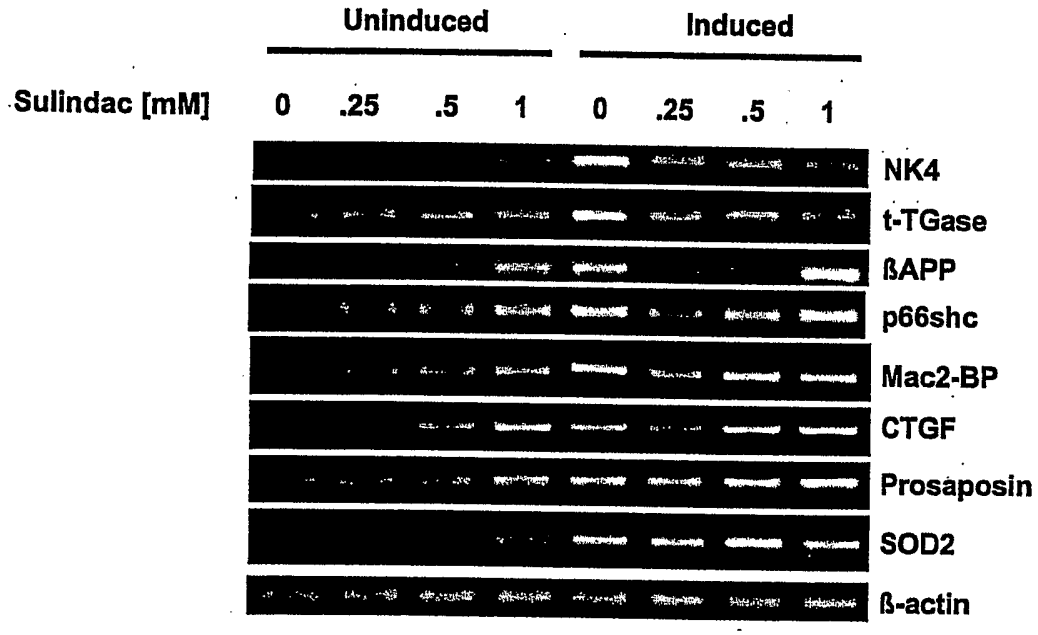


Fig. 9

Figure 11

RT-PCR analysis of LuNK4p21 cells uninduced or induced with IPTG for two days in the presence of the indicated concentrations of Sulindac



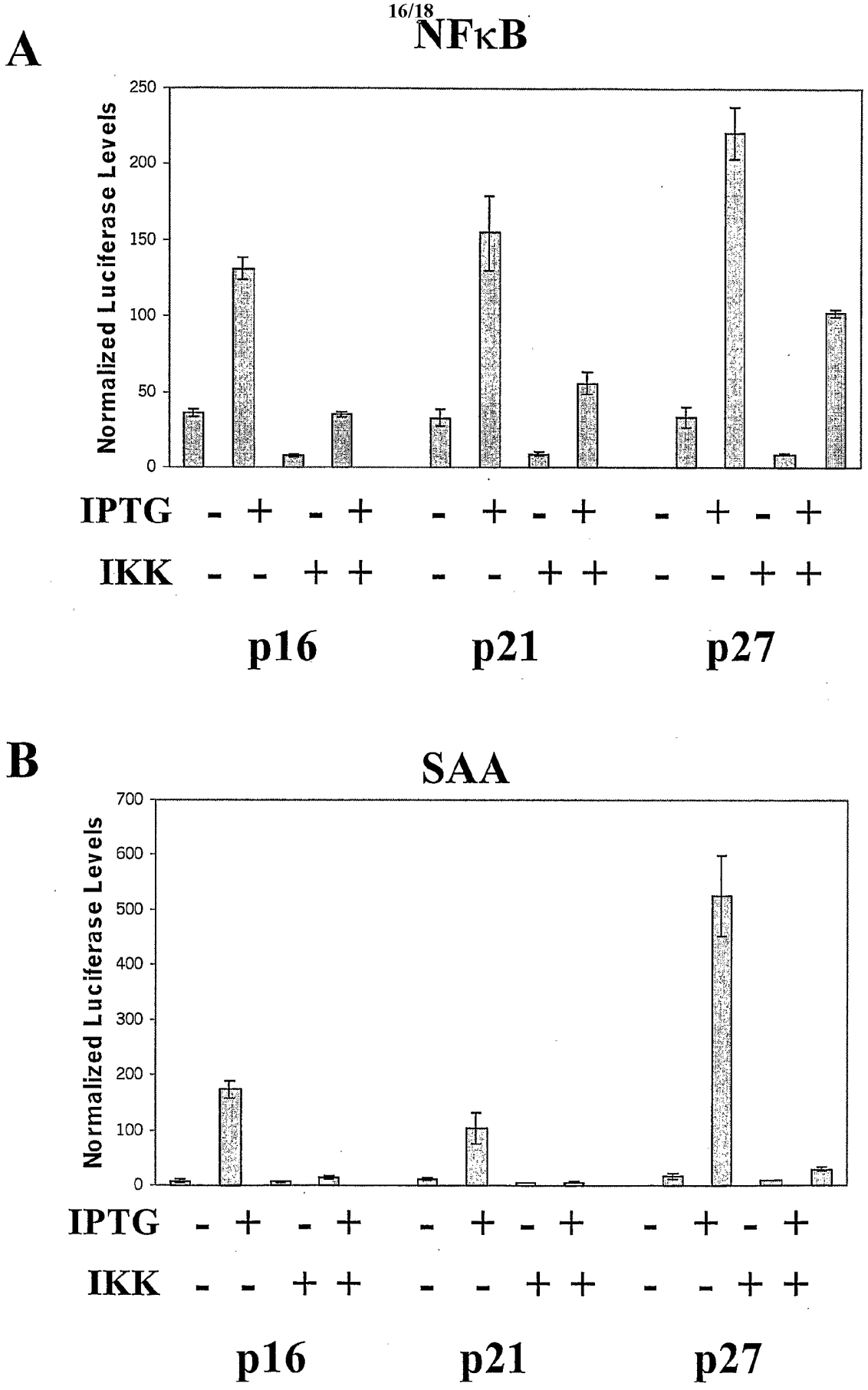
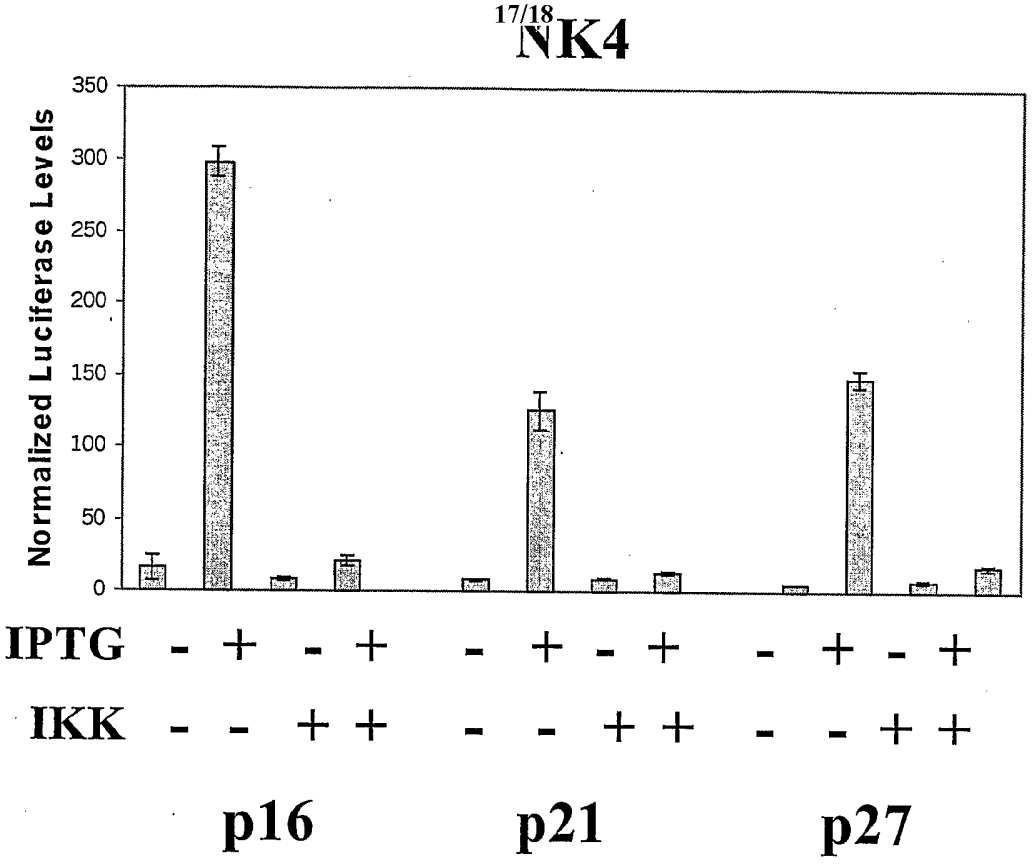

A
NFκB
Normalized Luciferase Levels
250
200
150
100
50
0
IPTG - + - + - + - + - + - + - +
IKK - - + + - - + + - - + +
p16
p21
p27
B
SAA
Normalized Luciferase Levels
700
600
500
400
300
200
100
0
IPTG - + - + - + - + - + - + - +
IKK - - + + - - + + - - + +
p16
p21
p27

FIGURE 12

C


NK4
Normalized Luciferase Levels
350
300
250
200
150
100
50
0
IPTG - + - + - + - + - + - + - +
IKK - - + + - - + + - - + +
p16
p21
p27

D

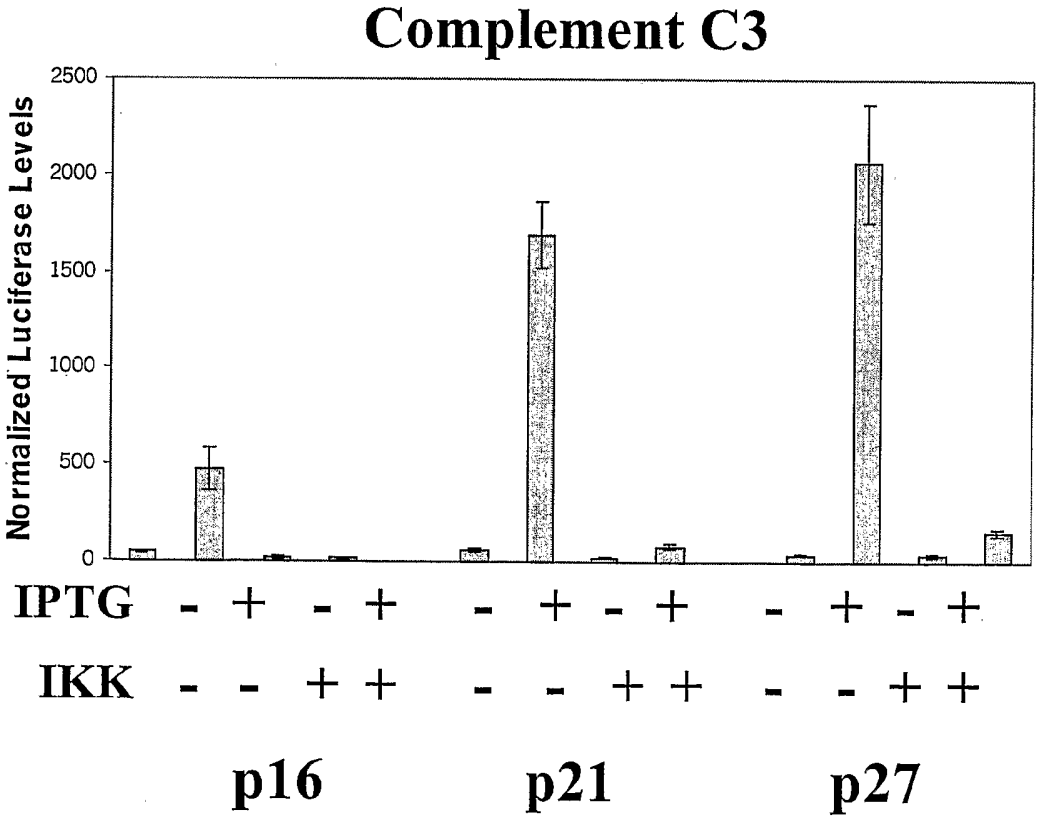

Complement C3
Normalized Luciferase Levels
2500
2000
1500
1000
500
0
IPTG - + - + - + - + - + - + - +
IKK - - + + - - + + - - + +
p16
p21
p27

FIGURE 12

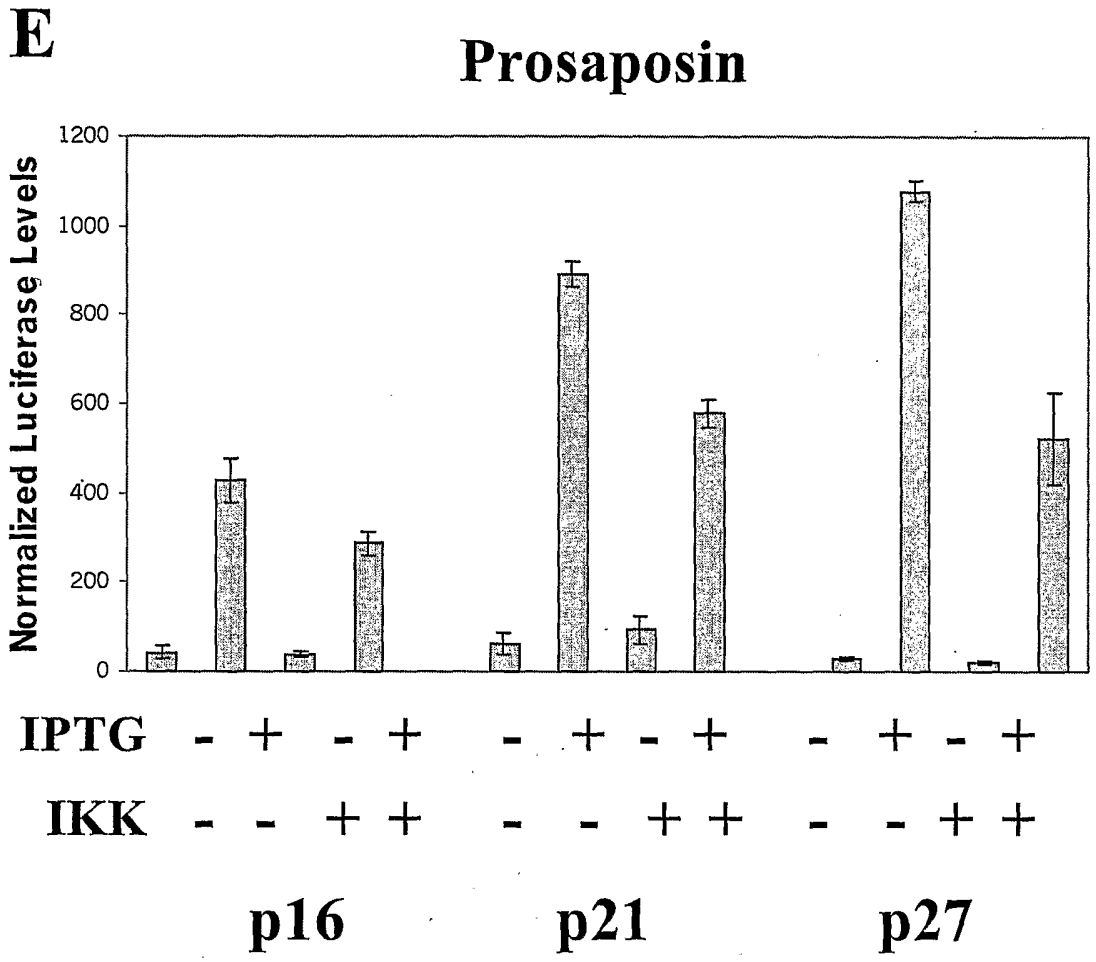

E
Prosaposin
Normalized Luciferase Levels
1200
1000
800
600
400
200
0
IPTG - + - + - + - + - + - + - +
IKK - - + + - - + + - - + +
p16
p21
p27

FIGURE 12

SEQUENCE LISTING

<110> Board of Trustees of the University of Illinois
Poole, Jason
Roninson, Igor

<120> Reagents And Methods For Identifying And Modulating Expression Of Genes Regulated By CDK Inhibitors

<130> 01-1156-B

<140> PCT/US02/
<141> 2002-02-01

<150> US 09/861,925
<151> 2002-05-21

<150> US 60/265,840
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<170> PatentIn version 3.0

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ttgttattac aaatgcctag cccctcaggg tatgaacatg gactggagaa gaagaaacca 180

gagttgctgc tatgtccacc agcctctctg catgtcctgg cctcagcccc cctgggctct 240

ggtactgacc catctctggc caccatgctc ctccataagc ctctgcagag ctaatctgac 300

cctgttgatg ttctcatgag agagtgatct gaatgccccc tgaacccctc cgtgataata 360

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ccgttttaaa gcttaaagct ttagacatga actcacaggg acttcagtca gggtcatctg 720

ccatgtggcc cagcagggcc catcctgagg aaatgaccgg tatagtcagg agctggctga 780

agagctgccc tcactccaca ccttccagca gcccaggtgc cgccatcacg gggctcccac 840

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tccattgcca aggcacagca aacctctctt gttcccatag gttacacaac tgggataaat 960

gacccgggat gaagaaacca [redacted] 1020

aatgacctgc agggactttc cccagggacc acatccagct tttcttccct cccaagagac 1080

cagcaaggct cactataaat agcagccacc tctccctggc agacagggac ccgcagctca 1140

gctacagcac agatcaggtg aggagcacac aaggagtgat ttttaaaact tactctgttt 1200

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acaaacaata caatttaata tcctaagagg atattgacat tagacaggta caaaagctct 120

agaaatgagg actttcctca gtgatgactt ttttcaccac caaagtcact caggcatcct 180

gacaagggta agtgagggga gcctccttgg aaaataaact cacttggata gtgaactcct 240

gcacatacct caaagcccat ctgaaatgtc ccctcctaca ggaagttttc cctgaccctc 300

caagaagcag agttctattt cactggggaa aacatttctt cttcttcttt tttttccctg 360

ccctgcacat gagctagaaa acatttcatg aaactgggag tttctgtgct gggctctgtc 420

cctcccccat tctacttccc ctccctcagc atggaagcct ctggaagtgg ggctctgact 480

cccagcctac agagagattc ctaggaagtg ttcgactgat aaacgcatgg ccaaaagtga 540

actggggatg aggtccaaga catctgcggt ggggggttct ccagacctta gtgttcttcc 600

actacaaagt gggtccaaca gagaaaggtc tgtgttcacc aggtggccct gaccctggga 660

gagtccaggg cagggtgcag ctgcattcat gctgctgggg aacatgccct caggttactc 720

accccatgga catgttggcc ccagggactg aaaagcttag gaaatggtat tgagaaatct 780

ggggcagccc caaaagggga gaggccatgg ggagaagggg gggctgagtg ggggaaagca 840

gagccagata aaaagccagc tccagcaggc gctgctcact cctccccatc ctctccctct 900

gtccctctgt ccctctgacc ctgcactgtc ccagcaccat gggacccacc tcaggtccca 960

gcctgctgct cctgctacta acccacctcc ccctggctct ggggagtccc atgtgagt 1018

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tgtgtttata aatgatatga atcaggagtg gtgcgaagag gatagggaaa aaaaaattct 180

atttggtgct ggaaatactg cgctttttt tttccttttt ttttttttct gcgagctgga 240

gtgtgccagc tttttcagac ggaggaatgc tgagtgtcaa ggggtcagga tcaatccggt 300

gtgagttgat gaggcaggaa ggtggggagg aatgcgagga atgtccctgt ttgtgtagac 360

tccattcagc tcattggcga gcgcgccgcc cggagcgtat aaaagcctcg gccgcccgcc 420

ccaaactcac acaacaactc ttccgctgag aggagacagc cagtgcgact ccaccctcca 480

gctcgacggc agccgccccg gccgacagcc ccgagacgac agcccggccg gtcccggtcc 540

ccacctccga ccaccgccag cgctccaggc cccgcgctcc ccgctcgccg ccaccgcgcc 600

ctcccgtccg cccgcagtgc caaccatgac cgccgccagt atgggccccg tccgcgtcgc 660

cttcgtggtc ctcctcgccc tctgcag 687

<210> 4
<211> 584
<212> DNA
<213> Homo sapiens
<220>
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gcaagaaaaa aattagtgaa taataaagga ctgaaccggt tcagagaagg cattcagcag 120

atgtttgcca gtcaaatgaa ttaaagtgtg aatgaatgaa actcgaggta gtgggtgaat 180

gtgtcccaag aatccagcga aacagggtct cccaggaggc gggactggaa gggtccggag 240

aggggccaca ggctcctggc ctttctaagc acaccaagtg cccagtcgcg gacccccggg 300

accaggatgc gctgacgacc cggctggcag gcgggtcctc gtgggcgagg cgagggaggc 360

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cgaggggatc tagagaagcc ggaggggagg aagcgcgagt ccgcggcccg ccccgttgcg 480

tcccacccac cgcgtccccc cccctccccc cccgctgcgg aaaagcggcc gcgggcggcg 540

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<213> Homo sapiens

<220>
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<223> Activin beta-A gene, regulatory sequence of 5' upstream region (A
N: D17357

<400> 5

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<223> prosaposin gene, 5'-flanking region, exon 1, and partial cds (AN: AF057307

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<223> 90K (Mac-2 BP) promoter region (AN: U91729)

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<213> Homo sapiens

<220>
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<223> galectin 3 (LGALS3) gene, exon 1 (AN: AF031421)

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<213> Homo sapiens

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<221> misc_feature
<223> manganese superoxide dismutase (SOD2) gene (AN: S77127)

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<221> misc_feature<223> granulin gene (AN: L32588)

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<213> Homo sapiens

<220>
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<223> p66SHC gene, 5' upstream (2.0 kb) region (AL_NT_004525)

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<223> TIMP-1 (tissue inhibitor of metalloproteinases-1) gene, promoter region (AN: D26513

<400> 20
agaaccggta cccatctcag agatttgttg tgagctttga gtgagataaa atatgctgag 60

tgcctggata tcagtaggtg ctgtataata tgccggctat ttgcctgtgt tatttgagac 120

cctggctttg ctcctggcca cctgagttcc agtctcagtt ctgccatgta ttgactctgt 180

gatcctgggt aagtcactta accactccgt gcctcagttt ccccgatttt gtattcctcc 240

cctttcacct gccttatctc cctccactgc tgctacttaa tttgtttcct ctctgccacc 300

cctcaccagc atgtcagaca tacaaaatca aggcattttt gtgtgcttgg cacacagtag 360

atgcacaata aatgttgaag ggctgaacta atttgggttt gagtcatagg gagacttggg 420

ggagtgtggg tgattggata gattctggag actttagggg actgggccgg gggaaatgcg 480

gcctctaagc tctcgctgag gcggcttgga aggaatagtg actgacgtgg aggtggggga 540

ggtggctggc ccggtcgagg cccagggaga gggagaggag gcgggtggga gaggaggagg 600

gtgtatctcc tttcgtcggc ccgccccttg gcttctgcac tgatggtggg tggatgagta 660

atgcatccag gaagcctgga ggcctgtggt ttccgcaccc gctgccaccc ccgcccctag 720

cgtggacatt tatcctctag cgctcaggcc ctgccgccat cgccgcagat ccagcgccca 780

gagagacacc agaggtacag 800

<210> 21
<211> 27
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense PCR primer for CC3 promoter (spec Table IIIa)

<400> 21
gctaagagga tattgacatt agacagg 27

<210> 22
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense PCR primer for CC3 promoter (spec Table IIIa)

<400> 22
aggggggaggt gggttagtag 20

<210> 23
<211> 22
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature<223> Sense primer for NK4 promoter (Table IIIa)

<400> 23
tggagctaga agagcccgta gg 22

<210> 24
<211> 21
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for NK4 promoter (Table IIIa)

<400> 24
gccaaaagtt caaggagcca a 21

<210> 25
<211> 23

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for SAA promoter (Table IIIa)

<400> 25
cagagttgct gctatgtcca cca 23

<210> 26
<211> 22
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for SAA promoter (Table IIIa)

<400> 26
cactccttgt gtgctcctca cc 22

<210> 27
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for beta-APP promoter (Table IIIa)

<400> 27
ttgctccttt ggttcgttct 20

<210> 28
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for beta-APP promoter (Table IIIa)

<400> 28
gctgccgagg aaactgac 18

<210> 29
<211> 28
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for t-TGase promoter (Table IIIa)

<400> 29
cccagggaga aatatccact gaagcaac 28

<210> 30
<211> 28

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for t-TGase promoter (Table IIIa)

<400> 30
tcgggcgggg gcggtggctc cttccact 28

<210> 31
<211> 25
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for CTGF promoter

<400> 31
gcctcttcag ctacctactt cctaa 25

<210> 32
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for CTGF promoter

<400> 32
cgaggaggac cacgaagg 18

<210> 33
<211> 21
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for integrin B3 promoter

<400> 33
gattggtctt gccctcaaca g 21

<210> 34
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for integrin B3 promoter

<400> 34
ccagcacagt cgcccaga 18

<210> 35
<211> 24

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for activin promoter

<400> 35
tgattccaat gtttttctaa aagg 24

<210> 36
<211> 23
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for activin promoter

<400> 36
gaatgtctaa agagctcaga agt 23

<210> 37
<211> 23
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for prosaposin promoter

<400> 37
ggtttaagca atttctggcc tct 23

<210> 38
<211> 25
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for prosaposin promoter

<400> 38
cgtctgactc tccgcagtct gcaat 25

<210> 39
<211> 25
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for Mac2-BP promoter

<400> 39
gtaaaactcc ctgatgattc cttct 25

<210> 40
<211> 22

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for Mac2-BP promoter

<400> 40
ctctgcagac tggtcctttg ac 22

<210> 41
<211> 22
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for GAL-3 promoter

<400> 41
tgtcttcaca aggtggaagt gg 22

<210> 42
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for GAL-3 promoter

<400> 42
ctggagggca gagcacag 18

<210> 43
<211> 25
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for Mn-SOD promoter

<400> 43
taccaaccct aggggtaaaa ataaa 25

<210> 44
<211> 22
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for Mn-SOD promoter

<400> 44
atgctgctag tgctggtgct ac 22

<210> 45
<211> 25

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for granulin promoter

<400> 45
gagactagga agccacttct ctttc 25

<210> 46
<211> 25
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for granulin promoter

<400> 46
ctggaatgct gtgttctttt ctact 25

<210> 47
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for p66shc promoter

<400> 47
gtggcagaca gggcactc 18

<210> 48
<211> 19
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for p66shc promoter

<400> 48
ctcctgagct gcctcaatg 19

<210> 49
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for NK4

<400> 49
ggtgtcagct cctccttgtc 20

<210> 50
<211> 20

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for t-TGase

<400> 50
actacaactc ggcccatgac 20

<210> 51
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for cathepsin B promoter

<400> 51
ctcccgagta gctgggatta 20

<210> 52
<211> 18
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for cathepsin B promoter

<400> 52
ccacgtgacc accgcgca 18

<210> 53
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for clusterin promoter

<400> 53
agcccttga cttctctcct 20

<210> 54
<211> 19
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for clusterin promoter

<400> 54
ctcctggcga cgccgcgtt 19

<210> 55
<211> 24

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for PSF promoter

<400> 55
aaagtgctgg gattagaggc gtga 24

<210> 56
<211> 28
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for PSF promoter

<400> 56
tatgtattgc taagggaagc tattggag 28

<210> 57
<211> 23
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for VEGF-C promoter

<400> 57
gttcttggat catcaggcaa ctt 23

<210> 58
<211> 19
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for VEGF-C promoter

<400> 58
gtggaaggac cgggggtgg 19

<210> 59
<211> 21
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Sense primer for TIMP-1 promoter

<400> 59
agaaccggta cccatctcag a 21

<210> 60
<211> 21

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Antisense primer for TIMP-1 promoter

<400> 60
ctgtacctct ggtgtctctc t 21

<210> 61
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for t-TGase

<400> 61
gccagtttgt tcaggtggtt 20

<210> 62
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for APP

<400> 62
ctcgttcctg acaagtgcaa 20

<210> 63
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for APP

<400> 63
tgttcagagc acacctctcg 20

<210> 64
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for p66(shc)

<400> 64
gagggtgtgg ttcggactaa 20

<210> 65
<211> 20

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for p66(shc)

<400> 65
gcccagaggt gtgatttgtt 20

<210> 66
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for CTFG

<400> 66
ggagagtcct tccagagcag 20

<210> 67
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for CTGF

<400> 67
atgtcttcat gctggtgcag 20

<210> 68
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for MAC2-BP

<400> 68
accatgagtg tggatgctga 20

<210> 69
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for MAC2-BP

<400> 69
acagggacag gttgaactgc 20

<210> 70
<211> 20

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for granulin

<400> 70
accacggacc tcctcactaa 20

<210> 71
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for granulin

<400> 71
acactgcccc tcagctacac 20

<210> 72
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for prosaposin

<400> 72
ccagagctgg acatgactga 20

<210> 73
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for prosaposin

<400> 73
gtcacctcct tcaccaggaa 20

<210> 74
<211> 20
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for SOD2

<400> 74
caaattgctg cttgtccaaa 20

<210> 75
<211> 20

<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for SOD2

<400> 75
catccctaca agtccccaaa 20

<210> 76
<211> 23
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical sense primer for beta-actin

<400> 76
gggaaatcgt gcgtgacatt aag 23

<210> 77
<211> 22
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Analytical antisense primer for beta-actin

<400> 77
tgtgttggcg tacaggtctt tg 22

<210> 78
<211> 180
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> 5x NFkB sequence

<400> 78
actcatcaat gtatcttatc atgtctggat ccaagctagg ggactttccg cttggggact 60

ttccgctggg gactttccgc tggggacttt ccgctgggga ctttccgcgg tgactctaga 120

gggtatataa tggaagctcg aattccagct tggcattccg gtactgttgg taaaatggaa 180

<210> 79
<211> 99
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> 1xNFkB sequence

<400> 79
ccaagctgag ctctggggac tttccgcggt gactctagag ggtatataat ggaagctcga 60

attccagctt ggcattccgg tactgttggt aaaatggaa 99

<210> 80
<211> 30
<212> DNA
<213> Artificial

<220>
<221> misc_feature
<223> 1xNFkB primer - sense

<220>
<221> misc_feature
<223> GC + SacI + NFkB + 8bp

<400> 80
gcgagctctg gggactttcc gcggtgactc 30

<210> 81
<211> 693
<212> DNA
<213> Simian virus 40

<220>
<221> misc_feature
<223> SV40 promoter

<400> 81
agatctgcgc agcaccatgg cctgaaataa cctctgaaag aggaacttgg ttaggtacct 60

tctgaggcgg aaagaaccag ctgtggaatg tgtgtcagtt agggtgtgga aagtccccag 120

gctccccagc aggcagaagt atgcaaagca tgcatctcaa ttagtcagca accaggtgtg 180

gaaagtcccc aggctcccca gcaggcagaa gtatgcaaag catgcatctc aattagtcag 240

caaccatagt cccgccccta actccgccca tcccgccccc aactccgccc agttccgccc 300

attctccgcc ccatggctga ctaatttttt ttatttatgc agaggccgag gccgcctcgg 360

cctctgagct attccagaag tagtgaggag gctttttgg aggcctaggc ttttgcaaaa 420

agcttgattc ttctgacaca acagtctcga acttaagctg cagaagttgg tcgtgaggca 480

ctgggcaggt aagtatcaag gttacaagac aggtttaagg agaccaatag aaactgggct 540

tgtcgagaca gagaagactc ttgcgtttct gataggcacc tattggtctt actgacatcc 600

actttgcctt tctctccaca ggtgtccact cccagttcaa ttacagctct taaggctaga 660

gtacttaata cgactcacta taggctagcc acc 693

<210> 82
<211> 1067
<212> DNA
<213> Cytomegalovirus

<220>
<221> misc_feature
<223> CMV promoter

<400> 82
agatcttcaa tattggccat tagccatatt attcattggt tatatagcat aaatcaatat 60

tggctattgg ccattgcata cgttgtatct atatcataat atgtacattt atattggctc 120

atgtccaata tgaccgccat gttggcattg attattgact agttattaat agtaatcaat 180

tacggggtca ttagttcata gcccatatat ggagttccgc gttacataac ttacggtaaa 240

tggcccgcct ggctgaccgc ccaacgaccc ccgcccattg acgtcaataa tgacgtatgt 300

tcccatagta acgccaatag ggactttcca ttgacgtcaa tgggtggagt atttacggta 360

aactgcccac ttggcagtac atcaagtgta tcatatgcca agtccgcccc ctattgacgt 420

caatgacggt aaatggcccg cctggcatta tgcccagtac atgaccttac gggactttcc 480

tacttggcag tacatctacg tattagtcat cgctattacc atggtgatgc ggttttggca 540

gtacaccaat gggcgtggat agcggtttga ctcacgggga tttccaagtc tccaccccat 600

tgacgtcaat gggagtttgt tttggcacca aaatcaacgg gactttccaa aatgtcgtaa 660

taaccccgcc ccgttgacgc aaatgggcgg taggcgtgta cggtgggagg tctatataag 720

cagagctcgt ttagtgaacc gtcagatcac tagaagcttt attgcggtag tttatcacag 780

ttaaattgct aacgcagtca gtgcttctga cacaacagtc tcgaacttaa gctgcagaag 840

ttggtcgtga ggcactgggc aggtaagtat caaggttaca agacaggttt aaggagacca 900

atagaaactg ggcttgtcga gacagagaag actcttgcgt ttctgatagg cacctattgg 960

tcttactgac atccactttg cctttctctc cacaggtgtc cactcccagt tcaattacag 1020

ctcttaaggc tagagtactt aatacgactc actataggct agccacc 1067

<210> 83
<211> 26
<212> DNA
<213> artificial

<220>
<221> misc_feature
<223> 1xNFkB primer - antisense

<220>
<221> misc_feature
<223> 18bp 5' of last NFkB site + SacI + GC

<400> 83
cggagctcag cttggatcca gacatg 26

<210> 84
<211> 26

<212> DNA
<213> atificial

<220>
<221> misc_feature
<223> 1x NFkB Primer - antisense complement

<400> 84
catgtctgga tccaagctga gctccg 26

<210> 85
<211> 540
<212> DNA
<213> Human immunodeficiency virus type 1

<400> 85
tggaagggct aattcactcc caacgaagac aagatatcct tgatctgtgg atctaccaca 60
cacaaggcta cttccctgat tagcagaact acacaccagg gccaggagtc agatatccac 120
tgacctttgg atggtgctac aagctagtac cagttgagcc agataaggta gaagaggcca 180
ataaaggaga gaacaccagc ttgttacacc ctgtgagcct gcatgggatg gatgaccctg 240
agagagaagt gttagagtgg aggtttgaca gccgcctagc atttcatcac gtggcccgag 300
agctgcatcg ggagtacttc aagaactgtt gacatcgagc ttgttacaag ggactttccg 360
ctggggactt tccagggagg cgtggcctgg gcgggactgg ggagtggcga gccctcagat 420
gctgcatata agcagctgct ttttgcctgt actgggtctc tctggttaga ccagatctga 480
gcctgggagc tctctggcta gctagggaac ccactgctta agcctcaata aagctgcctt 540

Table I

Genes downregulated by p21 induction

A. p21-inhibited genes identified by UniGemV array:

Genes	Accession No.	Balanced Diff. Expr.	Confirmed by[a]
Associated with mitosis:			
CDC2	X05360	2.5	R, W
CKsHs1 (CDC2 kinase)	X54941	5.5	R
PLK1 (polo-like kinase)	U01038	5.1	R,W
XCAP-H condensin homolog	D38553	6	R
CENP-A (centromere protein A)	U14518	5.3	R
CENP-F (centromere protein F)	U30872	2.5	R
MAD2	U65410	6.6	R, W
BUBR1	AF053306	5.9	R
MCAK (mitotic centromere-associated kinesin)	U63743	3.8	R
HSET kinesin-like protein	AL021366	3.6	R
CHL1 helicase	U75968	3.3	R
AIK-1 (aurora/IPL1-related kinase)	D84212	4.6	R
AIM-1 (AIK-2; aurora/IPL1-related kinase)	AF004022	10.2	R
PRC1 (protein regulating cytokinesis 1)	AF044588	12.6	R, W
Citron kinase	H10809	2.7	R
Lamin B1	L37747	7	
Lamin B2	M94362	2.7	
LAP-2 (lamin-associated protein 2)	U18271	4.6	R
MPP2 (M phase phosphoprotein 2)	U74612	3.7	R
MPP5 (M phase phosphoprotein 5)	X98261	3.7	
Associated with DNA replication, segregation and chromatin assembly:			
Thymidine kinase 1	K02581	2.9	R
Thymidylate synthase	X02308	3.9	R
Uridine phosphorylase	X90858	2.5	
Ribonucleotide reductase M1	X59543	4.6	R
Ribonucleotide reductase M2	X59618	10.7	R
CDC47 homolog (MCM7)	D55716	9.6	R
CDC21 homolog (MCM4)	X74794	2.7	R
CDC45 homolog (Porc-PI)	AJ223728	4.1	R
HsORC1 (origin recognition complex 1)	U40152	2.7	R
DNA polymerase α	X06745	2.8	R
Replication factor C (37-kD subunit)	M87339	2.6	
B-MYB	X13293	9.1	
HPV16 E1 protein binding protein	U96131	3.7	
Topoisomerase IIα	J04088	8.6	R
Chromatin assembly factor-I (p60 subunit)	U20980	2.7	R
High-mobility group chromosomal protein 2	X62534	3.7	R
High-mobility group chromosomal protein 1	D63874	3.8	R
Histone H2A.F/Z variant	AA203494	2.8	
Associated with DNA repair:			
XRCC9	U70310	3.6	R
RAD54 homolog	X97795	5.4	R
HEX1 5'-3' exonuclease (RAD2 homolog)	AF042282	5.2	R
ATP-dependent DNA ligase I	M36067	2.5	R
RAD21 homolog	D38551	2.9	R

Table I

Associated with transcription and RNA processing:			
Putative transcription factor CA150	AF017789	2.8	
Transcriptional coactivator ALY	AF047002	3.3	
WHSC1/MMSET (SET domain protein)	AA401245	2.9	
NN8-4AG (SET domain protein)	U50383	2.8	
EZH2 (enhancer of zeste homolog 2)	U61145	2.8	
PTB-associated splicing factor	X70944	2.5	
AU-rich element RNA-binding protein AUF1	U02019	2.8	
U-snRNP-associated cyclophilin	AF016371	2.8	
Other genes:			
3-phosphoglycerate dehydrogenase	AF006043	4.8	
L-type amino acid transporter, subunit LAT1	M80244	4.1	R
Hyaluronan-mediated motility receptor	U29343	4	
Phorbolin I (PKC-inducible)	U03891	3.9	
PSD-95 binding family protein	D13633	3.7	R
HTRIP (TNF receptor component)	U77845	3.6	
NAD-dependent methylenetetrahydrofolate dehydrogenase	X16396	3.4	
Membrane glycoprotein 4F2 antigen heavy chain	J02939	3.2	
Mucin-like protein	D79992	3.2	
MAC30 (differentially expressed in meningiomas)	L19183	2.9	
P52rIPK (regulator of interferon-induced protein kinase)	AF007393	2.8	
Putative phosphoserine aminotransferase	AA192483	2.8	
Glucose 6-phosphate translocase	Y15409	2.7	
Calcyclin binding protein	AF057356	2.6	
Ornithine decarboxylase 1	X16277	2.6	R
Trophinin assisting protein (tastin)	U04810	2.5	
Acyl-coenzyme A cholesterol acyltransferase	L21934	2.5	
Pinin/SDK3	Y10351	2.5	
Genes with unknown function:			
EST	AA975298	2.7	
EST	AA034414	2.5	
EST	AA482549	2.5	

B. p21-inhibited genes identified by RT-PCR:

Genes	Accession No.	UniGemV result[b]
Cyclin A1	U66838	IS
Cyclin B1	M25753	IS
CDC25A	NM_001789	A
Dihydrofolate reductase	J00140	1.5
ING1	NM_005537	A

[a]Abbreviations: R, RT-PCR; W, western blotting

[b]Abbreviations: IS, insufficient signal; A, absent from the array

Table II

Genes upregulated by p21 induction

Genes	Accession No	Balanced Diff Expr	Confirmed by[a]
Secreted proteins and proteins associated with extracellular matrix:			
Fibronectin 1	X02761	5.7	R
Plasminogen activator inhibitor, type I	M14083	3.7	R, N
Plasminogen activator, tissue type	M15518	2.8	Z
Laminin β2	X79683	2.1	
Desmocollin 2a/bb	X56807	3.5	
Podocalyxin-like protein	U97519	2	
Activin A (inhibin βA)	J03634	2	R
Galectin 3 (Mac-2)	AB006780	2.4	N
Mac-2 binding protein	L13210	2	R, N
Prosaposin	J03077	2.9	N
CTGF (connective tissue growth factor)	M92934	3.3	N
Granulin/epithelin	AF055008	2.1	N
Cathepsin B	L04288	2.4	N
Tissue transglutaminase	M55153	2.5	R, N, W
P37NB (slit homolog)	U32907	2.1	
Serum amyloid A protein precursor	M26152	4	R, N, W
Alzheimer's disease amyloid A4 protein precursor	D87675	2	R, N
Complement C3 precursor	K02765	5.9	R, N
Testican	X73608	2.1	N
Integrin β3	M35999	2.1	R, N
Lysosomal proteins:			
N-acetylgalactosamine-6-sulfate sulfatase	U06088	2.3	N
Acid alpha-glucosidase	X55079	2.4	N
Acid lipase A (cholesterol esterase)	X76488	2.1	N
Lysosomal pepstatin-insensitive protease (CLN2)	AF017456	2.5	
Mitochondrial proteins:			
Superoxide dismutase 2	X07834	3.5	R, N, W
Metaxin	J03060	3.4	
2,4-dienoyl-CoA reductase	U78302	2	
Other genes associated with stress response and signal transduction:			
Ubiquitin-conjugating enzyme (UbcH8)	AF031141	2	
Ubiquitin-specific protease 8	D29956	2	
RTP/Cap43/Drg1/Ndr1 (inducible by nickel, retinoids, homocysteine and ER stress)	D87953	2.5	
C-193 muscle ankyrin-repeat nuclear protein (cytokine-inducible)	X83703	3	
LRP major vault protein associated with multidrug resistance	X79882	2.2	N
β-arrestin related HHCPA78 homolog (upregulated by vitamin D3)	S73591	4.1	N
R-RAS	M14949	2.4	
RAB 13 small GTPase	X75593	2.2	
P66 SHC (ski oncogene)	U73377	2	N
MK-STYX (MAP kinase phosphatase-like protein)	N75168	2	
H73 nuclear antigen/MA-3 apoptosis-related/TIS (topoisomerase-inhibitor suppressed)	U96628	2.4	

Table II

Other genes:			
Natural killer cells protein 4	M59807	4.4	R
TXK tyrosine kinase (T-cell specific)	L27071	3.8	
X-linked PEST-containing transporter	U05321	2.1	
AMP deaminase 2	M91029	2	N
FIP2/HYPL huntingtin-interacting protein	AF061034	2	
DNASE I homolog	X90392	2.5	N
Transcription factor 11	X77366	2	
Histone H2A.2	L19779	2.8	
Histone H2B	AL021807	2.4	
Genes with unknown function:			
23808	AF038192	2.1	
CGI-147	AA307912	2.1	N
EST	W69120	2.8	
EST	AI026140	2.5	
EST	AA218982	2.4	
EST	W63684	2	

*Abbreviations: R, RT-PCR; N, northern hybridization; W, western blotting; Z, zymography