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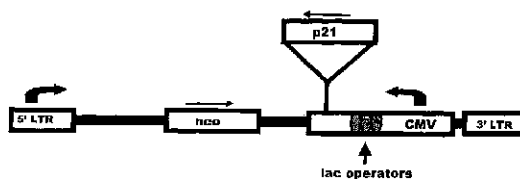
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(54) 【発明の名称】 CDK阻害剤によって調節される遺伝子発現を同定及び調節するための試薬及び方法

(57) 【要約】

本発明は、サイクリン依存性キナーゼ阻害剤によって誘導される遺伝子である、癌及び老化関連疾病に關与する遺伝子の誘導を阻害する化合物を同定するための方法及び試薬を提供する。

IPTG-regulated retroviral vector LNP21CO3



## 【特許請求の範囲】

## 【請求項 1】

サイクリン依存性キナーゼ阻害剤によって誘導される哺乳類遺伝子に由来するプロモータに機能的に連結された、レポーター遺伝子をコードする組み換え発現作成物。

## 【請求項 2】

レポーター遺伝子が、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、クロラムフェニコールアセチルトランスフェラーゼ、ガラクトシダーゼ、緑色蛍光蛋白質、またはアルカリホスファターゼをコードすることを特徴とする、請求項 1 に記載の組み換え発現作成物。

## 【請求項 3】

プロモータが、CDK 阻害剤によって誘導されるヒト遺伝子に由来するプロモータであることを特徴とする、請求項 1 に記載の組み換え発現作成物。

## 【請求項 4】

プロモータが、表 I I に示されるヒト遺伝子に由来するプロモータであることを特徴とする、請求項 3 に記載の組み換え発現作成物。

## 【請求項 5】

プロモータが、血清アミロイド A (配列番号: 1)、補体 C 3 (配列番号: 2)、結合組織増殖因子 (配列番号: 3)、インテグリン - 3 (配列番号: 4)、アクチピン A (配列番号: 5)、ナチュラルキラー細胞蛋白質 4 (配列番号: 6)、プロサポシン (配列番号: 7)、Mac 2 結合蛋白質 (配列番号: 8)、ガレクチン - 3 (配列番号: 9)、スーパーオキシドジスムターゼ 2 (配列番号: 10)、グラニューリン/エピテリン (配列番号: 11)、p 6 6<sup>s h c</sup> (配列番号: 12)、カテプシン B (配列番号: 14)、アミロイド前駆体蛋白質 (配列番号: 15)、組織トランスグルタミナーゼ (t - T G アーゼ; 配列番号: 16)、クラステリン (配列番号: 17)、プロスタサイクリン刺激因子 (配列番号: 18)、血管内皮細胞増殖因子 - C (配列番号: 19)、及びメタロプロテアーゼ - 1 の組織阻害剤 (配列番号: 20) に由来するプロモータであることを特徴とする、請求項 4 に記載の組み換え発現作成物。

## 【請求項 6】

プロモータが、ヒトナチュラルキラー細胞蛋白質 4 (配列番号: 6)、血清アミロイド A (配列番号: 1)、補体 C 3 (配列番号: 2)、組織トランスグルタミナーゼ (t - T G アーゼ; 配列番号: 16)、アミロイド前駆体蛋白質 (配列番号: 15)、またはプロサポシン (配列番号: 7) に由来するプロモータであることを特徴とする、請求項 4 に記載の組み換え発現作成物。

## 【請求項 7】

組み換え発現作成物は p L u N K 4 であることを特徴とする、請求項 4 に記載の組み換え発現作成物。

## 【請求項 8】

請求項 1、2、3、4、5、6 または 7 に記載の組み換え発現作成物を含む哺乳類細胞。

## 【請求項 9】

A . T . C . C 受託番号 P T A 3 3 8 1 ( H T 1 0 8 0 L u N K 4 9 2 1 ) で識別される、請求項 8 の哺乳類細胞。

## 【請求項 10】

組み換え発現作成物の発現が N F B によって調節されることを特徴とする、請求項 8 に記載の哺乳類細胞。

## 【請求項 11】

哺乳類 CDK 阻害剤遺伝子をコードする第 2 の組み換え発現作成物をさらに含むことを特徴とする、請求項 8 に記載の哺乳類細胞。

## 【請求項 12】

CDK 阻害剤の発現が該哺乳類細胞内で実験的に誘導されることを特徴とする、請求項 11 に記載の哺乳類細胞。

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## 【請求項 13】

哺乳類CDK阻害剤遺伝子をコードする組み換え発現作成物が、組み換え発現作成物からのCDK阻害剤の発現が組み換え細胞を、誘導可能なプロモータからの転写を誘導する誘導剤に接触させることにより、あるいは当該プロモータから転写を阻害する物質を除去することによって媒介される、誘導可能なプロモータの転写制御下にあることを特徴とする、請求項11に記載の哺乳類細胞。

## 【請求項 14】

哺乳類CDK阻害剤遺伝子が、ヒトp21またはそのCDK結合断片であることを特徴とする、請求項13に記載の哺乳類細胞。

## 【請求項 15】

哺乳類CDK阻害剤遺伝子が、ヒトp16またはそのCDK結合断片であることを特徴とする、請求項13に記載の哺乳類細胞。

## 【請求項 16】

哺乳類CDK阻害剤遺伝子が、マウスまたはヒトp27遺伝子またはそのCDK結合断片であることを特徴とする、請求項13に記載の哺乳類細胞。

## 【請求項 17】

細菌ラクトースリプレッサーをコードする組み換え発現作成物をさらに含み、その転写が哺乳類プロモータによって制御され、哺乳類CDK阻害剤遺伝子をコードする組み換え発現作成物がラクトースリプレッサー応答プロモータ要素を含み、CDK阻害剤遺伝子の転写が前記ラクトースリプレッサー応答プロモータ要素によって制御されること、及び組み換え発現作成物からのCDK阻害剤遺伝子の発現が組み換え細胞をラクトースリプレッサー特異的誘導剤と接触させることによって媒介されることを特徴とする、請求項13に記載の哺乳類細胞。

## 【請求項 18】

細胞がヒトHT1080線維肉腫細胞であることを特徴とする、請求項8に記載の哺乳類細胞。

## 【請求項 19】

細胞がヒトHT1080線維肉腫細胞であることを特徴とする、請求項11に記載の哺乳類細胞。

## 【請求項 20】

細胞がヒトHT1080線維肉腫細胞であることを特徴とする、請求項17に記載の哺乳類細胞。

## 【請求項 21】

第2の発現作成物がLNp21CO3であることを特徴とする、請求項11に記載の哺乳類細胞。

## 【請求項 22】

A.T.C.C受託番号PTA1664(HT1080 p21-9)で識別される、請求項21に記載の哺乳類細胞。

## 【請求項 23】

第2の発現作成物がLNp16RO2であることを特徴とする、請求項11に記載の哺乳類細胞。

## 【請求項 24】

A.T.C.C受託番号\_\_\_\_\_ (HT1080 p16-5)で識別される、請求項23に記載の哺乳類細胞。

## 【請求項 25】

第2の発現作成物がLNp27RO2であることを特徴とする、請求項11に記載の哺乳類細胞。

## 【請求項 26】

A.T.C.C受託番号\_\_\_\_\_ (HT1080 p27-2)で識別される、請求項25に記載の哺乳類細胞。

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## 【請求項 27】

ラクトースリプレッサー特異的誘導剤が - ガラクトシダーゼであることを特徴とする、請求項 17 に記載の哺乳類細胞。

## 【請求項 28】

哺乳類細胞内で CDK 阻害剤によって誘導される遺伝子の誘導を阻害する化合物の同定方法であって、以下の工程からなる方法：

(a) 請求項 8 に記載の組み換え哺乳類細胞を、当該化合物の存在下または非存在下において哺乳類細胞内で CDK 阻害剤によって誘導される遺伝子の発現を誘導する条件下で培養する工程と；

(b) 化合物の存在下での前記細胞におけるレポーター遺伝子の発現と、化合物の非存在下での前記細胞におけるレポーター遺伝子の発現とを比較する工程；および

(c) 化合物の非存在下よりも化合物の存在下の方がレポーター遺伝子発現が低い場合に、当該化合物が CDK 阻害剤によって誘導される遺伝子の誘導を阻害すると同定する工程。

## 【請求項 29】

前記細胞内で CDK 阻害剤の発現を誘導する条件下で、細胞が培養されることを特徴とする、請求項 28 の方法。

## 【請求項 30】

CDK 阻害剤が p21、p27 または p16 か、またはその CDK 結合断片であることを特徴とする、請求項 29 の方法。

## 【請求項 31】

細胞が哺乳類 CDK 阻害剤遺伝子をコードする第 2 の組み換え発現作成物をさらに含むことを特徴とする、請求項 28 の方法。

## 【請求項 32】

哺乳類 CDK 阻害剤遺伝子をコードする第 2 の組み換え発現作成物は誘導可能なプロモータの転写制御下にあり、組み換え発現作成物からの CDK 阻害剤の発現は、組み換え細胞を、誘導可能なプロモータからの転写を誘導する誘導剤に接触させることにより、あるいは当該プロモータからの転写を阻害する物質を除去することによって媒介されることを特徴とする、請求項 31 の方法。

## 【請求項 33】

哺乳類 CDK 阻害剤遺伝子がヒト p21 またはその CDK 結合断片であることを特徴とする、請求項 32 の方法。

## 【請求項 34】

哺乳類 CDK 阻害剤遺伝子がヒト p16 またはその CDK 結合断片であることを特徴とする、請求項 32 の方法。

## 【請求項 35】

哺乳類 CDK 阻害剤遺伝子がヒト p27 遺伝子またはその CDK 結合断片であることを特徴とする、請求項 32 の哺乳類細胞。

## 【請求項 36】

細胞がヒト HT1080 線維肉腫細胞であることを特徴とする、請求項 32 の方法。

## 【請求項 37】

細菌ラクトースリプレッサーをコードする組み換え発現作成物をさらに含み、その転写が哺乳類プロモータによって制御され、哺乳類 CDK 阻害剤遺伝子をコードする組み換え発現作成物がラクトースリプレッサー応答プロモータ要素を含み、CDK 阻害剤遺伝子の転写が前記ラクトースリプレッサー応答プロモータ要素によって制御されること、及び組み換え発現作成物からの CDK 阻害剤遺伝子の発現が組み換え細胞をラクトースリプレッサー特異的誘導剤と接触させることによって媒介されることを特徴とする、請求項 32 の方法。

## 【請求項 38】

細胞遺伝子発現の CDK 阻害剤媒介性誘導を阻害する化合物を同定するため方法であって

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、以下の工程からなる方法：

- ( a ) 哺乳類細胞において C D K 阻害剤の発現を生み出す工程；
- ( b ) 化合物の存在下において、該細胞について、C D K 阻害剤によって発現が調節される細胞遺伝子の発現における検定する工程；および
- ( c ) 工程 ( b ) の細胞遺伝子の発現の変化の程度が化合物の存在下の方が小さい場合に、その化合物を、細胞遺伝子発現の C D K 阻害剤媒介性調節の阻害剤として同定する工程。

【請求項 39】

C D K 阻害剤が p 16 , p 27 または p 21 であることを特徴とする、請求項 38 の方法。

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【請求項 40】

哺乳類細胞が誘導可能な異種プロモータの転写制御下にある哺乳類 C D K 阻害剤をコードする組み換え発現作成物を含み、組み換え発現作成物からの C D K 阻害剤の発現は、組み換え細胞を、誘導可能プロモータからの転写を誘導する誘導剤と接触させるか、あるいはそのようなプロモータからの転写を阻害する物質を除去することによって媒介されることを特徴とする、請求項 39 の方法。

【請求項 41】

C D K 阻害剤が p 16 であることを特徴とする、請求項 40 の方法。

【請求項 42】

C D K 阻害剤が p 21 であることを特徴とする、請求項 40 の方法。

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【請求項 43】

C D K 阻害剤が p 27 であることを特徴とする、請求項 40 の方法。

【請求項 44】

細胞遺伝子の発現が p 21 によって誘導されることを特徴とする、請求項 38 の方法。

【請求項 45】

細胞遺伝子の発現が p 16 によって誘導されることを特徴とする、請求項 38 の方法。

【請求項 46】

細胞遺伝子の発現が p 27 によって誘導されることを特徴とする、請求項 38 の方法。

【請求項 47】

細胞遺伝子が表 I I に示されることを特徴とする、請求項 38 の方法。

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【請求項 48】

細胞遺伝子が表 I I に示されることを特徴とする、請求項 40 の方法。

【請求項 49】

細胞遺伝子の発現が免疫学的試薬を用いて検出されることを特徴とする、請求項 38 の方法。

【請求項 50】

細胞遺伝子の発現が、細胞遺伝子産物の活性をアッセイすることにより検出されることを特徴とする、請求項 38 の方法。

【請求項 51】

細胞遺伝子の発現が、相補的核酸へのハイブリダイゼーションによって検出されることを特徴とする、請求項 38 の方法。

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【請求項 52】

哺乳類細胞において細胞遺伝子発現の C D K 阻害剤媒介性誘導を阻害する化合物を同定するための方法であって、以下の工程からなる方法：

- ( a ) 哺乳類細胞を化合物の存在下または非存在下において試薬で処理するか、あるいは哺乳類細胞を、老化を誘導する条件下で培養する工程；
- ( b ) 哺乳類細胞について、C D K 阻害剤遺伝子発現によって誘導される遺伝子の誘導をアッセイする工程；および
- ( c ) C D K 阻害剤によって誘導される遺伝子の誘導の程度が、化合物の非存在下よりも化合物の存在下の方が小さければ、当該化合物を、細胞遺伝子発現の C D K 阻害剤媒介

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性誘導の阻害剤と同定する工程。

【請求項 5 3】

C D K 阻害剤が p 2 1、p 1 6 または p 2 7 であることを特徴とする、請求項 5 2 の方法。

【請求項 5 4】

遺伝子が表 I I に示されていることを特徴とする、請求項 5 2 の方法。

【請求項 5 5】

細胞遺伝子の発現が免疫学的試薬を用いて検出されることを特徴とする、請求項 5 2 の方法。

【請求項 5 6】

細胞遺伝子の発現が細胞遺伝子産物の活性をアッセイすることによって検出されることを特徴とする、請求項 5 2 の方法。

【請求項 5 7】

細胞遺伝子の発現が相補的核酸へのハイブリダイゼーションによって検出されることを特徴とする、請求項 5 2 の方法。

【請求項 5 8】

哺乳類細胞において細胞遺伝子発現の C D K 阻害剤媒介性誘導を阻害する化合物を同定する方法で、以下の工程からなる方法：

( a ) 化合物の存在下または非存在下において哺乳類細胞を試薬に接触させるか、あるいは哺乳類細胞を老化を誘導する条件下で培養する工程であって、細胞は C D K 阻害剤によって発現が調節される哺乳類遺伝子に対するプロモータの転写制御下にあるレポーター遺伝子を含む工程；

( b ) 細胞について、レポーター遺伝子の発現の変化をアッセイする工程；および

( c ) レポーター遺伝子発現の変化の程度が、化合物の非存在下よりも化合物の存在下で小さい場合に、その化合物を細胞遺伝子発現の C D K 阻害剤媒介性誘導の阻害剤として同定する工程。

【請求項 5 9】

C D K 阻害剤が p 2 1、p 1 6 または p 2 7 であることを特徴とする、請求項 5 8 の方法。

【請求項 6 0】

遺伝子が表 I I に示されていることを特徴とする、請求項 5 8 に記載の方法。

【請求項 6 1】

細胞遺伝子の発現が免疫学的試薬を用いて検出されることを特徴とする、請求項 5 8 の方法。

【請求項 6 2】

細胞遺伝子の発現が細胞遺伝子産物の活性をアッセイすることによって検出されることを特徴とする、請求項 5 8 の方法。

【請求項 6 3】

細胞遺伝子の発現が相補的核酸へのハイブリダイゼーションによって検出されることを特徴とする、請求項 5 8 の方法。

【請求項 6 4】

細胞遺伝子発現の C D K 阻害剤媒介性誘導を阻害するための方法であって、細胞を、請求項 2 8 に記載の方法に従って得られた化合物と接触させる工程を含む方法。

【請求項 6 5】

細胞遺伝子発現の C D K 阻害剤媒介性誘導を阻害するための方法であって、細胞を、請求項 3 8 に記載の方法に従って得られた化合物と接触させる工程を含む方法。

【請求項 6 6】

細胞遺伝子発現の C D K 阻害剤媒介性誘導を阻害するための方法であって、細胞を、請求項 5 2 に記載の方法に従って得られた化合物と接触させる工程を含む方法。

【請求項 6 7】

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細胞遺伝子発現のCDK阻害剤媒介性誘導を阻害するための方法であって、細胞を、請求項58に記載の方法に従って得られた化合物と接触させる工程を含む方法。

【請求項68】

細胞遺伝子発現のCDK阻害剤媒介性誘導を阻害するための方法であって、細胞を、有効量のNF B活性を阻害する化合物と接触させる工程を含む方法。

【請求項69】

CDK阻害剤によって誘導される遺伝子誘導を伴う動物における疾病を治療するための方法であって、動物に、NF B活性を阻害する有効量の非ステロイド系抗炎症剤(NSAID)を投与する工程を含む方法。

【請求項70】

疾病が大腸癌以外の癌であることを特徴とする、請求項69に記載の方法。

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【請求項71】

疾病が腎不全であることを特徴とする、請求項69に記載の方法。

【請求項72】

疾病はアルツハイマー病であり、NSAIDはアスピリンまたはサリチル酸塩以外であることを特徴とする請求項69に記載の方法。

【請求項73】

疾病はアテローム性動脈硬化症であり、NSAIDはアスピリン以外であることを特徴とする請求項69に記載の方法。

【請求項74】

疾病は関節炎であり、NSAIDはアスピリン、スリダクまたはサリチル酸塩以外であることを特徴とする請求項69に記載の方法。

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【請求項75】

哺乳類細胞における老化の病原性結果に関与する遺伝子を阻害する化合物であって、該化合物が、

(a) 哺乳類細胞を化合物の存在下で試薬で処理するか、あるいは哺乳類細胞を老化を誘導する条件下で培養する工程；

(b) 哺乳類細胞について、CDK阻害剤遺伝子発現によって誘導される遺伝子の誘導をアッセイする工程；および

(c) CDK阻害剤によって誘導される遺伝子の誘導の程度が化合物の存在下の方が小さいならば、その化合物を老化の阻害剤と同定する工程

を有する方法によって得られることを特徴とする化合物。

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【請求項76】

CDK阻害剤がp21、p16またはp27であることを特徴とする、請求項69の化合物。

【請求項77】

哺乳類細胞におけるCDK阻害剤によって誘導される遺伝子産物の産生を阻害する化合物であって、該化合物が、

(a) 哺乳類細胞を化合物の存在下で試薬で処理するか、あるいは哺乳類細胞をCDK阻害剤の発現を誘導する条件下で培養する工程；

(b) 哺乳類細胞について、CDK阻害剤遺伝子発現によって誘導される遺伝子の誘導をアッセイする工程；

(c) CDK阻害剤によって誘導される遺伝子の誘導の程度が化合物の存在下の方が小さいならば、その化合物をCDK阻害剤誘導の阻害剤と同定する工程

を有する方法によって得られることを特徴とする化合物。

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【請求項78】

CDK阻害剤はp21、p16またはp27であることを特徴とする、請求項77の化合物。

【請求項79】

哺乳類細胞において抗アポトーシスまたは分裂促進因子の産生を阻害するための方法であ

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って、細胞を、C D K 阻害剤による遺伝子発現の誘導を阻害する化合物と接触させる工程を含む方法。

【請求項 8 0】

哺乳類細胞が間質線維芽細胞であることを特徴とする、請求項 7 9 の方法。

【請求項 8 1】

化合物が N F B 阻害剤または p 3 0 0 / C P B 阻害剤であることを特徴とする、請求項 7 9 に記載の方法。

【請求項 8 2】

C D K 阻害剤によって誘導される遺伝子発現に伴う疾病の効果を防止または改善するために動物を治療するための方法であって、これを必要とする動物に、請求項 2 8、3 8、5 2 または 5 8 の方法に従って同定される化合物の薬学的組成物を、治療上有効な用量で投与する工程を含む方法。

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【請求項 8 3】

哺乳類細胞において C D K 阻害剤によって誘導される遺伝子の発現を阻害または防止する方法であって、哺乳類細胞を、請求項 2 8、3 8、5 2 または 5 8 の方法に従って同定される化合物の、C D K 阻害剤によって誘導される遺伝子の発現を阻害または防止するのに有効な量と、接触させる工程を含む方法。

【請求項 8 4】

動物において C D K 阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、当該治療が必要な動物に N F B を投与することを含む方法。

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【請求項 8 5】

N F B 阻害剤が非ステロイド系抗炎症化合物であることを特徴とする、請求項 8 4 の方法。

【請求項 8 6】

動物がヒトであることを特徴とする、請求項 8 5 の方法。

【請求項 8 7】

動物において C D K 阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項 2 8 の方法によって得られる化合物を投与することを含む方法。

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【請求項 8 8】

動物がヒトであることを特徴とする、請求項 8 7 の方法。

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【請求項 8 9】

動物において C D K 阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項 3 8 の方法によって得られる化合物を投与することを含む方法。

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【請求項 9 0】

動物はヒトであることを特徴とする、請求項 8 9 の方法。

【請求項 9 1】

動物において C D K 阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項 5 2 の方法によって得られる化合物を投与することを含む方法。

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【請求項 9 2】

動物はヒトであることを特徴とする、請求項 9 1 の方法。

【請求項 9 3】

動物において C D K 阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項 5 8 の方法によって得られる化合物を投与することを含む方法。

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【請求項 9 4】

動物がヒトであることを特徴とする、請求項 9 3 の方法。

【請求項 9 5】

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動物においてCDK阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項75の方法によって得られる化合物を投与することを含む方法。

【請求項96】

動物がヒトであることを特徴とする、請求項95の方法。

【請求項97】

動物においてCDK阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項28、38、52または58の方法によって得られる化合物を投与することを含む方法。

【請求項98】

動物がヒトであることを特徴とする、請求項97の方法。

【請求項99】

動物においてCDK阻害剤によって誘導される遺伝子の誘導を選択的に阻害するための方法であって、動物に請求項77に記載の化合物を投与することを含む方法。

【請求項100】

動物がヒトであることを特徴とする、請求項99の方法。

【発明の詳細な説明】

【技術分野】

【0001】

(発明の背景)

本願は2001年2月1日に提出された米国仮出願番号60/265,840、および2001年5月21日に提出された米国特許出願番号09/861,925に優先権を主張する。

【0002】

本願は国立保健研究所からの許可、No. R01CA62099を得て出願している。政府は本発明における一定の権利を有する。

【0003】

(発明の分野)

本発明は、細胞老化ならびに老化に伴う細胞性遺伝子発現の変化に関する。とりわけ、本発明は、老化の開始時に細胞内で誘導されるサイクリン依存性キナーゼ(CDK)阻害剤と呼ばれるクラスの細胞性遺伝子産物によって発現が調節される遺伝子の同定に関する。より詳細には、本発明は上記のようなCDK阻害剤によって発現が誘導される遺伝子である細胞老化のマーカーを提供する。本発明は、細胞老化の病的結果を阻害する化合物の存在下において、CDK阻害剤により上記マーカー遺伝子の誘導の阻害を検出することにより当該化合物を同定するための方法を提供する。また、実験的に誘導可能なp21、p16またはp27などの様々な細胞性CDK阻害剤をコードする組み換え発現作成物を含む組み換え哺乳類細胞である試薬、ならびに、内在性または外来性的実験的に誘導可能なCDK阻害剤によって発現が誘導される遺伝子に対するプロモータの緊縮制御下にて、レポーター遺伝子を発現する組み換え発現作成物を含む組み換え哺乳類細胞も提供される。

【背景技術】

【0004】

(関連技術の概要)

細胞周期の進行は、サイクリン依存性キナーゼ(CDKs)として知られる一組のセリン/スレオニンキナーゼによって大いに調節されている。CDK阻害剤として知られる特定の蛋白質群は、CDKsと相互作用してこれを阻害することにより、様々な生理学的状況において細胞周期の捕捉を引き起こす(Sieleckiら, 2000年, J. Med. Chem. 第43巻: 1~18頁、及びその中の引例を参照)。CDK阻害剤には2つのファミリーが存在する。Cip/Kipとして知られる1つ目のファミリーには、p21<sup>Waf1/Cip1/Sdi1</sup>, p27<sup>Kip1</sup>及びp57<sup>Kip2</sup>が含まれる。2つ目のファミリーであるInk4には、p16<sup>Ink4A</sup>, p15<sup>Ink4b</sup>, p18<sup>Ink</sup>

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<sup>4c</sup> 及び p19<sup>Ink4d</sup> が含まれる。特定の CDK 阻害剤の発現は、様々な因子によって活性化される。例えば、接触阻害は p27 及び p16 発現を誘導し (Dietrichら, 1997年, *Oncogene* 第15巻: 2743~2747頁)、TGF などの細胞外抗分裂促進因子は p15 発現を誘導し (Reynisdottirら, 1995年, *Genes Dev.* 第9巻: 1831~1845頁)、血清の不足は p27 発現を誘導し (Polyakら, 1994年, *Genes Dev.* 第8巻: 9~22頁)、紫外線照射は p16 発現を誘導する (Wangら, 1996年, *Cancer Res.* 第56巻: 2510~2514頁)。さらに、上述のすべての処理は、様々なかたちの DNA 損傷とともに、既知の CDK 阻害剤の中でも最も多面性のある p21 の発現を誘導する (Dotto, 2000年, *BBA Rev. Cancer* 第1471巻: M43-M56)。

#### 【0005】

本発明の分野で特に重要なことは、CDK 阻害剤のうち p21 と p16 の2つは、哺乳類細胞における老化の進行に密接に関与している。複製老化 (Alcortaraら, 1996年, *Proc. Natl. Acad. Sci. USA* 第93巻: 13742~13747頁) 及び損傷によって誘導され、加速される老化 (Robles & Adami, 1998年, *Oncogene* 第16巻: 1113~1123頁) の開始時には、p21 誘導によって細胞の増殖が捕捉される。しかしながら、この p21 発現の急増は一時的なものであり、次に p16 の安定した活性化が起こり、このことが老化細胞における増殖停止の維持を担っていると考えられる。p21 のノックアウト (Brownら, 1997年, *Science*, 第277巻: 831~834頁) または p16 のノックアウト (Serranoら, 1996年, *Cell* 第85巻: 27~37頁) により、老化の開始が遅延または阻害される。さらに、p21 または p16 のいずれかの異所性過剰発現は、正常細胞及び腫瘍細胞の両方において老化の表現型マーカーに随伴した増殖停止を誘導する (Vogtら, 1998年, *Cell Growth Differ.* 第9巻: 139~146頁; McConnellら, 1998年, *Curr. Biol.* 第8巻: 351~354頁; Fangら, 1999年, *Oncogene* 第18巻: 2789~2797頁)。

#### 【0006】

p21 は、CDK と結合してこれを阻害する蛋白質として (Harperら, 1993年, *Cell* 第75巻: 805~816頁)、野生型 p53 によって上方制御される遺伝子として (e1-Deiryら, 1993年, *Cancer Res.* 第55巻: 2910~2919頁)、及び老化線維芽細胞において過剰発現される増殖阻害遺伝子として (Nodaら, 1994年, *Exp. Cell Res.* 第211巻: 90~98頁)、当該技術分野において別々に同定されている。p53 によって制御される増殖の停止において極めて重要な役割を有することから、p21 は通常は腫瘍サプレッサーと見なされている。それにもかかわらず、ヒトの癌において p21 変異が見られることは希であり (Hall & Peters, 1996年, *Adv. Cancer Res.* 第68巻: 67~108頁)、p21 ノックアウトマウスは正常に発育し、腫瘍形成速度の増加を見せることはない (Dengら, 1995年, *Cell* 第82巻: 675~684頁)。

#### 【0007】

p21 の細胞レベルは、DNA 損傷剤や分化剤を含む様々な刺激に反応して増大する。これらの反応のいくつかは、p53 による p21 遺伝子の転写活性化を介するが、p21 は様々な p53 非依存性因子によっても制御される (Gartel と Tyner, 1999年, *Exp. Cell Res.* 第227巻: 171~181頁に概説されている)。

#### 【0008】

p21 の一過性誘導は、細胞による DNA 損傷の修復を許容する一過性停止、ならびに、DNA 損傷または発癌性 RAS (Serranoら, 1997年, *Cell* 第88巻: 593~602頁) によって正常な線維芽細胞 (DiLeonardoら, 1994年, *Genes Develop.* 第8巻: 2540~2551頁; Robles & Adam

i, 1998年, *Oncogene* 第16巻: 1113~1123頁)及び腫瘍細胞(Changら, 1999年, *Cancer Res.* 第59巻: 3761~3767頁)において誘導される永久的増殖停止(「加速された老化」とも呼ばれる)を含む様々な形の損傷誘導性増殖停止を媒介する。p21発現の急増は、老化線維芽細胞の複製老化の間の最終増殖停止(Nodaら, 1994年, 同書中; Alcorraら, 1996年, *Proc. Natl. Acad. Sci. USA* 第93巻: 13742~13747頁; Steinら, 1999年, *Mol. Cell. Biol.* 第19巻: 2109~2117頁)ならびに有糸分裂後細胞の最終分化(El-Deiryら, 1995年, 同書中; Gartelら, 1996年, *Exp. Cell Res.* 第246巻: 280~289頁)の開始と同時に起こる。

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## 【0009】

p21自体は転写因子ではないものの、細胞遺伝子発現に対して細胞機能における役割を果たすであろう間接的な効果を有している(Dotto, 2000年, *BBA Rev. Cancer* 第1471巻: M43~M56及びその中の引例)。p21によるCDK阻害の結果の1つにRbの脱リン酸化があり、この脱リン酸化により、DNA複製及び細胞周期の進行に関与する多くの遺伝子を制御するE2F転写因子が阻害される(Nevins, 1998年, *Cell Growth Differ.* 第9巻: 585~593頁)。p21発現細胞(p21+/+)とp21非発現細胞(p21-/-)との比較から、p21が細胞周期進行に関与するいくつかの遺伝子の照射誘導性阻害に関係していると思われてきた(de Toledoら, 1998年, *Cell Growth Differ.* 第9巻: 887~896頁)。p21によるCDK阻害の別の結果としては、NF- $\kappa$ Bを含む多くの誘導可能な転写因子を増強する、転写補因子ヒストンアセチルトランスフェラーゼp300の刺激があげられる(Perkinsら, 1988年, *Science* 第275巻: 523~527頁)。p300の活性化は、遺伝子発現に対して多面的な効果を有しているのかもしれない(Snowden&Perkins, 1988年, *Biochem. Pharmacol.* 第55巻: 1947~1954頁)。p21はCDK以外の多くの転写調節因子及び補調節因子、例えばJNKキナーゼ類、アポトーシスシグナル調節キナーゼ1、Myc等との相互作用を介して遺伝子発現に影響を及ぼしているのかもしれない(Dotto, 2000年, *BBA Rev. Cancer* 1471: M43-M56)。上記相互作用は、対応する経路によって制御される遺伝子の発現に影響を及ぼすのかもしれない。

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## 【0010】

本発明に特に関係する別のCDK阻害剤としては、Serranoらによって記載されているヒトの蛋白質であるp16<sup>INK4A</sup>があげられる(1993年, *Nature* 366巻: 704~707頁)。上述のように、p16は哺乳類細胞における老化に不可欠な調節因子である。これは真正腫瘍サプレッサであるとともに、ヒト癌において最もよく変異する遺伝子の1つである(Hall&Peters, 1996年, *Adv. Cancer Res.* 第68巻: 67~108頁)。p16はCDK4及びCDK6を直接的に阻害することが知られており、CDK2も同様に間接的に阻害するのかもしれない(McConnellら, 1999年, *Molec. Cell Biol.* 第19巻: 1981~1989頁)。

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## 【0011】

本発明に特に関連する別のCDK阻害剤として、p27<sup>Kip1</sup>があげられる。p27は当初、TGF- $\beta$ またはロバスタチンとの接触阻害によって増殖が停止された細胞内において、CDK2の阻害剤として同定された(Hengstlerら, 1994年, *Proc. Natl. Acad. Sci. USA* 第91巻: 5291~5295頁; Polyakら, 1994年, *Cell* 第78巻: 59~66頁)。p27は、分化、血清不足、浮遊状態での増殖、及び他の因子に応答して細胞増殖停止を媒介する。ヒト癌におけるp27発現のレベルは、正常な組織に対して頻繁に変化(増加減少)する(Philipp-Staheliら, 2001年, *Exp. Cell Res.* 第264巻: 148~161

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頁)。p 27は、老化に繋がる経路の1つにおいて、腫瘍サプレッサP T E Nと協力することが示唆されてきた(BringoldとSerrano, 2000年, Exp. Gerontol. 第35巻: 317~329頁)。

【発明の開示】

【発明が解決しようとする課題】

【0012】

本技術分野においては、p 21、p 16またはp 27などのCDK阻害剤遺伝子の誘導によって発現が制御される遺伝子を同定する必要性が残されている。本技術分野においては、細胞老化、発癌及び老化に関連する疾病に対する化合物の効果を調べるためのターゲットを開発する必要性もある。

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【課題を解決するための手段】

【0013】

(発明の概要)

本発明はCDK阻害剤遺伝子発現の誘導によって発現が制御される遺伝子を同定するための試薬及び方法を提供する。本発明は、発癌や老化関連疾病などの細胞老化の病原性結果を防止するための合理的ドラッグデザインにおける第1段階として、細胞遺伝子発現に対するp 21、p 27及びp 16などのCDK阻害剤の効果を阻害する化合物を同定するための試薬及び方法も提供する。

【0014】

第1の態様において、本発明は誘導可能なCDK阻害剤遺伝子を含む哺乳類細胞を提供する。好ましい実施形態において、CDK阻害剤遺伝子はp 21、p 16またはp 27をコードする。好ましい実施形態において、哺乳類細胞は、誘導可能なp 21遺伝子または誘導可能なp 16遺伝子または誘導可能なp 27遺伝子をコードする組み換え発現作成物を含んでいる。より好ましくは、前記作成物は、誘導可能なプロモータの転写制御下にある、p 21、最も好ましくはヒトp 21をコードするヌクレオチド配列を含んでいる。代替実施形態において、前記作成物は、CDK結合ドメインを含む、より好ましくはp 21のアミノ酸配列の1~78のアミノ酸を含むアミノ末端部分をコードするヌクレオチド配列を含んでいる。さらなる実施形態において、前記作成物は、誘導可能なプロモータの転写制御下にある、p 16、最も好ましくはヒトp 16をコードするヌクレオチド配列を含んでいる。さらなる実施形態において、前記作成物は、誘導可能なプロモータの転写制御下にある、p 27、好ましくはヒトp 27またはマウスp 27をコードするヌクレオチド配列を含んでいる。好ましい実施形態において、上記のような作成物内の誘導可能なプロモータの誘導は、誘導可能なプロモータから転写を誘導する、誘導剤、最も好ましくは生理学的に中立の誘導剤に細胞を接触させること、あるいは上記プロモータからの転写を阻害する物質を除去することによって行うことができる。好ましい細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態においては、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒトHT 1080線維肉腫細胞系列の細胞及びその類縁体である。

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【0015】

本発明の第1の態様の他の実施形態において、組み換え発現作成物を含む組み換え哺乳類細胞が提供され、該組み換え発現作成物においては、レポーター遺伝子が、CDK阻害剤、最も好ましくはp 21、p 16またはp 27によって発現が調節される細胞遺伝子から取り出したプロモータの転写制御下におかれている。好ましい実施形態において、プロモータはp 21、p 16またはp 27などのCDK阻害剤によって発現が誘導される細胞遺伝子から得られる。上記の実施形態において、プロモータは最も好ましくは表II内に示される遺伝子に由来するものであるが、当業者であればCDK阻害剤によって発現が誘導される遺伝子であればどんな遺伝子に由来するプロモータでも上記作成物において有益に使用できることを認識するであろう。最も好ましくは、プロモータは血清アミロイドA(配列番号: 1)、補体C3(配列番号: 2)、結合組織増殖因子(配列番号: 3)、インテグリン - 3(配列番号: 4)、アクチビンA(配列番号: 5)、ナチュラルキラー細

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胞蛋白質 4 (配列番号: 6)、プロサポシン (配列番号: 7)、Mac 2 結合蛋白質 (配列番号: 8)、ガレクチン - 3 (配列番号: 9)、スーパーオキシドジスムターゼ 2 (配列番号: 10)、グラニユリン / エピテリン (配列番号: 11)、p 6 6<sup>s h c</sup> (配列番号: 12)、カテプシン B (配列番号: 14)、 $\alpha$ -アミロイド前駆体蛋白質 (配列番号: 15)、組織トランスグルタミナーゼ (t - T G アーゼ; 配列番号: 16)、クラステリン (配列番号: 17)、プロスタサイクリン刺激因子 (配列番号: 18)、血管内皮細胞増殖因子 - C (配列番号: 19)、及びメタロプロテアーゼ - 1 の組織阻害剤 (配列番号: 20) から得られる。本発明の組み換え発現作成物を含む好ましいレポーター遺伝子としては、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、クロラムフェニコールアセチルトランスフェラーゼ、 $\beta$ -ガラクトシダーゼ、緑色蛍光蛋白質またはアルカリフォスファターゼが挙げられる。

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#### 【0016】

さらなる好ましい実施形態において、本発明は、CDK 阻害剤、最も好ましくは p 2 1、p 1 6 または p 2 7 によって発現が調節される、哺乳類遺伝子に対するプロモータの転写制御下にある、レポーター遺伝子をコードする第 1 の組み換え発現作成物と、哺乳類の CDK 阻害剤遺伝子をコードする第 2 の組み換え発現作成物とを含む哺乳類細胞を提供し、該哺乳類細胞において、CDK 阻害剤の発現は哺乳類細胞内で実験的に誘導される。好ましい実施形態において、CDK 阻害剤遺伝子は p 2 1、p 1 6 または p 2 7 である。好ましい実施形態において、哺乳類 CDK 阻害剤遺伝子をコードする組み換え発現作成物は、誘導可能な異種プロモータの転写制御下にあり、組み換え発現作成物からの CDK 阻害剤の発現は、組み換え細胞を、誘導可能なプロモータからの転写を誘導する誘導剤に接触させるか、あるいは、そのようなプロモータからの転写を阻害する物質を除去することにより媒介される。好ましくは、前記作成物は、p 2 1、最も好ましくはヒト p 2 1 をコードするヌクレオチド配列を含んでいる。他の実施形態において、前記作成物は、CDK 結合ドメインを含む、より好ましくは p 2 1 のアミノ酸配列の 1 ~ 7 8 のアミノ酸を含むアミノ末端部分をコードするヌクレオチド配列を含んでいる。代替の好ましい実施形態において、前記作成物は、p 1 6、最も好ましくはヒト p 1 6 をコードするヌクレオチド配列を含んでいる。代替の好ましい実施形態において、前記作成物は、p 2 7、好ましくはヒト p 2 7 またはマウス p 2 7 をコードするヌクレオチド配列を含んでいる。レポーター遺伝子をコードする第 2 の組み換え発現作成物の好ましい実施形態において、プロモータは、p 2 1、p 1 6 または p 2 7 などの CDK 阻害剤によって発現が制御される細胞遺伝子に由来する。これらの実施形態において、プロモータは最も好ましくは表 I I に示された遺伝子に由来する。本発明の第 2 の組み換え発現作成物を含む好ましいレポーター遺伝子としては、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、クロラムフェニコールアセチルトランスフェラーゼ、 $\beta$ -ガラクトシダーゼ、緑色蛍光蛋白質またはアルカリフォスファターゼが挙げられる。特に好ましい実施形態においては、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト HT 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。レポーター遺伝子または CDK 阻害剤によって誘導される内在性遺伝子の産物は、免疫学的試薬を用いて、遺伝子産物の活性を検定するか、相補的核酸へのハイブリダイゼーションによって検出されることが好ましい。

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#### 【0017】

第 2 の態様において、本発明は、哺乳類細胞における分裂促進または抗アポトーシス因子の CDK 阻害剤誘導性発現を阻害する化合物を同定するためのスクリーニング方法を提供する。好ましい実施形態において、本方法は、化合物の存在下または非存在下の細胞において、CDK 阻害剤、最も好ましくは p 2 1、p 1 6 または p 2 7 の発現を誘導する工程と、馴化培地中で分裂促進または抗アポトーシス化合物、またはそれらの複数の発現を比較する工程とを含む。CDK 阻害剤の阻害剤効果は、馴化培地中で当該化合物の非存在時よりも存在時の方が分裂促進または抗アポトーシス化合物またはそれらの複数の量が少なくなっていることによって確認する。本発明の本態様において提供される方法において、あらゆる CDK 阻害剤発現細胞が有用であるが、p 2 1、p 1 6 または p 2 7 を発現する

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細胞が最も好ましく、そのような細胞内での p 2 1、p 1 6 または p 2 7 の発現は、内在性 p 2 1、p 1 6 または p 2 7 を誘導するか、あるいは、本発明に従う p 2 1、p 1 6 または p 2 7 をコードする誘導可能な発現作成物を含む細胞を用いることによって達成することができる。好ましい細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞が挙げられる。特に好ましい実施形態においては、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト HT 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。分裂促進または抗アポトーシス化合物の発現は、免疫学的試薬を用いて、遺伝子産物の活性を検定するか、相補的核酸へのハイブリダイゼーションによって検出される。

#### 【0018】

代替実施形態において、本発明は、哺乳類細胞において分裂促進または抗アポトーシス因子の CDK 阻害剤誘導性発現を阻害する化合物を同定するための方法を提供し、該哺乳類細胞は、p 2 1、p 1 6 または p 2 7 などの CDK 阻害剤によって誘導される分裂促進または抗アポトーシス因子をコードする細胞遺伝子のプロモータの転写制御下にある、レポーター遺伝子をコードする組み換え発現作成物を含んでいる。好ましい実施形態において、プロモータとしては、結合組織増殖因子 (CTGF; 配列番号: 3)、アクチビン A (配列番号: 5)、グラニユリン/エピテリン (配列番号: 11)、ガレクチン-3 (配列番号: 9)、プロサポシン (配列番号: 7)、クラスチリン (配列番号: 17)、プロスタサイクリン刺激因子 (配列番号: 18)、血管内皮細胞増殖因子-C (配列番号: 19)、及びメタロプロテアーゼ-1 の組織阻害剤 (配列番号: 20) に対するプロモータが挙げられる。好ましいレポーター遺伝子としては、特に限定されないが、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、ガラクトシダーゼ、アルカリフォスファターゼ、及び緑色蛍光蛋白質が挙げられる。これらの実施形態において、レポーター遺伝子発現の CDK 阻害剤媒介性誘導の阻害を用いて、CDK 阻害剤発現細胞における分裂促進または抗アポトーシス因子の誘導を阻害する化合物を同定する。

#### 【0019】

この態様において、本発明は、哺乳類細胞において、分裂促進または抗アポトーシス因子または化合物の産生を阻害するための方法であって、分裂促進または抗アポトーシス因子に細胞を接触させる工程を含む方法も提供し、該方法において、前記化合物は本発明の本態様の前述の方法によって同定される。好ましい実施形態において、分裂促進または抗アポトーシス因子の産生が阻害されている、阻害性化合物と接触された哺乳類細胞は線維芽細胞であり、最も好ましくは間質線維芽細胞である。好ましい実施形態において、前記化合物は核因子- $\kappa$ B (NF- $\kappa$ B) 活性または発現の阻害剤である。

#### 【0020】

第3の態様において、本発明は、細胞遺伝子発現の CDK 阻害剤媒介性誘導を阻害する化合物を同定するための方法を提供する。これらの方法は、哺乳類細胞内で CDK 阻害剤遺伝子の発現を誘導もしくは生み出す工程と、CDK 阻害剤によって発現が誘導される細胞遺伝子の発現に変化をもたらすための化合物の存在下で細胞を検定する工程と、当該化合物の非存在時よりも当該化合物の存在時の方が細胞遺伝子の発現の変化の度合いが少ない場合に、細胞遺伝子発現の CDK 阻害剤媒介性誘導を阻害する化合物を同定する工程とを含む。好ましい実施形態において、CDK 阻害剤は p 2 1、p 1 6 または p 2 7 である。好ましい実施形態において、細胞遺伝子は CDK 阻害剤によって誘導され、細胞遺伝子発現の誘導を阻害する化合物は、CDK 阻害剤を当該化合物の非存在下で発現させた場合に検出されるよりも低いレベルで遺伝子が発現していることを検出することにより検出される。本発明方法の本態様の好ましい実施形態において、CDK 阻害剤は p 2 1、p 1 6 または p 2 7 である。好ましい実施形態において、前記遺伝子は表 I I に示されている。さらなる代替実施形態において、本方法は、CDK 阻害剤によって発現が誘導される遺伝子から得られるプロモータの転写制御下、レポーター遺伝子を含む組み換え哺乳類細胞を用いて実施される。CDK 阻害剤によって誘導される遺伝子に由来するプロモータを含む作成物を用いる場合、化合物が CDK 阻害剤媒介性遺伝子発現制御を阻害もしくは妨害する

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時には、レポーター遺伝子産物は当該化合物の非存在時よりも存在時の方が低いレベルで産生される。本発明方法の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。これらの実施形態において、プロモータは最も好ましくは、表IIに示される遺伝子に由来する。最も好ましくは、プロモータは、血清アミロイドA（配列番号：1）、補体C3（配列番号：2）、結合組織増殖因子（配列番号：3）、インテグリン-3（配列番号：4）、アクチビンA（配列番号：5）、ナチュラルキラー細胞蛋白質4（配列番号：6）、プロサポシン（配列番号：7）、Mac2結合蛋白質（配列番号：8）、ガレクチン-3（配列番号：9）、スーパーオキシドジスムターゼ2（配列番号：10）、グラニューリン/エピテリン（配列番号：11）、p66<sup>shc</sup>（配列番号：12）、カテプシンB（配列番号：14）、 $\alpha$ -アミロイド前駆体蛋白質（配列番号：15）、組織トランスグルタミナーゼ（t-TGアーゼ；配列番号：16）、クラステリン（配列番号：17）、プロスタサイクリン刺激因子（配列番号：18）、血管内皮細胞増殖因子-C（配列番号：19）、及びメタロプロテアーゼ-1の組織阻害剤（配列番号：20）から得られる。本発明の組み換え発現作成物を含む好ましいレポーター遺伝子としては、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、クロラムフェニコールアセチルトランスフェラーゼ、 $\beta$ -ガラクトシダーゼ、緑色蛍光蛋白質またはアルカリフォスファターゼが挙げられる。他の好ましい実施形態において、細胞は、CDK阻害剤によって発現が制御される哺乳類遺伝子に対するプロモータの転写制御下にある、レポーター遺伝子をコードする第1の組み換え発現作成物と、哺乳類のCDK阻害剤遺伝子をコードする第2の組み換え発現作成物とを含み、CDK阻害剤の発現は哺乳類細胞内で実験的に誘導される。レポーター遺伝子またはCDK阻害剤によって誘導される内在性遺伝子の産物は、免疫学的試薬を用いて、遺伝子産物の活性を検定するか、相補的核酸へのハイブリダイゼーションによって検出されることが好ましい。

#### 【0021】

第4の態様において、本発明は、哺乳類細胞における老化の病原性結果を阻害する化合物を同定するための方法を提供し、該方法において、該病原性結果は少なくとも部分的に、CDK阻害剤によって誘導される遺伝子の発現によって媒介される。これらの方法は、化合物の存在下で哺乳類細胞を試薬とともに処理するか、CDK阻害剤遺伝子発現を誘導する条件下で哺乳類細胞を培養する工程と、哺乳類細胞に対して、CDK阻害剤によって誘導された遺伝子の誘導を検定する工程と、CDK阻害剤によって誘導される遺伝子の発現が化合物の非存在時よりも化合物の存在時に誘導の程度が小さい場合に、当該化合物を老化または老化の病原性結果の阻害剤と同定する工程とを含む。本発明方法の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましい実施形態において、遺伝子は表IIに示されている。さらなる代替実施形態において、本方法は、CDK阻害剤によって発現が調節される遺伝子に由来するプロモータの転写制御下にあるレポーター遺伝子を含む組み換え哺乳類細胞を用いて実施される。これらの実施形態において、CDK阻害剤によって誘導される遺伝子に由来するプロモータを含む作成物を用いて、化合物の非存在時よりも存在時のほうがレポーター遺伝子の産物が低いレベルで産生されることは、当該化合物が細胞老化の病原性結果の阻害剤である場合に検出される。本発明方法の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。プロモータは好ましくは、表IIに示される遺伝子に由来する。プロモータは最も好ましくは、血清アミロイドA（配列番号：1）、補体C3（配列番号：2）、結合組織増殖因子（配列番号：3）、インテグリン-3（配列番号：4）、アクチビンA（配列番号：5）、ナチュラルキラー細胞蛋白質4（配列番号：6）、プロサポシン（配列番号：7）、Mac2結合蛋白質（配列番号：8）、ガレクチン-3（配列番号：9）、スーパーオキシドジスムターゼ2（配列番号：10）、グラニューリン/エピテリン（配列番号：11）、p66<sup>shc</sup>（配列番号：12）、カテプシンB（配列番号：14）、 $\alpha$ -アミロイド前駆体蛋白質（配列番号：15）、組織トランスグルタミナーゼ（t-TGアーゼ；配列番号：16）、クラステリン（配列番号：17）、プロスタサイクリン刺激因子（配列番号：18）、血管内皮細胞増殖因子-C（配列番号：19）、及びメ

タロプロテアーゼ - 1 の組織阻害剤 (配列番号 : 20) に由来する。他の好ましい実施形態において、細胞は、CDK 阻害剤によって発現が制御される哺乳類遺伝子に対するプロモータの転写制御下にある、レポーター遺伝子をコードする第 1 の組み換え発現作成物と、哺乳類の CDK 阻害剤遺伝子をコードする第 2 の組み換え発現作成物とを含み、CDK 阻害剤の発現は哺乳類細胞内で実験的に誘導される。本発明方法の本態様の好ましい実施形態において、CDK 阻害剤は p 21、p 16 または p 27 である。特に好ましい実施形態においては、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト HT 1080 線維肉腫細胞系列の細胞及びその類縁体である。レポーター遺伝子または CDK 阻害剤によって誘導される内在性遺伝子の産物は、免疫学的試薬を用いて、遺伝子産物の活性を検定するか、相補的核酸へのハイブリダイゼーションによって検出されることが好ましい。

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**【0022】**

第 5 の態様において、本発明は、発癌または老化関連疾病などの細胞老化の病原性結果を阻害するための方法を提供し、該方法は細胞を、本発明の前記態様において提供される方法を用いて判定される老化または老化の病原性結果を防止する化合物と接触させる工程を含む。

**【0023】**

第 6 の態様において、本発明は本明細書に開示される本発明の方法のいずれかを用いて同定される化合物を提供する。

**【0024】**

第 7 の態様において、本発明は CDK 阻害剤による遺伝子発現の誘導を阻害または防止するための方法を提供する。好ましい実施形態において、本発明は、細胞を、CDK 阻害剤による遺伝子発現の誘導を阻害または防止する化合物を同定するための新奇の方法によって同定される化合物と接触させる工程を含む。好ましい実施形態において有効量の化合物が、薬学的に許容される担体または他の薬剤を用いて医薬組成物中に調合され、動物、最も好ましくは CDK 阻害剤誘導性遺伝子発現によって引き起こされる疾病に罹った動物に投与される。好ましい実施形態において、疾病は癌、アルツハイマー病、腎臓病、関節炎またはアテローム性動脈硬化症である。好ましい実施形態において、本方法は NF B 阻害剤である化合物を利用する。

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**【0025】**

本発明の具体的な好ましい実施形態については、以下の特定の実施形態のより詳細な説明及び請求項から明らかになるであろう。

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**【発明を実施するための最良の形態】****【0026】**

(好ましい実施例の詳細な説明)

本発明は、CDK 阻害剤誘導性細胞老化及び老化の病原性結果の媒介に関与する遺伝子を同定するための試薬及び方法、ならびに、哺乳類細胞内において老化及び老化の病原性結果を阻害することのできる化合物を提供する。とりわけ、細胞老化に関与し、CDK 阻害剤 p 21、p 27 または p 16 によって誘導される遺伝子を同定するための試薬及び方法の実施形態が提供される。

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**【0027】**

本発明の目的のために、用語「CDK 阻害剤」は、サイクリン依存性キナーゼ阻害の生化学的活性を有する哺乳類遺伝子のファミリーのメンバーを包含するものとする。この定義内に明白に含まれるものとしては、CDK 阻害剤 p 15、p 14、p 18、また特に p 21、p 16 または p 27 が挙げられ、後の 3 つは本発明の試薬及び方法の特に好ましい実施形態である。

**【0028】**

本発明の目的のために、「細胞 (単数)」または「細胞 (複数)」は等価であるものとし、特に技術上公知のように培養され維持された哺乳類細胞のインビトロ培養物を包含するものとする。

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## 【0029】

本発明の目的のために、「細胞遺伝子」を複数で記載する場合、これは2つ以上の遺伝子のみならず単一の遺伝子も包含するものとする。細胞遺伝子発現の調節の影響または、細胞遺伝子に由来するプロモータの転写制御下にあるレポーター作成物は、第1の遺伝子において検出して、その影響を第2の遺伝子または任意の数のさらなる遺伝子またはレポーター遺伝子作成物を試験することにより再現することができることは、当業者によって理解されるであろう。あるいは、2つまたはそれ以上の遺伝子またはレポーター遺伝子作成物の発現を本発明の範囲内において同時にアッセイすることができる。

## 【0030】

本明細書において、「馴化培地」という用語は、分裂促進または抗アポトーシス因子を含むCDK阻害剤発現細胞の増殖によって規定された細胞培養培地を包含するものとする。馴化培地は、哺乳類細胞培養培地中で、最も好ましくは血清添加剤を含まない合成培地中でCDK阻害剤発現細胞を培養することにより、好ましい実施形態において生産される。任意のCDK阻害剤発現細胞を前記馴化培地の生産に有用に用いることができ、そのような細胞内でのCDK阻害剤発現は内在性CDK阻害剤を誘導することにより（たとえばDNA損傷剤による処理、イオン化または紫外線照射または接触阻害）、あるいは、本発明による誘導可能なCDK阻害剤発現作成物を含む細胞を用いて、生理学的に中立な誘導剤中で細胞を培養することによって達成することができる。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましい細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態においては、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒトHT1080線維肉腫細胞系列の細胞及びその類縁体である。

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## 【0031】

本発明の目的のために、「老化」という用語は、正常細胞の増殖寿命の最後に起こるか、若しくは細胞毒性薬物、DNA損傷または他の細胞傷害に反応して正常細胞または腫瘍細胞内で起こるような、増殖因子によって回復されないDNA複製及び細胞増殖の永久的中止を含むものと理解される。

## 【0032】

老化は、哺乳類細胞内で多数の方法で誘導することができる。1つめは、インピボまたはインピトロのいずれかにおける正常な細胞の増殖の正常な結果である。正常な細胞が老化する前に経験する細胞分裂、経過、世代の数は限られている。正確な数は、細胞の型と起源の種類によって異なる（HayflickとMoorhead、1961年、Exp. Cell Res. 第25巻：585～621頁）。任意の細胞型において老化を誘導するための別の方法としては、殆どの抗癌剤、照射、及び細胞分化剤などの細胞毒性薬剤での処理がある。Changら、1999年、Cancer Res. 第59巻：3761～3767頁を参照のこと。老化は、当該細胞内に腫瘍サプレッサ遺伝子を形質導入することによりあらゆる哺乳類細胞（p53、p21、p16またはRb等）において即座に誘導することができる。Sugrueら、1997年、Proc. Natl. Acad. Sci. USA 第94巻：9648～9653頁；Uhrbomら、1997年、Oncogene 第15巻：505～514頁；Xuら、1997年、Oncogene 第15巻：2589～2596頁；Vogtら、1998年、Cell Growth Differ. 第9巻：139～146頁を参照のこと。

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## 【0033】

本発明の目的のために、「老化の病原性結果」という用語は、癌、アテローム性動脈硬化症、アルツハイマー病、アシロイドーシス、腎臓病及び関節炎などの疾病を包含するものとする。

## 【0034】

本発明の試薬には、CDK阻害剤遺伝子、最も好ましくはp21、p16またはp27の発現を誘導することのできる、任意の哺乳類細胞、好ましくは齧歯類または霊長類細胞、

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より好ましくはマウス細胞及び最も好ましくはヒト細胞が含まれ、そのような遺伝子は遺伝子工学によって導入された内在性遺伝子または外来性遺伝子のいずれかである。実施例では、誘導可能な p 2 1、p 2 7 及び p 1 6 遺伝子をコードする組み換え発現作成物を含む組み換え哺乳類細胞を開示しているが、これらの実施形態は実験上の設計の選択と便宜の問題にすぎず、本発明は p 2 1、p 2 7 または p 1 6 などの内在性 C D K 阻害剤遺伝子の誘導も完全に包含するものと理解されたい。

【0035】

好ましい実施形態において、本発明は、誘導可能な哺乳類 p 2 1 遺伝子をコードする組み換え発現作成物を含む哺乳類細胞を提供する。好ましい実施形態において、p 2 1 遺伝子は、引用により本願に組み込まれる米国特許第 5, 4 2 4, 4 0 0 号に記載のヌクレオチド及びアミノ酸配列を有するヒト p 2 1 である。代替実施形態において、p 2 1 遺伝子は、好ましくは（参照により本願に組み込まれる米国特許第 5, 8 0 7, 6 9 2 号に開示されるように）天然のヒト p 2 1 蛋白質のアミノ酸残基 1 ~ 7 8 を含み、より好ましくは天然のヒト p 2 1 蛋白質のアミノ酸 2 1 ~ 7 1 からなる C D K 結合ドメインを含む（N a k a n i s h i ら、1 9 9 5 年、E M B O J . 第 1 4 巻：5 5 5 ~ 5 6 3 頁）ヒト p 2 1 遺伝子のアミノ末端部分である。好ましい宿主細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト H T 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。最も好ましい細胞系列は、2 0 0 0 年 4 月 6 日に A m e r i c a n T y p e C u l t u r e C o l l e c t i o n ( 在米国バージニア州マナサス) に受託番号 P T A 1 6 6 4 として寄託された、H T 1 0 8 0 p 2 1 - 9 として識別される H T 1 0 8 0 線維肉腫細胞系列類縁体である。

【0036】

代替の好ましい実施形態において、本発明は誘導可能な哺乳類 p 1 6 遺伝子をコードする組み換え発現作成物を含む哺乳類細胞を提供する。好ましい実施形態において、p 1 6 遺伝子は、N C B I R e f S e q N M \_ 0 0 0 0 7 7 及び N P \_ 0 0 0 0 6 8 に記載されるヌクレオチド及びアミノ酸配列を有するヒト p 1 6 である。好ましい宿主細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト H T 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。最も好ましい細胞系列は、2 0 0 2 年 1 月 3 1 日に A m e r i c a n T y p e C u l t u r e C o l l e c t i o n ( 在米国バージニア州マナサス) に受託番号 \_\_\_\_\_ として寄託された、H T 1 0 8 0 p 1 6 - 5 として識別される H T 1 0 8 0 線維肉腫細胞系列類縁体である。

【0037】

代替の好ましい実施形態において、本発明は誘導可能な哺乳類 p 2 7 遺伝子をコードする組み換え発現作成物を含む哺乳類細胞を提供する。好ましい実施形態において、p 2 7 遺伝子は、N C B I R e f S e q N M \_ 0 0 4 0 6 4 及び N P \_ 0 0 4 0 5 5 に記載されるヌクレオチド及びアミノ酸配列を有するヒト p 2 7 または N C B I R e f S e q N M \_ 0 0 9 8 7 5 及び N P \_ 0 3 4 0 0 5 に記載されるヌクレオチド及びアミノ酸配列を有するマウス p 1 6 である。好ましい宿主細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト H T 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。最も好ましい細胞系列は、2 0 0 2 年 1 月 3 1 日に A m e r i c a n T y p e C u l t u r e C o l l e c t i o n ( 在米国バージニア州マナサス) に受託番号 \_\_\_\_\_ として寄託された、H T 1 0 8 0 p 2 7 - 2 として識別される H T 1 0 8 0 線維肉腫細胞系列類縁体である。

【0038】

組み換え発現作成物は、当業者によって理解されるように適当な哺乳類細胞内へ導入することができる。前記作成物の好ましい実施形態は、伝達性ベクター、より好ましくはウィ

ルスベクター、最も好ましくは技術上知られているように、レトロウィルスベクター、アデノウィルスベクター、アデノ関連ウィルスベクター、及びワクシニアウィルスベクターにおいて生産される。一般的には、MOLECULAR VIROLOGY: A PRACTICAL APPROACH、(DavisonとElliot編)、オックスフォード大学出版局：ニューヨーク、1993年を参照のこと。

【0039】

さらなる好ましい実施形態において、本発明の組み換え細胞は、誘導可能なCDK阻害剤遺伝子をコードする作成物を含んでおり、遺伝子は誘導可能なプロモータの転写制御下にある。より好ましい実施形態において、誘導可能なプロモータは、誘導剤によって効果の調節が可能なトランス作用因子に反応を示す。この誘導剤は、温度及び最も好ましくは誘導剤の有無など、実験的に操作されることのできるいかなる因子であってもよい。好ましくは、誘導剤は化学的化合物、最も好ましくはトランス作用因子に特異的な生理学的に中立の化合物である。本願に開示されるような誘導可能なプロモータを含む作成物の使用時には、組み換え発現作成物からのCDK阻害剤の発現は、組み換え細胞を、誘導可能なプロモータからの転写を誘導する誘導剤と接触させることによって、あるいはそのようなプロモータからの転写を阻害する物質を除去することによって媒介される。本発明方法の本態様の好ましい実施形態において、CDK阻害剤はp21、p27またはp16である。細胞培養の温度を上昇させることによって活性化することができる熱ショックプロモータ、ならびに、より好ましくはtetプロモータやその同起源のtetリプレッサーなどのプロモータ/因子対、及びその哺乳類転写因子との融合物(米国特許第5,654,168号、第5,851,796号、及び第5,968,773号に開示されるような)、ならびにラクトースオペロンの細菌lacプロモータとその同起源のlacリプレッサー蛋白質を含む、様々な誘導可能なプロモータ及び同起源のトランス作用因子が従来技術において知られている。好ましい実施形態において、組み換え細胞はlacIリプレッサー蛋白質と、1つまたは複数のlac応答要素を含むプロモータの制御下にあるヒトp21をコードする組み換え発現作成物を発現し、p21の発現は、細胞を生理学的に中立な誘導剤であるイソプロピルチオ-βガラクトシドと接触させることにより誘導することができる。この好ましい実施形態において、lacIリプレッサーは、3'SS(Stratagene, カリフォルニア州ラホーヤから市販)として識別される組み換え発現作成物によってコードされる。代替の好ましい実施形態において、組み換え細胞はlacIリプレッサー蛋白質と、1つまたは複数のlac応答要素を含むプロモータの制御下にあるヒトp16をコードする組み換え発現作成物を発現し、p16の発現は、細胞を生理学的に中立な誘導剤であるイソプロピルチオ-βガラクトシドと接触させることにより誘導することができる。lacIリプレッサーは、3'SS組み換え発現作成物(Stratagene)によってコードされる。代替の好ましい実施形態において、組み換え細胞はlacIリプレッサー蛋白質と、1つまたは複数のlac応答要素を含むプロモータの制御下にあるヒトp27をコードする組み換え発現作成物を発現し、p27の発現は、細胞を生理学的に中立な誘導剤であるイソプロピルチオ-βガラクトシドと接触させることにより誘導することができる。lacIリプレッサーは、3'SS組み換え発現作成物(Stratagene)によってコードされる。

【0040】

本発明は、レポーター遺伝子がp21、p16またはp27などのCDK阻害剤によって発現が調節されるプロモータの転写制御下にある、組み換え発現作成物も提供する。これらは、CDK阻害剤によって発現が誘導される遺伝子を含む。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましい実施形態において、プロモータはCDK阻害剤発現によって発現が誘導または増大される遺伝子に由来し、これらのプロモータは表IIに示されている。最も好ましくは、プロモータは血清アミロイドA(配列番号:1)、補体C3(配列番号:2)、結合組織増殖因子(配列番号:3)、インテグリン-3(配列番号:4)、アクチピンA(配列番号:5)、ナチュラルキラー細胞蛋白質4(配列番号:6)、プロサボシン(配列番号:7)、Ma

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c 2 結合蛋白質 (配列番号: 8)、ガレクチン - 3 (配列番号: 9)、スーパーオキシドジスムターゼ 2 (配列番号: 10)、グラニューリン / エピテリン (配列番号: 11)、p 6 6<sup>s h c</sup> (配列番号: 12)、カテプシン B (配列番号: 14)、 $\alpha$ -アミロイド前駆体蛋白質 (配列番号: 15)、組織トランスグルタミナーゼ (t - T G アーゼ; 配列番号: 16)、クラスτεリン (配列番号: 17)、プロスタサイクリン刺激因子 (配列番号: 18)、血管内皮細胞増殖因子 - C (配列番号: 19)、及びメタロプロテアーゼ - 1 の組織阻害剤 (配列番号: 20) から得られる。これらのレポーター遺伝子は、C D K 阻害剤遺伝子発現の効果に対する感受性が高く便利な指示薬として用いられ、哺乳類細胞内で C D K 阻害剤発現の効果を抑害する化合物を容易に同定することを可能にする。これらの作成物に対する宿主細胞としては、細胞内で C D K 阻害剤遺伝子発現の誘導が可能であればどのような細胞でもよいが、上述のような誘導可能な C D K 阻害剤遺伝子を含む組み換え発現作成物も含んだ細胞が好ましい。本発明の本態様の実施において有用なレポーター遺伝子としては、特に限定されないが、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、クロラムフェニコールアセチルトランスフェラーゼ、 $\beta$ -ガラクトシダーゼ、緑色蛍光蛋白質またはアルカリフォスファターゼが挙げられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒト H T 1 0 8 0 線維肉腫細胞系列の細胞及びその類縁体である。最も好ましい細胞系列は、2001年5月17日に American Type Culture Collection (在米国バージニア州マナサス) に受託番号 P T A - 3 3 8 1 として寄託された、H T 1 0 8 0 / L U N K 4 p 2 1 として識別される H T 1 0 8 0 線維肉腫細胞系列類縁体である。

#### 【0041】

好ましい実施形態において、本発明による細胞は、C D K 阻害剤によって発現が調節される哺乳類遺伝子に対するプロモータの転写制御下にあるレポーター遺伝子をコードする第1の組み換え発現作成物と、哺乳類 C D K 阻害剤遺伝子をコードする第2の組み換え発現作成物との両方を含み、これにより C D K 阻害剤の発現は哺乳類細胞内において実験的に誘導可能である。本発明の本態様の好ましい実施形態において、C D K 阻害剤は p 2 1、p 1 6 または p 2 7 である。代替実施形態において、本発明は、C D K 阻害剤によって発現が調節される哺乳類遺伝子に対するプロモータの転写制御下にあるレポーター遺伝子をコードする組み換え発現作成物を含む哺乳類細胞を提供し、プロモータは、血清アミロイド A (配列番号: 1)、補体 C 3 (配列番号: 2)、結合組織増殖因子 (配列番号: 3)、インテグリン - 3 (配列番号: 4)、アクチビン A (配列番号: 5)、ナチュラルキラー細胞蛋白質 4 (配列番号: 6)、プロサポシン (配列番号: 7)、M a c 2 結合蛋白質 (配列番号: 8)、ガレクチン - 3 (配列番号: 9)、スーパーオキシドジスムターゼ 2 (配列番号: 10)、グラニューリン / エピテリン (配列番号: 11)、p 6 6<sup>s h c</sup> (配列番号: 12)、カテプシン B (配列番号: 14)、 $\alpha$ -アミロイド前駆体蛋白質 (配列番号: 15)、組織トランスグルタミナーゼ (t - T G アーゼ; 配列番号: 16)、クラスτεリン (配列番号: 17)、プロスタサイクリン刺激因子 (配列番号: 18)、血管内皮細胞増殖因子 - C (配列番号: 19)、及びメタロプロテアーゼ - 1 の組織阻害剤 (配列番号: 20) をコードする遺伝子から得られる。本発明の本態様の好ましい実施形態において、C D K 阻害剤は p 2 1、p 1 6 または p 2 7 である。

#### 【0042】

本発明は、哺乳類細胞における分裂促進または抗アポトーシス因子の C D K 阻害剤誘導性発現を抑害する化合物を同定するためのスクリーニング方法を提供する。好ましい実施形態において、分裂促進または抗アポトーシス因子の C D K 阻害剤誘導性発現の阻害剤であることを同定しようとしている化合物の存在下または非存在下、哺乳類細胞培養内で C D K 阻害剤発現が誘導される。化合物は、該化合物の存在下または非存在下、細胞内で C D K 阻害剤の発現を誘導し、分裂促進または抗アポトーシス因子、あるいはそれらの複数の発現の程度を比較することにより、分裂促進または抗アポトーシス因子の発現の量が減っている化合物を阻害剤として同定する。本発明の本態様の好ましい実施形態において、C D K 阻害剤は p 2 1、p 1 6 または p 2 7 である。前記馴化培地を作成するためにはあら

ゆるCDK阻害剤発現細胞が有用であり、そのような細胞内でのCDK阻害剤発現は、内在性CDK阻害剤を(DNA損傷剤や他の細胞毒性化合物で処理したり、イオン化またはUV照射、あるいは接触阻害などにより)誘導することにより、あるいは本発明による誘導可能なCDK阻害剤発現作成物を含む細胞を用いて、該細胞を生理学的に中立な誘導剤中で培養することによって達成される。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましい細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒトHT1080線維肉腫細胞系列の細胞及びその類縁体である。本発明の特に好ましい実施形態に従う例示的細胞系列は、2000年4月6日にAmerican Type Culture Collection(在米国バージニア州マナサス)に受託番号PTA1664として寄託された、HT1080 p21-9として識別されるHT1080線維肉腫細胞系列類縁体である。例示的細胞集団は、2000年10月10日にAmerican Type Culture Collection(在米国バージニア州マナサス)に受託番号PTA-2580として寄託された、HT1080/LNp16RO2として識別されるヒトHT1080線維肉腫類縁体である。本発明のこの特定の好ましい実施形態による他の例示的細胞系列は、\_\_\_にAmerican Type Culture Collection(在米国バージニア州マナサス)に受託番号\_\_\_として寄託された、HT1080 p16-5として識別されるHT1080線維肉腫細胞系列類縁体である。本発明のこの特定の好ましい実施形態による他の例示的細胞系列は、\_\_\_にAmerican Type Culture Collection(在米国バージニア州マナサス)に受託番号\_\_\_として寄託された、HT1080 p27-2として識別されるHT1080線維肉腫細胞系列類縁体である。

#### 【0043】

代替実施形態において、本発明は、哺乳類細胞において分裂促進または抗アポトーシス因子のCDK阻害剤誘導性発現を阻害する化合物を同定するための方法を提供し、該哺乳類細胞は、CDK阻害剤によって誘導される分裂促進または抗アポトーシス因子をコードする細胞遺伝子のプロモータの転写制御下、レポーター遺伝子をコードする組み換え発現作成物を含んでいる。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましいプロモータとしては、結合組織増殖因子(CTGF; 配列番号: 3)、アクチビンA(配列番号: 5)、エピテリン/グラニユリン(配列番号: 11)、ガレクチン-3(配列番号: 9)、プロサポシン(配列番号: 7)、クラステリン(配列番号: 17)、プロスタサイクリン刺激因子(配列番号: 18)、血管内皮細胞増殖因子-C(配列番号: 19)、及びメタロプロテアーゼの組織阻害剤(配列番号: 20)に対するプロモータが挙げられる。好ましいレポーター遺伝子としては、特に限定されないが、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、-ガラクトシダーゼ、アルカリフォスファターゼ、及び緑色蛍光蛋白質が挙げられる、これらすべては市販されている。これらの実施形態においては、細胞内でCDK阻害剤の発現が誘導され、レポーター遺伝子の発現の程度が、化合物が存在する場合と存在しない場合とで比較される。阻害剤は、当該化合物の非存在時よりも当該化合物の存在時の方がレポーター遺伝子の発現量が減るような化合物であるとみなされる。本発明の本態様においてはあらゆるCDK阻害剤発現細胞が有用であり、そのような細胞内でのCDK阻害剤発現は、内在性CDK阻害剤を(DNA損傷剤や他の細胞毒性化合物で処理したり、イオン化またはUV照射、あるいは接触阻害などにより)誘導することにより、あるいは本発明による誘導可能なCDK阻害剤発現作成物を含む細胞を用いて、該細胞を生理学的に中立な誘導剤中で培養することによって達成される。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。好ましい細胞としては、哺乳類細胞、好ましくは齧歯類または霊長類細胞、より好ましくはマウスまたはヒト細胞があげられる。特に好ましい実施形態は、線維肉腫細胞、より好ましくはヒト線維肉腫細胞、最も好ましくはヒトHT1080線維肉腫細胞系列の細胞及びその類縁体である。最も好ましい細胞系

列は、2001年5月17日にAmerican Type Culture Collection (在米国バージニア州マナサス)に受託番号PTA 3381として寄託された、HT1080/LUNK4p21として識別されるHT1080線維肉腫細胞系列類縁体である。

【0044】

本発明は、細胞老化の病原性結果を阻害する化合物を同定するための方法を提供し、該方法により、化合物がCDK阻害剤によって発現が誘導される遺伝子の誘導を阻害するかどうかを判定することによって当該化合物の効果が検定される。本発明の方法の実施にあたっては、CDK阻害剤を誘導することのできる培養哺乳類細胞が、例えばイオン化または紫外線照射、あるいは接触阻害処理または細胞毒性薬剤による処理、あるいはCDK阻害剤をコードする伝達性ベクターによる形質導入により、阻害剤遺伝子を誘導するように処理される。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。より好ましくは、細胞をIPTGに接触させることによりp21を誘導することのできるHT1080 p21-9細胞(2000年4月6日に受託番号PTA1664として、American Type Culture Collection, 在米国バージニア州マナサスに寄託)、または、IPTGによってp16を誘導することができるHT1080 p16-5細胞(2002年1月31日に受託番号\_\_\_\_\_)として、American Type Culture Collection, 在米国バージニア州マナサスに寄託)、またはIPTGによってp27を誘導することのできるHT1080 p27-2細胞(2002年1月31日に受託番号\_\_\_\_\_として、American Type Culture Collection, 在米国バージニア州マナサスに寄託)が用いられる。典型的には、細胞は適当な培養培地(例えば、HT1080類縁体に対しては、10%ウシ胎仔血清(FCS)を添加したDMEM)中で培養される。HT1080 p21-9、HT1080 p16-5またはHT1080 p27-2細胞においては、CDK阻害剤遺伝子発現は、IPTGを約50 $\mu$ Mの濃度で培養培地に添加することにより誘導される。典型的には、CDK阻害剤は、本発明の方法に従って試験される化合物の存在下または非存在下にて上記細胞内において誘導される。次にCDK阻害剤が誘導されている細胞からmRNAを単離し、CDK阻害剤によって調節されている遺伝子の発現を分析する。化合物の存在下でCDK阻害剤が誘導されている細胞における発現と、化合物の非存在下でCDK阻害剤が誘導されている細胞における発現とを比較し、この差異を用いて、本願に記載の方法に従って細胞遺伝子発現に影響を及ぼしている化合物を同定する。ある実施形態においては、細胞遺伝子発現は、(例えば、Genome Systems, Inc., 在ミズーリ州セントルイスから)市販されているようなオリゴヌクレオチドまたは細胞cDNAのマイクロアレイを用いて分析される。代替の実施形態において、CDK阻害剤によって誘導されることが知られている遺伝子を検定する。遺伝子発現は、CDK阻害剤によって調節される遺伝子の1つまたは複数について、細胞mRNAまたは蛋白質のいずれかを分析することによって検定することができる。本発明の本態様の好ましい実施形態において、CDK阻害剤はp21、p16またはp27である。最も好ましくは、これらの検定に用いられる遺伝子は、表IIに示されている遺伝子である。

【0045】

代替実施形態において、そのような化合物がCDK阻害剤に向けられた実験的操作とは無関係に同定された。そのような検定において、細胞は、特に限定されないが、細胞毒性薬剤による処理、照射または細胞分化剤、または腫瘍サプレッサ遺伝子の導入を含む上記に開示した任意の方法で老化を誘導するように処理される。CDK阻害剤によって誘導される遺伝子の発現を、試験化合物の存在下または非存在下において分析した。最も好ましくは、遺伝子発現分析のために上記で検討したmRNA及び蛋白質アッセイの類を用い、これらの検定では表IIに示される遺伝子を用いる。

【0046】

代替実施形態において、CDK阻害剤が誘導される細胞は、CDK阻害剤によって誘導さ

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れる細胞遺伝子のプロモータの転写制御下にあるレポーター遺伝子をコードする組み換え発現作成物をさらに含む。本発明の本態様の好ましい実施形態において、CDK阻害剤は、p21、p16またはp27である。好ましい実施形態において、細胞遺伝子は、CDK阻害剤によって誘導される遺伝子であり、プロモータは、表IIに示される遺伝子に由来するものである。そのような遺伝子に対するプロモータとしては、例えば、血清アミロイドA（配列番号：1）、補体C3（配列番号：2）、結合組織増殖因子（配列番号：3）、インテグリン-3（配列番号：4）、アクチビンA（配列番号：5）、ナチュラルキラー細胞蛋白質4（配列番号：6）、プロサポシン（配列番号：7）、Mac2結合蛋白質（配列番号：8）、ガレクチン-3（配列番号：9）、スーパーオキシドジスムターゼ2（配列番号：10）、グラニューリン/エピテリン（配列番号：11）、p66<sup>shc</sup>（配列番号：12）、カテプシンB（配列番号：14）、-アミロイド前駆体蛋白質（配列番号：15）、組織トランスグルタミナーゼ（t-TGアーゼ；配列番号：16）、クラスチリン（配列番号：17）、プロスタサイクリン刺激因子（配列番号：18）、血管内皮細胞増殖因子-C（配列番号：19）、及びメタロプロテアーゼ-1の組織阻害剤（配列番号：20）が知られている。好ましいレポーター遺伝子としては、特に限定されないが、ホタルルシフェラーゼ、ウミシイタケルシフェラーゼ、-ガラクトシダーゼ、アルカリフォスファターゼ、及び緑色蛍光蛋白質が挙げられる、これらすべては市販されている。

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#### 【0047】

本発明は、細胞老化及び老化の病原性結果に関連する遺伝子、またはCDK阻害剤によって誘導される細胞老化の作用を媒介する遺伝子を同定するための方法も提供する。CDK阻害剤の誘導は、老化、最終分化及び細胞損傷に対する応答に伴う細胞増殖停止の不可欠な部分であることが分かっている。後述の実施例において記載するように、cDNAアレイハイブリダイゼーションからは、これらの効果が遺伝子発現におけるp21誘導性変化であることが示された。p21は細胞老化及び老化に関連するか、アテローム性動脈硬化症、アルツハイマー病、アミロイドーシス、腎臓病及び関節炎を含む老化関連疾病に關与していた遺伝子を選択的に誘導した。これらの知見は、有機体内でのp21誘導の累積的効果が、癌及び老化関連疾病の病原性に寄与しているかもしれないことを示唆する。さらに、多数のp21によって活性化される遺伝子は、細胞増殖及びアポトーシスに対して潜在的なパラクリン有する分泌蛋白質をコードしている。この見解と一致して、p21によって誘導される細胞からの馴化培地は分裂促進及び抗アポトーシス活性を示した。

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#### 【0048】

さらに、以下の実施例において示される結果からは、p16またはp27の誘導発現は、p21遺伝子発現とよく似ていること、またp21遺伝子発現によって発現が調節される同様の遺伝子は、p16またはp27遺伝子発現によっても調節されることが実証された（図6を参照のこと）。したがって、本発明の方法は、内在性p16またはp27遺伝子の誘導によって、あるいはp16またはp27をコードする誘導可能な発現作成物を含む組み換え細胞において、p16またはp27遺伝子発現が誘導される細胞も含むように拡大される。

#### 【0049】

観察されたCDK阻害剤誘導、特に遺伝子発現に対するp21、p16及びp27誘導の効果は、細胞老化及び有機体の老化に伴う変化と多くの相関性を示した。これらの相関性のいくつかは、CDK阻害剤によって阻害される遺伝子の解析から得られる。老化線維芽細胞は、p21誘導時に観察されたように、低いレベルでRbを発現することが報告されている（Steinら、1999年、Mol. Cell. Biol. 第19巻：2109～2117頁）。また、CDK阻害剤によって阻害された3つの遺伝子CHL1、CDC21及びRAD54は、ヘリカーゼファミリーのメンバーをコードしていることも興味深い。ヘリカーゼ群の別の蛋白質の欠失が、早期老化に伴う臨床状態であるウェルナー症候群の原因であり、細胞レベルでは、培養中の細胞の老化を加速させることが確認されている（Grayら、1997年、Nature Genet. 第17巻：100～103頁

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【0050】

しかしながら、老化表現型との最も強い相関性は、その多くが複製老化または有機体老化のあいだにレベルを増加させることが知られている、CDK阻害剤によって誘導される遺伝子の同定から得られる。細胞外基質（ECM）蛋白質の過剰発現は、複製老化のよく知られた顕著な特徴であり、このグループ内の2つのCDK阻害剤誘導性遺伝子、フィブロネクチン1及びプラスミノゲン活性化因子阻害剤1（PAI-1）が、細胞老化に頻繁に関与している（CrisofaloとPignolo、1996年、Exp. Gerontol. 第31巻：111～123頁に概説）。老化線維芽細胞内で過剰発現されていると報告された他のCDK阻害剤誘導性遺伝子としては、組織型プラスミノゲン活性化因子（t-PA；Westら、1996年、Exp. Gerontol. 第31巻：175～193頁）、カテプシンB（diPaololaら、1992年、Exp. Cell Res. 第201巻：500～505頁）、インテグリン 3（Hashimotoら、1997年、Biochem. Biophys. Res. Commun. 第240巻：88～92頁）及びAPP（Adlerら、1991年、Proc. Natl. Acad. Sci. USA 第88巻：16～20頁）が挙げられる。t-PA及びPAI-1（Hashimotoら、1987年、Thromb. Res. 第46巻：625～633頁）、カテプシンB（Bernsteinら、1990年、Brain Res. Bull. 第24巻：43～549頁）アクチピンA（Loriaら、1998年、Eur. J. Endocrinol. 第139巻：487～492頁）、プロサポシン（Mathurら、1994年、Biochem. Mol. Biol. Int. 第34巻：1063～1071頁）、APP（Ogomoriら、1988年、J. Gerontol. 第43巻：B157～B162）、SAA（RosenthalとFranklin、1975年、J. Clin. Invest. 第55巻：746～753頁）、及びt-TGアーゼ（Singhalら、1997年、J. Invest. Med. 第45巻：567～575頁）を含む、いくつかのCDK阻害剤誘導性蛋白質の発現は、有機体の老化と相関性があることが示されている。

【0051】

最も一般的に用いられている細胞老化のマーカーはSA-gal活性（Dimriら、1995年、Proc. Natl. Acad. Sci. USA 第92巻：9363～9367頁）である。この遺伝子は、IPTG処理したHT1080 p21-9細胞において強く高められる（Changら、1999年、Oncogene 第18巻：4808～4818頁）。SA-galは、活性の向上と、リソソーム-ガラクトシダーゼとは異なる局在性を見せると示唆されていたが（Dimriら、1995年、同書中）、他の研究では、老化細胞内でリソソーム活性が高まると記述されている（CristofaloとKabakijan、1975年、Mech. Aging Dev. 第4巻：19～28頁）。N-アセチルガラクトサミン-6-硫酸スルファターゼ（GALNS）、カテプシンB、酸-グルコシダーゼ、酸リパーゼA及びリソソームペプスタチン非感受性プロテアーゼを含む5つのリソソーム酵素が表IIに示されている。p21は、ミトコンドリア蛋白質SOD2、メタジン及び2,4-ジエノイル-CoAレダクターゼに対する遺伝子も上方制御したが、これは、老化細胞において過剰発現される様々なミトコンドリア遺伝子の報告と相関する（Doggettら、1992年、Mech. Aging Dev. 第65巻：239～255頁；Kodamaら、1995年、Exp. Cell Res. 第219巻：82～86頁；Kumazakiら、1998年、Mech. Aging Dev. 第101巻：91～99）。

【0052】

目立ったこととして、我々がp21、p16またはp27によって誘導されることを見つけた多くの遺伝子の産物は、アルツハイマー病、アミロイドーシス、アテローム性動脈硬化症及び関節炎と関連していた。したがってAPPはアルツハイマーのアミロイド斑の主要成分である-アミロイドペプチドになる。補体C3（Veerhuisら、1995年

、Virchows Arch. 第426巻：603～610)及びAMPデアミナーゼ (Simsら、1998年、Neurobiol. Aging 第19巻：385～391頁)もまた、アルツハイマー病において役割を果たすことが示唆されていた。また、p21によって最も迅速に誘導され、細胞分化、発癌、アポトーシス及び老化の多面的メディエータであることが記載されている (Parkら、1999年、J. Gerontol. A Biol. Sci. 第54巻：B78～B83)。t-TGアーゼが、アルツハイマー病とアミロイドーシスの両方に付随するプラークの形成に関与していること (DudekとJohnson、1994年、Brain Res. 第651巻：129～133頁)は特に興味深いことである。アミロイドーシスは、アテローム性動脈硬化症、変形性関節症及び関節リウマチにも関与していると思なされる、別のCDK阻害剤誘導性遺伝子産物であるSAAの堆積によるものである (JensenとWhitehead、1998年、Biochem. J. 第334巻：489～503頁)。CDK阻害剤によって上方制御された他の2つの分泌蛋白質、CTGF及びガレクチン3は、アテローム性動脈硬化症に関与している (Oemarら、1997年、Circulation 第95巻：831～839頁; Nachtigalら、1998年、Am. J. Pathol. 第152巻：1199～1208頁)。さらに、カテプシンB (Howieら、1985年、J. Pathol. 第145巻：307～314頁)、PAI-1 (Cerinicら、1998年、Life Sci. 第63巻：441～453頁)、フィブロネクチン (Chevalier、1993年、Semin. Arthritis Rheum. 第22巻：307～318頁)、GALNS及びMac-2結合蛋白質 (Sekiら、1998年、Arthritis Rheum. 第41巻：1356～1364頁)は、変形性関節症及び/または関節リウマチに関与していた。さらに、ECM蛋白質における老化に係る変化、例えばPAI-1発現の増加などは、皮膚及び他の組織の構造に年齢特異的な劣化をもたらすと提案されている (Campisi、1998年、J. Invest. Dermatol. Symp. Proc. 第3巻：1～5頁)。老化細胞によるフィブロネクチン産生の増加は、ECMフィブロネクチン網目構造の密度を増大させて、これが老化した固体における損傷の治癒を遅らせることに寄与と示唆されている (Albinら、1988年、Coll Relat. Res. 第8巻：23～37頁)。

### 【0053】

p21及びp21誘導可能な遺伝子は、糖尿病腎症及び慢性腎不全にも関与している。Kuanら (1998年、J. Am. Soc. Nephrol. 第9巻：986～993頁)は、p21が、糖尿病腎症のインビトロモデルである、グルコース誘導性メサンギウム細胞肥大の条件下で誘導されることを発見した。Megyesiら (1996年、Am. J. Physiol. 第271巻：F1211～1216)は、p21がいくつかの急性腎不全の動物モデルにおいて、インビボで誘導され、このp21の誘導がp53に依存しないことを実証した。この病原性プロセスにおけるp21の機能的役割は、p21(-/-)マウスが実験的糖尿病の条件下で糸球体肥大を発症しないことを発見したA1-Douahjiら (1999年、Kidney Int. 第56巻：1691～1699頁)、及び、p21(-/-)マウスが腎臓の部分切除後にも慢性腎不全を発症しないことを示したMegyesiら (1999年、Proc Natl Acad Sci USA. 第96巻：10830～10835頁)によって実証されている。際立ったものとしては、Murphyら (1999年、J. Biol. Chem. 第274巻：5830～5834頁)は、Kuanら (1998年、J. Am. Soc. Nephrol. 第9巻：986～993頁)によって用いられたものと同じインビトロモデルを用いて研究を行い、メサンギウム細胞の肥大には、本願においてp21によって誘導されることが示されたいくつかの遺伝子の上方制御が関与していることを報告した。これらには、CTGF、フィブロネクチン及びプラスミノゲン活性化因子阻害剤1が含まれる。後者の研究は、CTGFが、モデル系におけるメサンギウム基質蓄積において機能的役割を果たすことも示した (Murphyら、1999年、J. Biol. Chem. 第274巻：5830～5834頁)。これらの結果は、腎不全の病原性において、p21及びp21によって媒介

される遺伝子発現の誘導が関与していることを示す。

【0054】

特に興味深いのは、最近になって酸化損傷を増強することが分かった遺伝子である p66<sup>sc</sup> の発現が p21 によって誘導され、p66(-/-) マウスがストレス耐性の増加と顕著な寿命の延長をみせた (Migliaccioら、1999年、Nature 第402巻: 309~313頁)。これらの知見は、遺伝子発現に対する p21 の影響が複数の疾病の病原性及び哺乳類の寿命の全般的制限に寄与しているかもしれないことを示唆する。

【0055】

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 治験を実施中の抗癌剤の主要な新しいクラスは、血管形成阻害剤である。これらの薬剤は腫瘍細胞ではなく、腫瘍によって分泌される血管形成因子により刺激される間質毛細管の増殖をターゲットとしている (最近の概説としては、Kerbel、2000年、Carcinogenesis 第21巻: 505~515頁を参照のこと)。しかしながら、脈管系は腫瘍増殖に必要とされる間質要素だけではない。複数の研究に、間質線維芽細胞もまたインビトロ及びインビボにおいて腫瘍細胞の増殖を支持すること、及び正常及び不死化線維芽細胞が、腫瘍形成を促進し癌細胞の死を阻害するパラクリンを分泌することが示されている (GregoireとLieubeau、1995年、Cancer Metastasis Rev. 第14巻: 339~350頁; Campsら、1990年、Proc. Natl. Acad. Sci. U.S.A. 第87巻: 75~79頁; Noelら、1998年、Int. J. Cancer 第76巻: 267~273頁; Olumiら、1998年、Cancer Res. 第58巻: 4525~4530頁)。上記のような因子は、線維芽細胞馴化培地中 (Chung、1991年、Cancer Metastasis Rev. 第10巻: 263~74頁) 及び共培養の研究において同定された。特に、Olumiら (1998年、Cancer Res. 第58巻: 4525~4530頁) は、前立腺癌細胞を正常な前立腺線維芽細胞と共培養すると、癌細胞死が強力に低下し、異種移植片腫瘍形成が促進されることを示した。線維芽細胞のパラクリン効果もまた、初期の前立腺上皮細胞発癌において実証されているのと同様に、腫瘍促進活性を有している (Olumiら、1999年、Cancer Res. 第59巻: 5002~5011頁)。これらの結果にも拘わらず、腫瘍関連線維芽細胞のパラクリン発癌性及び腫瘍刺激活性は、薬理的介入に対するターゲットとして利用されたことはなかった 20  
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 。本発明は、そのような間質線維芽細胞からの分裂促進の生産を阻害することができ、腫瘍細胞の増殖を阻害する手だてを与える化合物を検出及び同定するための方法を提供する。

【0056】

最近になって、このパラクリン腫瘍促進活性は、p21及びp16の誘導を伴うプロセスである、正常なヒト線維芽細胞の複製老化のあいだに選択的に高められることが示された (Krtolicaら、2000年、Proc. Amer. Assoc. Can. Res. 第41巻、Abs. 448)。間質組織の腫瘍促進効果は、間質線維芽細胞における高レベルでp21を生産する処理 (Meyerら、1999年、Oncogene 第18巻: 5795~5805頁) である、イオン化照射 (Barcellos-Hoff及びRavani、2000年、Cancer Res. 第60巻: 1254~60頁) によって、マウスの哺乳類発癌モデルにおいても誘導されることが示された。これらの結果は、p21過剰発現細胞の馴化培地において本願において開示されるパラクリン抗アポトーシス及び分裂促進活性が、同様の生物学的現象を示す可能性が非常に高いことを示している。 40

【0057】

本願において開示される結果は、CDK阻害剤誘導が癌または老化関連疾病の発症の可能性を増大させるであろう様式で、細胞遺伝子発現に影響を及ぼすことを示している。CDK阻害剤発現の急増は、正常な複製老化においてのみならず、細胞損傷に反応しても起こり、いずれの場合も、CDK阻害剤誘導の好ましくない効果が老化依存的に蓄積すると予 50

想される。

【0058】

したがって、本発明は細胞老化の病原性結果に伴う遺伝子の誘導を阻害することのできる化合物、特に老化の間に誘導される遺伝子、及び特にCDK阻害剤発現によって誘導される遺伝子を同定する方法を提供する。そのような化合物は、遺伝子発現のCDK阻害剤媒介性誘導に対する当該化合物の効果によって老化関連疾病を防止、遅延または逆行される能力を示すと予想される。

【0059】

本発明の1つの実施形態は、p21、p16またはp27などのCDK阻害剤によって誘導される遺伝子発現を阻害するための方法を提供する。好ましい実施形態において、そのような阻害は、細胞を核因子 - B (NF B) の活性、発現または核転座を阻害する有効量の化合物と接触させることによって達成される。当業者であれば、NF B活性を以下の少なくとも3つの様式で阻害できることを理解するであろう。第1に、NF Bヘテロダイマーを構成している遺伝子のいずれかの転写、プロセッシング及び/または翻訳を下方制御または阻害すること、第2には、細胞内でのI Bの発現及び/または活性の不活性化を阻害することに依存する可能性のある、細胞質から核へのNF Bの転座を阻害すること、第3には、NF Bそのものの活性を阻害することである。本発明は、NF B活性を阻害することにより、前記いずれかまたは全ての様式でCDK阻害剤による遺伝子の誘導を阻害する方法も包含する。技術上知られているNF B阻害剤の例としては、N - 複素環カルボキシイミド誘導體（例えば、国際出願公開WO01/02359に開示されるような）；アニリド化合物（例えば、国際出願公開WO01/15603に開示されるような）；4 - ピリミジノアミノインダン誘導體（例えば、国際出願公開WO00/05234に開示されるような）；4H - 1 - ベンゾピラン - 4 - オン誘導體（例えば、日本出願JP11193231に開示されるような）；キサンチン誘導體（例えば、日本出願JP9227561に開示されるような）；カルボキシアルケニルベンゾキノン及びカルボキシアルケニルナフトール誘導體（例えば、日本出願JP7291860に開示されるような）；ジスルフィド及びその誘導體（例えば、国際出願公開WO99/40907に開示されるような）；プロテアーゼ阻害剤（例えば、欧州出願公開EP652290に開示されるような）；フルルビプロフェン、タリドミド、デキサメタゾン、ピロリジンジチオカルバメート、ジメチルフマル酸、メサリジン、ピモベンダン、スルファサラジン、クロロゲン酸メチル、クロロメチルケトン、コハク酸 - トコフェロール、テボキサリン、ならびに、アスピリン、サリチル酸ナトリウム及びスリダクを含むある種の非ステロイド抗炎症剤 (NSAID) が挙げられる。

【0060】

以下の実施例は、本発明の特定の好ましい実施形態をさらに説明することを意図しており、本質を限定するものではない。

【実施例1】

【0061】

誘導可能なp21遺伝子を含む哺乳類細胞の生産

ヒト繊維肉腫細胞系列HT1080 p21 - 9の組み換え類縁体を、基本的にChangら(1999年、Oncogene 第18巻: 4808 ~ 4818頁、参照により本願に組み込まれる)に従って生産した。この細胞系列は、イソプロピル - チオガラクトシド (IPTG) によって調節されるプロモータの転写制御下にあるp21コーディング配列を含むものとした。上記細胞を十分な量のIPTGの存在下で培養して、内在性p21遺伝子の誘導が誘発するかもしれないあらゆる付加的効果の非存在下において、研究対象とするp21発現が連続して起こるようにすることにより、p21の発現を誘導することができる。この細胞系列は、2000年4月6日にAmerican Type Culture Collection (ATCC: 在米国バージニア州マナサス) に受託番号PTA1664として寄託されている。

【0062】

簡単に説明すると、マウス狭宿主性レトロウイルス受容体及びプラスミド3' S S ( S t r a t a g e n e ) によってコードされる改変細菌 l a c I リプレッサーを発現する H T 1 0 8 0 のサブ系列 ( C h a n g と R o n i n s o n 、 1 9 9 6 年、 G e n e 第 3 3 巻 : 7 0 3 ~ 7 0 9 頁、参照により本願に組み込まれる ) を、図 1 に構造が示されている組み換えレトロウイルス L N p 2 1 C O 3 を含むレトロウイルス粒子に感染させた。このレトロウイルスベクターは、レトロウイルスの長い末端反復プロモータの転写制御下にある細菌ネオマイシン耐性遺伝子 ( n e o ) を含んでいる。 p 2 1 をコードする配列を n e o 遺伝子の転写方向とは逆向きに、かつ改変ヒトサイトメガロウイルスプロモータの制御下にクローン化した。詳細には、 C M V プロモータは、細胞内で発現される l a c I リプレッサーに感受性のプロモータからの発現を行う細菌 l a c オペレータ配列を 3 回反復して含んでいる。 L N p 2 1 C O 3 は、 p 2 1 をコードする配列を含む 4 9 2 b p の D N A 断片を、親ベクター L N X C O 3 の N o t I 及び B g 1 I I 部位にクローニングすることにより構築した ( C h a n g と R o n i n s o n 、 同書中に開示 ) 。

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#### 【 0 0 6 3 】

感染後、 L N p 2 1 C O 3 X ベクターに感染した細胞は、 4 0 0  $\mu$  g / m L の G 4 1 8 ( B R L - G B C O , 在メリーランド州ガイザースバーグ ) の存在下で細胞を培養することにより選択した。クローン系列 H T 1 0 8 0 p 2 1 - 9 は、 L N p 2 1 C O 3 によって変換された G 4 1 8 耐性細胞系列から、クローン細胞系列が得られるまでの終点希釈によって得た。

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#### 【 実施例 2 】

#### 【 0 0 6 4 】

#### 細胞増殖アッセイ

実施例 1 のようにして生産した H T 1 0 8 0 p 2 1 - 9 を用いて、細胞内で p 2 1 が発現された場合に細胞増殖にどのような変化が起こるかを調べるための細胞増殖アッセイを行った。

#### 【 0 0 6 5 】

H T 1 0 8 0 p 2 1 - 9 細胞における L N p 2 1 C O 3 ベクターからの p 2 1 の発現は、細胞を 1 0 % ウシ胎仔血清 ( H y c l o n e 、 在ユタ州ローガン ) 及び I P T G を含有する D M E M 培地中で培養することによって誘導した。上記アッセイの結果が図 2 A 及び図 2 B に示されている。図 2 A は、 5 0  $\mu$  M I P T G の存在下で培養した細胞における p 2 1 蛋白質産生の経時変化を示している。 p 2 1 遺伝子の発現は、培養培地に I P T G を導入してから 6 ~ 1 2 時間のあいだ増加し、導入後約 2 4 時間で発現はピークに達した。細胞を I P T G 含有培地から除去すると、 p 2 1 の発現は上昇時とほぼ同程度に迅速に低下し、 I P T G の除去から約 2 4 時間後に導入前のレベルに戻った ( 図 2 B ) 。

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#### 【 0 0 6 6 】

I P T G の存在下における細胞増殖は、以下の 3 つの方法でアッセイした。<sup>3</sup> H - チミジン取り込み ( 「ラベリング指標」とよぶ ) の測定 ; 顕微鏡による培養中の分裂細胞の数の観察 ( 「分裂指標」とよぶ ) ; 及び細胞周期の異なる部分における培養細胞の分布の判定 ( 「細胞周期分布」とよぶ ) 。

#### 【 0 0 6 7 】

<sup>3</sup> H - チミジン取り込みアッセイは、実質的に D i m r i ら ( 1 9 9 5 年、 P r o c . N a t l . A c a d . S c i . U S A 第 9 2 巻 : 9 3 6 3 ~ 9 3 6 7 頁 ) に記載されているようにして実施した。細胞を <sup>3</sup> H - チミジンの存在下で 3 時間培養し、オートラジオグラフィによって分析した。 D N A の複製は、培養培地に I P T G を添加してから 9 時間までに完全に停止していることがオートラジオグラフィによって確認された。分裂指標は、 5  $\mu$  g / m L の 4 , 6 - ジアミノ - 2 - フェニルインドール ( D A P I ) で染色した後、顕微鏡観察して分裂中の細胞を計算することによって決定し、画像は L e i c a D M I R B 蛍光顕微鏡及び V a y t e k ( 在アイオワ州フェアフィールド ) イメージングシステムによって収集した。顕微鏡によって検出可能な分裂細胞は、 1 4 時間の I P T G 処理によって上記培養から消失した。

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## 【0068】

細胞周期分布は、Becton Dickinson FACS Sortを用いて、Jordanら(1996年、Cancer Res. 第56巻: 816~825頁)に記載されているように、ヨウ化プロピジウムで染色した後、DNA含量のFACS分析を用いて判定した。細胞周期分布は、IPTG処理の24時間後に安定した。この時間までに、IPTG処理細胞の42~43%が、それぞれG1及びG2において進行が停止され、約15%の細胞がS期DNA含量とともに進行が停止されていた。IPTG処理したHT1080 p21-9細胞もまた、SA-gal活性(Changら、1999年、同書中)だけでなく、形態的老化マーカー(大きく平坦になり、粒度が増大)を示した。これらの結果は、p21の誘導発現が、細胞周期の捕捉と細胞老化に特徴的な他の様々な変化のどちらも生み出すことを示している。 10

## 【実施例3】

## 【0069】

p21 遺伝子発現によって調節される遺伝子発現の解析

実施例2に開示した結果は、p21誘導の形態学的及び細胞周期の結果は、遺伝子発現における複数の変化を反映していることを示唆した。細胞遺伝子発現に対するp21誘導の効果は、以下のようにして調べた。

## 【0070】

未処理のHT1080 p21-9細胞及び50µmのIPTGで3日間処理した細胞から、ポリ(A)<sup>+</sup>RNAを単離した。poly(A)<sup>+</sup>RNAからcDNAを調製し、4,000個を超える配列を変えた既知のヒト遺伝子と3,000個のESTを含むヒトUniGEM V cDNAマイクロアレイを用いた(Genome System, Inc. (在ミズーリ州セントルイス)によって実施されるように)分別ハイブリダイゼーションのためのプローブとして用いた。2,500個を超える遺伝子とESTが、未処理HT1080 p21-9細胞及びIPTG処理したHT1080 p21-9細胞のどちらから得たプローブとも測定可能なハイブリダイゼーションシグナルを見せた。発現量差(2.5)で下方制御された遺伝子または発現量差が2.0で上方制御された遺伝子をそれぞれ表I及びIIに示す。 20

## 【0071】

これらの遺伝子のうちの69個の発現は、RT-PCRまたはノーザンハイブリダイゼーションによって個々に調べた。RT-PCRは基本的にNoonanら(1990年、Proc. Natl. Acad. Sci. USA 第87巻: 7160~7164頁)に記載されるようにして実施した。ノーザンハイブリダイゼーションのためのプローブは、マイクロアレイ内に存在するcDNAクローンのインサートから取り出し、当該cDNAはGenome Systems, Inc.より入手した。さらに、p21によって調節されたいくつかの遺伝子産物の発現の変化は、イムノブロットティングによって解析した。イムノブロットティングのためには以下の一次抗体を用いた。Cdc2に対するマウスモノクローナル抗体(Santa Cruz)、サイクリンA(NeoMarkers)、Plk1(Zymed)及びRb(PharMingen); MAD2に対するウサギポリクローナル抗体(BadCo)、p107(Santa Cruz)、CTGF(Fisp-12; Dr. L. Lauより寄贈)、Prc1(Dr. W. JiangとDr. T. Hunterより寄贈)及びトポイソメラーゼII(Ab0284; Dr. W. T. Beckより寄贈)、及びSOD2に対するヒツジモノクローナル抗体(Calbiochem)。使用したホースラディッシュペルオキシダーゼ(HRP)複合化二次抗体は、ヤギ抗マウス及びヤギ抗ウサギIgG(Santa Cruz)及びウサギ抗ヒツジIgG(KPL)である。全ての試料中の蛋白質濃度は、BioRad蛋白質アッセイキットによる測定後、均一にした。イムノブロットティングは、標準的な手法によって実施し、シグナルはLumiGlo(KPL)を用いて化学発光により検出した。 30 40

## 【0072】

これらの結果は図3A~図3Cに示されている。38/39下方制御遺伝子及び27/3 50

0 上方制御遺伝子について、上述のマイクロアレイアッセイによって予測される遺伝子発現の変化を確認した。被験遺伝子の殆どに対してノーザンハイブリダイゼーションまたは RT-PCR 観察されたシグナルの差異 (図 3 A ~ 図 3 C) は、cDNA アレイから判定された発現量差の値よりも高いようであった (表 I 及び II)。このことは、cDNA アレイハイブリダイゼーションが遺伝子発現に対する p 2 1 の効果の大きさを過小評価する傾向にあることを示唆している。6 個の下方制御された遺伝子及び 4 個の上方制御された遺伝子の発現の変化については、イムノプロットティング (図 3 B) またはザイモグラフィ (図示せず) によって蛋白質レベルでも試験し、すべての被験ケースについて確認を行った。

#### 【0073】

遺伝子発現における p 2 1 によって媒介される変化は、短期効果と、p 2 1 によって誘導される細胞増殖の捕捉に続く長期効果からなることを認めた。この目的のために、IPTG の添加及び除去後の、p 2 1 によって阻害された (図 3 B) 及び p 2 1 によって誘導された遺伝子 (図 3 C) のサブセットの RNA レベルの経時変化を調べた。Rb リン酸化 (電気泳動度によって示される) の p 2 1 によって誘導された変化、及び Rb 及び cDNA アレイにより p 2 1 によって阻害されたいくつかの蛋白質の細胞レベルの p 2 1 によって誘導された経時変化を解析するために、イムノプロットティングを用いた。これらの結果は図 3 B に示されている。Rb は IPTG の添加後 6 時間で早くも脱リン酸化されていることが分かった。さらに、Rb 蛋白質レベルは、12 ~ 24 時間の間に急激に減少したが (図 3 B に示される)、Rb mRNA レベルには顕著な変化は全く見られなかった (図示せず)。同様の減少が Rb 関連蛋白質 p 107 に対しても観察された (図 3 A に示される)。

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#### 【0074】

##### 1. p 2 1 によって阻害される遺伝子発現

p 2 1 によって阻害された全て被験遺伝子は、p 2 1 誘導及び放出に対して迅速な反応を見せた。これらの遺伝子のうちの 5 個 (トポイソメラーゼ II、ORC1、PLK1、PRC1 及び XRC9) は、RNA 及び蛋白質レベルの両方に、IPTG の添加後 4 ~ 8 時間の間に顕著な阻害を示した (図 3 B)。このパターンは、細胞増殖の捕捉と脱リン酸化と平行しており、「即時反応」と呼ばれる。他の p 2 1 によって阻害された遺伝子 (CDC2 または CHF R など) は、DNA 複製及び分裂の停止より僅かに遅れて「早期反応」を示し、mRNA レベルの大きな増加は IPTG を添加してから 12 時間後にしか検出されなかった。すべての p 2 1 によって阻害された遺伝子は、細胞がまだ増殖捕捉されており、DNA 複製及び分裂の再開前である、IPTG の除去から 12 ~ 16 時間後に発現を一時停止した (図 3 B)。この分析は、p 2 1 によって阻害された遺伝子の発現の変化が、p 2 1 誘導及び放出の短期効果であり、細胞増殖の捕捉及び回復の結果ではないことを実証した。

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#### 【0075】

まとめると、69 個の遺伝子と 3 個の EST が、cDNA マイクロアレイによって、p 2 1 誘導性細胞において、2.5 ~ 12.6 の発現量差で下方制御されることを確認した (表 I A)。細胞周期の進行に関与し、我々の個別のアッセイによって IPTG 処理細胞において下方制御されることが確認されているさらに 5 個の遺伝子が表 I B に挙げられている。cDNA アレイによって同定された下方制御された遺伝子は高い割合で (69 のうち 43)、分裂、DNA 複製、分離及び修復、及びクロマチン構築が伴い、このことは p 2 1 によって媒介される遺伝子発現の阻害が高度に選択的な性質であることを示している。

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#### 【0076】

p 2 1 によって下方制御された遺伝子の最大のグループは、分裂のシグナリング、実行及び制御に関与していた。いくつかの p 2 1 によって阻害された遺伝子は、DNA 複製及び分離、クロマチン構築及び DNA 修復に関与している。これらの遺伝子のいくつかは、ヌクレオチド生合成に関与する酵素をコードし、他の蛋白質は DNA 複製に関与している。いくつかの p 2 1 によって阻害される遺伝子は、DNA 修復に関与している。これらの結

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果は、治療的介入に対するターゲットになりうるであろう、p 2 1 誘導性の増殖捕捉の細胞プログラムの成分を発見する機会を示唆するものである。

【 0 0 7 7 】

2 . p 2 1 によって誘導される遺伝子発現

p 2 1 発現によって抑制される遺伝子に加えて、上述のアッセイでは p 2 1 によって誘導される遺伝子も検出した。p 2 1 によって誘導される遺伝子の遺伝子発現のパターンは図 3 C に示されている。p 2 1 によって阻害される遺伝子とは対照的に、p 2 1 によって上方制御される遺伝子は、I P T G の添加から 4 8 時間後、すなわちすべての細胞において増殖の捕捉が起こった後だけに発現の増大を見せた。1 つの被験遺伝子、組織トランスグルタミナーゼ ( t - T G アーゼ ) のみが、I P T G の添加から 1 2 時間後に検出可能な増加を見せたが、その発現が最大に達したのは 4 8 時間であった ( 図 3 C に示されるように ) 。さらに、すべての被験遺伝子 ( t - T G アーゼ以外 ) の発現の増大は、I P T G からの遊離後少なくとも 3 日間は持続し、細胞周期の回復後の持続していた ( 図示せず ) 。この「遅い反応」動態は、このような遺伝子の p 2 1 誘導が、p 2 1 によって媒介される増殖進行の停止に対して遅れた効果であることを示した。

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【 0 0 7 8 】

4 8 個の既知の遺伝子及び 6 個の E S T または未知の機能を有する遺伝子が、p 2 1 によって誘導された細胞において、2 . 0 ~ 7 . 8 の発現量差で上方制御されることが確認された ( 表 I I ) 。このグループに同定可能な遺伝子は非常に高い割合で ( 2 0 / 4 8 ) 、細胞外基質 ( E C M ) 成分 ( 例えば、フィブロネクチン 1 、ラミニン 2 、M a c - 2 結合蛋白質 ) 、他の分泌蛋白質 ( 例えば、アクチン A 、結合組織増殖因子、血清アミロイド A ) 、または E C M 受容体 ( インテグリン 3 など ) をコードしている。これらの分泌蛋白質のうちいくつかは、p 2 1 によって誘導される細胞内蛋白質の大きなグループとともに ( 表 I I ) 、様々な形のストレス反応において誘導されるか、あるいはストレス関連の信号伝達において役割を果たすことが知られている。顕著なものとしては、我々が p 2 1 によって誘導されることを見いだした多くの遺伝子は、細胞老化、有機体の老化、または様々な老化関連疾病においても上方制御され、このことから p 2 1 媒介性遺伝子誘導の抑制が、そのような疾病の発症を防止する手だてを与えることが示された。下記の実施例 5 に開示するように、いくつかの p 2 1 によって誘導される遺伝子は、パラクリン抗アポトーシス及び分裂促進活性を有する分泌因子をコードし、p 2 1 によって誘導される細胞からの馴化培地は、p 2 1 によって上方制御される遺伝子の性質によって予測される 2 つの生物学的効果、すなわち細胞増殖の刺激とアポトーシスの抑制を示す。この発見は、p 2 1 の「パラクリン」効果が、隣接細胞に対する腫瘍促進効果を介して発癌に寄与している可能性を示唆するものである。このことは、p 2 1 媒介性遺伝子誘導の抑制が、抗発癌効果を達成する手だてを与えるかもしれない可能性をもたらす。

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【 実施例 4 】

【 0 0 7 9 】

I P T G 処理した H T 1 0 8 0 p 2 1 - 9 細胞と血清不足 H T 1 0 8 0 p 2 1 - 9 細胞との比較による、p 2 1 誘導の特異性の同定

細胞増殖の捕捉の結果である可能性の高い細胞遺伝子発現における p 2 1 誘導性変化の正体を、以下のようにして確かめた。

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【 0 0 8 0 】

細胞を血清を含まない培地中で 4 日間培養することによって生み出した血清不足により、H T 1 0 8 0 p 2 1 - 9 細胞において増殖の捕捉 ( 静止 ) を誘導した。I P T G 処理した H T 1 0 8 0 p 2 1 - 9 細胞とは異なり、血清不足細胞においては、細胞波老化形態をみせず、非常に弱い S A - g a l 発現しか示さなかった。血清不足細胞における p 2 1 レベルは、I P T G 処理細胞で見られる 1 5 ~ 2 0 倍の増加とは対照的に、約 2 倍にしか増加しなかった。図 3 D は、血清を含まない培地中で 4 日間または 5 0 μ M I P T G 存在下で 3 日間後に増殖が阻害された H P 1 0 8 0 p 2 1 - 9 細胞における、p 2 1 によって阻害された遺伝子と p 2 1 によって誘導された遺伝子の発現を上述のようにして R

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T-PCR解析した結果を示している。培養培地が50 μM IPTGを含んでいた場合にHT1080 p21-9細胞において完全に阻害された遺伝子は、血清不足細胞においても阻害されたが、これらの遺伝子の大半はIPTG処理細胞におけるよりも受けた阻害の程度は小さかった。

#### 【0081】

p21によって発現が誘導される遺伝子は、3つの明確に区別できるパターンを見せた。第1のグループは、発現が静止細胞においても老化細胞と同様に強く誘導される遺伝子である。これらにはガレクチン-3、スーパーオキシドジスムターゼ2、補体C3、及びプロサポシンが含まれ、これらの誘導が細胞増殖の捕捉の結果であったこと、または上記遺伝子がわずかに高められたp21レベルに対して極めて敏感であったことが示される。第2のグループは、静止細胞においては上方制御されたが、老化細胞においてはそれほど強く上方制御されなかった遺伝子である。これらの遺伝子には、フィブロネクチン-1、Mac2結合蛋白質及びアルツハイマー前駆体蛋白質血清アミロイドAが含まれる。第3のグループは、静止細胞においては検出可能に誘導されることはないが、老化細胞においては強く誘導された遺伝子である。これらの遺伝子には、CTGF、プラスミノゲン活性化因子阻害剤1、組織トランスグルタミナーゼまたはナチュラルキラー細胞マーカー蛋白質NK4、インテグリン 3及びアクチビンAが含まれる。

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#### 【0082】

血清不足による静止の誘導に対するある遺伝子の反応と、p21のIPTG誘導性過剰発現を介した細胞老化との間の差異から、これらの遺伝子は老化の診断マーカーであると同等とされた。さらに、p21発現細胞と静止細胞との間での発現を比較することにより、新奇の老化マーカーを同定することもできる。

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#### 【実施例5】

#### 【0083】

#### 分裂促進因子を含む馴化培地の生産と分裂促進活性アッセイ

p21によって上方制御された数個の遺伝子(表II)は、CTGF(Bradhamら、1991年、J. Cell Biol. 第114巻:1285~1294頁)、アクチビンA(Sakuraiら、1994年、J. Biol. Chem. 第269巻:14118~14122頁)、エピテリン/グラニユリン(Shoyabら、1990年、Proc. Natl. Acad. Sci. USA 第87巻:7912~7916頁)及びガラクチン-3(Inoharaら、1998年、Exp. Cell Res. 第245巻:294~302頁)を含む増殖因子として作用する分泌蛋白質をコードする。さらに、ガレクチン-3(Akahaniら、1997年、Cancer Res. 第57巻:5272~5276頁)及びプロサポシン(Hiraiwaら、1997年、Proc. Natl. Acad. Sci. USA 第94巻:4778~4781頁)は、抗アポトーシス活性を有することが示されている。パラクリン抗アポトーシスまたは分裂促進活性は、cDNAマイクロアレイハイブリダイゼーションにおける発現量差値が1.8~1.9であったために表IIに挙げられていない、いくつかのp21誘導可能な遺伝子産物に対しても報告されている。上記の値は、この表に含めるかどうか、あるいはRT-PCRによる検証のために用いた、任意に選んだ最小値2.0を下回っている。上記蛋白質は、クラステリン(Koch-BrandtとMorgans、1996年、Prog. Mol. Subcell. Biol. 第16巻:130~149頁)、プロスタサイクリン刺激因子(PSF)(Yamauchiら、1994年、Biochem. J. 第303巻:591~598頁)、血管内皮細胞増殖因子-C(VEGF-C)(Joukovら、1996年、EMBO J. 第15巻:290~298頁)、ゲルソリン(Ohtsuら、1996年、EMBO J. 第16巻:4650~4656頁)及びメタロプロテアーゼ1の組織阻害剤(TIMP-1)(Liら、1999年、Cancer Res. 第59巻:6267~6275頁)である。

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#### 【0084】

分泌された分裂促進及び抗アポトーシス因子のp21による誘導を検証するために、IP

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TG処理したHT1080 p21-9細胞からの馴化培地を試験して、これらが細胞増殖及びアポトーシスに対して効果を有するかどうかを調べた。これらの実験において、馴化培地は、DMEM/10%FCSを含む15cmのプレートあたり $10^6$ 個のHT1080 p21-9細胞をプレATINGすることにより調製した。翌日、IPTGを最終濃度が $50\mu\text{M}$ となるように添加し、3日後にこの培地を、0.5%FCS及び $50\mu\text{M}$  IPTGを添加したDMEMに交換した。2日後(IPTG処理して3~5日目)、この馴化培地を回収し、使用時まで4で長くとも15日間まで保存した。対照培地は、IPTGを含まないDMEM/0.5%FCSを、IPTG処理細胞と同じ密度まで増殖させた未処理細胞に添加し、その2日後に培地を回収することにより調製した。

#### 【0085】

遅く増殖するヒト線維肉腫細胞系列HS15.Tを用いて、馴化培地内の分裂促進活性を検出した。分裂促進活性アッセイのためには両方のタイプの馴化培地、ならびに、分裂促進活性をテストするためには新鮮な培地及び、馴化培地と新鮮培地の1:1混合物を用いた。これらの実験において、馴化培地には1%または2%FCSを添加した。簡単に述べると、HS15.T細胞を12ウェルプレートに1ウェルあたり15,000細胞となるようにプレATINGした。2日後、これらの細胞を異なるタイプの培地中で培養した。細胞は馴化培地中で60時間増殖させ、 $^3\text{H}$ -チミジンを $3.13\mu\text{Ci/mL}$ の濃度で添加して24時間インキュベートした。その後、細胞を回収して、 $^3\text{H}$ -チミジン取り込みをMoscaら(1992年、Mol.Cell.Biol、第12巻:4375~4383頁)に記載されるようにして決定した。

#### 【0086】

このアッセイにおいて新鮮培地にIPTGを添加しても全く効果はなかった。新鮮培地とHT1080 p21-9細胞からの馴化培地のあいだに有意な細胞増殖の差異はみられなかった。これに対し、IPTG処理細胞からの馴化培地では、 $^3\text{H}$ -チミジンの取り込みが3倍にも上昇していた。IPTG処理細胞からの馴化培地によるHS15.Tの増殖刺激は、メチレンブルー染色によっても検出可能である。

#### 【0087】

アポトーシスに対するこの馴化培地の効果も調べた。これらの実験では、E1Aで不死化したマウス胚線維芽細胞系列C8を用いた。この細胞系列は、様々な刺激によってアポトーシスの誘導を非常に受けやすい(Loweら、1994年、Science 第266巻:807~810頁;Nikiforovら、1996年、Oncogene 第13巻:1709~1719頁)。上記様々な刺激としては、血清不足(Loweら、1994年、Proc.Natl.Acad.Sci.USA 第91巻:2026~2030頁)が含まれる。アポトーシスは、6cmのプレートにつき $3\times 10^5$ 個のC8細胞をプレATINGし、翌日に培地を、0.4%血清を添加した新鮮培地または馴化培地(新鮮な血清を添加していない)に交換することにより分析した。DNA含量分析及びDAPI染色は、24時間後及び48時間後に実施し、低血清培地において48時間後に相対細胞数をメチレンブルー染色によって測定した(Perryら、1992年、Mutat.Res. 第276巻:189~197頁)。

#### 【0088】

低血清新鮮培地または、IPTG処理細胞または未処理細胞からの馴化培地の添加によって、C8細胞におけるアポトーシスが迅速に誘導された。このことはDAPI染色後の大多数の細胞において検出可能な細胞脱離とアポトーシス形態とによって(図示せず)証明された。しかしながら、48時間後にまだ付着したままの細胞のメチレンブルー染色によって測定されたように、IPTG処理細胞からの馴化培地は、新鮮培地及び未処理細胞からの馴化培地に比較して、細胞の生存率を強力に高めていた。p21誘導性細胞からの馴化培地の効果は、培地交換から24時間後及び48時間後の付着及び浮遊C8細胞混合物に対して実施した、DNA含量のFACS分析においてさらに明らかであった。他の多くの細胞系列とは違って、C8細胞のアポトーシスでは、DNAの量が減少(サブ-G1)した細胞はわずかしか生産されず、このことはG2/MDNA含量を有した細胞の選択的

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消失によって特徴づけられる (Nikiforovら、1996年、同書中)。IPTG 処理細胞からの馴化培地における血清不足細胞は、G2/M画分を維持しており、血清豊富培地中で増殖している対照細胞に似た細胞周期プロファイルを見せた。IPTGを単独で添加した場合、C8細胞におけるアポトーシスに対して全く効果を有しなかった。したがって、HT1080細胞におけるp21の誘導は、p21によってレギュレートされなかった遺伝子の性質によって予測されるように、分裂促進及び抗アポトーシス因子の分泌をもたらす。

#### 【実施例6】

#### 【0089】

誘導可能な p16<sup>Ink4A</sup> または p27<sup>Kip1</sup> 遺伝子を含む哺乳類細胞の生産 10  
 誘導可能なCDK阻害剤 p16<sup>Ink4A</sup> (CDK4/6を優先的に阻害する; Seranoら、Nature 第16巻、704~707頁、1993年) または p27<sup>Kip1</sup> (CDK2を優先的に阻害する; Blainら、J. Biol. Chem. 第272巻、25863~25872頁、1997年) を含む哺乳類細胞系列を、誘導可能なp21含有細胞系列の生産に関して実施例1に記載したようにして生産した。細菌lacI遺伝子をコードし、マウス狭宿主性レトロウイルス受容体を発現する組み換え発現作成物を含む、ヒトHT1080線維肉腫細胞系列の組み換え類縁体 (HT1080 3'SS6; ChangとRoninson、1996年、Gene 第183巻: 137~142頁) を用いて、誘導可能な系列を作成した。p16の誘導可能発現のために、ヒトp16の471bpコーディング配列を含むDNA断片 (参照により本願に組み込む、米国特許第5,889,169号に記載されるように) をIPTGによって調節されるレトロウイルスベクターLNxRO2 (ChangとRoninson、1996年、Gene 第183巻: 137~142頁) 中にクローン化した。このレトロウイルスベクターは、レトロウイルスの長い末端反復プロモータの転写制御下にある細菌のネオマイシン耐性遺伝子(neo)を含み、G418(BRL-GIBCO)を用いた選択が可能である。結果として得られるLNp16RO2と呼ぶ作成物が図4に模式的に描かれている。p27の誘導可能発現のために、同一のLNxRO2ベクター内にマウスp27cDNAを保有するベクターLNp27RO2(NCBI RefSeq NM\_009875)が開発され、Kokontisら(1998年、Mol. Endocrinol. 第12巻、941~953頁)によって記載されている。このベクターはDr. N. Hay(イリノイ大学、シカゴ)から提供された。 20

#### 【0090】

従来レトロウイルス感染方法を用いて、LNp16RO2及びLNp27RO2作成物を個別にHT1080 3'SS細胞に導入した。細胞を400µg/mL G418(BRL-GIBCOより入手)の存在下で培養することにより、感染細胞を選択した。G418によって選択されたLNp16RO2形質導入細胞の集団をHT1080/LNp16RO2と名付けた。この細胞集団は、2000年10月10日にAmerican Type Culture Collection(ATCC; バージニア州マナサス)に寄託されており、受託番号PTA-2580が与えられている。 30

#### 【0091】

この細胞集団をサブクローン化し、20個のクローン細胞系列を単離し、IPTG誘導可能増殖阻害について調べた。最も強い増殖阻害を示した細胞系列をHT1080 p16-5と名付けた。この細胞系列は、2002年1月31日にAmerican Type Culture Collection(ATCC; バージニア州マナサス)に寄託されており、受託番号\_\_\_\_\_が与えられている。図5Aは、50µM IPTGを添加したときの、HT1080 p16-5細胞の細胞周期分布の変化を示したものである。異なる細胞周期にある細胞画分は、Becton Dickinson FACS ortを用いて、Jordanら(1996年、Cancer Res. 第56巻: 816~825頁)に記載されるようにヨウ化プロピジウムによる染色の後、DNA含量のFACS分析を用いて決定した。細胞周期分布はIPTG処理から24時間後に安定した。 40

この時間までに I P T G 処理細胞の 93% が G 1 に捕捉されていた。このような G 1 捕捉は、p 1 6 による C D K 4 / 6 の阻害によるもの予想される。

【 0 0 9 2 】

同様に、G 4 1 8 によって選択された L N p 2 7 R O 2 形質導入細胞の集団をサブクローン化し、38個のクローン細胞系列を単離し、I P T G 誘導可能な増殖阻害について調べた。最も強い増殖阻害を見せた細胞系列を H T 1 0 8 0 p 2 7 - 2 と名付けた。この細胞系列は、2002年1月31日に American Type Culture Collection (A.T.C.C.; バージニア州マナサス) に寄託されており、受託番号 \_\_\_\_\_ が与えられている。図 5 B は、50 μM I P T G を添加したときの、H T 1 0 8 0 p 2 7 - 2 細胞の細胞周期分布の変化を示したものである。細胞周期分布は I P T G 処理から 2 4 時間後に安定した。この時間までに I P T G 処理細胞の 8 9 % が G 1 に捕捉されていた。このような G 1 捕捉は、p 1 6 による C D K 4 / 6 の阻害によるもの予想される。

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【 実施例 7 】

【 0 0 9 3 】

p 2 1 誘導可能遺伝子の発現に対する p 1 6 及び p 2 7 の効果

実施例 6 に記載されるように I P T G によって誘導可能な p 1 6 または p 2 7 遺伝子を保持する、H T 1 0 8 0 類縁体、H T 1 0 8 0 p 1 6 - 5 及び H T 1 0 8 0 p 2 7 - 2 を用いて、以下のように遺伝子発現アッセイを行った。

【 0 0 9 4 】

50 μM I P T G の存在下または非存在下で 3 日間培養したこれらの細胞系列から R N A を得た。次にこれらの R N A 試料を用いて R T - P C R アッセイを行った。この R T - P C R アッセイは、標準化のために  $\beta$  - ミクログロブリンではなく  $\beta$  - アクチンを用いたことを除いては、基本的に上述の実施例 3 に記載されるようにして実施した。p 2 1 によって誘導されることが示された上述の 1 8 個の遺伝子について、これらの細胞の I P T G 処理によって誘導された p 1 6 または p 2 7 遺伝子発現の効果を調べた。試験対象遺伝子としては、アルツハイマー病、アミロイドーシス、関節炎、アテローム性動脈硬化症、及び誘導された p 2 1 発現に関して上述したようなパラクリンアポトーシス及び分裂促進効果に参与している遺伝子を含めた。p 1 6 に対する結果は図 6 A に、p 2 7 に対する結果は図 6 B に示されている。p 2 1 によって誘導されるすべての被験遺伝子は、I P T G 誘導性 p 1 6 発現によっても誘導され、試験対象遺伝子の殆ど全て ( t - P A 及び C T G F 以外 ) が、p 2 7 によっても誘導された。図 6 に示される結果は、P 1 6 または p 2 7 発現が p 2 1 発現に対して検出可能な効果を一切有しなかったことも示している。

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【 実施例 8 】

【 0 0 9 5 】

p 2 1 応答プロモータによるレポーター遺伝子発現を含む組み換え発現作成物の生産

プロモータ - レポーター作成物は、以下のような N K 4 、 S A A 、 補体 C 3 ( C C 3 ) 、 プロサボシン、 A P P 及び t - T G アーゼを含む、いくつかの p 2 1 誘導可能ヒト遺伝子のプロモータから調製した。C C 3 遺伝子のプロモータ領域は、ヒトゲノム配列において ( N C B I 受託番号 M 6 3 4 2 3 . 1 ) C C 3 c D N A ( V i k ら、1991年、B i o c h e m i s t r y 第 3 0 巻 : 1 0 8 0 ~ 1 0 8 5 頁 ) の 5 ' 末端に隣接していることが確認された。N K 4 遺伝子のプロモータ領域は、ヒトゲノム配列 ( 受託番号 A J 0 0 3 1 4 7 ) において、N K 4 c D N A ( 受託番号 M 5 9 8 0 7 ) の 5 末端に隣接していることが確認された。以前に説明した S A A 遺伝子 ( E d b r o o k e ら、1989年、M o l . C e l l . B i o l . 第 9 巻 : 1 9 0 8 ~ 1 9 1 6 頁 ) のプロモータは、ヒトゲノム配列 ( 受託番号 M 2 6 6 9 8 ) 内で確認された。A P P 遺伝子のプロモータ領域は、ヒトゲノム配列 ( 受託番号 X 1 2 7 5 1 ) において、A P P c D N A ( 受託番号 X M 0 0 9 7 1 0 ) の 5 ' 末端に隣接することが確認された。t - T G アーゼ遺伝子のプロモータ領域は、ヒトゲノム配列 ( 受託番号 Z 4 6 9 0 5 ) において、t - T G アーゼ c D N A ( 受託番号 M 5 5 1 5 3 ) の 5 ' 末端に隣接することが確認された。プロモータ特異的 D

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NAのポリメラーゼ鎖長延長反応(PCR)増幅は、HT1080 p21-9細胞からのゲノムDNAを鋳型に用いて実施した。PCRは、PfuTurbo DNAポリメラーゼ(Stratagene)及び表II Iaにあげたプライマーセットを用いて実施した。それぞれのプライマーセットに対するPCR条件は、表II Ibに記載されている。本実施例に開示されるような使用遺伝子プロモータを含む、プロモータCDK阻害剤によって誘導されるいくつかの遺伝子からのプロモータ配列の増幅のためのプライマーセットは、表II Icに挙げられている。

#### 【0096】

得られたPCR産物はTOPO TAクローニングベクターpCR2.1/TOPO(SAA, CC3, APP及びt-TGアーゼに対して)、またはpCRII/TOPO(NK4に対して)にクローン化した。これらの作成物をシーケンシングによって確認し、プロモータを正しい向きで含んだKpnI-XhoI断片を、標準的な組み換え遺伝子技術(Sambrookら、同書中)を用いて、ホタルルシフェラーゼ-レポーターベクターpGL2ベシク(Promega, 在ウィスコンシン州マディソン)のKpnI及びXhoI部位に挿入した。プロサポシンプロモータの480bp配列を含み、ホタルルシフェラーゼ発現を駆動するクローンは、Sunら(1999年、Gene 第218巻、37~47頁)に記載されており、Dr. Grabowski(子供病院医療センター、在オハイオ州シンシナティ)より提供された。

#### 【0097】

それぞれのプロモータ作成物に対するプラスミドクローンについてh、一過性トランスフェクションアッセイによってp21調節を調べた。HT1080 p21-9細胞の一過性トランスフェクションは、本質的にBio-Radプロトコールに記載されているようにして、エレクトロポレーションによって実施した。それぞれのエレクトロポレーションに対して、HT1080 p21-9細胞は、10%FC2血清を添加し、ペニシリン、ストレプトマイシン及びグルタミンを含有したDMEMを用いて15cmプレートに95%の集密になるまで増殖させた。次に細胞をトリプシン処理し、DMEMまたはOpti-MEM培地(GibcoBRL)に再懸濁し、IEC HN-SII遠心分離器中、10分間、1,000rpmでスピンドウンした。遠心分離に続いて、培地を吸引して、細胞を1mLあたり1800万~2000万細胞の濃度になるように、再度Opti-MEMに懸濁した。400µLの細胞懸濁液(約700万~800万細胞)を4cm溝のエレクトロポレーションキュベット(Bio-Rad)に移した。10~20µgのプロモータ-ルシフェラーゼ作成物を細胞に加えた。いくつかの実験においては、標準化のために、CMVプロモータから細菌-ガラクトシダーゼを発現する対照プラスミドpCMVbgalを、1:10の比率で混合液に加えた。他の実験においては、CMVプロモータからウミシイタケルシフェラーゼを発現するベクターpRL-CMVを1:20のモル比で加えることによって標準化を行い、ホタルルシフェラーゼ及びウミシイタケルシフェラーゼの活性は、二重ルシフェラーゼEssayキット(Promega)を用いて同一試料中で測定した。エレクトロポレーションは、960µFDにセットされたキャパシタンス拡張器を備え、27~30の値を与える、0.22ボルトのBio-Rad Gene Pulserを用いて実施した。予備実験において、エレクトロポレーション後の細胞の生存率及び付着は約33%であると判定された。細胞は、12ウェルプレート内に1ウェルあたり約50,000付着細胞の初期密度となるようにプレティングした(同一のものを3つ作成)。細胞を3~6時間のあいだおいた後、培地を吸引して、50µM IPTGを含むまたは含まない新鮮培地に交換した。2~4日後、細胞をリン酸緩衝生理食塩水で2回洗浄し、300µLの1xPassive Lysis BufferまたはReporter Lysis Buffer(Promega)中に回収した。溶解物を10,000gで簡単に遠心してペレットデブリスとし、50µLのアリコートホタルルシフェラーゼアッセイ(Promega)で用いるために新鮮なチューブに移した。ルシフェラーゼ活性は、52.1%の感度のTurner 20/20照度計を用い手、遅延時間5秒、第2積分時間10~15秒として測定した。

## 【 0 0 9 8 】

図 7 は代表的な実験の結果を示したものである。トランスフェクション細胞における p 2 1 誘導の 2 ~ 4 日後、p 2 1 によって誘導された遺伝子のプロモータ作成物からの発現は、NK 4 に対しては約 7 . 0 倍、S A A に対しては 3 . 7 倍、C C 3 に対しては 1 2 . 5 倍、プロサポシンに対しては 3 . 0 倍、A P P に対しては 2 . 6 倍、t - T G アーゼに対しては 2 . 3 倍増加していた。これらの結果は、p 2 1 がこれらの遺伝子の発現を、当該遺伝子のプロモータを調節することにより上方制御すること、及びそのような遺伝子のプロモータ作成物が遺伝子発現の p 2 1 によって媒介される調節に対するアッセイに使用可能であることを示した。そうしたアッセイは、下記実施例 9 に記載するように、p 2 1 によって媒介される遺伝子活性化を阻害する化合物を同定するのに用いることができる。

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## 【 表 1 】

表 IIIa. プライマー配列

プロモータ	センスプライマー (5'→3')	アンチセンスプライマー (5'→3')
CC3	GCTAAGAGGATATTGACATTAGA CAGG (配列番号 : 21)	AGGGGGAGGTGGGTTAGTAG (配列番号 : 22)
NK4	TGGAGCTAGAAGAGCCCGTAGG (配列番号 : 23)	GCCAAAAGTTCAAGGAGCCAA (配列番号 : 24)
SAA	CAGAGTTGCTGCTATGTCCACCA (配列番号 : 25)	CACTCCTTGTGTGCTCCTCACC (配列番号 : 26)
βAPP	TTGCTCCTTTGGTTCGTTCT (配列番号 : 27)	GCTGCCGAGGAAACTGAC (配列番号 : 28)
t-TG アーゼ	CCCAGGGAGAAATATCCACTGAA GCAAC (配列番号 : 29)	TCGGGCGGGGGCGGTGGCTCCT TCCACT (配列番号 : 30)

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## 【 表 2 】

表 IIIb. PCR条件

プロモータ	変性	アニーリング	延長	サイクル数	産物サイズ
CC3	95°, 1 分	63°, 1 分	72°, 1 分 40 秒	31	1018 bp
NK4	94°, 1 分	65°, 1 分	72°, 1 分 40 秒	32	877 bp
SAA	94°, 1 分	68°, 1 分	72°, 1 分 40 秒	32	1000 bp
βAPP	94°, 1 分	62.9°, 1 分	72°, 1 分 40 秒	30	623 bp
t-TGアーゼ	94°, 1 分	66.5°, 1 分	72°, 1 分 40 秒	33	1600 bp

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## 【 表 3 】

表 IIIc. プライマー配列

識別番号	遺伝子名	上流 (センス、5'-3')	下流 (アンチセンス、5'-3')
1	SAA	cagagttgctgctatgtccacca (配列番号: 25)	cactcctgtgtctctcacc (配列番号: 26)
2	補体 C 3	ccctaaaggatattgacattagacaag (配列番号: 21)	agggggaggtgggttagtag (配列番号: 22)
3	CTGF	gcctctcagctactactctctctaa (配列番号: 31)	cgaggaggaccaccgaagg (配列番号: 32)
4	インテグリン B 3	Gattgtctgtccctcaacag (配列番号: 33)	ccagcacagctgcccaaga (配列番号: 34)
5	アクチビン	tgattccaatgtttctaaagg (配列番号: 35)	gaatgtctaaaggctcagaagt (配列番号: 36)
6	NK4	tggagctagaagagcccgtagg (配列番号: 23)	gccaaaagttcaaggagccaag (配列番号: 24)
7	プロサポニン	ggfttaagcaattctgycctct (配列番号: 37)	cgctgactctccgcagctcgaat (配列番号: 38)
8	Mac2-BP	gtaaaactccctgctgctctctct (配列番号: 39)	ctctgcagactgtctcttggac (配列番号: 40)
9	GAL-3	tgcttcacaagggtggaagtgg (配列番号: 41)	ctggagggcagagaccag (配列番号: 42)
10	MnSOD	taccaaacctiaggggtaaaaaataaa (配列番号: 43)	atgtctctagtgtctgtgtctac (配列番号: 44)
11	グラニューリン	gagactiaggaaagccactctctcttc (配列番号: 45)	ctggaaatgtctgtctctctctact (配列番号: 46)
12	p66shc	gtggcagacacagggcactc (配列番号: 47)	ctccctgagctgcctcaatg (配列番号: 48)
14	カテプシン B	Ctcccgagtagcttgggatta (配列番号: 51)	ccacgctgaccaccggcga (配列番号: 52)
15	βAPP	ttgctccttggctctctct (配列番号: 27)	gctgcccaggaacaatgac (配列番号: 28)
16	t-TGアーゼ	cccagggagaataatccactgaaagaac (配列番号: 29)	tcgggcccgggcccgtgtgctctctcact (配列番号: 30)
17	クラステリン	agccccctgactctctctct (配列番号: 53)	ctccctggcgacgccggctt (配列番号: 54)
18	PSF	aaaagctctgggattagagggcttga (配列番号: 55)	tatgtattgtctaagggaagctattggag (配列番号: 56)
19	VEGF-C	gttcttgatcatcagcgaactt (配列番号: 57)	gttggaaagaccgggggttgg (配列番号: 58)
20	TIMP-1	agaaccggtaccatctcaga (配列番号: 59)	ctgtactctctgtgtctctct (配列番号: 60)

p 2 1 誘導可能なレポーター作成物で安定的にトランスフェクトされた細胞の生産

p 2 1 によって調節されるルシフェラーゼ発現を有する安定的にトランスフェクトされた細胞系列を開発するために、実施例 8 に記載し、p L u N K 4 と名付けた N K 4 プロモータ - ルシフェラーゼ作成物を、I P T G 誘導可能 p 2 1 を保持する H T 1 0 8 0 p 2 1 - 9 細胞に、選択可能なマーカとしてのプロマイシン N - アセチルトランスフェラーゼを保持する p B a b e P u r o とのコトランスフェクションにより導入した。トランスフェクションは、L I P O F E C T A M I N E 2 0 0 0 ( L i f e T e c h n o l o g i e s , I n c . 在メリーランド州ガイザースバーグ) を用い、p L u N K 4 と p B a b e P u r o の比を 1 0 : 1 として実施した。安定なトランスフェクタントを 1 μ g / m L プロマイシンを用いて 5 日間かけて選択した。5 4 個のプロマイシン耐性細胞系列を単離し、5 0 μ M I P T G の存在下及び非存在下における、ルシフェラーゼ活性を調べた ( L u c i f e r a s e A s s a y S y s t e m , P r o m e g a を使用)。

【 0 1 0 0 】

このアッセイは以下のようにして実施した。細胞を 1 2 ウェルプレートに、ペニシリン / ストレプトマイシン、グルタミン、及び 1 0 % ウシ胎仔血清 ( F C S ) を含有する培地 1 m L あたり、4 0 , 0 0 0 個 / ウェルの密度でプレティングした。付着後、細胞を時間を変えながら、5 0 μ M I P T G で処理するか、もしくは未処理のままにした。次にルシフェラーゼ活性を上述の実施例 8 に記載されるようにして測定した。さらに別のアリコート細胞溶解物から取り出し、B i o - R a d 蛋白質アッセイキット ( B r a d f o r d アッセイ) を用いて蛋白質濃度を測定した。各試料に対するルシフェラーゼ活性は、蛋白質含量に正規化し、ルシフェラーゼ活性 / 1 μ g 蛋白質として表現した。すべてのアッセイは 3 回ずつ行い、その平均と標準偏差が示されている。

【 0 1 0 1 】

5 4 個の被験細胞系列のうち 2 1 個が、測定可能なルシフェラーゼ活性を示したが、H T 1 0 8 0 L u N K 4 p 2 1 と名付けたわずか 1 個の細胞系列だけが、I P T G 非存在下よりも I P T G 存在下でより高いルシフェラーゼ発現を見せた。p 2 1 L u N K 4 細胞系列を用いて実施したアッセイの結果が図 8 A 及び図 8 B に示されている。図 8 A は、2 4 時間の I P T G 処理後のルシフェラーゼ発現の I P T G 用量依存性を示し、図 8 B は 5 0 μ M I P T G を添加時のルシフェラーゼ発現の経時変化を示している。この分析から、殆どの誘導はわずか 5 μ M の I P T G とわずか 1 7 時間の処理時間を用いて達成できることが分かった。

【 0 1 0 2 】

これらの結果は、レポーター遺伝子転写の p 2 1 誘導に関与する安定的にトランスフェクトされた細胞系列を生産するために、p L u N K 4 レポーター作成物を使用できることを実証した。このような作成物及び細胞は、p 2 1 によって媒介される遺伝子活性化を阻害する化合物を同定するためのスクリーニングアッセイの基礎を与える。ルシフェラーゼ誘導に必要とされる比較的短い時間 ( 約 1 7 時間 ) 、ならびに I P T G 処理細胞におけるルシフェラーゼレベルの顕著な増加 ( 約 3 倍 ) は、L u N K 4 9 2 1 細胞系列を、p 2 1 の誘導効果を阻害するであろう化合物の高処理能スクリーニングに適したものにすることに違いない。同様の ( 潜在的にはより良好な ) 誘導性を有する他の細胞系列もまた、L u N K 4 p 2 1 を得るために用いた本願に開示の方法によって開発することができる。実施例 8 に記載した結果は、同様のタイプのスクリーニングが、安定的にトランスフェクトされた細胞系列ではなく p 2 1 誘導可能遺伝子のプロモータ作成物を用いた一過性トランスフェクションアッセイを用いても行えることを実証している。ルシフェラーゼ発現に基づく高処理能スクリーニングのための方法は、技術上周知である ( 一過性トランスフェクションに基づくアッセイの最近の例としては、S t o r z ら、1 9 9 9 年、A n a l y t . B i o c h e m . 第 2 7 6 巻 : 9 7 ~ 1 0 4 頁を参照。また安定的にトランスフェクトされる細胞系列に基づくスクリーニングの例としては、R o o s ら、2 0 0 0 年、V i r o l o g y 第 2 7 3 巻 . 3 0 7 ~ 3 1 5 頁を参照のこと ) 。これらの細胞及びアッセイを用いて同定された化合物は、老化関連遺伝子の p 2 1 によって媒介される誘導を阻害または防止

する治療剤を開発するために有用である。

【実施例 10】

【0103】

一過性トランスフェクションアッセイにおいて p21 媒介性誘導を阻害するための、NF B 及び p300 / CBP 阻害剤の利用

p21 誘導可能遺伝子のプロモータ配列を調べたところ、NK4 を含むこれらのプロモータの多くが、既知または潜在的な NF B 結合部位を含んでいることが判明した。いくつかの p21 によって誘導された遺伝子が、NF B によって正に調節されることが知られている。このような遺伝子としては、スーパーオキシドジスムターゼ 2 (SOD2) (Jones ら、1997 年、Mol. Cell. Biol. 第 17 巻: 6970 ~ 6981 頁)、t-TGアーゼ (Mirza ら、1997 年、Amer. J. Physiol. 第 272 巻: G281 ~ G288)、アルツハイマー - アミロイド前駆体蛋白質 (APP) (Grilli ら、1996 年、J. Biol. Chem. 第 271 巻: 15002 ~ 15007 頁) 及び炎症性蛋白質血清アミロイド A (SAA) (Jensen と Whitehead、1998 年、Biochem J. 第 334 巻: 489 ~ 503) が挙げられる。p21 は以前に一過性トランスフェクション実験によって、NF B 依存性転写を活性化することが示されている (Perkins ら、1997 年、Science 第 275 巻: 523 ~ 527 頁)。この p21 の効果は、転写補因子 p300 及び CBP の刺激によるものであることが示されており (Perkins ら、1997 年、Science 第 275 巻: 523 ~ 527 頁)、p300 / CBP または関連する転写補因子の活性化が、上方制御される遺伝子のいくつかに対して p21 の効果に関与している可能性がある。したがって、NF B または p300 / CBP は、p21 による転写の誘導を潜在的に阻害するのかもしれない。

【0104】

HT1080 p21-9 細胞における IPTG 誘導可能な p21 発現が、NF B の転写活性を刺激するかどうかを判定するために、我々は一過性トランスフェクションアッセイを用いて、Stratagene より市販されているプラスミド pNF B-Luc からのルシフェラーゼ発現の p21 誘導の効果調べた。このプラスミドは、5 つの NF B コンセンサス配列を含む人工プロモータからホタルルシフェラーゼを発現する。pNF B-Luc からのルシフェラーゼ発現に対する、NF B の遺伝子阻害剤の効果の評価するために、20 µg の pNF B-Luc プラスミド (モル比 1:2) を、NF B を選択的に阻害する I B キナーゼ (DiDonato ら、1996 年、Mol. Cell. Biol. 第 16 巻: 1295 ~ 1304 頁) の優性変異体を発現するプラスミド MAD3 (a.k.a. PRC / アクチン-HA-IKK) (Dr. M. Karin、カリフォルニア大学サンディエゴ校より提供) と混合した。以後このプラスミドは IKK と呼ぶ。pNFkB-Luc からのルシフェラーゼ発現に対する p300 / CBP 阻害の効果判定のために、プラスミド pNFkB-Luc を、別のアッセイにおいて、C 末端を欠失したアデノウィルス E1A 蛋白質 { CR2 (120-140) } に対する欠失遺伝子を発現するベクターと混合した。C 末端を欠失した E1A (E1A CR2 と呼ばれる) は、p300 / CBP 及び関連する因子 (PCAF など) を阻害するが、E1A の C 末端ドメインのターゲットである Rb は阻害しないことが知られている (Chakravarti ら、1999 年、Cell 第 96 巻: 393 ~ 403 頁)。負の対照として、pNF B-Luc を、E1A N / CR2 と呼ばれ、C 末端と N 末端の両方に欠失を有した { N (2-36) } 機能的に不活性な形の E1A と混合した。E1A CR2 及び E1A N / CR2 作成物は、Dr. V. Ogrzyzko (NICHD, NIH) より提供された。pNFkB-Luc と IKK、E1A CR2 または E1A N / CR2 との混合物は、実施例 8 に記載されるように、エレクトロポレーションによって HT1080 p21-9 細胞にトランスフェクトした (標準化のための pRL-CMV プラスミドをさらに加えて)。エレクトロポレーション後、同数のトランスフェクト細胞を 50 µM IPTG で処理するか、未処理のまま 3 日間おいた (同一実験につき 3 回実

施)。ホタルルシフェラーゼ活性を測定し、それぞれのトランスフェクト試料において、(IPTGの非存在下で)測定されたウミシイタケルシフェラーゼ活性に対して標準化した。

#### 【0105】

この分析の結果が図9Aに示されている。負の対照(E1A N / CR2)と混合したpNFkB-Lucは、IPTGの存在下において15倍もの誘導を示し、HT1080 p21-9細胞におけるNF B転写活性の増加が実証された。pNFkB-Lucを阻害剤と混合すると、IPTG処理細胞または未処理細胞においてルシフェラーゼの発現がほぼ完全に廃止され、この阻害剤の有効性が実証された。E1A CR2は、IKKよりと同様ではあるがより弱い効果しか有しておらず、HT1080 p21-9細胞におけるNF B活性に対するp300/CBPの必要性が示唆された(図9A)。

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#### 【0106】

6個のp21誘導可能遺伝子に対して、プロモータ-ルシフェラーゼ作成物を用いて同様の分析を行った。SAAに対する結果は図9Bに、プロサポシンに対する結果は図9Cに、APPに対する結果は図9Dに、t-TGアーゼに対する結果は図9Eに、補体Cに対する結果は図9Fに、NK4に対する結果は図9Gに示されている。IKKとE1A CR2のいずれも、IPTGの存在下においてすべての被験プロモータの誘導を阻害したことから、p21によるこれらのプロモータの調節が、部分的にp300/CBP及びNF Bを介していることが分かった。しかしながら、定量的には、これらの阻害剤の効果はプロモータ間で異なっていた。SAA(図9B)及びNK4(図9G)のプロモータの基底及びIPTG刺激性発現はいずれも、NF Bと殆ど同等の強さでIKK及びE1A CR2によっても阻害された。これとは対照的に、上記阻害剤は、プロサポシン(図9C)、APP(図9D)、t-TGアーゼ(図9E)または補体C3(図9F)のプロモータからの基底発現に対して、殆どまたは全く効果を示さなかったが、IPTGの存在下ではこれらのプロモータの誘導を阻害した。これらの結果は、p300/CBPとNF Bがすべての被験プロモータのp21による誘導に関与していることを示す。

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#### 【実施例11】

#### 【0107】

#### p21媒介性遺伝子誘導を阻害するための非ステロイド抗炎症剤の使用

臨床用途において最も研究の進んでいるNF B阻害剤は、アスピリン、サリチル酸ナトリウム及びスリダクなどの、ある種の非ステロイド抗炎症剤(NSAID)である。(KoppとGhosh、1994年、Science 第265巻:956~959頁; Yinら、1998年、Nature 第396巻:77~80頁; Yamamotoら、1999年、J. Biol. Chem. 第274巻:27307~27314頁)。上記実施例9に記載のLuNK4 p21細胞系列を用いて、これらの細胞系列におけるp21によるルシフェラーゼ活性の誘導を、NF B阻害活性を有するNSAIDによって阻害することができるかどうかを調べた。

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#### 【0108】

ルシフェラーゼ活性アッセイは、実質的に実施例9に記載されるように実施した。ルシフェラーゼ活性は、50 μM IPTGの存在下または非存在下で16時間インキュベートし、20 mMサリチル酸ナトリウム、1 mMスリダク、または10 mMアスピリンの存在下または非存在下でさらに20時間処理した後に測定した。さらに、NF Bを阻害しない2つのNSAID、すなわちインドメタシンとイブプロフェン(それぞれ25 μMで)についても試験した(Yamamotoら、1999年、同書中)。NSAID濃度は、これらの薬剤が抗炎症特性に必要とする、患者の血清中の当該薬剤の薬理的濃度に基づいたものとした(Yinら、1998年、同書中)。

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#### 【0109】

これらのアッセイの結果が図10に示されている。IPTGは、NSAIDの非存在下においてルシフェラーゼ活性を約3~4倍増加させたが、この誘導はサリチル酸塩、スリダクまたはアスピリンの存在下ではほぼ完全に無効化されていた。これとは対照的に、イ

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ンドメタシン及びイブプロフェンは、I P T Gによるルシフェラーゼの誘導に顕著な違いを与えなかった。

【0110】

N F B阻害性NSADが、NK4プロモータからの転写の誘導だけでなく、内在性のp21誘導可能な遺伝子のRNA発現も阻害したかどうかを確かめるため、LuK4p21細胞を6ウェルプレートに1ウェルあたり125,000細胞となるようにプレATINGし、250 $\mu$ M、500 $\mu$ Mまたは1mM濃度のスリダクの存在下または非存在下にて、50 $\mu$ M I P T G処理するか、または未処理のまま48時間(p21誘導可能遺伝子の最大刺激に必要とされる時間; Changら、2000年、Proc. Natl. Acad. Sci. USA 第97巻: 4291~4296頁)おいた。このインキュベーション後、Qiagen RNeasy Mini Kitを用いて細胞からRNAを抽出し、いくつかのp21誘導可能遺伝子の相対RNAレベルは、cDNA標準化のために $\beta$ -ミクログロビンではなく、アクチンを用いたことを除いては、基本的にNoonanら(1990年、Proc. Natl. Acad. Sci. USA 第87巻: 7160~7164頁)に記載されるようにして、逆転写-PCR(RT-PCR)によって決定した。被験遺伝子のそれぞれに対するPCRプライマーの配列が表IVaに示されている。PCRサイクルは以下の通りである。1サイクル目は、変性のための3分間と、アニーリングのための30秒間と、延長のための2分間とからなり、残りのサイクルは、変性のための30秒間と、アニーリングのための30秒間と、延長のための1分間からなるものとした。PCRサイクルの温度条件及びPCR産物のサイズは、表IVbに示されている。

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【0111】

RT-PCR分析の結果が図11に示されている。NK4(このNK4のプロモータLuNK4p21細胞においてルシフェラーゼ発現を駆動するために用いられた)に対しては、スリダクの添加はI P T G非存在下における遺伝子発現に僅かな効果しか与えなかったが、すべてのスリダク濃度で、I P T G存在下におけるNK4 RNAレベルの用量依存性低下が見られた。非常によく似た結果がt-TGアーゼRNAを用いた場合にも得られた。他のすべての被験遺伝子を用いた場合に、スリダクはI P T G非存在下において遺伝子発現の用量依存性低下を与えた。この効果の結果として、四隣ダクの最高試験用量(1mM)では、I P T G存在下における遺伝子発現が低下しなかったが、それより低いスリダク用量においてはI P T G効果に顕著な低下が見られた。特に、I P T Gの効果は、APP遺伝子に対しては250及び500 $\mu$ Mスリダクによって軽減されたが、p66<sup>shc</sup>、CTGF及びMac2結合蛋白質(Mac2-BP)に対しては250 $\mu$ Mスリダクでしか軽減されなかった。どの被験スリダク濃度も、プロサポシンまたはスーパーオキシドジスムターゼ2(SOD2)のI P T G誘導性RNAレベルに顕著な低下を与えなかった。プロサポシンに対するスリダク効果の欠如は、プロサポシンプロモータに対するIKK阻害剤の穏やかな効果と一致するものである(上記実施例10を参照)。したがって、スリダクの中用量(250 $\mu$ M)では、被験遺伝子の殆どに対してp21の転写誘導能が阻害される。

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【表4】

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表IVa. プライマー配列

遺伝子	センスプライマー(5'→3')	アンチセンスプライマー(5'→3')
NK4	AGCACCAGGCCATAGAAAGA (配列番号:13)	GGTGTTCAGCTCCTCCTTGTC (配列番号:49)
T-TGアーゼ	ACTACAACCTCGGCCCATGAC (配列番号:50)	GCCAGTTTGTTCAGGTGGTT (配列番号:61)
BAPP	CTCGTTCCTGACAAGTGCAA (配列番号:62)	TGTTTCAGAGCACACCTCTCG (配列番号:63)
P66 <sup>shc</sup>	GAGGGTGTGGTTCGGACTAA (配列番号:64)	GCCCAGAGGTGTGATTGTGTT (配列番号:65)
CTGF	GGAGAGTCCTTCCAGAGCAG (配列番号:66)	ATGTCTTCATGCTGGTGCAG (配列番号:67)
MAC2-BP	ACCATGAGTGTGGATGCTGA (配列番号:68)	ACAGGGACAGGTTGAACTGC (配列番号:69)
グラニューリン	ACCACGGACCTCCTCACTAA (配列番号:70)	ACACTGCCCTCAGCTACAC (配列番号:71)
プロサポシン	CCAGAGCTGGACATGACTGA (配列番号:72)	GTCACCTCCTTCACCAGGAA (配列番号:73)
SOD2	CAAATTGCTGCTTGCCAAA (配列番号:74)	CATCCCTACAAGTCCCAAAA (配列番号:75)
B-アクチン	GGGAAATCGTGC GTGACATTAA G (配列番号:76)	TGTGTTGGCGTACAGGTCTTTG (配列番号:77)

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【表5】

表IVb. PCR温度(°C)

遺伝子	変性	アニーリング	延長	サイクル数	産物サイズ
NK4	94	58	72	24	481
T-TGアーゼ	94	58	72	24	499
B-APP	94	58	72	20	500
p66 <sup>shc</sup>	94	58	72	22	514
CTGF	94	64	72	28	499
MAC2-BP	94	58	72	21	517
グラニューリン	94	64	72	25	446
プロサポシン	94	58	72	21	500
SOD2	94	58	72	23	505
βアクチン	94	60	72	17	275

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【0112】

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上記の結果から、p21誘導可能な遺伝子のプロモータからのレポーター発現のp21媒介性誘導との干渉のアッセイによって、発癌及び老化関連疾病に伴う遺伝子のp21媒介性誘導を阻害する物質を同定することができることが実証された。特に、LuNK4 p21細胞系列を用いたプロモータに基づくアッセイにおいて有効な阻害剤であると最小に同定された物質(スリダク)は、いくつかの老化関連遺伝子のp21による誘導を阻害することが発見された。これらの結果は、さらに、NF-κB阻害剤として有効なNSAIDが、CDK阻害剤による老化関連疾病の誘導を防止することができることを実証した。

【0113】

CDK阻害剤による転写の誘導を阻害する物質は、アルツハイマー病、アミロイドーシス、アテローム性動脈硬化症及び関節炎を含む老化関連疾病の化学的予防または発症の遅延

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に対して臨床的に有用であるかもしれない。さらに、そのような化合物は、当該化合物の有する分泌増殖因子（CTGFなど）の発現への効果を通して癌治療または予防における有用性を有するかもしれない。実際に、NF- $\kappa$ B阻害活性を有するNSAIDに対しての入手可能な臨床データは、この用途を支持している。したがって、スリダク、アスピリン及びサリチル酸塩を含むいくつかのNSAIDが、大腸癌や様々な種類の癌における化学予防価値を有することが示され、大腸ポリープの消失を促進していた（「CANCER. PRINCIPLES & PRACTICE OF ONCOLOGY」、DeVitaら編、Lippincott-Raven：フィラデルフィア、599～607頁の中の、Leeら、1997年、「アスピリンとその他の非ステロイド性抗炎症薬の使用と癌発生の危険性」）。アスピリン及び他のNSAIDを使用しても、アルツハイマー病の危険性が低下されることが示されている（Stewartら、1997年、Neurology、第48巻：626～632頁）。長期のアスピリン治療によって、アテローム性動脈硬化症の発生率が低下するという報告もある（Sloop、1998年、Angiology第49巻：827～832頁）。最後に、スリダクは、関節炎の治療において臨床効果が証明されている最も一般的に用いられている薬剤の1つである（Brogdenら、1978年、Drugs第16巻：97～114頁）。NSAIDのこれらの有益な効果のいくつかは、それらの持つシクロオキシゲナーゼ2阻害剤としての活性によるものであることが示されているが（Pennisi、1998年、Science第280巻：1191～1192頁）、本願に開示した結果は、これらの臨床活性が、おそらくは上記化合物のNF- $\kappa$ B阻害活性を介した、p21によって誘導される遺伝子発現の阻害によるのではないかという可能性を示唆する。本発明によって提供されるアッセイ及びスクリーニング系により、当業者はこの活性の向上のために様々なNSAID誘導体を試験することができるようになる。さらに、これらの結果は、NF- $\kappa$ B及びp300/CBP阻害剤の一般的なカテゴリーを、癌及び老化関連疾病の化学予防または治療のための物質として用いることの基礎を提供する。

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#### 【実施例12】

##### 【0114】

##### p16及びp27によるp21誘導可能遺伝子のプロモータの刺激

実施例7において実証したように、p21誘導可能遺伝子の発現は、他のCDK阻害剤、p16<sup>INK4A</sup>及びp27<sup>KIP1</sup>によっても上方制御される。p21誘導可能な遺伝子のプロモータが、これらのCDK阻害剤によって刺激されるかどうかを判定するために、実施例8に記載のpNF- $\kappa$ B及びプロモータ-ルシフェラーゼ作成物を、実施例6に記載されている、p16のIPTG誘導可能発現を有する（PT1080 p16-5）またはp27のIPTG誘導可能発現を有する（PT1080 p27-2）HT1080類縁体にトランスフェクトした。次にこれらのプロモータの発現に対するIPTGの効果を、実施例8のp21-誘導可能系列に対して記載したようにして分析した。

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##### 【0115】

pNF- $\kappa$ B-LucからのNF- $\kappa$ B-依存性発現は、p16（図12A）またはp27（図13A）の誘導によって強く刺激された。p27の場合、NF- $\kappa$ Bに対して観察された誘導の特異性は、基底及びIPTG誘導発現の両方を強く阻害したIKK阻害剤とのコトランスフェクションによっても実証された（図13A）。これらの結果は、p21のような上記CDK阻害剤がNF- $\kappa$ B活性を刺激することを実証する。さらに、p21誘導可能遺伝子のすべての被験プロモータは、p16またはp27によっても上方制御された。特に、IPTGによって誘導されるp16発現は、補体C3（図12B）、SAA（図12C）、t-TGアーゼ（図12D）及びNK4（図12E）のプロモータからのレポーター発現の誘導も導いた。IPTGによって誘導されるp27発現は、補体C3（図13B）、APP（図13C）、t-TGase（図13D）及びNK4（図13E）のプロモータを強く誘導した。これらの発見は、p21誘導可能プロモータが、p21によってだけでなく、p16やp27などの他のCDK阻害剤によっても活性化されることを示している。

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## 【 0 1 1 6 】

上記開示は本発明のある特定の実施形態を強調するものであって、これらに対するすべての変更または代替は、添付の請求項に記載される本発明の精神及び範囲に含まれると理解すべきである。

## 【 表 6 】

表 I

p 21 誘導によって下方制御される遺伝子

A. UniGamVアレイにより確認されたp21によって阻害される遺伝子:

遺伝子	受託番号	発現量差	確認方法 <sup>a</sup>
有糸分裂に関連するもの:			
CDC2	X05360	2.5	R, W
CKsHs1 (CDC2キナーゼ)	X54941	5.5	R
PLK1 (ポロ様キナーゼ)	U01038	5.1	R, W
XCAP-H コンデンシン相同体	D38553	6	R
CENP-A (動原体蛋白質 A)	U14518	5.3	R
CENP-F (動原体蛋白質 F)	U30872	2.5	R
MAD2	U65410	6.6	R, W
BUBR1	AF053306	5.9	R
MCAK (有糸分裂動原体関連キネシン)	U63743	3.8	R
HSET キネシン様蛋白質	AL021366	3.6	R
CHL1 ヘリカーゼ	U75968	3.3	R
AIK-1 (オーロラ/IPL1関連キナーゼ)	D84212	4.6	R
AIM-1 (AIK-2; オーロラ/IPL1関連キナーゼ)	AF004022	10.2	R
PRC1 (蛋白質調節性細胞質分裂 1)	AF044588	12.6	R, W
シトロンキナーゼ	H10809	2.7	R
ラミンB 1	L37747	7	
ラミンB 2	M94362	2.7	
LAP-2 (ラミン関連蛋白質 2)	U18271	4.6	R
MPP2 (M期リン蛋白質 2)	U74612	3.7	R
MPP5 (M期リン蛋白質 5)	X98261	3.7	
DNA複製、分離及びクロマチン構築に関連するもの:			
チミジンキナーゼ 1	K02581	2.9	R
チミジレートシンターゼ	X02308	3.9	R
ウリジノスフオリラーゼ	X90858	2.5	
リボヌクレオチドリダクターゼM1	X59543	4.6	R
リボヌクレオチドリダクターゼM2	X59618	10.7	R
CDC47 相同体 (MCM7)	D55716	9.6	R
CDC21 相同体 (MCM4)	X74794	2.7	R
CDC45 相同体 (Porc-Pl)	AJ223728	4.1	R
HsORC1 (開始点認識複合体 1)	U40152	2.7	R
DNAポリメラーゼ $\alpha$	X06745	2.8	R
複製因子 C (37-kDサブユニット)	M87339	2.6	
B-MYB	X13293	9.1	
HPV16 E1 蛋白結合性蛋白質	U96131	3.7	
トポイソメラーゼII $\alpha$	J04088	8.6	R
クロマチン構築因子-1 (p60サブユニット)	U20980	2.7	R
高移動性群染色体蛋白質 2	X62534	3.7	R
高移動性群染色体蛋白質 1	D63874	3.6	R
ヒストンH2A, F/Z変異体	AA203494	2.8	
DNA修復に関連するもの:			
XRCC9	U70310	3.6	R
RAD54 相同体	X97795	5.4	R
HEX1 5'-3'エキソヌクレアーゼ (RAD2相同体)	AF042282	5.2	R
ATP依存性DNAリガーゼ I	M36067	2.5	R
RAD21 相同体	D38551	2.9	R

表 I

転写及びRNAプロセッシングに関連するもの :

推定転写因子CA150	AF017789	2.8	
転写補助活性化因子ALY	AF047002	3.3	
WHSC1/MMSET (SETドメイン蛋白質)	AA401245	2.9	
NN8-4AG (SETドメイン蛋白質)	U50383	2.8	
EZH2 (zeste相同体2エンハンサー)	U61145	2.8	
PTB関連スプライシング因子	X70944	2.5	10
AUリッチ要素RNA結合蛋白質AUF1	U02019	2.8	
U-snRNP-関連シクロフィリン	AF016371	2.8	

その他の遺伝子 :

3-ホスホグリセリン酸デヒドロゲナーゼ	AF006043	4.8	
L型アミノ酸輸送体、サブユニットLAT1	M80244	4.1	R
ヒアルロナン媒介性運動性受容体	U29343	4	
ホルボリンI (PKC誘導可)	U03891	3.9	
PSD-95 結合ファミリー蛋白質	D13633	3.7	R
HTRIP (TNF受容体要素)	U77845	3.6	
NAD-依存性メチレンテトラヒドロ葉酸デヒドロゲナーゼ	X18396	3.4	
膜糖蛋白質4F2抗原重鎖	J02939	3.2	20
ムチン様蛋白質	D79992	3.2	
MAC30 (髄膜腫において差次的に発現)	L19183	2.9	
P52HPK (インターフェロンにより誘導される蛋白質キナーゼの調節因子)	AF007393	2.8	
推定ホスホセリンアミノトランスフェラーゼ	AA192483	2.8	
グルコース6リン酸トランスロカーゼ	Y15409	2.7	
カルサイクリン結合蛋白質	AF057356	2.6	
オルニチンデカルボキシラーゼ1	X16277	2.6	R
トロフィニン補助蛋白質 (タスチン)	U04810	2.5	
アシル補酵素Aコレステロールアシルトランスフェラーゼ	L21934	2.5	
ピニン/SDK3	Y10351	2.5	

機能が未知の遺伝子 :

EST	AA975298	2.7	30
EST	AA034414	2.5	
EST	AA482549	2.5	

B. RT-PCRにより同定されたp21によって阻害される遺伝子

遺伝子	受託番号	UniGemV 結果 <sup>b</sup>	
サイクリンA1	U66838	IS	
サイクリンB1	M25753	IS	
CDC25A	NM_001789	A	
ジヒドロ葉酸レダクターゼ	J00140	1.5	
ING1	NM_005537	A	40

<sup>a</sup> 略称 : R, RT-PCR; W, ウェスタンブロットイング<sup>b</sup> 略称 : IS, シグナル不十分 ; A, アレイに不在

【表 7】

表II

## p21誘導によって上方制御される遺伝子

遺伝子	受託番号	発現量差	確認方法 <sup>a</sup>	
分泌蛋白質及び細胞外マトリックスに関連する蛋白質:				
フィブロネクチン	X02761	5.7	R	10
I型プラスミノゲンアクチベータ阻害剤	M14083	3.7	R, N	
組織型プラスミノゲンアクチベータ阻害剤	M15518	2.8	Z	
ラミニンβ2	X79683	2.1		
テースモコリン2a/bb	X56807	3.5		
ホトカリキシン様蛋白質	U97519	2		
アクチビンA(インヒビンβA)	J03634	2	R	
ガレクチン3(Mac-2)	AB006780	2.4	N	
Mac-2結合蛋白質	L13210	2	R, N	
プロサリン	J03077	2.9	N	
CTGF(結合組織増殖因子)	M92934	3.3	N	
グラーニュリン/エビテリン	AF055008	2.1	N	
カテプシンB	L04288	2.4	N	20
組織トランスグルタミナーゼ	M55153	2.5	R, N, W	
P37NB(スリット相同体)	U32907	2.1		
血清アミロイドA蛋白質前駆体	M26152	4	R, N, W	
アルツハイマー病アミロイドA4蛋白質前駆体	D87675	2	R, N	
補体C3前駆体	K02765	5.9	R, N	
テスチカン	X73608	2.1	N	
インテグリンβ3	M35999	2.1	R, N	
リソソーム蛋白質:				
N-アセチルガラクトサミン-6-硫酸スルファターゼ	U06088	2.3	N	
酸α-グルコシダーゼ	X55079	2.4	N	
酸リパーゼA(コレステロールエステラーゼ)	X76488	2.1	N	
リソソームβ-スタチン非感受性プロテアーゼ(CLN2)	AF017456	2.5		30
ミトコンドリア蛋白質:				
スーパーオキシドジスムターゼ2	X07834	3.5	R, N, W	
メタキシン	J03060	3.4		
2,4-ジエノイル-CoAリクターゼ	U78302	2		
ストレス応答及び信号伝達に関連する他の遺伝子:				
ユビクチン共役酵素(UbcH8)	AF031141	2		
ユビクチン特異的プロテアーゼ8	D29956	2		
RTP/Cap43/Drp1/Ndr1(ニッケル、レノイト、 ホモシステイン及びERストレスによって誘導可能)	D87953	2.5		
C-193筋肉アンキリンリピート核蛋白質(サイトカイン誘導性)	X83703	3		
多剤耐性に関連するLRP major vault蛋白質				
β-アレスチン関連HHCPA78相同体(ヒタミンド3に よって上方制御される)	X79882	2.2	N	40
	S73591	4.1	N	
R-RAS	M14949	2.4		
RAB13小GTPアーゼ	X75593	2.2		
P66SHC(ski腫瘍遺伝子)	U73377	2	N	
MK-STYX(MAPキナーゼホスファターゼ様蛋白質)	N75168	2		
H73核抗原/MA-3アホトシス関連/TIS (トポイソメラーゼ阻害剤によって抑制)	U96628	2.4		

表II

他の遺伝子:			
フェルリチン細胞蛋白質4	M59807	4.4	R
TXKプロシナーゼ (T細胞特異的)	L27071	3.8	
X共役PEST含有輸送体	U05321	2.1	
AMP <sup>+</sup> アミナーゼ 2	M91029	2	N
FIP2/HYPLハンチン相互作用蛋白質	AF061034	2	
DNASEI相同体	X90392	2.5	N
転写因子11	X77366	2	
ヒストンH2A. 2	L19779	2.8	
ヒストンH2B	AL021807	2.4	
機能が未知の遺伝子:			
23808	AF038192	2.1	
CGI-147	AA307912	2.1	N
EST	W89120	2.8	
EST	AI026140	2.5	
EST	AA218982	2.4	
EST	W63684	2	

<sup>a</sup>略称: R, RT-PCR; N, ノーザンハイブリダイゼーション; W, ウェスタンブロットニング; Z, ザイモグラフィ

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#### 【図面の簡単な説明】

【0117】

【図1】ヒトHP1080線維肉腫細胞系列変種HT1080 p21-9を生産するために用いたIPTGによって調節されるレトロウイルスベクターLNp21CO3の模式図。

【図2】図2Aは、50μM IPTGを添加した後の、p21誘導の経時変化を示したグラフであり、p21のレベルはELISAによって測定されている。図2Bは、IPTG除去後のp21減衰の経時変化を示したグラフ。

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【図3A】図3Aは、RT-PCR実験(左)、細胞mRNA発現のノーザンブロット分析(中央)及び表示された遺伝子の発現のIPTG誘導性変化に対するイムノブロットイングアッセイ(右)のゲル電気泳動パターンの写真であり、「C」は対照の未処理HT1080 p21-9細胞であり、「I」は50μM IPTGで3日間処理した細胞である。2-ミクログロブリン(2-M)をRT-PCRに対する標準化対照として用い、S14リボソーム蛋白質をノーザンハイブリダイゼーションのために用いた。

【図3B】図3Bは、IPTGを添加及び除去した場合の表示したp21によって阻害される遺伝子の発現における経時変化を示す、RG-PCR実験(左)及びイムノブロットイング分析(右)のゲル電気泳動の写真。

【図3C】図3Cは、IPTGを添加及び除去した場合の表示したp21によって阻害される遺伝子の発現における経時変化を示す、RG-PCR実験(左)及びイムノブロットイング分析(右)のゲル電気泳動の写真。

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【図3D】図3Dは、未処理の対照HT1080 p21-9細胞(C)、血清不足静止細胞(Q)及びIPTG処理老化細胞(I)の間での遺伝子発現の比較。

【図4】ヒトHP1080線維肉腫細胞系列変種HT1080/LNp16RO2を生産するために用いたIPTGによって調節されるレトロウイルスベクターLNp16RO2の模式図。

【図5】50μM IPTGを添加時の、HT1080 p16-5(図5A)またはHT1080 p27-2(図5B)細胞の細胞周期分布の変化を示した模式図。

【図6】HT1080 p16-5細胞におけるp16(図6A)またはHT1080

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p 2 7 - 2 細胞における p 2 7 ( 図 6 B ) の I P T G 誘導性発現時における、表示された遺伝子の発現の I P T G 誘導性変化を検出するための R T - P C R 実験のゲル電気泳動パターンの写真であり、「 - 」は対照細胞であり、「 + 」は 5 0 μ M I P T G で 3 日間処理した細胞である。 - アクチンを R T - P C R のための標準化対照として用いた。

【図 7】 H T 1 0 8 0 p 2 1 - 9 細胞における p 2 1 誘導の、示された p 2 1 誘導可能遺伝子のプロモータによって駆動されるルシフェラーゼレポーター遺伝子の発現に対する影響を示した図。 5 0 μ M I P T G の存在下または非存在下で 2 日間 ( プロサボシンプロモータの場合 ) または 3 日間 ( 他の全てのプロモータの場合 ) 培養した後、一過性トランスフェクションの後にアッセイは実施した。アッセイは、 3 回 ( プロサボシンの場合 ) または 4 回 ( 他の全てのプロモータの場合 ) 実施した。

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【図 8】 2 4 時間の I P T G 処理後の L u N K 4 p 2 1 細胞系列におけるルシフェラーゼ発現の I P T G 用量依存性と ( 図 8 A ) 、 5 0 μ M I P T G 添加時のルシフェラーゼ発現の経時変化 ( 図 8 B ) を示すグラフである。

【図 9】 N F B 依存性プロモータ ( 図 9 A ) または表示した p 2 1 誘導可能遺伝子のプロモータ ( 図 9 B ~ 図 9 G ) によって駆動されるルシフェラーゼレポーター遺伝子の発現に対する、 H T 1 0 8 0 p 2 1 - 9 細胞内の p 2 1 誘導の影響を示している。プロモータ - レポーター作成物は、 N F B の優勢な阻害剤 ( I K K ) 、 p 3 0 0 / C B P を阻害する C 欠失 E 1 A 変異体 ( E 1 A C R 2 ) 、または E 1 A の非機能性 N 及び C 欠失変異体 ( E 1 A N / C R 2 ) を発現するベクターと、 1 : 2 のモル比で混合した。ルシフェラーゼレベルは、 I P T G の存在下または非存在下において 3 日後に測定し、コトランスフェクトされた p R L - C M V プラスミドから発現されたウミシイタケルシフェラーゼのレベルまたは ( 図 9 C 内 ) 細胞蛋白質のレベルのいずれかによって標準化した。実験は 3 回実施した。

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【図 1 0】 I P T G の存在下及び非存在下で様々な量の N S A I D とともにインキュベートした L u N K 4 p 2 1 細胞におけるルシフェラーゼ活性の棒グラフ。

【図 1 1】 様々な量のスリダクによる表示された遺伝子の発現における I P T G に誘導性変化の阻害を検出するための、 L u N K 4 p 2 1 を用いた R T - P C R 実験のゲル電気泳動パターンの写真。 - アクチンを R T - P C R のための標準化対照として用いた。

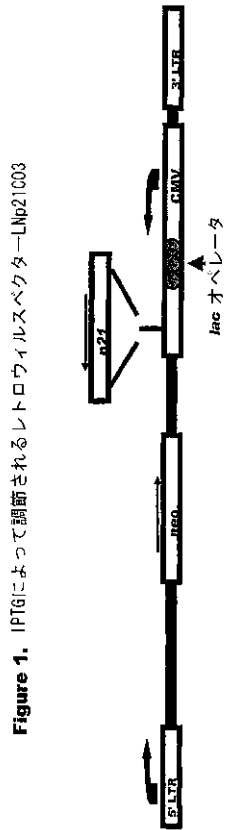
【図 1 2】 H T 1 0 8 0 p 1 6 - 5 細胞における、 N F B 依存性プロモータ ( 図 1 2 A ) または表示した p 2 1 誘導可能な遺伝子のプロモータ ( 図 1 2 B ~ 1 2 E ) によって駆動されるルシフェラーゼレポーター遺伝子の発現に対する、 p 1 6 誘導の影響を示す図。ルシフェラーゼレベルは、 I P T G の存在下または非存在下において 3 日後に測定し、コトランスフェクトされた p R L - C M V プラスミドから発現されたウミシイタケルシフェラーゼのレベルによって標準化した。図 1 2 A 及び図 1 2 E の実験は 3 回ずつ実施し、図 1 2 B 、 1 2 C 及び 1 2 D は 1 点のみで実施した。

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【図 1 3】 H T 1 0 8 0 p 2 7 - 2 細胞における、 N F B 依存性プロモータ ( 図 1 3 A ) または表示した p 2 1 誘導可能な遺伝子のプロモータ ( 図 1 3 B ~ 1 3 E ) によって駆動されるルシフェラーゼレポーター遺伝子の発現に対する、 p 2 7 誘導の影響を示す図。図 1 3 A において、プロモータ - レポーター作成物は、 N F B の優勢な阻害剤 ( I K K ) を発現するベクターと、 1 : 2 のモル比で混合した。ルシフェラーゼレベルは、 I P T G の存在下または非存在下において 3 日後に測定し、コトランスフェクトされた p R L - C M V プラスミドから発現されたウミシイタケルシフェラーゼのレベルによって標準化した。すべての実験は 3 回実施した。

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【 図 1 】



【 図 2 】

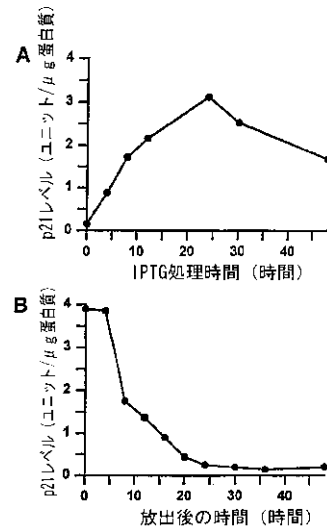
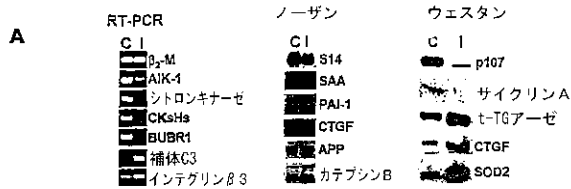


Fig. 2

【 図 3 A 】



【 図 3 C 】

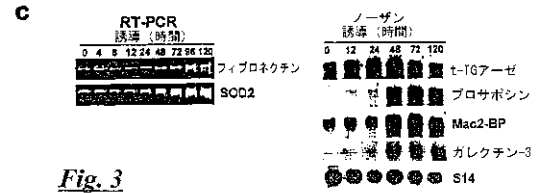
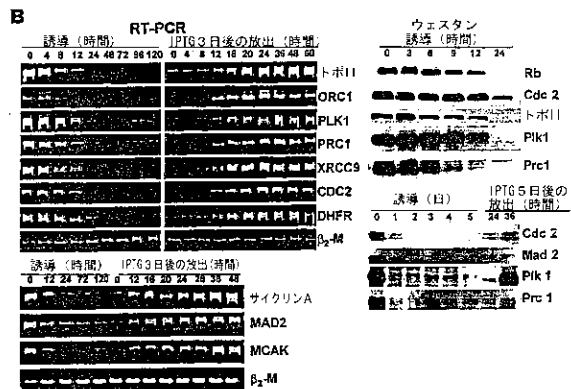
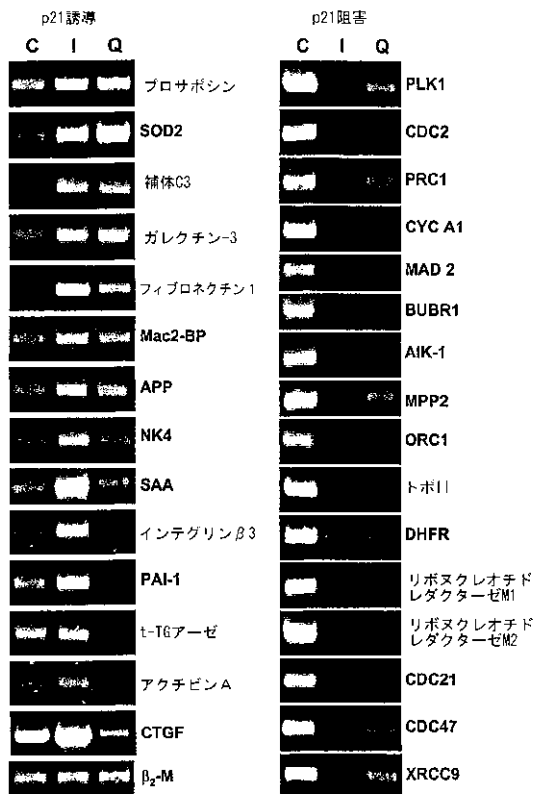


Fig. 3

【 図 3 B 】



【 図 3 D 】



C. 対照未処理細胞; I. IPTG処理; Q. 血清不足 (静止状態) 細胞

Fig. 3D

【 図 5 】

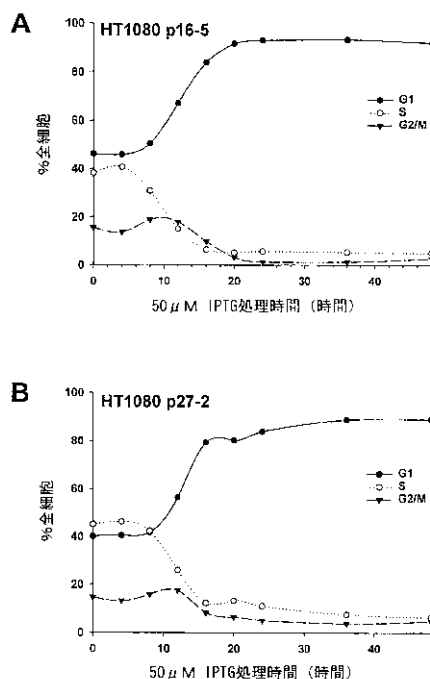


Fig. 5

【 図 6 】

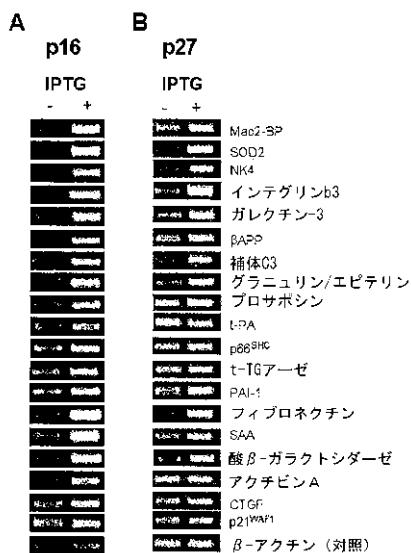


Fig. 6

【 図 7 】

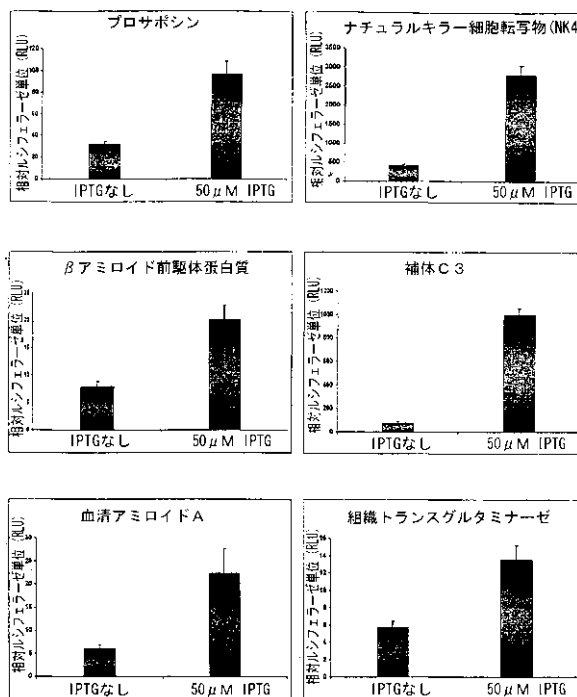
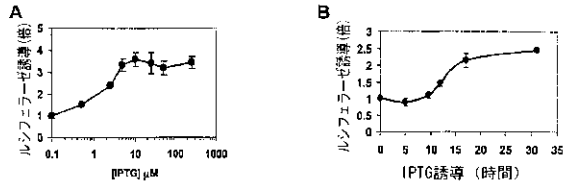


Figure 7

【 図 8 】

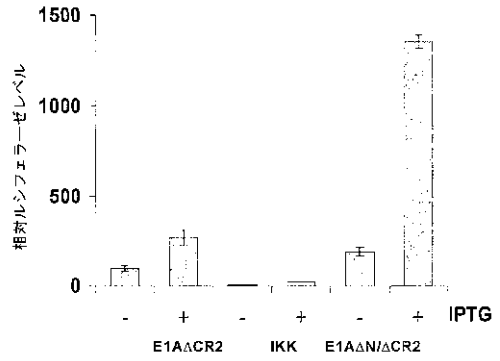
Figure 8

HT1080 LuNK4p21細胞におけるルシフェラーゼ発現のIPTG  
用量依存性(24時間処理)(A)及び経時変化(50 μM IPTG)(B)



【 図 9 A . B 】

A. 核因子-κB



B. 血清アミロイドA

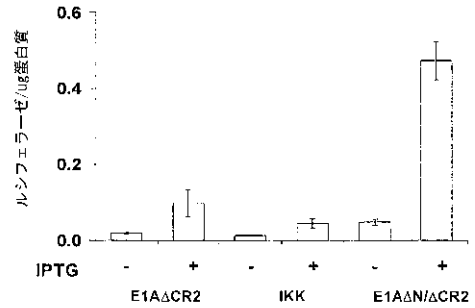
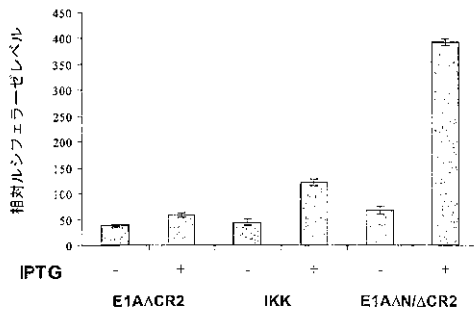


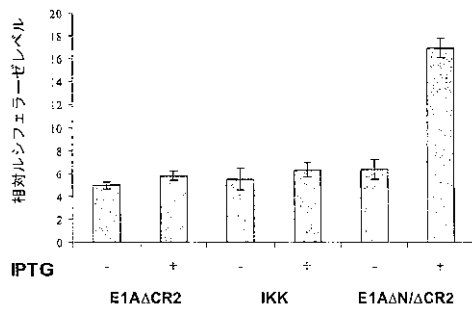
Fig. 9

【 図 9 C . D 】

C. プロサポシン

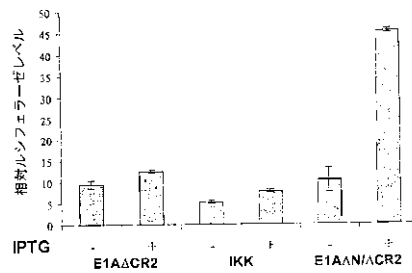


D. β-アミロイド前駆体蛋白質



【 図 9 E . F 】

E. 組織トランスグルタミナーゼ



F. 補体C3

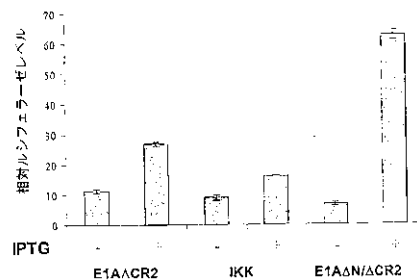


Fig. 9

Fig. 9.

【 図 9 G 】

G. NK細胞転写物 4 (NK4)

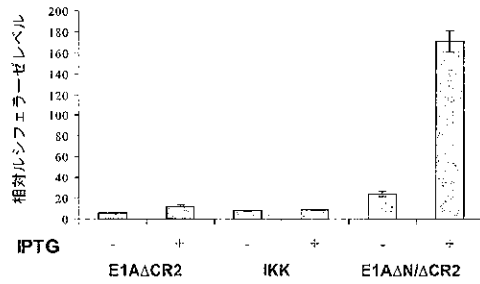
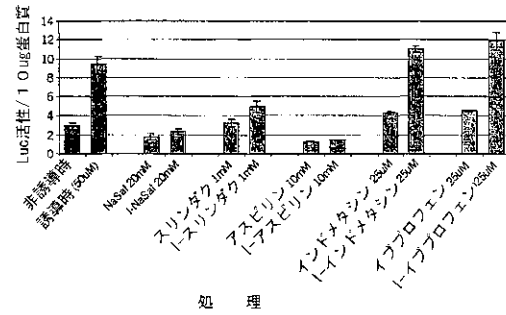


Fig. 9

【 図 1 0 】

Figure 10

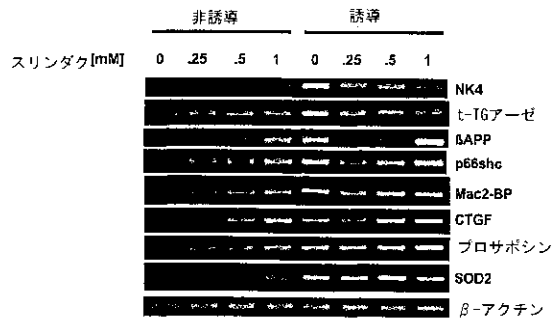
非処理または50  $\mu$ M IPTG処理 (1) における、HT1080 LuNK4p21細胞でのルシフェラーゼ発現に対する表示NSAIDの効果



【 図 1 1 】

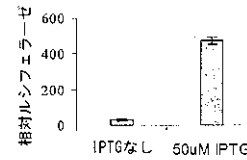
Figure 11

表示濃度のスリンダク存在下、IPTGによる非誘導時または誘導時のLuNK4p21細胞のRT-PCR分析

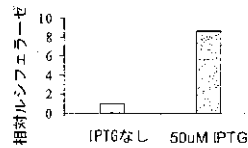


【 図 1 2 】

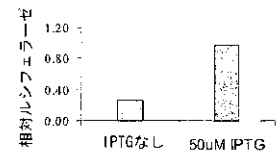
A. 核因子- $\kappa$ B



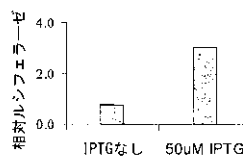
B. 補体C3



C. 血清アミロイドA



D. 組織トランスグルタミナーゼ



E. NK細胞転写物 4 (NK4)

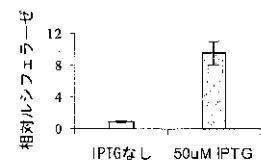


Fig. 12

【 図 1 3 】

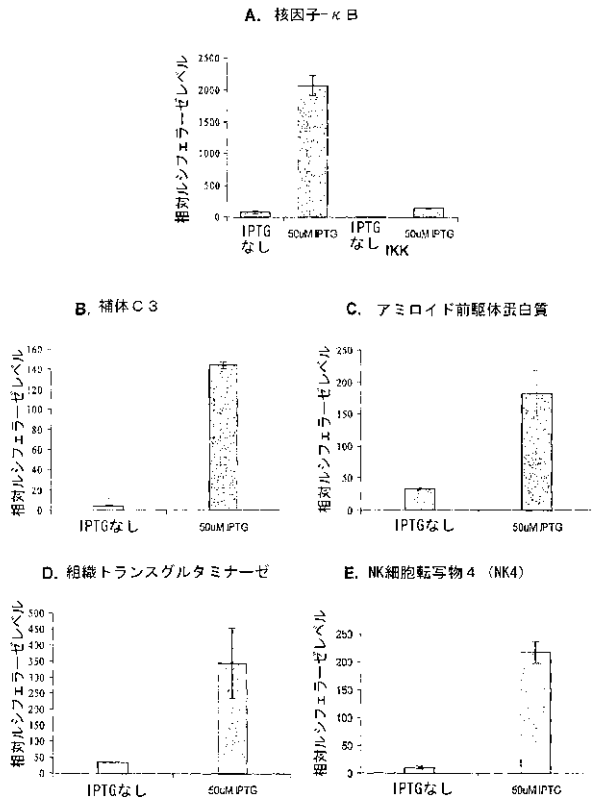


Fig. 13

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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 cancer and age related diseases, such genes being induced by cyclin dependent kinase inhibitors.

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

5

**BACKGROUND OF THE INVENTION**

This application claims priority to U.S. Provisional Application Serial No.: 60/265,840, filed February 1, 2001, and U.S. patent application Serial No: 09/861,923, filed May 21, 2001.

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This application was supported by a grant from the National Institutes of Health, No: R01CA62099. The government may have certain rights in this invention.

**1. Field Of The Invention**

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This invention is related to cellular senescence and changes in cellular gene expression that accompany senescence. 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. More specifically, the invention provides markers of cellular senescence 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 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.

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## 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 Siewicki *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<sup>Waf1/Cip1/Ink4</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>. The second family, Ink4, includes p16<sup>Ink4A</sup>, p15<sup>Ink2</sup>, p18<sup>Ink4c</sup>, and p19<sup>Ink4B</sup>. 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 TGF $\alpha$  induce p15 expression (Reynisdottir *et al.*, 1995, *Genes Dev.* **9**: 1831-1845), serum starvation induces p27 expression (Poiyak *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* **147**: 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 (Aicardi *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

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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).

5 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.* **53**: 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  
10 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  
15 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  
20 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.*,  
25 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.*,

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1994, *ibid.*; Alcorla *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<sup>+/+</sup>) and p21-nonexpressing cells (p21<sup>-/-</sup>) 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 result of CDK inhibition by 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<sup>INK4A</sup>; 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

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well (McCConnell *et al.*, 1999, *Molec. Cell Biol.* 19: 1981-1989).

Still another CDK inhibitor of particular relevance to the present invention is p27<sup>Kip1</sup>. p27 was initially identified as an inhibitor of CDK2 in cells that had been growth arrested by contact inhibition, TGF- $\beta$  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 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, such as carcinogenesis 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

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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, 5 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 10 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 15 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.

20 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 25 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; however,

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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  $\beta$ -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), granulim/epithelin (SEQ ID NO: 11), p66<sup>del</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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). 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

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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. 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

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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), elastatin (SEQ ID NO: 12), 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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -galactosidase, alkaline phosphatase and green

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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.

5 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-  
10 apoptotic factors is inhibited are fibroblasts, most preferably stromal fibroblasts. In preferred embodiments, the compounds are inhibitors of nuclear factor (kappa-B (NF $\kappa$ B) activity or expression.

In a third aspect, the invention provides methods for identifying compounds that inhibit CDK inhibitor-mediated induction of cellular gene expression. These methods  
15 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  
20 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  
25 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

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11. 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. 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  $\beta$ -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), granulins/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), elastin (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).

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

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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. 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. 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  $\beta$ -3 (SEQ ID NO: 4), activin A

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(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), granulim/epithelin (SEQ ID NO: 11), p66<sup>luc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15),

5 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). 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

10 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

15 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.

20 In a fifth 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.

25 In a sixth aspect, the invention provides compounds that are identified using any of the methods of the invention as disclosed herein.

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In a seventh 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 NF $\kappa$ B inhibitors.

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

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#### 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.

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

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

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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  $\mu$ M IPTG.  $\beta$ 2-microglobulin ( $\beta$ 2-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  $\mu$ M IPTG.

Figures 6A and 6B are photographs of gel electrophoresis patterns of RT-PCR

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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  $\mu$ M IPTG.  $\beta$ -actin was used as a normalization control for RT-PCR.

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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  $\mu$ M IPTG. The assays were carried out in triplicate (for prosaposin) or in quadruplicate (for all the other constructs).

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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  $\mu$ M IPTG (Figure 8B).

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Figures 9A through 9G illustrate the effects of p21 induction in HT1080 p21-9 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 9A) or by the promoters of the indicated p21-inducible genes (Figures 9B through 9G). The promoter-reporter constructs were mixed at a molar ratio 1:2 with vectors expressing a dominant inhibitor of NF $\kappa$ B (IKK), C-truncated I $\kappa$ B mutant that inhibits p300/CBP (E1A $\Delta$ CR2), or non-functional N- and C-truncated version of I $\kappa$ B (E1A $\Delta$ N/ $\Delta$ CR2). Luciferase levels were measured after 3 days in the presence or absence of IPTG and normalized either by the levels of Renilla luciferase expressed from the co-transfected pRL-CMV plasmid or (in Fig. 9C) by the level of cellular

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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.

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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;  $\beta$ -actin was used as a normalization control for RT-PCR.

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Figures 12A through 12E illustrate the effects of p16 induction in H11080 p16-5 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 12A) or by the promoters of the indicated p21-inducible genes (Figures 12B through 12E). 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. The experiments in Fig. 12A and Fig. 12E were carried out in triplicates, and in Figs. 12B, 12C and 12D in single points.

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Figures 13A through 13E illustrate the effects of p27 induction in H11080 p27-2 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 13A) or by the promoters of the indicated p21-inducible genes (Figures 13B through 13E). In Fig. 13A, the promoter-reporter construct was mixed at a molar ratio 1:2 with a vector expressing a dominant inhibitor of NF $\kappa$ B (IKK). 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.

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**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

This invention provides reagents and methods for identifying genes involved in mediating CDK inhibitor-induced cellular senescence and pathogenic consequences of senescence, and compounds capable of inhibiting senescence and pathogenic consequences of senescence in mammalian cells. Particularly provided are embodiments of such reagents and methods for identifying genes involved in cellular senescence and 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.

As used herein, the term "conditioned media" is intended to encompass cell culture media conditioned by growth of CDK inhibitor-expressing cells that contains

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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 exogenous 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 EIT-080 fibrosarcoma cell line and derivatives thereof.

15 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.

20 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,

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*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.

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

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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  
5 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  
10 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  
15 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. \_\_\_\_\_.

In alternative preferred embodiments, the invention provides mammalian cells containing a recombinant expression construct encoding an inducible mammalian p27  
20 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  
25 rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma

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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.

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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, (Davidson & Hilloff, ed.), Oxford University Press: New York, 1993.

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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

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known in the prior art, including heat shock promoters then 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 *lacI* repressor protein. In a preferred embodiment, the recombinant cell expresses the *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* 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 *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* repressor is encoded by the 3'SS recombinant expression construct (Stratagene). In alternative preferred embodiments, the recombinant cell expresses the *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* repressor is encoded by the 3'SS recombinant expression construct (Stratagene).

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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. 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  $\beta$ -3 (SEQ ID NO: 4), actin 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), granulins/epithelins (SEQ ID NO: 11), p56<sup>lck</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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). 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

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most preferably cells of the 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.

5 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  
10 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  
15 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  $\beta$ -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),  
20 granulit/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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-1 (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20). In preferred embodiments of this aspect of the  
25 invention, the CDK inhibitor is p21, p16 or p27.

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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

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human HT1080 fibrosarcoma derivative identified as HT1080/Np16RO2, 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 HT1080 fibrosarcoma cell line derivative identified as HT:080 p16-5, deposited on \_\_\_\_\_ with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. \_\_\_\_\_. Another exemplary cell line according to this particularly preferred embodiment of the invention is an HT1080 fibrosarcoma cell line derivative identified as HT1080 p27-2, deposited on \_\_\_\_\_ with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. \_\_\_\_\_.

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), epinephrine/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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -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

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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

5 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

10 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

15 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

20 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

25 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 LP1G (deposited

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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. \_\_\_\_\_) 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. \_\_\_\_\_) 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 50 $\mu$ M. 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.

In alternative embodiments, such compounds are identified independently of CDK inhibitor-directed experimental manipulation. In such assays, cells are treated to

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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. 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  $\beta$ -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), galactin-3 (SEQ ID NO: 9), superoxide dismutase 2 (SEQ ID NO: 10), granulins/epithelin (SEQ ID NO: 11), p66<sup>cas</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -galactosidase, alkaline phosphatase and green fluorescent protein, all of which are commercially available.

The invention also provides methods for identifying genes associated with cellular senescence and pathogenic consequences of senescence or that mediate the

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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

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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).

5           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  $\beta 3$  (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 (Ogomi *et al.*, 1988, *J. Gerontol.* 43: B157-B162), SAA (Rosenthal & Franklin, 1975, *J. Clin. Invest.* 55: 746-753) and t-UGase (Singhal *et al.*, 1997, *J. Invest. Med.* 45: 567-575).

          The most commonly used marker of cell senescence is the SA- $\beta$ -gal activity (Dimiri *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92: 9363-9367). This gene is strongly elevated in DDC-treated IM1080 p21-9 cells (Chang *et al.*, 1999, *Oncogene* 18: 4808-

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481E). SA- $\beta$ -gal was suggested to represent increased activity and altered localization of the lysosomal  $\beta$ -galactosidase (Dimri *et al.*, 1995, *ibid.*), and other studies have described elevated lysosome activities in senescent cells (Cristofalo & Kabakjian, 1975, *Mech. Aging Dev.* **4**: 19-28). Five lysosomal enzymes appear in Table II, including N-acetylgalactosamine-6- sulfate sulfatase (GALNS), cathepsin B, acid  $\alpha$ -glucosidase, acid lipase A and lysosomal pepstatin-insensitive protease. p21 also upregulated genes for mitochondrial proteins SOD2, metaxin and 2, 4-dienoyl-CoA reductase, which correlates with reports of different mitochondrial genes overexpressed 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  $\beta$ -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 tTGase, 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 (Jersem & Whitehead, 1998, *Biochem. J.* **334**: 489-503). Two other CDK inhibitor upregulated secreted proteins, C1GH and galectin 3 are involved in atherosclerosis (Oczar *et al.*, 1997, *Circulation* **95**: 831-839; Nachtigal *et al.*, 1998, *Am. J. Pathol.* **152**: 1199-1208). In addition, cathepsin

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13 (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  
5 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  
10 (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-  
15 F1216) 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-Dounhji *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*  
20 *USA.* 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  
25 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

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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<sup>shc</sup>, 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; Nue *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

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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.

5 This paracrine tumor-promoting activity was recently shown to be selectively increased during replicative senescence of normal human fibroblasts (Ktcolica *et al.*, 2000, Proc. Amer. Assoc. Can. Res. 41, Abs. 448), a process that involves induction of p21 and p16. 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  
10 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.

15 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  
20 age-dependent manner.

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  
25 the capacity to prevent, retard or reverse age-related diseases by their effects on CDK inhibitor-mediated induction of gene expression.

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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 (NF $\kappa$ B). It will be understood by those with skill in the art that NF $\kappa$ B 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 NF $\kappa$ B heterodimer; second, inhibiting translocation of NF $\kappa$ B from the cytoplasm to the nucleus, which can depend on inhibiting inactivation of I $\kappa$ B expression and/or activity in cells; and third, by inhibiting the activity of NF $\kappa$ B itself. This invention encompasses methods for inhibiting NF $\kappa$ B activity, and thereby inhibiting induction of genes by CDK inhibitors, in any and all of these ways. Examples of NF $\kappa$ B 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); carboxyalkenylbenzoquinone 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, dimethyl fumarate, mesalazine, pimobendan, sulfasalazine, methyl chlorogenate, chloromethylketone, alpha-tocopherol succinate, tepoxaline, and certain non-steroidal

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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**

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15  
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- $\beta$ -thiogalactoside (IPTG). Expression of p21 can be induced by culturing these cells in the presence of a sufficient amount of IPTG, thereby permitting the sequelae 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 FTA 1664.

20  
25  
Briefly, a subline of HT1080 expressing a murine ecotropic retrovirus receptor and a modified bacterial *lacI* repressor encoded by the plasmid 3'8S (Stratagene) (described in Chang & Robinson, 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 *lacI* repressor expressed in the cell. LNp21CO3 was constructed by

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cloning a 492bp fragment of DNA comprising the p21 coding sequence into the *NotI* and *BglII* 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.

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**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.

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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).

20

Cell growth in the presence of IPTG was assayed in three ways: measuring <sup>3</sup>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

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cycle distribution").

<sup>3</sup>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 <sup>3</sup>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 HCT1080 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**

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The results disclosed in Example 2 suggested that the morphological and cell cycle consequences of p21 induction could reflect multiple changes in gene expression.

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The effects of p21 induction on cellular gene expression were examined as follows.

Poly(A)<sup>+</sup> RNA was isolated from untreated HT1080 p21<sup>-9</sup> cells and from cells that were treated for 3 days with 50  $\mu$ M IPTG. cDNA was prepared from the poly(A)<sup>+</sup> 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<sup>-9</sup> 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), Pro 1 (a gift of Drs. W. Jiang and T. Hunter), and topoisomerase II $\alpha$  (Ab0284; a gift of Dr. W.T. Beck), and sheep polyclonal antibody against SOH2 (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).

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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 II $\alpha$ , ORC1, PLK1, PRC1 and XRCC9)

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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 *cc1* growth arrest and Rb dephosphorylation. Other p21-inhibited genes (such as CDC2 or DIFER) 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.

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## 2. Gene expression induced by p21

In addition to genes repressed by p21 expression, the assays described above  
5 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  
10 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.

15 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  $\alpha 2$ , Mac-2 binding protein), other  
secreted proteins (e.g. activin A, connective tissue growth factor, serum amyloid A), or  
20 ECM receptors (such as integrin  $\beta 3$ ). 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,  
25 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

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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.

5 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**  
**10 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.

15 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- $\beta$ -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. 5D shows RT-PCR analysis performed as  
20 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  $\mu$ M IPTG. Genes that were completely inhibited in HT1080 p21-9 cells when the culture media contained 50  $\mu$ M IPTG were also inhibited in serum-starved cells, but most of these genes were inhibited to a lesser extent than in IPTG-treated cells.  
25

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

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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

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minimum value of 2.0 that we have used for inclusion into this Table or verification by RT-PCR. These proteins are clusterin (Kocá-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), gel'solin (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  $10^5$  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 50 $\mu$ M, and this media was replaced three days later with DMEM supplemented with 0.5% FCS and 50 $\mu$ M 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  $\mu$ Ci/ml, was added and incubated for 24 hrs.

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Cells were then collected and their  $^3\text{H}$ -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  $^3\text{H}$ -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* 12: 1709-1719), including serum starvation (Lowe *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91: 2026-2030). Apoptosis was analyzed by plating  $3 \times 10^5$  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

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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.

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**EXAMPLE 6**  
**Production of Mammalian Cell comprising**  
**Inducible p16<sup>INK4A</sup> or p27<sup>Kip1</sup> Genes**

Mammalian cell lines comprising inducible CDK inhibitors p16<sup>INK4A</sup> (which preferentially inhibits CDK4/6; Serrano *et al.*, *Nature* 16, 704-707, 1993) or p27<sup>Kip1</sup> (which preferentially inhibits CDK2; Blain *et al.*, *J. Biol. Chem.* 272, 25863-25872, 1997) 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 3'5S6; 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

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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 LNXRO2 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).

5 The LNP16RO2 and LNP27RO2 constructs were introduced individually into HT1080 3T3 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, 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 15 31, 2002 in the American Type Culture Collection (A.T.C.C.), Manassas, VA and given Accession Number \_\_\_\_\_. 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 20 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 25 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

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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 \_\_\_\_\_. Figure 5B shows changes in the cell cycle distribution of HT1080 p27-2 cells upon the addition of 50  $\mu$ M 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 gene 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 50 $\mu$ M IPTG for three days. These RNA samples were then used in RT-PCR assays performed essentially as described above in Example 3, except that  $\beta$ -actin rather than  $\beta$ -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.

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**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-  
10 inducible human genes, including NK4, SAA, Complement C3 (CC3), prosaposin,  
 $\beta$ APP 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  
15 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  $\beta$ APP gene was identified in the human genome  
sequence (Accession number X12751) as adjacent to the 5' end of  $\beta$ APP cDNA  
20 (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  
25 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

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pCR2.1/TOPO (for SAA, CC3,  $\beta$ APP and t-TGase) or pCR2/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  $\mu$ l of cell suspension (approximately 7 to 8 million cells) was transferred to a 4 cm gap electroporation cuvette (Bio-Rad). 10-20  $\mu$ g of the promoter-luciferase construct was added to the cells. In some experiments, a control plasmid pCMV $\beta$ gal expressing bacterial  $\beta$ -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 Assay kit (Promega). Electroporations were

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performed using Bio-Rad Gene Pulser at 0.22 volts, with a capacitance extender set to 960 $\mu$ FD, providing a  $\tau$  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  $\mu$ M IPTG. 2 to 4 days later, cells were washed twice with phosphate-buffered saline and collected in 300  $\mu$ L of 1x Passive Lysis Buffer or Reporter Lysis Buffer (Promega). The lysate was centrifuged briefly at 10,000 g to pellet debris, and 50  $\mu$ L 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  $\beta$ APP, 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' $\rightarrow$ 3')	Antisense primer (5' $\rightarrow$ 3')
CC3	GCTAAGAGGATATTGACATTAGACAGG (SEQ ID NO: 21)	AGGGGGAGGTGGGTTAGTAG (SEQ ID NO: 22)
NK4	TGGAGCTAGAGAGCCCGTAGG (SEQ ID NO: 23)	GCCAAAAGTTCAAGGAGCCAA (SEQ ID NO: 24)
SAA	CAGAGTTGCTGCTATGTCACCA (SEQ ID NO: 25)	CACTCCTTGTGTGCTCCTCACC (SEQ ID NO: 26)
$\beta$ APP	TTGCTCCTTTGGTTCGTTCT (SEQ ID NO: 27)	GCTGCCGAGGAAACTGAC (SEQ ID NO: 28)

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t-TGase	CCCAGGGAGAAATCCACTGAA GCAAC (SEQ ID NO: 29)	TCGGCGGGGGCGGTGGCTCCCT TCCACT (SEQ ID NO: 30)
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5 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	1600 bp
BAPP	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



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**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<sup>-9</sup> 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 3A and 3B. Fig. 3A shows the IPTG dose dependence of luciferase expression after 24 hrs of IPTG treatment, and Fig. 3B shows the time course of luciferase expression upon the addition of 50 µM IPTG. This analysis shows that

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most of the induction can be achieved using as little as 5  $\mu$ M IP1G and a treatment period as short as 17 hrs.

5 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 IP1G 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.

25 **EXAMPLE 10**  
**Use of NF $\kappa$ B 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 NF $\kappa$ B binding sites.

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Several p21-induced genes are known to be positively regulated by NF $\kappa$ B, 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  $\beta$ -amyloid  
precursor protein (APP) (Grilli *et al.*, 1996, *J. Biol. Chem.* **271**: 15002-15007) and the  
5 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 NF $\kappa$ B-dependent transcription (Perkins *et al.*, 1997, *Science* **275**: 523-527).  
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  
10 p300/CBP or related transcription cofactors may be responsible for the effect of p21 on  
some of the upregulated genes. Thus, inhibitors of NF $\kappa$ B or p300/CBP may potentially  
prevent the induction of transcription by p21.

To determine if IF1 $\gamma$ -inducible p21 expression in HT1080 p21-9 cells stimulates  
the transcriptional activity of NF $\kappa$ B, we have used transient transfection assays to  
15 investigate the effect of p21 induction on luciferase expression from the plasmid  
pNF $\kappa$ B-Luc, commercially available from Stratagene. This plasmid expresses firefly  
luciferase from an artificial promoter containing five NF $\kappa$ B consensus sequences. To  
evaluate the effects of genetic inhibitors of NF $\kappa$ B on luciferase expression from pNF $\kappa$ B-  
Luc, 20  $\mu$ g of the latter plasmid were mixed (at a molar ratio 1:2) with a plasmid MAD3  
20 (a.k.a. pRC/pactin-HA-IKK $\alpha$ ) that expresses a dominant mutant of I $\kappa$ B kinase  $\alpha$  that  
selectively inhibits NF $\kappa$ B (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 pNF $\kappa$ B-Luc, the latter plasmid was similarly mixed in another assay  
25 with a vector expressing a truncated gene for adenoviral E1A protein with a C-terminal

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deletion ( $\Delta$ CR2 (120-140)). The C-truncated E1A (termed E1A $\Delta$ CR2) 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 ( $\Delta$ N(2-36)), termed E1A $\Delta$ N/ $\Delta$ CR2. The E1A $\Delta$ CR2 and E1A $\Delta$ N/ $\Delta$ CR2 constructs were provided by Dr. V. Ogryzko (NICHD, NIH). The mixtures of pNFkB-Luc with IKK, E1A $\Delta$ CR2 or E1A $\Delta$ N/ $\Delta$ CR2 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  $\mu$ M 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 9A. pNFkB-Luc mixed with the negative control (E1A $\Delta$ N/ $\Delta$ CR2) showed up to 15-fold induction in the presence of IPTG, demonstrating an increase in NFkB 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. E1A $\Delta$ CR2 had a similar but weaker effect than IKK, suggesting the requirement of p300/CBP for NFkB activity in HT1080 p21-9 cells (Fig. 9A).

The same analysis was carried out using promoter-luciferase constructs for six p21-inducible genes. The results for SAA are shown in Figure 9B, for prosaposin in Figure 9C, for  $\beta$ AAPP in Figure 9D, for tTGase in Figure 9E, for complement C3 in Figure 9F, and for NK4 in Figure 9G. Both IKK and E1A $\Delta$ CR2 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 NFkB. Quantitatively,

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however, the effects of these inhibitors varied among the promoters. Both basal and IPTG-stimulated expression of the promoters of SAA (Fig. 9B) and NK4 (Fig. 9G) was inhibited by IKK and ELAΔCR2 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. 9C), βA2M (Fig. 9D), t-TGase (Fig. 9E), or complement C3 (Fig. 9F), but interfered with the induction of these promoters in the presence of IPTG. These results indicate that p300/CBP and NFκB are involved in the induction of all the tested promoters by p21.

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**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 LuNK-p21 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.

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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.*).

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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 NF $\kappa$ B-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  $\mu$ M 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  $\mu$ M, 500  $\mu$ M 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  $\beta$ -actin rather than  $\beta$ -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.

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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  $\mu$ M sulindac for the APP gene, but only by 250  $\mu$ M sulindac for *p66<sup>Bcl-2</sup>*, 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  $\mu$ M) inhibits the ability of p21 to induce transcription for most of the tested genes.

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Table IVa. Primer sequences

GENE	SENSE PRIMER (5'→3')	ANTISENSE PRIMER (5'→3')
NK4	AGCACCAGGCCATAGAAAGA (SEQ ID NO: 13)	GGTGTACAGCTCCTCTGTGTC (SEQ ID NO: 49)
T-TGASE	ACTACAACCTCGGCCCATGAC (SEQ ID NO: 50)	GCCAGTTTGTTCAGGTGGTT (SEQ ID NO: 61)
BAPP	CTCGTTCCTGACAAGTGCAA (SEQ ID NO: 62)	TGTTACAGAGCACACCTCTCG (SEQ ID NO: 63)
P66 <sup>shc</sup>	GAGGGTGTGGTTCGGACTAA (SEQ ID NO: 64)	GCCCAAGAGTGTGATTTGTT (SEQ ID NO: 65)
CTGF	GGAGAGTCCTCCAGAGCAG (SEQ ID NO: 66)	ATGTCTTCATGCTGGTGCAG (SEQ ID NO: 67)
MAC2-BP	ACCATGAGTGTGGATGCTGA (SEQ ID NO: 68)	ACAGGGACAGGTGAACTGC (SEQ ID NO: 69)
GRANULIN	ACCACGGACCTCTCACTAA (SEQ ID NO: 70)	ACACTGCCCTCAGCTACAC (SEQ ID NO: 71)
PROSPOIN	CCAGAGCTGGACATGACTGA (SEQ ID NO: 72)	GTCACCTCCTCACCAGGAA (SEQ ID NO: 73)
SOD2	CAAATTGCTGCTTGTCAAA (SEQ ID NO: 74)	CATCCCTACAAGTCCCAAA (SEQ ID NO: 75)
B-ACTIN	GGGAAATCGTGGTGACATTAA G (SEQ ID NO: 76)	TGTGTGGCGTACAGGTCTTIG (SEQ ID NO: 77)

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Table IVb. PCR temperatures (in °C)

Gene	Denaturation	Annealing	Extension	Cycles	Product size
NK4	94	58	72	24	481
5-TGase	94	58	72	24	499
B-APP	94	58	72	20	500
p66 <sup>shc</sup>	94	58	72	22	514
CTGF	94	64	72	28	499
MAC2-3P	94	58	72	21	517
Granulin	94	64	72	25	446
Prosaposin	94	58	72	21	500
SOD2	94	58	72	23	505
$\beta$ -actin	94	60	72	17	275

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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 LeuNK4p21 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 NF $\kappa$ B 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 NF $\kappa$ B-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,

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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 NF $\kappa$ B-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 NF $\kappa$ B 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<sup>INK4A</sup> and p27<sup>KIP1</sup>. To determine if the promoters of p21-inducible genes are stimulated by the latter CDK inhibitors, pNF $\kappa$ B-Luc and several of the promoter-luciferase constructs described in Example 8 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. The effect of IPTG on the expression of these promoters was then analyzed as described for the p21-inducible line in Example 8.

NF $\kappa$ B-dependent expression from pNF $\kappa$ B-Luc was strongly stimulated by the induction of either p16 (Fig. 12A) or p27 (Fig. 13A). In the case of p27, the specificity of the observed induction for NF $\kappa$ B was also demonstrated by cotransfection with the IKK inhibitor, which strongly inhibited both basal and IPTG-induced expression (Fig. 13A). These results demonstrate that these CDK inhibitors, like p21, stimulate NF $\kappa$ B activity. Furthermore, all the tested promoters of p21-inducible genes were also upregulated by p16 or p27. In particular, IPTG-induced p16 expression led to the induction of reporter expression from the promoters of Complement C3 (Fig. 12B), SAA

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(Fig. 12C), t-TGase (Fig. 12D) and NK4 (Fig. 12E). IPTG-induced p27 expression strongly induced the promoters of Complement C3 (Fig. 13B),  $\beta$ APP (Fig. 13C), t-TGase (Fig. 13D) and NK4 (Fig. 13E). These findings indicate that p21-inducible promoters are activated not only by p21 but also by other CDK inhibitors, such as p16 and p27.

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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.

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Table I

Genes downregulated by p21 induction

*A. p21-inhibited genes identified by UniGeneV array:*

Genes	Accession No.	Balanced Diff. Expr.	Confirmed by*
<i>Associated with mitosis:</i>			
CDC2	X05360	2.9	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)	U14516	5.3	R
CENP-F (centromere protein F)	U00572	2.5	R
MAD2	U65410	6.6	R,W
BUBR1	AF033306	5.9	R
MCAK (mitotic centromere-associated kinesin)	U63743	3.8	R
HSET kinesin-like protein	AL021365	3.6	R
CHL1 helicase	U75958	3.3	R
AIK-1 (aurora/PL1-related kinase)	D84212	4.8	R
AIK-1 (AIK-2; aurora/PL1-related kinase)	AF064022	10.2	R
PRC1 (protein regulating cytokinesis 1)	AF044555	12.0	R,W
Citron kinase	H10909	2.7	R
Lamin B1	U37747	7	
Lamin B2	M94262	2.7	
LAP-2 (lamin-associated protein 2)	U18271	4.5	R
MPP2 (M phase phosphoprotein 2)	U74612	3.7	R
MPP5 (M phase phosphoprotein 5)	X96261	3.7	
<i>Associated with DNA replication, segregation and chromatin assembly:</i>			
Thymidine kinase 1	K02581	2.9	R
Thymidylate synthase	X02308	3.9	R
Uridine phosphorylase	X50259	2.5	
Ribonucleotide reductase M1	X58543	4.6	R
Ribonucleotide reductase M2	X59018	19.7	R
CDC47 homolog (MCM7)	D55716	9.6	R
CDC21 homolog (MCM4)	X74734	2.7	R
CDC48 homolog (Polo-Pl)	AJ223728	4.1	R
HsORC1 (origin recognition complex 1)	U40152	2.7	R
DNA polymerase $\alpha$	X06745	2.8	R
Replication factor C (37-kD subunit)	M87339	2.6	
B-MYB	X13293	5.1	
HPV18 E1 protein binding protein	U96131	3.7	
Topoisomerase IIa	J04088	8.8	R
Chromatin assembly factor-I (p80 subunit)	U20930	2.7	R
High-mobility group chromosomal protein 2	X62534	3.7	R
High-mobility group chromosomal protein 1	D63874	3.6	K
Histone H2A.F/Z variant	AA203484	2.8	
<i>Associated with DNA repair:</i>			
XRCC9	U70310	3.6	R
RAD54 homolog	X57795	5.4	R
HEX1 5'-3' exonuclease (RAD2 homolog)	AF042282	5.2	R
ATP-dependent DNA ligase I	M36057	2.9	R
RAD21 homolog	D38551	2.9	R

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Table I

<i>Associated with transcription and RNA processing:</i>		
Putative transcription factor CA150	AF017789	2.3
Transcriptional coactivator ALY	AF047002	3.3
WHSC1/MMSET (SET domain protein)	AA401245	2.9
NNS-AG (SET domain protein)	U50333	2.8
EZH2 (enhancer of zeste homolog 2)	U81145	2.8
PYB-associated splicing factor	X70944	2.5
AU-rich element RNA-binding protein AUF1	U02018	2.8
U-snRNP-associated cyclophilin	AF016371	2.9
<i>Other genes:</i>		
3-phosphoglycerate dehydrogenase	AF008043	4.8
L-type amino acid transporter, subunit LAT1	AB0244	4.1
Hyaluronan-mediated motility receptor	U23343	4
Proctolin I (PKC-inducible)	U03881	3.9
PSD-95 binding family protein	D13033	3.7
HTRIP (TNF receptor component)	U77945	3.6
NAD-dependent methyltetrahydrofolate dehydrogenase	X18356	3.4
Membrane glycoprotein 4F2 antigen heavy chain	J02939	3.2
Mucin-like protein	D79962	3.2
MNAC30 (differentially expressed in meningiomas)	L19183	2.9
P521PK (regulator of interferon-induced protein kinase)	AF007363	2.8
Putative phosphoserine aminotransferase	AA192463	2.8
Glucose 6-phosphate translocase	Y18409	2.7
Calcyclin binding protein	AF067356	2.6
Citrimine decarboxylase 1	X18277	2.6
Trophin1 assisting protein (tastin)	U04810	2.5
Acyl-coenzyme A:cholesterol acyltransferase	L31934	2.3
Pinin/SRK3	Y10351	2.5
<i>Genes with unknown function:</i>		
EST	AA875258	2.7
EST	AA034414	2.5
EST	AA482548	2.5
<b>B. p24-Inh(b)led genes identified by RT-PCR:</b>		
<b>Gene</b>	<b>Accession No.</b>	<b>UniGeneV result<sup>b</sup></b>
Cyclin A1	U66828	IS
Cyclin B1	M26753	IS
CDC28A	NM_001788	A
Dihydrofolate reductase	J03140	1.5
ING1	NM_005537	A

\*Abbreviations: R, RT-PCR; W, western blotting

\*Abbreviations: IS, insufficient signal; A, absent from the array

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Table II

Genes upregulated by p21 induction			
Genes	Accession No	Balanced Diff Ester	Confirmed by <sup>a</sup>
<i>Secreted proteins and proteins associated with extracellular matrix:</i>			
Fibronectin 1	X02761	5.7	R
Plasminogen activator inhibitor, type 1	M14083	3.7	R, N
Plasminogen activator, tissue type	M15519	2.9	Z
Laminin β2	X79683	2.1	
Desmocollin 2αbb	X66807	3.5	
Podocalyxin-like protein	U97519	2	
Activin A (Intibin βA)	J03934	2	R
Galectin 3 (Mac-2)	AB006760	2.4	N
Mac-2 binding protein	L13210	2	R, N
Procollagen	J03077	2.9	N
CTGF (connective tissue growth factor)	M62934	3.3	N
Granulin/epithelin	AF055008	2.1	N
Cathepsin B	L04289	2.4	N
Tissue transglutaminase	M65153	2.5	R, N, W
FGFB (fibrinogen)	U32907	2.1	
Serum amyloid A protein precursor	M26182	4	R, N, W
Alzheimer's disease amyloid A4 protein precursor	D87675	2	R, N
Complement C3 precursor	K02753	5.9	R, N
Tenascin	X73608	2.1	N
Integrin β3	M35998	2.1	R, N
<i>Lysosomal proteins:</i>			
N-acetylgalactosamine 6-sulfate sulfatase	U08088	2.3	N
Acid alpha-glucosidase	X65078	2.4	N
Acid lipase A (cholesterol esterase)	X79486	2.1	N
Lysosomal pepsinogen-insensitive protease (CLN2)	AF017458	2.5	
<i>Mitochondrial proteins:</i>			
Superoxide dismutase 2	X07634	3.5	R, N, W
Melanin	J03060	3.4	
2,4-dienoyl-CoA reductase	U78302	2	
<i>Other genes associated with stress response and signal transduction:</i>			
Ubiquitin-conjugating enzyme (UbcH6)	AF031141	2	
Ubiquitin-specific protease 5	C02698	2	
RTP/Cap43/Crg1/Nsr1 (inducible by nickel, retinoids, homocysteine and ER stress)	D67963	2.5	
C-153 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-RAF	M14949	2.4	
RAV 13 small GTPase	X76583	2.2	
P66 SHC (sh1 oncogene)	U73377	2	N
MK-STYX (MAP kinase phosphatase-like protein)	U75158	2	
H73 nuclear antigen/MA-3 apoptosis-related/TIS (topoisomerase-inhibitor suppressed)	U95628	2.4	

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Table II

<i>Other genes:</i>			
Natural killer cell protein 4	M69807	4.4	R
TKK tyrosine kinase (T-cell specific)	L27071	3.8	
X-linked PEST-containing transporter	U05324	2.1	
AMP deaminase 2	B91022	2	N
FIP2/HYPL huntingtin-interacting protein	AF061034	2	
DNASE I homolog	X90382	2.5	N
Transcription factor 11	X77386	2	
Histone H2A.2	L19779	2.8	
Histone H2B	AL021807	2.4	
<i>Genes with unknown function:</i>			
23809	AF038192	2.1	
CGI-147	AA307912	2.1	N
EST	W89120	2.8	
EST	A026140	2.5	
EST	AA218982	2.4	
EST	W83684	2	

<sup>a</sup>Abbreviations: R, RT-PCR; N, northern hybridization; W, western blotting; Z, zymography

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**WE CLAIM:**

1. A recombinant expression construct encoding a reporter gene operably linked to a promoter from a mammalian gene induced by a cyclin-dependent kinase inhibitor.
- 5 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 gene induced by a CDK inhibitor.
- 10 4. A recombinant expression construct according to Claim 3, wherein the promoter is a promoter from a human gene identified in Table II.
5. A recombinant expression construct according to Claim 4, 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  $\beta$ -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), granulins/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), prostacyclin stimulating factor (t-H; SEQ ID NO: 18), vascular endothelial growth factor-C (SEQ ID NO: 19) and tissue inhibitor of metalloproteinase-1 (SEQ ID NO: 20).
- 20 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

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amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), tissue transglutaminase (SEQ ID NO: 16),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), or prosaposin (SEQ ID NO: 7).

7. A recombinant expression construct according to claim 4, wherein the  
5 recombinant expression construct is pL.uNK4.

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 (H11080 L.uNK4/p21).

10. A mammalian cell according to Claim 8 wherein expression of the recombinant expression construct is modulated by NF $\kappa$ B.

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.  
15

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  
20 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.

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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.
- 5 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  
10 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  
15 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.
- 20 21. The mammalian cell of claim 21, 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).

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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. \_\_\_\_\_ (HT1080 p16-5).
- 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. \_\_\_\_ (HT1080 p27-2).
27. The mammalian cell of claim 17, wherein the lactose repressor-specific  
10 inducing agent is a  $\beta$ -galactoside.
28. A method for identifying a compound that inhibits induction of 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 genes induced by a CDK inhibitor in mammalian  
15 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  
20 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.

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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 is further comprises a second recombinant expression construct encoding a mammalian CDK inhibitor gene.

5 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  
10 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.

15 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.

20 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

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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.

5 38. A method for identifying a compound that inhibits CDK inhibitor-mediated induction of 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
- 10 (c) identifying the compound as an inhibitor of CDK inhibitor-mediated modulation of 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  
15 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.

20 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.

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44. The method of claim 38, wherein expression of the cellular gene is induced by p21.
45. The method of claim 38, wherein expression of the cellular gene is induced by p16.
- 5 46. The method of claim 38, wherein expression of the cellular gene is induced by p27.
47. The method of claim 38, wherein the cellular gene is identified in Table II.
48. The method of claim 40, wherein the cellular gene is identified in Table II.
- 10 49. The method of claim 38, wherein expression of the cellular gene is detected using an immunological reagent.
50. The method of claim 38, wherein expression of the cellular gene is detected by assaying for an activity of the cellular gene product.
- 15 51. The method of claim 38, where expression of the 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 cellular gene expression in a mammalian cell, the method comprising the steps of:
- 20 (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 genes that are induced by

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CDK inhibitor gene expression; and

(c) identifying the compound as an inhibitor of CDK inhibitor-mediated induction of 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.

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

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

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

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

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induction of 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.

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 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 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 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 cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 58.

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68. A method for inhibiting CDK inhibitor-mediated induction of cellular gene expression, the method comprising the step of contacting the cell with an effective amount of a compound that inhibits NF $\kappa$ B 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 NF $\kappa$ B 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 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 genes that are induced by CDK inhibitor gene expression; and

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(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.

5 77. A compound that inhibits production of 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;

10

(b) assaying the mammalian cell for induction of 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.

15

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.

20 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/CBP inhibitor.

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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.

5

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.

10

84. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering an NF- $\kappa$ B inhibitor to an animal in need of such treatment.

85. A method of claim 84, wherein the NF- $\kappa$ B inhibitor is a non-steroidal anti-inflammatory compound.

15

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

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

20

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

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

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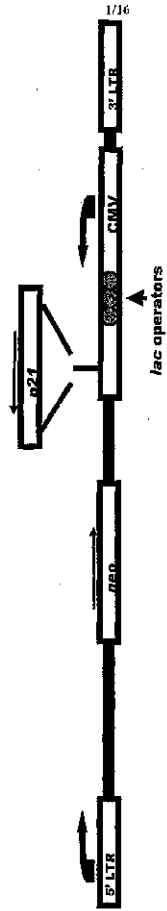
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90. The method of claim 89, wherein the animal is a human.
91. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 52.
- 5 92. The method of claim 91, wherein the animal is a human.
93. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 53.
94. The method of claim 93, wherein the animal is a human.
- 10 95. A method for selectively inhibiting induction of 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 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.
- 15 98. The method of claim 97, wherein the animal is a human.
99. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound according to claim 77.
- 20 100. The method of claim 99, wherein the animal is a human.

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Figure 1. IPTG-regulated retroviral vector LNp21C03



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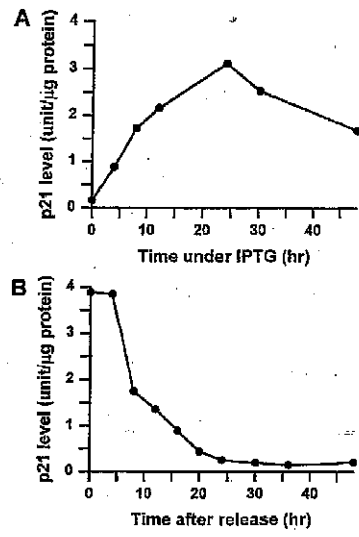
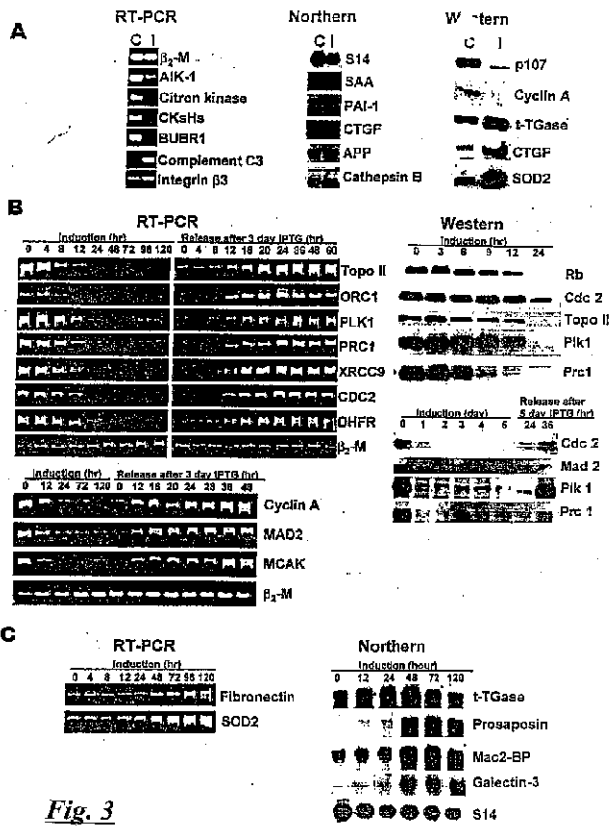


Fig. 2

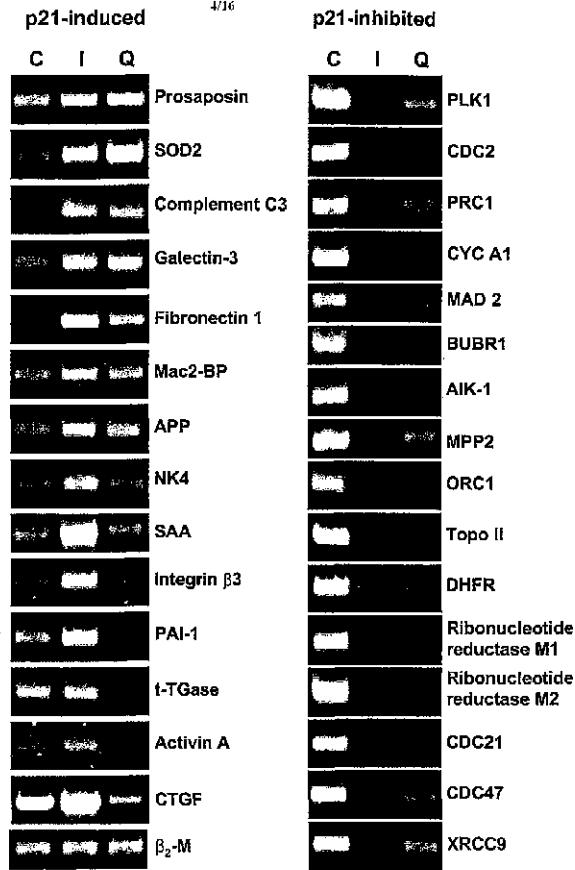


*Fig. 3*

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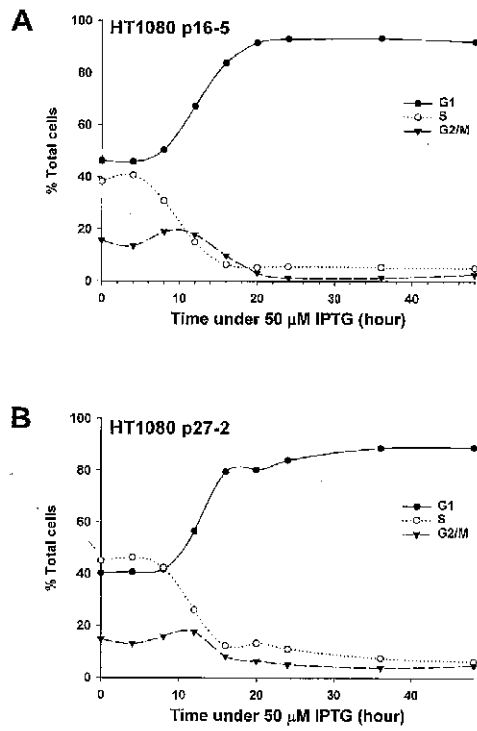
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C, control untreated cells; I, IPTG-treated, Q, serum-starved (quiescent) cells

Fig. 3D



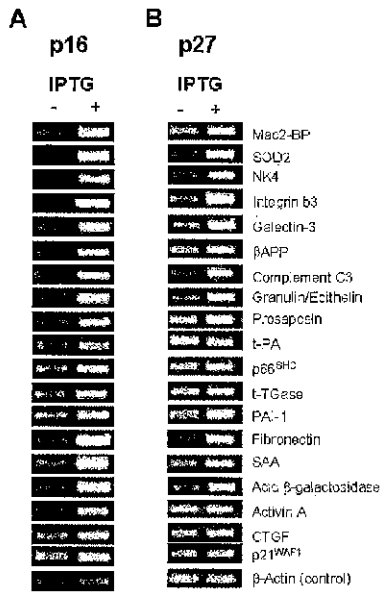


*Fig. 5*

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**Fig. 6**

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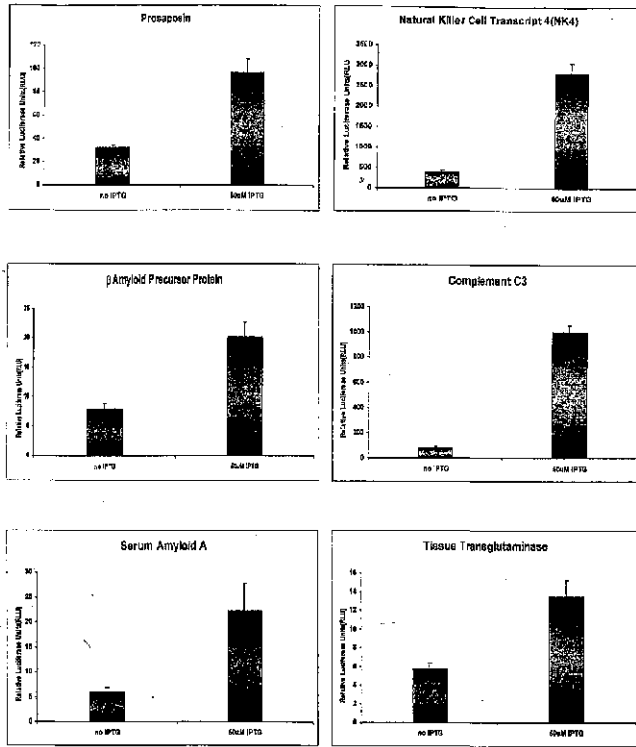


Figure 7

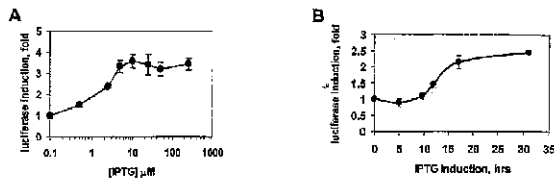
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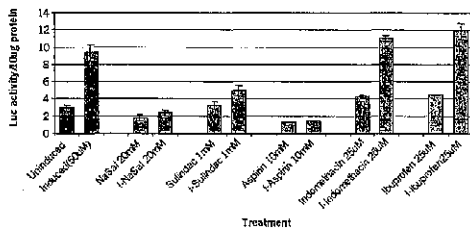
**Figure 8**

IPTG dose-dependence (24 hrs treatment) (A) and time course (50  $\mu$ M 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  $\mu$ M IPTG

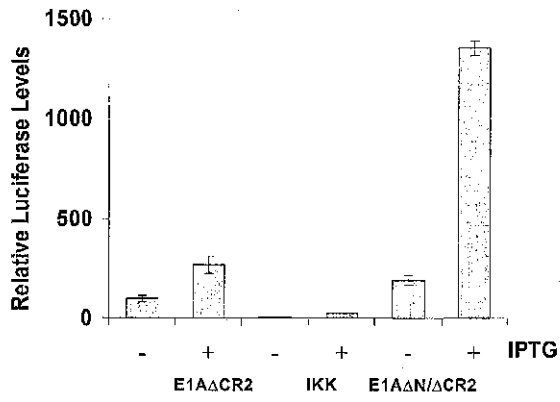


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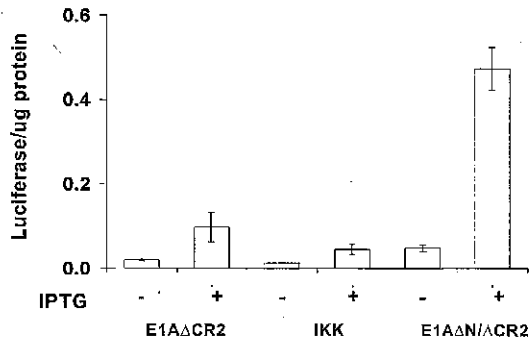
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### B. Serum Amyloid A



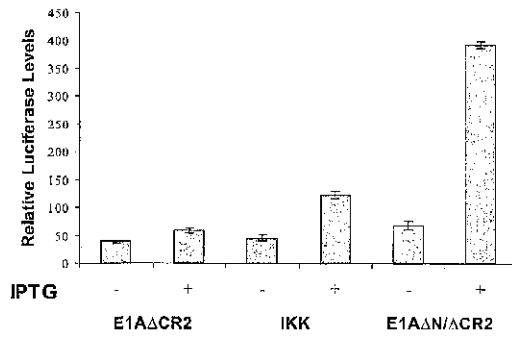
**Fig. 9**

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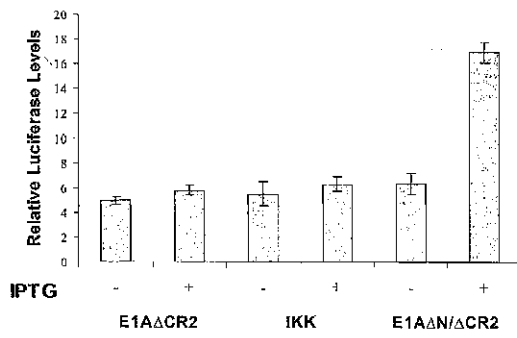
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### C. Prosaposin



### D. $\beta$ -Amyloid Precursor Protein



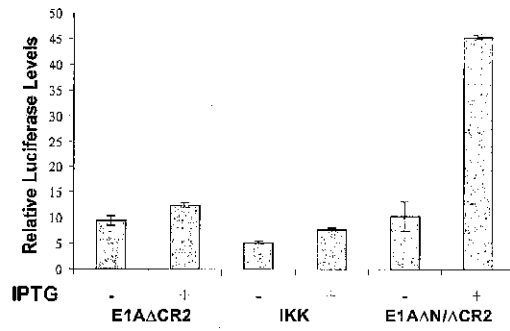
*Fig. 9.*

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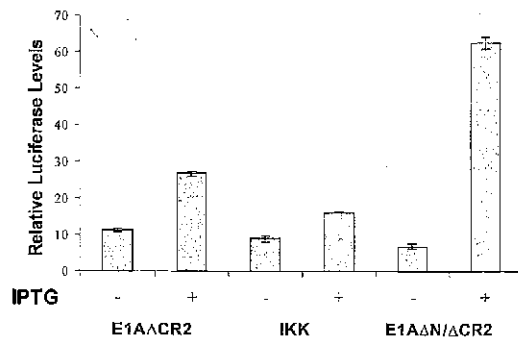
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### E. Tissue Transglutaminase

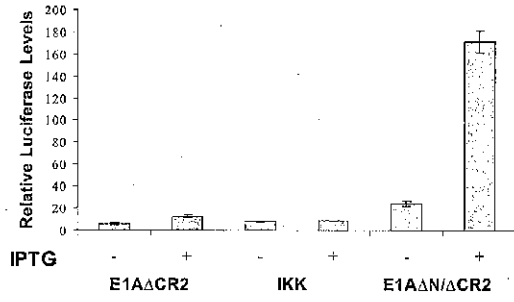


### F. Complement C3



*Fig. 9*

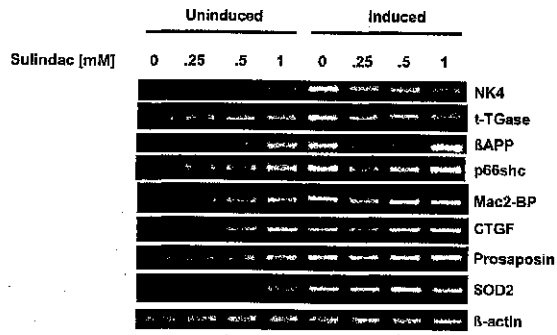
**G. NK Cell Transcript 4 (NK4)**



***Fig. 9***

**Figure 11**

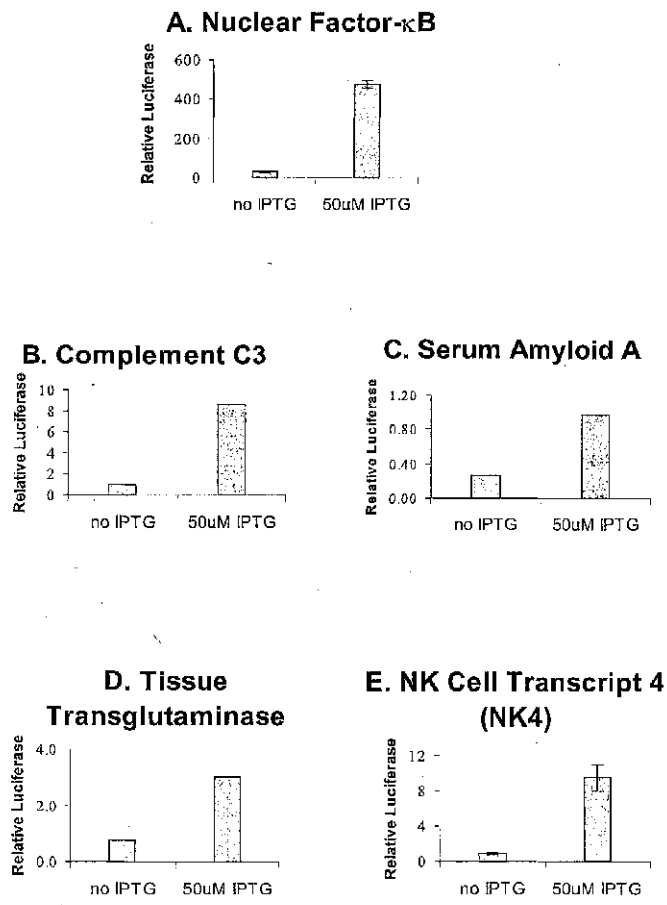
RT-PCR analysis of LuNK4p21 cells uninduced or induced with IPTG for two days in the presence of the indicated concentrations of Sulindac



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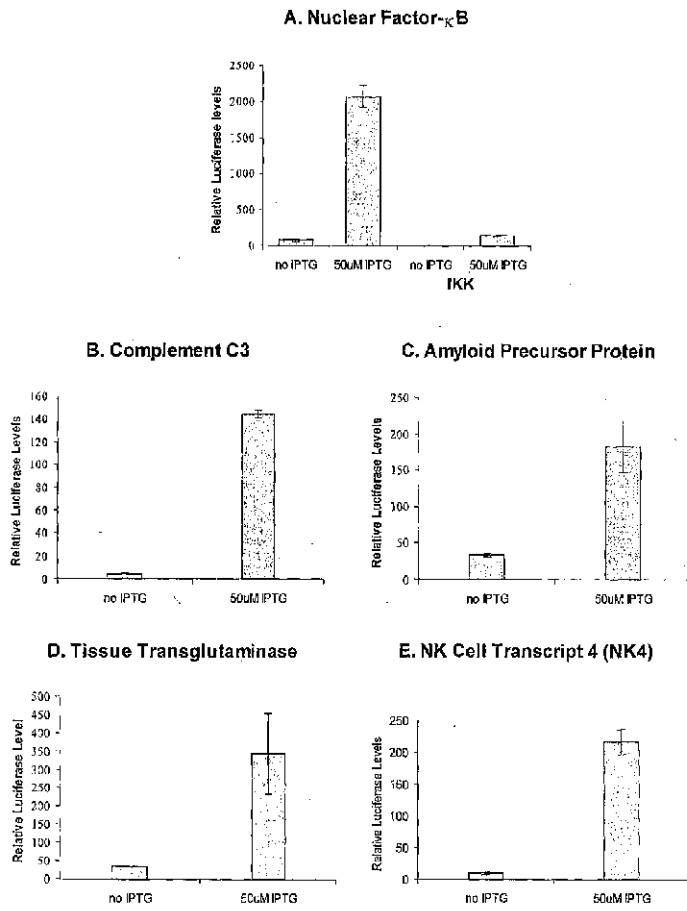


**Fig. 12**

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**Fig. 13**

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 Chang, Bey-Dia

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<210> 16
<211> 1741
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<222> Tissue: transglutaminase gene, promoter region and 5' UTR (AM: 013920)

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<100> 16
aaccttccac cagctggagg caccagtttc tgcacacata cctataaaa cggccaatta 60
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gtttgaccca gggagacaca tccactgaag caaacagggt tcttttccct gagccatctg 180
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gttttgaaa aagtgcgggc caagcccctg cggcctctct ctctccggga aacctccca 360
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atgaaaggat gagggaagg ataggagggg gggtaggtga tgcattgggtg agtggatgga 660
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atcggagggat taatcgggtt tgcatacaqa agggacatgg gttgtgtctgt ctttggaggt 780
ggtctctgat gctgtacct gactccatgc ctgcattgtt gctaacctt gactgagcau 840
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g 1741

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<210> 17
<211> 1440
<212> DNA
<213> Homo sapiens
<214>
<215> misc_feature
<216> Clusterin (APC3, SGP-2, SP-40, CRPM-2) protein gene (AM: M63376)

```

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4495 17
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cctcaggcca aactgattag tctcaggcca cctcctctgt agggctcaca ctgactctt 120
atctaacctt tctcaggcca gctcctctgt cctccactta gctcaggcca ttgactctt 180
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gggactctgt ctctctctgt tctcaggcca gctcctctgt tctcaggcca 300

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agaagtact gcaactccag ggttctaga ttgcccctc ctgcccggg ggagggytgg 540
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<210> 18
<211> 2000
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<222> Prothymosin stimulating factor (PSF, IGFBP-7, mac25) gene, 5' up
stream (2.0 kb) region (AN: AC022483)
<400> 18
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gctctctcc gctctctcc caaacctcc ggtctctcc tctctctcc tctctctcc 240
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<210> 19  
 <211> 1127

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<213> DNA
<213> Homo sapiens

<220>
<221> misc feature
<223> Vascular endothelial growth factor C gene, partial cds and 5' up
stream region (AM: AF020333)

<400> 19
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<210> 20
<211> 800
<213> DNA
<213> Homo sapiens

<220>
<221> misc feature
<223> TIMP-1 (tissue inhibitor of metalloproteinases-1) gene, promoter
region (AM: U26574)

<400> 20
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tgcctggata tcaatagatg ctgtataata tgcggctat ttgcctgtgt tatttgagac 120
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gatctctgggt aagtcactta cccactccgt gcttcagttt ccccgatctt gctttctctc 240
cccttcaact gactatctc cctccactga tgcactaa ttgtttctct ctctgcctcc 300
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gagagacacc agaggtacag 800

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

<220>
<221> misc_feature
<222> Sense PCR primer for CCS promoter (spec Table IIIa)

<400> 21
gctaaaggga tattgacat agacagg 27

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

<220>
<221> misc_feature
<222> AntiSense PCR primer for CCS promoter (spec Table IIIa)

<400> 22
agggggaggg gggttactag 20

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

<220>
<221> misc_feature<222> Sense primer for NK4 promoter (Table IIIa)

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<400> 23
ggagcctaga agagcctgta 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 caaggagca 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
cagagllgat gatatyfca 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
caactcttgh gctctctca cc                22

<210> 27
<211> 20
<212> DNA
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<220>
<221> misc_feature
<223> Sense primer for beta APP promoter (Table IIIa)

<400> 27
ttgcctcttt ggtctggttt                26

<210> 28
<211> 19
<212> DNA
<213> Homo sapiens

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

<400> 28
gctgdcgagg aaatggac                               18

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

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

<400> 29
ccccgggggg aatatccact gaagcaac                     28

<210> 30
<211> 28
<212> DNA
<213> Homo sapiens

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

<400> 30
tcgggggggg gagtggtct cttccact                       28

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

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

<400> 31
gcttctctag ctactactt cctaa                          25

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

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

<400> 32
cggggggggc cagyaag                                     18

<210> 33

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<210> 31
<211> 33
<212> DNA
<213> Homo sapiens

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

<400> 33
gatttggtatt ggcctcaaca g 31

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

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

<400> 34
caagcaacagc cgcctccaga 18

<210> 35
<211> 24
<212> DNA
<213> Homo sapiens

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

<400> 35
tgattccaat gttttcttaa aagg 24

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

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

<400> 36
gaattctctaa agagctcaga agt 23

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

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

<400> 37

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<210> 24
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<213> Homo sapiens

<220>
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<223> Antisense primer for prorepsin promoter

<400> 38
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<210> 39
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<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
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<210> 59
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<400> 59
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<210> 60
<211> 21
<212> DNA
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<400> 60
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<210> 61
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<213> Homo sapiens
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<210> 70
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<210> 73
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<223> Analytical antisense primer for beta-actin

<400> 77
tgtgtttggc tacaggctt tg                22
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>21, 27, 29, 50, 78</u> , line <u>18, 5, 4, 17, 4</u> (respectively).	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depository institution AMERICAN TYPE CULTURE COLLECTION	
Address of depository institution (including postal code and country) 10801 UNIVERSITY BOULEVARD MANASSAS, VIRGINIA 20110-2203 UNITED STATES OF AMERICA	
Date of deposit 31 January 2002 (31.01.02)	Accession Number: PTA-4020
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
For receiving Office use only <input type="checkbox"/> This sheet was received with the international application	For International Bureau use only <input checked="" type="checkbox"/> This sheet was received by the International Bureau on: <u>19-JUNE-2002</u> Authorized officer <u>HOMERO HERANDEZ</u>
Authorized officer	

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**INDICATIONS RELATING TO A DEPOSITED MICROORGANISM**

(PCT Rule 154a)

A. The indications made below relate to the microorganism referred to in the description on page <u>22, 27, 29, 51, 76</u> , line <u>5, 10, 6, 2, 8</u> (respectively)	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depository institution AMERICAN TYPE CULTURE COLLECTION	
Address of depository institution (including postal code and country) 10801 UNIVERSITY BOULEVARD MANASSAS, VIRGINIA 20110-2208 UNITED STATES OF AMERICA	
Date of deposit 31 January 2002 (31.01.02)	Accession Number PTA-4021
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is contained on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
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<input type="checkbox"/> For receiving Office use only This sheet was received with the international application	<input type="checkbox"/> For International Bureau use only This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

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- (74) Agent: NOONAN, Kevin, E.; McDonnell Boehnen Lihbert & Baggett, 300 South Wacker Drive, Chicago, IL 60606 (US).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GR, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SF, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
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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 cancer and age-related diseases, such genes being induced by cyclin-dependent kinase inhibitors.



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## 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 Stielecki 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<sup>Waf1/Cip1/Ink4a</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup>. The second family, Ink4, includes p16<sup>Ink4a</sup>, p15<sup>Ink4b</sup>, p18<sup>Ink4c</sup>, and p19<sup>Ink4d</sup>. Expression of specific CDK inhibitors is activated by different factors. For example, contact inhibition induces p27 and p16 expression (Dietrich *et al.*, 1991, *Oncogene 15: 2723-2747*), extracellular anti-mitogenic factors such as TGF $\beta$  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, *EBM Rev. Cancer 1371: 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

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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).

5 p21 has been independently identified in the rat 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.* 53: 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  
10 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  
15 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 Garai & Tyner, 1999, *Exp. Cell Res.* 227: 171-181).

Transient induction of p21 mediates different forms of damage-induced growth  
20 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.*,  
25 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.*,

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1994, *ibid.*; Alcortas *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 (Dutta, 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 result of CDK inhibition by p21 is stimulation of the transcription cofactor histone acetyltransferase p300, that enhances many inducible transcription factors including NFkB (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 (Dutta, 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<sup>INK4</sup>, 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

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well (McConnell *et al.*, 1999, *Molec. Cell Biol.* **19**: 1981-1989).

Still another CDK inhibitor of particular relevance to the present invention is p27<sup>Kip1</sup>. p27 was initially identified as an inhibitor of CDK2 in cells that had been growth arrested by contact inhibition, TGF- $\beta$  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 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, such as carcinogenesis 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

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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, 5 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 10 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 15 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.

20 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 25 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; however,

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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  $\beta$ -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), granulins/epithelins (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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). 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

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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. 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

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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 IIT1080 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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -galactosidase, alkaline phosphatase and green

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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  
5 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-  
10 apoptotic factors is inhibited are fibroblasts, most preferably stromal fibroblasts. In preferred embodiments, the compounds are inhibitors of nuclear factor  $\kappa$ B (NF $\kappa$ B) activity or expression.

In a third aspect, the invention provides methods for identifying compounds that inhibit CDK inhibitor-mediated induction of cellular gene expression. These methods  
15 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  
20 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  
25 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

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11. 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. 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  $\beta$ -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), granulins/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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).

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

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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  
5 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  
10 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  
15 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 modulated by a CDK inhibitor. In these embodiments, production of the product of the reporter gene at lesser levels in the presence than the  
20 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. The promoter most preferably is  
25 derived from serum amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), connective tissue growth factor (SEQ ID NO: 3), integrin  $\beta$ -3 (SEQ ID NO: 4), activin A

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(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), granulisin/epithelin (SEQ ID NO: 11), p66<sup>hhv</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15),  
5 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). 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  
10 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  
15 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.

20 In a fifth 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.

25 In a sixth aspect, the invention provides compounds that are identified using any of the methods of the invention as disclosed herein.

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In a seventh 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 NF- $\kappa$ B inhibitors.

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

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the IPTG-regulated retroviral vector L.Np21CO3 used to produce the human HT1080 fibrosarcoma cell line variant HT1080 p21-9.

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

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

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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  $\mu$ M IPTG.  $\beta$ 2-microglobulin ( $\beta$ 2-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  $\mu$ M IPTG.

Figures 6A and 6B are photographs of gel electrophoresis patterns of RT-PCR

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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  $\mu$ M IPTG.  $\beta$ -actin was used as a normalization control for RT-PCR.

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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  $\mu$ M IPTG. The assays were carried out in triplicate (for prosaposin) or in quadruplicate (for all the other constructs).

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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  $\mu$ M IPTG (Figure 8B).

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Figures 9A through 9G illustrate the effects of p21 induction in HT1080 p21-9 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 9A) or by the promoters of the indicated p21-inducible genes (Figures 9B through 9G). The promoter-reporter constructs were mixed at a molar ratio 1:2 with vectors expressing a dominant inhibitor of NF $\kappa$ B (IKK), C-truncated E1A mutant that inhibits p300/CBP (E1A $\Delta$ CR2), or non-functional N- and C-truncated version of E1A (E1A $\Delta$ N/ $\Delta$ CR2). Luciferase levels were measured after 3 days in the presence or absence of IPTG and normalized either by the levels of Renilla luciferase expressed from the co-transfected pRL-CMV plasmid or (in Fig. 9C) by the level of cellular

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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 NSA(1)s.

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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;  $\beta$ -actin was used as a normalization control for RT-PCR.

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Figures 12A through 12E illustrate the effects of p16 induction in HT1080 p16-5 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 12A) or by the promoters of the indicated p21-inducible genes (Figures 12B through 12E). 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. The experiments in Fig. 12A and Fig. 12E were carried out in triplicates, and in Figs. 12B, 12C and 12D in single points.

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Figures 13A through 13E illustrate the effects of p27 induction in HT1080 p27-2 cells on the expression of luciferase reporter genes driven by the NF $\kappa$ B-dependent promoter (Figure 13A) or by the promoters of the indicated p21-inducible genes (Figures 13B through 13E). In Fig. 13A, the promoter-reporter construct was mixed at a molar ratio 1:2 with a vector expressing a dominant inhibitor of NF $\kappa$ B (IKK). 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.

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**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

This invention provides reagents and methods for identifying genes involved in mediating CDK inhibitor-induced cellular senescence and pathogenic consequences of senescence, and compounds capable of inhibiting senescence and pathogenic consequences of senescence in mammalian cells. Particularly provided are 5  
embodiments of such reagents and methods for identifying genes involved in cellular senescence and 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. 10

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. 15

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. 20

As used herein, the term "conditioned media" is intended to encompass cell culture media conditioned by growth of CDK inhibitor-expressing cells that contains 25

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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.* 22: 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,

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*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.* 2: 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.

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

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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  
5 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  
10 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  
15 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. \_\_\_\_\_.

In alternative preferred embodiments, the invention provides mammalian cells  
20 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\_004053 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  
25 rodent or primate cells, and more preferably mouse or human cells. Particularly preferred embodiments are fibrosarcoma cells, more preferably human fibrosarcoma

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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.

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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. (Davisor. & Elliott, ed.), Oxford University Press: New York, 1993.

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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

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known in the prior art, including heat shock promoters that 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 *lacI* repressor protein. In a preferred embodiment, the recombinant cell expresses the *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* 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 *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* repressor is encoded by the 3'SS recombinant expression construct (Stratagene). In alternative preferred embodiments, the recombinant cell expresses the *lacI* 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- $\beta$ -galactoside. In this preferred embodiment, the *lacI* repressor is encoded by the 3'SS recombinant expression construct (Stratagene).

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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. 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  $\beta$ -3 (SEQ ID NO: 4), actin 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), granulocyte protein (SEQ ID NO: 11), p66<sup>ink</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), cholesteryl (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). 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

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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/JNK4p21, deposited on May 17, 2001 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. PTA-3381.

5 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  
10 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  
15 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  $\beta$ -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),  
20 granuline/epithelin (SEQ ID NO: 11), p66<sup>lac</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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). In preferred embodiments of this aspect of the  
25 invention, the CDK inhibitor is p21, p16 or p27.

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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 HT1080 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

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human HT1080 fibrosarcoma derivative identified as HT1080/L.Np16R02, 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 HT1080 fibrosarcoma cell line derivative identified as HT1080 p16-5, deposited on \_\_\_\_\_ with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. \_\_\_\_\_.

Another exemplary cell line according to this particularly preferred embodiment of the invention is an HT1080 fibrosarcoma cell line derivative identified as HT1080 p27-2, deposited on \_\_\_\_\_ with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. \_\_\_\_\_.

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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -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

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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 5 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 10 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 15 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 20 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 25 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

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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. \_\_\_\_\_) are used in which p16 can be induced with IPTG, or HT1080 p27-2 cells  
5 (deposited on January 31, 2002 with the American Type Culture Collection, Manassas, Virginia U.S.A. under Accession No. \_\_\_\_\_) 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  
10 by adding IPTG to the culture media at a concentration of about 50 $\mu$ M. 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  
15 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,  
20 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.

25 In alternative embodiments, such compounds are identified independently of CDK inhibitor-directed experimental manipulation. In such assays, cells are treated to

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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. 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  $\beta$ -3 (SEQ ID NO: 4), actin 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), granulir/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), tissue transglutaminase (t-TGase; SEQ ID NO: 16), clusterin (SEQ ID NO: 17), proinflammatory 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). Preferred reporter genes include but are not limited to firefly luciferase, Renilla luciferase,  $\beta$ -galactosidase, alkaline phosphatase and green fluorescent protein, all of which are commercially available.

The invention also provides methods for identifying genes associated with cellular senescence and pathogenic consequences of senescence or that mediate the

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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

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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).

5 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 I 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  $\beta 3$  (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 (Loriz *et al.*, 1998, *Eur. J. Endocrinol.* 139: 487-492), prosaposin (Mathur *et al.*, 1994, *Biochem. Mol. Biol. Int.* 24: 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).

25 The most commonly used marker of cell senescence is the SA- $\beta$ -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-

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4818). SA- $\beta$ -gal was suggested to represent increased activity and altered localization of the lysosomal  $\beta$ -galactosidase (Dimri *et al.*, 1995, *ibid.*), and other studies have described elevated lysosome activities in senescent cells (Cristofalo & Kabakjian, 1975, *Mech. Aging Dev.* 4: 19-28). Five lysosomal enzymes appear in Table II, including N-acetylgalactosamine-6-sulfate sulfatase (GALNS), cathepsin B, acid  $\alpha$ -glucosidase, acid lipase A and lysosomal pepstatin-insensitive protease. p21 also upregulated genes for mitochondrial proteins SOD2, metaxin and 2,4-dienoyl-CoA reductase, which correlates with reports of different mitochondrial genes overexpressed in senescent cells (Doggett *et al.*, 1992, *Mech. Aging Dev.* 63: 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  $\beta$ -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 TGF $\alpha$ , 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; Nachthyal *et al.*, 1998, *Am. J. Pathol.* 152: 1199-1208). In addition, cathepsin

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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 (Sekic *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. Invest. Dermatol. Symp. Proc.* 2: 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-Douhji *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

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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.

5 Of special interest, p21 induced expression of p66<sup>shc</sup>, 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 Lienbeau, 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).  
25 Despite these results, this paracrine carcinogenic and tumor-stimulating activity of

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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.

5 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 p16. The tumor-promoting effect of stromal tissue was also shown in a mouse mammary carcinogenesis model to be induced by ionizing radiation (Barcellos-Ruff and  
10 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.

15 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  
20 age-dependent manner.

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  
25 the capacity to prevent, retard or reverse age-related diseases by their effects on CDK inhibitor-mediated induction of gene expression.

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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 (NF $\kappa$ B). It will be understood by those with skill in the art that NF $\kappa$ B 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 NF $\kappa$ B heterodimer; second, inhibiting translocation of NF $\kappa$ B from the cytoplasm to the nucleus, which can depend on inhibiting inactivation of I $\kappa$ B expression and/or activity in cells; and third, by inhibiting the activity of NF $\kappa$ B itself. This invention encompasses methods for inhibiting NF $\kappa$ B activity, and thereby inhibiting induction of genes by CDK inhibitors, in any and all of these ways. Examples of NF $\kappa$ B 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); carboxyalkenylbenzoquinone 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: EP657290); flurbiprofen, thalidomide, dexamethasone, pyrrolidine dithiocarbamate, dimethylfumarate, mesalazine, piroxicam, sulfasalazine, methyl chlorogenate, chloromethylketone, alpha-tocopherol succinate, tepoxaline, and certain non-steroidal

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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**

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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- $\beta$ -thiogalactoside (IPTG). Expression of p21 can be induced by culturing these cells in the presence of a sufficient amount of IPTG, thereby permitting the sequence 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.

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Briefly, a subline of HT1080 expressing a murine ecotropic retrovirus receptor and a modified bacterial *lacI* 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 *lacI* repressor expressed in the cell. LNp21CO3 was constructed by

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cloning a 492bp fragment of DNA comprising the p21 coding sequence into the *NcoI* and *BglII* sites of the parent vector, LNXCO3 (disclosed in Chang & Robinson, *ibid.*).

After infection, cells infected with the L.Np21CO3X 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 L.Np21CO3 transduced, G418-resistant cell lines by end-point dilution until a clonal cell line was obtained.

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#### 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.

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p21 expression from the L.Np21CO3 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).

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Cell growth in the presence of IPTG was assayed in three ways: measuring <sup>3</sup>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

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cycle distribution").

<sup>3</sup>H-thymidine incorporation assays were performed substantially as described by Dinici *et al.* (1995, *Proc. Natl. Acad. Sci. USA* **92**: 9363-9367). Cells were cultured in the presence of <sup>3</sup>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 H11080 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.

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The effects of p21<sup>w</sup> induction on cellular gene expression were examined as follows.

Poly(A)<sup>+</sup> RNA was isolated from untreated HT1080 p21-9 cells and from cells that were treated for 3 days with 50  $\mu$ M IPTG. cDNA was prepared from the poly(A)<sup>+</sup> 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), CTFE (Fisp-12; a gift of Dr. L. Lau), Prc 1 (a gift of Drs. W. Jiang and T. Hunter), and topoisomerase II $\alpha$  (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).

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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).

i. 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 II $\alpha$ , ORC1, PLK1, PRC1 and XRCC9)

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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.

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## 2. Gene expression induced by p21

In addition to genes repressed by p21 expression, the assays described above  
5 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  
10 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.

15 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  $\alpha 2$ , Mac-2 binding protein), other  
secreted proteins (e.g. activin A, connective tissue growth factor, serum amyloid A), or  
20 ECM receptors (such as integrin  $\beta 3$ ). 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,  
25 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

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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.

5 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**

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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- $\beta$ -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  $\mu$ M IPTG. Genes that were completely inhibited in HT1080 p21-9 cells when the culture media contained 50  $\mu$ M IPTG were also inhibited in serum-starved cells, but most of these genes were inhibited to a lesser extent than in IPTG-treated cells.

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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

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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.

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

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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

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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 (Ohsu *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 50 $\mu$ M, and this media was replaced three days later with DMEM supplemented with 0.5% FCS and 50 $\mu$ M 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  $\mu$ Ci/ml was added and incubated for 24 hrs.

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Cells were then collected and their  $^3\text{H}$ -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  $^3\text{H}$ -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  $3 \times 10^3$  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

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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.

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**EXAMPLE 6**  
**Production of Mammalian Cell comprising**  
**Inducible p16<sup>INK4A</sup> or p27<sup>KIP1</sup> Genes**

Mammalian cell lines comprising inducible CDK inhibitors p16<sup>INK4A</sup> (which preferentially inhibits CDK4/6; Serrano *et al.*, *Nature* 16, 704-707, 1993) or p27<sup>KIP1</sup> (which preferentially inhibits CDK2; Blain *et al.*, *J.Biol. Chem.* 272, 25863-25872, 1997) 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 3'5S6; 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

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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 Kokonts *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<sup>SS</sup> 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 inhibitor 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 \_\_\_\_\_. 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

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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 \_\_\_\_\_. Figure 5B shows changes in the cell cycle distribution of HT1080 p27-2 cells upon the addition of 50  $\mu$ M IPTG. Cell cycle distribution stabilized after 24 hrs of IPTG treatment, by which time 89% of IPTG-treated cells were arrested in G<sub>1</sub>. Such G<sub>1</sub> 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**

10 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 15 50 $\mu$ M IPTG for three days. These RNA samples were then used in RT-PCR assays performed essentially as described above in Example 3, except that  $\beta$ -actin rather than  $\beta_2$ -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 20 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 25 Figure 6 also illustrate that p16 or p27 expression has no detected effect on p21 expression.

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**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-  
10 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  
15 A1093147) as adjacent to the 5' end of NK4 cDNA (Accession number M39807). 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  
20 (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  
25 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

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pCR2.1/TOPO (for SAA, CC3,  $\beta$ APP 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 HIT1080 p21-9 cells was carried out by electroporation, essentially as described in the Bio-Rad protocols. For each electroporation, HIT1080 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 a 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  $\mu$ l of cell suspension (approximately 7 to 8 million cells) was transferred to a 4 cm gap electroporation cuvette (Bio-Rad). 10-20  $\mu$ g of the promoter-luciferase construct was added to the cells. In some experiments, a control plasmid pCMVgal expressing bacterial  $\beta$ -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 Assay kit (Promega). Electroporations were

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performed using Bio-Rad Gene Pulser at 0.22 volts, with a capacitance extender set to 960 $\mu$ FID, providing a  $\tau$  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  $\mu$ M IPTG. 2 to 4 days later, cells were washed twice with phosphate-buffered saline and collected in 300  $\mu$ L of 1x Passive Lysis Buffer or Reporter Lysis Buffer (Promega). The lysate was centrifuged briefly at 10,000 g to pellet debris, and 50  $\mu$ L 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  $\beta$ APP, 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 (SEQ ID NO: 21)	AGGGGGAGGTGGGTTAGTAG (SEQ ID NO: 22)
NK4	TGGAGCTAGAAGAGCCCGTAGG (SEQ ID NO: 23)	GCCAAAAGTTCAAGGAGCCAA (SEQ ID NO: 24)
SAA	ICAGAGTTGCTGCTATGTCCACCA (SEQ ID NO: 25)	CACTCCTTGTGTGCTCCTCACC (SEQ ID NO: 26)
$\beta$ APP	TTGCTCCTTTGGTTCCGTCT (SEQ ID NO: 27)	GCTGCCGAGGAAAACCTGAC (SEQ ID NO: 28)

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t-TGase	CCGAGGGAGAAATATCCACTGAA GCAAC (SEQ ID NO: 29)	TCCGGCGGGGGCGGTGGCTCC TCCACT (SEQ ID NO: 30)
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Table III. 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	1090 bp
$\beta$ APP	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



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**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 H1080 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  $\mu$ g/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  $\mu$ M 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  $\mu$ M 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/ $\mu$ g 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 H1080 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  $\mu$ M IPTG. This analysis shows that

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most of the induction can be achieved using as little as 5  $\mu$ M 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 NF $\kappa$ B 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 NF $\kappa$ B binding sites.

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Several p21-induced genes are known to be positively regulated by NF $\kappa$ B, including superoxide dismutase 2 (SOD2) (Jones *et al.*, 1997, *Mol. Cell. Biol.* 17: 6970-6981), tTGase (Mirza *et al.*, 1997, *Amer. J. Physiol.* 272: G281-G288), Alzheimer's  $\beta$ -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 NF $\kappa$ B-dependent transcription (Perkins *et al.*, 1997, *Science* 275: 523-527). 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 NF $\kappa$ B 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 NF $\kappa$ B, we have used transient transfection assays to investigate the effect of p21 induction on luciferase expression from the plasmid pNF $\kappa$ B-Luc, commercially available from Stratagene. This plasmid expresses firefly luciferase from an artificial promoter containing five NF $\kappa$ B consensus sequences. To evaluate the effects of genetic inhibitors of NF $\kappa$ B on luciferase expression from pNF $\kappa$ B-Luc, 20  $\mu$ g of the latter plasmid were mixed (at a molar ratio 1:2) with a plasmid MA103 (a.k.a. pRC/pactin-HA-IKK $\alpha$ ) that expresses a dominant mutant of I $\kappa$ B kinase  $\alpha$  that selectively inhibits NF $\kappa$ B (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 pNF $\kappa$ B-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

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deletion ( $\Delta$ CR2 (120-140)). The C-truncated E1A (termed E1A $\Delta$ CR2) 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: 393-405). 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 ( $\Delta$ N(2-36)), termed E1A $\Delta$ N/ $\Delta$ CR2. The E1A $\Delta$ CR2 and E1A $\Delta$ N/ $\Delta$ CR2 constructs were provided by Dr. V. Ogryzko (NICHD, NIH). The mixtures of pNFkB-Luc with IKK, E1A $\Delta$ CR2 or E1A $\Delta$ N/ $\Delta$ CR2 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  $\mu$ M 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 9A. pNFkB-Luc mixed with the negative control (E1A $\Delta$ N/ $\Delta$ CR2) showed up to 15-fold induction in the presence of IPTG, demonstrating an increase in NFkB 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. E1A $\Delta$ CR2 had a similar but weaker effect than IKK, suggesting the requirement of p300/CBP for NFkB activity in HT1080 p21-9 cells (Fig. 9A).

The same analysis was carried out using promoter-luciferase constructs for six p21-inducible genes. The results for SAA are shown in Figure 9B, for prosepisin in Figure 9C, for  $\beta$ AAPP in Figure 9D, for  $\alpha$ -TGase in Figure 9E, for complement C3 in Figure 9F, and for NK4 in Figure 9G. Both IKK and E1A $\Delta$ CR2 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 NFkB. Quantitatively,

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however, the effects of these inhibitors varied among the promoters. Both basal and IPTG-stimulated expression of the promoters of SAA (Fig. 9B) and NK4 (Fig. 9G) was inhibited by IKK and B2ACR2 almost as strongly as that of NF $\kappa$ B. In contrast, these inhibitors had little or no effect on the basal expression from the promoters of prosaposin (Fig. 9C),  $\beta$ APP (Fig. 9D), t-Tase (Fig. 9E), or complement C3 (Fig. 9F), but interfered with the induction of these promoters in the presence of IPTG. These results indicate that p300/CBP and NF $\kappa$ B are involved in the induction of all the tested promoters by p21.

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

The best-studied NF $\kappa$ B 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 NF $\kappa$ B-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  $\mu$ M 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 NF $\kappa$ B: indomethacin and ibuprofen (at 25  $\mu$ M 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.*).

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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 NF $\kappa$ B-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  $\mu$ M 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  $\mu$ M, 500  $\mu$ M 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  $\beta$ -actin rather than  $\beta_2$ -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  $\beta$ -TGase RNA.

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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  $\mu$ M sulindac for the APP gene, but only by 250  $\mu$ M sulindac for p66<sup>Shc</sup>, 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  $\mu$ M) inhibits the ability of p21 to induce transcription for most of the tested genes.

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## A B O U T E L E M E N T S

GENE	SENSE PRIMER (5'→3')	ANTISENSE PRIMER (5'→3')
NK4	AGCACCAGGCCATAGAAAGA (SEQ ID NO: 13)	GGTGTGAGCTCCTCCTTGTC (SEQ ID NO: 49)
UTGASE	ACTACAACTCGGCCCATGAC (SEQ ID NO: 50)	GCCAGTTTGTTCAGGTGGTT (SEQ ID NO: 51)
BAPP	CTCGTTCCYGACAAGTGCAA (SEQ ID NO: 62)	TGTTGAGAGCACACCTCTCG (SEQ ID NO: 63)
P66 <sup>SHC</sup>	GAGGGTGTGGTTCGGACTAA (SEQ ID NO: 64)	GCCCAGAGGTGTGATTGTGTT (SEQ ID NO: 65)
CTGF	GGAGAGTCCITCCAGAGCAG (SEQ ID NO: 66)	ATGTCTTCATGCTGGTGCAG (SEQ ID NO: 67)
MAC2-BP	ACCATGAGTGTGGATGCTGA (SEQ ID NO: 68)	ACAGGGACAGGTGAACTGC (SEQ ID NO: 69)
GRANULIN	ACCACGGACCTCCTCACTAA (SEQ ID NO: 70)	ACACTGCCCTCAGCTACAC (SEQ ID NO: 71)
PROSAPOSIN	CCAGAGCTGGACATGACTGA (SEQ ID NO: 72)	GTCACCTCCTCACCAGGAA (SEQ ID NO: 73)
SOD2	CAAATTGCTGCTTGTCCAAA (SEQ ID NO: 74)	CAATCCGTACAAGTCCCAAAA (SEQ ID NO: 75)
B-ACTIN	GGGAAATCGTGCGTGACATTAA (SEQ ID NO: 76)	TGTGTTGGCGTACAGGTCCTTG (SEQ ID NO: 77)

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TABLE 1: PCR temperatures (in °C.)

Gene	Denaturation	Annealing	Extension	Cycles	Product size
NK1	94	58	72	24	481
t-TGase	94	58	72	24	499
B-AIP	94	58	72	20	500
p66 <sup>shc</sup>	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
$\beta$ -actin	94	60	72	17	275

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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 NF $\kappa$ B 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 NF $\kappa$ B-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 (Broegden *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 (Pernisi,

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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 NF $\kappa$ B-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 NF $\kappa$ B 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<sup>INK4A</sup> and p27<sup>KIP1</sup>. To determine if the promoters of p21-inducible genes are stimulated by the latter CDK inhibitors, pNF $\kappa$ B-Luc and several of the promoter-luciferase constructs described in Example 8 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. The effect of IPTG on the expression of these promoters was then analyzed as described for the p21-inducible line in Example 8.

NF $\kappa$ B-dependent expression from pNF $\kappa$ B-Luc was strongly stimulated by the induction of either p16 (Fig. 12A) or p27 (Fig. 13A). In the case of p27, the specificity of the observed induction for NF $\kappa$ B was also demonstrated by cotransfection with the IKK inhibitor, which strongly inhibits both basal and IPTG-induced expression (Fig. 13A). These results demonstrate that these CDK inhibitors, like p21, stimulate NF $\kappa$ B activity. Furthermore, all the tested promoters of p21-inducible genes were also upregulated by p16 or p27. In particular, IPTG-induced p16 expression led to the induction of reporter expression from the promoters of Complement C3 (Fig. 12B), SAA

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(Fig. 12C), t-TGase (Fig. 12D) and NK4 (Fig. 12E). IPFG-induced p27 expression strongly induced the promoters of Complement C3 (Fig. 13B), BAPP (Fig. 13C), t-TGase (Fig. 13D) and NK4 (Fig. 13E). These findings indicate that p21-inducible promoters are activated not only by p21 but also by other CDK inhibitors, such as p16 and p27.

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.

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Table I

## Genes downregulated by p21 induction

## A. p21-inhibited genes identified by UniGemV array:

Genes	Accession No.	Balanced Diff. Error	Confirmed by <sup>1</sup>
<b>Associated with mitosis:</b>			
CDC2	X05360	2.5	R, W
CKsHs1 (CDC2 kinase)	X54941	5.5	R
PLK1 (pole-like kinase)	U01028	5.1	R, W
XCAP-H condensin homology	D38563	6	R
CENP-A (centromere protein A)	U14518	5.3	R
CENP-F (centromere protein F)	U30872	2.5	R
MAD2	U85410	6.6	R, W
BUBR1	AF053306	5.9	R
MCAK (mitotic centromere-associated kinesin)	U53743	3.6	R
HSET kinesin-like protein	AL021368	3.6	R
CHL1 helicase	U75960	1.3	R
AIK-1 (aurora/PL1-related kinase)	D84212	4.6	R
AIM-1 (AIK-2; aurora/PL1-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	R
Lamin B2	M94362	2.7	R
LAP2 (lamin-associated protein 2)	U18271	4.9	R
MPP2 (M phase phosphoprotein 2)	U74512	3.7	R
MPP5 (M phase phosphoprotein 5)	X98261	3.7	R
<b>Associated with DNA replication, segregation and chromatin assembly:</b>			
Thymidine kinase 1	K02581	2.9	R
Thymidylate synthase	X02308	3.9	R
Uridine phosphorylase	X60658	2.5	R
Ribonucleotide reductase M1	X55543	4.9	R
Ribonucleotide reductase M2	X49616	10.7	R
CDC47 homolog (MCM7)	D55716	9.8	R
CDC21 homolog (MCM4)	X74794	2.7	R
CDC45 homolog (Fov-Pi)	AJ223728	4.1	R
HsORC1 (origin recognition complex 1)	U40152	2.7	R
DNA polymerase $\alpha$	X06745	2.8	R
Replication factor C (37-kD subunit)	M87339	2.6	R
B-MYB	X13293	9.1	R
HPV16 E1 protein binding protein	U96131	3.7	R
Topoisomerase I $\alpha$	J04089	8.6	R
Chromatin assembly factor-1 (p65 subunit)	U20980	2.7	R
High-mobility group chromosomal protein 2	X52534	3.7	R
High-mobility group chromosomal protein 1	D53874	3.6	R
Histone H2A-F1Z variant	AA263494	2.8	R
<b>Associated with DNA repair:</b>			
XRCC9	U70310	3.6	R
RAD54 homolog	X87795	5.4	R
HEX1 5'-3' exonuclease (RAD2 homolog)	AF042282	5.2	R
ATP-dependent DNA ligase I	M3667	2.5	R
RAD21 homolog	D38551	2.9	R

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Table I

<i>Associated with transcription and RNA processing:</i>			
Pulative transcription factor CA150	AF017789	2.8	
Transcriptional coactivator ALY	AF047002	3.3	
WHSC1/MSSET (SET domain protein)	AA401245	2.9	
NNB-1AG (SET domain protein)	U60389	2.8	
EZH2 (enhancer of zeste homolog 2)	U61145	2.8	
PTB-associated splicing factor	X70044	2.5	
AU-rich element RNA-binding protein AUF1	U02019	2.3	
U-snRNP-associated cyclophilin	AF016371	2.8	
<i>Other genes:</i>			
3-phosphoglycerate dehydrogenase	AF006043	4.8	
L-type amino acid transporter, subunit LAT1	M60244	4.1	R
Hyaluronan-mediated motility receptor	U29343	4	
Phorbol 1 (PKC-inducible)	U03891	3.9	
PSD-95 binding family protein	D13633	3.7	R
HTRIP (TNF receptor component)	U77645	3.5	
NAD-dependent methyltetrahydrofolate dehydrogenase	X16366	3.4	
Membrane glycoprotein 4F2 antigen heavy chain	J02939	3.2	
Mucin-like protein	D78992	3.2	
MAC30 (differentially expressed in meningiomas)	L19183	2.9	
P52IPK (regulator of Interferon-induced protein kinase)	AF007293	2.8	
Pulative phosphoserine aminotransferase	AA132483	2.8	
Glucose 6-phosphate translocase	Y15409	2.7	
Calycalin binding protein	AF067356	2.6	
Ornithine decarboxylase 1	X16277	2.6	R
Trophinin assisting protein (tastin)	U04810	2.5	
Acyl-coenzyme A cholesterol acyltransferase	L21934	2.5	
PitrmrSDK3	Y10381	2.5	
<i>Genes with unknown function:</i>			
EST	AA975298	2.7	
EST	AA034414	2.5	
EST	AA402549	2.5	

**B. p21-inhibitor genes identified by RT-PCR:**

<u>Gene</u>	<u>Accession No.</u>	<u>UniGemV result<sup>a</sup></u>
Cyclin A1	U66830	IS
Cyclin B1	M25753	IS
CDC25A	NM_001739	A
Dihydrofolate reductase	J03140	1.5
ING1	NM_005637	A

\*Abbreviations: R, RT-PCR; W, western blotting

<sup>a</sup>Abbreviations: IS, insufficient signal; A, absent from the array

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Table II

Genes upregulated by p21 induction			
Genes	Accession No	Balanced Diff Expr	Confirmed by <sup>a</sup>
<i>Sacrosid proteins and proteins associated with extracellular matrix:</i>			
Fibronectin 1	X02761	5.7	R
Plasminogen activator inhibitor, type 1	M14883	3.7	R, N
Plasminogen activator, tissue type	M15518	2.8	Z
Laminin β2	X79683	2.1	
Desmoglein 2a/bb	X66807	3.5	
Podocalyxin-like protein	U97519	2	
Activin A (inhibin βA)	J03634	2	R
Galectin 3 (Mac-2)	AB008780	2.4	N
Muc-2 binding protein	L13210	2	R, N
Prospodin	J03077	2.9	N
CTGF (connective tissue growth factor)	M92934	3.3	N
Granulin/epithelin	AF066208	2.1	N
Cathepsin B	L04268	2.4	N
Tissue transglutaminase	M52153	2.5	R, N, W
PS7NB (alt. homolog)	U32907	2.1	
Serum amyloid A protein precursor	M26152	4	R, N, W
Alzheimer's disease amyloid A <sub>4</sub> protein precursor	D87676	2	R, N
Complement C3 precursor	K02765	5.9	R, N
Testican	X73808	2.1	N
Integrin β3	M35959	2.1	R, N
<i>Lysosomal proteins:</i>			
N-acetylgalactosamine-6-sulfate sulfatase	U09089	2.3	N
Acid alpha-glucosidase	X55079	2.4	N
Acid lipase A (cholesterol esterase)	X78495	2.1	N
Lysosomal peptidase-inactivator protease (CLN2)	AF017458	2.5	
<i>Mitochondrial proteins:</i>			
Superoxide dismutase 2	X07934	3.5	R, N, W
Metaxin	J03080	3.4	
2,4-dienoyl-CoA reductase	U78302	2	
<i>Other genes associated with stress response and signal transduction:</i>			
Ubiquitin-conjugating enzyme (UbcH8)	AF031141	2	
Ubiquitin-specific protease 8	D26956	2	
RTP/Cap43/Drg1/Net1 (inducible by nickel, retinoids, homocysteine and ER stress)	D87553	2.5	
C-153 muscle ankyrin-repeat nuclear protein (cytokine-inducible)	X83703	3	
LRP major vault protein associated with multidrug resistance	X79882	2.2	N
β-arrestin related Hic/CPA7B homolog (upregulated by vitamin D3)	S73591	4.1	N
R-RAS	M14949	2.4	
RAB 13 small GTPase	X75893	2.2	
P68 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	

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Table II

<i>Other genes:</i>			
Natural killer cells protein 4	M59807	4.4	R
TKK tyrosine kinase (T-cell specific)	L27071	3.8	
X-linked PEST-containing transporter	U05321	2.1	
AMP deaminase 2	M01029	2	N
FIP2/HYPL huntingtin-interacting protein	AF061034	2	
DNASE I homolog	X90362	2.5	N
Transcription factor 11	X77366	2	
Histone H2A.2	L19779	2.8	
Histone H2B	AL021807	2.4	
<i>Genes with unknown function:</i>			
23808	AF038152	2.1	
OGI-147	AA307912	2.1	N
EST	W89120	2.8	
EST	AI028140	2.5	
EST	AA218992	2.4	
EST	W83684	2	

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

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## WE CLAIM:

1. A recombinant expression construct encoding a reporter gene operably linked to a promoter from a mammalian gene induced by a cyclin-dependent kinase inhibitor.
- 5 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 gene induced by a CDK inhibitor.
- 10 4. A recombinant expression construct according to Claim 3, wherein the promoter is a promoter from a human gene identified in Table II
5. A recombinant expression construct according to Claim 4, 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  $\beta$ -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), granulim/epithelin (SEQ ID NO: 11), p66<sup>shc</sup> (SEQ ID NO: 12), cathepsin B (SEQ ID NO: 14),  $\beta$ -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).
- 20 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

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amyloid A (SEQ ID NO: 1), complement C3 (SEQ ID NO: 2), tissue transglutaminase (SEQ ID NO: 16),  $\beta$ -amyloid precursor protein (SEQ ID NO: 15), or prosaposin (SEQ ID NO: 7).

7. A recombinant expression construct according to claim 4, wherein the  
5 recombinant expression construct is pL.uNK4.

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 NF $\kappa$ B.

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.  
15

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.  
20

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

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- 15           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.
- 5           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  
10           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  
15           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.
- 20           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. HTA 1664 (HT1080 p21-9).

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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. \_\_\_\_\_ (HT1080 p16-5).
- 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. \_\_\_\_\_ (HT1080 p27-2).
- 10 27. The mammalian cell of claim 17, wherein the lactose repressor-specific inducing agent is a  $\beta$ -galactoside.
28. A method for identifying a compound that inhibits induction of 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 genes induced by a CDK inhibitor in mammalian cells in the presence and absence of a compound;
- 15 (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.
- 20 29. The method of Claim 28, wherein the cell is cultured under conditions that induce expression of a CDK inhibitor: in said cell.

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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 is further comprises a second recombinant expression construct encoding a mammalian CDK inhibitor gene.
- 5 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  
10 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.
- 15 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.
- 20 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

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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.

5 38. A method for identifying a compound that inhibits CDK inhibitor-mediated induction of 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

10 (c) identifying the compound as an inhibitor of CDK inhibitor-mediated modulation of 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  
15 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.

20 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.

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44. The method of claim 38, wherein expression of the cellular gene is induced by p21.
45. The method of claim 38, wherein expression of the cellular gene is induced by p16.
- 5 46. The method of claim 38, wherein expression of the cellular gene is induced by p27.
47. The method of claim 38, wherein the cellular gene is identified in Table II.
48. The method of claim 40, wherein the cellular gene is identified in Table II.
- 10 49. The method of claim 38, wherein expression of the cellular gene is detected using an immunological reagent.
50. The method of claim 38, wherein expression of the cellular gene is detected by assaying for an activity of the cellular gene product.
- 15 51. The method of claim 38, where expression of the 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 cellular gene expression in a mammalian cell, the method comprising the steps of:
- 20 (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 genes that are induced by

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CDK inhibitor gene expression; and

(e) identifying the compound as an inhibitor of CDK inhibitor-mediated induction of 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.

5

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.

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

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

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

15 58. A method for identifying a compound that inhibits CDK inhibitor-mediated induction of 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 gene whose expression is modulated by a CDK inhibitor;

20

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

(c) identifying the compound as an inhibitor of CDK inhibitor-mediated

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induction of 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.

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 cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 23.

65. A method for inhibiting CDK inhibitor-mediated induction of cellular gene expression, the method comprising the step of contacting the cell with a compound produced according to the method of claim 23.

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

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68. A method for inhibiting CDK inhibitor-mediated induction of cellular gene expression, the method comprising the step of contacting the cell with an effective amount of a compound that inhibits NF $\kappa$ B 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 NF $\kappa$ B 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 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 genes that are induced by CDK inhibitor gene expression; and

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- (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.
- 5 77. A compound that inhibits production of 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  
10 inhibitor;
- (b) assaying the mammalian cell for induction of genes that are induced by CDK inhibitor gene expression; and
- (c) identifying the compound as an inhibitor of CDK inhibitor induction if  
15 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.
- 20 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 NF- $\kappa$ B inhibitor or a p300/CIPB inhibitor.

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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  
5 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  
10 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-  
15 inflammatory compound.
86. The method of claim 85, wherein the animal is a human.
87. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 28.  
20
88. The method of claim 87, wherein the animal is a human.
89. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 38.

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90. The method of claim 89, wherein the animal is a human.
91. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound produced by the method of claim 52.
- 5 92. The method of claim 91, wherein the animal is a human.
93. A method for selectively inhibiting induction of 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.
- 10 95. A method for selectively inhibiting induction of 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 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.
- 15 98. The method of claim 97, wherein the animal is a human.
99. A method for selectively inhibiting induction of genes induced by a CDK inhibitor in an animal, comprising administering to the animal a compound according to claim 77.
- 20 100. The method of claim 99, wherein the animal is a human.

**IPTG-regulated retroviral vector LNp21CO3**

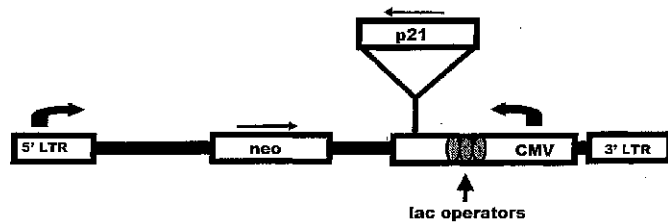


FIG. 1

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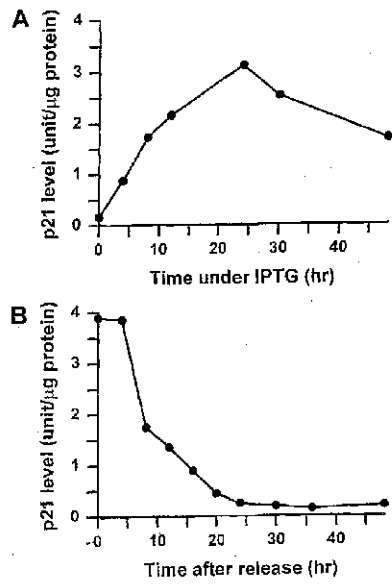
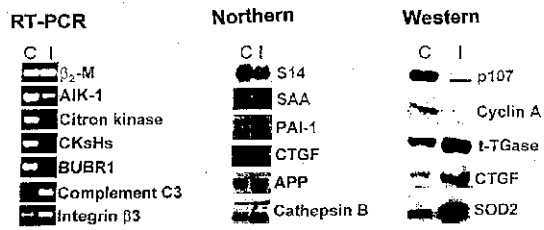


FIG. 2

FIG. 3A

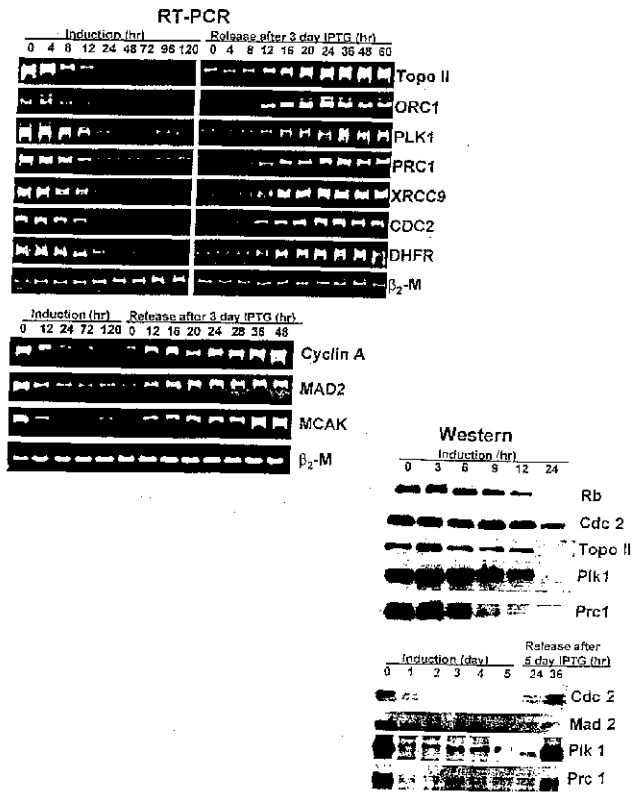


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FIG. 3B

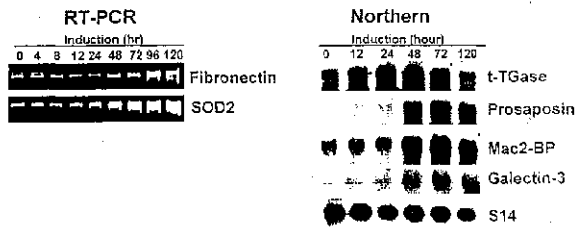


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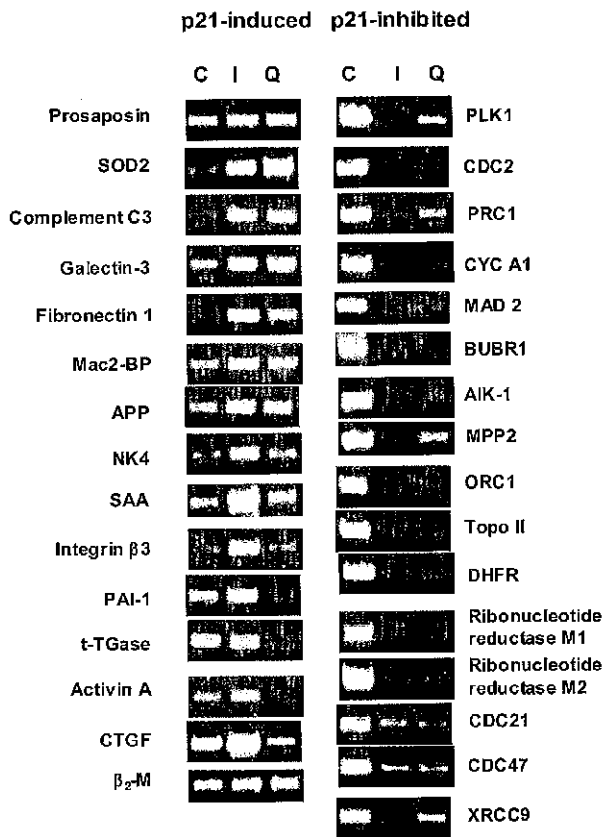
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FIG. 3C



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

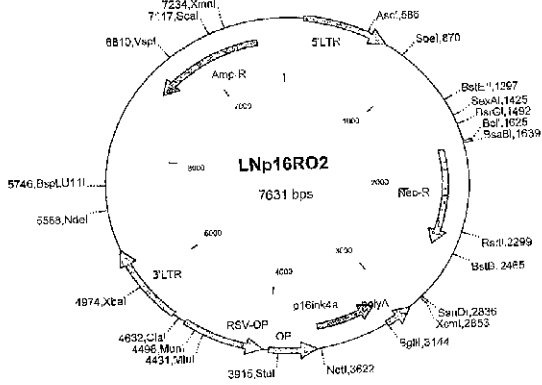
Fig. 3D

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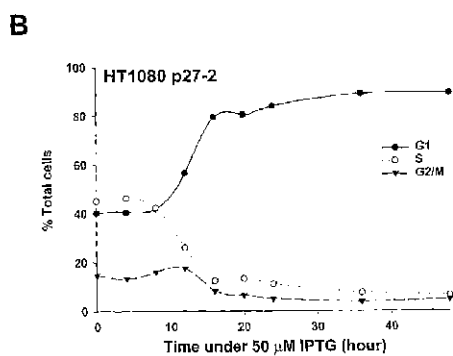
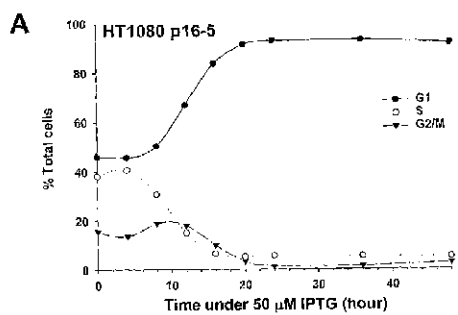
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FIG. 4



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FIG. 5



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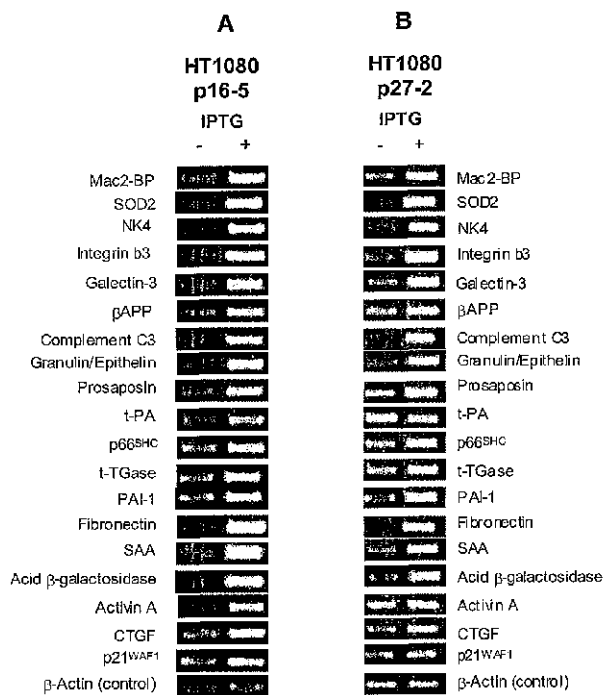
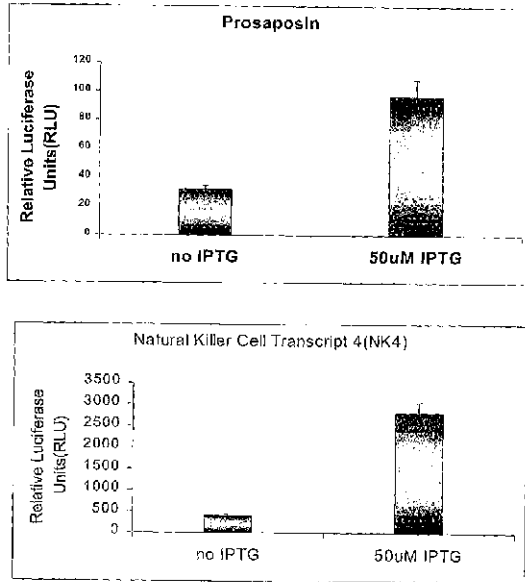


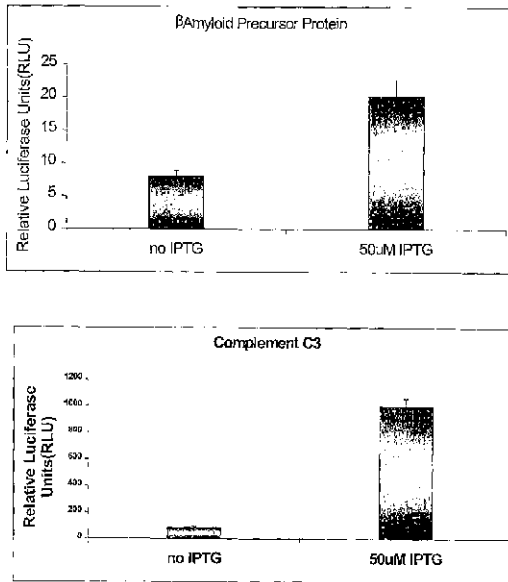
Fig. 6

FIG. 7A



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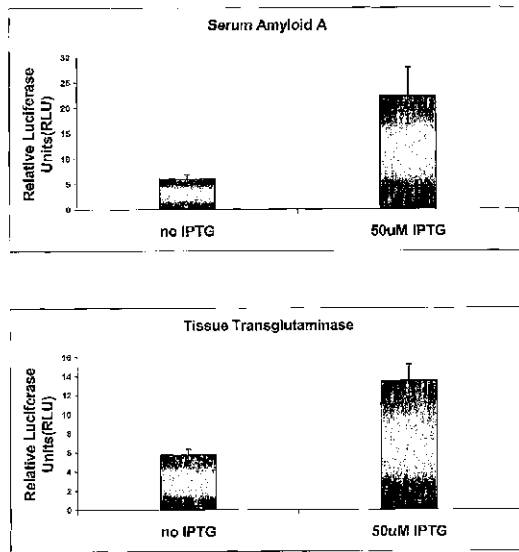
FIG. 7B



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FIG. 7C

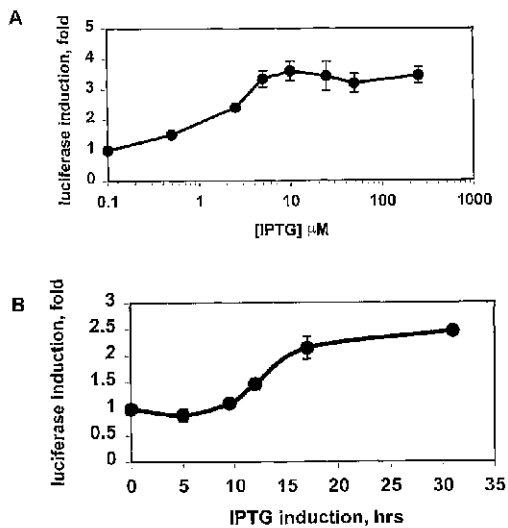


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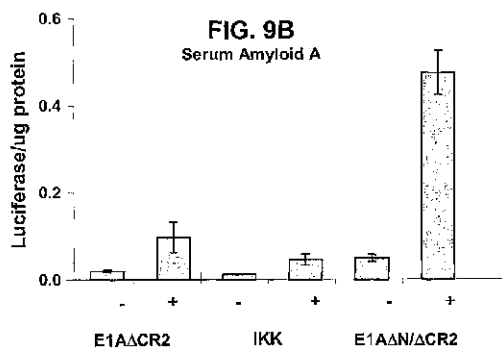
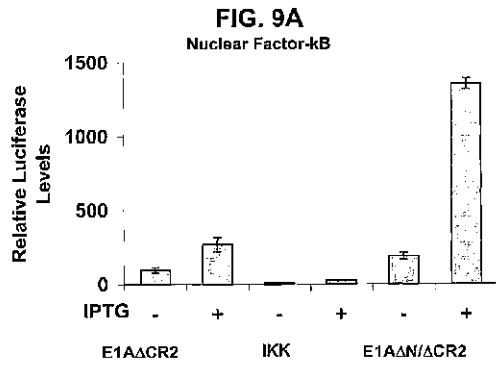
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FIG. 8



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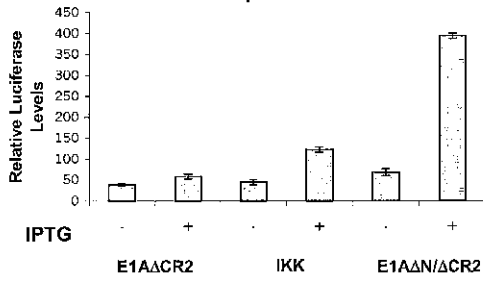
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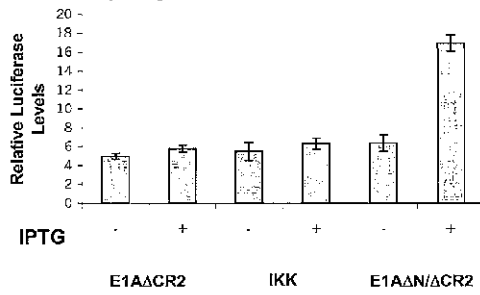
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**FIG. 9C**  
**Prosaposin**

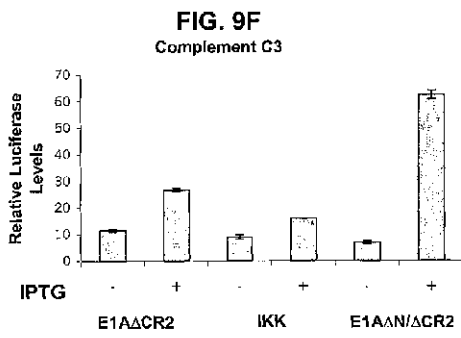
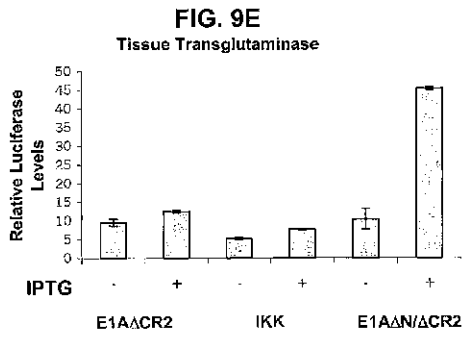


**FIG. 9D**  
**β-Amyloid Precursor Protein**



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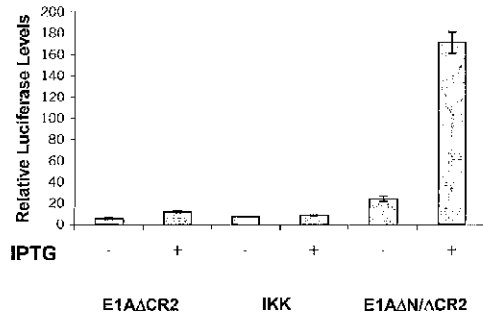


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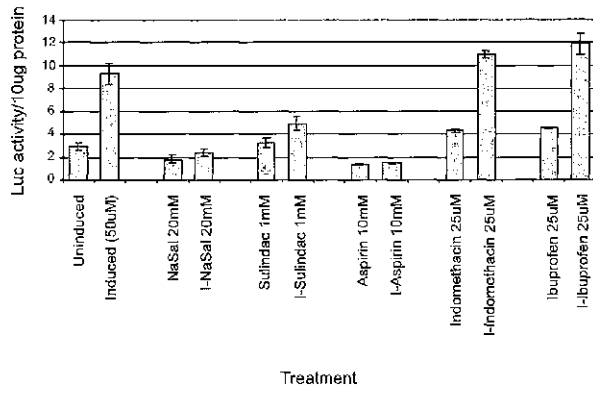
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**FIG 9G**  
**NK Cell Transcript 4 (NK4)**



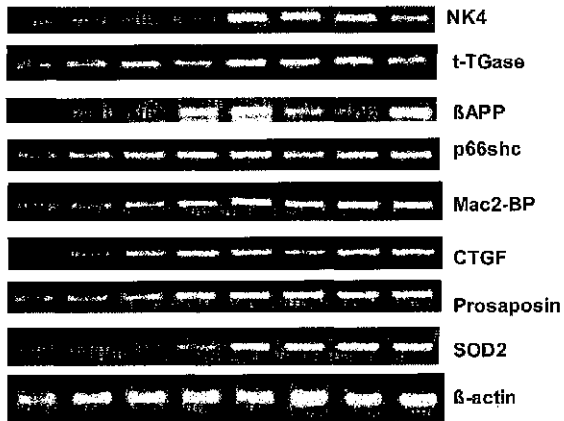
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FIG. 10



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

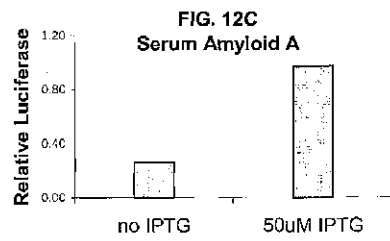
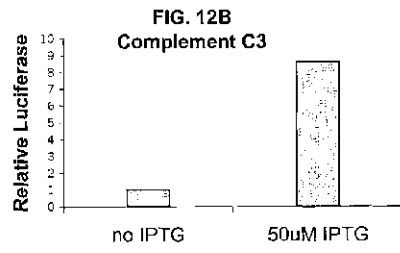
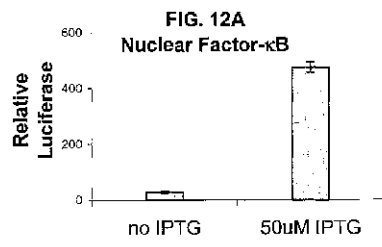
FIG. 11



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

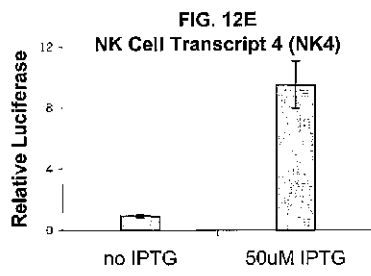
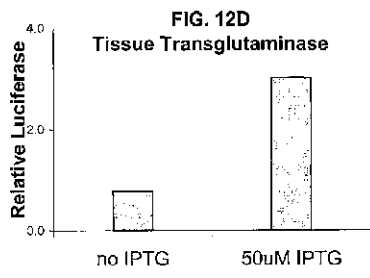
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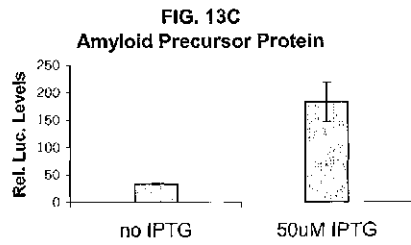
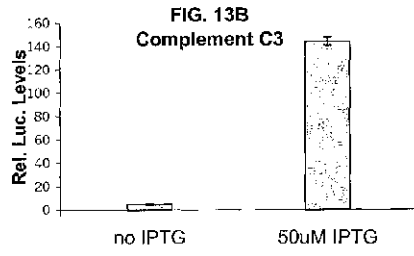
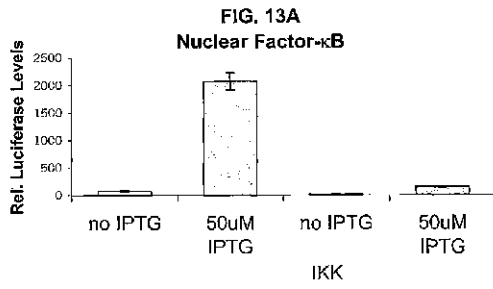
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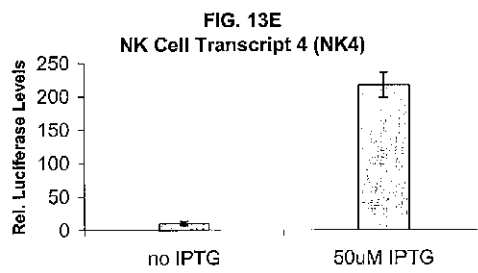
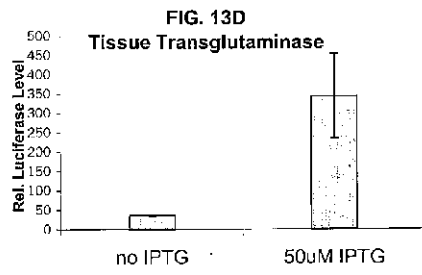
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 CAGCAGACA AGAGTACC CACCTTACC TGCCTGATG CTGGGCAGT CCCCAGCTG 420  
 GGTGCTATT CACGACCA CACTCTATT CTCCTAGGT GGGGTCCAG GACTAGCTG 480  
 GGTGAGCAG AAGTCCACC CTCACATTG TCTTGGTTC AGGAGCAAC TATGAAAG 540  
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 CCGATTAAA GCTTAAAGT CTAGACATG ACCACAGGG ACTTAGTCA GGTCACTGT 720  
 CCACTGGCC CAGAGGCCG CACTCCAGG AACGACCGT TATAGTCAG AGCTGGCTG 780  
 AGAGCCGCC CACTCTACA CTTCCAGCA GCCCAGTGC CGCCALCAG GGGCTCACC 840

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<210> 2
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<212> DNA
<213> Homo sapiens

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<220>
<221> misc feature
<222> Human complement C3 gene, exon 1 (AK H63423)

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<210> 3
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<212> DNA

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<221> misc_feature
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atttggctgt ggaataactg agcttttttt tctccctttt tttttttctt gctgactcga 240
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<210> 4
<211> 584
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<222> Interleukin beta-3 subunit gene, promoter region (AK: U28832)
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ctgtttctcc gtctaacgaa tttaactgtg aatgaatgaa actcagccta gtaggtgcat 180
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<210> 5  
 <211> 760  
 <212> DNA  
 <213> Homo sapiens  
 <220>  
 <221> misc\_feature  
 <222> Activin beta-A gene, regulatory sequence of 5' upstream region (A  
 N: D17357

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aatgbctaac actggaaaaa ccagtcctcc gctgagctct gotgatccca accaatccca 180
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taattccag gaacacac acactcagat gcagagagaa tcccaatatt gataagtgc 660
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```

<210> 6  
 <211> 1140  
 <212> DNA  
 <213> Homo sapiens  
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 <222> NK4 gene, regulatory sequence of 5' upstream region (AN: D17257)

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<400> 6
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<210> 7
<211> 360
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
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AF057307

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<211> 3271
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> 90K (Mac-2 BP) promoter region (AN: U91729)

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<210> 9
<211> 1403
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<222> galactin 3 (GALS3) gene, exon 1 (AF071421)

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<210> 10
<211> 839
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
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<210> 11
<211> 2877
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature<222> granulosa gene (AM: 632588)

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ggctctccc atctctgc tgcctacc caaacaaa tccgctctc caggctctg 900
gggctcagc caggcacc cctcagctg atgtctnag gctggcgg tcccaattc 960
ctctctctc ctctcacc acaagccc cctccccc accctccta cctctctc 1020
ttctctatt aaatgggca cagagctg cgtctgctc atgcaagg taaggctc 1080
agggccagg acctcgcg tctctctc gactggca caccctgg ctaccctc 1140
gagggctc ccagctgca cagagctc gctctccc tctcagag cccagctcc 1200
cactcagc ctctctctg agccagca gclallcg ctgctgctg gcccctcc 1260
ccccccccc gctctcag cggccccc cctcggcl' ccagagct cctctctc 1320
tccagctc tctctctg tgcctctg agctctcag ccccccac cgtctctc 1380
ccgctcagc cgtctctc cggctctc cactctcc cccctcgg cctcagct 1440

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<210> 18
<211> 2000
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<223> Prostaglandin stimulating factor (PSF, IGF1R-7, mac25) gene, 5' up
stream (2.0 kb) region (AI: AC022483)

<400> 18
gatgccagc atctctctc ggtggcag tctctgac tgcctctc tctctctc 60
ttttagctt atggggag gtaggact gactagga cggatctc actcctct 120
accgcaagc gggccagc aaccatct agaaccaat caagcagg ctggctcc 180
gctctcagc gctctctc caaacatt gggctctc ttttaaaaa aaagctaa 240
aatcagct ctataaata tcaatctc tctctcctc atctctct tttataaat 300

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aalacacaaa tatlalalal: ttattccaaa laaalalca tbtgaaaz gbaaglaal 350
aatagtaatt ttccaaatg: agaaatgctg gcatatatta caaacctca cacattttta 430
aaacacataa ttttcaatca ttacatgccc aaacacatc caaacatttt ttggtatatt 480
tataatgatt aataatcttt aaaaatata: aaaactaatc tgaattaaa atatacagaa 540
gagcaactgg cttttttttt tttttgagat ggaattttgc tttttttgca caggtgcaag 600
tgaatagggc tgaatctggc caactgcaac ctccgctctc tgggtcttga caatctctct 660
gcctcggact cctgagtagc tgggatyca gcaacccacc accatgtctg gctagtcttt 730
gtatttttag tagaaaaggg gtttcaactg gtgcccagc ctggctctga actcctgacc 780
taaagtgatc tgcacacac agcctccccc gctaatcttt gtattttta gaaagatgg 840
ggtttacaca tgttggccag gctggctttg aactccgac cttaagtgat cgcacactt 900
cagcccaaca aatgctcggg attagagggc tcatcactc gcccacact canaagaaac 960
atttfaatt: gtacttggc tgaacacat: taatattt aatolaala taattatgta 1020
ctcaattac tactactac atgtattac taatgaatc gtatagagc ttgacataa 1080
gaaacatctc caggcaattt tggcatttct gtgccaactc tgggtgctct ttctcaatg 1140
aacagacatc gtaacacaa ggcctcaga tggctcaga gctgagctga tctgattggc 1200
tgaataaatc gtggcaatc caacacagat gggcttctg tgggtgatac tgcacagatc 1260
tatgacctac aaatcagaa atgtgacca atcttatttt gtgacactcc catcaaaaa 1320
tatatgcta ttatgcta ataattgctc aactctcc atcaagtata ttctgatag 1380
tagcaaatct ttttttcaac caggtatcaa tgaagactga atcttctatt taacatgca 1440
caactctgat gatgggaagc attttctgaa gactagcttt tggctccaga cattcaaac 1500
tctattctcc ctccactact caactatatt ctctggtgct ggcacagctg gacacgtta 1560
tannnannct tgaacattgg ctctgcaact tgggtctatc acactagatg agttggctca 1620
atgggatctg gcaatatttt ggaactctct cctacacaa gagggcctgt aatagctaa 1680
ctaacatata agcgaactgc aaacacata aatatactgc actcaatcca aacttctatg 1740
alcccactct caagttgctc ttagttagat gcaaaaaatg cctgcaacca actctactct 1800
actgaatagc actgtatggc tgaagagctc agcaggaan gacagctatc ttanacaat 1860
gccttaaaa taactttatc ttcaaatg tacaancca catggctctc ggaactctct 1920
gttagggctc ctcaaggggt gctgcaaggc gcaactgact gtaaaactgt atctccaaa 1980
gtttccctta gcaatata 2000

```

<210> 19  
<211> 2027

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<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> Vascular endothelial growth factor 2 gene, partial cds and 5' ups
stream region (AK: AF020494)

<400> 15
gtctcttgat catcaggcaa ctttcaacta cacagccaa gggagagagg ggaacccctcc 63
gaggtcccaat aggtctctct gaactagtaa tcaacttttt ccaaaccttg agcaaggctc 123
tggggggccag gctgcccga gggagcaca gaactcagg gggcccacg acaaaagccc 180
ggtccctccc acccaacggc gccacglltc tcccgcgag agtggctaa gystgylcy 240
atcacctctc aaytcggatc cgcctaacgc cagcccccgg actgaaactg cccctccgga 300
cgcctcgtctc cgtcaggaga caggcccccg gaggagaga tccagagggg gctgagggga 360
ggtcggcccg ccggggagga gggcaggga ccggggagcc ccaggagagc gcttccgag 420
ggagagtagc agggggagga agcaggcctc aggcagctc cctccctcgg ccgttttcc 480
tcacataagc gcaagcagag ggcgcctcag tcatgcccgc cccctccgac cgcaccccgc 540
gcccgcaccc ctacagccgg cagcctctcg aggtctctcg gccgagggc tccagggccc 600
cgtcgtcgtcc agtcggcccg gccgcccctc tcccgcctcc ggcaccgccc ccagcgcctc 660
cgcctcagcgc ccagcggccc ggtctctctc attcgggga aggggagcga gaaggagagc 720
gagggtctcg gcccgtttag agggcctgaa catcgggggg cgtctcggtg tcccgcctcc 780
cgcctctcca aaaaactata ccgagcggga ccggggccc gctctctctc gctctcagct 840
cactctcagg gctcccaata cggagagctc ggaagtcagg ctctctctga gctttttacc 900
tgaacaccac cacccttccc cggcacctgc tgggggggg agctggaaag ttgggagcgc 960
ggagcaccgg acccgtctcc gccgcccctg gctgcccag gggggctcgc cgggagagag 1020
ccggggagga gggaccagga gggcccggcg gctcgcagg gggcagcagg ccccccaccc 1080
tgcaccgccc agcagacagg tcccacccc ccgctctctc caccctg 1127

```

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<212> 20
<213> 500
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> TIMP-1 (tissue inhibitor of metalloproteinase-1) gene, promoter
region (AK: D25513)

<400> 20
ggagaccggta cccactctag agatctgttg tgggtcttga ctgagataaa atatgtagag 60

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tgcctggaaa cawgtaggly ctgtalaala tggggctat. Pggmtdgtgt. Lauffggagc 120
ctgggtttg ctctgggca ccgaggttcc agctccagtt ctgcccagta ttgactctgt 180
gacccggggc aagtcactta acnacctccg gccctagatt ccccgatllc gnati.ccrcc 240
ccttccacc ccattatctc cctccactgc tggactcnaa ttgtllccct ctctgcccac 300
cctcaacgc atgtccagca taccnaatca aggcattttt ctgtgcttgg cacacactca 360
atgcacata aatgttgaag ggtcgaacta atttggcttt gactcabaq cacacttggc 420
ggaglglygg tgattggata gactctggag actttagggg actgggcccg gggcaactgc 480
gctctaaag cctccctgag gggcttggc agaatagtg actgactgc aggtggggga 540
gctggctggc cgggctggg cccggggga ggggagggc cgggctggga ggggagggg 600
gtgtatctcc ttctgtggc cggcccttg gctctggcc tgatggggg tggatgggtc 660
atgcatccan gaagcctgca ggcctggctt cctccacc ccgcccacc ccgcccctag 720
cctggcatt tatctctag cgtccagcc ctgcccacc ccgcccagat ccagcgcaca 780
ggagcctacc agagglacag 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
gctggggggc tcttgcatt ggaragg 27

```

```

<210> 22
<211> 26
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Antisense PCR primer for CC4 promoter (spec Table IIIa)

```

```

<400> 22
ccgggcaagc gggctraytag 26

```

```

<210> 23
<211> 22
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature<223> Sense primer for NK4 promoter (Table IIIa)

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<400> 23
cggagctaga agagucocga gg                22

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

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

<400> 24
eccaaaagct gaaggagcca a                21

<210> 25
<211> 23
<212> DNA
<213> Homo sapiens

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

<400> 25
caqaattgct gataiqtcga cca            23

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

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

<400> 26
cactcactgct gtagcactca cc            22

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

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

<400> 27
tgcctccttt ggctcgttct                20

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

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

<400> 18
gctgccgagg aacttgac                               18

<210> 20
<211> 28
<212> DNA
<213> Homo sapiens

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

<400> 29
ccnagggaga aatctccact gaagcaac                   28

<210> 30
<211> 28
<212> DNA
<213> Homo sapiens

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

<400> 30
tcnngcnnnn nngqtegtc ctccact                     28

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

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

<400> 31
gcctctctcag ctactactt cctaa                       25

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

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

<400> 32
cgaggagggc cactgaagg                               18

<210> 33

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

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

<400> 21
gatttggtctt gcccctcaaa g                21

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

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

<400> 34
ccagcacaagc ccccacaga                18

<210> 35
<211> 24
<212> DNA
<213> Homo sapiens

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

<400> 35
tgactccact gttttctaa aagg                24

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

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

<400> 36
gcaagctctaa agagctcaga cgt                23

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

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

<400> 37

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ggtttaagca atttctggcc tct                23
<210> 30
<211> 25
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Antisense primer for prosaposin promoter
<400> 38
cgcctgactc tccgcagtc gccat                25
.
<210> 39
<211> 25
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> sense primer for Mac2-BF promoter
<400> 39
gtaaactcc cggcagctc ctctt                25
.
<210> 40
<211> 22
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Antisense primer for Mac2-BF promoter
<400> 40
ctctgcagac tggcctctg ac                22
.
<210> 41
<211> 22
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Sense primer for GAL 3 promoter
<400> 41
cgtcttcaca aggtggaagt gg                22
.
<210> 42
<211> 18
<212> DNA
<213> Homo sapiens
<220>

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<221> misc_feature
<223> Antisense primer for GAT-3 promoter

<400> 42
ctggagggtca gggcacaq                18

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

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

<400> 43
taccacacct aagggtaaaa ataa                25

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

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

<400> 44
atgctgctag tctggtgct ac                22

<210> 45
<211> 23
<212> DNA
<213> Homo sapiens

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

<400> 45
gagactagga agcacttct ctltc                23

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

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

<400> 46
ctggaaagct ggtttcttt ctact                25

<210> 47
<211> 18

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<212> DNA
<213> Homo sapiens

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

<400> 47
qtggcagcagc gggcgcctc                                16

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

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

<400> 48
ctctcagcct gctctcctg                                  15

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

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

<400> 49
gggtctcagct cctccttctc                                20

<210> 50
<211> 20
<212> DNA
<213> Homo sapiens

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

<400> 50
actacacacc ggcacctgac                                  20

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

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

<400> 51
ctcccagcta gctgggatta                                  20

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

<220>
<221> misc_feature
<222> Antisense primer for cathepsin B promoter

<400> 52
ccacgctgacg aatggagca                18

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

<220>
<221> misc_feature
<222> Sense primer for clusterin promoter

<400> 53
agacacctga ctctctctct                20

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

<220>
<221> misc_feature
<222> Antisense primer for clusterin promoter

<400> 54
ctctctggga ggcgcgctt                19

<210> 55
<211> 24
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> Sense primer for F5F promoter

<400> 55
taagtgctgg gcttagaggg atam                24

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

<220>
<221> misc_feature

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<223> Antisense primer for FGF promoter
<400> 56
tatctattac taaggggaagc tattggag          28

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

<220>
<221> misc_feature
<223> Sense primer for VEGF-C promoter
<400> 57
gtctctggat 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
ctggaaaggac cgggggagg                    19

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

<220>
<221> misc_feature
<223> Sense primer for TIMP-1 promoter
<400> 59
agaaaccgta 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
ctgtactctc ggtgtctctc t                  21

<210> 61
<211> 20
<212> DNA

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<213> Homo sapiens
<220>
<221> misc_feature
<223> Analytical antisense primer for h-TGase
<400> 51
gccagtttgt tcaagtgtytt                20

<210> 52
<211> 20
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Analytical sense primer for APP
<400> 52
ctcgttctctg acaagtgcaa                20

<210> 53
<211> 20
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Analytical antisense primer for APP
<400> 53
tgttcagagc acaacctctc                20

<210> 54
<211> 20
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Analytical sense primer for p65(shc)
<400> 54
gagggtgctgg ttctggactaa                20

<210> 55
<211> 20
<212> DNA
<213> Homo sapiens
<220>
<221> misc_feature
<223> Analytical antisense primer for p56(shc)
<400> 55
gccccagagct gctgatttqll                20

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

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

<400> 66
ggagagttccc tccagagcag 30

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

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

<400> 67
atgtcttcatt gctgctgcag 20

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

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

<400> 68
atccttcagtg cggcagctga 20

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

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

<400> 69
acagggaccag gttcgaactgc 20

<210> 70
<211> 20
<212> DNA
<213> Homo sapiens

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

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<400> 70
atcacggacc tcccaactaa                20

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

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

<400> 71
aacctcacc tccgctacac                20

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

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

<400> 72
ccagaccctgg acatgactga                20

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

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

<400> 73
gtacactact tcaccaggaa                20

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

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

<400> 74
caaatgctg ctugtcataa                20

<210> 75
<211> 20
<212> DNA
<213> Homo sapiens

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<220>
<221> misc_feature
<222> Analytical antisense primer for ROP2
<400> 75
catccctaca agtcccaaa                20

<210> 76
<211> 34
<212> DNA
<213> Homo sapiens

<220>
<221> misc_feature
<222> Analytical sense primer for beta-actin
<400> 76
gggcaatcgt gctgacatt aag                33

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

<220>
<221> misc_feature
<222> Analytical antisense primer for beta actin
<400> 77
tgtgttggg tacaggttt tg                22
```

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 130b)

A. The indications made below relate to the microorganisms referred to in the description on page <u>21, 27, 29, 50, 76</u> , line <u>18, 5, 4, 17, 4</u> (respectively)	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depository institution AMERICAN TYPE CULTURE COLLECTION	
Address of depository institution (including postal code and country) 10801 UNIVERSITY BOULEVARD MANASSAS, VIRGINIA 20110-2209 UNITED STATES OF AMERICA	
Date of deposit 31 January 2002 (31.01.02)	Accession Number # 1A-4020
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
For receiving Office use only <input type="checkbox"/> This sheet was received with the international application Authorized officer	For International Bureau use only <input checked="" type="checkbox"/> This sheet was received by the International Bureau on: <u>19-JUNE-2002</u> Authorized officer <u>HONGRO HERMANSEZ</u>

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 133a)

<p>A. The indications made below relate to the microorganism referred to in the description on page <u>22, 27, 29, 51, 78</u>, line <u>5, 10, 5, 2, 9</u> (respectively).</p>			
<p>B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span></p> <p>Name of depository institution AMERICAN TYPE CULTURE COLLECTION</p> <p>Address of depository institution (including postal code and country) 10501 UNIVERSITY BOULEVARD MANASSAS, VIRGINIA 20108-2208 UNITED STATES OF AMERICA</p> <table border="1" style="width: 100%;"> <tr> <td style="width: 50%;">Date of deposit 31 January 2002 (31.01.02)</td> <td style="width: 50%;">Accession Number PTA-4021</td> </tr> </table>		Date of deposit 31 January 2002 (31.01.02)	Accession Number PTA-4021
Date of deposit 31 January 2002 (31.01.02)	Accession Number PTA-4021		
<p>C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span></p>			
<p>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)</p>			
<p>E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)</p> <p>The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")</p>			
<p style="text-align: center;">For receiving Office use only</p> <p><input type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer</p>	<p style="text-align: center;">For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>		

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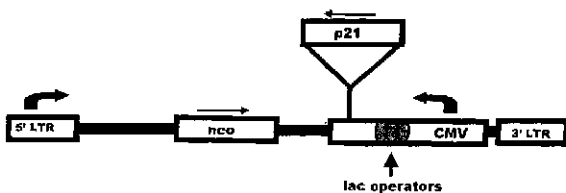
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- (72) Inventors: and
- (75) Inventor/Applicants (for US only): POOLF, Jason [US/US]; 918 S. Bell Avenue, #2, Chicago, IL 60612
- (74) Agent: NOONAN, Kevin E., McDonnell Boehnen Hulbert & Bergoff, 300 South Wacker Drive, Chicago, IL 60606 (US)
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Published: with international search report before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

[Continued on next page]

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

**IPTG-regulated retroviral vector LNP21CO3**



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

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*with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

**Previous Correction:**  
see PCT Gazette No. 44/2002 of 31 October 2002, Section II

**(88) Date of publication of the international search report:**  
13 November 2003

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**(15) Information about Correction:**

## 【 国際調査報告 】

INTERNATIONAL SEARCH REPORT		International application no. PCT/US 02/02784
A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12Q1/68 C12N5/10 C12N15/63		
According to International Patent Classification (IPC) or to both national classifications and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12Q C12N		
Documentation searched other than "minimum" documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and where practical, search terms used) EPO-Internal, WPI Data, EMBASE, PAJ, BIOSIS, SEQUENCE SEARCH		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category <sup>1</sup>	Category of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 61751 A (CHANG BEY DIH ;UNIV ILLINOIS (US); RONINSON IGOR B (US)) 19 October 2000 (2000-10-19) the whole document	1-63
P, X	WO 01 38532 A (TRUSTEES OF THE UNIVERSITY OF ;CHANG BEY DIH (US); RONINSON IGOR B) 31 May 2001 (2001-05-31) the whole document	1-63
<input type="checkbox"/> Further documents are listed in the continuation of box C <input checked="" type="checkbox"/> Patent family members are listed in annex		
<sup>1</sup> Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *P* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claimed or which is cited to establish the publication date of another claim or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *E* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to inform of the prior art or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken into account *Y* document of particular relevance; the claimed invention can be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *S* document member of the same patent family		
Date of the actual completion of the international search 4 September 2003		Date of making of the international search report 02.10.03
Name and mailing address of the ISA European Patent Office, P.O. 5012 Patentstr. 2 NL - 2280 HV The Hague Tel: (+31-70) 905-2000, Tx: 31 651 epo nl Fac: (+31-70) 905-3016		Authorized officer Chavanne, F

Form PCT/ISBA/11 (September 2003) July 2002

INTERNATIONAL SEARCH REPORT	
International Application No. PCT/US 02/02784	
<b>Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)</b>	
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:	
1.	<input checked="" type="checkbox"/> Claims Nos.: because they relate to subject matter not required to be searched by the Authority, namely: see FURTHER INFORMATION sheet PCT/ISA/210
2.	<input checked="" type="checkbox"/> Claims Nos.: 64-68, 77, 78, 82, 83, 87-94, 97-100 because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: see FURTHER INFORMATION sheet PCT/ISA/210
3.	<input type="checkbox"/> Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).
<b>Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)</b>	
This International Searching Authority found multiple inventions in this International application, as follows:	
see additional sheet	
As a result of the prior review under R. 40.2(e) PCT, no additional fees are to be refunded.	
1.	<input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2.	<input type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, this Authority did not require payment of any additional fee.
3.	<input checked="" type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.: 1-63 (partially)
4.	<input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims. It is covered by claims Nos.:
<b>Remark on Protest</b>	
<input checked="" type="checkbox"/> The additional search fees were accompanied by the applicant's protest.	
<input type="checkbox"/> No protest accompanied the payment of additional search fees.	

International Application No. PCT/US 02 02784

## FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-68, 77, 78, 82, 83, 87-94, 97-100 (partially)

Invention 1:

A recombinant expression construct encoding a reporter gene operably linked to a promoter from a serum amyloid A, a mammalian cell comprising said construct, method for identifying compounds by culturing said cell.

2. Claims: 1-68, 77, 78, 82, 83, 87-94, 97-100 (partially)

Inventions 2-4:

A recombinant expression construct encoding a reporter gene operably linked to a promoter from complement C3, beta-amyloid precursor protein or prosaposin, respectively, a mammalian cell comprising said construct, method for identifying compounds by culturing said cell.

3. Claims: 1-6, 8, 10-68, 77, 78, 82, 83, 87-94, 97-100 (partially)

Inventions 5 and 6:

A recombinant expression construct encoding a reporter gene operably linked to a promoter from a human natural killer cell protein 4 or tissue transglutaminase, respectively, a mammalian cell comprising said construct, method for identifying compounds by culturing said cell.

4. Claims: 1-5, 8, 10-68, 77, 78, 82, 83, 87-94, 97-100 (partially)

Inventions 7-19:

A recombinant expression construct encoding a reporter gene operably linked to a promoter from connective tissue growth factor, integrin beta-3, activin A, Mac2 binding protein, galectin-3, superoxide dismutase 2, granulysin/epithelin, p66shc, cathepsin B, clusterin, prostacyclin stimulating factor, vascular endothelial growth factor-C or tissue inhibitor of metalloproteinase-1, respectively, a mammalian cell comprising said construct, method for identifying compounds by culturing said cell.

5. Claims: 69-74, 84-86 (completely)

International Application No. PCT/US 02 02784

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Invention 20:

Method for treating a disease in an animal comprising the administration of a non-steroidal anti-inflammatory drug (NSAID).

6. Claims: 75, 76, 95, 96 (completely)

## Invention 21:

Compound that inhibit genes associated with pathogenic consequences of senescence in mammalian cells, and methods of treatment using said compound.

7. Claims: 79-81 (completely)

## Invention 22:

Method for inhibiting the production of anti-apoptotic or mitogenic factors in a mammalian cell.

International Application No. PCT/US 02 02784

## FURTHER INFORMATION CONTINUED FROM PCTISA/ 210

## Continuation of Box I.1

Although claims 64-68, 82, 83, 87-94 and 97-100 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 38-63 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

## Continuation of Box I.2

Claims Nos.: 64-68, 77, 78, 82, 83, 87-94, 97-100

Present claims 64-68 relate to methods based on the use of undefined compounds. In fact, said claims refer to compounds produced according to the claim 38, whereas claim 38 does not mention the production of any compound. The claims lack clarity and conciseness within the meaning of Article 6 PCT to such an extent as to render a meaningful search of the claims impossible.

Present claims 77 and 78 refer to a compound defined by a result to be achieved and by means of a process. In the present case, claims 77 and 78 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small portion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search of the claims is impossible.

Present claims 82, 83, 87-94 and 97-100 relate to methods. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is not to be found. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search is impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT  
 Information on patent family members

Intern. application No.  
 PCT/US 02/02784

Patent document cited in search report	Publication date	Patent family members:	Publication date
WO 0061751 A	19-10-2000	AU 4079000 A	14-11-2000
		AU 8010800 A	04-06-2001
		CA 2365908 A1	19-10-2000
		CA 2392495 A1	31-05-2001
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WO 0138532 A2	31-05-2001		

Intern. PCT/US 02/02784 (family members only) (1/06)

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A 6 1 P 29/00	A 6 1 P 35/00	
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G 0 1 N 33/15	G 0 1 N 33/53	M
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发明人	プール、ジェイソン ロニンソン、イゴアビー。 チャン、バイ-ディ		
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F-TERM分类号	2G045/AA35 2G045/AA40 2G045/BA11 2G045/BB50 2G045/DA12 2G045/DA13 2G045/FB02 4B024/AA01 4B024/AA11 4B024/CA02 4B024/CA04 4B024/DA02 4B024/FA02 4B024/HA14 4B063/QA18 4B063/QQ43 4B063/QR62 4B063/QR80 4B063/QS34 4B065/AA90 4B065/AB04 4B065/BA02 4B065/CA44 4B065/CA46 4C084/AA02 4C084/AA17 4C084/BA44 4C084/DC50 4C084/NA14 4C084/ZA16 4C084/ZA45 4C084/ZA81 4C084/ZA96 4C084/ZB11 4C084/ZB21 4C084/ZB26 4C084/ZC02 4C084/ZC20 4C084/ZC41 4C206/AA01 4C206/AA02 4C206/JA19 4C206/MA01 4C206/NA14 4C206/ZA16 4C206/ZA45 4C206/ZA81 4C206/ZA96 4C206/ZB11 4C206/ZB21 4C206/ZB26 4C206/ZC02 4C206/ZC20 4C206/ZC41		
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优先权	60/265840 2001-02-01 US 09/861925 2001-05-21 US		
其他公开文献	JP2004535775A		

#### 摘要(译)

本发明提供了用于鉴定抑制与癌症和年龄有关的疾病有关的基因的诱导的化合物的方法和试剂，这些基因是由细胞周期蛋白依赖性激酶抑制剂诱导的。