(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 December 2010 (29.12.2010)

PCT

(10) International Publication Number
WO 2010/148447 A1

(51) **International Patent Classification**:
C12N 15/12 (2006.01) *G01N 33/50* (2006.01)
C12Q 1/68 (2006.01)

(21) **International Application Number**:
PCT/AU2010/000790

(22) **International Filing Date**:
23 June 2010 (23.06.2010)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
2009902906 23 June 2009 (23.06.2009) AU

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/148447 A1

(54) **Title**: A NOVEL REGULATOR OF CELLULAR SENESCENCE

(57) **Abstract**: Provided herein are methods for regulating whether a cell undergoes apoptosis or senescence, comprising modulating the level of expression an/or activity of SENEX in the cell, wherein increasing the expression and/or activity of SENEX relative to normal endogenous levels induces cellular senescence and decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis. Methods for the diagnosis and treatment of senescence-associated and apoptosis-associated disorders are also provided.

A novel regulator of cellular senescence

Field of the Invention

The present invention relates generally to a novel senescence-inducing gene, designated herein *SENEX*, and the polypeptide (SENEX) encoded thereby. More particularly the invention relates to the ability of SENEX to regulate the progression of a cell into either an apoptotic pathway or senescence pathway, and thus the invention provides methods for the modulation of apoptosis and senescence by modulation of the expression and/or activity of SENEX.

Background of the Invention

Somatic cells have a finite proliferative capacity. Cellular senescence and apoptosis each constitute major pathways for the regulation of cell proliferation. In some circumstances these processes are beneficial, for example in the suppression of tumourigenesis and limiting disease progression more generally (see, for example, Collado *et al.*, 2005; Krizhanovsky *et al.*, 2008). However senescence is also implicated in the aetiology of a number of disorders such as age-related diseases.

The recognition and impact of senescence on the vascular system is only just emerging. Increased numbers of senescent endothelial cells are found in mature atherosclerotic plaques, in vessels from diabetic patients, in post-angioplastic restenotic vessels, in coronary vessels of patients with ischaemic heart disease, and in hypertensive patients (Voghel *et al.*, 2007). Furthermore, endothelial progenitor cells in diabetics undergo heightened senescence which contributes to the impaired neovascularisation seen in diabetes. Senescent endothelial cells have also been identified in the tumour vasculature in glioma. However the causes and results of these senescent endothelial cells in the different pathologies have not been clearly defined.

The recognition of senescent cells relies on a number of specific criteria. The cells exit the cell cycle although remain viable, they exhibit a large flattened morphology and show accumulation of senescence associated β-galactosidase (SA-β-gal) activity. Furthermore, they show altered genetic profiles which may be cell type specific. There are two broad forms of senescence, replicative and stress induced. Replicative senescence is mediated through the shortening of telomeres that occurs after each cell division. This shortening eventually registers as DNA damage and triggers ATM activation and initiates a program of cell cycle arrest. Stress induced premature senescence (SIPS) may be induced by oncogenes, by oxidative stress, or by suboptimal culture conditions and occurs independent of a change in telomere length. Senescence is mediated through the p53 pathway which transactivates the cyclin dependent kinase inhibitor p21, or through the p16 pathway to inhibit the cyclin dependent kinases 2 and 4, preventing the phosphorylation of Rb and thus silencing genes involved in proliferation.

Programmed cell death is an evolutionary conserved pathway, the activation of which leads to an energy-dependent cell suicide mechanism. The best characterized programmed cell death pathway is caspase-dependent cell death, or apoptosis. Apoptosis involves the sequential activation of a group of proteases (caspases) and involves a co-ordinated process of morphological and biochemical changes including cell shrinkage, nuclear condensation and DNA fragmentation. Also characteristic of apoptosis is altered patterns of expression of a range of proteins, including those of the Bcl_2 family, which has both pro-apoptotic (Bak, Bax, etc.) and anti-apoptotic members (Bcl_2, Bcl_x).

In endothelial cells excessive proliferation activates the replicative senescence pathway. In addition oxidative stress with the concomitant generation of reactive oxygen species (ROS) appears to be a common theme in many senescence-associated vascular diseases. ROS accumulate with age, are involved in age associated degenerative diseases and in tumour development. Furthermore, ROS induce cell senescence and apoptosis. Indeed there is a common theme in terms of signaling pathways and phenotypic changes seen during aging, senescence and cancer development. Of the functional ROS, H_2O_2 is perhaps the most important since it is also a known signaling molecule, involved in the proliferative response to growth factors such as platelet-derived growth factor and epidermal growth factor but can also induce apoptosis and senescence.

Given the impact of senescence on the biological function and in particular the potential for its manipulation in damaged or aged vasculature there is considerable interest in the identification of suitable targets and therapeutic approaches for the modulation of sensescence. Similarly, in view of the fact that aberrant cell growth and/or proliferation is associated with a wide variety of disease conditions there is increasing interest in the therapeutic application of apoptosis inducers. The ability to modulate either or both of senescence and apoptosis, and to regulate the anti-proliferative pathway adopted by a given cell or cell type would be highly advantageous in designing appropriate and effective therapies for specific conditions and specific individuals.

Described herein is the identification of a novel gene, designated *SENEX*, which has the dual capacity to act as a regulator of stress-induced premature senescence and apoptosis. As described herein, modulation of the levels of *SENEX* are crucial in determining vascular function.

Summary of the Invention

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

According to a first aspect of the invention there is provided a method for regulating whether a cell undergoes apoptosis or senescence, the method comprising modulating the level of expression an/or activity of SENEX in the cell, wherein increasing the expression and/or activity of SENEX relative to normal endogenous levels induces cellular senescence and decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

Typically the cell is a eukaryotic cell, and more typically a mammalian cell. In an embodiment the cell is an endothelial cell.

In an embodiment the senescence is stress-induced premature senescence. The method may be used in the treatment or prevention of a senescence-associated disorder or a condition associated with, or characterised by, aberrant or otherwise unwanted cell growth and/or proliferation. In an embodiment the senescence-associated disorder or condition is atherosclerosis.

According to a second aspect of the present invention there is provided a method of regulating senescence in a cell, the method comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell.

In an embodiment the senescence is stress-induced premature senescence.

Typically the cell is a eukaryotic cell, and more typically a mammalian cell. In an embodiment the cell is an endothelial cell. The cell may be contacted with the agent *in vitro*, *ex vivo* or *in vivo*.

The cell may be located in, or derived from, a subject in which inducing or inhibiting cellular senescence is desirable. For example, the method may comprise inducing senescence in the cell by increasing the level of expression and/or activity of SENEX relative to normal endogenous levels. By way of example, the subject may be suffering from, or predisposed to, cancer, a vascular disease or an inflammatory condition. Where senescence is abnormally upregulated the method may comprise normalising the levels of SENEX by contacting the cell with an agent capable of inhibiting SENEX expression and/or activity. By way of example, the subject may be suffering from, or predisposed to, a senescence-associated disorder such as, for example, atherosclerosis.

In an embodiment regulating senescence comprises inducing cellular senescence, the method comprising contacting the cell with an effective amount of SENEX or a polynucleotide encoding the same in order to increase or upregulate SENEX expression. The SENEX polypeptide may comprise the amino acid sequence set forth in SEQ ID NO:1 or a fragment, variant or homologue thereof. The polynucleotide encoding SENEX may comprise a nucleotide sequence as set forth in SEQ ID NO:2, or a fragment, variant or homologue thereof. In an alternate embodiment, SENEX expression may be

increased by contacting the cell with an effective dose of an agent capable of upregulating SENEX expression such as, for example, hydrogen peroxide.

In an embodiment regulating senescence comprises inhibiting or reversing aberrant or abnormally upregulated cellular senescence, the method comprising contacting the cell with an effective amount of an inhibitor of SENEX expression and/or activity to normalise levels of SENEX. In an embodiment the inhibitor may be an anti-SENEX antibody or antisense construct. In an alternate embodiment, SENEX expression may be downregulated by contacting the cell with an effective dose of an agent such as, for example, hydrogen peroxide.

According to a third aspect of the invention there is provided a method of regulating apoptosis in a cell, the method comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell, wherein decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

Typically the cell is a eukaryotic cell, and more typically a mammalian cell. In an embodiment the cell is an endothelial cell. The cell may be contacted with the agent *in vitro*, *ex vivo* or *in vivo*.

The cell may be located in, or derived from, a subject in which inducing or inhibiting apoptosis is desirable. For example, the method may comprise inducing apoptosis in the cell by decreasing the level of expression and/or activity of SENEX relative to normal endogenous levels. Where apoptosis is abnormally upregulated the method may comprise normalising the levels of SENEX by contacting the cell with an agent capable of increasing SENEX expression and/or activity.

In an embodiment regulating apoptosis comprises inducing apoptosis, the method comprising contacting the cell with an effective amount of an inhibitor of SENEX expression and/or activity. By way of example, the method may be used for the treatment of conditions associated with aberrant or otherwise unwanted cell growth and/or proliferation, chronic conditions associated with a dysfunctional vascular system and inflammatory conditions.

In an embodiment regulating apoptosis comprises inhibiting apoptosis, the method comprising contacting the cell with an effective amount of SENEX or a polynucleotide encoding the same to normalise levels of SENEX. The SENEX polypeptide may comprise the amino acid sequence set forth in SEQ ID NO:1 or a fragment, variant or homologue thereof. The polynucleotide encoding SENEX may comprise a nucleotide sequence as set forth in SEQ ID NO:2, or a fragment, variant or homologue thereof. By way of example, the method may be used for the treatment of apoptosis-associated disorders.

According to a fourth aspect of the invention there is provided a method of inhibiting or preventing

inflammation, the method comprising administering to a subject in need thereof an effective amount of an agent capable of upregulating the expression and/or activity of SENEX in at least one cell of the subject, relative to normal endogenous levels.

The method may comprise administering an effective amount of SENEX or a polynucleotide encoding the same in order to increase or upregulate SENEX expression.

The method may be used for the treatment or prevention of an inflammatory condition. The inflammatory condition may be a chronic inflammatory condition.

According to a fifth aspect of the invention there is provided the use of SENEX as an anti-inflammatory agent.

Also provided are compositions for use in accordance with the methods defined hereinbefore, the compositions comprising agents capable of upregulating or downregulating the expression and/or activity of SENEX.

A sixth aspect of the invention provides a method for determining disease control in a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising:

(a) obtaining a biological sample from the subject; and

(b) determining the level of expression and/or activity of SENEX in the sample relative to normal endogenous levels,

wherein the level of SENEX expression and/or activity relative to normal endogenous levels is indicative of disease control in the subject.

The method may further comprise monitoring disease control over time in the subject comprising: repeating steps (a) and (b) at least once over a period of time; and determining whether the SENEX levels change over the period of time.

A seventh aspect of the invention provides a method for evaluating the efficacy of a treatment regime in a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising:

(a) treating the subject with a treatment regime for a period sufficient to evaluate the efficacy of the regime;

(b) obtaining a biological sample from the subject;

(c) determining the level of expression and/or activity of SENEX in the sample;

(d) repeating steps (b) and (c) at least once over a period of time; and

(e) determining whether the SENEX levels change over the period of time,

wherein a change in the level of expression and/or activity of SENEX is indicative of a change in disease control in the subject and the degree of efficacy of the treatment regime.

The treatment regime may comprise the administration of one or more drugs, medications, supplements or medical devices aimed at directly treating the disorder, early intervention for the disorder, management of residual symptoms of the disorder, prevention of relapse, or overcoming treatment resistance in the subject.

An eighth aspect of the invention provides a method for designing a suitable treatment regime for a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising monitoring the level of expression and/or activity of SENEX in the subject in the presence or absence of a treatment regime for treating the disorder and adjusting the identity, timing and/or intensity of the treatment regime so as to normalise the level expression and/or activity of SENEX.

A ninth aspect of the invention provides a method for treating a subject suffering from a senescence- or apoptosis-associated disorder comprising administering to the subject a treatment regime designed in accordance with the eighth aspect.

A tenth aspect of the invention provides a method for identifying a compound suitable for treating a senescence- or apoptosis-associated disorder, the method comprising the steps of:

(a) isolating at least one cell from a subject suffering from a a senescence- or apoptosis-associated disorder;
(b) determining the level of expression and/or activity of SENEX in the at least one cell;
(c) contacting the at least one cell with a candidate compound; and
(d) determining the level of expression and/or activity of SENEX,

wherein normalisation of the level expression and/or activity of SENEX between steps (b) and (d) is indicative of the ability of the compound to treat the senescence- or apoptosis-associated disorder.

An eleventh aspect of the invention provides a method for diagnosing a senescence- or apoptosis-associated disorder in a subject, or predicting the susceptibility of a subject to such a disorder, the method comprising:

(a) obtaining a biological sample from the subject; and
(b) determining the level of expression and/or activity of SENEX in the sample,

wherein increased levels of SENEX expression and/or activity relative to normal endogenous levels is diagnostic or predictive of a senescence-associated disorder, whereas decreased levels of SENEX relative to normal endogenous levels is diagnostic or predictive of an apoptosis-associated disorder.

The method may further comprise comparing the level of expression and/or activity of SENEX in the sample with the level from one or more control samples. The control samples may be derived from one or more individuals known not to suffer from a senescence- or apoptosis-associated disorder, or alternatively known to suffer from a specific, diagnosed senescence- or apoptosis-associated disorder.

Brief Description of the Drawings

Embodiments of the invention are described herein, by way of non-limiting example only, with reference to the following drawings.

Figure 1. **A.** Human umbilical vein endothelial cells (HUVECs) were infected with empty vehicle (EV) (i) or antisense *SENEX* (ii) containing adenovirus. After 24 h cells were plated onto Matrigel, and capillary tube formation was observed over a 24 h time course. Photographs taken 12 h after plating are shown. Data shown is representative of 3 independent experiments. Bars =100 μm. **B.** HUVECs were transfected with control siRNA or siRNA against *SENEX*. After 24 h total RNA was extracted and subjected to reverse transcriptase followed by Q-RT-PCR. mRNA expression of *SENEX* was measured and standardized to Cyclophilin A. Results are the mean +/-SEM of 15 replicates from five HUVEC lines. ** $p< 0.001$ compared to siRNA control. **C.** HUVECs were transfected with control siRNA or siRNA against *SENEX*. After 24 h whole protein lysates were harvested and *SENEX* protein levels were measured using western blotting. β-actin was used as a loading control. Data shown is representative of 3 independent experiments.

Figure 2. A. HUVECs transfected with the control (con) or siRNA for *SENEX* were assessed for apoptosis using the Caspase 3 activity assay. The fold change relative to the control is given. Results are mean +/- SEM of 4 independent experiments, ***$p<0.001$ compared to Control after 24 h. **B.** HUVECs were plated in labtek slides, transfected with control siRNA (i) or siRNA against *SENEX* (ii). After 24 h cells were stained with Dapi to visualize apoptosis. Data shown is representative of 2 independent experiments. Bars= 90μm. **C.** Expression profile of *SENEX* mRNA during *in vitro* capillary tube formation. Total RNA was harvested at the time points shown from cells undergoing angiogenesis in a 3D collagen assay, and virtual northern blots were probed with *SENEX* cDNA fragment. **D.** Blots were quantified using a phosphor imager and standardized to cyclophilin A. This is representative of 3 independent experiments. **E.** A microarray profile was generated for the *SENEX* clone on the microarray slide. Plotted is the fold induction (log2) with respect to *time 0* (*y*-axis) versus time (in hours; *x*-axis).

Figure 3. A. Expression levels of *SENEX* protein in HUVECs at 24 and 48 h after infection with empty vehicle (EV) or *SENEX* (S) adenovirus and detected by western blot analysis. Protein molecular masses (in kD) appear on the right. Data shown is representative of 5 independent experiments. **B.** HUVECs were infected with EV or *SENEX* adenovirus. After 24 h cells were plated onto Matrigel, and capillary tube formation was observed over a 24 h time course. Photographs taken 12 h after plating are shown. Data shown is representative of 3 independent experiments. **C.** HUVECs were infected with EV (i) or *SENEX* (ii) containing adenovirus. Infected cells are visualized with green fluorescent protein (GFP). Photographs were taken after 48 h. Arrows mark large senescent cells. Bar=220 μm. **D.** Enlarged area of cells from B. Bar=80 μm. **E.** Phase contrast photograph of a number of enlarged cells. Bar=80 μm.

Figure 4. A. HUVECs were infected with EV (i) or *SENEX* (ii) containing adenovirus. After 72 h cells were stained for acidic β-galactosidase. Bars 220 μm. **B.** HUVECs were transfected with control (i) or *SENEX* (ii) expressing plasmid using Amaxa nucleofector kit. Bar=90 μm. **C.** High power view of a senescent cell induced by transfection of *SENEX*.

Figure 5. A. *SENEX* overexpression inhibits endothelial cell proliferation. HUVECs infected with EV (white bars) or *SENEX* (black bars) containing adenovirus were assessed for cell proliferation after 4 days using the MTS assay. OD_{490nm} for cells at Day 0 and Day 3 are given. Results are the mean +/- SEM of 4 replicates of each group. Data shown is representative of 3 independent experiments. *$p<0.05$ compared to EV on Day 4. **B.** HUVECs were infected with *SENEX* and EV adenovirus for 24 h. Total protein was used for western blotting with eNOS antibodies. β-actin was used as a loading control. Data shown is a representative of 3 independent experiments.

Figure 6. HUVECs were infected with *SENEX* (R365A) mutant construct and EV containing adenovirus. Cells were visualized 48 h after infection. Large flattened and vacuolated cells were seen in the *SENEX* mutant overexpressing cells and at a similar frequency to the wild type *SENEX*.

Figure 7. A. HUVECs were infected with EV (white bars) or *SENEX* (black bars) containing adenovirus. Photographs were taken at 24 h time intervals for 4 days. The number of senescent cells, based on an enlarged morphology, were counted and presented as a percentage of total cells counted. At least 1000 cells were counted for each of the three individual HUVEC lines. The mean +/-SEM is shown. **$p<0.01$ and *** $p<0.001$ compared to EV. **B.** HUVECs were infected with *SENEX* (*S*) or EV (EV) containing adenovirus. Cells were harvested after 24, 48 and 72 h of culture and telomere length was measured by southern blot analysis. **C.** Replicative senescence in HUVECs was induced through constant passaging and the cells were harvested when the senescent morphology was evident in the majority of cells. mRNA expression was measured by Q-RT-PCR of COX2, IL-1α, PAI-1 and *SENEX* and standardized to cyclophilin A. The mean +/-SEM of 6 replicates from two lines of HUVECs is shown. Early passage cells (white bar) and late passage senescent cells (black bar). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to early passage. **D.** HUVECs were infected with *SENEX* (black bars) and EV (white bars) containing adenovirus. After 2-4 days when at least 50% senescence was seen, the cells were harvested and mRNA levels determined for PAI-1, COX-2 and IL-1α by Q-RT-PCR. There were no significant differences seen between the EV and *SENEX* groups. Data shown is representative of 2 independent experiments.

Figure 8. A. HUVECs were infected with *SENEX* and EV containing adenovirus for 24 h. Total protein was used for western blotting with p53 and p21 antibodies. β-actin was used as a loading control. Analysis of each protein was performed in 3-5 different HUVEC lines. **B.** HUVECs were infected with *SENEX* or EV adenovirus for 24 and 72 h. Total RNA was extracted and Q-RT-PCR used to determine levels of p16 standardized to Cyclophilin A. Data shown is a representative of 4 independent

experiments. Results are the mean +/-SEM of 3 replicates of each group. *p<0.05 compared to EV. **C.** HUVECs were infected with *SENEX* and EV adenovirus for 24 h. Total protein was used for western blotting using p16 or phosphorylated Rb specific antibodies. β-actin was used as a loading control. Data shown is representative of 3-4 independent experiments performed using different endothelial cell isolates.

Figure 9. A. HUVECs were stimulated with 100 μM H_2O_2 for 2 h and then placed in normal HUVEC medium for 24 h (ii) or kept in normal HUVEC medium for 24 h (i). Cells were then stained for β galactosidase activity. Bar= 100 μm. **B.** HUVECs were stimulated with 10 μM or 100 μM H_2O_2 for 2 h and then placed in normal HUVEC medium for 24 h. *SENEX* expression was measured by western blot with β-actin used as a loading control. Data shown is representative of 3 independent experiments. **C.** HUVECs were treated with 500 μM H_2O_2 for 6 h. *SENEX* expression was measured by western blot with β-actin as a loading control. Data shown is representative of 3 independent experiments.

Figure 10. A. Cells were treated with 10 ng/ml of TNFα in normal HUVEC medium for 24 h. Protein lysates were harvested at 6 and 24 h and the *SENEX* protein levels were measured using western blotting. β-actin was used as a loading control. Data shown is representative of 3 independent experiments. **B.** HUVECs were treated with 10 ng/ml of TNFα in normal HUVEC medium for 24h. After 6 and 24 h RNA was extracted and subjected to reverse transcriptase followed by Q-RT-PCR. MRNA expression of SENEX was measured and standardized to cyclophilin A. Results are the mean +/-SEM of 12 replicates from three HUVEC lines. * P<0.05 compared to TNFα treated cells. **C.** HUVECs were infected with *SENEX* and EV adenovirus for 24 h. They were treated with 10 ng/ml of TNFα in normal HUVEC medium for 24 h or cultured in serum free HUVEC medium for 24 h or left untreated. Protein lysates were harvested and apoptosis measured using a Caspase 3 activity assay. The mean +/-SEM of 6 replicates from three lines of HUVECs is shown. *p<0.05 and ***p<0.001 compared to EV. **D.** HUVECs were cultured in varying concentrations of serum for 24 h. After 6 and 24 h, RNA was extracted and mRNA expression of *SENEX* was measured and standardized to Cyclophilin A. Results are the mean +/-SEM of 18 replicates from six HUVEC lines.

Figure 11. A. HUVECs were plated on fibronectin coated labtek slides at $6x10^4$ for 24 h, infected with *SENEX* and EV containing adenovirus and after 48 h stimulated with TNFα (5 ng/ml) for 5 h. Adhesion of neutrophils was then assessed (ii). Data shown is representative of 3 independent experiments. Arrows show senescent cells from the corresponding fluorescence view (i). Bar= 220 μm. **B.** HUVECs were treated as in (A). Adhesion of mononuclear cells was then assessed (ii). Data shown is a representative of 3 independent experiments. Arrows show senescent cells from the corresponding fluorescence view (i). Bar= 220 μm. **C.** From the photographs taken in (A) counts were made of the number of adherent neutrophils on a senescent cell (black bar). Neutrophils were then counted in the same surface area on neighbouring nonsenescent cells (white bar). Data shown is representative of 102 senescent cells and the corresponding area of non-senescent cells from three HUVEC lines.

p<0.001 compared to EV. **D.** From the photographs in (B) counts were taken of the number of adherent mononuclear cells on a senescent cell (black bar). Mononuclear cells were then counted in the same surface area on neighbouring non-senescent cells (white bar). Data shown is representative of 42 senescent cells from three HUVEC lines. p<0.001 compared to EV. **E.** HUVEC monolayers seeded on a transwell membrane were infected with EV (white bars or *SENEX* (black bars) containing adenovirus. Passage of FITC-dextran through the membrane in response to thrombin was tested 48 h later. Data shown is representative of 3 independent experiments, showing the average of four transwells per condition +/- SEM. * p<0.05 compared to EV group with thrombin. **F.** HUVECs were infected with *SENEX* or EV containing adenovirus. Cells were replated after 24 h and left for a further 24 h. TNFα (5 ng/ml) was added for 24 h. Surface expression of VCAM-1 was analyzed by flow cytometry. Graphs show the expression of VCAM- 1 on untreated EV infected cells (red), untreated *SENEX* infected cells (blue), TNFα treated EV infected cells (green) and TNFα treated *SENEX* infected cells (black). Controls for each assay included the absence of primary antibody and the incubation of the cells with an isotype-matched, non-relevant antibody (not shown). Data shown is representative of 2 independent experiments performed on separate HUVEC lines.

Figure 12. HUVECs were stimulated with 100 μM H_2O_2 for 2 h and then placed in normal HUVEC medium for 24 h. They were then replated into labtek slides and 24 hours later stimulated with 5ng/ml of TNF for 5 hours then stained for E selectin (**A**) or VCAM (**B**) Bar= 220 μm . Phase contast photos of the area photographed for adhesion molecule expression is given in the left hand panels. **C**. Senescent and non senescent cells were analysed for cell surface expression of E selectin and VCAM. The mean pixel intensity per cell was measured using Image J for senescent cells (black bar) and for neighbouring non senescent cells (white bar). The E selectin data is a representative of the mean +/-SEM of 33 senescent cells and non-senescent cells from two HUVEC lines. *** p<0.001 compared to non senescent cells. The VCAM data is a representative of the mean +/-SEM of 17 senescent cells and the corresponding area of non-senescent cells from two HUVEC lines. *** p<0.001 compared to non senescent cells. for 2 h and then placed in normal HUVEC medium for 24 h. They were then replated into labtek slides and 24 hours later stimulated with 5ng/ml of TNF for 5 hours then stained for E selectin (A) or VCAM (B) Bar= 220 μm . Phase contast photos of the area photographed for adhesion molecule expression is given in the left hand panels. **D**. Total RNA was extracted from the aortos of male ApoE-/- mice that were on a Western diet for 1 (open columns) and 5 months (filled columns). The RNA was subjected to reverse transcriptase followed by Q RT PCR, as described in materials and methods. mRNA expression of SEN1 was assessed and standardized to HRPT, PBGD and TIE2. quantification of *SENEX* levels standardized to the three housekeeping genes.

The subject specification contains amino acid and nucleotide sequence information prepared using the programme PatentIn Version 3.4, presented herein in a Sequence Listing. Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc.

Specifically, the amino acid sequence of human SENEX is provided in **SEQ ID NO:1** and the nucleotide sequence of human *SENEX* is provided in **SEQ ID NO:2**. **SEQ ID NOs:3 to 14** provide the sequences of various oligonucleotide primers used in the Examples.

Detailed Description of the Invention

In the context of this specification, the term "activity" as it pertains to a protein, polypeptide or polynucleotide means any cellular function, action, effect or influence exerted by the protein, polypeptide or polynucleotide, either by a nucleic acid sequence or fragment thereof, or by the protein or polypeptide itself or any fragment thereof. The cellular function, action, effect or influence may be effected by the protein, polypeptide or polynucleotide may be exerted directly or indirectly.

As used herein the term "associated with" when used in the context of a disease or condition "associated with" either apoptosis or senescence means that the disease or condition may result from, result in, be characterised by, or otherwise associated with apoptosis or senescence. Thus, the association between the disease or condition and apoptosis or senescence may be direct or indirect and may be temporally and/or spatially separated.

As used herein, the term "disease control" means the status of the disease or disorder, typically in light of intervention to treat the disease or disorder. Thus "disease control" describes the range and severity of symptoms and conditions experienced and suffered by patients as a result of their disorder. Disease control effectively provides a measure at a given point in time of the disease status of an individual, reflecting both current therapeutic treatment regimes used by the individual and the individual's recent experiences.

As used herein the term "effective amount" includes within its meaning a non-toxic but sufficient amount or dose of an agent or compound to provide the desired effect. The exact amount or dose required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

It will be understood that as used herein the term "expression" may refer to expression of a polypeptide or protein, or to expression of a polynucleotide or gene, depending on the context. The polynucleotide may be coding or non-coding. Expression of a polynucleotide may be determined, for example, by measuring the production of RNA transcript levels. Expression of a protein or polypeptide may be determined, for example, by immunoassay using an antibody(ies) that bind with the polypeptide.

The term "inhibiting" and variations thereof such as "inhibition" and "inhibits" as used herein do not necessarily imply the complete inhibition of the specified event, activity or function. Rather, the

inhibition may be to an extent, and/or for a time, sufficient to produce the desired effect. Inhibition may be prevention, retardation, reduction or otherwise hindrance of the event, activity or function. Such inhibition may be in magnitude and/or be temporal in nature. In particular contexts, the terms "inhibit" and "prevent", and variations thereof may be used interchangeably.

In the context of this specification, the term "inhibitor" refers to any agent or action capable of inhibiting either or both the expression or activity of SENEX, either directly or indirectly. Accordingly the inhibitor may operate directly or indirectly on the SENEX polypeptide, the corresponding mRNA or gene, or alternatively act via the direct or indirect inhibition of any one or more components of a SENEX - associated pathway. Such components may be molecules activated, inhibited or otherwise modulated prior to, in conjunction with, or as a consequence of SENEX activity. Thus, the inhibitor may operate to prevent transcription, translation, post-transcriptional or post-translational processing or otherwise inhibit the activity of SENEX or a component of a SENEX - associated pathway in any way, via either direct or indirect action. The inhibitor may for example be nucleic acid, peptide, any other suitable chemical compound or molecule or any combination of these. It will be understood that in indirectly impairing the activity of SENEX or a component of a SENEX - associated pathway, the inhibitor may effect the activity of molecules which regulate, or are themselves subject to regulation or modulation by, SENEX or a component of a SENEX - associated pathway.

Reference to "normal endogenous levels" in one context should be understood as a reference to the normal levels of expression and/or activity of SENEX, either polypeptide or polynucleotide, in normally dividing, non-senescent, or non-apoptotic cell, either of a particular cell type, tissue, individual or group of individuals. At the individual level, for example, it will be appreciated by those skilled in the art that this "normal endogenous level" is likely to correspond to a range of levels, as opposed to a singularly uniform discrete level, due to differences between cohorts of individuals. By "cohort" is meant a cohort characterised by one or more features which are also characteristic of the subject who is undergoing treatment. These features include, but are not limited to, age, gender or ethnicity, for example. Accordingly, reference herein to elevated or reduced SENEX levels relative to normal endogenous levels is a reference to increased or decreased SENEX levels relative to either a discrete level which may have been determined for healthy non-diseased cells of the individual, cells of normal individuals who are representative of the same cohort as the individual being treated, or relative to a defined range which corresponds to that expressed by a population of individuals corresponding to those from a range of different cohorts. It will also be appreciated that in the context of the inducement or inhibition of senescence or apoptosis in a cell, reference to "normal endogenous levels" should be understood as a reference to the level of SENEX in cells of a subject in which senescence or apoptosis (as appropriate) is normally regulated.

As used herein the term "polypeptide" means a polymer made up of amino acids linked together by peptide bonds. The terms "polypeptide" and "protein" are used interchangeably herein, although for the

purposes of the present invention a "polypeptide" may constitute a portion of a full length protein. The term "polynucleotide" as used herein refers to a single- or double-stranded polymer of deoxyribonucleotide, ribonucleotide bases or known analogues or natural nucleotides, or mixtures thereof. In some contexts in the present specification the terms "polynucleotide" and "nucleic acid molecule" are used interchangeably.

As used herein "*SENEX*" refers to the apoptosis- and senescence-associated gene described herein and "SENEX" refers to the protein or polypeptide encoded by this gene. Whilst typically referring to the gene and polypeptide as found in humans, or to derivatives, fragments or variants thereof, those skilled in the art will appreciate that homologues of human SENEX from other species are also contemplated and encompassed by the present disclosure. The cDNA encoding human SENEX is located in the National Center for Biotechnology Information (NCBI) database as RefSeq Accession No. NM033515 (GenBank Accession No. AB053293) and is referred to in RefSeq as ARHGAP18 and MacGAP.

The term "subject" as used herein refers to mammals and includes humans, primates, livestock animals (eg. sheep, pigs, cattle, horses, donkeys), laboratory test animals (eg. mice, rabbits, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animals (eg. foxes, kangaroos, deer). Typically, the mammal is human or a laboratory test animal. Even more typically, the mammal is a human.

Reference to "susceptibility" should be understood as a reference to both determining whether any existing symptoms associated with or indicative of a senescence- or apoptosis-associated disorder experienced by an individual are linked to abnormal SENEX levels as described herein and to determining whether individuals who have not experienced symptoms indicative of a senescence- or apoptosis-associated disorder nevertheless exhibit a predisposition or risk thereto. Thus, depending on the particular circumstances of a particular subject, the term "susceptibility" should be understood to mean vulnerability to a senescence- or apoptosis-associated disorder or having an increased likelihood of development of a senescence- or apoptosis-associated disorder in the future.

As used herein the terms "treating", "treatment", "preventing" and "prevention" refer to any and all uses which remedy a condition or symptoms, prevent the establishment of a condition or disease, or otherwise prevent, hinder, retard, or reverse the progression of a condition or disease or other undesirable symptoms in any way whatsoever. Thus the terms "treating" and "preventing" and the like are to be considered in their broadest context. For example, treatment does not necessarily imply that a patient is treated until total recovery. In conditions which display or a characterized by multiple symptoms, the treatment or prevention need not necessarily remedy, prevent, hinder, retard, or reverse all of said symptoms, but may prevent, hinder, retard, or reverse one or more of said symptoms. In the context of some disorders, methods of the present invention involve "treating" the disorder in terms of reducing or ameliorating the occurrence of a highly undesirable event associated with the disorder or an irreversible outcome of the progression of the disorder but may not of itself prevent the initial occurrence

of the event or outcome. Accordingly, treatment includes amelioration of the symptoms of a particular disorder or preventing or otherwise reducing the risk of developing a particular disorder.

Described herein is the identification and functional characterization of a gene that acts as a regulator of cellular senescence and apoptosis. The gene, designated *SENEX*, for senescence gene (based on the Latin, *senex* for old age), is demonstrated to provide a unique gatekeeper function in the stress-induced premature senescence (SIPS) and apoptosis pathways in endothelial cells. This is the first gene described which has the dual capacity to regulate this arm of cellular responses and suggests that modulation of the levels of *SENEX* are crucial in determining vascular function.

As exemplified herein, *SENEX*-induced senescent cells exhibit the established senescence criteria of inhibited proliferation, a flattened, large vacuolated cellular morphology, polyploidy, positive staining with the senescence marker β galactosidase and a reduction in the endothelial specific nitric oxide synthase, eNOS. *SENEX* is also shown to be highly regulated during *in vitro* angiogenesis, being downregulated at a time of lumen formation where apoptosis is involved and upregulated when vessels are being stabilized. Moreover, *SENEX*-induced senescent endothelial cells are not activated by TNFα to support neutrophil or mononuclear cell adhesion and display a significant inhibition of their inflammatory state indicating an anti-inflammatory effect of SENEX. The findings described herein of *SENEX* mRNA induction at sites of atherosclerosis suggest a relevant physiological role to limit or attenuate the chronic inflammatory response that is characteristic of such atherosclerotic regions. Furthermore, although senescence was originally considered to be a mechanism, together with apoptosis, for controlling cell proliferation and malignant transformation, the data described herein suggest that senescence plays a broader role in disease progression or resolution.

Accordingly, provided herein are methods for regulating whether a cell undergoes apoptosis or senescence, comprising modulating the level of expression an/or activity of SENEX in the cell, wherein increasing the expression and/or activity of SENEX relative to normal endogenous levels induces cellular senescence and decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

Also provided are methods of regulating senescence in a cell, comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell, wherein increasing the expression and/or activity of SENEX relative to normal endogenous levels induces cellular senescence. Further provided are methods of regulating apoptosis in a cell, comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell, wherein decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

In addition to increasing and decreasing levels of expression and/or activity of SENEX relative to normal endogenous levels in order to achieve a desired outcome, for example inducing senescence or

apoptosis in a cell, respectively, also contemplated herein is the modulation of SENEX levels by upregulating or downregulating SENEX levels in order to approach normal endogenous levels. In this way, a switch can be induced such that senescence can be inhibited or reversed by normalising upregulated levels of SENEX, and similarly apoptosis can be inhibited or reversed by normalising downregulated levels of SENEX. In "normalising" SENEX levels, it should be understood that the level need not necessarily be fully normalised in order to achieve the desired outcome, although complete normalisation may be a preferred result in some circumstances.

In accordance with embodiments disclosed herein, cells to which methods and compositions pertain may be any cell in which SENEX is expressed. In an embodiment the cell is an endothelial cell, although those skilled in the art will appreciate that the scope of the present disclosure is not so limited and other cell types are also contemplated.

Embodiments disclosed herein find application in a range of therapeutic settings, providing novel treatment and prophylactic options for senescence-associated disorders, apoptosis-associated disorders and diseases and conditions characterised by, or associated with, aberrant or otherwise unwanted cell growth and/or proliferation. Thus, depending on the circumstances, there is a wide range of clinical settings in which inducing or inhibiting sensescence, inducing or inhibiting apoptosis, and regulating the switch between senescence and apoptosis may be desirable.

In terms of downregulating, inhibiting or reversing senescence or apoptosis, embodiments disclosed herein find application in the treatment and prevention of senescence-associated and apoptosis-associated disorders. In the present context, senescence-associated disorders include any disorder which is fully or partially mediated by the induction or maintenance of a non-proliferating or senescent state in a cell or a population of cells in an individual or that is otherwise related to this non-proliferating or senescent state. Examples include coronary disease (including atherosclerosis), impaired wound healing, immune dysfunction, age-related tissue or organ decline, Alzheimer's disease, liver cirrhosis and immuno-senescence caused by chronic infection. Similarly, in the present context, an apoptosis-associated disorder is one that is fully or partially mediated by the induction of an apoptotic cell death way or otherwise related thereto. Examples include neurodegenerative diseases (such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis), haematologic diseases (such as aplastic anaemia) and tissue damage (such as myocardial infarction and ischaemia).

Circumstances in which it may be desirable to induce senescence or apoptosis in a cell by modulating the expression and/or activity of SENEX include in the treatment of cancer (for example in the suppression of tumourigenesis), vascular diseases (such as stenosis, restenosis, vascular complications associated with diabetes), airways inflammatory diseases (such as asthma, COPD, acute respiratory distress syndrome), other inflammatory diseases (such as rheumatoid arthritis, inflammatory bowel disease, diabetes, vasculitis), autoimmune diseases such as lupus, and viral infections.

In addition to therapeutic and prophylactic applications, it may be desirable to induce or upregulate the occurrence of senescence or apoptosis for example in an *in vitro* model or an animal model, in order to facilitate an outcome such as providing a system for screening for the effectiveness of adjunctive therapies, prophylactic therapies or for otherwise facilitating the ongoing analysis of diseases and conditions associated with senescence and apoptosis.

Also provided herein is the use of SENEX as an anti-inflammatory agent and methods for the inhibition of inflammation in a subject comprising administering to the subject an effective amount of an agent capable of upregulating the expression and/or activity of SENEX in at least one cell of the subject, relative to normal endogenous levels. Inflammatory conditions to which these embodiments may be applied include chronic inflammatory conditions such as airways inflammatory diseases bronchial asthma, COPD, acute respiratory distress syndrome rheumatoid arthritis, inflammatory bowel disease, diabetes and vasculitis.

The above-listed disorders, diseases, and conditions are provide by way of example only, and those skilled in the art will appreciate that the methods and compositions disclosed herein are not limited to their application to these exemplary indications, but are ore generally applicable to the treatment or prevention of any disorder, disease, and condition in which the inducement or inhibition of senescence or apoptosis may be desirable.

It should be understood that the methods disclosed can be performed either *in vitro* or *in vivo*. Although methods are typically to therapeutically or prophylactically treat an individual *in vivo* in order to achieve a desired clinical outcome, it should nevertheless be understood that it may be desirable that a method of the invention be applied in an *in vitro* or *ex vivo* environment. For *ex vivo* treatment cells may, for example, be removed from an individual, transfected with a SENEX polynucleotide, or fragment, variant or homologue thereof, and re-introduced into the individual in need of treatment. The cells to be transfected may be autologous or allogeneic.

As defined herein, reference to SENEX should be understood as a reference to all forms of the polypeptide molecule and to functional derivatives and homologues thereof. This includes, for example, any isoforms which may arise from alternative splicing of the subject SENEX mRNA or functional mutants or polymorphic variants of these proteins.

Embodiments of the invention contemplate the administration of SENEX, or a fragment, variant or homologue thereof. The SENEX may be derived from a human and may comprise an amino acid sequence as set forth in SEQ ID NO: 1, or be encoded by a polynucleotide comprising a nucleotide sequence as set forth in SEQ ID NO: 2. The SENEX may be administered as a polypeptide or

polynucleotide. The present invention also contemplates the use of fragments, variants and homologues of human SENEX.

The polynucleotide may be natural, recombinant or synthetic and may be obtained by purification from a suitable source or produced by standard recombinant DNA techniques such as those well known to persons skilled in the art, and described in, for example, Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press (the disclosure of which is incorporated herein by reference). Where a polynucleotide encoding SENEX is administered, the polynucleotide is typically present in a vector operably linked to suitable regulatory sequences capable of providing for the expression of the coding sequence by a cell. The term "regulatory sequence(s)" includes promoters and enhancers and other expression regulation signals. These may be selected to be compatible with the cell for which the expression vector is designed. Mammalian promoters, such as β-actin promoters and the myosin light chain promoter may be used. However, other promoters may be adopted to achieve the same effect. These alternate promoters are generally familiar to the skilled addressee. The vector containing a *SENEX* polynucleotide may be episomal such that the polynucleotide contained therein is maintained extra-chromosomally, thereby eliminating any potential detrimental effects of chromosomal integration. Alternatively, the vector may enable or facilitate integration of the *SENEX* polynucleotide into the genome. The choice of an episomal or integrating vector will depend largely on the particular circumstances, for example the cell type to be transfected and whether transient or long term expression of SENEX is desired.

The term "variant" as used herein refers to substantially similar sequences. Generally, polypeptide sequence variants possess qualitative biological activity in common. A variant may take any form and may be naturally or non-naturally occurring. A variant polypeptide sequence may be a derivative of a sequence as disclosed herein, which derivative comprises the addition, deletion, or substitution of one or more amino acids. For example, variants of the human SENEX disclosed herein may possess about 70% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. The variant may comprise amino acid sequences having at least about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence set forth in SEQ ID NO: 1. The term "variant" encompasses polypeptide sequences modified from those disclosed herein by any suitable means.

Variants may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterised by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in a sequence has been removed and a different

residue inserted in its place. Additions to amino acid sequences include fusions with other peptides, polypeptides or proteins, as detailed above. Modifications may be made to the *SENEX* polynucleotide sequence, for example via the insertion or deletion of one or more codons, such that modified derivatives of the SENEX polypeptide are generated. Such modifications are also included within the scope of the term "variant". For example, modifications may be made so as to enhance the biological activity or expression level of SENEX or to otherwise increase the effectiveness of the polypeptide to achieve the desired result.

As used herein a "homologue" means that the molecule is derived from a species other than that which is being treated in accordance with the method of the present invention. This may occur, for example, where it is determined that a species other than that which is being treated produces a form of SENEX which exhibits similar and suitable functional characteristics to that of the SENEX which is naturally produced by the subject undergoing treatment.

Similarly, the terms fragment, variant and homologue as used herein are also applicable to nucleotide sequences with similar scope as defined above.

Particular embodiments disclosed herein contemplate the administration of one or more agents capable of inhibiting or reducing the expression and/or activity of SENEX. Such inhibitors may directly or indirectly effect SENEX expression and may act at the level of the *SENEX* gene or any products thereof including mRNA (precursor or mature message) or polypeptide. The inhibitor may be, for example, a proteinaceous or non-proteinaceous molecule that modulates the transcription and/or translation of the gene or a functional portion thereof (such as a promoter region), or alternatively that modulates the transcription and/or translation of an alternative gene or functional portion thereof, which alternative gene or gene product directly or indirectly modulates the expression of SENEX. The inhibitory agent may be an antagonist. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing SENEX from carrying out its normal biological functions. For the present purposes, the term "antagonist" is used hereinafter to refer to inhibitors of polypeptide activity and expression.

A variety of suitable antagonists may be employed and the scope of the invention is not limited by the selection of any one particular molecule or compound. Suitable antagonists include antibodies, such as monoclonal antibodies, and antisense nucleic acids which prevent transcription or translation of genes or mRNA. Modulation of expression may also be achieved utilising antigens, RNA, ribosomes, DNAzymes, aptamers, antibodies or molecules suitable for use in cosuppression.

Suitable antibodies include, but are not limited to polyclonal, monoclonal, chimeric, humanised, single chain, Fab fragments, and a Fab expression library. Antibodies may act as antagonists of a SENEX polypeptide, or fragment, variant or homologue thereof. Typically, antibodies are prepared from discrete regions or fragments of the polypeptide. Methods for the generation of suitable antibodies will be

readily appreciated by those skilled in the art. For example, a suitable monoclonal antibody may be prepared using the hybridoma technology described in Antibodies-A Laboratory Manual, Harlow and Lane, eds., Cold Spring Harbor Laboratory, N.Y. (1988), the disclosure of which is incorporated herein by reference.

Suitable antisense constructs for use in accordance with the present disclosure include antisense polynucleotides and oligonucleotides, small interfering RNAs (siRNAs) and catalytic antisense nucleic acid constructs. For example, a reduction in SENEX expression may be achieved by the introduction of the *SENEX* polynucleotide in antisense orientation or the introduction of specific antisense oligonucleotides. Suitable antisense oligonucleotides may be prepared by methods well known to those of skill in the art. Typically oligonucleotides will be chemically synthesized on automated synthesizers. Those skilled in the art will readily appreciate that antisense oligonucleotides need not display 100% sequence complementarity to the target sequence. One or more base changes may be made such that less than 100% complementarity exists whilst the oligonucleotide retains specificity for its target and retains antagonistic activity against this target. Suitable antisense oligonucleotides include morpholinos where nucleotides comprise morpholine rings instead of deoxyribose or ribose rings and are linked via phosphorodiamidate groups rather than phosphates.

An alternative antisense technology, known as RNA interference (RNAi), see, eg. Chuang *et al.* (2000) *PNAS USA* 97: 4985) may be used, according to known methods in the art (for example Hammond *et al.* (2000) *Nature* 404: 293-296; Bernstein *et al.* (2001) *Nature* 409: 363-366; Elbashir *et al* (2001) *Nature* 411: 494-498; WO 99/49029 and WO 01/70949, the disclosures of which are incorporated herein by reference), to inhibit the expression or activity of nucleic acid molecules. RNAi refers to a means of selective post-transcriptional gene silencing by destruction of specific RNA by small interfering RNA molecules (siRNA). The siRNA is generated by cleavage of double stranded RNA, where one strand is identical to the message to be inactivated. Double-stranded RNA molecules may be synthesised in which one strand is identical to a specific region of the target transcript and introduced directly. Alternatively corresponding dsDNA can be employed, which, once presented intracellularly is converted into dsRNA. Methods for the synthesis of suitable molecules for use in RNAi and for achieving post-transcriptional gene silencing are known to those of skill in the art.

A further means of inhibiting the expression or activity of SENEX may involve introducing catalytic antisense nucleic acid constructs, such as ribozymes, which are capable of cleaving a SENEX mRNA transcript. Ribozymes are targeted to and anneal with a particular sequence by virtue of two regions of sequence complementarity to the target flanking the ribozyme catalytic site. After binding the ribozyme cleaves the target in a site-specific manner. The design and testing of ribozymes which specifically recognise and cleave SENEX mRNA sequences can be achieved by techniques well known to those in the art (for example Lieber and Strauss, (1995) *Mol. Cell. Biol.* 15:540-551, the disclosure of which is incorporated herein by reference).

If desired, agents for use in accordance with the present invention may be fused to other compounds, including peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, agents may be fused to molecules to facilitate localisation to the airway tissue.

Pharmaceutical compositions

Agents may be administered in accordance with the present invention in the form of pharmaceutical compositions, which compositions may comprise one or more pharmaceutically acceptable carriers, excipients or diluents. Such compositions may be administered in any convenient or suitable route such as by parenteral (e.g. subcutaneous, intraarterial, intravenous, intramuscular), oral (including sublingual), nasal or topical routes. In circumstances where it is required that appropriate concentrations of the desired agent are delivered directly to the site in the body to be treated, administration may be regional rather than systemic. Regional administration provides the capability of delivering very high local concentrations of the desired agent to the required site and thus is suitable for achieving the desired therapeutic or preventative effect whilst avoiding exposure of other organs of the body to the compound and thereby potentially reducing side effects.

It will be understood that the specific dose level of a composition of the invention for any particular individual will depend upon a variety of factors including, for example, the activity of the specific agents employed, the age, body weight, general health and diet of the individual to be treated, the time of administration, rate of excretion, and combination with any other treatment or therapy. Single or multiple administrations can be carried out with dose levels and pattern being selected by the treating physician. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of agent may be administered per kilogram of body weight per day. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

Examples of pharmaceutically acceptable carriers or diluents are demineralised or distilled water; saline solution; vegetable based oils such as peanut oil, safflower oil, olive oil, cottonseed oil, maize oil, sesame oil, arachis oil or coconut oil; silicone oils, including polysiloxanes, such as methyl polysiloxane, phenyl polysiloxane and methylphenyl polysolpoxane; volatile silicones; mineral oils such as liquid paraffin, soft paraffin or squalane; cellulose derivatives such as methyl cellulose, ethyl cellulose, carboxymethylcellulose, sodium carboxymethylcellulose or hydroxypropylmethylcellulose; lower alkanols, for example ethanol or iso-propanol; lower aralkanols; lower polyalkylene glycols or lower alkylene glycols, for example polyethylene glycol, polypropylene glycol, ethylene glycol, propylene glycol, 1,3-butylene glycol or glycerin; fatty acid esters such as isopropyl palmitate, isopropyl myristate or ethyl oleate; polyvinylpyrridone; agar; carrageenan; gum tragacanth or gum acacia, and petroleum jelly. Typically, the carrier or carriers will form from 10% to 99.9% by weight of the compositions.

Pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. The formulation must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of superfactants. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a

disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

The present invention contemplates combination therapies, wherein agents as described herein are coadministered with other suitable agents that may facilitate the desired therapeutic or prophylactic outcome. For example, in the context of cancer, one may seek to maintain ongoing anti-cancer therapies such as chemotherapy or radiotherapy whilst employing agents in accordance with embodiments of the present invention to inhibit or reduce tumour angiogenesis and/or tumour metastasis. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. By "sequential" administration is meant a time difference of from seconds, minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

Provided herein are methods for diagnosing senescence- and apoptosis-associated disorders, or predicting the susceptibility of a subject to such disorders, comprising obtaining a biological sample from a subject and determining the level of expression and/or activity of SENEX in the sample, wherein increased levels of SENEX expression and/or activity relative to normal endogenous levels is diagnostic or predictive of a senescence-associated disorder, whereas decreased levels of SENEX relative to normal endogenous levels is diagnostic or predictive of an apoptosis-associated disorder.

Embodiments disclosed herein also provide methods for evaluating disease control, monitoring disease control in a subject over time, evaluating the efficacy of a treatment or disease management regime and designing an appropriate treatment or disease management regime for an individual. Determination of disease control in a subject, in particular the monitoring of control over time, can facilitate decision making with respect to the most appropriate intervention or treatment regime for an individual subject. The treatment regime will typically be tailored so as to obtain normalisation in the level of expression and/or activity of SENEX, either increasing SENEX levels in the case of apoptosis-associated disorders and decreasing SENEX levels in the case of senescence-associated disorders. For example, this may comprise introducing a new treatment regime or modifying an existing regime with a view to improving

disease symptoms or other parameters. The modification of a regime may be modification with respect to any one or more of a variety of factors, such as the nature of any existing medication, the dosage thereof, the timing of administration and/or any supplementary disease management strategies. Such decision making with respect to treatment regimes will vary from case to case and the determination of the most appropriate strategy is well within the expertise and experience of those skilled in the art.

A treatment regime for the treatment of a senescence- and apoptosis-associated disorder in a subject as disclosed herein may involve administration of any medications commonly utilised in the treatment of the particular disorder in question and/or may involve a variety of other physical medical, psychological and/or psychiatric treatments. In the case of drug administration, the treatment regime may comprise the administration of a number of drugs simultaneously, sequentially, or in combination with each other. The type of drug(s) administered, dosage, and the frequency of administration can be determined by those directing the administration of the drugs in accordance with accepted medical principles, and will typically depend on factors such as the severity of the disease, the age and weight of the subject, the medical history of the subject, other medication being taken by the subject, existing ailments and any other health related factors normally considered when determining treatments.

In determining disease control, evaluating the efficacy of a treatment or disease and in diagnosing senescence- and apoptosis-associated disorders, or predicting susceptibility to such disorders reference may be had to one or more control samples. In this context, the term "control" or "control sample" may refer to one or more biological samples from individuals or groups of individuals diagnosed as not having the senescence- or apoptosis-associated disorder(s) for which a subject is being assessed, or alternatively from individuals or groups of individuals diagnosed as having a specific senescence- or apoptosis-associated disorder. A "control sample" may comprise the compilation of data from one or more individuals whose diagnosis as a "control" for the purposes of the present invention has been confirmed. That is, for the purposes of practicing embodiments of the present invention samples to be used as controls need not be specifically or immediately obtained for the purpose of comparison with the sample(s) obtained from the subject under assessment.

The present invention also provides kits suitable for use in accordance with the methods of the invention. Such kits may include for example diagnostic kits for assaying biological samples, comprising an agent for detecting SENEX, or encoding nucleic acid molecules, and reagents useful for facilitating the detection by the agent(s). Further means may also be included, for example, to receive a biological sample. The agent(s) may be any suitable detecting molecule. Kits according to the present invention may also include other components required to conduct the methods of the present invention, such as buffers and/or diluents. The kits typically include containers for housing the various components and instructions for using the kit components in the methods of the present invention.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations

and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the present application. Further, the reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

The present invention is further described by reference to the following non-limiting examples.

Examples

General methods

Human umbilical vein endothelial cells (HUVECs)

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described (Meyer *et al.*, 1997) and cultured in gelatin-coated flasks in medium 199 (M199) with Earles' balanced salts and 0.68 mM glutamine, 20 mM HEPES, 20% FCS, 15 ug/ml endothelial cell growth supplement (ECGS; BD Biosciences), 50 U/ml penicillin, 50 U/ml streptomycin, and 15ug/ml heparin. HUVECs were used between passage 1 - 3.

For immunoprecipitation and immunoblotting experiments, HUVECs were lysed in ice-cold lysis buffer (50 mM Tris.HC1, pH 7.4, with 1% NP-40, 150 mM NaCl, 2 mM EGTA, 1 mM $NaVO_4$, 100 mM NaF, 10 mM $Na_4P_2O_7$, and protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of protein were loaded onto 10% polyacrylamide gels, separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim milk powder and 0.1% Triton-X 100 in phosphate-buffered saline (PBS), and probed with mouse monoclonal anti-beta actin (Sigma-Aldrich), monoclonal mouse anti $21^{Cip1/WAF1}$ (Zymed Laboratories), mouse monoclonal anti-human p53 (Invitrogen), mouse monoclonal anti-eNOS/NOS Type III (BD Biosciences), rabbit polyclonal anti Phospho-Rb (Ser807/811) (Cell Signaling Technologies), rabbit polyclonal anti-p 16 (C-20): sc-468 (Santa Cruz Biotechnology). After washing, membranes were incubated with anti-rabbit or anti-mouse secondary antibody and reactive bands were detected by chemiluminescence (ECL Western Blotting Detection Reagents; (Amersham Pharmacia Biotech)).

For DAPI staining HUVECs were plated on fibronectin coated labtek slides (Invitro Technologies) at $6x10^4$ for 24 h. They were then fixed with 100% methanol and stained with Dapi (1 µg/ml) (Sigma-Aldrich) for 15 mins at 37°C, then washed and mounted.

For cell cycle analysis, detached cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at -20°C for 30 mm and stained at 37°C for 30 mm in 1 ml of PBS containing 20 µg/ml propidium iodide and 20 ig/ml RNase A. Cells were analyzed using a FACS Canto, FACS Diva for data acquisition (BD Biosciences) and FlowJo software (Tree Star) for data analysis.

DNA manipulation and mRNA analysis

Virtual Northern Blot analysis and generation of microarray data was as decribed in Hahn *et al.* 2005.

The AdEasy system (Qbiogene) was used to produce recombinant adenovirus carrying human *SENEX* (*SENEX*) or empty vector (EV) according to the Qbiogene Version 1.4 AdEasy Vector system manual. Equivalent plaque forming units (pfu/cell) were adjusted to yield a similar level of green fluorescent protein (GFP) expression as determined by FACS analysis.

PCDNA3-*SENEX* constructs were transfected into HUVECs using the Amaxa nucleofector kit according to the manufacturer's protocol (Amaxa Biosystems). siRNA transfection of HUVECs was achieved using validated Stealth siRNAs (50 nM (Invitrogen)) in parallel with corresponding non-specific Stealth siRNA negative control (Invitrogen). The cells were transfected using HiPerFect transfection reagent according to the manufacturer's protocol (Qiagen).

For RT-PCR, total RNA was isolated using the RNeasy mini prep kit (QIAGEN). Two µg RNA was DNase I treated (Promega) and then reverse transcribed in a final volume of 20 µl using Superscript II RT (Invitrogen) according to the manufacturer's instructions. After reverse transcription, sample volumes were increased to 200 µl with TE buffer, and 2 µl of the cDNA was used for subsequent quantitative PCR. Primers are as follows:

Cyclophilin A

Forward – GGCAAATGCTGGACCCAACACAAA (SEQ ID NO:3)

Reverse – CTAGGCATGGGAGGGAACAAGGAA (SEQ ID NO:4)

SENEX

Forward - TTGCTCTGTTTTCCAGATTGGA (SEQ ID NO:5)

Reverse - GCCCCAGTGCTTGAGGCT (SEQ ID NO:6)

PAII

Forward - GGTAGGGCACAAAGATGGATGA (SEQ ID NO:7)

Reverse - CCCAGGCTGGTCTTGAACTC (SEQ ID NO:8)

IL-1α

Forward - TTACCTGGGCATTCTTGTTTCA (SEQ ID NO:9)

Reverse - CAGTGGTCTCATGGTTGTCAAAGT (SEQ ID NO:10)

Cox2

Forward - TGTTATTAACATTGATCTGCTGACAAAA (SEQ ID NO:11)

Reverse - ACACATTTGTCTGAGGCACTGAA (SEQ ID NO:12)
p16
Forward - TGCCTTTTCACTGTGTTGGAGTT (SEQ ID NO:13)
Reverse – GCAAGAAATGCCCACATGAAT (SEQ ID NO:14)

The threshold cycle values for all genes were normalized to the threshold cycle values determined for *Cyclophilin A*. All real-time RT-PCR assays were repeated at least three times from independent RNA preparations.

For analysis of telomere length, genomic DNA was extracted from cells using a DNA extraction kit (Qiagen). Twenty µg of DNA was digested with *Hin*fI and *Rsa*I (Boehringer Mannheim). The digested DNA was quantified by nanodrop (Thermo Scientific) and 1.0 µg was electrophoresed through a 0.8% agarose gel in lx Tris-acetate-EDTA (TAE) buffer at 2 V/cm for 17 h. The gel was dried at 60°C for 2 h, denatured for 30-60 mm in 0.5 M NaOH and 1 .5 M NaCl and neutralized for 30-60 min in 1 M Tris-HCl, pH 8.0 and 1.5 M NaCl. The gel was then hybridized to a [γ-^{32}P]dATP 5' end-labeled telomeric oligonucleotide probe [γ-^{32}P-(TTAGGG)$_3$]. Hybridization and washing were carried out as previously described (Counter *et al.*, 1992). The gel was autoradiographed on Kodak XAR-5 X-ray film for 12-24 h at room temperature.

Cellular function and activity assays

Replicative senescence - HUVECs were maintained under subconfluent conditions at all times and were passaged every 3-4 days. During passaging cells were lifted using 0.5% (w/v) trypsin and between 0.6x10^6 and 1x10^6 cells were replated onto fresh 75-cm^2 flasks.

Matrigel tube formation assay- The capillary tube formation assay was performed as previously described (Gamble *et al.*, 1999). Briefly, 6.4x10^4 cells/160 µl HUVEC medium were plated onto 100µl of gelled Matrigel (Becton Dickinson) in 96 well plates and visualized over the following 24 h.

Cell proliferation assay - HUVECs were plated at 3x10^3 cells per well in 96-well plates and cell numbers assessed with the MTS assay (Promega,) on Day 0 and Day 3.

β-galactosidase assay - Acidic β-galactosidase activity was detected using the manufacturer's method (Cell Signaling Technology).

Caspase 3 activity assay - Cell lysates were prepared and the caspase 3 assay was performed essentially as given in the manufacturer's protocol (Calbiochem-Novabiochem). Fluorescence was measured at excitation and emission wavelengths of 385 nm and 460 nm, and normalized for the protein concentration.

Transwell permeability assay - Polycarbonate membrane (3µm) transwells (Corning Incorporated) were

coated with 50 µg/ml fibronectin. Three x10^5 passage two HUVECs in complete medium were added to each well and incubated for 24 h after which another 1x10^5 cells were added to each well to produce a confluent monolayer. After 6 h, non-adhered cells were removed and the monolayer was infected with adenovirus (EV or *SENEX*) at an MOI sufficient to give 100% infection but without toxicity. After 48 h, the medium in both upper and lower wells was replaced with HUVEC medium containing 2% serum. To the upper well, 300 ng of FITC-dextran (Sigma-Aldrich) was added that contained 1.0U/ml thrombin (Sigma-Aldrich) where appropriate. 40 µl of medium was removed from the bottom well of each assay well after 50 minutes and fluorescence was quantified using a Wallac luminescence spectrometer (excitation 485 nm, emission 530 nm).

Neutrophil and mononuclear cell adhesion assay - Neutrophils and mononuclear cells were prepared from fresh blood from healthy volunteers. Blood was dextran sedimented, cells were separated by Histopaque (Sigma-Aldrich) gradient centrifugation. The buffy coat was collected and purified to obtain mononuclear cells and the neutrophils were purified from the cell pellet with hypotonic lysis of the remaining red cells. HUVECs were plated on fibronectin coated labtek slides (Invitro technologies) at 6x10^4 for 24 h. Monolayers cultured on the slides were preincubated with TNFα (5 ng/ml) for 5 h before the assay, and then washed. Neutrophils and mononuclear cells (10^6) were added to the HUVECs. After 60 min the wells were washed and photographed and counts of adherent cells were made.

Statistics

Statistical analyses using a two-tailed Student's *t* test and 2 way ANOVA with Bonferroni posttest were performed using Prism software (version 4; GraphPad Software, Inc.). Data that satisfy confidence levels of $P < 0.05$, 0.01 or 0.001 are noted. Data are presented as means ±SEM, unless otherwise noted.

Example 1 – Isolation of *SENEX*

A PCR based suppression subtractive hybridization approach was used to isolate genes involved in the process of capillary tube formation, as an *in vitro* model of angiogenesis. Using HUVECs as starting material, virtual Northern Blot analysis and generation of microarray data was as described in Hahn *et al.* 2005.

One clone was identified as having an interesting and unique expression pattern in that it was down regulated during the stage of lumen formation but upregulated thereafter. The cDNA was isolated and cloned from endothelial cells (EC) and a search of the National Center for Biotechnology Information (NCBI) (February 2004) identified a 3305-bp cDNA (RefSeq Accession No.NM033515, GenBank Accession No. AB053293) that encoded a hypothetical protein of 663 aa (75 kD). Western blot analysis showed a molecular weight of approximately 80kD suggesting possible modification (such as glycosylation). This protein contains a putative RhoGAP domain spanning amino acids 340-520 but does not contain any other known protein domains. The gene has been classified in RefSeq as

ARHGAP18 or MacGAP. The gene is herein referred to as *SENEX*, and the encoded protein as SENEX. The nucleotide sequence is shown in SEQ ID NO:2 and the amino acid sequence of the encoded polypeptide sequences is shown in SEQ ID NO:1.

Example 2 – *SENEX* in angiogenesis and apoptosis

To determine whether *SENEX* is important in endothelial cell function, HUVECs were infected with adenovirus containing constructs of *SENEX* in the antisense orientation or empty vector (EV) as control, and analyzed for effects on capillary tube formation on Matrigel.

The EV control cells formed tubes normally (Figure 1Ai). In contrast, cells infected with antisense SENEX-containing adenovirus failed to form capillary tubes (Fig 1Aii) with changes evident as early as 45 min after plating. Although they initially aligned, the antisense infected cells failed to join and were unable to form tubes and the cells appeared to undergo apoptosis. This apoptosis was confirmed either with antisense adenovirus (data not shown) or with the use of siRNA where there was a depletion of *SENEX* mRNA expression by 70% (Figure 1B) and at the protein level by 75% (Figure 1C).

Such depletion caused a >2 fold increase in apoptosis as measured by caspase 3 expression (Figure 2A) and confirmed with DAPI stain (Figure 2B). Thus *SENEX* expression is essential for EC survival. Interestingly, the mRNA expression profile as determined by Virtual Northern blots for the expression of this gene during tube formation showed that it was downregulated at the 3-6 hour time points (Figure 2C and 2D) where apoptosis is known to occur and contribute to lumen formation.

Example 3 – SENEX induces senescence

Using the adenovirus system for gene delivery overexpression of *SENEX* of 5-10 fold after 24 hours and 15-25 fold after 48 hours was routinely achieved compared to basal levels of *SENEX* when the dose of virus was adjusted to achieve an infectivity of 18 pfu per cell (Figure 3A). This level of overexpression did not effect capillary tube formation when measured within 48 hours of infection with the adenovirus (Figure 3B). However, under normal culture conditions large flattened cells became apparent which often contained large vacuoles and exhibited polyploidy (Figure 3C, D and E), reminiscent of senescent cells.

To confirm the presence of senescent cells, the cells were stained for the classic marker of senescence, senescence associated β galactosidase (SA-β-gal). Infection with *SENEX*-containing adenovirus significantly increased the number of HUVECs with SA-®-gal activity compared with the EV (Figure 4A). This induction of senescence was independent of the use of adenovirus since a similar morphology in the cells was obtained when overexpression was achieved through transfection of plasmid (Figure 4B and 4C).

Overexpression of *SENEX* inhibited the proliferation of HUVECs (Figure 5A) consistent with senescent cells that enter a state of irreversible growth arrest. There was a 61 ± 4% reduction in cell numbers (n = 4) with a 5.8+/- 2% increase in cells in the G1 phase and 3+/-1% decrease in the S and G2 phases as assessed by flow cytometry at 24 hours after transfection, confirming the arrest is at the G1 phase of the cell cycle. *SENEX*-induced senescent cells also showed the characteristic increase in eNOS expression (Figure 5B) as has been reported previously for senescent endothelial cells (Kumazaki *et al.*, 1993). Thus, on the basis of morphology, expression of β-gal, cell cycle arrest and eNOS expression, the present data demonstrates that overexpression of *SENEX* induces senescence in endothelial cells.

SENEX is a member of the RhoGAP family of proteins. To determine whether the GAP domain is essential for *SENEX* induced senescence, an R365A mutant was generated. This mutation eliminated the Rho activity (data not shown). However, overexpression of this mutant protein was still able to confer the senescence phenotype on HUVECs (Figure 6) suggesting that the GAP domain is not essential for this aspect of SENEX function.

Overexpression of *SENEX* induced senescence within 24 hours using gene delivery through adenovirus (Figure 7A) or by transient transfection. The senescent cells are long lived compared to normal HUVECs as the *SENEX* overexpressing cells have been maintained out to 2 months. Given the rapidity of this induction, it is unlikely that it is a replicative form of senescence and this was confirmed using two criteria. First, the inventors investigated telomere length by Southern blot analysis in *SENEX* induced senescent EC. Cells were infected with adenovirus containing either EV or *SEEN*. 72 hours later, senescent cells were enriched by light trypsinisation and then analysed for telomere length. It was found that there was no change in telomere length of HUVECs (Figure 7B) overexpressing *SENEX* compared to control EV. Second, replicative senescence was induced in the HUVECs by repeatedly passaging the cells. Senescence was evident after 15-20 passages. These replicative cells displayed increased expression of 3 genes considered markers of replicative senescent endothelial cells, plasminogen inhibitor-1 (PAI-1), interleukin-1< (IL-<) and cycloxygenase 2 (COX2). However, there was no concurrent increase in *SENEX* expression (Figure 7C). Furthermore, when senescence was induced by *SENEX*, there was no significant change in the levels of PAI-1, COX2 or IL-1α (Figure 7D). Together, these results suggest that *SENEX* is not involved in the replicative senescence pathway.

The p53/p21 pathway and the p16/Rb pathway have been implicated in senescence induction. HUVECs were harvested 48 hours after infection with the *SENEX* adenovirus at a time when 40-50% of the cells displayed a senescent morphology. Immunoblotting showed that there was no change in the protein expression of p53 or p21 (Figure 8A). *SENEX* overexpression did however induce an increase in both the mRNA (Figure 8B) and protein levels for p16 (Figure 8C), and there was a decrease in the protein expression of the hyperphosphorylated Rb (Figure 8C). These results indicate that *SENEX* activates the p16/pRb pathway.

Example 4 – H_2O_2 induces SENEX expression and senescence

H_2O_2, a ROS implicated in cardiovascular disease and in cancer, is an inducer of senescence when delivered in a subcytotoxic dose. The senescence that results from H_2O_2 occurs through the oxidative stress pathway, without a shortening in telomere length, similar to that seen for *SENEX*. For this reason, the inventors investigated whether H_2O_2 regulates *SENEX* expression.

Subcytotoxic doses of H_2O_2 (10μM and 100μM) were administered to HUVECs for 2 hrs and the cells cultured in fresh normal medium for a further 24-48 hours. H_2O_2 induced senescence as judged by ®-gal staining (Figure 9A). Furthermore, these cells showed an increase in *SENEX* protein (Figure 9B). Conversely, high dose H_2O_2 is known to induce endothelial cell apoptosis (confirmed herein – data not shown). As shown in Figure 9C, high dose H_2O_2 (500 μM) is also associated with an inhibition in the expression of *SENEX*. In summary, the results presented herein demonstrate that a reduction in *SENEX* levels *per se* induced apoptosis in endothelial cells.

The inventors have also demonstrated that *SENEX* is a TNFα responsive gene. TNFα treatment caused a down regulation of *SENEX* expression (Figure 10A and B) and induced EC apoptosis as measured by caspase 3 levels (Figure 10C). However, overexpression of *SENEX* protected against this TNFα induced apoptosis. Interestingly, *SENEX* overexpression did not protect against serum deprivation (Figure 10C) nor did the level of *SENEX* change with serum stimulation (Figure 10D).

Considering the data described in Examples 2 to 4, the fact that overexpression of *SENEX* induces senescence whereas depletion induces apoptosis highlights the fact that the *SENEX* dosage (or a more complicated change in its subcellular distribution) governs a balance between these two vital cellular mechanisms. The changes induced by oxidative stress in the form of H_2O_2 support this notion, since low doses of H_2O_2 induce *SENEX* and senescence, whereas higher doses inhibit SENEX expression and induce apoptosis.

Example 5 – SENEX induced senescent EC show anti-inflammatory properties

To determine the inflammatory phenotype of the *SENEX* induced endothelial cells, the inventors tested the capacity of HUVECs to support neutrophil adhesion. As shown in Figure 11A, there was a striking lack of neutrophil adhesion to TNFα-stimulated morphologically enlarged senescent HUVECs. HUVECs which had been transfected with *SENEX* but did not show the change in cell size in general displayed levels of neutrophil attachment similar to that seen with control cells. Quantification based on the number of neutrophils attached per large senescent cell versus non-enlarged EC occupying the same area showed a 75% inhibition in the capacity of neutrophils to adhere to senescent HUVECs (Figure 11A and 11C). There was no preferential binding of the neutrophils to the junctions of the senescent cells and little or no neutrophil transmigration across the senescent HUVECs was observed (data not shown).

A similar lack of mononuclear cell adhesion was also observed (Figure 11B and 11D). Neutrophil attachment is mediated predominantly through the adhesion molecule E selectin. The *SENEX* induced senescent cells displayed little or no cell surface expression of E selectin or VCAM in response to TNFα stimulation. Furthermore FLOW analysis also demonstrated a significant reduction in expression levels of VCAM (Figure 11F) and E selectin (data not shown).

Inflammation is also associated with an increase in the permeability of the endothelium. As measured by the passage of FITC-dextran across the monolayer, the *SENEX*-induced senescent HUVECs had a lower response to the permeability inducing agent thrombin than did the control EV infected cells (Figure 11E). There was no alteration in the levels of PAR-1, the receptor for thrombin with *SENEX* overexpression (data not shown). Together, these results demonstrate that *SENEX*-induced senescent endothelial cells have a profound inhibition of their inflammatory state although the mechanism does not appear to be through the classic regulators of adhesion.

Similar to *SENEX*-induced senescent cells, H_2O_2 induced senescent cells also displayed this anti-inflammatory phenotype as there was no cell surface expression of E selectin or VCAM following TNF stimulation (Figure 12A,B,C) confirming the biological importance of the program induced by SENEX. Together, these results demonstrate that *SENEX* induced senescent endothelial cells have a profound inhibition of their inflammatory state. Increases in oxidative stress are associated with atherosclerosis and are postulated to lead to endothelial cell damage and aging. Furthermore, senescent endothelial cells, as judged by β galactosidase positivity, have been detected in human atherosclerotic plaques and in vascular cells in injured rabbit carotid arteries. To determine whether SENEX is regulated during atherosclerosis, the inventors investigated the mRNA levels in the aortic region of apoE gene knockout mice fed a Western diet. The animals were perfused with cold phosphate-buffered saline. The aortas (thoracic and abdominal) were excised and immediately transferred into RNAlater (Ambion), stored for 24 hours at 4°C, and then stored at -80°C. Aortas from four (1-month time point) and five animals each (5-months time point) were pooled to obtain n=5 and n=2 separate pools for the 1-month and 5-months time point, respectively. Total RNA was isolated from each pool of samples, cDNA prepared, and quantitative reverse-transcription polymerase chain reaction performed as described previously (Choy *et al.*, 2005). No change was seen in *SENEX* levels after 1 month of Western diet. However after 5 months on the diet there was an increase in the levels of *SENEX* in relation to PBGD, HRPT or Tie2 (Figure 12D).

Example 6 – SENEX in human disease

The neoangiogenic vessels from human endarterectomy samples were analysed for SENEX expression by immunohistochemistry using a polyclonal antibody raised against a peptide fragment of SENEX. From 25 individual samples analysed, all had positive staining for SENEX expression of the endothelial cells in the neoangiogenic vessels found within the necrotic core of the samples (data not shown).

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Claims

1. A method for regulating whether a cell undergoes apoptosis or senescence, the method comprising modulating the level of expression an/or activity of SENEX in the cell, wherein increasing the expression and/or activity of SENEX relative to normal endogenous levels induces cellular senescence and decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

2. The method of claim 1 wherein the cell is an endothelial cell.

3. The method of claim 1 or 2 wherein the senescence is stress-induced premature senescence.

4. The method of any one of claims 1 to 3 wherein the method is used in the treatment or prevention of a senescence-associated disorder or a condition associated with, or characterised by, aberrant or otherwise unwanted cell growth and/or proliferation.

5. The method of claim 4 wherein the senescence-associated disorder or a condition is atherosclerosis.

6. A method of regulating senescence in a cell, the method comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell.

7. The method of claim 6 wherein the senescence is stress-induced premature senescence.

8. The method of claim 6 or 7 wherein the cell is an endothelial cell.

9. The method of any one of claims 6 to 8 wherein the cell is contacted with the agent *in vitro*, *ex vivo* or *in vivo*.

10. The method of any one of claims 6 to 9 wherein the cell is located in, or derived from, a subject in which inducing or inhibiting cellular senescence is desirable.

11. The method of any one of claims 6 to 10, comprising inducing senescence in the cell by increasing the level of expression and/or activity of SENEX relative to normal endogenous levels.

12. The method of claim 11 wherein the subject suffers from, or is predisposed to, cancer, a vascular disease or an inflammatory condition.

13. The method of claim 11 or 12, comprising contacting the cell with an effective amount of SENEX or a polynucleotide encoding the same in order to increase or upregulate SENEX expression.

14. The method of claim 13 wherein SENEX polypeptide comprises the amino acid sequence set forth in SEQ ID NO:1 or a fragment, variant or homologue thereof.

15. The method of claim 13 wherein the polynucleotide encoding SENEX comprises a nucleotide sequence as set forth in SEQ ID NO:2, or a fragment, variant or homologue thereof.

16. The method of any one of claims 6 to 10, comprising, where senescence is abnormally upregulated, normalising the levels of SENEX by contacting the cell with an agent capable of inhibiting SENEX expression and/or activity.

17. The method of claim 16 wherein the subject suffers from, or is predisposed to, a senescence-associated disorder.

18. The method of claim 17 wherein the senescence-associated disorder is atherosclerosis.

19. The method of claim 16 or 17, comprising inhibiting or reversing aberrant or abnormally upregulated cellular senescence, comprising contacting the cell with an effective amount of an inhibitor of SENEX expression and/or activity to normalise levels of SENEX.

20. The method of claim 19 wherein the inhibitor is an anti-SENEX antibody or antisense construct.

21. A method of regulating apoptosis in a cell, the method comprising contacting the cell with an agent capable of modulating the expression and/or activity of SENEX in the cell, wherein decreasing the expression and/or activity of SENEX relative to normal endogenous levels induces apoptosis.

22. The method of claim 21 wherein the cell is an endothelial cell.

23. The method of claim 21 or 22 wherein the cell is contacted with the agent *in vitro*, *ex vivo* or *in vivo*.

24. The method of any one of claims 19 to 21 wherein the cell is located in, or derived from, a subject in which inducing or inhibiting apoptosis is desirable.

25. The method of any one of claims 21 to 24, comprising inducing apoptosis in the cell by decreasing the level of expression and/or activity of SENEX relative to normal endogenous levels.

26. The method of claim 25, comprising contacting the cell with an effective amount of an inhibitor of SENEX expression and/or activity.

27. The method of claim 25 or 26 wherein the method is for the treatment of conditions associated with aberrant or otherwise unwanted cell growth and/or proliferation, chronic conditions associated with a dysfunctional vascular system and inflammatory conditions.

28. The method of any one of claims 21 to 24, comprising, where apoptosis is abnormally upregulated, normalising the levels of SENEX by contacting the cell with an agent capable of increasing SENEX expression and/or activity.

29. The method of claim 28 comprising contacting the cell with an effective amount of SENEX or a polynucleotide encoding the same to normalise levels of SENEX.

30. The method of claim 29 wherein the SENEX polypeptide comprises the amino acid sequence set forth in SEQ ID NO:1 or a fragment, variant or homologue thereof.

31. The method of claim 29 wherein the polynucleotide encoding SENEX comprises a nucleotide sequence as set forth in SEQ ID NO:2, or a fragment, variant or homologue thereof.

32. The method of any one of claims 28 to 31 for the treatment of apoptosis-associated disorders.

33. A method of inhibiting or preventing inflammation, the method comprising administering to a subject in need thereof an effective amount of an agent capable of upregulating the expression and/or activity of SENEX in at least one cell of the subject, relative to normal endogenous levels.

34. The method of claim 33 comprising administering an effective amount of SENEX or a polynucleotide encoding the same in order to increase or upregulate SENEX expression.

35. The method of claim 33 or 34 for the treatment or prevention of an inflammatory condition.

36. The method of claim 35 wherein the inflammatory condition is a chronic inflammatory condition.

37. Use of SENEX as an anti-inflammatory agent.

38. A method for determining disease control in a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising:

(a) obtaining a biological sample from the subject; and

(b) determining the level of expression and/or activity of SENEX in the sample relative to normal endogenous levels,

wherein the level of SENEX expression and/or activity relative to normal endogenous levels is indicative of disease control in the subject.

39. The method of claim 38, further comprising monitoring disease control over time in the subject comprising: repeating steps (a) and (b) at least once over a period of time; and determining whether the SENEX levels change over the period of time.

40. A method for evaluating the efficacy of a treatment regime in a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising:

(a) treating the subject with a treatment regime for a period sufficient to evaluate the efficacy of the regime;

(b) obtaining a biological sample from the subject;

(c) determining the level of expression and/or activity of SENEX in the sample;

(d) repeating steps (b) and (c) at least once over a period of time; and

(e) determining whether the SENEX levels change over the period of time,

wherein a change in the level of expression and/or activity of SENEX is indicative of a change in disease control in the subject and the degree of efficacy of the treatment regime.

41. The method of claim 40 wherein the treatment regime comprises the administration of one or more drugs, medications, supplements or medical devices aimed at directly treating the disorder, early intervention for the disorder, management of residual symptoms of the disorder, prevention of relapse, or overcoming treatment resistance in the subject.

42. A method for designing a suitable treatment regime for a subject suffering from a senescence- or apoptosis-associated disorder, the method comprising monitoring the level of expression and/or activity of SENEX in the subject in the presence or absence of a treatment regime for treating the disorder and adjusting the identity, timing and/or intensity of the treatment regime so as to normalise the level expression and/or activity of SENEX.

43. A method for treating a subject suffering from a senescence- or apoptosis-associated disorder comprising administering to the subject a treatment regime designed in accordance with the eighth aspect.

44. A method for identifying a compound suitable for treating a senescence- or apoptosis-associated disorder, the method comprising the steps of:

(a) isolating at least one cell from a subject suffering from a a senescence- or apoptosis-associated disorder;

(b) determining the level of expression and/or activity of SENEX in the at least one cell;

(c) contacting the at least one cell with a candidate compound; and

(d) determining the level of expression and/or activity of SENEX,

wherein normalisation of the level expression and/or activity of SENEX between steps (b) and (d) is indicative of the ability of compound to treat the senescence- or apoptosis-associated disorder.

45. A method for diagnosing a senescence- or apoptosis-associated disorder in a subject, or predicting the susceptibility of a subject to such a disorder, the method comprising:

(a) obtaining a biological sample from the subject; and

(b) determining the level of expression and/or activity of SENEX in the sample,

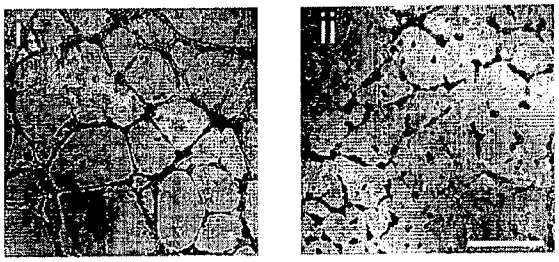
wherein increased levels of SENEX expression and/or activity relative to normal endogenous levels is diagnostic or predictive of a senescence-associated disorder, whereas decreased levels of SENEX relative to normal endogenous levels is diagnostic or predictive of an apoptosis-associated disorder.

46. The method of claim 45, further comprising comparing the level of expression and/or activity of SENEX in the sample with the level from one or more control samples.

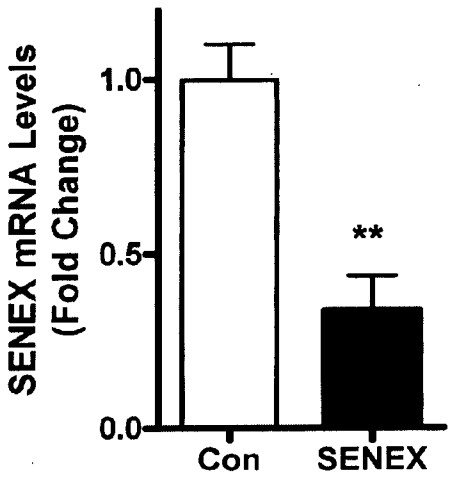
47. The method of claim 46 wherein the control sample(s) is derived from one or more individuals known not to suffer from a senescence- or apoptosis-associated disorder, or known to suffer from a specific, diagnosed senescence- or apoptosis-associated disorder.

FIGURE 1

A


i
ii

B


SENEX mRNA Levels
(Fold Change)
1.0
0.5
0.0
**
Con
SENEX

C


SENEX
β-actin
80kD
42kD

FIGURE 2

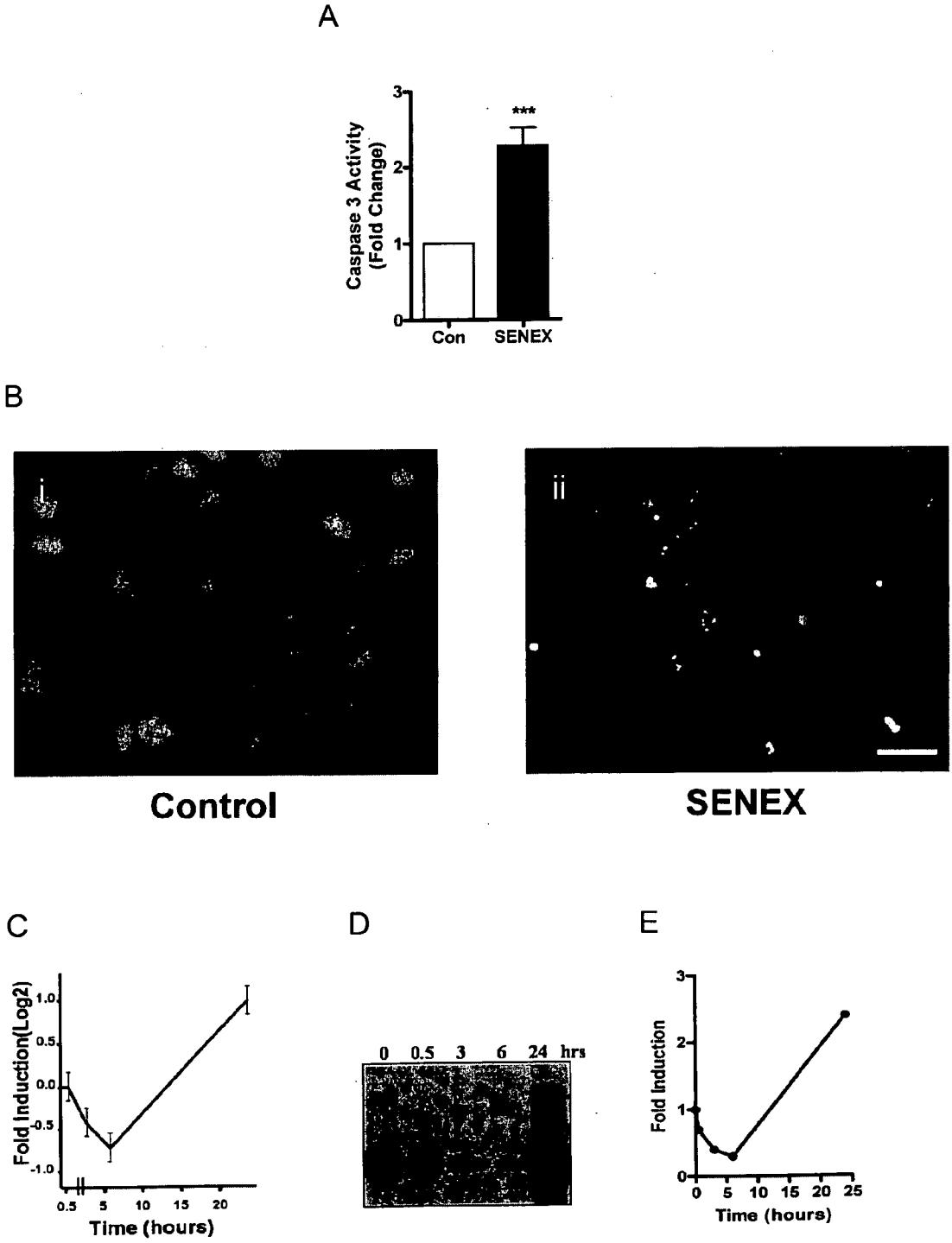

A
Caspase 3 Activity (Fold Change)
***
Con
SENEX
B
i
ii
Control
SENEX
C
Fold Induction(Log2)
Time (hours)
D
0 0.5 3 6 24 hrs
E
Fold Induction
Time (hours)

FIGURE 3

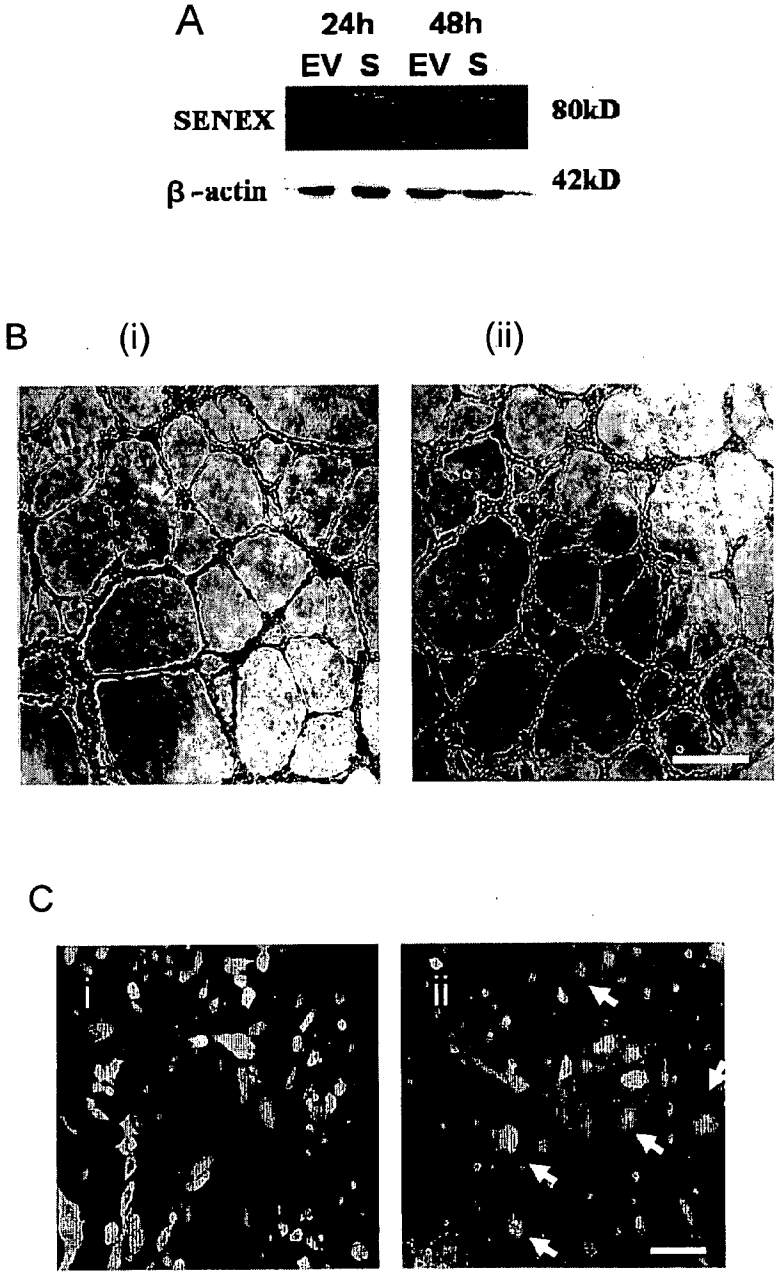
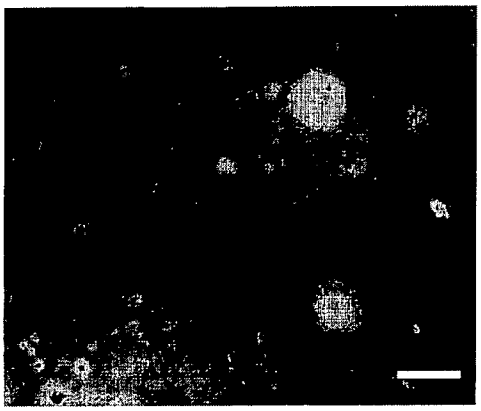


FIGURE 3 (cont'd)

D



E

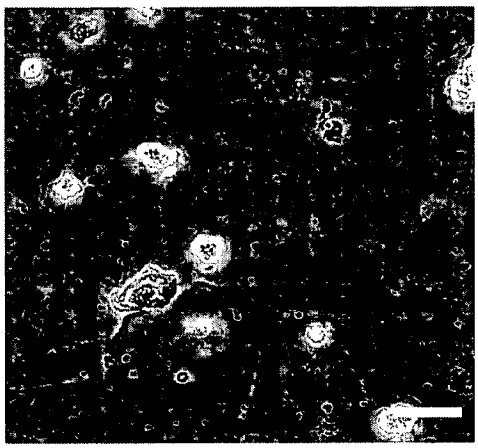


FIGURE 4

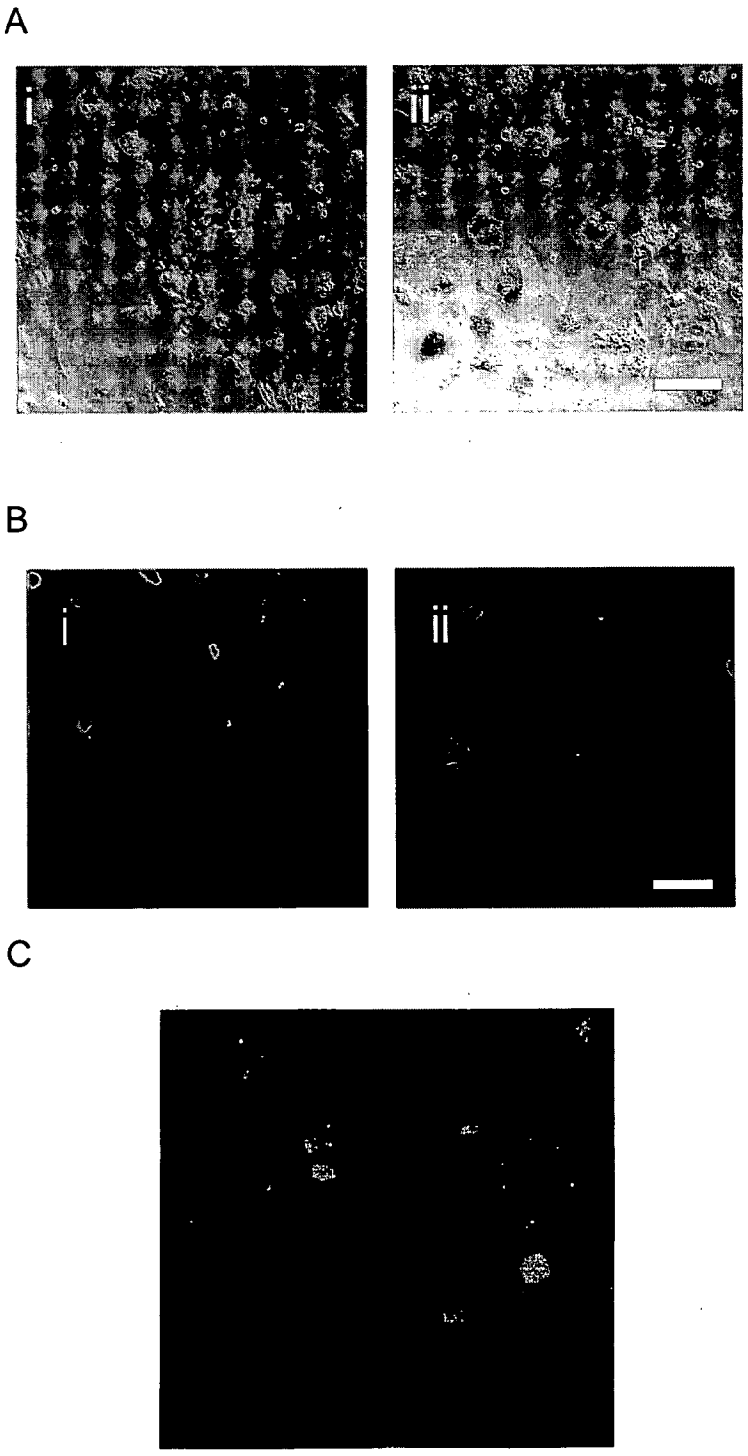


FIGURE 5

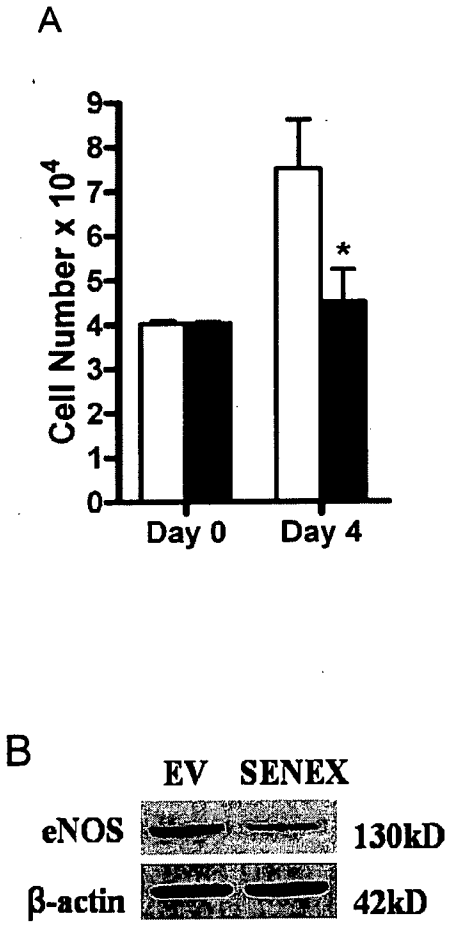

A
Cell Number x 10^4
0
1
2
3
4
5
6
7
8
9
*
Day 0
Day 4
B
EV
SENEX
eNOS
130kD
β-actin
42kD

FIGURE 6

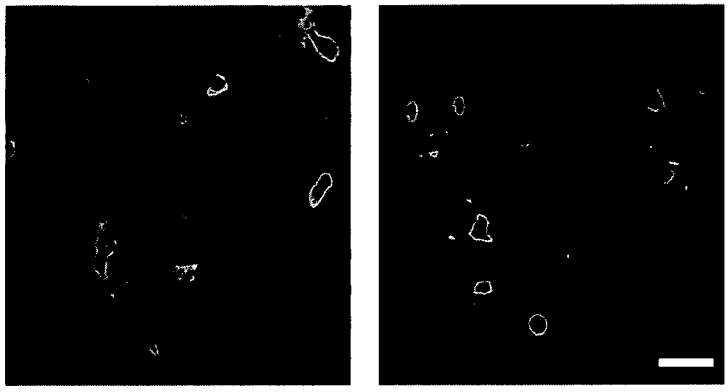


FIGURE 7

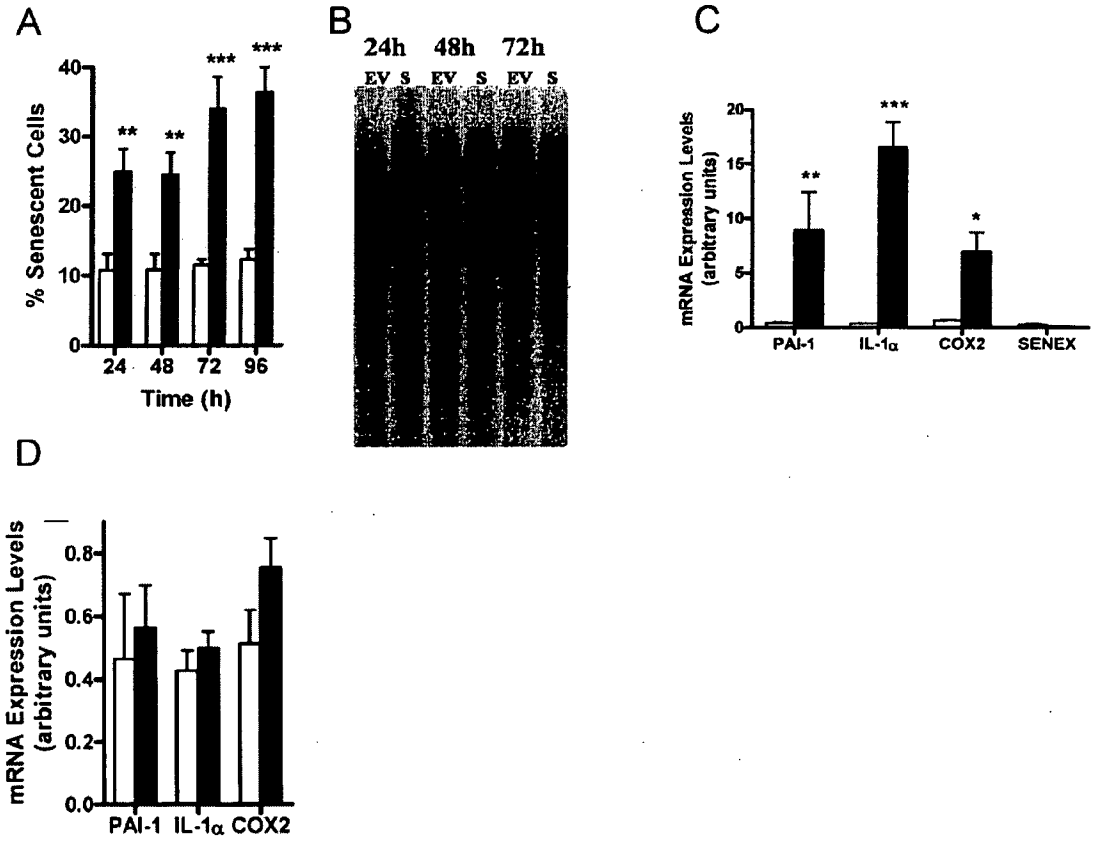


FIGURE 8

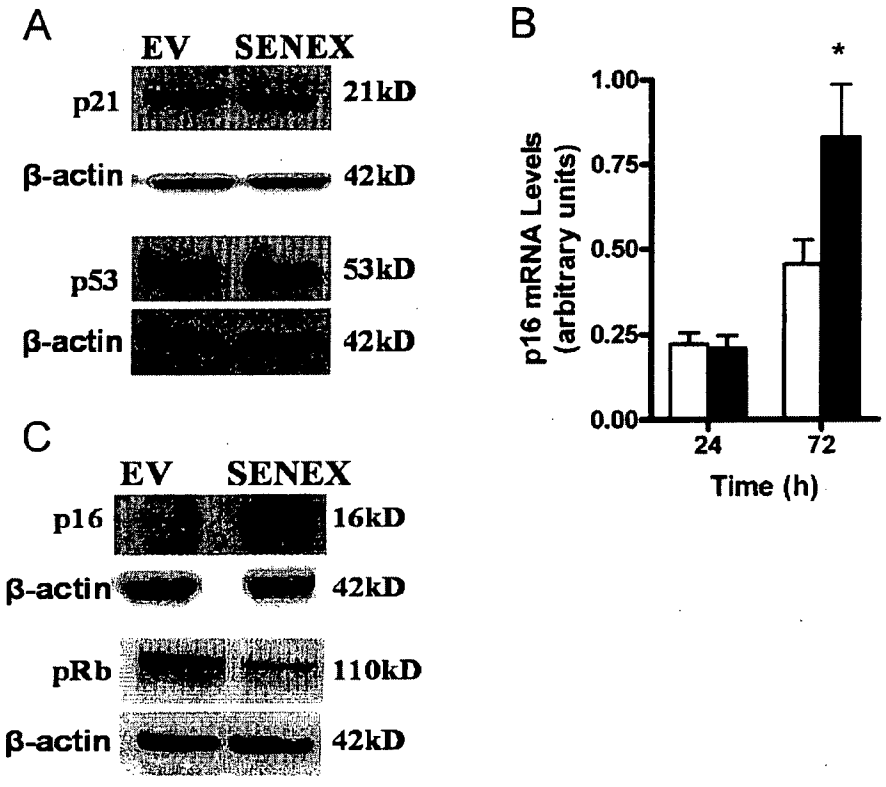

A
EV
SENEX
p21
21kD
β-actin
42kD
p53
53kD
β-actin
42kD
B
*
1.00
0.75
0.50
0.25
0.00
p16 mRNA Levels
(arbitrary units)
24
72
Time (h)
C
EV
SENEX
p16
16kD
β-actin
42kD
pRb
110kD
β-actin
42kD

FIGURE 9

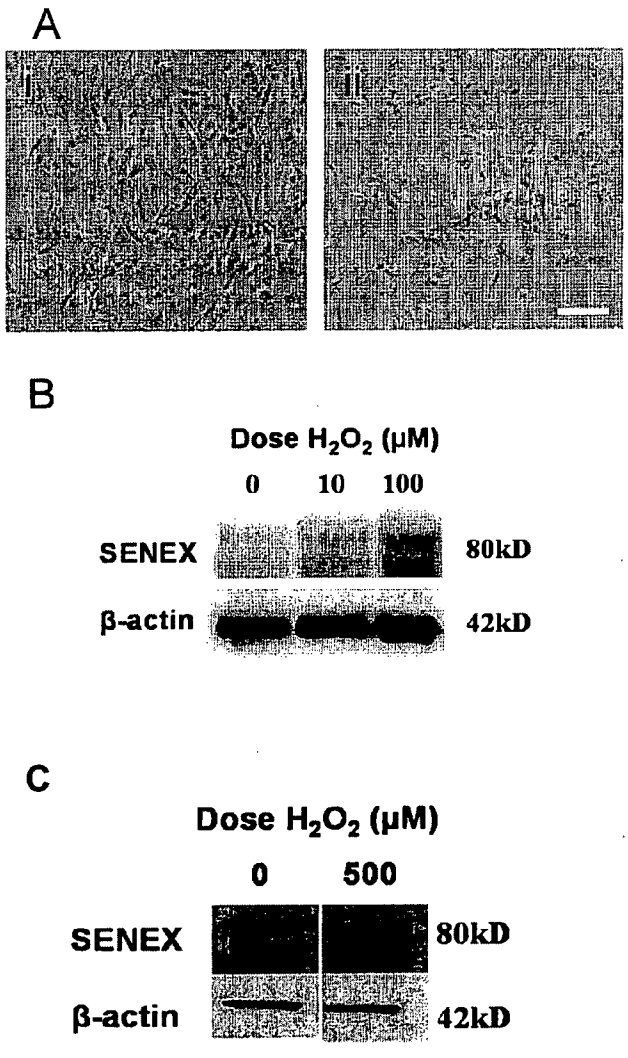

A
i
ii
B
Dose H_2O_2 (μM)
0
10
100
SENEX
80kD
β-actin
42kD
C
Dose H_2O_2 (μM)
0
500
SENEX
80kD
β-actin
42kD

FIGURE 10

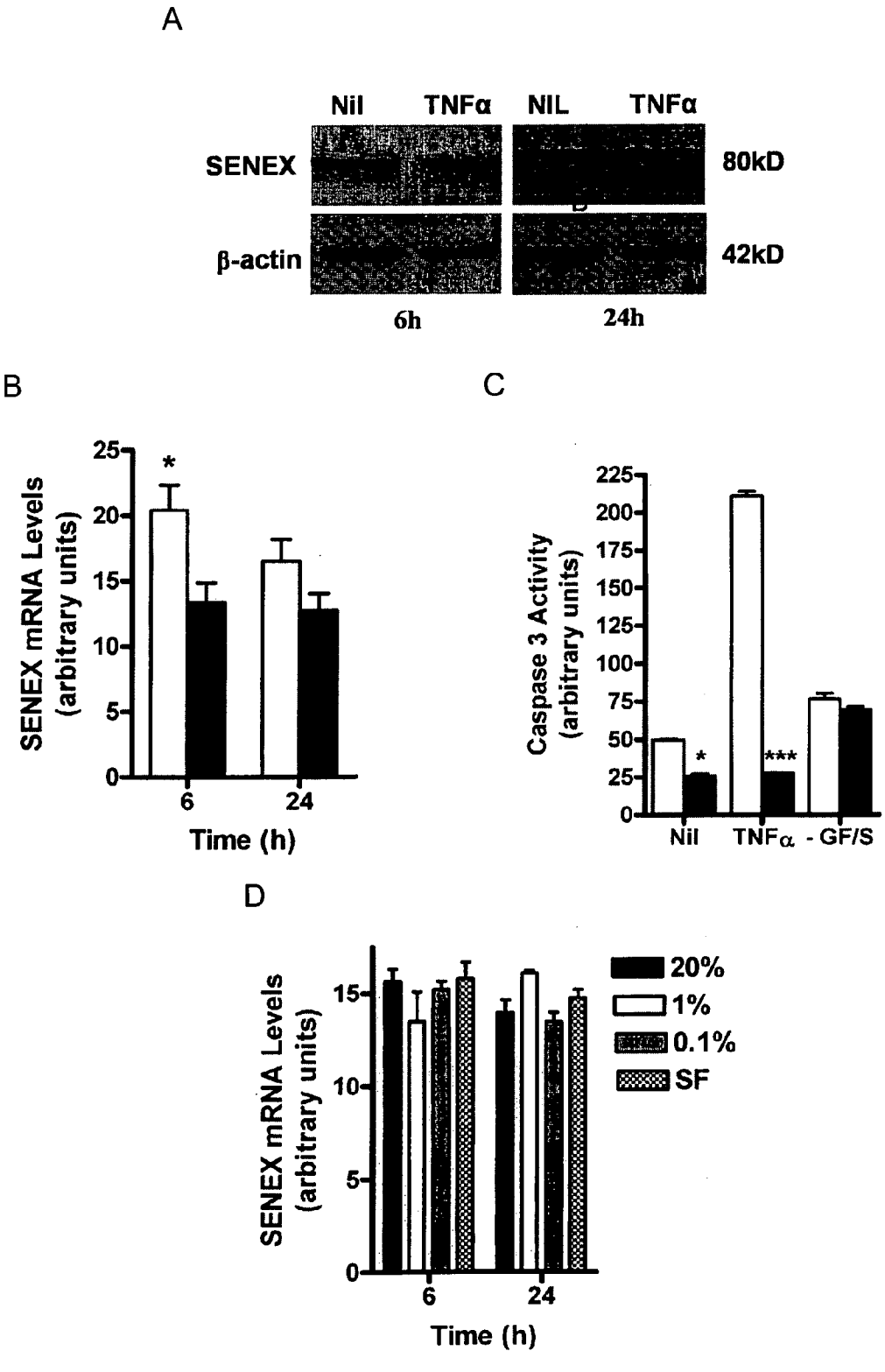
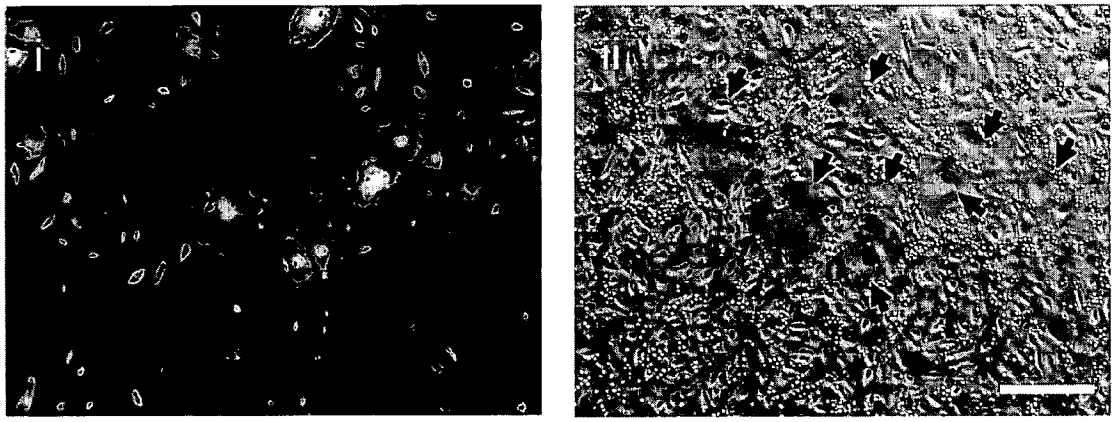

A
Nil
TNFα
NIL
TNFα
SENEX
80kD
β-actin
42kD
6h
24h
B
SENEX mRNA Levels (arbitrary units)
25
20
15
10
5
0
*
6
24
Time (h)
C
Caspase 3 Activity (arbitrary units)
225
200
175
150
125
100
75
50
25
0
*
***
Nil
TNFα
- GF/S
D
SENEX mRNA Levels (arbitrary units)
15
10
5
0
6
24
Time (h)
20%
1%
0.1%
SF

FIGURE 11

A



B

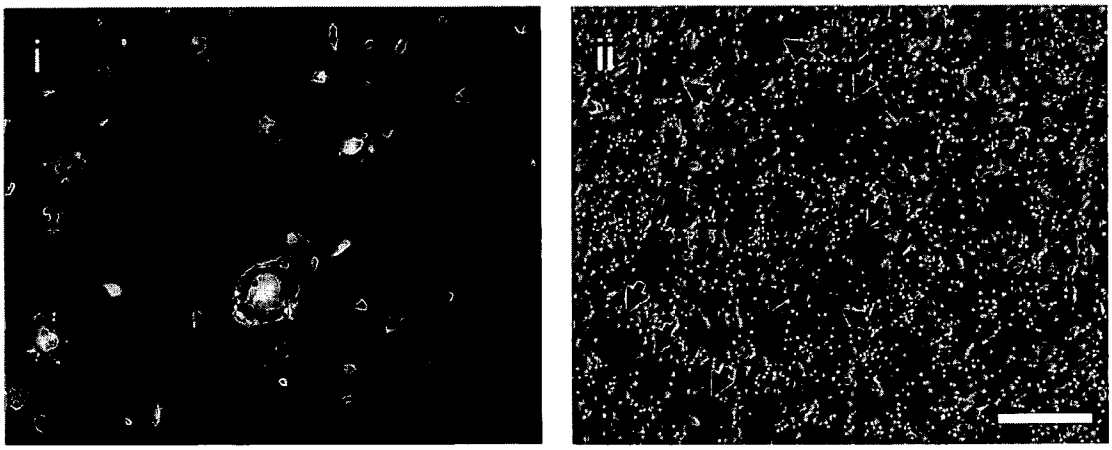


FIGURE 11 (cont'd)

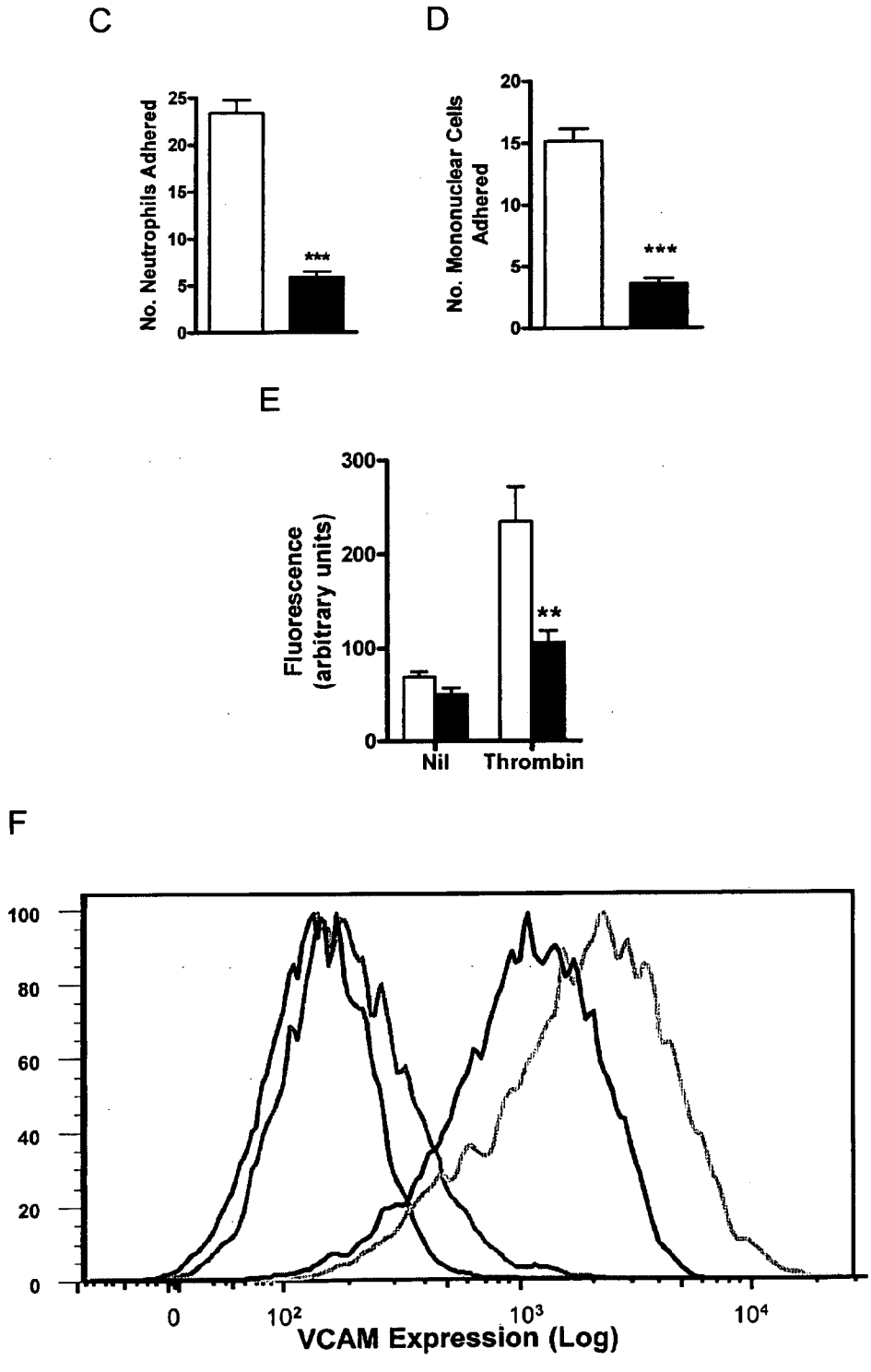
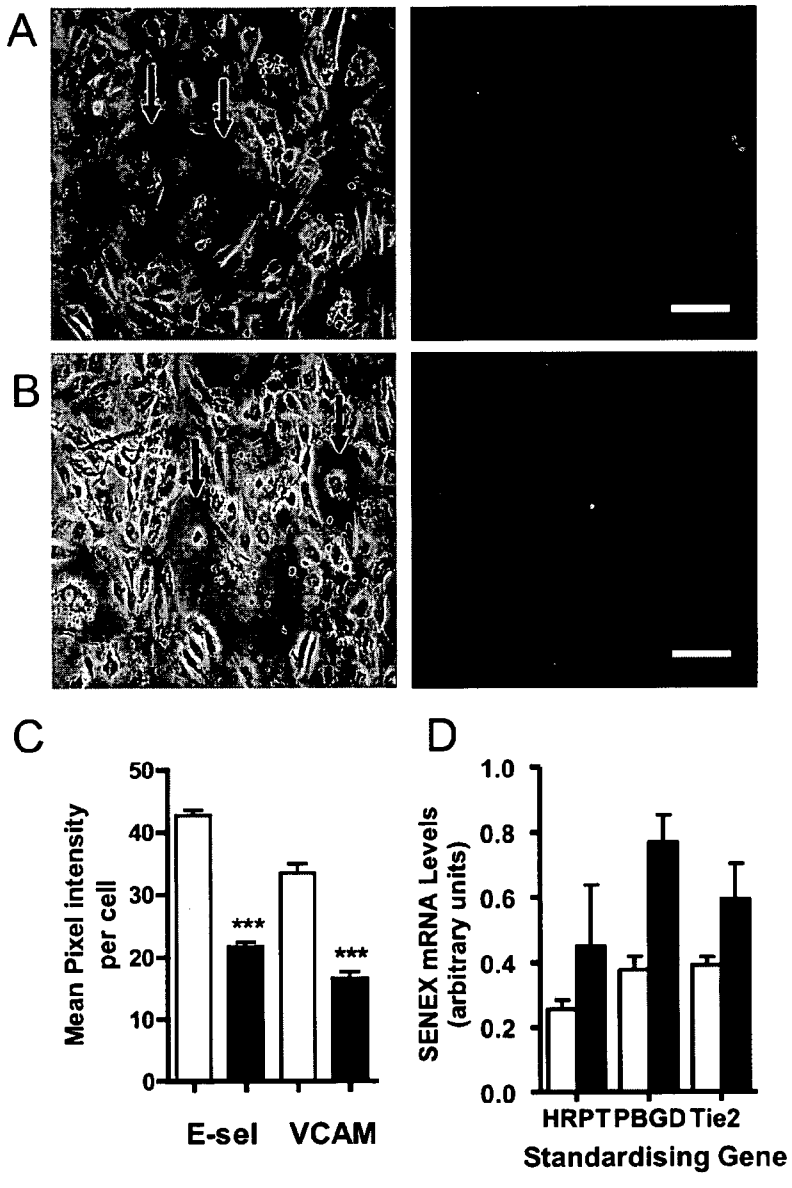


FIGURE 12


A
B
C
Mean Pixel intensity per cell
50
40
30
20
10
0
***
***
E-sel
VCAM
D
SENEX mRNA Levels (arbitrary units)
1.0
0.8
0.6
0.4
0.2
0.0
HRPT
PBGD
Tie2
Standardising Gene

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/000790

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 15/12 (2006.01) *C12Q 1/68* (2006.01) *G01N 33/50* (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS, BIOSIS, WPI, EPODOC, (senescence, apoptosis, disease, and similar terms)
GENOMEQUEST (SEQ ID NO: 1)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/019258 A2 (GENENTECH, INC.) 3 March 2005 (Fig 4141 and 4143, page 4 lines 35-page 5 line 10, page 5 lines 34-39, page 68 lines 17-19)	33-42 and 44-47
X	WO 2002/032962 A2 (MILENNIUM PHARMACEUTICALS, INC.) 25 April 2002 ([0018], [0178]-[0189], [0374], [0375], [0437], SEQ ID NO: 14)	33-42 and 44-47
X	WO 2001/025433 A2 (GENENTECH, INC; CURAGEN CORPORATION) 12 April 2001 (page 3 lines 11-23, page 11 lines 26-34, page 45, SEQ ID NO: 14)	38-42 and 44-47

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" 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 understand the principle 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 alone

"Y" document of particular relevance; the claimed invention cannot 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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 August 2010	- 9 SEP 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. +61 2 6283 7999	Authorized officer **ANITA PREMKUMAR** AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 2572

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2010/000790

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 43
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:
The claims do not comply with Rule 6.2(a) because they rely on references to the description.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ 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.:

4. ☐ 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.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2010/000790

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	2005019258	EP	1654278	EP	2014675	EP	2182006
		US	2007184444	US	2010034817	WO	2005016962
WO	2002032962	AU	30831/02	US	2002197695	US	6979564
WO	2001025433	AU	79946/00	AU	2005248940	CA	2382859
		EP	1224282	US	2005032693		

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX