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(71) Applicant *(for all designated States except US)*: **TELOGENE INC.** [CA/CA]; Institut de Pharmacologie de Sherbrooke, 3001, 12e Avenue Nord, Fleurimont, Quebec J1H 5N4 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **CHABOT, Benoit** [CA/CA]; 386 Victoria, Sherbrooke, Quebec J1H 3J2 (CA). **BOUCHARD, Louise** [CA/CA]; 3110, des Topazes, Fleurimont, Quebec J1G 4P8 (CA). **LABRECQUE, Pascale** [CA/CA]; 2654, Place de l'Alcove, St-Elie d'Orford, Quebec J0B 2S0 (CA). **PATRY, Caroline** [CA/CA]; 643, Drapeau, Sherbrooke, Quebec J1L 2H8 (CA). **WELLINGER, Raymund** [CA/CA]; 1490, Desjardins, Sherbrooke, Quebec J1J 1G2 (CA).

(74) Agents: **ROBINSON, Christopher, J.** et al.; Smart & Biggar, Box 11560 Vancouver Centre, Suite 2200, 650 West Georgia Street, Vancouver, British Columbia V6B 4N8 (CA).

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(54) **Title:** METHODS AND COMPOSITIONS FOR TREATING NEOPLASIA RELATING TO HNRNP A1 AND A2 NUCLEIC ACID MOLECULES

(57) **Abstract:** The present invention provides therapeutic and diagnostic methods for neoplasia treatment relating to hnRNP Al and hnRNP A2 nucleic acid molecules.

METHODS AND COMPOSITIONS FOR TREATING NEOPLASIA RELATING TO hnRNP A1 AND A2 NUCLEIC ACID MOLECULES

Background of the Invention

5 The invention features methods and compositions for treating neoplasia.

Telomeres are found at the ends of vertebrate chromosomes and are comprised of variable numbers of TTAGGG repeats in double-stranded form followed by a single-stranded overhang of G-rich repeats. The size of the overhang is estimated to be approximately 150-300 nucleotides in length and at 10 least a portion of this extension invades the preceding double-stranded telomeric DNA to form a T-loop. The mammalian proteins TRF1 and TRF2 bind directly to double-stranded telomeric DNA and are important for telomere biogenesis. Proteins that interact specifically with the single-stranded repeats include the hnRNP A1 and A2 proteins, as well as the recently discovered hPot1 protein.

15 The ribonucleoprotein enzyme telomerase directs the synthesis of telomeric repeats onto the G-rich strand, a process that counteracts the loss of sequence that occurs at each cell division. A gradual loss of telomeric sequences is thought to lead to cellular senescence. Mutagenic events resulting in mutant cells that are able to maintain stable telomeres may precede the development of neoplasia. In 20 approximately 85% of all tumors, stabilized telomeres are thought to be a direct consequence of the reactivation of the telomerase enzyme. Distinct mechanisms involving other pathways (ALT) have also been uncovered. Telomere function is absolutely essential for the growth of cancer cells, irrespective of their origin. Consequently, many studies aimed at reversing the neoplastic phenotype of cells 25 have targeted the activity of proteins involved in telomere biogenesis.

For example, the expression of a catalytically inactive form of telomerase in human cancer cell lines was shown to promote telomere shortening, ultimately leading to growth arrest and cell death. The use of telomerase inhibitors to promote telomere shortening in cancer cells is also being explored. It should be 30 noted that the longer the telomeres are when telomerase inhibitors are administered, the more divisions a cancer cell sustains before telomeres reach a

critical length that elicits genomic instability. Meanwhile, alternative pathways for telomere maintenance may arise and bypass the requirement for telomerase function, thereby neutralizing the effect of telomerase inhibitors.

Proteins involved in the capping function of telomeres are another attractive target for therapeutic intervention. The capping function is likely to be mediated, at least in part, by proteins that recognize the single-stranded G-rich extension at the ultimate end of chromosomes. The enzyme telomerase is probably not essential for capping because stable chromosomes exist in the absence of telomerase. Strategies that interfere with the capping function of telomeres in cancer cells may lead to rapid growth cell arrest and cell death. The double-stranded DNA binding telomeric protein, TRF2, likely plays a role in capping, based on its function in T-loop formation and in the ability of a dominant negative version of TRF2 to promote chromosome fusions and rapid p53-dependent programmed cell death.

hnRNP Proteins

hnRNP proteins are some of the most abundant nuclear proteins in mammalian cells. There are over 20 hnRNP proteins in human cells that associate with precursor mRNAs. Many of these influence pre-mRNA processing and other aspects of mRNA metabolism and transport. The best-characterized hnRNP protein, hnRNP A1, plays a role in the control of pre-mRNA splicing. hnRNP A1 also binds with high-affinity to telomeric single-stranded DNA sequences, and can interact simultaneously with telomerase RNA *in vitro*. HnRNP A1 may interact simultaneously with telomeric DNA and the human telomerase RNA *in vitro*. Importantly, defective A1 expression in mouse erythroleukemic cells produces short telomeres whose length is increased when normal levels of hnRNP A1 are restored or when UP1, a smaller version of A1 that is defective in alternative splicing function, is expressed. Overexpressing A1 also elicits telomere elongation in human HeLa cells.

A close homolog of hnRNP A1 is the hnRNP A2 protein (A2), which shares 69% amino acid identity with hnRNP A1. Although hnRNP A2 can bind specifically to single-stranded telomeric sequence *in vitro*, its role in telomere biogenesis has not yet been confirmed. For both A1 and A2, less abundant splice
5 variants have been described, $A1^B$ and B1, respectively. Interestingly, A1 is overexpressed in colon cancers, and the A2/B1 proteins have been used as early markers for lung cancer.

In approximately 85% of all tumors, stabilized telomeres are thought to be a direct consequence of the reactivation of the telomerase enzyme. Telomere
10 function is absolutely essential for the growth of neoplastic cells. Given that more than 1 in 2 Americans will develop a neoplasia during their lifetime, and approximately 556,500 Americans will die of neoplasia in 2003 efficient methods for the treatment of neoplasia are urgently needed.

15 Summary of the Invention

The present invention features methods and compositions for treating neoplasia.

In one aspect, the invention provides a method of inducing cell death in a cell by inhibiting the expression of hnRNP A1 and hnRNP A2 nucleic acid
20 molecules or polypeptides. In one embodiment, the method involves administering to the cell (i) a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A1 and (ii) a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A2, where the
25 nucleic acid molecules are administered in an amount sufficient to reduce the expression of endogenous hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins. In a preferred embodiment, the administered nucleic acid molecules are stably expressed in the cell. In another preferred embodiment, the cell is a neoplastic cell. In another preferred embodiment, the neoplastic cell is a
30 mammalian cell (e.g., a human cell). In another preferred embodiment, the human

cell is *in vivo*. In another preferred embodiment, the cell death is caused by telomere uncapping. In another preferred embodiment, the method is sufficient to induce apoptosis in a neoplastic cell, but not in a normal cell.

In another aspect the invention provides a method of treating a subject
5 having a neoplasm, the method comprising administering to a cell of the subject (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A1 and (ii) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A2, where the administering decreases
10 expression of hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins in a cell of the subject. In preferred embodiments, the administering specifically induces cell death in a neoplastic cell of the subject, but does not induce cell death in a normal cell of the subject. In another preferred embodiment, the cell death is caused by telomere uncapping. In other embodiments, the subject has bladder,
15 blood, bone, brain, breast, cartilage, colon kidney, liver, lung, lymph node, nervous tissue, ovary, pancreatic, prostate cancer, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, or vaginal cancer. In another preferred embodiment, the method is administered in combination with any standard cancer therapy.

20 In a related aspect, the invention provides a method of decreasing the length of single-stranded telomere extensions of chromosomes in a cell, the method comprising administering to a cell (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNPA1 and (ii) a nucleic acid molecule comprising at least one strand that is
25 complementary to at least a portion of a nucleic acid sequence of hnRNPA2, where the administering decreases expression of hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins. In one embodiment, the cell death is the result of increased telomere or chromosome fusion.

In another aspect, the invention features a pharmaceutical composition comprising a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A1.

In another aspect, the invention features a pharmaceutical composition comprising a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of SEQ ID NO:30.

In another aspect, the invention features a pharmaceutical composition comprising (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A1 and (ii) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A2. In preferred embodiments, the nucleic acid molecule is a dsRNA, siRNA, shRNA, or antisense nucleic acid molecule. In other preferred embodiments, the siRNA of (i) has 100% nucleic acid sequence identity to at least 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides of SEQ ID NO:30. In another preferred embodiment, the nucleic acid molecule is an antisense. In preferred embodiments the antisense nucleic acid molecule of (i) is complementary to at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides of SEQ ID NO:29 and the antisense nucleic acid molecule of (ii) is complementary to to at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides of SEQ ID NO:30.

In a related aspect, the invention provides a pharmaceutical composition containing at least one pair of double stranded nucleic acid molecules selected from the group consisting of any one or more of the following SEQ ID NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26 in a pharmaceutically acceptable carrier. In a preferred embodiment, pharmaceutical composition contains at least two pairs of nucleic acid molecules selected from the group consisting of SEQ ID NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26.

In another aspect, the invention features a pharmaceutical composition comprising one antisense nucleic acid molecule selected from the group consisting of any one or more of the following SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26.

In another aspect, the invention features a kit for the treatment of a neoplasia in a patient comprising at least one pair of double stranded nucleic acid molecules selected from the group consisting of any one or more of the following SEQ ID NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26.

In a related aspect, the invention features a kit for the treatment of a neoplasia in a patient comprising at least one antisense nucleic acid molecule selected from the group consisting of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26.

In another aspect, the invention features a method of diagnosing a patient as having, or having a propensity to develop, a neoplasia, the method comprising determining the level of expression of an hnRNPA1 or hnRNPA2 nucleic acid molecule or polypeptide in a patient sample, where an increased level of expression relative to the level of expression in a control sample, indicates that the patient has or has a propensity to develop a neoplasia. In one embodiment, the method involves determining the level of expression of the hnRNPA1. In another embodiment, the method involves determining the level of expression of the hnRNPA2 nucleic acid molecule. In a preferred embodiment, method involves determining the level of expression of hnRNPA1 and hnRNPA2 nucleic acid molecules. In another preferred embodiment, the method involves determining the level of expression of the hnRNPA1 polypeptide, the hnRNPA2 polypeptide, or the hnRNPA1 polypeptide and the hnRNPA2 polypeptide. In one embodiment, the level of expression is determined in an immunological assay.

5 In another aspect, the invention features a diagnostic kit for the diagnosis of a neoplasia in a patient comprising a nucleic acid sequence, or fragment thereof, and at least one of an hnRNP A1 and an hnRNP A2 nucleic acid molecule.

In another aspect, the invention features a method of identifying a candidate compound that ameliorates a neoplasia, the method comprising contacting a cell that expresses a hnRNPA1 and an hnRNPA2 nucleic acid molecule with a candidate compound, and comparing the level of expression of the nucleic acid molecule in the cell contacted by the candidate compound with the level of expression in a control cell not contacted by the candidate compound, where a decrease in expression of the hnRNP A1 or hnRNP A2 nucleic acid molecule identifies the candidate compound as a candidate compound that ameliorates a neoplasia. In one embodiment, the decrease in expression is a decrease in transcription. In another embodiment, the decrease in expression is a decrease in translation.

In another aspect, the invention features a method of identifying a candidate compound that ameliorates a neoplasia, the method comprising contacting a cell that expresses an hnRNP A1 or hnRNP A2 polypeptide with a candidate compound, and comparing the level of expression of the polypeptide in the cell contacted by the candidate compound with the level of polypeptide expression in a control cell not contacted by the candidate compound, where a decrease in the expression of the hnRNP A1 or hnRNP A2 polypeptide identifies the candidate compound as a candidate compound that ameliorates a neoplasia. In one embodiment, the decrease in expression is assayed using an immunological assay, an enzymatic assay, or a radioimmunoassay.

In another aspect, the invention features a method of inducing cell death in a cell by inhibiting the expression of an hnRNP A2 nucleic acid molecule or polypeptide. In a preferred embodiment, the method involves administering to the cell a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A2, where the nucleic acid molecule is administered in an amount sufficient to reduce the expression of an hnRNP A2 nucleic acid molecule or protein. In another preferred embodiment, the administered nucleic acid molecules are double stranded nucleic acid molecules.

In another aspect, the invention features a vector comprising a nucleic acid molecule positioned for expression, where the nucleic acid molecule encodes a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A1.

5 In another aspect, the invention features a vector comprising a nucleic acid molecule positioned for expression, where the nucleic acid molecule encodes a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A2.

In another aspect, the invention features a vector comprising a nucleic acid
10 molecule positioned for expression, where the nucleic acid molecule encodes (i) a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A1, and (ii) a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A2.

15 In another aspect, the invention features a method of using the nucleic acid molecules of the previous aspects to induce apoptosis in a cell. In preferred embodiments, the cell is a neoplastic cell. In other preferred embodiment, the neoplastic cell is in a human.

In another aspect, the invention features a method of using a nucleic acid
20 molecule of any previous aspect to treat a subject having a neoplasm. In a preferred embodiment, the subject is a human.

In another aspect, the invention features a method of using the nucleic acid molecule of any of the previous aspects to decrease the length of single-stranded telomere extensions of chromosomes in a cell.

25 In various preferred embodiments of any of the above aspects, the nucleic acid molecules are dsRNAs, siRNAs, shRNAs, or anti-sense nucleic acid molecules. In other preferred embodiments of any of the above aspects, the nucleic acid molecules are stably expressed in a cell (e.g., a mammalian, human, or neoplastic cell). In preferred embodiments of any of the above aspects, the human

cell is *in vivo*. In other embodiments of the above aspects, cell death is caused by telomere uncapping.

In other preferred embodiments of any of the above aspects, the siRNA has 85%, 90%, 95%, or 100% nucleic acid sequence identity to at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides of SEQ ID NO:29 or 30. In other embodiments of any of the above aspects, the antisense nucleic acid molecule is 85%, 90%, 95%, or 100% complementary to at least 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, or 500 nucleotides of SEQ ID NO:29 or 30.

By "antisense" is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand, or mRNA, of an hnRNP A1 or hnRNP A2 gene. Preferably, the antisense nucleic acid molecule is capable of decreasing the expression of hnRNP A1 or hnRNP A2 in a cell by at least 10%, 20%, 30%, 40%, or more preferably by at least 50%, 60%, 70%, or 75%, or even by as much as 80%, 90%, or 95% relative to an untreated control cell. Preferably an antisense nucleic acid includes from about 8 to 30 nucleotides. An antisense nucleic acid may also contain at least 10, 15, 20, 25, 30, 40, 60, 85, 120, or more consecutive nucleotides that are complementary to a hnRNP A1 or hnRNP A2 mRNA or DNA, and may be as long as a full-length hnRNP A1 or hnRNP A2 gene or mRNA. The antisense nucleic acid molecule may contain a modified backbone, for example, phosphorothioate, phosphorodithioate, or other modified backbones known in the art, or may contain non-natural internucleoside linkages.

By "candidate compound" is meant any nucleic acid molecule, polypeptide, or other small molecule, that is assayed for its ability to alter gene or protein expression levels, or the biological activity of a gene or protein by employing one of the assay methods described herein. Candidate compounds include, for example, peptides, polypeptides, synthesized organic molecules, naturally occurring organic molecules, nucleic acid molecules, and components thereof.

By "cell death" is meant apoptosis. Apoptosis is a highly regulated form of cell death characterized by one or more of the following features: cell shrinkage,

membrane blebbing, internucleosomal DNA cleavage, and chromatin condensation culminating in cell fragmentation.

By "differentially expressed" is meant a difference in the expression level of a nucleic acid molecule or polypeptide. This difference may be either an increase or a decrease of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% in expression, relative to a reference or to control expression.

By "effective amount" is meant an amount sufficient to arrest, ameliorate, or inhibit the continued proliferation, growth, or metastasis (e.g., invasion, or migration) of a neoplasia.

By "neoplastic cell" is meant a cell multiplying or growing in an abnormal, uncontrolled manner. A neoplastic cell grows in conditions that would inhibit the proliferation of a normal cell.

By "decreasing telomere length" is meant reducing the overall number of terminal repeats (TTAGGG) found in the telomere. In general the overall length of a shortened telomere, as used herein, includes telomeres from 3kB to 12kB, more preferably 5kB to 10 kB, most preferably 5kB to 8kB. In general the rate of telomere shortening will range from 20 to 200 nucleotides per population doubling, with a more preferable rate of 30 to 150 nucleotides per population doubling, and a most preferable rate of 40 to 100 nucleotides per population doubling.

By "decreasing single-stranded G-rich strand telomeric overhang" is meant reducing the number of single-stranded TTAGGG repeats found at the very 3'-end of chromosomes.

The preferred length of telomere 3' single stranded G-rich overhang is 50 to 400 nucleotides and more preferably 125 to 275 nucleotides (Cimino-Reale et al., Nucl. Acids Res. 29:e35, 2001; Wright et al., Genes and Dev. 11:2801-2809, 1997).

By "dsRNA" is meant a ribonucleic acid molecule having both a sense and an anti-sense strand.

By "hnRNP A1 nucleic acid molecule" is meant a nucleic acid molecule (e.g., DNA, cDNA, genomic, mRNA, RNA, dsRNA, antisense RNA, shRNA) substantially identical to GenBank accession number NM_002136 (SEQ ID NO:29).

5 By "hnRNPA2 nucleic acid molecule" is meant a nucleic acid molecule (e.g., DNA, cDNA, genomic, mRNA, RNA, dsRNA, antisense RNA, shRNA) that is substantially identical to GenBank accession number NM_002137 (SEQ ID NO:30).

By "hnRNP A1" polypeptide is meant a polypeptide encoded by an hnRNP 10 A1 nucleic acid sequence. Such polypeptides belong to the A/B subfamily of ubiquitously expressed hnRNPs.

By "hnRNP A2 polypeptide" is meant a protein encoded by an hnRNP A2 nucleic acid molecule. Such polypeptides belong to the A/B subfamily of ubiquitously expressed hnRNPs.

15 By "inhibit" is meant to decrease preferably by 20%, 30%, or 40%, more preferably by 50%, 60%, or 70%, most preferably by 80%, 90%, or even 100%.

By "substantially complementary" is meant a nucleic acid sequence that is 70%, 80%, 85%, 90% or 95% complementary to at least a portion of a reference nucleic acid sequence.

20 By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 75%, but preferably 85%, more preferably 90%, most preferably 95%, or even 99% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 20 amino acids, preferably at least 30 amino acids, more preferably at least 40 amino 25 acids, and most preferably 50 amino acids. For nucleic acids, by "substantially identical" is also meant "substantially complementary." For nucleic acids, the length of comparison sequences will generally be at least 60 nucleotides, preferably at least 90 nucleotides, and more preferably at least 120 nucleotides.

Sequence identity is typically measured using sequence analysis software 30 with the default parameters specified therein (e.g., Sequence Analysis Software

Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "neoplasm" is meant an abnormal tissue that grows by a rapid, uncontrolled cellular proliferation and continues to grow after the stimuli that initiated the new growth cease. Neoplasms show partial or complete lack of structural organization and functional coordination with the normal tissue, and usually form a distinct mass of tissue, which may be either benign or malignant.

By "neoplasia" is meant a disease characterized by the pathological proliferation of a cell or tissue. Neoplasia growth is typically uncontrolled and progressive, and occurs under conditions that would not elicit, or would cause cessation of, multiplication of normal cells. Neoplasias can affect a variety of cell types, tissues, or organs, including but not limited to an organ selected from the group consisting of bladder, bone, brain, breast, cartilage, glia, esophagus, fallopian tube, gallbladder, heart, intestines, kidney, liver, lung, lymph node, nervous tissue, ovaries, pancreas, prostate, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, and vagina, or a tissue or cell type thereof. Neoplasias include cancers, such as sarcomas, carcinomas, or plasmacytomas (malignant tumor of the plasma cells).

By "reduce or inhibit" is meant the ability to cause an overall decrease preferably of 20% or greater, more preferably of 50% or greater, and most preferably of 75% or greater, in the level of protein or nucleic acid, detected by the aforementioned assays, as compared to samples not treated with RNAi. This reduction or inhibition of RNA or protein expression can occur through targeted mRNA cleavage or degradation.

By "RNA interference (RNAi)" is meant the administration of a nucleic acid molecule (e.g., antisense, shRNA, siRNA, dsRNA), regardless of length, that inhibits the expression of an hnRNP A1 or hnRNP A2 gene. Typically, the administered nucleic acid molecule contains one strand that is complementary to
5 the coding strand of an mRNA of an hnRNP A1 or hnRNP A2 gene. RNAi is a form of post-transcriptional gene silencing initiated by the introduction of double-stranded RNA (dsRNA) or antisense RNA. Preferably, RNAi is capable of decreasing the expression of hnRNP A1 or hnRNP A2 in a cell by at least 10%, 20%, 30%, or 40%, more preferably by at least 50%, 60%, or 70%, and most
10 preferably by at least 75%, 80%, 90%, 95% or more. The double stranded RNA or antisense RNA is at least 10, 20, or 30 nucleotides in length. Other preferred lengths include 40, 60, 85, 120, or more consecutive nucleotides that are complementary to a hnRNP A1 or hnRNP A2 mRNA or DNA, and may be as long as a full-length hnRNP A1 or hnRNP A2 gene or mRNA. The double stranded
15 nucleic acid may contain a modified backbone, for example, phosphorothioate, phosphorodithioate, or other modified backbones known in the art, or may contain non-natural internucleoside linkages. In one preferred embodiment, short 21, 22, 23, 24, or 25 nucleotide double stranded RNAs are used to down regulate hnRNP A1 or hnRNP A2 expression. Such RNAs are effective at down-regulating gene
20 expression in mammalian tissue culture cell lines (Elbashir et al., *Nature* 411:494-498, 2001, hereby incorporated by reference). The further therapeutic effectiveness of this approach in mammals was demonstrated *in vivo* by McCaffrey et al. (*Nature* 418:38-39. 2002). The nucleic acid sequence of an hnRNP A1 or hnRNP A2 gene can be used to design small interfering RNAs that
25 will inactivate an hnRNP A1 or hnRNP A2 gene and that may be used, for example, as therapeutics to treat a variety of neoplasias.

By "small interfering RNAs (siRNAs)" is meant an isolated dsRNA molecule, preferably greater than 10 nucleotides in length, more preferably greater than 15 nucleotides in length, and most preferably 18, 19, 20, 21, 22, 23, 24, or 25
30 nucleotides in length that is used to identify the target gene or mRNA to be

degraded. A range of 19-25 nucleotides is the most preferred size for siRNAs. siRNAs can also include short hairpin RNAs in which both strands of an siRNA duplex are included within a single RNA molecule. siRNA includes any form of dsRNA (proteolytically cleaved products of larger dsRNA, partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA) as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution, and/or alteration of one or more nucleotides. Such alterations can include the addition of non-nucleotide material, such as to the end(s) of the 21 to 23 nucleotide RNA or internally (at one or more nucleotides of the RNA). In a preferred embodiment, the RNA molecule contains a 3'hydroxyl group. Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs of RNA. siRNAs of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate RNAi. As used herein "mediate RNAi" refers to the ability to distinguish or identify which RNAs are to be degraded.

By "telomerase" is meant the enzyme responsible for the addition of TTAGGG repeats to the ends of telomeres.

By "telomere" is meant the end section of a eukaryotic chromosome, composed of several hundred terminal repeats of the sequence TTAGGG.

By a "therapeutic amount" is meant an amount of a compound, alone or in combination with known therapeutics, that is sufficient to inhibit neoplasia growth, progression, or metastasis *in vivo*. The effective amount of an active compound(s) used to practice the present invention for therapeutic treatment of neoplasms (i.e., neoplasia) varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. An effective amount of an hnRNP A1 or hnRNP A2 therapeutic for the treatment of neoplasia is as little as 0.005, 0.01, 0.02, 0.025, 0.05, 0.075, 0.1, 0.133 mg per dose, or as much

as 0.15, 0.399, 0.5, 0.57, 0.6, 0.7, 0.8, 1.0, 1.25, 1.5, 2.0 or 2.5 mg per dose. The dose may be administered once a day, once every two, three, four, seven, fourteen, or twenty-one days. The amount administered to treat neoplasia is based on the activity of the therapeutic compound. It is an amount that is sufficient to effectively reduce cell proliferation, tumor size, neoplasia progression, or metastasis. It will be appreciated that there will be many ways known in the art to determine the therapeutic amount for a given application. For example, the pharmacological methods for dosage determination may be used in the therapeutic context.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of Drawings

Figure 1 shows a Western blot of hnRNP A1 and hnRNP A2 expression in siRNA transfected HeLaS3 cells. Cells from the cervical carcinoma HeLaS3 cell line were seeded in 6-well plates (65,000 cells/well) and were transfected at 24 and 48 hours. Control samples were treated with oligofectamine in the absence of siRNA. Cells were collected 96 hours after the first transfection. Ponceau S-staining of the nitrocellulose membrane was used to confirm that equal amounts of protein were loaded in each lane. (not shown). The hnRNP A1, hnRNP A2 proteins, and their respective spliced isoforms $A1^B$ and B1 were revealed with the anti-A1/A2 antibody. A1#1-A1#7: sense and antisense siRNAs targeting the human hnRNP A1 mRNA; A2#1-A2#5: sense and antisense siRNAs targeting the human hnRNP A2 mRNA; A1#1M: control siRNA containing a mismatched version of A1#1, control: lipofectamine without siRNA. (Note: these abbreviations have this meaning throughout the figures).

Figure 2 is a histogram showing cell growth of siRNA transfected HeLaS3 cells. The siRNA targeted either hnRNP A1 (A1#1-A1#7, A1 mismatched control A1#M) or hnRNP A2 (A2#1-A2#5). 96 hours post-transfection, adherent cells were photographed and both adherent and floating cells were harvested and

counted. Cell viability was evaluated by trypan blue dye exclusion. The hatched area indicates that cells show the characteristic morphology associated with apoptosis.

Figure 3 shows micrographs of siRNA-transfected HeLaS3 cells under
5 phase contrast microscopy (200X magnification). Control: lipofectamine; siA1: siRNA targeting hnRNP A1; siA1M: mismatch hnRNP A1 control; siA2: siRNA targeting hnRNP A2. (Note: these abbreviations have this meaning throughout the figures).

Figure 4A upper panel shows a Western blot analysis with a monoclonal
10 antibody that recognizes both the 33 kDa inactive pro-caspase-3 as well as the activated 20 kDa form found in apoptotic cells. HeLaS3 cells were transfected as described above and cells were harvested 96 hours after the first transfection. Figure 4A lower panel shows a western analysis performed on the same protein samples with an antibody that recognizes the PARP enzyme, which is a substrate
15 for the activated caspase-3.

Figure 4B shows a TUNEL assay on HeLaS3 cells treated with lipofectamine (control) a combination of siRNAs targeting hnRNP A1 and A2 (siA1 +siA2), a mismatch control combination (siA1M + siA2) or staurosporin.

Figure 4C shows DNA content in siRNA-treated cells that were fixed and
20 stained with propidium iodide prior to DNA content analysis by cytometry. "n" refers to the haploid DNA content. Note that the appearance of subG$_1$ DNA associated with apoptosis is seen in HeLa S3 cells.

Figure 5A shows the results of an oligonucleotide ligation assay to measure the length of telomeric single-stranded extensions. Seventy-two hours after the
25 first transfection, HeLaS3 cells were harvested and cellular DNA was extracted. The oligonucleotide ligation assay was performed using 5 μg of cellular DNA and the ligation products were resolved on a sequencing gel, detected by autoradiography. The gel was scanned, and the histogram beside the gel shows the band intensity of the scanned image. Lane 1:combination of A1#1 and A2#1; lane
30 2: A1#1M and A2.

Figure 5B is a graph showing the quantitation of the oligonucleotide ligation assay of Figure 5A. Similar results were seen 48 hours after the first transfection. The image was analyzed using Quantity One quantification software™ (Bio-Rad). The value of the intensity of each band of the ligation
5 products ladder was normalized by dividing for the number of concatenated oligonucleotide probes in the band. This value was then normalized to the total intensity and plotted as relative frequency of the 3'-overhang length.

Figure 5C provides a measurement of the telomeric single-stranded 3'-overhang in HeLaS3 cells treated with staurosporine (lane 2) for 24 hours or with
10 DMSO as control (lane 1). The gel was scanned, and the histogram beside the gel shows the band intensity of the scanned image.

Figure 5D provides a quantitation of the telomeric probe ligation products of the assay ligation assay shown in Figure 5C. The image was analyzed using Quantity One quantification software™ (Bio-Rad). The value of the intensity of
15 each band of the ligation products ladder was normalized by dividing for the number of concatenated oligonucleotide probes in the band. This value was then normalized to the total intensity and plotted as relative frequency of the 3'-overhang length.

Figure 6 is a histogram showing the effect of varying siRNA concentrations
20 targeting hnRNP A1 and hnRNP A2 on HeLaS3 cell viability. HeLaS3 cells were seeded in 6-well plates (65,000 cells/well) 24 hours before transfection. Cells were transfected twice with the indicated concentrations of siRNA and cell viability analysis was performed using Trypan Blue dye exclusion assays 96 hours after transfection. NT: Non-transfected; control: lipofectamine without siRNA;
25 lamin A/C.

Figure 7A is a graph showing cell viability measurements of HeLaS3 cells at various time points after siRNA transfection. HeLaS3 cells were seeded in 6-well plates (65,000 cells/well) 24 hours before transfection. Cells were transfected with siRNA at 24 hours and 48 hours and cell viability analysis was performed

using Trypan Blue exclusion assays at 72 hours, 96 hours, 120 hours, and 144 hours after the first transfection.

Figure 7B shows hnRNPA1 and hnRNP A2 protein expression in HeLaS3 cell extracts assayed after siRNA transfection. Extracts from 40,000 cells transfected as above were harvested at 72 hours, 96 hours, 120 hours, and 144 hours after transfection, separated by SDS-PAGE, transferred to a membrane, and immunoblotted using a polyclonal antibody against $A1/A2/A1^B/B1$. Oligofect: control transfection without siRNA.

Figure 8 is A Western blot showing the impact of treatment with siRNAs on hnRNP A1 and hnRNP A2 expression in a HCT116 cancer cell line.

Figure 9A is a histogram showing the effect of siRNA targeting hnRNP A1 and A2 on HCT116 colorectal carcinoma cell line on cell growth. The bottom portion shows a western analysis of the A1/A2 expression.

Figure 9B shows photomicrographs of siRNA treated HCT116 cells. Seventy-two hours post transfection, cells were harvested and processed to determine the impact on hnRNP A1 hnRNP A2 expression on the phenotype.

Figure 10 is a Western blot showing the impact of treatment with siRNAs on hnRNP A1 and hnRNP A2 expression in the HT1080 fibrosarcoma cancer cell line.

Figure 11A is a histogram and Western blot showing the effect of siRNA transfection on cell growth and hnRNP A1 and hnRNP A2 expression in HT1080 cells. siA1M + siA2: mismatch control combination; control: lipofectamine treatment without siRNA present; siA1 + siA2: siRNA combination targeting hnRNP A1 and hnRNP A2.

Figure 11B shows photomicrographs of HT1080 cells transfected with the indicated siRNAs. siA1M +siA2: mismatch control combination; control is lipofectamine treatment without siRNA present; siA1 + siA2: siRNA combination targeting hnRNP A1 and hnRNP A2.

Figure 12A is a histogram showing the effect of siRNA targeting hnRNP A1 and A2 on the growth of the MCF-7 breast cancer cell line. The bottom portion shows a western analysis of the hnRNP A1 and A2 expression.

Figure 12B shows photomicrographs of siRNA treated MCF-7 cells.
5 Seventy-two hours post transfection, cells were harvested and processed to determine the impact on hnRNP A1 hnRNP A2 expression on the phenotype.0

Figure 13A is a histogram and Western blot showing the effect of siRNA transfection on cell growth and hnRNP A1 and hnRNP A2 expression in CCD-18Co cells.

10 Figure 13B shows photomicrographs of CCD-18Co cells transfected with the indicated siRNAs. siA1M +siA2: mismatch control combination; Control is lipofectamine treatment without siRNA present; siA1 + siA2: siRNA combination targeting hnRNP A1 and hnRNP A2.

Figure 14A is a histogram and Western blot showing the effect of siRNA
15 transfection on cell growth and hnRNP A1 and hnRNP A2 expression in mortal BJ cells.

Figure 14B shows photomicrographs of mortal BJ cells transfected with the indicated siRNAs.

Figure 15A is a graph and Western blot showing the effect of siRNA
20 transfection on cell growth and hnRNP A1 and hnRNP A2 expression in HIEC cells.

Figure 15B shows photomicrographs of immortalized HIEC cells transfected with the indicated siRNAs.

Figure 16A is a graph and Western blot showing the effect of siRNA
25 transfection on cell growth and hnRNP A1 and hnRNP A2 expression in immortalized BJ-TIELF cells.

Figure 16B shows photomicrographs of immortalized BJ-TIELF cells transfected with the indicated siRNAs.

Figure 17 shows DNA content analysis after RNAi on BJ-TIELF cells.

Figure 18 is a table showing the effects of RNAi on hnRNP A1 and hnRNP A2 expression in various cell lines.

Figure 19 shows micrographs of hnRNP A1 and hnRNP A2 expression in cancer and normal tissues. Immunohistochemistry analysis of hnRNP A1 and
5 hnRNP A2 expression in lung tissue from (A) normal patient and (B) a patient with lung adenocarcinoma. Immunohistochemistry analysis of hnRNP A1 and hnRNP A2 expression in a pancreatic tissue from (C) normal patient and (D) patient with pancreatic adenocarcinoma. Magnification, 40X.

10 Detailed Description of the Invention

The invention provides methods and compositions for treating and preventing neoplasia.

As reported in more detail below, we have discovered that mammalian hnRNP A1 and A2 proteins, which bind to single-stranded extensions within
15 telomeres, are expressed at high levels in a variety of human cancers and that inhibiting hnRNP A1 and hnRNP A2 expression promotes rapid apoptotic cell death specifically in neoplastic cells.

We used RNA interference mediated by small interfering RNAs (siRNAs) to reduce levels of hnRNP A1 and hnRNP A2 proteins in human cancer cell lines.
20 This treatment promoted specific and rapid cell death by apoptosis in cell lines derived from cervical, colon, breast, ovarian and brain cancer. Cancer cell lines that lack p53 or that express a defective p53 protein were also sensitive to an siRNA-mediated decrease in hnRNP A1 and HNRNP A2 expression. Remarkably, comparable decreases in the expression of hnRNP A1 and HNRNP
25 A2 in several mortal human fibroblastic and epithelial cell lines did not elicit cell death.

hnRNP A1 and hnRNP A2 Expression in Human Cancer

We examined the relationship between hnRNP A1 and hnRNP A2
30 expression and different types of human cancers. In addition, we determined the

effect of alterations in hnRNP A1 and/or A2 protein levels on the growth of neoplastic and normal mortal cell lines using RNA interference (RNAi) to reduce the expression of hnRNP A1 and hnRNP A2 proteins in human cell lines. RNAi is a recently discovered method of post-transcriptional gene silencing. In RNAi, at
5 least one small double-stranded RNAs (siRNAs) corresponding to at least a portion of a gene of interest is introduced into a mammalian cell to elicit the degradation of a corresponding mRNA. RNAi represents a powerful tool for regulating gene function.

Our results on the expression profile of A1 and A2 identified these proteins
10 as potential markers for many types of tumors. Most importantly, we showed that a combined reduction in hnRNP A1 and A2 expression promoted apoptosis in all cancer cell lines tested. A similar decrease in hnRNP A1 and hnRNP A2 protein levels in normal mortal cell lines had no significant effect on cell growth. Without being tied to a particular model, our results suggest that hnRNP A1 and hnRNP A2
15 proteins are mammalian telomeric capping factors, and demonstrate that inhibiting hnRNP A1 and hnRNP A2 expression is a powerful and specific approach to prevent or inhibit the growth of neoplastic cells.

Effects of RNAi on HeLaS3 cell growth and protein hnRNP A1 and hnRNP
20 A2 RNAi in HeLaS3 cells

If hnRNP A1 and hnRNP A2 proteins are involved in the formation of a telomeric cap, inhibiting their expression should result in uncapping, cell growth arrest, and rapid cell death. To test this hypothesis, we needed to promote a specific reduction in the level of A1 and /or A2 proteins in human cancer cells.
25 We accomplished this using siRNAs to carry out RNA interference assays.

Optimal conditions for siRNA transfection were identified using a fluorescent oligonucleotide and siRNA complementary to lamin A/C in HeLaS3 cells. We designed a variety of 19 base pair double-stranded RNAs containing a 2-nucleotide extension at the 3' end and corresponding to portions of the A1 and
30 A2 mRNAs.

A1#1: 5'-UGGGGAACGCUCACGGACUdTdT-3' (SEQ ID NO: 1)
3'-dTdTACCCCUUGCGAGUGCCUGA-5' (SEQ ID NO: 2)

A1#1M: 5'-UGGGGAACCGUCACGGACUdTdT-3' (SEQ ID NO: 3)
5 3'-dTdTACCCCUUGGGAGUGCCUGA-5' (SEQ ID NO: 4)

A1#2: 5'-UGAGAGAUCCAAACACCAAdTdT-3' (SEQ ID NO: 5)
3'-dTdTACUCUCUAGGUUUGUGGUU-5' (SEQ ID NO: 6)

10 **A1#3:** 5'-GCGCUCCAGGGGCUUUGGGdTdT-3' (SEQ ID NO: 7)
3'-dTdTCGCGAGGUCCCCGAAACCC-5' (SEQ ID NO: 8)

A1#4: 5'-UCGAAGGCCACACAAGGUGdTdT-3' (SEQ ID NO: 9)
3'-dTdTAGCUUCCGGUGUGUUCCAC-5' (SEQ ID NO: 10)

15

A1#5: 5'-AUCAUGACUGACCGAGGCAdTdT-3' (SEQ ID NO: 11)
3'-dTdTUAGUACUGACUGGCUCCGU-5' (SEQ ID NO: 12)

A1#6: 5'-CUUUGGUGGUGGUCGUGGAdTdT-3' (SEQ ID NO: 13)
20 3'-dTdTGAAACCACCACCAGCACCU-5' (SEQ ID NO: 14)

A1#7: 5'-UUUUGGAGGUGGUGGAAGCdTdT-3' (SEQ ID NO: 15)
3'-dTdTAAAACCUCCACCACCUUCG (SEQ ID NO: 16)

25 **A2#1:** 5'-GCUUUGAAACCACAGAAGAdTdT-3' (SEQ ID NO: 17)
3'-dTdTCGAAACUUUGGUGUCUUCU-5' (SEQ ID NO: 18)

A2#2: 5'-CCACAGAAGAAAGUUUGAGdTdT-3' (SEQ ID NO: 19)
3'-dTdTGGUGUCUUCUUUCAAACUC-5' (SEQ ID NO: 20)

30

A2#3: 5'-GAAGCUGUUUGUUGGCGGAdTdT-3' (SEQ ID NO: 21)
3'-dTdTCUUCGACAAACAACCGCCU-5' (SEQ ID NO: 22)

A2#4: 5'-AUUUCGGACCAGGACCAGGdTdT-3' (SEQ ID NO: 23)
35 3'-dTdTUAAAGCCUGGUCCUGGUCC-5' (SEQ ID NO: 24)

A2#5: 5'-CUUUGGUGGUAGCAGGAAC-3' (SEQ ID NO: 25)
3'-dTdTGAAACCACCAUCGUCCUUG-5' (SEQ ID NO: 26)

40 Each of these RNAs was tested as follows.

Double-stranded siRNAs complementary to a portion of A1 or A2 were individually introduced into HeLaS3 cells by performing two successive transfections with an A1 and an A2 siRNA (20 nM). The second transfection was

performed 24-hours after the first. Seven different siRNAs complementary to a portion of A1 and five siRNAs complementrary to a portion of A2 were tested. Control samples were treated with oligofectamine in the absence of siRNA. As an additional negative control, the siRNA A1-1M was used. This control contained a
5 mismatched version of A1-1 having a mutation at two adjacent positions (GC to CG).

Cells were counted after Trypan blue staining and cell growth was evaluated by calculating the number of cell divisions (expressed as the number of population doublings) 96 hours after the first transfection.

10

hnRNP A1 and A2 protein expression in siRNA transfected cells

Ninety-six hours after the first transfection, total proteins were isolated and the abundance of A1 and A2 proteins was assessed by western analysis using a rabbit polyclonal antibody that binds A1, A2, and their lower abundance splice
15 isoforms, $A1^B$ and B1 (Figure 1).

Protein extracts from cells transfected with siRNAs targeting either hnRNP A1 or hnRNP A2 (A1-1, A1-2, A1-5 and A1-6) showed a marked reduction in the protein expression level of A1. All siRNAs against A2, with the exception of A2-4, promoted a strong decrease in A2 protein level. siRNA A1-1M did not promote
20 a reduction in hnRNP A1. Thus, we identified several siRNAs that reduced the expression of hnRNP A1 and A2.

Cell Growth Assays in siRNA A1 and A2 transfected cells

To determine whether the treatment of HeLaS3 cells with siRNAs that
25 target A1 and A2 affected cell growth (Figure 2), we transfected HeLaS3 cells with individual siRNAs, combinations of siRNAs, and control mixtures. Adherent and non-adherent cells were collected and counted 96 hours after the first transfection. We also assessed gross cellular morphology by microscopic inspection (Figure 3). Individual siRNAs that decreased either A1 or A2
30 expression levels did not affect cell growth nor did they change cell morphology.

Combinations of siRNAs that promoted a reduction in the abundance of both hnRNP A1 and A2 (siRNAs A1-1/A2-1 and A1-5/A2-5) affected cell growth and cell morphology. In fact, the morphology of cells treated with these combinations that targeted hnRNP A1 and hnRNP A2 resembled apoptotic cells. In some experiments, the reduction in cell growth was less apparent, but the majority of the cells examined were round and loosely adherent. We attribute the variations in cell growth between experiments to differences in the timing of cell death.

Trypan blue exclusion staining indicated that the majority of the cells treated with siRNA combinations targeting both hnRNP A1 and hnRNP A2 always produced increased numbers of dead cells relative to cells treated with individual siRNAs targeting hnRNP A1 or hnRNP A2. Pairs of siRNAs that affected only hnRNP A1 or hnRNP A2 did not elicit these effects (e.g. A1-6/A2-4). Likewise, the mismatch control siRNA (A1-1M/A2-1) pair, which promoted a decrease in hnRNP A2 protein levels, but did not produce a decrease in hnRNP A1 protein levels, did not affect cell growth and cell morphology. Thus, specific combinations of siRNAs that targeted both hnRNP A1 and hnRNP A2 (A1-1/A2-1 siRNA), were effective at reducing A1 and A2 protein expression and at promoting cell death. The experiment shown in Figure 2 was conducted at a concentration of 80 nM for individual siRNA and a total concentration of 80 nM when pairs of siRNAs (40 nM of each) were used. This experiment was repeated many times (n>10) with identical results (data not shown). Although mixtures of siRNAs at 20 nM were active, lower concentrations did not efficiently reduce cell viability. A fifty percent decrease in the level of hnRNP A1 and hnRNP A2 protein levels relative to untreated cells almost invariably promoted cell death. Treatment with individual siRNA targeting hnRNP A1 or hnRNP A2 had no effect on cell growth when tested at a concentration of 120 nM, 210 nM and 300 nM, 300 nM being the highest concentration tested.

We also tested HeLaS3 cells grown at low concentrations of serum. Under these conditions, the number of cell divisions for the control mixture remained low

(less than 3 population doublings in 96 hours), but specific siRNA-induced cell death was as dramatic (data not shown). The reduction in cell growth, the change in cell morphology and the results of differential staining for live cells using trypan blue all suggested that siRNAs combinations targeting both hnRNP A1 and
5 hnRNP A2 promoted cell death.

Apoptotic Assays

To confirm that this cell death was occurring by apoptosis, we carried out a variety of assays, including PARP, pro-caspase-3 cleavage, and DNA content
10 analysis assays (Figures 4A-4C). The siRNA combination targeting both hnRNP A1 and hnRNP A2 (A1-1/A2-1) resulted in cell death by apoptosis as assayed by pro-caspase 3 protein cleavage.

DNA content analysis indicated that a characteristic subG1 increase due to DNA fractionation was observed with the siRNA combination that targeted
15 hnRNP A1 and hnRNP A2 (A1-1/A2-1), but not with the siRNA mismatch combination control (A1-1M/A2-1) (Figure 4C). We also carried out TUNEL assays (Figure 4B) that specifically stain apoptotic cells. These assays indicated that more than 70% of the HeLa cells were TUNEL-positive when treated with the A1-1/A2-1 siRNAs. Less than 0.1% of cell treated with the control A1-1M/A2-1
20 siRNA combination (Figure 4B) were TUNEL-positive. Thus, apoptotic analyses indicated that a reduction in hnRNP A1 and hnRNP A2 expression in HeLaS3 cells promoted apoptosis.

The rapid cell death elicited by siRNAs targeting hnRNP A1 and hnRNP A2 was consistent with these proteins functioning as telomeric capping proteins.
25 If this is the case, one would predict that a reduction in hnRNP A1 and hnRNP A2 levels would result in a decrease in the length of single-stranded G-rich extension on telomeres. To determine whether the single-stranded extensions were shortened when hnRNP A1 and hnRNP A2 levels were reduced by siRNA treatment, we performed a telomere oligonucleotide ligation assay (T-OLA)
30 (Figure 5A and 5B). This assay characterized the size distribution of G-rich

extensions in HeLaS3 cells treated with siRNAs combinations targeting hnRNP A1 and A2 and control siRNAs. HeLaS3 cells treated for 72 hours with the siRNA combination targeting both hnRNP A1 and hnRNP A2 exhibited a difference in the size distribution of ligated telomeric oligonucleotides (Figure 5A) relative to cells treated with the control siRNA mismatch control combination (A1-1M/A2-1). A decrease in hnRNP A1 and A2 expression was associated with shorter telomeric extensions (Figures 5C and 5D). The same result was observed at 48-hours post-transfection (data not shown). Most importantly, we did not observe a similar change in the length of the G-rich extensions when HeLaS3 cells were treated for 48-hours with staurosporine, an inducer of apoptosis.

Comparison of varying concentration of siRNA on RNAi efficacy.

HeLa cells were seeded in 6-well plates (65,000 cells/well) and after 24-hours they were transfected with combinations of siRNA targeting both hnRNP A1 and hnRNP A2 (A1#1 and A2#1 or A1#2 and A2#1) using the methods described below. At 96-hours, Trypan blue dye exclusion assays for cell viability were performed. Final concentrations of siRNA of 1 nM and 2 nM were inefficient at reducing cell viability (Figure 6). Final concentrations of siRNAs of 100 nM and 10 nM were approximately equivalent in their ability to reduce cell viability (Figure 6; Note: the hatched area indicates that the cells presented an altered morphology characteristic of apoptotic cells). The 10 nM siRNA combination concentration was slightly less effective.

Time course of siRNA treatment on HeLaS3 cells

HeLaS3 cells were seeded in 6-well plates (65,000 cells/well) and were transfected at 24 and 48 hours with the indicated combinations of siRNA (80 nM). At each time point indicated, Trypan blue dye exclusion assays for cell viability were performed. Maximal cell death was seen 96 hours after the first transfection (Figure 7A).

Whole cell extracts from 40,000 cells taken at the indicated time points were analyzed by western blotting using a polyclonal antibody against A1/A2/A1^B/B1. The extracts from cells transfected with siRNA combinations that targeted both hnRNP A1 and A2 (A1#1 and A2#1) showed reduced protein expression beginning at 72 hours with a maximal reduction achieved by 144 hours after the first transfection (Figure 7B, top panel). The extracts from cells transfected with siRNA A1#2 and A2#1 showed reduced protein expression beginning at 72 hours and almost no detectable protein expression at 144 hours (Figure 7B, lower panel). Ponceau S-staining of the nitrocellulose membrane was used in both conditions to confirm equal protein loading.

hnRNP A1 and A2 -targeted RNAi promotes apoptosis in a variety of cancer cell lines

The effectiveness of RNAi in reducing levels of A1 and A2 in HeLaS3 cells and their effect on cell viability was assayed in cell lines derived from a variety of human cancers.

Colorectal carcinoma

We first tested the effect of individual or combinations of hnRNP A1 and A2 siRNAs on the colorectal carcinoma cell line HCT116 (Figure 8). Individual or combinations of siRNAs targeting hnRNP A1 and/or hnRNP A2 were applied twice to HCT 116. Cell viability was measured at 72-hours post-transfection. Similar to what was observed for HeLaS3 cells, treatment with individual siRNAs promoted a reduction in the targeted protein (Figure 8), but only the combinations of siRNAs targeting both hnRNP A1 and A2 affected the growth and morphology of HCT116 cells (Figures 9A and 9B). Cells transfected with the mismatched control combination A1-1M/A2-1, showed a reduction in A2, but they did not change morphology (Figure 9B). The apoptotic phenotype was confirmed by testing for PARP and pro-caspase-3 cleavage (data not shown). Thus, specific combinations of siRNAs targeting both hnRNP A1 and hnRNP A2 effectively inhibited A1 and A2 protein expression and promoted the death of HCT116 cells.

Similar results were obtained with the colorectal carcinoma cell line HT29, which expresses a mutated p53 (data not shown). These results indicate that the siRNA hnRNP A1 and hnRNP A2-mediated apoptosis occurs independently of p53.

Fibrosarcoma

5 The effect of siRNA-mediated reduction in hnRNP A1 and A2 expression was also tested in the fibrosarcoma cell line HT1080 (Figure 10). These assays were carried out as described for HCT116. The siRNA-mediated reduction in hnRNP A1 and A2 expression correlated with a reduction in protein expression (Figure 10), cell growth (Figure 11A) and a change in cell morphology that is
10 characteristic of apoptosis (Figure 11B). The DNA content analysis revealed an increase in cells in the subG1 category (data not shown). This was consistent with apoptosis-mediated chromatin fractionation.

Breast carcinoma, ovarian carcinoma, and glioblastoma

Additional cancer cell lines that were tested include the breast carcinoma
15 cell line, MCF-7 (Figure 12), the ovarian carcinoma cell line, PA-1 and the metastatic ovarian carcinoma SK-OV-3 (provided by Claudine Rancourt), and the glioblastoma cell line, U373 (generously supplied by David Fortin). In all cases, treatment with the hnRNP A1 and A2 combination siRNA pair, A1-1/A2-1, elicited a marked reduction in the expression of hnRNP A1 and A2 polypeptides
20 that was accompanied by a reduction in cell growth and a phenotypic change characteristic of apoptosis. Treatment with individual siRNAs or with the siRNA mismatch control combination (A1-1M/A2-1) displayed no phenotypic changes even when they produced a reduction in hnRNP A1 or A2 expression.

Reduced expression of hnRNP A1 and hnRNP A2 does not affect the growth of mortal cell lines

To evaluate the impact of treatment with siRNAs that target hnRNP A1 and hnRNP A2 expression in normal cells, we used three mortal cell lines: colonic
5 myofibroblasts CCD-18Co (Figure 13), foreskin fibroblasts BJ (Figure 14), and the epithelial intestinal cell line HIEC (Figure 15)(supplied by Jean-François Beaulieu). We also used the BJ-TIELF cell line (Figures 16 and 17) that is immortalized, but is an otherwise apparently normal version of the BJ line, expressing the catalytic component (hTERT) of human telomerase (kindly
10 provided by James Smith, Baylor College of Medicine, Texas).

The BJ-TIELF cell line is immortal because it expresses the catalytic subunit of telomerase. Cells were seeded in 6-well were transfected twice with the indicated siRNA alone (80 nM) or with combinations of siRNA (40 nM each siRNA for a total concentration of 80 nM). Control cells were treated with
15 oligofectamine in the absence of siRNA. Trypan blue dye exclusion assays for cell viability were performed 72 hours after the first transfection and cell growth was evaluated (expressed in population doublings). Western analysis was carried out with the polyclonal antibody against A1/A2/A1^{B}/B1. Ponceau S-staining of the nitrocellulose membrane was used to confirm equal protein loading of all lanes
20 (not shown). At 96 hours post-transfection, adherent cells were photographed and both adherent and floating cells were harvested and counted.

Cell viability was evaluated by trypan blue dye exclusion (Figure 16A) and morphology was evaluated using phase contrast microscopy (200X magnification) (Figure 16B). DNA content analysis of BJ-TIELF cells treated with siRNA
25 against hnRNP A1 and hnRNP A2 was carried out (Figure 17). The 96 hour-post transfection profile is compared with a parallel treatment of HeLaS3 cells.

All these mortal cells express hnRNP A1 and A2 proteins (Figure 18). As noted previously, hnRNP A1 and A2 expression drops when mortal cells approach senescence (Hubbard et al., Exp Cell Res. 218: 241-247, 1995). The immortal BJ-

TIELF cell line consistently expressed higher levels of hnRNP A1 and hnRNP A2 proteins than was observed even in early passages of BJ cells.

RNA interference assays with siRNAs targeting hnRNP A1, A2, or both reduced the corresponding protein level. This decrease was comparable to the decrease observed in similarly treated cancer cell lines (Figure 18). In contrast to our results with cancer cell lines, described above, the mortal cell lines tolerated a reduction in hnRNP A1 and hnRNP A2 expression, but no significant effects on cell growth and morphology were observed. Even the growth of immortal, but non-transformed, BJ-TIELF cells was not affected by siRNA treatment that decreased hnRNP A1 and A2 expression levels by 50% of the level observed in untreated cells. In all cases examined, cell cycle analysis of the DNA content indicated no subG1 increases. We concluded that mortal human cell lines tolerate very well a reduction in hnRNP A1 and A2 proteins imposed by RNA interference in contrast to cancer cell lines.

A1 and A2 RNAi effects on cell growth and protein expression in human cell lines.

A number of normal and cancerous human cell lines were treated with siRNA targeting either hnRNP A1 or hnRNP A2 alone (A1#1, A1#1M, A2#1) or with siRNA combinations targeting both hnRNP A1 and hnRNP A2 (A1#1 and A2#1) or with an A1 mismatch control in combination with an siRNA targeting A2 (A1#1M and A2#1). In each case cells were transfected once at 24 hours and once at 48 hours, and cells were harvested at a timepoint following transfection that allowed for at least 3 to 4 population doublings following the first transfection. Cell growth was measured and protein expression ascertained as described herein. The proportion of apoptotic cells was measured using standard assays. A reduction in hnRNP A1 and A2 expression resulted in extensive cell death in all human cancer cell lines tested, independent of their p53 status. Interestingly, in all the normal human cell lines tested, the reduction in A1 and A2 expression never resulted in massive induction of cell death, although in some normal cell lines the

siRNA combination that targeted both hnRNP A1 and hnRNP A2 (A1#1 and A2#1) resulted in a slight reduction in cell growth rate and in slight morphological changes.

5 **hnRNP A1 and hnRNP A2 expression in cancer and normal tissues**

We used rabbit polyclonal antibodies to investigate the expression of hnRNP A1 and A2 in various human cancer biopsies and normal cell types. Immunohistochemistry was performed with an anti-A1 antibody that binds the A1 and A1B proteins, and with an anti-A1/A2 antibody that binds $A1/A1^{B}/A2/B1$

10 proteins.

Table I shows hnRNP A1 and hnRNP A2 expression in cancer tissues. The cancer screen was performed on 8 different human cancer types. Three different biopsies per cancer type were analyzed using the rabbit polyclonal anti-A1 and anti A1/A2 sera. The overall results of the nuclear expression of hnRNA A1 and A2 is

15 reported with a note in superscript (^{c}) indicating the status of hnRNP A1 and hnRNP A2 expression in the cytoplasm. Expression levels are reported as follows: Strong: +++, Moderate: ++, Low: +, Very low: +/-, Negative: -.

TABLE I

Tumor	**Sample**	**A1/A2 expression[1]**
Breast cancer	1	$+++^{c++}$
	2	$+++^{c++}$
	3	+++
Colon carcinoma	1	$+++^{c+++}$
	2	$+^{c+++}$
	3	$+++^{c++}$
Lung adenocarcinoma	1	$+/-^{c+}$
	2	$++^{c++}$
	3	+++
Small Cell Lung carcimoma	1	$++^{c++}$
	2	$++^{c++}$
	3	++
Ovary carcinoma	1	$++^{c+}$
	2	++
	3	$+++^{c++}$
Pancreas carcinoma	1	$+/-^{c+++}$
	2	$+++^{c+/-}$
	3	$++^{c++}$
Prostate carcinoma	1	$++^{c++}$
	2	$-^{c++}$
	3	++
Skin melanoma	1	++
	2	$++^{c++}$
	3	+/-

[1]Expression levels: Strong: +++, Moderate: ++, Low: +, Very low: +/-, Negative: -.

Table II shows hnRNP A1 and hnRNP A2 expression in normal tissues. The normal tissue screen was performed on 10 different normal human tissues (one sample per tissue) using both an the anti-A1 and an the anti-A1/A2 sera. Two different sections of the same tissue sample were independently treated with each serum. Results are given for the cell types that were observed in each section. The overall results of the nuclear expression of hnRNP A1 and hnRNP A2 is reported with a note in superscript (^{c}) indicating the status of hnRNP A1 and hnRNP A2 expression in the cytoplasm.

TABLE II

Tissue	Cell type	A1/A2 expression[1]
Brain	neurons (some)	++
	neutrophils	$-^{c+/-}$
	astrocytes, microglia, oligodendrocytes, endothelium, vascular smooth muscle	-
Heart	cardiac myocytes, endothelial cells, vascular smooth muscle, fibroblasts	-
Kidney	endothelium, thick and thin loop of Henle, glomerular capillary and collecting duct endothelium, vascular smooth muscle	+/++
	Bowman's capsule epithelium, podocytes, proximal and distal convoluted tubules	+/++
	mesanglial cells	-
Liver	hepatocytes, endothelium, lymphocytes, vascular smooth muscle	+
	bile duct	++
	fibroblasts	-
	macrophages, Kupffer cells	$-^{c+}$
Lungs	pneumocytes, fibroblasts, endothelium, mesothelium	+
	alveolar macrophages	$-^{c++}$
Pancreas	endothelium, vascular smooth muscle, fibroblasts, adipocytes	-
	peripheral islets cells	$-^{c+++}$
	acinar epithelium	$+/-^{c++}$
	pancreatic duct	+/-
Skeletal muscle	myocytes	$+^{c++}$
	vascular smooth muscle	$-^{c+/-}$
	endothelium	+
	fibroblasts	-
Skin	squamous epiuthelium (basal layer)	++/+++
	squamous epithelium (nucleated layer), superficial dermal fibroblasts, endothelium, lymphocytes	+
	stratum lucidum, eccrine sweet glands	$-^{c+/-}$
	subcutaneous glands	-
	vascular smooth muscle	$-^{c++}$
	mast cells	$-^{c+}$
Small Intestine	neuroendocrine cells, epithelium (bases of crypts)	$-^{c+}$

	villi columnar epithelium, lymphocytes	+
	goblet cells, Schwann cells	-
	macrophages	++[c+]
	smooth muscle	+/-[c++]
	fibroblasts, ganglion cells, endothelium	+/-
Spleen	smooth muscle, macrophages	-[c+]
	lymphocytes, mesothelium	+/++
	fibroblasts	-
	neutrophils	+
	endothelial cells	-[c++]

[1]Expression levels are reported as follows: Strong: +++, Moderate: ++, Low: +, Very low: +/-, Negative: -.

Most normal tissues examined expressed low or undetectable levels of hnRNP A1 and hnRNP A2 proteins, except for the basal layer of the skin, which expressed high levels of A1. Low, or occasional, A1 expression was observed in some neurons, kidney epithelia and endothelium, liver Kuppfer cells, macrophages, bile duct, neuroendocrine tissue, macrophages, crypt cells of the small intestine, lymphocytes, and mesothelium of the spleen.

Higher expression of hnRNP A1 and hnRNP A2 proteins was observed in tumor cells relative to normal cells (Table II and Fig. 19). This expression profile identifies A1 and A2 as a useful markers for cancer detection. The functional association that links hnRNP A1 with telomere biogenesis suggests that A1 plays a crucial role in maintaining the transformed state of neoplastic cells, possibly via its role as a telomeric capping factor. Several reports have documented a high level of expression of A2, and its spliced isoform B1, in lung cancer (Zhou et al., J. Biol. Chem. 271: 10760-10766, 1996; Sueoka et al., Cancer Res. 59: 1404-1407, 1999).

Recent studies have also identified A2/B1 as early markers for pancreatic and breast cancers (Yan-Sanders et al., Cancer Lett, 183: 215-220, 2002; Zhou et al., Breast Cancer Res Treat 66: 217-224, 2001). Given the amino acid sequence identity between A1 and A2, and the fact that both bind telomeric repeats *in vitro*, it appeared that these proteins are functional homologues. Consistent with this view, hnRNP A1 and hnRNP A2 control *in vitro* alternative pre-mRNA splicing in a very similar manner (Hutchison et al., J Biol Chem. 277:29745-52, 2002).

Distinct sets of multiple heterogenous nuclear ribonucleoprotein (hnRNP) A1 binding sites control 5' splice site selection in the hnRNP A1 pre-mRNA.

hnRNP A1 and hnRNP A2 expression in cancerous and benign tissues

5 Figure 19 shows an immunohistological analysis using anti-hnRNP A1 or anti-hnRNP A1/A2 antiserum in benign and cancerous breast (Figure 19A) and pancreatic tissues (Figure 19B).

Therapeutic applications

10 The successful treatment of cancer depends on the identification of therapeutic targets whose expression is restricted to cancer cells and which function to promote or permit unlimited cell growth. Although varied targets have been identified in different types of cancer, there are very few examples of factors that play a ubiquitous role in virtually all types of cancers. The identification of 15 telomeric factors whose expression is restricted to cancer cells would represent a major advance towards novel cancer therapeutic strategies because the maintenance of functional telomeres is essential for cancer cell division, regardless of the mechanisms leading to the development of a cancer.

One promising cancer target is the enzyme telomerase. While telomerase is 20 not expressed in most normal human tissues, except for some highly regenerating tissue types, it is expressed in nearly 85% of all cancers. Although treatments that abrogate telomerase function in cancer cells will likely have health benefits, their success will likely depend on the length of the telomeres present in the cancer cells at the time of treatment, because telomeres must gradually shorten until they reach 25 a critically small length that is incompatible with cell division. In the experiments described herein, we have identified the hnRNP A1 and A2 proteins as targets for cancer therapeutics. While it was known that hnRNP A1 and A2/B1 proteins were expressed at high levels in colon and lung cancers, respectively, we have now shows that moderate to high levels of hnRNP A1 proteins are detected in breast, 30 lung, colon, prostate, ovary, pancreas and skin cancers. Levels of hnRNP A1 and

hnRNP A2 proteins in normal tissues are generally much lower than that observed in cancer cells. Only the basal layer of the skin displayed expressed high levels of hnRNP A1 and A2 proteins.

Remarkably, we found that cancer cell lines from many different origins were all sensitive to decreases in the levels of hnRNP A1 and A2 proteins. The RNAi-mediated reduction in hnRNP A1 and A2 expression levels usually elicited the death of cancer cells by apoptosis within 96 hours. A reduction in hnRNP A1 or A2 protein alone did not induce apoptosis, possibly because A1 and A2 are functional homologues that can compensate for one another. In other experiments, we had observed that a mouse erythroleukemic cell line severely deficient in A1 had short telomeres, whose size increased when A1 expression levels were increased.

It is likely that hnRNP A2, which is normally expressed at a slightly higher level in these cells (data not shown), partially compensated for reductions in A1 function, when A1 alone was targeted, allowing the cells to survive. Thus, in a situation where A1 and A2 are expressed in equimolar amounts, it may be virtually impossible to reduce the global level of hnRNP A1 and hnRNP A2 by 50% by targeting either A1 or A2 alone. Only by targeting A1 and A2 in combination is it possible to achieve a global reduction in hnRNP A1 and hnRNP A2 levels and to inhibit cell growth. Still, it is possible that in some cell types, hnRNP A1 and A2 expression may be independently controlled. For example, some cancer cells may express higher levels of hnRNP A1 and lower levels or no hnRNP A2. In such cell types, targeting either A1 or A2 individually might inhibit cell growth and induce programmed cell death.

The rapidity with which cancer cells die following treatment with hnRNP A1 and A2 -specific siRNAs is consistent with hnRNP A1 and A2 proteins acting as telomeric capping factors. Consistent with this view, we have now shown that reduced hnRNP A1 and A2 expression is accompanied by a decrease in the length of the telomeric single-stranded G-rich extensions. Because this shortening can be detected between 48 and 72 hours after siRNA treatment, a reduction in the size of

telomeric overhangs may be triggering cell growth arrest that would in turn elicit cancer cell apoptosis. Most importantly, this decrease in the length of G-rich extensions is not observed when cells are treated with the apoptotic inducer staurosporine, suggesting that the degradation of single-stranded telomeric repeats is not an obligatory feature associated with apoptosis. This provides further evidence to support the conclusion that a decrease in hnRNP A1 and A2 expression is directly responsible for a decrease in the length of the G-rich extension. It is also very interesting to note that the apoptosis induction appears independent of the status of p53 expression since p53 null and p53 mutant cell lines were equally sensitive to RNAi against hnRNP A1 and A2. In contrast, apoptosis triggered by a dominant negative mutation in the telomeric factor TRF2 required the presence of wild-type p53 protein. These results suggest that mutated TRF2 and reduced levels of hnRNP A1 and A2 trigger different events that lead to apoptosis.

In sharp contrast, the siRNA-mediated reduction in hnRNP A1 and A2 levels in mortal cell lines did not affect cell division and did not induce cell death. The fact that these "normal" cell lines are resistant to decreases in hnRNP A1 and A2 expression is intriguing and suggests that differences exist in the telomere capping structure of cancer cells and normal cells. This is not entirely unexpected given that telomerase is usually expressed in cancer cells but is usually not expressed in normal cells. Telomerase expression, and other factors, may lead to differences in the size of the single-stranded G-rich extensions, possibly affecting the identity and function of capping factors. The hPot1 protein has recently been shown to associate with human telomeric single-stranded extensions. Future studies should clarify the expression profile of hPot1 and its contribution to the telomere capping function in normal and cancer cells.

In summary, we have demonstrated that decreased expression of both hnRNP A1 and A2 caused programmed cell death in a variety of cancer cell lines, including p53-compromised cells. Our findings establish hnRNP A1 and hnRNP A2 as drug targets in cancer therapeutics and provide a strong rationale for the

development of strategies aimed at abrogating the expression or function of hnRNP hnRNP A1 and hnRNP A2 proteins in cancer cells. Such approaches are particularly attractive given that hnRNP A1 and hnRNP A2 are expressed at low levels in normal tissues, and that reducing hnRNP A1 and hnRNP A2 levels in
5 mortal cell lines does not significantly effect their cell growth or survival.

Methods

Transfection

The day before transfection, exponentially growing cells were trypsinized,
10 counted, and seeded in 6-well plates so that they were 30-50% confluent on the day of transfection. See below for the appropriate number of cells and media for each cell line; note that some cell lines need FBS whereas for other cell lines it is important not to add FBS. Antibiotics were avoided at the time of plating and during transfection; cell cultures below 20 passages were always selected.
15

Cell line	Cell number/well	Culture media
HeLa S3	65,000	DMEM + **10% FBS**
HCT116	65,000	McCoys5A + **10% FBS**
HT-29	50,000	McCoys5A + **10% FBS**
MCF7	100.000	MEM Earle's Salt **w/o FBS**
HT1080	50,000	MEM Earle's Salt **w/o FBS**
HIEC	100,000	OPTI-MEM I + **5% FBS**
BJ	100,000	αMEM **w/o FBS**
BJ-TIELF	50,000	αMEM **w/o FBS**
18Co	100,000	MEM Earle's Salt **w/o FBS**

Cells were incubated overnight at 37° C/5% CO_2. On the day of transfection, mix #1 was prepared for each well and incubated at room temperature
20 for 5 to 10 minutes:

Mix #1
10μl of siRNA (8 μM stock prepared by diluting the 50 μM stock) + 175μl OPTI-MEM I (Invitrogen Cat. #51985-034).

Transfection reagent for each well (mix #2) was also prepared:

Mix #2
4 μl Oligofectamine (Invitrogen Cat. #12252-011) + 11 μl OPTI-MEM I.

5 Mix #2 was added to mix #1, mixed gently, and incubated at room temperature for 20 minutes. The culture media was removed and 800 μl of fresh media was added to each well (use the same media as for the overnight culture). The complex was mixed and overlayed onto the cells. The final concentration of siRNA was 80 nM. The cells were incubated with the mixed compound for 4 h at 10 37° C/5% CO_2. 1.0 ml of growth media containing 2 times the normal concentration of serum was added without removal of the transfection mixture. The cells were incubated at 37° C/5% CO_2. A second, identical transfection was performed 24h after the first one.

Cell viability, cell growth and protein expression were assayed 48-144 15 hours after the first transfection. Depending on the cell line and the analysis, the incubation time varied as described below.

Cell line	Incubation time before protein expression assay	Incubation time before cell viability assay
HeLa S3	72-144 h	72-144 h
HCT116	48-72 h	72 h
HT-29	48-96 h	96 h
MCF7	96 h	96 h
HT1080	96 h	96 h
HIEC	96-168 h	96-168 h
BJ	96-168 h	96-168 h
BJ-TIELF	96-168 h	96-168 h
18Co	72-168 h	72-168 h

Measurement of cell viability by Trypan blue dye exclusion assay:

For each well of transfected cells, the culture media was transferred into a 20 2.0 ml microfuge. Cells were centrifuged (quick spin) to recover the cells that were in suspension and the supernatant was discarded. The adherent cells of each well were rinsed with 400 μl of PBS/EDTA (170 mM NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.5 mM EDTA, 0.0015% phenol red). PBS/EDTA

was transfered to the 2.0 ml micro tube containing the corresponding pellet of floating cells. 300 µl of .06% trypsin in PBS/EDTA was added and incubated for 5 minutes. The trypsinized cells were recovered and transferred to the corresponding 2.0 ml microtube. Each well was rinsed with 400 µl PBS/EDTA,
5 and transferred to the cell suspension. Final volume was determined 50 µl of cell suspension was mixed with 50 µl of trypan blue stain. The trypan blue mix was loaded into the chamber of a hemacytometer and the living (unstained) and dead (blue) cells were counted. The number of cells contained in the total recovered volume was determined. The suspensions were combined, centrifuged for 1 min.
10 and the supernatant was discarded. Cell pellets were resuspended in 100 µl Laemmli Buffer, sonicated to reduce viscosity, and incubated for 3 min in a boiling water bath. Protein concentration was measured on a 10µl aliquot by the method of Lowry. Samples were stored at –20°C until they were used for Western blot analysis (15 to 25 µg/lane on SDS-PAGE) of protein expression.

15

Measurement of cell growth:

Cell growth was measured by calculating the number of population doublings since transfection using the equation: $PD = \log (N_f/N_0)/\log 2$ where:

PD: number of population doublings

20 N_f: Final number of cells (living and dead cells as counted after trypan blue exclusion).

N_0: number of cells at the time of transfection (average number of 110,000 for HeLaS3 cells; 150,000 for HCT116 cells; 80,000 for MCF7 cells)

25 *Anti-hnRNP antibodies*

Rabbit polyclonal sera raised against either a peptide unique to the hnRNP A1 protein peptide sequence: (ASASSSQRGR) or against a peptide common to both hnRNP A1 and A2 proteins (KEDTEEHHLRDYFE) was used to carry out the immunohistochemical studies. Peptide synthesis and antibody production was
30 carried out by the Eastern Quebec Proteomic Center (Quebec City). The

specificity of each serum was confirmed by ELISA and western analyses.

Immunohistochemistry

The normal tissue screen was performed on 10 different normal human tissues (brain, heart, kidney, liver, lung, pancreas, skeletal muscle, skin, small intestine and spleen) using both sera. Two different sections of the same tissue sample were independently treated with each serum. The cancer screen was performed on 8 different human cancer types (breast carcinoma, colon carcinoma, lung adenocarcinoma, lung small cell carcinoma, ovary carcinoma, pancreas carcinoma, prostate carcinoma and skin melanoma). Three different samples per cancer type were screened using the anti-A1 and the anti-A1/A2 sera. Immunohistochemistry was conducted by LifeSpan BioSciences Inc. (Seattle, WA).

Cell culture

HeLaS3, HCT 116, HT-1080, MCF-7 and CCD-18Co cells were from the American Type Culture Collection. BJ foreskin normal fibroblasts were kindly provided by James Smith (Baylor College of Medicine, Houston). HIEC cells were from Jean-François Beaulieu (Université de Sherbrooke, Québec). PA-1 and SK-OV-3 cells were provided by Claudine Rancourt (Université de Sherbrooke, Québec). U387 were kindly supplied by David Fortin (Université de Sherbrooke, Québec). HeLaS3 and U-373 MG cells were grown in DMEM supplemented with 10% FBS. HCT 116 cells were grown in McCoy's 5A media supplemented with 10% FBS. BJ and BJ-TIELF cells were grown in αMEM supplemented with 10% FBS. HIEC cells were grown in Opti-MEM I supplemented with 5% FBS. PA-1 and SK-OV-3 cells were grown in DMEM-F12 supplemented with 10% FBS. MCF-7 cells were grown in EMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids and 10 μg/ml bovine insulin. HT-1080 and CCD-18Co cells were grown in αMEM supplemented with 10% FBS, Earle's salt, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids.

siRNAs

Oligonucleotides were purchased from Dharmacon Research, Inc. (Lafayette, CO). The nucleic acid sequences to be targeted were identified as follows. The mRNA sequence to be targeted was BLAST searched against the
5 human genome to ensure that only one human gene was targeted by each siRNA. Seven siRNAs targeting the human hnRNP A1 mRNA (GenBank accession number NM_002136) were tested. They covered nucleotides 107 to 127 from the start codon (A1-1), 135 to 155 (A1-2), 154 to 174 (A1-3), 217 to 237 (A1-4), 404 to 424 (A1-5), 601 to 621 (A1-6) and 757 to 777 (A1-7). Five siRNAs were
10 directed at the hnRNP A2 mRNA (GenBank accession number NM_002137) and were from nucleotides 48 to 68 (A2-1), 57 to 77 (A2-2), 298 to 318 (A2-3), 615 to 635 (A2-4) and 922 to 942 (A2-5). Prior to transfection, siRNA duplexes were prepared by annealing complementary pairs of oligonucleotides. Duplex formation was verified by fractionating a portion of the mixture on a 2% agarose
15 gel. The final concentration of the siRNA duplex was 50 μM in 20 mM KCl, 6 mM HEPES-KOH pH 7.5 and 0.2 mM $MgCl_2$. This mixture was stored frozen in aliquots at –80° C.

The sequence of the siRNAs are

A1#1 5'-UGGGGAACGCUCACGGACUdTdT-3' (sense), 3'-
20 dTdTACCCCUUGCGAGUGCCUGA-5' (antisense),

A1#1M: 5'-UGGGGAACCGUCACGGACUdTdT-3' (sense), 3'-dTdTACCCCUUGGCAGUGCCUGA-5' (antisense),

A1#2: 5'-UGAGAGAUCCAAACACCAAdTdT-3' (sense), 3'-dTdTACUCUCUAGGUUUGUGGUU-5' (antisense),

25 A1#3: 5'-GCGCUCCAGGGGCUUUGGGdTdT-3' (sense), 3'-dTdTCGCGAGGUCCCCGAAACCC-5' (antisense),

A1#4: 5'-UCGAAGGCCACACAAGGUGdTdT-3' (sense) 3'-dTdTAGCUUCCGGUGUGUUCCAC-5' (antisense),

A1#5: 5'-AUCAUGACUGACCGAGGCAdTdT-3' (sense), 3'-
30 dTdTUAGUACUGACUGGCUCCGU-5' (antisense),

A1#6: 5'-CUUUGGUGGUGGUCGUGGAdTdT-3' (sense), 3'-dTdTGAAACCACCACCAGCACCU-5' (antisense),

A1#7: 5'-UUUUGGAGGUGGUGGAAGCdTdT-3' (sense), 3'-dTdTAAAACCUCCACCACCUUCG-5' (antisense),

5 A2#1: 5'-GCUUUGAAACCACAGAAGAdTdT-3' (sense), 3'-dTdTCGAAACUUUGGUGUCUUCU-5' (antisense),

A2#2: 5'-CCACAGAAGAAAGUUUGAGdTdT-3' (sense), 3'-dTdTGGUGUCUUCUUUCAAACUC-5' (antisense),

A2#3: 5'-GAAGCUGUUUGUUGGCGGAdTdT-3' (sense), 3'-

10 dTdTCUUCGACAAACAACCGCCU-5' (antisense),

A2#4: 5'-AUUUCGGACCAGGACCAGGdTdT-3' (sense), 3'-dTdTUAAAGCCUGGUCCUGGUCC-5' (antisense),

A2#5: 5'-CUUUGGUGGUAGCAGGAACdTdT-3' (sense), 3'-dTdTGAAACCACCAUCGUCCUUG-5' (antisense).

15 *Transfection*

The day before transfection, exponentially growing cells were trypsinized and seeded into 6-well plates. Transfection was performed on 30 to 50% confluent cells using Oligofectamine™ according to the manufacturer's instructions and at the indicated siRNA concentrations: HeLaS3 (80 nM), HCT 116 (20 or 40 nM),

20 HCT 116 p53- (40 nM), HT-1080 (20 nM), PA-1 (10 nM), U-373 MG (10 nM), SK-OV-3 (20 nM) HIEC (80 nM), BJ (80 nM), BJ-TIELF (80 nM), and CCD-18Co (80 nM). Briefly, the siRNAs (in 10 μl) were mixed with 175 μl of OPTI-MEM-I (Invitrogen) while Oligofectamine™ was mixed with OPTI-MEM-I (4 μl and 11 μl, respectively). The transfection reagent and the siRNAs were then mixed

25 and incubated at room temperature for 20 minutes before being applied to cells. A second transfection at the same concentration of siRNAs was always conducted 24 hours later.

Protocols for TUNEL assay and DNA content analysis

At the indicated time following the first transfection, both adherent and

30 floating cells were harvested and counted. Cell viability was evaluated by trypan

blue dye exclusion. The number of population doublings post-transfection was calculated for each sample using the equation: PD = log (Nf/N0)/log 2.

TUNEL labeling was performed using the ApopTag kit ™(Intergen, S7110), according to the manufacturer's instructions. Briefly, adherent cells were fixed with 2% formaldehyde in PBSA for 1 hour at 4°C and permeabilized in pre-cooled ethanol:acetic acid (2:1) for 5 minutes at -20°C. The reaction buffer containing the TdT enzyme was incubated on cells for 90 minutes at 37°C in a wet chamber to create tails with digoxigenin-dNTP. The TdT products were detected using anti-digoxigenin conjugated with fluorescein for 30 minutes in a wet chamber at room temperature. Propidium iodide (0.5 μg/ml) was used as a nuclear couterstain to visualize the whole cell population. The cells were visualized by fluorescence microscopy.

For DNA content analysis, both floating and adherent cells were recovered, fixed in 80% cold ethanol, stand at room temperature for 5 minutes and stored at -20°C (could be stored up to two weeks). The cells were washed with PBSA and treated with RNAse A for 30 minutes at 37°C (20 μg RNAse A, 5 mM EDTA, 0.5% BSA in PBSA). The cells were stained with propidium iodide (50 μg) for 5 minutes at room temperature and read on a Becton Deckinson FACScan™ using the CellQuest™ software. For each sample, at least 10,000 cells were analyzed for DNA content.

Western blotting

Whole cell extracts were prepared by lysing cells in Laemmli sample buffer. Equal amounts of each sample (15 to 25 μg) were loaded onto a polyacrylamide gel. Western blotting was performed according to standard protocols using the following dilutions for primary antibodies: 1:5000 for the anti-A1/A2 antibodies; 1:500 for the anti-PARP antibodies (Biosource, AHF0262); 1:100 for the active caspase-3 antibodies (Chemicon, AB3623); and 1:500 for the anti-pro-caspase-3 antibody (Biosource, AHZ0052).

Telomere G-tail extension analysis

The T-OLA assay was carried out as described in Cimino-Reale et al., Nucl. Acids Res. 29:e35, 2001. Briefly, genomic DNA was prepared from by standard cell lysis protocols. Oligonucleotide $(CCCTAA)_3$ was end-labeled and
5 phosphorylated by T4 polynucleotide kinase in the following reaction mixture: 0.16 μM of oligonucleotide, 1.6 μM of [γ-32P]ATP (3000 Ci/mmole, 10 mCi/ml), 70 mM Tris pH 7.6, 10 mM MgCl2, 5 mM DTT and 20U of T4 polynucleotide kinase in a final volume of 50 μl. The reaction was allowed to proceed for 40 minutes at 37°C, then 1 μl of 0.1 M ATP and a further 10U of kinase were added
10 before another 20 minutes incubation period. The enzyme was then heat-inactivated at 65°C for 20 minutes. The oligonucleotide was precipitated with ethanol and dissolved in water. Hybridization was conducted in a 20 μl volume containing 10 μg of undenatured DNA, 0.5 pmole of oligonucleotide, 20 mM Tris pH 7.6, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM
15 nicotinamide adenine dinucleotide (NAD) and 0.1% Triton X-100 in a 0.5 ml PCR tube at 50°C for 12 to 14 hours. Forty units of thermostable Taq ligase (New England Biolabs) and 2 μl of fresh 10 mM NAD stock were added and the ligation reaction was allowed to proceed for 5 hours at the same temperature. Reactions were ended by adding 30 μl of water and by phenol-chloroform extraction.
20 Samples were ethanol-precipitated and dissolved in 6 μl of TE buffer. Three μl of each reaction was mixed with 4 μl of formamide dye, denatured by heating at 90°C and quenched on ice before loading onto a 8% acrylamide-urea gel. Gel were exposed to an autoradiography film before the ligation products were scanned and quantified.

RNA interference

RNAi is a form of post-transcriptional gene silencing initiated by the introduction of double-stranded RNA (dsRNA). Short twenty-one to twenty-five nucleotide double-stranded RNAs are effective at down-regulating gene expression in nematodes (Zamore et al., *Cell* 101: 25-33) and in mammalian tissue culture cell lines (Elbashir et al., *Nature* 411:494-498, 2001, hereby incorporated by reference). The further therapeutic effectiveness of this approach in mammals was demonstrated *in vivo* by McCaffrey et al. (*Nature* 418:38-39. 2002). The nucleic acid sequence of a mammalian gene, such as A1 or A2, can be used to design small interfering RNAs (siRNAs) that will inactivate A1 or A2 target genes that have the specific 21 to 25 nucleotide RNA sequences used. siRNAs may be used, for example, as therapeutics to treat a neoplasia.

Provided with the sequence of a mammalian gene, dsRNAs may be designed to inactivate target genes of interest and screened for effective gene silencing, as described herein. In addition to the dsRNAs disclosed herein, additional dsRNAs may be designed using standard methods.

The specific requirements and modifications of dsRNA are described in PCT application number WO 01/75164 (incorporated herein by reference). While dsRNA molecules can vary in length, it is most preferable to use siRNA molecules that are 21- to 23- nucleotide dsRNAs with characteristic 2- to 3- nucleotide 3' overhanging ends, preferably these are (2'-deoxy)thymidine or uracil. The siRNAs typically comprise a 3' hydroxyl group. Alternatively, single stranded siRNAs or blunt ended dsRNA are used. In order to further enhance the stability of the RNA, the 3' overhangs are stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine. Alternatively, substitution of pyrimidine nucleotides by modified analogs e.g. substitution of uridine 2-nucleotide overhangs by (2'-deoxy)thymide is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl group significantly enhances the nuclease resistance of the overhang in tissue culture medium.

siRNA molecules can be obtained through a variety of protocols including chemical synthesis or recombinant production using a Drosophila *in vitro* system. They can be commercially obtained from companies such as Dharmacon Research Inc. or Xeragon Inc., or they can be synthesized using commercially available kits
5 such as the *Silencer*™ siRNA Construction Kit from Ambion (catalog number 1620) or HiScribe™ RNAi Transcription Kit from New England BioLabs (catalog number E2000S).

Alternatively siRNA can be prepared using any of the methods set forth in PCT number WO01/75164 (incorporated herein by reference) or using standard
10 procedures for *in vitro* transcription of RNA and dsRNA annealing procedures as described in Elbashir S.M. et al. (*Genes & Dev.,* 15:188-200, 2001). siRNAs are also obtained as described in Elbashir S.M. et al. by incubation of dsRNA that corresponds to a sequence of the target gene in a cell-free Drosophila lysate from syncytial blastoderm Drosophila embryos under conditions in which the dsRNA is
15 processed to generate siRNAs of about 21 to about 23 nucleotides, which are then isolated using techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate the 21-23nt RNAs and the RNAs can then be eluted from the gel slices. In addition, chromatography (e.g. size exclusion chromatography), glycerol gradient centrifugation, and affinity purification with
20 antibody can be used to isolate the 21 to 23 nucleotide RNAs.

Short hairpin RNAs (shRNAs) can also be used for RNAi as described in Yu et al. or Paddison et al. (*Proc. Natl. Acad. Sci USA,* 99:6047-6052, 2002; *Genes & Dev,* 16:948-958, 2002; incorporated herein by reference). shRNAs are designed such that both the sense and antisense strands are included within a single
25 RNA molecule and connected by a loop of nucleotides (3 or more). shRNAs can be synthesized and purified using standard *in vitro* T7 transcription synthesis as described above and in Yu et al. (supra). shRNAs can also be subcloned into an expression vector that has the mouse U6 promoter sequences which can then be transfected into cells and used for *in vivo* expression of the shRNA.

30

Introduction of dsRNA into cells

The success of RNAi depends on a number of factors including dsRNA sequence selection and design, the cells being used, transfection reagents and transfection conditions. A variety of methods are available for transfection, or introduction, of dsRNA into mammalian cells. For example, there are several commercially available transfection reagents including but not limited to: TransIT-TKO™ (Mirus, Cat. # MIR 2150), Transmessenger™ (Qiagen, Cat. # 301525), and Oligofectamine™ (Invitrogen, Cat. # MIR 12252-011). Protocols for each transfection reagent are available from the manufacturer.

The concentration of dsRNA used for each target and each cell line varies but in general ranges from 0.05 nM to 500 nM, more preferably 0.1nM to 100 nM, and most preferably 1 nM to 50 nM. If desired, cells can be transfected multiple times, using multiple dsRNAs to optimize the gene-silencing effect.

Stable expression of siRNA

DNA template methods are used to create and deliver siRNA molecules (reviewed in T. Tuschl, *Nature Biotechnology*, 20:446-448, 2002). The siRNA template is cloned into RNA polymerase III transcription units, which normally encode the small nuclear RNA U6 or the human RNAse P RNA H1. These expression cassettes allow for the expression of both sense and anti-sense RNA. Expression cassettes are also available for the stable expression of small hairpin RNAs (see Brummelkamp et al., *Science* 296: 550-553, 2002; Paddison et al., *Genes & Dev.* 16:948-958, 2002; Paul et al., *Nature Biotechnol.* 20:505-508, 2002; and Yu et al., *Proc. Natl. Acad. Sci. USA* 99(9):6047-6052.

The endogenous expression of siRNA or shRNAs from introduced DNA templates is thought to overcome some limitations of exogenous delivery, in particular the transient loss of phenotype. In fact, stable cell lines have been obtained using these expression cassettes allowing for a stable loss of function phenotype (Miyagishi M. and Taira K., *Nature Biotech.*, 20:497-500, 2002; Brummelkamp T.R. et al., *Science*, 296:550-553, 2002). shRNAs can also be

expressed stably using a mouse U6 promoter based expression vector. If desired, stable cell lines for RNAi of A1 and/or A2 can be generated using the above techniques.

5 **Assays for evaluating gene silencing effect**

In general, cells are incubated for 5 hours to 7 days after transfection of siRNA and then harvested for analysis. mRNA and protein expression can be analyzed using any of a variety of art known methods including but not limited to northern blot analysis, RNAse protection assays, luciferase or ß-gal reporter 10 assays, and western blots.

Cell Types

RNAi is used to downregulate gene or protein expression of A1 and/or A2 in virtually any mammalian cell expressing A1 or A2. These cells include, but are 15 not limited to, HeLaS3, HCT116, CCD18Co, BJ, BJ-TIELF, HIEC, NIH3T3, BHK-21, CHO-K1, primary human mammary epithelial cells, and neoplastic cells, which express higher levels of A1 than differentiating tissues (Biamonti et al. J. Mol. Biol. 230: 77-89, 1993).

20 **Assays for evaluating promotion of cell death**

The effectiveness of A1 and/or A2 RNAi in promoting cell death is assayed using any assay systems known in the art, including but not limited to, standard cell growth assays, trypan blue staining for cell survival, TUNEL assays, flow cytometry analysis, detection of apoptotis markers by western blot, or any other 25 assay for apoptosis.

Assays for evaluating telomere length

The effectiveness of A1 and/or A2 RNAi in modulating telomere length can be assayed using virtually any assay for telomere length known in the art, 30 including, but not limited to, Southern blotting with oligonucleotides that are

homologous to telomeric sequences in order to measure telomere restriction fragment (TRF) length or Oligonucleotide Ligation Assays (OLA) to measure the telomeric G-rich strand 3'single-stranded overhang.

5 **Diagnostics**

Expression levels of particular nucleic acids or polypeptides may be correlated with a particular disease state, and thus are useful in diagnosis. Oligonucleotides or longer fragments derived from hnRNP A1 or hnRNP A2 may be used as probes to assay the expression levels of an endogenous hnRNP A1 or
10 hnRNP A2 in a biological sample (e.g., isolated cell, isolated tissue, biopsy specimen, or biological fluid) from a subject (e.g., patient). Biological samples showing increased levels of hnRNP A1 and/or hnRNP A2 relative to a corresponding control sample diagnose the patient as having or having a propensity to develop a neoplasia (e.g., lung cancer, colon cancer, kidney cancer,
15 bone cancer, breast cancer, prostate cancer, uterine cancer, ovarian cancer, liver cancer, pancreatic cancer, brain cancer, lymphoma, melanoma, myeloma, adenocarcinoma, thymoma, plasmacytoma, or any other neoplasm). Preferably, a subject having a neoplasia or having a propensity to develop a neoplasia will show an increase in the expression of at least one of hnRNP A1 or hnRNP A2.
20 In another embodiment, an antibody that specifically binds an hnRNP A1 and/or hnRNP A2 polypeptide may be used for the diagnosis of a neoplasia. A variety of protocols for measuring an alteration in the expression of such polypeptides are known, including immunological methods (such as ELISAs and RIAs), and provide a basis for diagnosing a neoplasia. An increase in the level of
25 an hnRNP A1 and/or hnRNP A2 polypeptide is diagnostic of a patient having a neoplasia.

In yet another embodiment, hybridization with PCR probes that are capable of detecting an hnRNP A1 and/or hnRNP A2 polynucleotide sequences, including genomic sequences, or closely related molecules, may be used to hybridize to a
30 nucleic acid sequence derived from a patient having a neoplasia. The specificity of

the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a conserved motif, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low), determine whether the probe hybridizes to a naturally occurring sequence, allelic variants, or other related sequences. Hybridization techniques may be used to identify mutations indicative of a neoplasia in an hnRNP A1 or hnRNP A2 gene, or may be used to monitor expression levels of these genes (for example, by Northern analysis (Ausubel et al., Ausubel *et al., Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001).

In yet another approach, a subject may be diagnosed for a propensity to develop a neoplasia by direct analysis of the sequence of an hnRNP A1 or hnRNP A2 nucleic acid molecule.

Screening Assays

As discussed above, the expression of an hnRNP A1 or hnRNP A2 gene is increased in neoplasia. Based on this discovery, compositions of the invention are useful for the high-throughput low-cost screening of candidate compounds to identify those that decrease the expression or biological activity of an hnRNP A1 and/or hnRNP A2 polypeptide whose expression is increased in a patient having a neoplasia.

Any number of methods are available for carrying out screening assays to identify new candidate compounds that inhibit the expression of an hnRNP A1 and/or hnRNP A2 polypeptide. In one working example, candidate compounds are added at varying concentrations to the culture medium of cultured cells expressing an hnRNP A1 or hnRNP A2 nucleic acid sequence. Gene expression is then measured, for example, by microarray analysis, Northern blot analysis (Ausubel et al., supra), or RT-PCR, using any appropriate fragment prepared from the nucleic acid molecule as a hybridization probe. The level of gene expression in the presence of the candidate compound is compared to the level measured in a control culture medium lacking the candidate molecule. A compound which

promotes an decrease in the expression of an hnRNP A1 or hnRNP A2 nucleic acid molecule, or a functional equivalent thereof, is considered useful in the invention; such a candidate compound may be used, for example, as a therapeutic to treat a neoplasia in a human patient.

5 In another working example, the effect of candidate compounds may be measured at the level of polypeptide production using the same general approach and standard immunological techniques, such as Western blotting or immunoprecipitation with an antibody specific for a polypeptide encoded by an hnRNP A1 or hnRNP A2 gene. For example, immunoassays may be used to 10 detect or monitor the expression of an hnRNP A1 or hnRNP A2 polypeptide in an organism. Polyclonal or monoclonal antibodies that are capable of binding to such a polypeptide may be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA assay) to measure the level of the polypeptide. Preferably, a candidate compound promotes a decrease in the expression or biological activity 15 of the polypeptide. Again, such a molecule may be used, for example, as a therapeutic to prevent, delay, ameliorate, or treat a neoplasia, or the symptoms of a neoplasia, in a human patient.

In yet another working example, candidate compounds may be screened for those that specifically bind to an hnRNP A1 or hnRNP A2 polypeptide. The 20 efficacy of such a candidate compound is dependent upon its ability to interact with such a polypeptide or a functional equivalent thereof. Such an interaction can be readily assayed using any number of standard binding techniques and functional assays (e.g., those described in Ausubel et al., supra). In one embodiment, a candidate compound may be tested *in vitro* for its ability to specifically bind a an 25 hnRNP A1 or hnRNP A2 polypeptide. In another embodiment, a candidate compound is tested for its ability to enhance the biological activity of an hnRNP A1 or hnRNP A2 polypeptide. The biological activity of an hnRNP A1 or hnRNP A2 is assayed using standard methods as described herein.

In another working example, an hnRNP A1 or hnRNP A2 nucleic acid 30 molecule is expressed as a transcriptional or translational fusion with a detectable

reporter, and expressed in an isolated cell (e.g., mammalian or insect cell) under the control of a heterologous promoter, such as an inducible promoter. The cell expressing the fusion protein is then contacted with a candidate compound, and the expression of the detectable reporter in that cell is compared to the expression of the detectable reporter in an untreated control cell. A candidate compound that decreases the expression of the detectable reporter is a compound that is useful for the treatment of a neoplasia. In preferred embodiments, the candidate compound decreases the expression of a reporter gene fused to an hnRNP A1 or hnRNP A2 nucleic acid molecule.

In one particular working example, a candidate compound that binds to an hnRNP A1 or hnRNP A2 polypeptide may be identified using a chromatography-based technique. For example, a recombinant polypeptide of the invention may be purified by standard techniques from cells engineered to express the polypeptide (e.g., those described above) and may be immobilized on a column. A solution of candidate compounds is then passed through the column, and a compound specific for the hnRNP A1 or hnRNP A2 polypeptide is identified on the basis of its ability to bind to the polypeptide and be immobilized on the column. To isolate the compound, the column is washed to remove non-specifically bound molecules, and the compound of interest is then released from the column and collected. Similar methods may be used to isolate a compound bound to a polypeptide microarray. Compounds isolated by this method (or any other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography). Compounds that are identified as binding to a polypeptide of the invention with an affinity constant less than or equal to 10 mM are considered particularly useful in the invention. Alternatively, any *in vivo* protein interaction detection system, for example, any two-hybrid assay may be utilized.

Potential antagonists include organic molecules, peptides, peptide mimetics, polypeptides, nucleic acids, and antibodies that bind to an hnRNP A1 or hnRNP A2 nucleic acid sequence or polypeptide.

Each of the DNA sequences listed herein may also be used in the discovery and development of a therapeutic compound for the treatment of neoplasia. The encoded protein, upon expression, can be used as a target for the screening of drugs. Additionally, the DNA sequences encoding the amino terminal regions of the encoded protein or Shine-Delgarno or other translation facilitating sequences of the respective mRNA can be used to construct sequences that promote the expression of the coding sequence of interest. Such sequences may be isolated by standard techniques (Ausubel et al., *supra*).

Small molecules of the invention preferably have a molecular weight below 2,000 daltons, more preferably between 300 and 1,000 daltons, and most preferably between 400 and 700 daltons. It is preferred that these small molecules are organic molecules.

Test extracts and compounds

In general, compounds that decrease hnRNP A1 or hnRNP A2 expression or biological activity are identified from large libraries of both natural products, synthetic (or semi-synthetic) extracts or chemical libraries, according to methods known in the art. Those skilled in the art will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modifications of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from, for example, Brandon Associates (Merrimack, NH), Aldrich Chemical (Milwaukee, WI), and Talon Cheminformatics (Acton, Ont.)

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including, but not limited to, Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceangraphics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A.
5 (Cambridge, MA). In addition, natural and synthetically produced libraries are produced, if desired, according to methods known in the art (e.g., by combinatorial chemistry methods or standard extraction and fractionation methods). Furthermore, if desired, any library or compound may be readily modified using standard chemical, physical, or biochemical methods.

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hnRNP A1 or hnRNP A2 Production

hnRNP A1 or hnRNP A2 polypeptides are useful in screening for candidate compounds that bind to such polypeptides and inhibit their biological activity. In general, polypeptides, such as hnRNP A1 or hnRNP A2, may be produced by
15 transformation of a suitable host cell, for example, a eukaryotic cell, with all or part of a polypeptide-encoding nucleic acid molecule, or a fragment thereof in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant
20 protein. Eukaryotic hnRNP A1 or hnRNP A2 peptide expression systems may be generated in which an hnRNP A1 or hnRNP A2 gene sequence is introduced into a plasmid or other vector, which is then used to transform living cells. Constructs in which the hnRNP A1 or hnRNP A2 cDNA containing the entire open reading frame inserted in the correct orientation into an expression plasmid may be used
25 for protein expression. Eukaryotic expression systems allow for the expression and recovery of hnRNP A1 or hnRNP A2 peptide fusion proteins in which the hnRNP A1 or hnRNP A2 peptide is covalently linked to a tag molecule that facilitates identification and/or purification. An enzymatic or chemical cleavage site can be engineered between the hnRNP A1 or hnRNP A2 peptide and the tag
30 molecule so that the tag can be removed following purification.

Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted hnRNP A1 or hnRNP A2 nucleic acid in the plasmid-bearing cells. They may also include an origin of replication sequence allowing for their autonomous replication within the host organism, sequences that encode genetic traits that allow vector-containing cells to be selected for in the presence of otherwise sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced that have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

The precise host cell used is not critical to the invention. A hnRNP A1 or hnRNP A2 polypeptide may be produced in any eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, such as Sf21 cells, or mammalian cells, such as NIH 3T3, HeLa, COS cells, or fibroblasts). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, see, e.g., Ausubel *et al., Current Protocols in Molecular Biology*, Wiley Interscience, New York, 2001). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel *et al.* (*supra*); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels *et al.*, 1985, Supp. 1987).

Native hnRNP A1 or hnRNP A2 can be isolated from human cells that produce it naturally, or from transgenic eukaryotic cells that have been engineered to express a recombinant hnRNP A1 or hnRNP A2 gene.

Once the appropriate expression vectors are constructed, they are introduced into an appropriate host cell by transformation techniques, such as, but not limited to, calcium phosphate transfection, DEAE-dextran transfection, electroporation, microinjection, protoplast fusion, or liposome-mediated transfection.

Once the recombinant polypeptide of the invention is expressed, it is isolated, e.g., using affinity chromatography. In one example, an antibody (e.g., produced as described herein) raised against a polypeptide of the invention may be attached to a column and used to isolate the recombinant polypeptide. Lysis and fractionation of polypeptide-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel *et al.*, *supra*). The recombinant protein can be purified by any appropriate techniques, including, for example, high performance liquid chromatography chromatography or other chromatographies (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short peptide fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful peptide fragments or analogs.

Therapeutic hnRNP A1 or hnRNP A2 RNAi

Neoplasms from any warm-blooded mammal may be treated using the methods of the invention. Neoplasms subject to such therapies include, but are not limited to, lung cancer, colon cancer, kidney cancer, bone cancer, breast cancer, prostate cancer, uterine cancer, ovarian cancer, liver cancer, pancreatic cancer, brain cancer, lymphoma, melanoma, myeloma, adenocarcinoma, thymoma, plasmacytoma, or any other neoplasm, such neoplasms are, preferably, characterized by having increased A1 and/or A2 expression. Of particular interest for using the dsRNA molecules of the invention are neoplasms associated with increased expression of the hnRNP gene product or expression of an altered gene product. Warm-blooded animals include, but are not limited to, humans, cows, horses, pigs, sheep, birds, mice, rats, dogs, cats, monkies, baboons, or other mammals.

hnRNP A1 or hnRNP A2 therapeutics for RNAi

The administration of hnRNP A1 or hnRNP A2 nucleic acid molecules for RNAi therapy (e.g., dsRNA, antisense RNA, or siRNA) may be provided to prevent or treat a neoplasm. Such nucleic acid molecules may be administered directly to a tissue or neoplasm or may be provided within an expression vector, such that the nucleic acid molecule mediating the RNAi is stably expressed.

For direct administration of hnRNP A1 or hnRNP A2 nucleic acid molecules for RNAi (e.g., dsRNA, antisense RNA, or siRNA) or mixtures thereof, nucleic acid molecules are provided in a unit dose form, each dose containing a predetermined quantity of such molecules sufficient to silence a target gene in association with a pharmaceutically acceptable diluent or carrier, such as phosphate-buffered saline, to form a pharmaceutical composition. In addition, the hnRNP A1 or hnRNP A2 nucleic acid molecules for RNAi may be formulated in a solid form and redissolved or suspended prior to use. The pharmaceutical composition may, optionally, contain other chemotherapeutic agents, antibodies, antivirals, and exogenous immunomodulators.

The route of administration may be intravenous, intramuscular, subcutaneous, topical, intradermal, intraperitoneal, intrathecal, *ex vivo*, and the like. Administration may also be by transmucosal or transdermal means, or the compound may be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the hnRNP A1 or hnRNP A2 nucleic acid molecule for RNAi is formulated into conventional oral administration forms, such as capsules, tablets and tonics. For topical administration, the nucleic acid molecules of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

In providing a mammal with the hnRNP A1 or hnRNP A2 nucleic acid molecules for RNAi, the dosage of administered nucleic acid molecules will vary depending upon such factors as the mammal's age, weight, height, sex, general medical condition, previous medical history, disease progression, tumor burden, and the like. The dose is administered as indicated. Other therapeutic drugs may be administered in conjunction with the nucleic acid molecules.

The efficacy of treatment using the nucleic acid molecules described herein may be assessed by determination of alterations in the concentration or activity of the DNA, RNA or gene product of A1 and A2, tumor regression, or a reduction of the pathology or symptoms associated with the neoplasm.

Nucleic acid therapy

Nucleic acid therapy is another therapeutic approach for preventing or ameliorating a neoplasia related to the increased expression of an hnRNP A1 and hnRNP A2 nucleic acid molecule. Expression vectors encoding anti-sense nucleic acid molecules, dsRNAs, siRNAs, or shRNAs can be delivered to cells that overexpress an endogenous hnRNP A1 and hnRNP A2 nucleic acid molecule. Such delivery results in the sustained expression of hnRNP A1 and hnRNP A2 nucleic acid molecules for RNAi. The nucleic acid molecules must be delivered to cells in need of RNAi (e.g., neoplastic cells) in a form in which they can be taken up by the cells and so that sufficient levels of RNAi nucleic acid molecules can be produced to decrease hnRNP A1 or A2 levels in a patient having a neoplasia.

Transducing viral (*e.g.*, retroviral, adenoviral, and adeno-associated viral) vectors can be used for somatic cell gene therapy, especially because of their high efficiency of infection and stable integration and expression (see, *e.g.*, Cayouette *et al.*, Human Gene Therapy 8:423-430, 1997; Kido *et al.*, Current Eye Research 15:833-844, 1996; Bloomer *et al.*, Journal of Virology 71:6641-6649, 1997; Naldini *et al.*, Science 272:263-267, 1996; and Miyoshi *et al.*, Proc. Natl. Acad. Sci. U.S.A. 94:10319, 1997). Other viral vectors that can be used include, for example, a vaccinia virus, a bovine papilloma virus, or a herpes virus, such as

Epstein-Barr Virus (also see, for example, the vectors of Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis *et al.*, BioTechniques 6:608-614, 1988; Tolstoshev *et al.*, Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta
5 *et al.*, Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller *et al.*, Biotechnology 7:980-990, 1989; Le Gal La Salle *et al.*, Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995). Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg *et al.*, N. Engl.
10 J. Med 323:370, 1990; Anderson *et al.*, U.S. Patent No. 5,399,346). Most preferably, a viral vector is used to express an hnRNP A1 or hnRNP A2 nucleic acid molecule capable of mediating RNAi.

Non-viral approaches can also be employed for the introduction of an RNAi therapeutic to a cell of a patient having a neoplasia. For example, a nucleic acid
15 molecule can be introduced into a cell by administering the nucleic acid in the presence of lipofection (Felgner *et al.*, Proc. Natl. Acad. Sci. U.S.A. 84:7413, 1987; Ono *et al.*, Neuroscience Letters 17:259, 1990; Brigham *et al.*, Am. J. Med. Sci. 298:278, 1989; Staubinger *et al.*, Methods in Enzymology 101:512, 1983), asialoorosomucoid-polylysine conjugation (Wu *et al.*, Journal of Biological
20 Chemistry 263:14621, 1988; Wu *et al.*, Journal of Biological Chemistry 264:16985, 1989), or by micro-injection under surgical conditions (Wolff *et al.*, Science 247:1465, 1990). Preferably the nucleic acid molecules are contained within plasmid vectors and are administered in combination with a liposome and protamine.

25 Nucleic acid molecule expression for use in RNAi gene therapy methods can be directed from any suitable promoter (*e.g.*, the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in specific cell types,
30 such as tumor cells, can be used to direct the expression of a nucleic acid. The

enhancers used can include, without limitation, those that are characterized as tissue- or cell-specific enhancers.

Combination Therapies

5 hnRNP A1 or A2 nucleic acids of polypeptides may be administered in combination with any other standard neoplasia therapy; such methods are known to the skilled artisan (e.g., Wadler *et al.*, Cancer Res. 50:3473-86, 1990), and include, but are not limited to, chemotherapy, hormone therapy, immunotherapy, radiotherapy, and any other therapeutic method used for the treatment of neoplasia.

10

Other Embodiments

From the foregoing description, it is apparent that variations and modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the

15 following claims.

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

20 What is claimed is:

Claims

1. A method of inducing cell death in a cell, said method comprising inhibiting the expression of hnRNP A1 and hnRNP A2 nucleic acid molecules or polypeptides.

5

2. The method of claim 1, wherein said method comprises administering to the cell (i) a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A1 and (ii) a nucleic acid molecule having at least one strand that is complementary to at least a

10 portion of the sequence of hnRNP A2, wherein said nucleic acid molecules are administered in an amount sufficient to reduce the expression of endogenous hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins.

3. The method of claim 1, wherein said administered nucleic acid

15 molecules are double stranded nucleic acid molecules.

4. The method of claim 1, wherein said administered nucleic acid molecules are siRNA nucleic acid molecules.

20 5. The method of claim 1, wherein said administered nucleic acid molecules are anti-sense nucleic acid molecules.

6. The method of claim 1, wherein said administered nucleic acid molecules are stably expressed in said cell.

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7. The method of claim 1, wherein said cell is a neoplastic cell.

8. The method of claim 7, wherein said neoplastic cell is a mammalian cell.

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9. The method of claim 8, wherein said mammalian cell is a human cell.

10. The method of claim 9, wherein said human cell is *in vivo*.

5

11. The method of claim 1, wherein said cell death is caused by telomere uncapping.

12. The method of claim 4, wherein said siRNA has 100% nucleic acid
10 sequence identity to at least 18 nucleotides of SEQ ID NOs:29 or 30.

13. The method of claim 4, wherein said siRNA has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NOs:29 or 30.

15 14. The method of claim 4, wherein said siRNA has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NOs:29 or 30.

15. The method of claim 5, wherein said antisense nucleic acid molecule is complementary to at least 10 nucleotides of SEQ ID NOs:29 or 30.

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16. The method of claim 2, wherein said antisense nucleic acid molecule is complementary to at least 20 nucleotides of SEQ ID NOs:29 or 30.

17. The method of claim 2, wherein said antisense nucleic acid molecule
25 is complementary to at least 30 nucleotides of SEQ ID NOs:29 or 30.

18. The method of claim 1, wherein said method is sufficient to induce apoptosis in a neoplastic cell, but not in a normal cell.

19. A method of treating a subject having a neoplasm, said method comprising administering to a cell of said subject (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A1 and (ii) a nucleic acid molecule comprising at
5 least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A2, wherein said administering decreases expression of hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins in a cell of said subject.

10 20. The method of claim 12, wherein said administered nucleic acid molecules are double stranded nucleic acid molecules.

21. The method of claim 12, wherein said administered nucleic acid molecules are siRNA nucleic acid molecules.

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22. The method of claim 12, wherein said administered nucleic acid molecules are anti-sense nucleic acid molecules.

23. The method of claim 12, wherein said administered nucleic acid
20 molecules are stably expressed in said cell.

24. The method of claim 12, wherein said administering specifically induces cell death in a neoplastic cell of said subject, but does not induce cell death in a normal cell of said subject.

25

25. The method of claim 17, wherein said cell death is caused by telomere uncapping.

26. The method of claim 12, wherein said subject has bladder, blood,
30 bone, brain, breast, cartilage, colon kidney, liver, lung, lymph node, nervous tissue,

ovary, pancreatic, prostate cancer, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, urogenital tract, ureter, urethra, uterus, or vaginal cancer.

5 27. The method of claim 12, wherein said method is administered in combination with any standard cancer therapy.

28. The method of claim 21, wherein said siRNA has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NOs:29 or 30.

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29. The method of claim 21, wherein said siRNA has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NOs:29 or 30.

30. The method of claim 21, wherein said siRNA has 100% nucleic acid
15 sequence identity to at least 20 nucleotides of SEQ ID NOs:29 or 30.

31. The method of claim 22, wherein said antisense nucleic acid molecule is complementary to at least 10 nucleotides of SEQ ID NOs:29 or 30.

20 32. The method of claim 22, wherein said nucleic acid molecule is complementary to at least 20 nucleotides of SEQ ID NOs:29 or 30.

33. The method of claim 22, wherein said nucleic acid molecule is complementary to at least 30 nucleotides of SEQ ID NOs:29 or 30.

25

34. A method of decreasing the length of single-stranded telomere extensions of chromosomes in a cell, said method comprising administering to a cell (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNPA1 and
30 (ii) a nucleic acid molecule comprising at least one strand that is complementary to

at least a portion of a nucleic acid sequence of hnRNPA2, wherein said administering decreases expression of hnRNP A1 and hnRNP A2 nucleic acid molecules or proteins.

5 35. The method of claim 21, wherein said administered nucleic acid molecules are double stranded nucleic acid molecules.

36. The method of claim 21, wherein said administered nucleic acid molecules are siRNA nucleic acid molecules.

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37. The method of claim 21, wherein said administered nucleic acid molecules are anti-sense nucleic acid molecules.

38. The method of claim 21, wherein said cell death is the result of

15 increased telomere or chromosome fusion.

39. A pharmaceutical composition comprising a nucleic acid molecule having at least one strand that is substantially complementary to at least a portion of the sequence of hnRNP A1.

20

40. The composition of claim 39, wherein said nucleic acid molecule is an siRNA nucleic acid molecule.

41. The composition of claim 40, wherein said siRNA has 100% nucleic

25 acid sequence identity to at least 18 nucleotides of SEQ ID NOs:29.

42. The composition of claim 40, wherein said siRNA has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NOs:29.

43. The composition of claim 40, wherein said siRNA has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NOs:29.

44. A pharmaceutical composition comprising a nucleic acid molecule 5 having at least one strand that is complementary to at least a portion of the sequence of SEQ ID NO:30.

45. The composition of claim 44, wherein said nucleic acid molecule is an siRNA.

10

46. The composition of claim 45, wherein said siRNA has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO: 30.

47. The composition of claim 45, wherein said siRNA has 100% nucleic 15 acid sequence identity to at least 19 nucleotides of SEQ ID NO: 30.

48. The composition of claim 45, wherein said siRNA has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO: 30.

20 49. The composition of claim 44, wherein said nucleic acid molecule is an antisense nucleic acid molecule.

50. The composition of claim 49, wherein said antisense nucleic acid molecule is 100% complementary to at least 10 nucleotides of SEQ ID NO: 30.

25

51. The method of claim 49, wherein said antisense nucleic acid molecule is 100% complementary to at least 20 nucleotides of SEQ ID NO: 30.

52. The method of claim 49, wherein said antisense nucleic acid 30 molecule is 100% complementary to at least 30 nucleotides of SEQ ID NOs: 30.

53. A pharmaceutical composition comprising (i) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a nucleic acid sequence of hnRNP A1 and (ii) a nucleic acid molecule comprising at least one strand that is complementary to at least a portion of a
5 nucleic acid sequence of hnRNP A2.

54. The composition of claim 53, wherein said nucleic acid molecules are double stranded nucleic acid molecules.

10 55. The composition of claim 53, wherein said nucleic acid molecules are siRNA nucleic acid molecules.

56. The composition of claim 53, wherein said nucleic acid molecules are anti-sense nucleic acid molecules.

15

57. The composition of claim 55, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:30.

20

58. The composition of claim 55, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:30.

25

59. The composition of claim 55, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:30.

30

60. The composition of claim 56, wherein the antisense of (i) is complementary to at least 10 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 10 nucleotides of SEQ ID NO:30.

5 61. The composition of claim 56, wherein the antisense of (i) is complementary to at least 20 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 20 nucleotides of SEQ ID NO:30.

62. The composition of claim 56, wherein the antisense of (i) is 10 complementary to at least 30 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 30 nucleotides of SEQ ID NO:30.

63. A pharmaceutical composition comprising at least one pair of double stranded nucleic acid molecules selected from the group consisting of SEQ ID 15 NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26 in a pharmaceutically acceptable carrier.

64. The pharmaceutical composition of claim 63, wherein said pair of 20 double stranded nucleic acid molecules is SEQ ID NOs:1 and 2.

65. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 3 and 4.

25 66. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 5 and 6.

67. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 7 and 8.

30

68. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 9 and 10.

69. The pharmaceutical composition of claim 63, wherein said pair of
5 double stranded nucleic acid molecules is SEQ ID NOs: 11 and 12.

70. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 13 and 14.

10 71. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 15 and 16.

72. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 17 and 18.

15

73. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 19 and 20.

74. The pharmaceutical composition of claim 63, wherein said pair of
20 double stranded nucleic acid molecules is SEQ ID NOs: 21 and 22.

75. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 23 and 24.

25 76. The pharmaceutical composition of claim 63, wherein said pair of double stranded nucleic acid molecules is SEQ ID NOs: 25 and 26

77. The pharmaceutical composition of claim 26, wherein said composition comprises at least two pairs of nucleic acid molecules selected from
30 the group consisting of SEQ ID NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10,

11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26.

78. A pharmaceutical composition comprising one antisense nucleic acid
5 molecule selected from the group consisting of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26.

79. A kit for the treatment of a neoplasia in a patient comprising at least one pair of double stranded nucleic acid molecules selected from the group consisting of SEQ ID NOs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14, 15 and 16, 17 and 18, 19 and 20, 21 and 22, 23 and 24, and 25 and 26.

80. A kit for the treatment of a neoplasia in a patient comprising at least one antisense nucleic acid molecule selected from the group consisting of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, and 26.

81. A method of diagnosing a patient as having, or having a propensity to develop, a neoplasia, said method comprising determining the level of expression of an hnRNPA1 or hnRNPA2 nucleic acid molecule or polypeptide in a patient sample, wherein an increased level of expression relative to the level of expression in a control sample, indicates that said patient has or has a propensity to develop a neoplasia.

82. The method of claim 81, wherein said method comprises determining the level of expression of said hnRNPA1.

83. The method of claim 81, wherein said method comprises determining the level of expression of said hnRNPA2 nucleic acid molecule.

84. The method of claim 81, wherein said method comprises determining the level of expression of hnRNPA1 and hnRNPA2 nucleic acid molecules.

85. The method of claim 81, wherein said method comprises determining the level of expression of said hnRNPA1 polypeptide.

86. The method of claim 81, wherein said method comprises determining the level of expression of said hnRNPA2 polypeptide.

87. The method of claim 81, wherein said level of expression is determined in an immunological assay.

88. A diagnostic kit for the diagnosis of a neoplasia in a patient comprising a nucleic acid sequence, or fragment thereof, and at least one of an hnRNP A1 and an hnRNP A2 nucleic acid molecule.

89. A method of identifying a candidate compound that ameliorates a neoplasia, said method comprising contacting a cell that expresses a hnRNPA1 and an hnRNPA2 nucleic acid molecule with a candidate compound, and comparing
5 the level of expression of said nucleic acid molecule in said cell contacted by said candidate compound with the level of expression in a control cell not contacted by said candidate compound, wherein a decrease in expression of said hnRNP A1 or hnRNP A2 nucleic acid molecule identifies said candidate compound as a candidate compound that ameliorates a neoplasia.

10

90. The method of claim 89, wherein said decrease in expression is a decrease in transcription.

91. The method of claim 89, wherein said decrease in expression is a decrease in translation.

92. A method of identifying a candidate compound that ameliorates a
5 neoplasia, the method comprising contacting a cell that expresses an hnRNP A1 or hnRNP A2 polypeptide with a candidate compound, and comparing the level of expression of said polypeptide in said cell contacted by said candidate compound with the level of polypeptide expression in a control cell not contacted by said candidate compound, wherein a decrease in the expression of said hnRNP A1 or
10 hnRNP A2 polypeptide identifies said candidate compound as a candidate compound that ameliorates a neoplasia.

93. The method of claim 92, wherein said decrease in expression is assayed using an immunological assay, an enzymatic assay, or a
15 radioimmunoassay.

94. A method of inducing cell death in a cell by inhibiting the expression of an hnRNP A2 nucleic acid molecule or polypeptide.

20 95. The method of claim 94, wherein said method comprises administering to the cell a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A2, wherein said nucleic acid molecule is administered in an amount sufficient to reduce the expression of an hnRNP A2 nucleic acid molecule or protein.

25

96. The method of claim 94, wherein said administered nucleic acid molecules are double stranded nucleic acid molecules.

97. The method of claim 94, wherein said administered nucleic acid
30 molecules are siRNA nucleic acid molecules.

98. The method of claim 94, wherein said administered nucleic acid molecules are anti-sense nucleic acid molecules.

99. The method of claim 94, wherein said administered nucleic acid
5 molecules are stably expressed in said cell.

100. The method of claim 94, wherein said cell is a neoplastic cell.

101. The method of claim 100, wherein said neoplastic cell is a
10 mammalian cell.

102. The method of claim 101, wherein said mammalian cell is a human cell.

15 103. The method of claim 102, wherein said human cell is *in vivo*.

104. The method of claim 94, wherein said cell death is caused by telomere uncapping.

20 105. A vector comprising a nucleic acid molecule positioned for expression, wherein said nucleic acid molecule encodes a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A1.

25 106. The vector of claim 105, wherein said encoded nucleic acid molecules are double stranded nucleic acid molecules.

107. The vector of claim 105, wherein said encoded nucleic acid molecules are siRNA nucleic acid molecules.

30

108. The vector of claim 107, wherein the siRNA has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:29.

109. The vector of claim 107, wherein the siRNA has 100% nucleic acid
5 sequence identity to at least 19 nucleotides of SEQ ID NO:29.

110. The vector of claim 107, wherein the siRNA has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO:29.

10 111. A vector comprising a nucleic acid molecule positioned for expression, wherein said nucleic acid molecule encodes a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A2.

15 112. The vector of claim 111, wherein said encoded nucleic acid molecules are double stranded nucleic acid molecules.

113. The vector of claim 111, wherein said encoded nucleic acid molecules are siRNA nucleic acid molecules.

20

114. The vector of claim 113, wherein the siRNA has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:30.

115. The vector of claim 113, wherein the siRNA has 100% nucleic acid
25 sequence identity to at least 19 nucleotides of SEQ ID NO:30.

116. The vector of claim 113, wherein the siRNA has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO:30.

117. The vector of claim 113, wherein said encoded nucleic acid molecule is an antisense nucleic acid molecule.

118. The vector of claim 117, wherein the antisense is complementary to
5 at least 10 nucleotides of SEQ ID NO:30.

119. The vector of claim 117, wherein the antisense is complementary to at least 20 nucleotides of SEQ ID NO:30.

10 120. The vector of claim 117, wherein the antisense is complementary to at least 30 nucleotides of SEQ ID NO:30.

121. A vector comprising a nucleic acid molecule positioned for expression, wherein said nucleic acid molecule encodes (i) a nucleic acid molecule
15 having at least one strand that is complementary to at least a portion of the sequence of hnRNP A1, and (ii) a nucleic acid molecule having at least one strand that is complementary to at least a portion of the sequence of hnRNP A2.

122. The vector of claim 122, wherein said nucleic acid molecule encodes
20 a dsRNA.

123. The vector of claim 122, wherein said nucleic acid molecule encodes a siRNA.

25 124. The vector of claim 122, wherein said nucleic acid molecule encodes an antisense RNA.

125. The composition of claim 123, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:29 and the

siRNA of (ii) has 100% nucleic acid sequence identity to at least 18 nucleotides of SEQ ID NO:30.

126. The composition of claim 123, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 19 nucleotides of SEQ ID NO:30.

127. The composition of claim 123, wherein the siRNA of (i) has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO:29 and the siRNA of (ii) has 100% nucleic acid sequence identity to at least 20 nucleotides of SEQ ID NO:30.

128. The composition of claim 124, wherein the antisense of (i) is complementary to at least 10 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 10 nucleotides of SEQ ID NO:30.

129. The composition of claim 124, wherein the antisense of (i) is complementary to at least 20 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 20 nucleotides of SEQ ID NO:30.

130. The composition of claim 124, wherein the antisense of (i) is complementary to at least 30 nucleotides of SEQ ID NO:29 and the antisense of (ii) is complementary to at least 30 nucleotides of SEQ ID NO:30.

131. A method of using the nucleic acid molecule of claim 105, 111, or 121 to induce apoptosis in a cell.

132. The method of claim 131, wherein said cell is a neoplastic cell.

133. The method of claim 132, wherein said cell is in a human.

134. A method of using the nucleic acid molecule of claim 105, 111, or 121 to treat a subject having a neoplasm.

5

135. The method of claim 134, wherein said subject is a human.

136. A method of using the nucleic acid molecule of claim 105, 111, or 121 to decrease the length of single-stranded telomere extensions of chromosomes

10 in a cell.

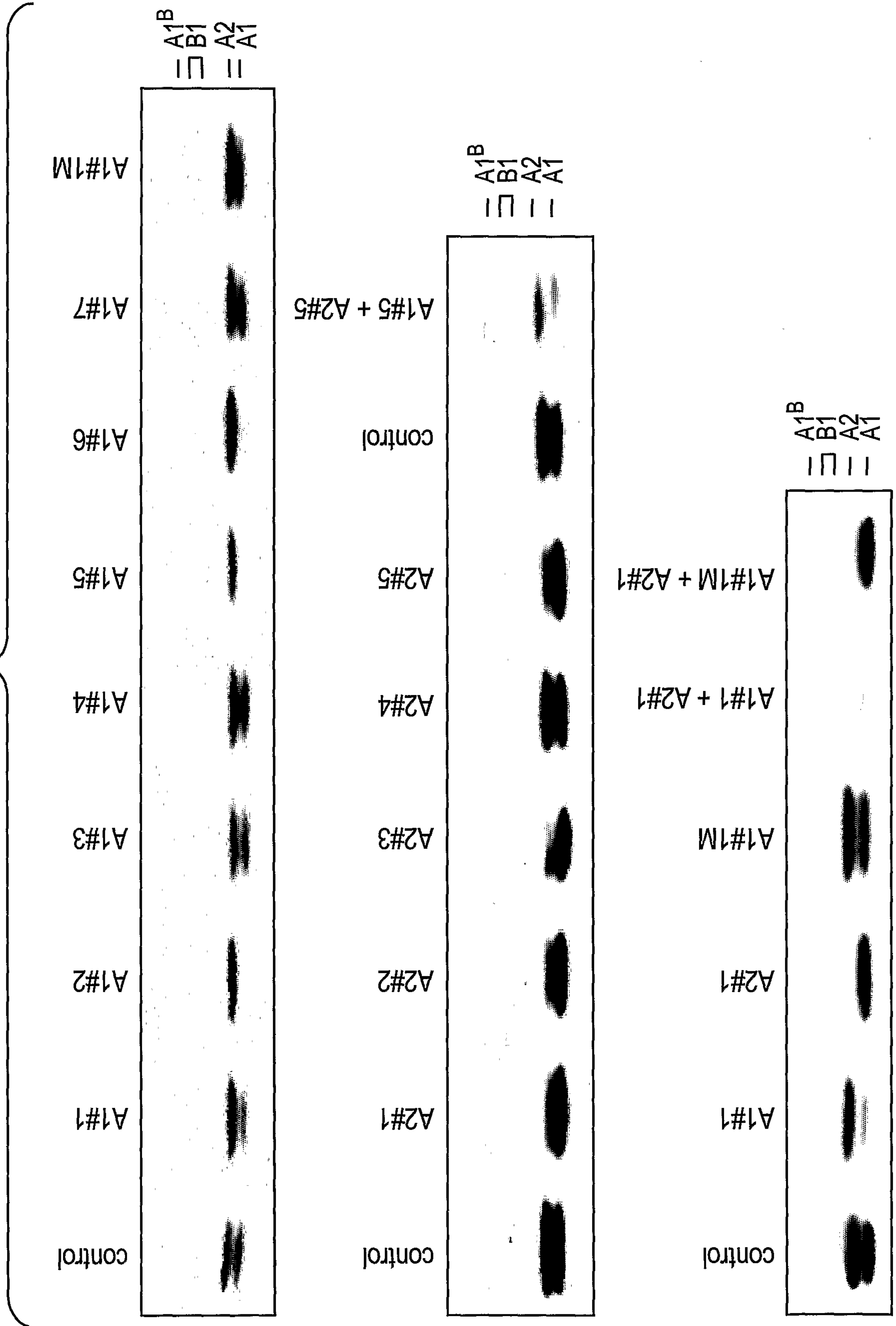


FIG. 1

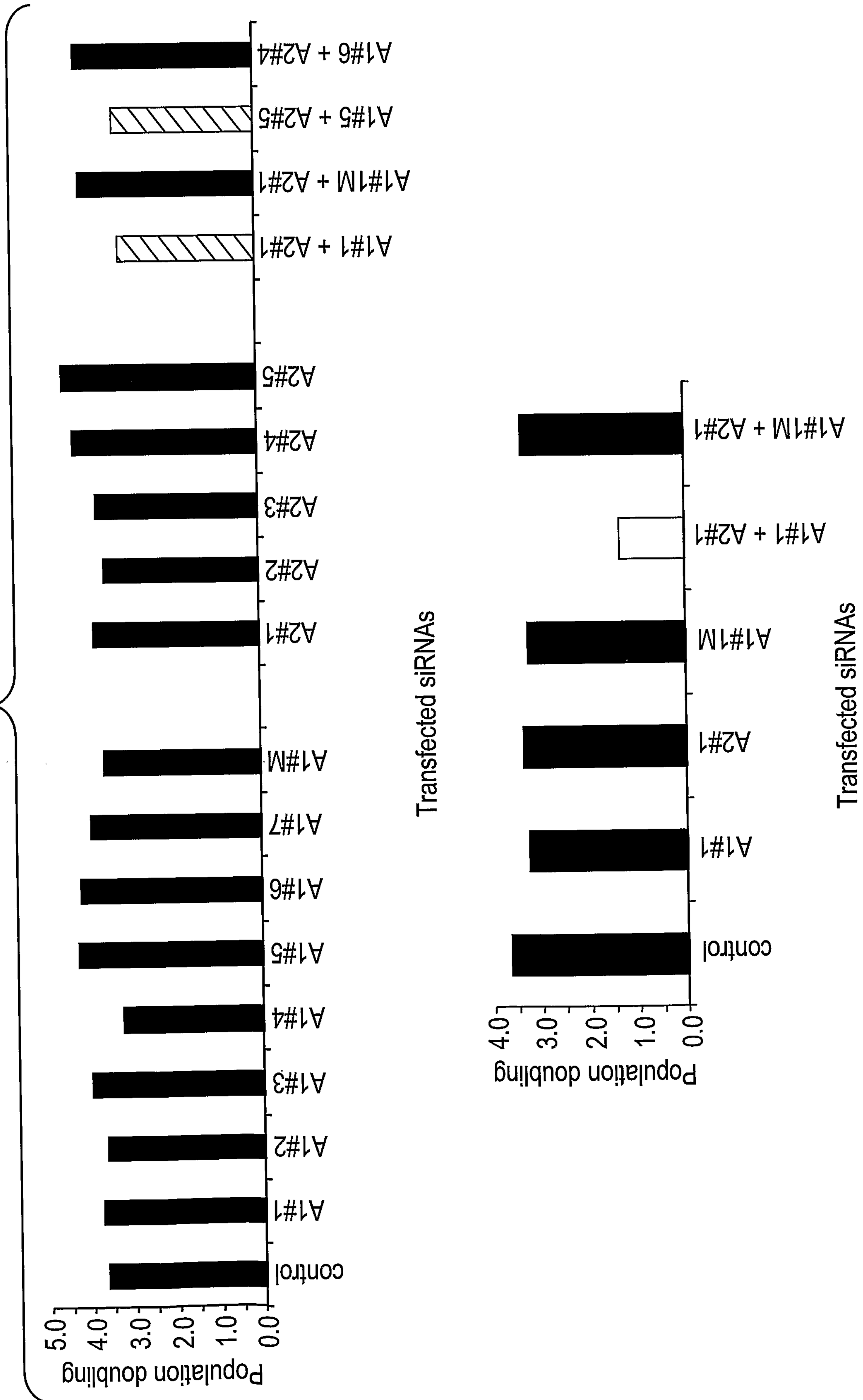


FIG. 2
Population doubling
5.0
4.0
3.0
2.0
1.0
0.0
control
A1#1
A1#2
A1#3
A1#4
A1#5
A1#6
A1#7
A1#M
A2#1
A2#2
A2#3
A2#4
A2#5
A1#1 + A2#1
A1#1M + A2#1
A1#5 + A2#5
A1#6 + A2#4
Transfected siRNAs
Population doubling
4.0
3.0
2.0
1.0
0.0
control
A1#1
A2#1
A1#1M
A1#1 + A2#1
A1#1M + A2#1
Transfected siRNAs

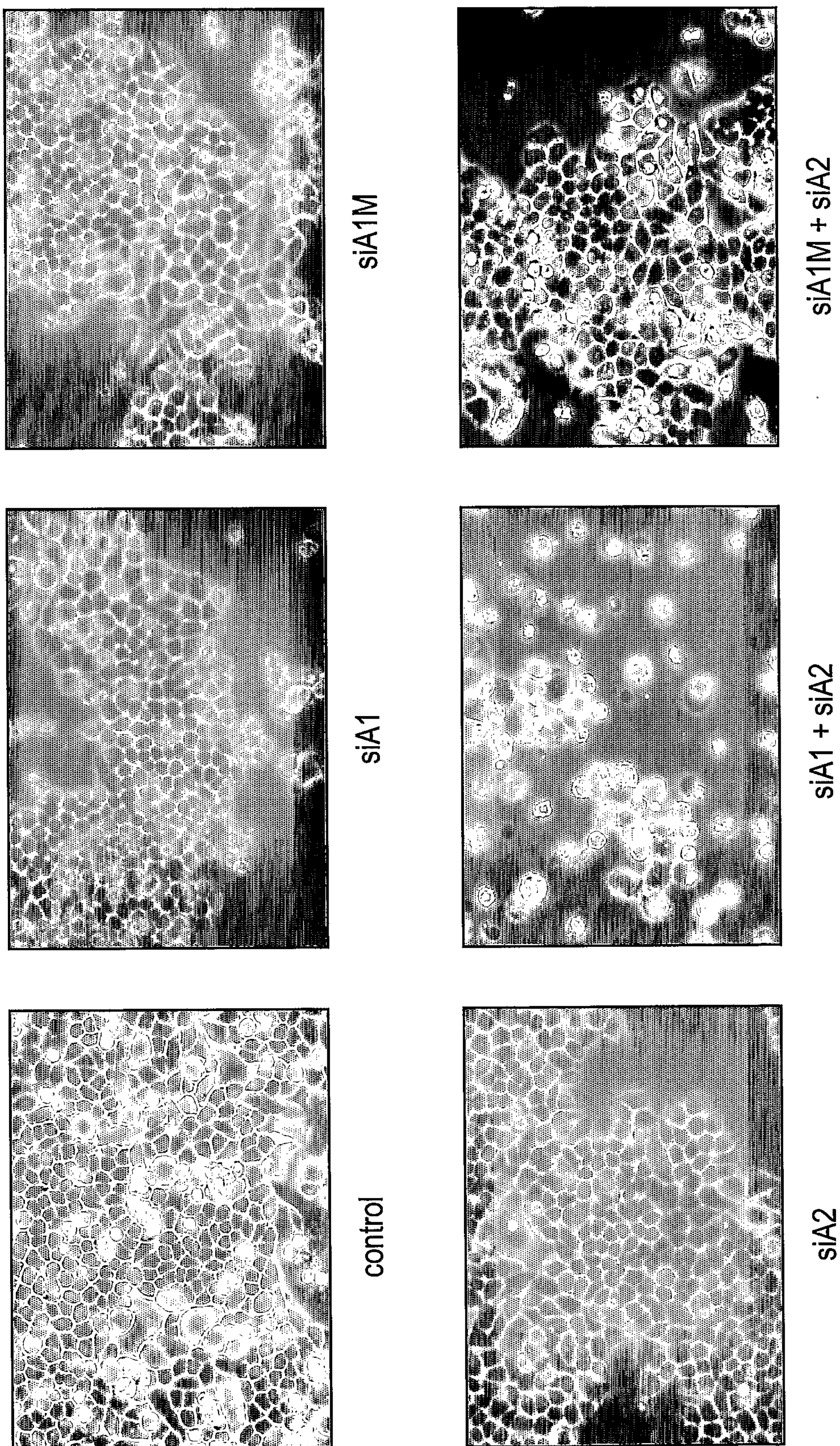


FIG. 3

FIG. 4C

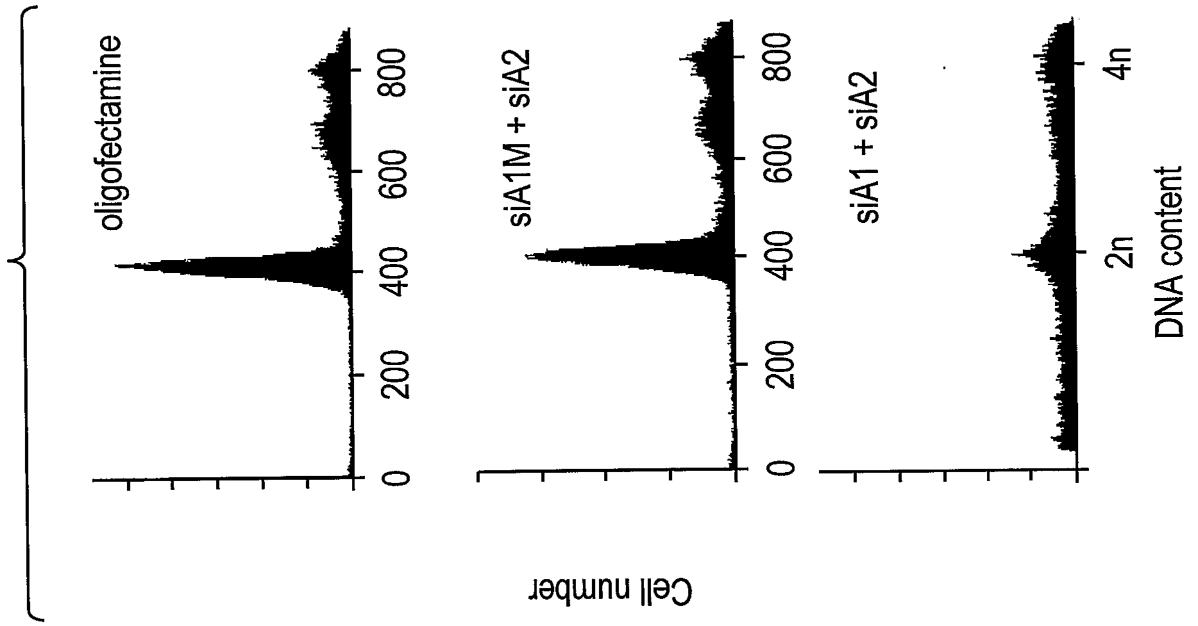


FIG. 4A

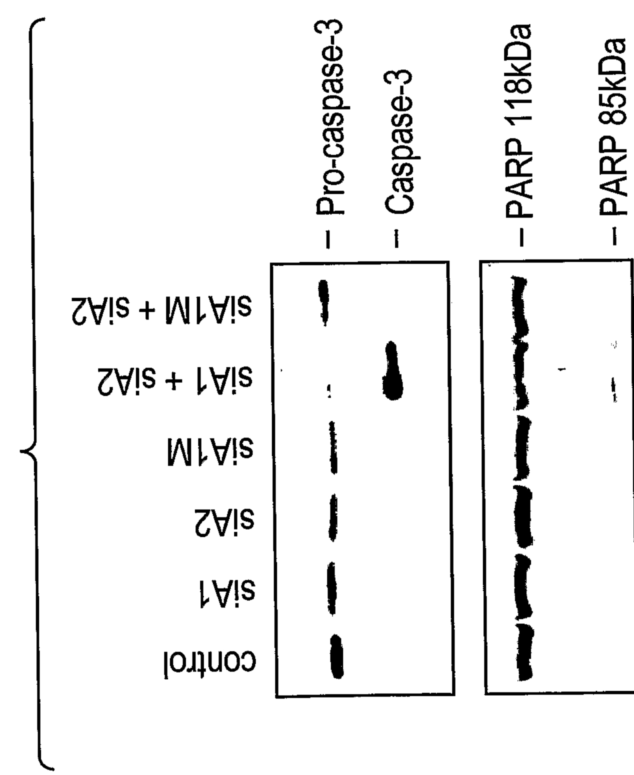
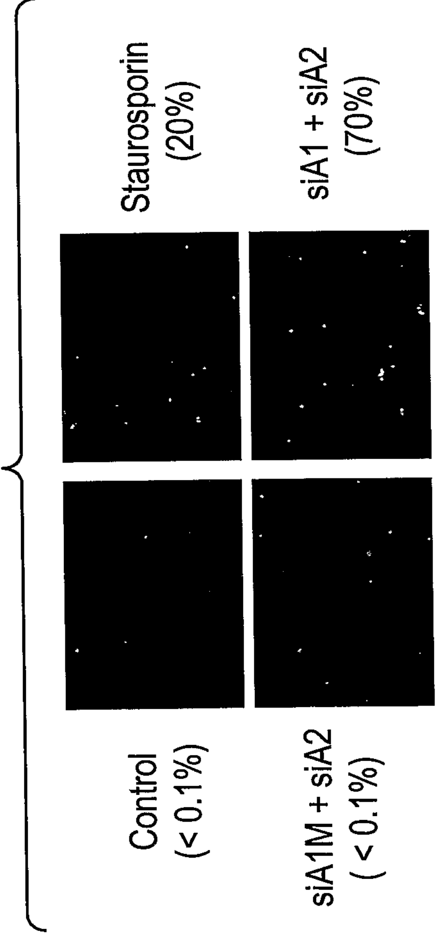


FIG. 4B



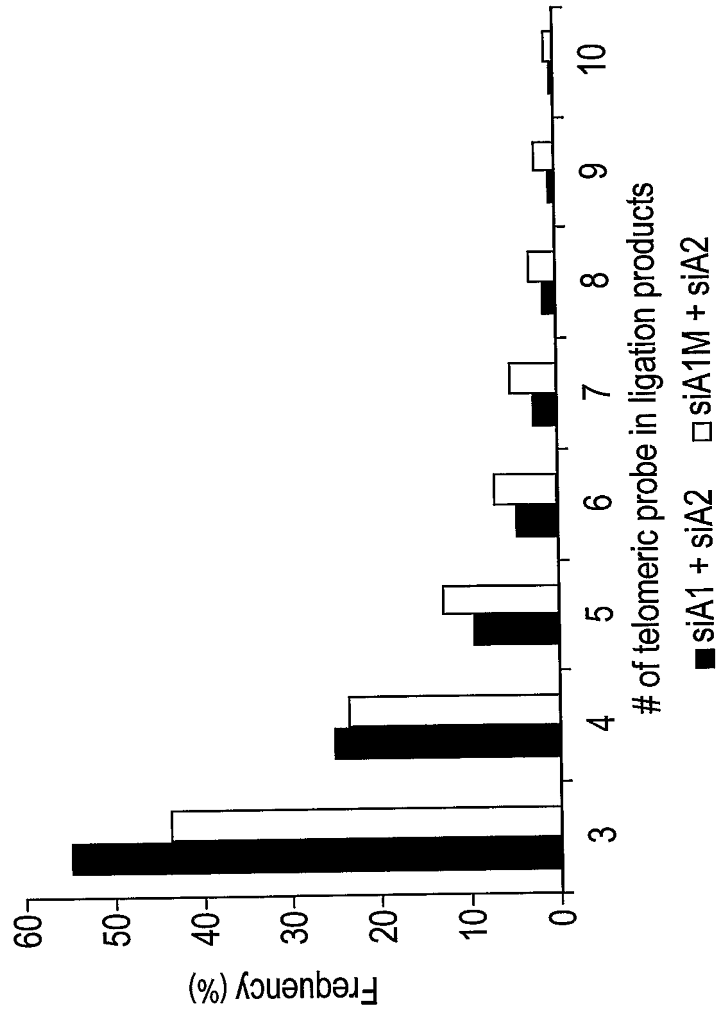


FIG. 5B

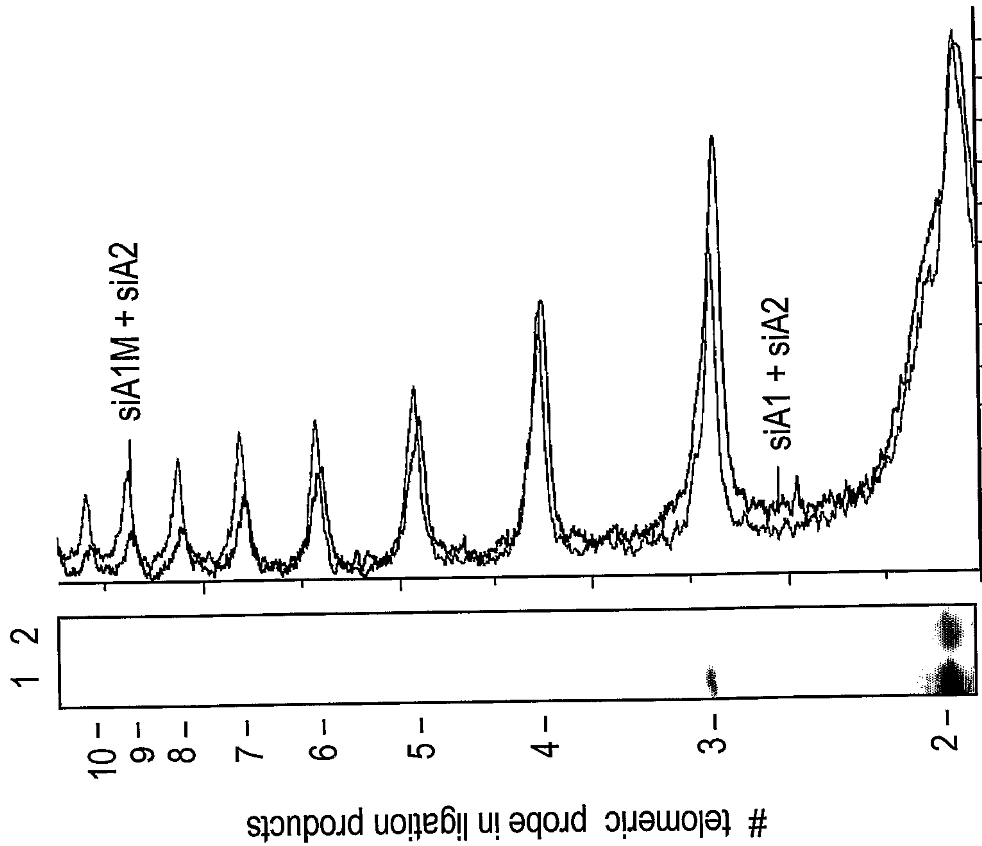


FIG. 5A

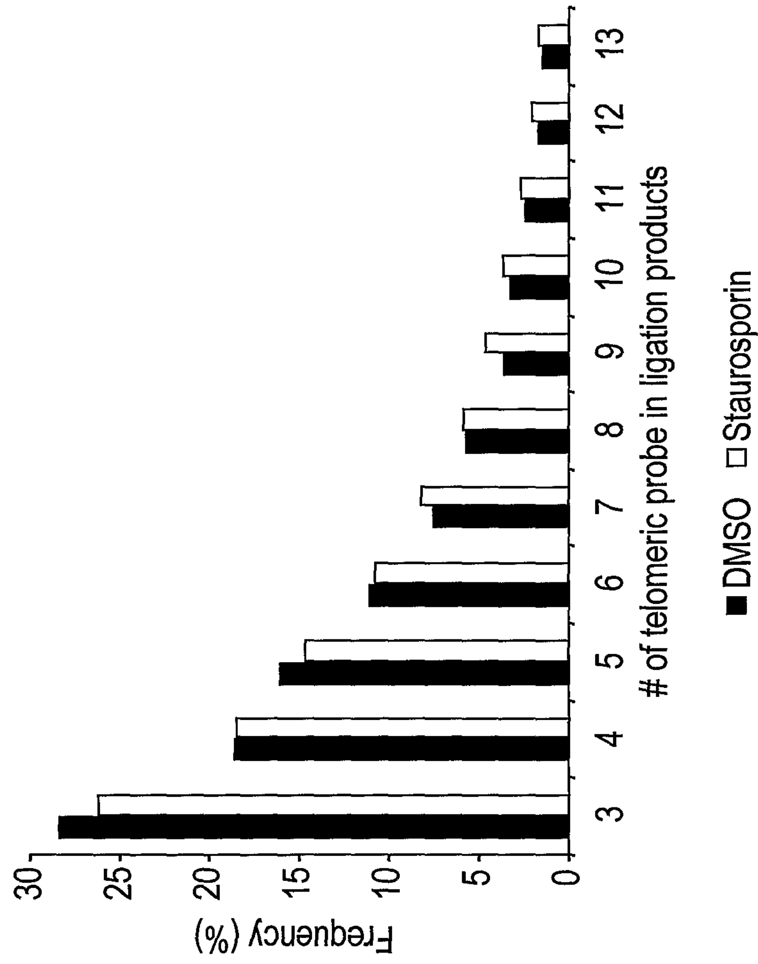


FIG. 5D
Frequency (%)
0
5
10
15
20
25
30
3
4
5
6
7
8
9
10
11
12
13
# of telomeric probe in ligation products
DMSO
Staurosporin

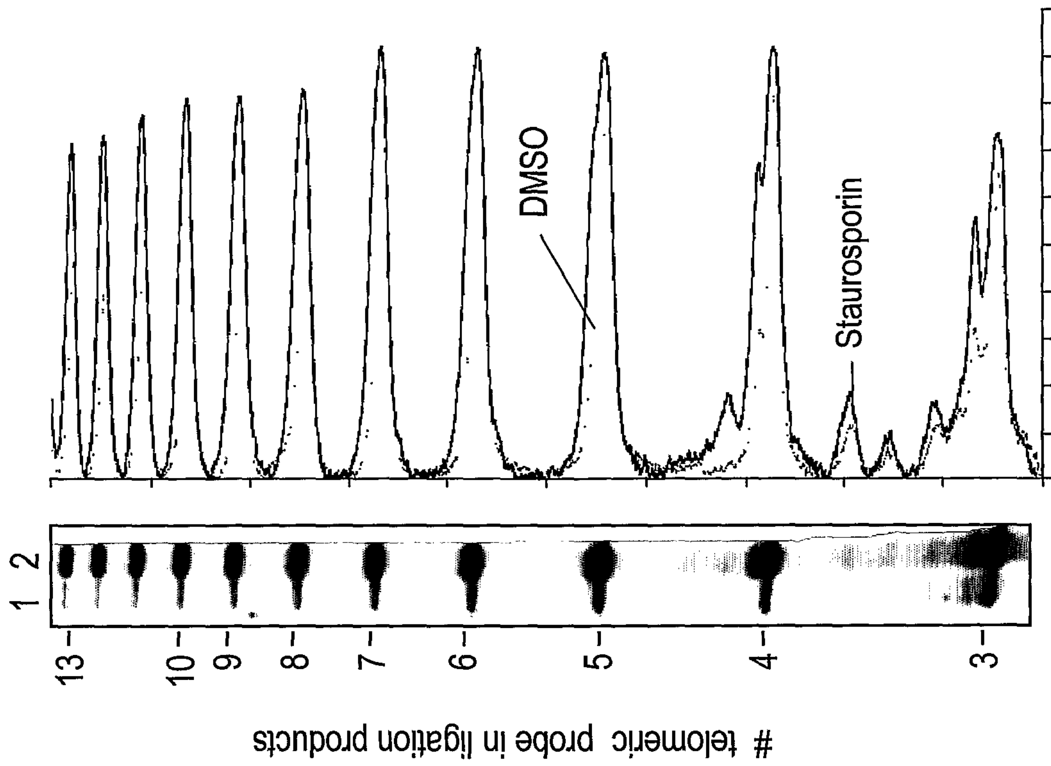
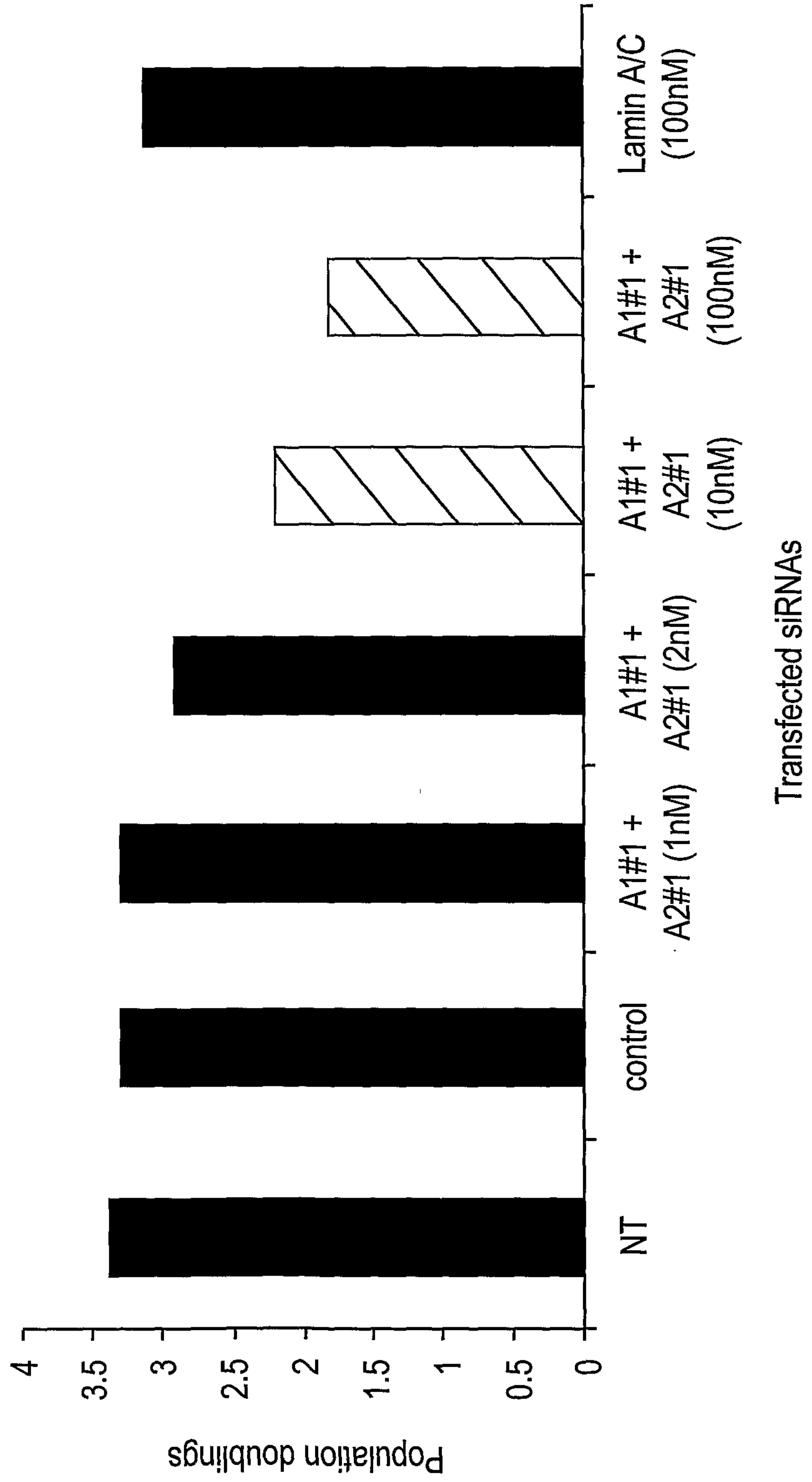


FIG. 5C
1 2
13
10
9
8
7
6
5
4
3
telomeric probe in ligation products
DMSO
Staurosporin

FIG. 6



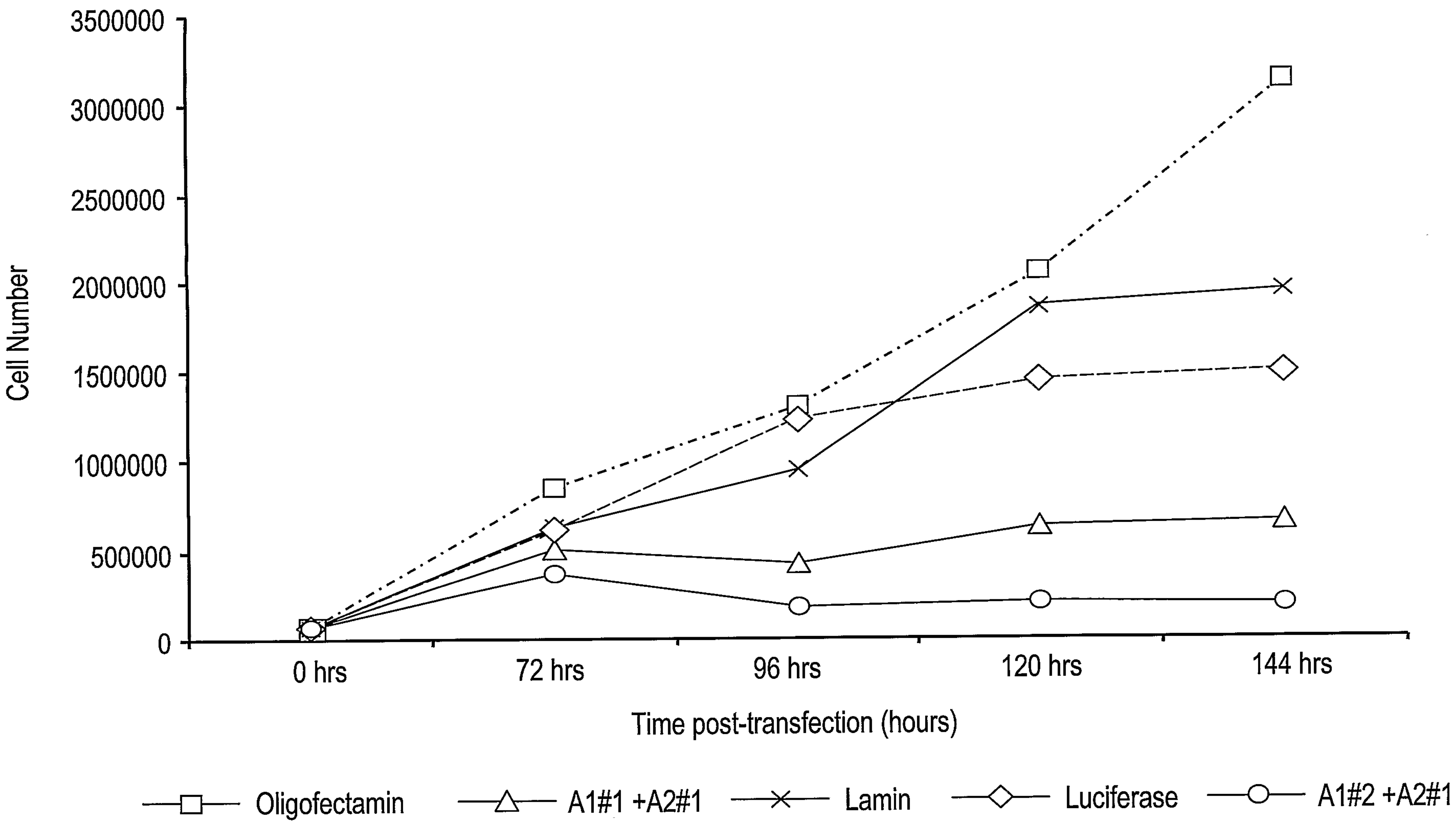


FIG. 7A
Time Course on HeLaS3 cells
Cell Number
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0 hrs
72 hrs
96 hrs
120 hrs
144 hrs
Time post-transfection (hours)
Oligofectamin
A1#1 +A2#1
Lamin
Luciferase
A1#2 +A2#1

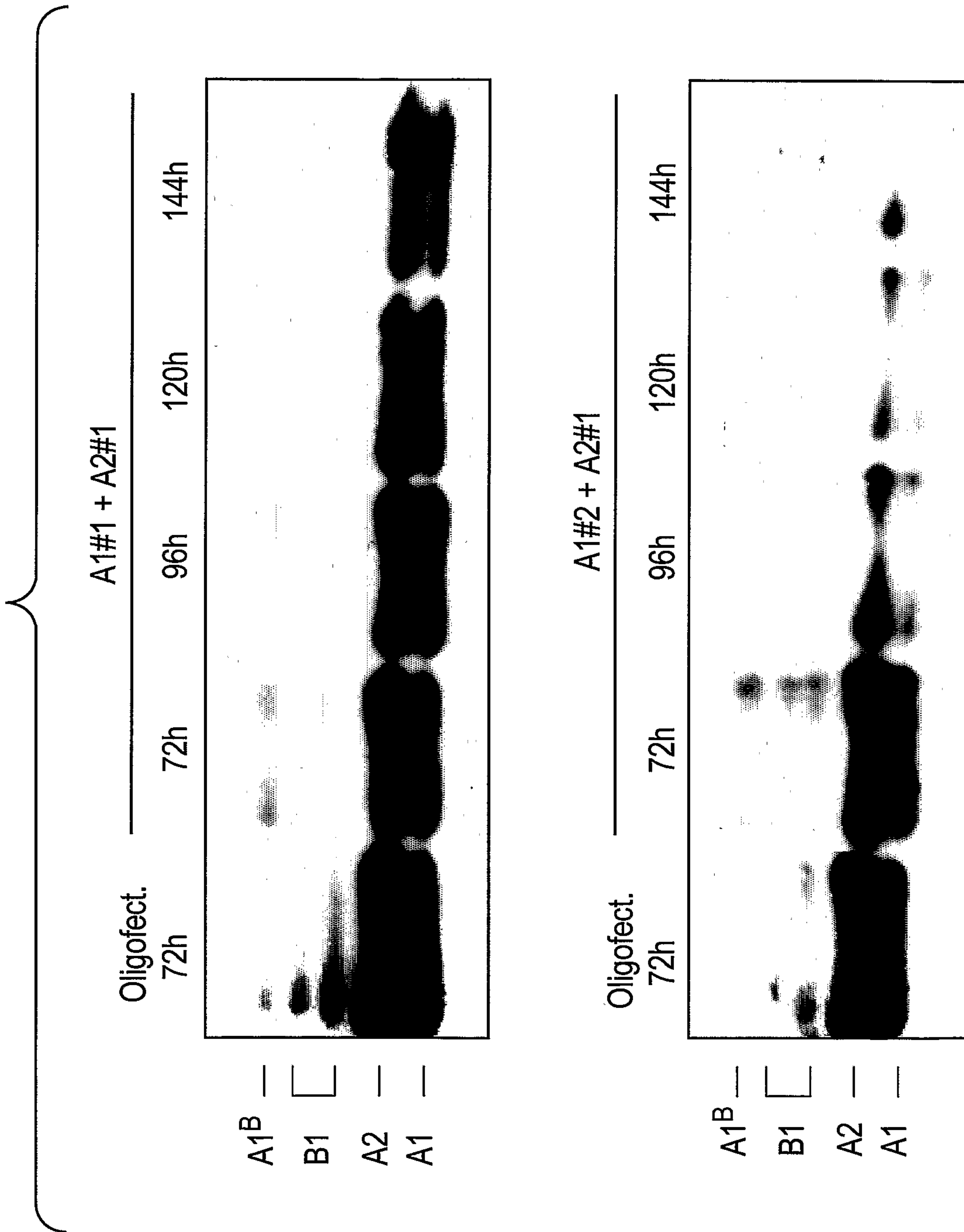


FIG. 7B

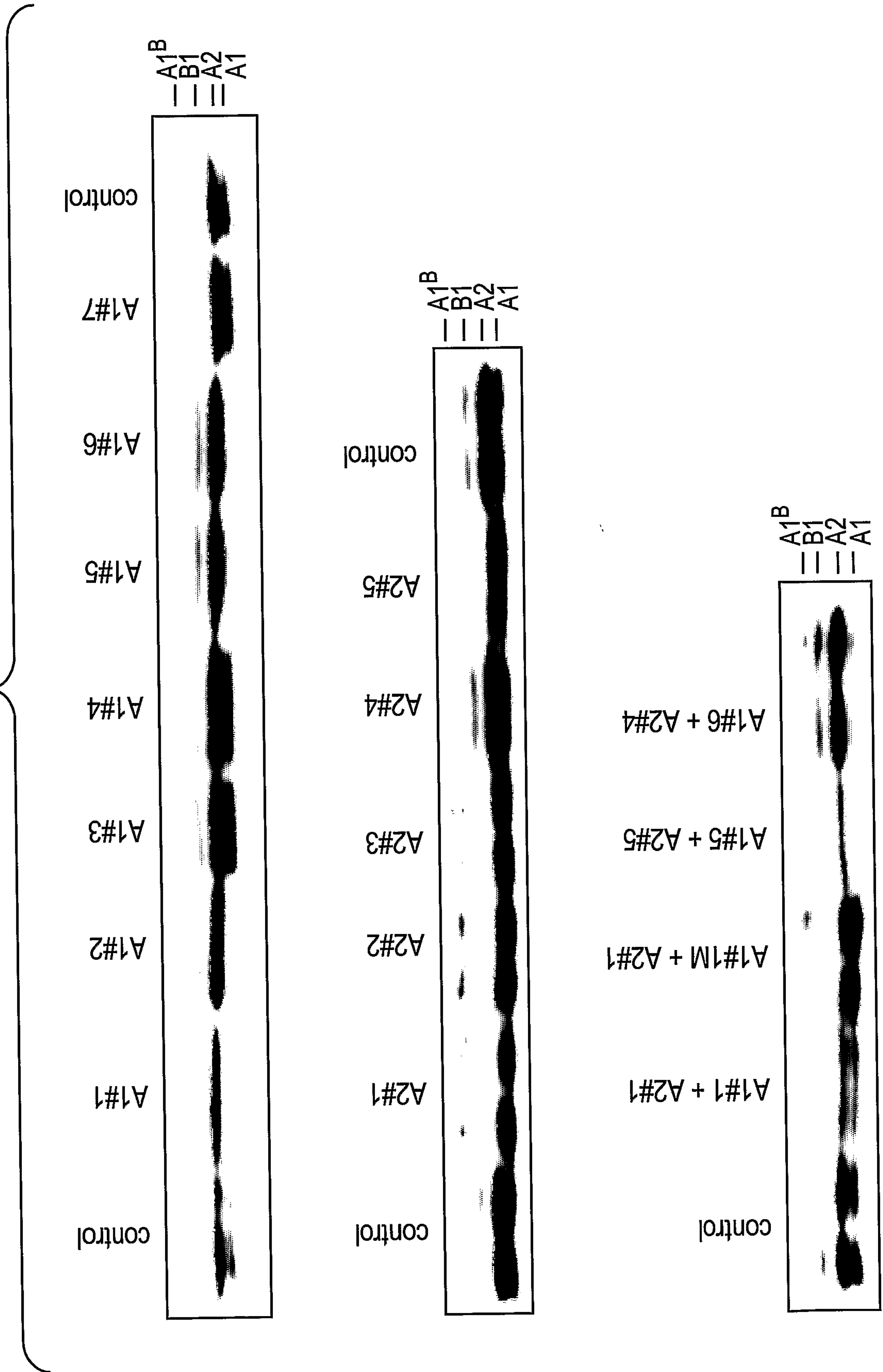


FIG. 8

FIG. 9B

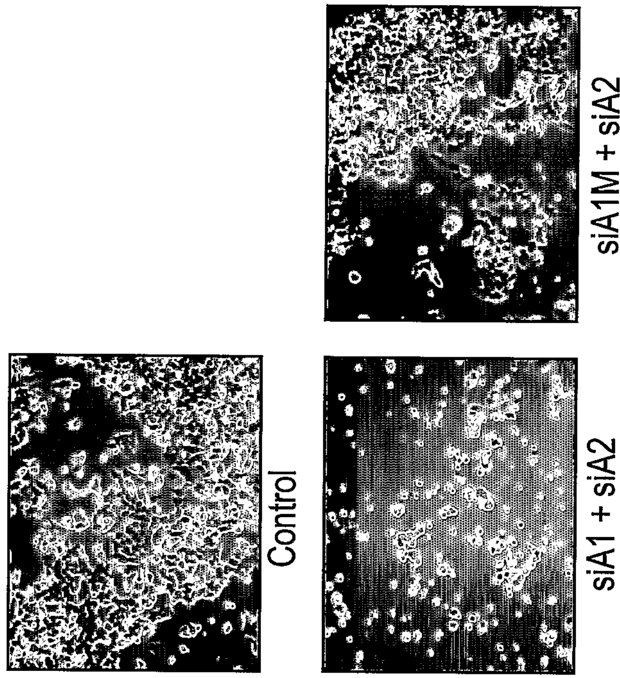
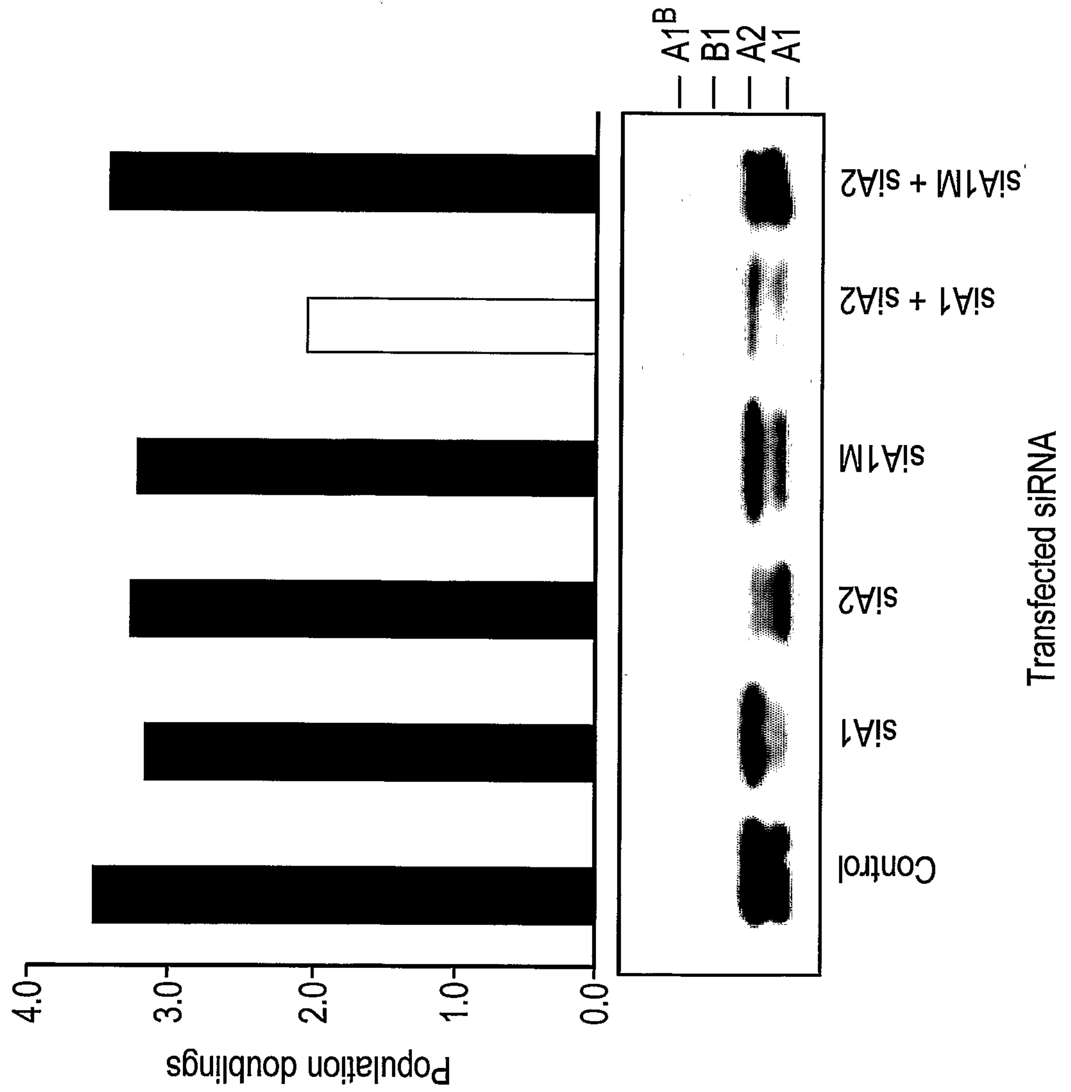


FIG. 9A



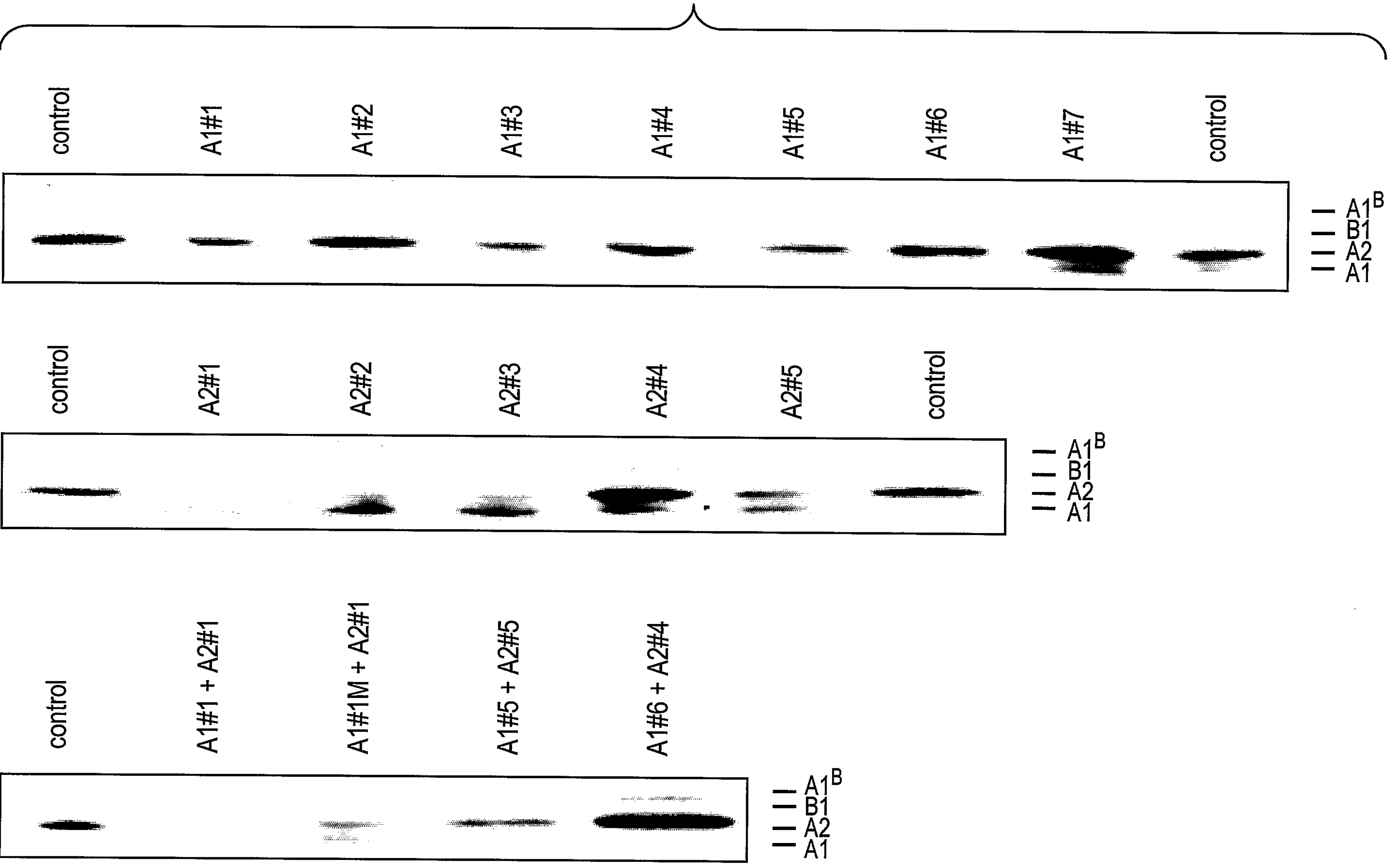


FIG. 10
control
A1#1
A1#2
A1#3
A1#4
A1#5
A1#6
A1#7
control
A1B
B1
A2
A1
control
A2#1
A2#2
A2#3
A2#4
A2#5
control
A1B
B1
A2
A1
control
A1#1 + A2#1
A1#1M + A2#1
A1#5 + A2#5
A1#6 + A2#4
A1B
B1
A2
A1

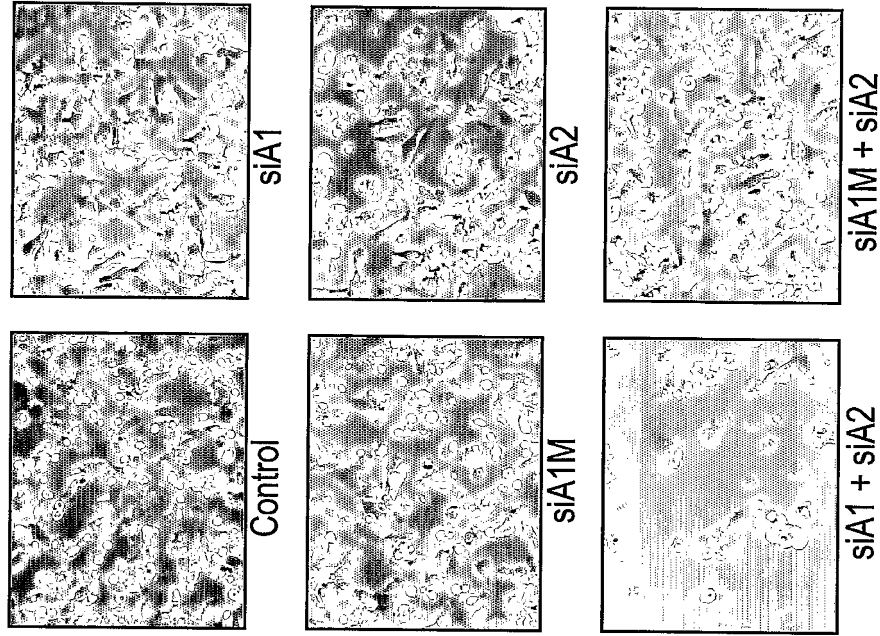


FIG. 11B
Control
siA1
siA1M
siA2
siA1 + siA2
siA1M + siA2

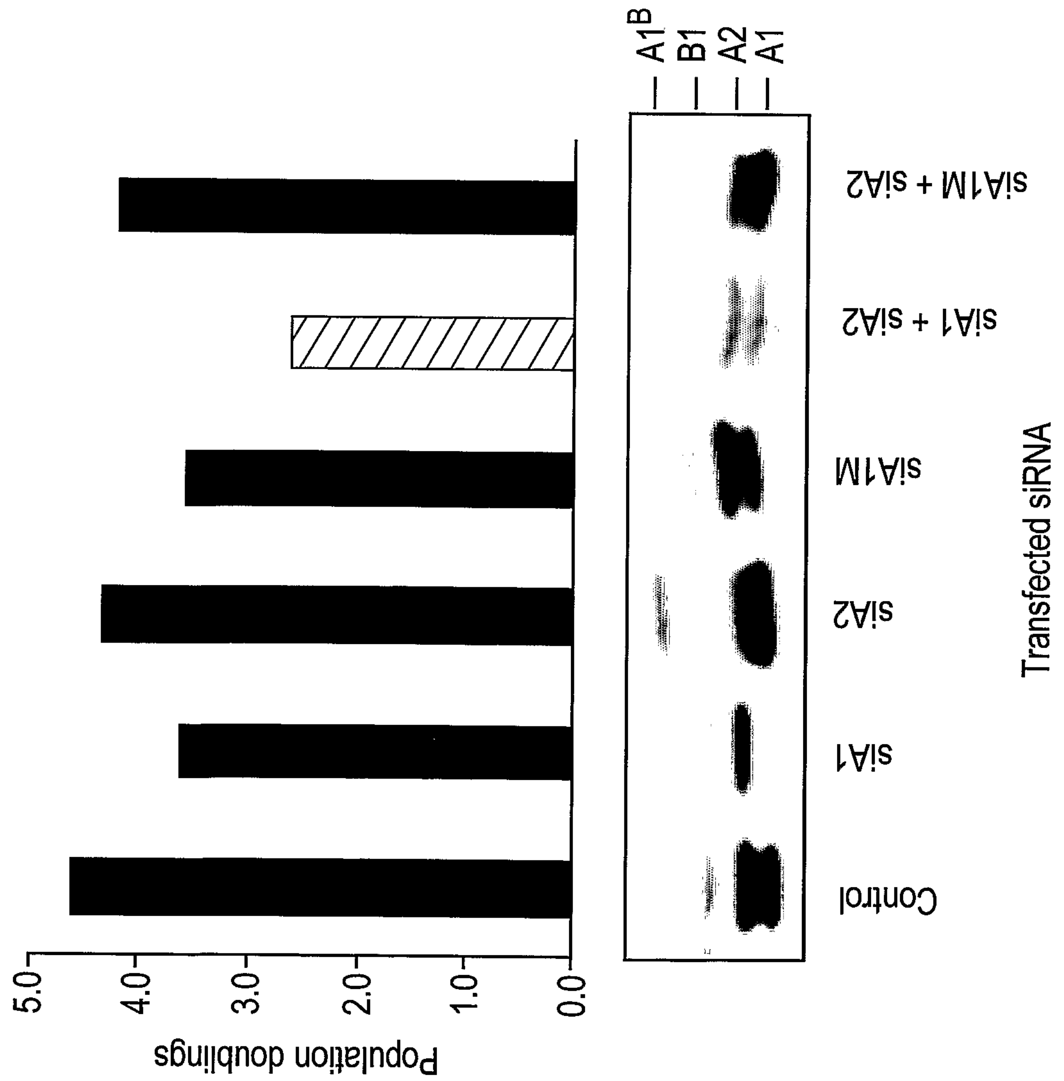


FIG. 11A
Population doublings
5.0
4.0
3.0
2.0
1.0
0.0
A1B
B1
A2
A1
Control
siA1
siA2
siA1M
siA1 + siA2
siA1M + siA2
Transfected siRNA

FIG. 12B

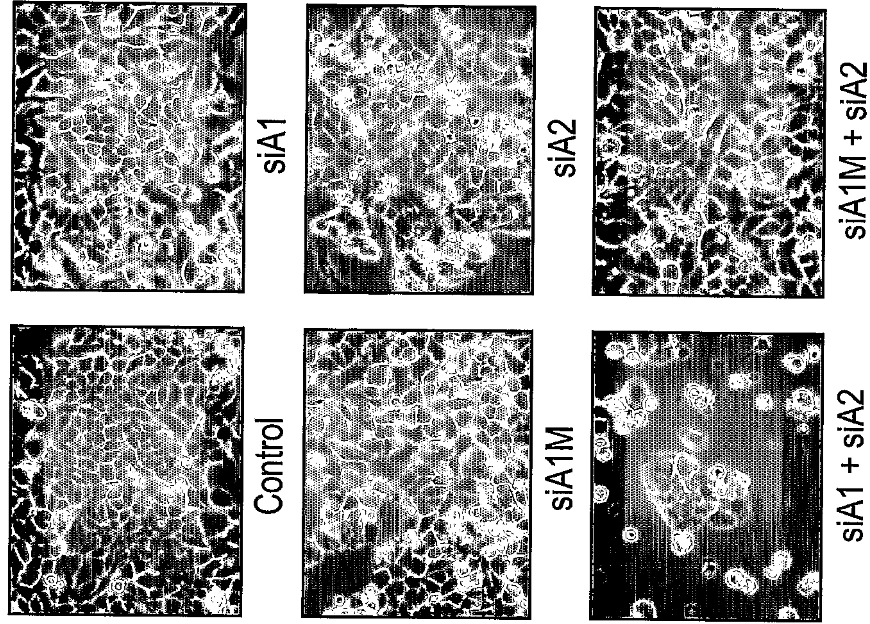
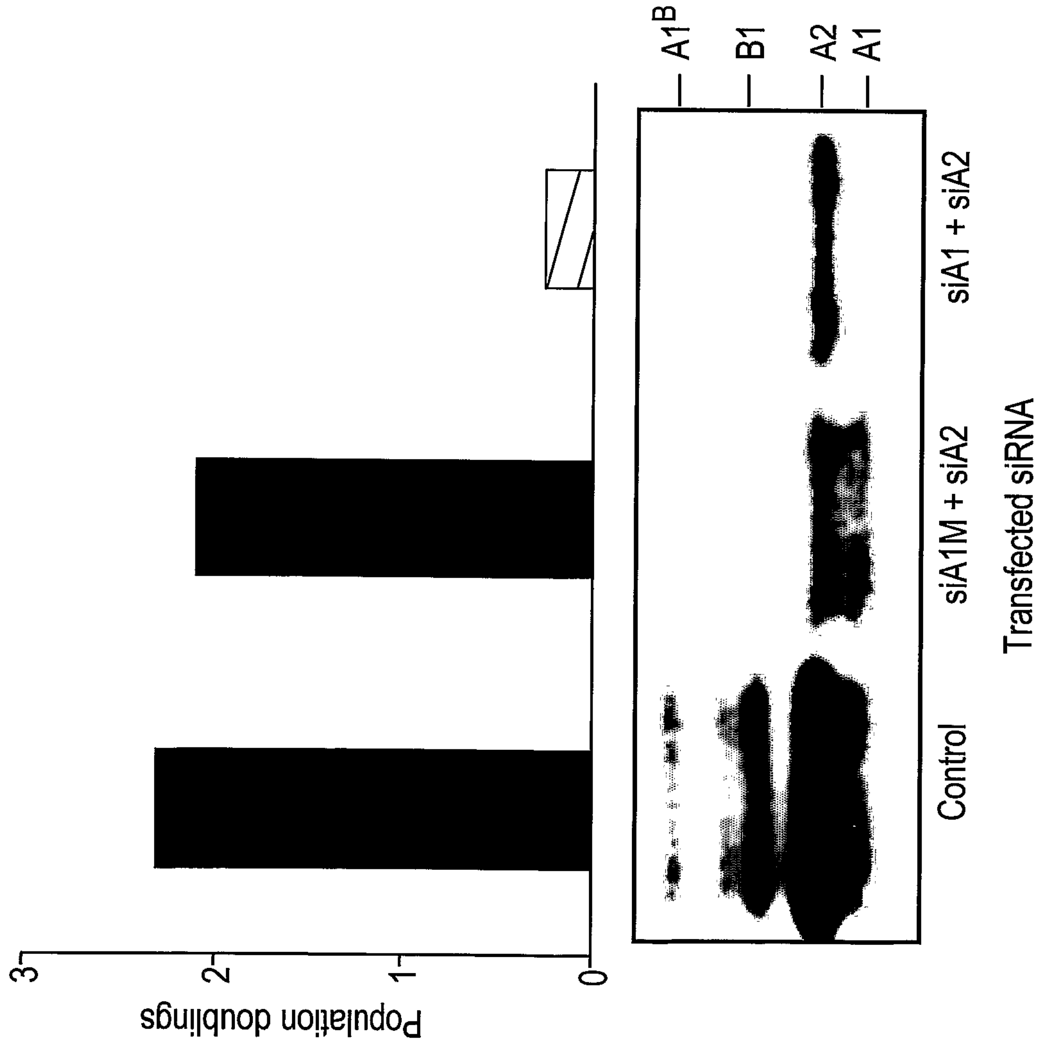


FIG. 12A



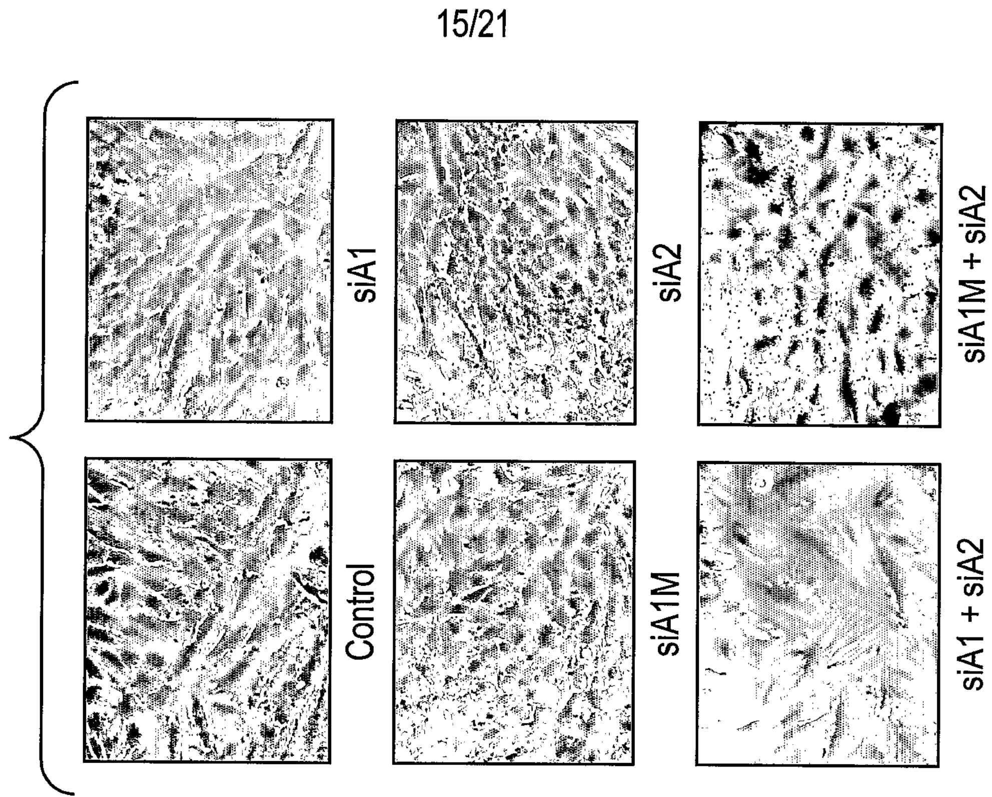


FIG. 13B

FIG. 13A

Population doublings
3
2
1
0
A1B
B1
A2
A1
Control
siA1
siA2
siA1M
siA1 + siA2
siA1M + siA2
Transfected siRNA

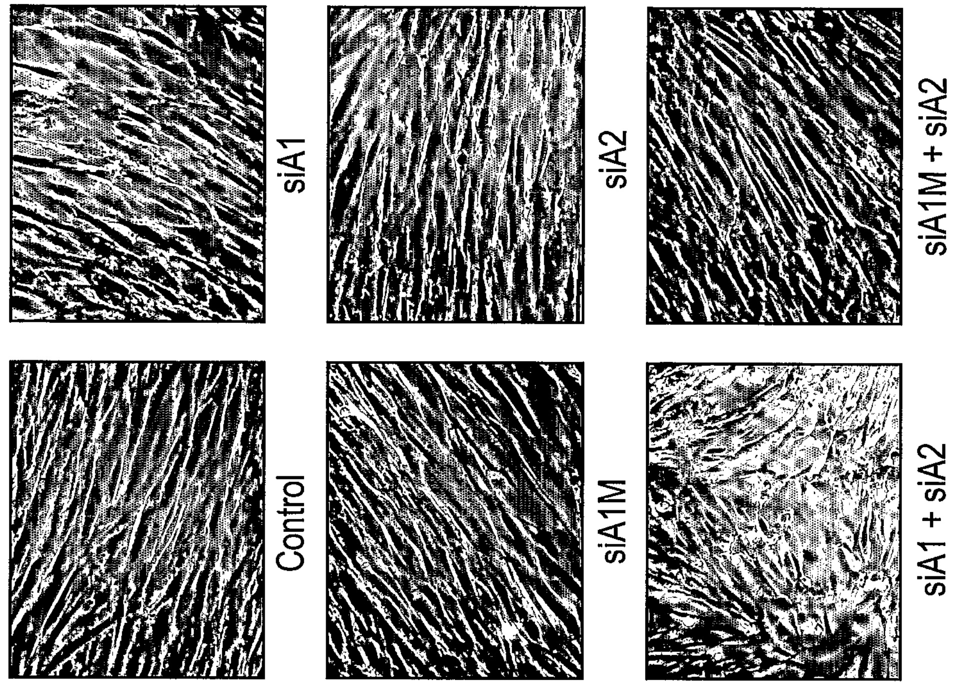


FIG. 14B
Control
siA1
siA1M
siA2
siA1 + siA2
siA1M + siA2

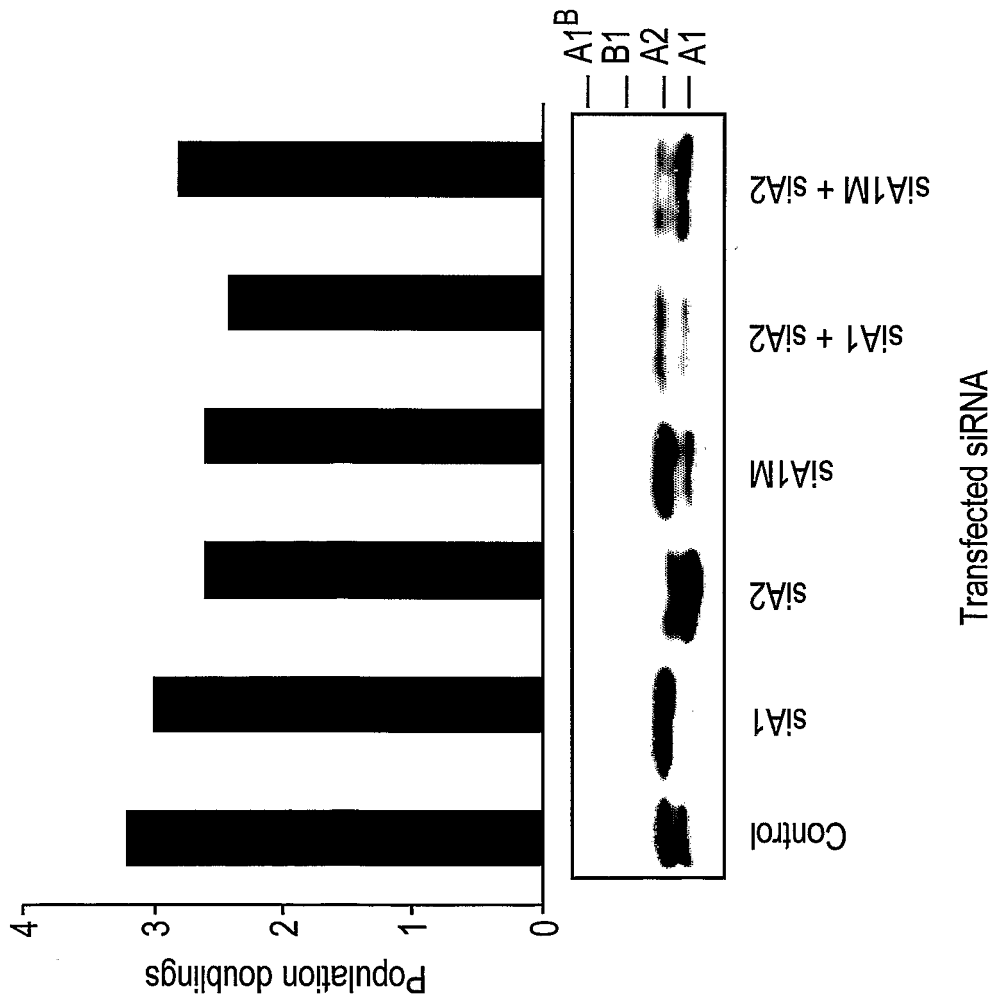


FIG. 14A
Population doublings
0
1
2
3
4
Control
siA1
siA2
siA1M
siA1 + siA2
siA1M + siA2
A1B
B1
A2
A1
Transfected siRNA

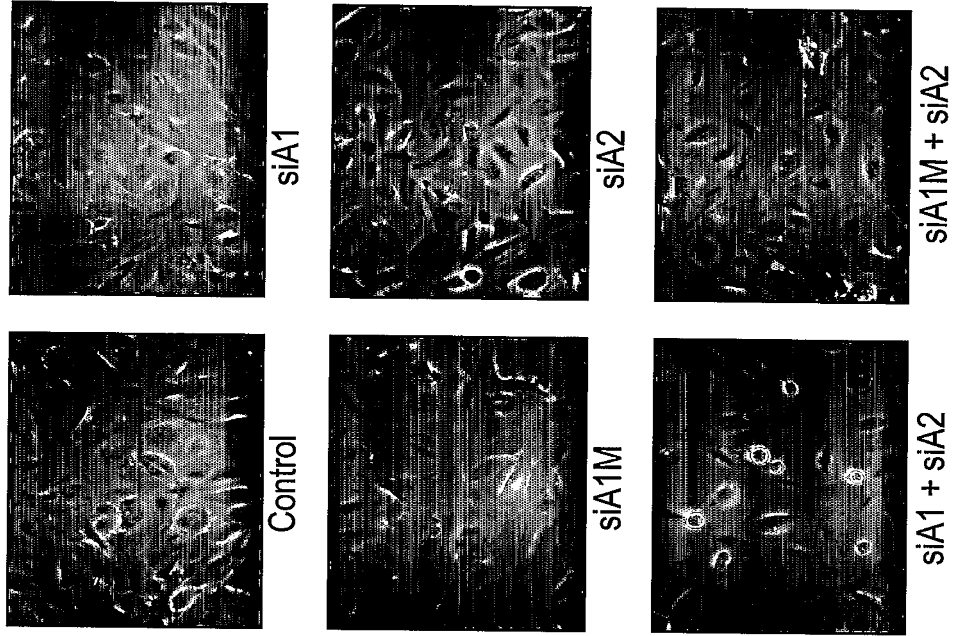


FIG. 15B

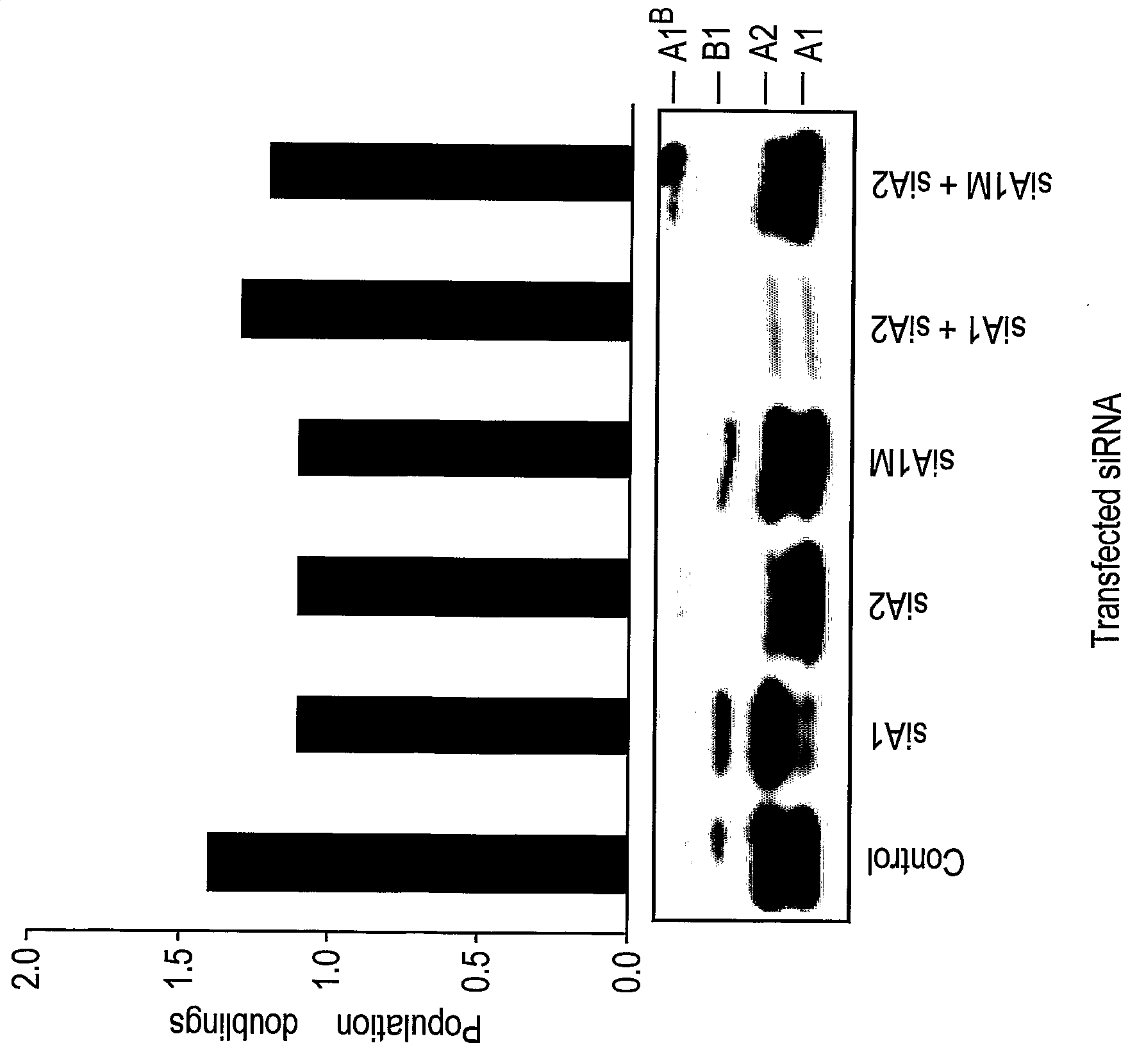


FIG. 15A

FIG. 16B

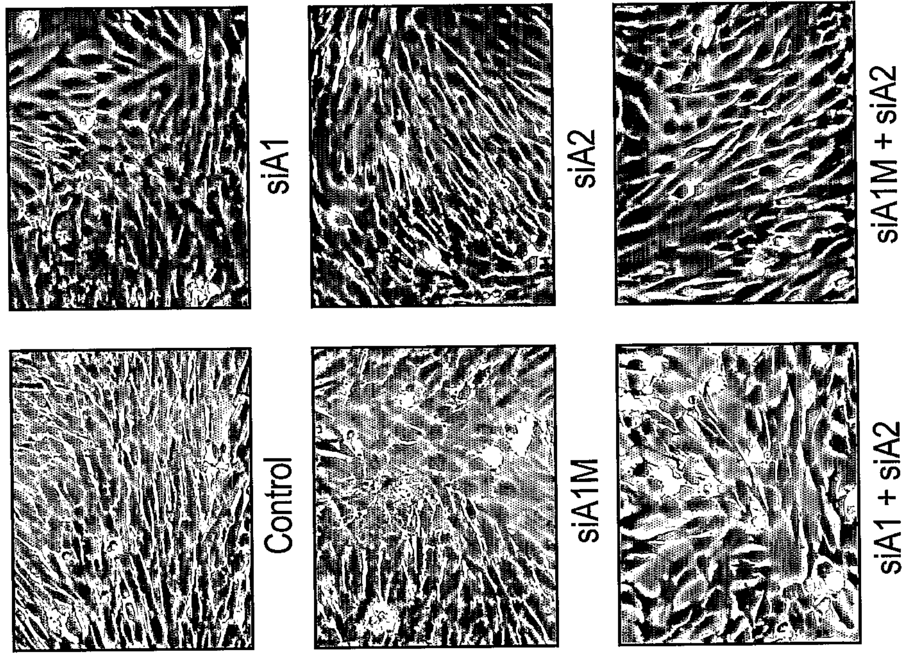


FIG. 16A

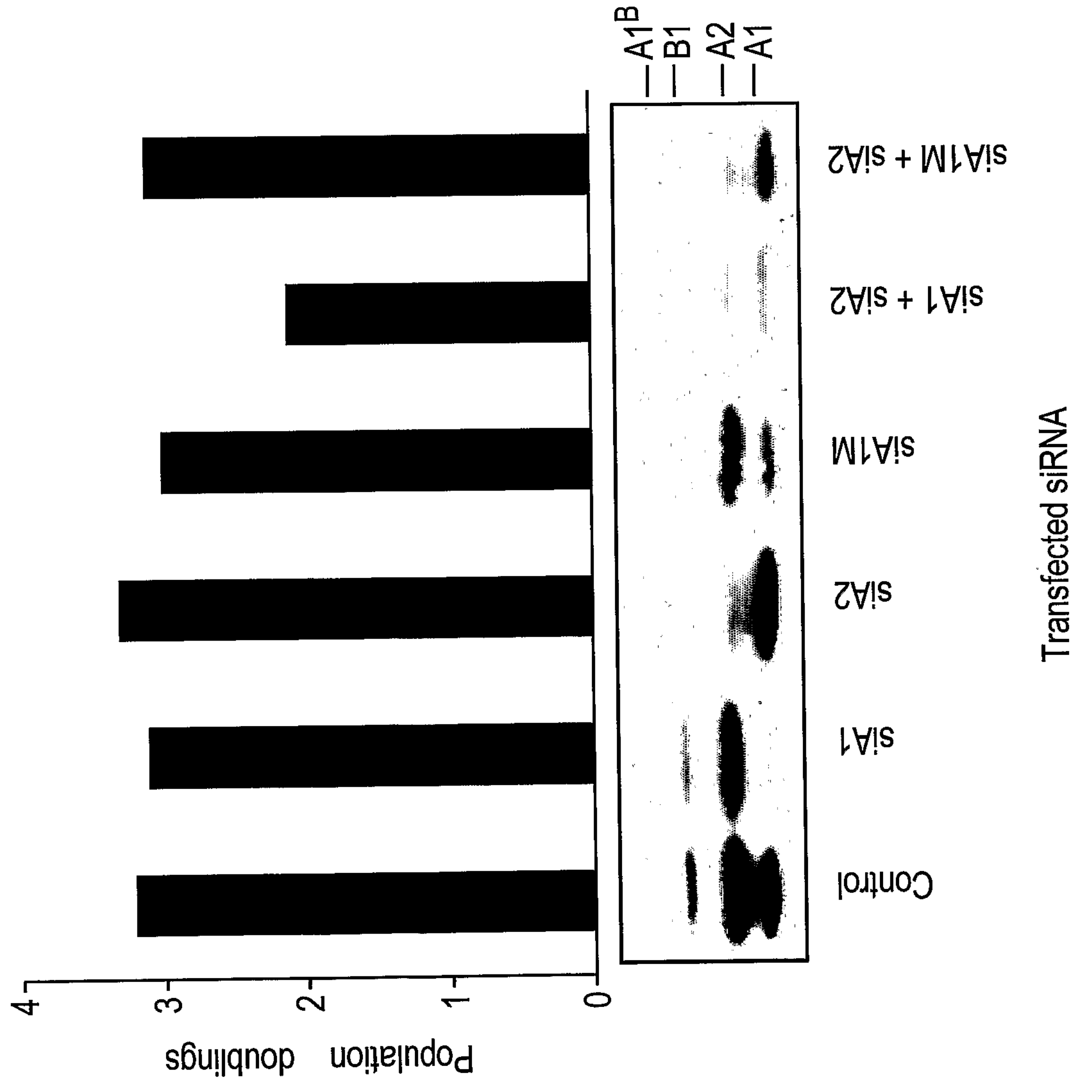


FIG. 17

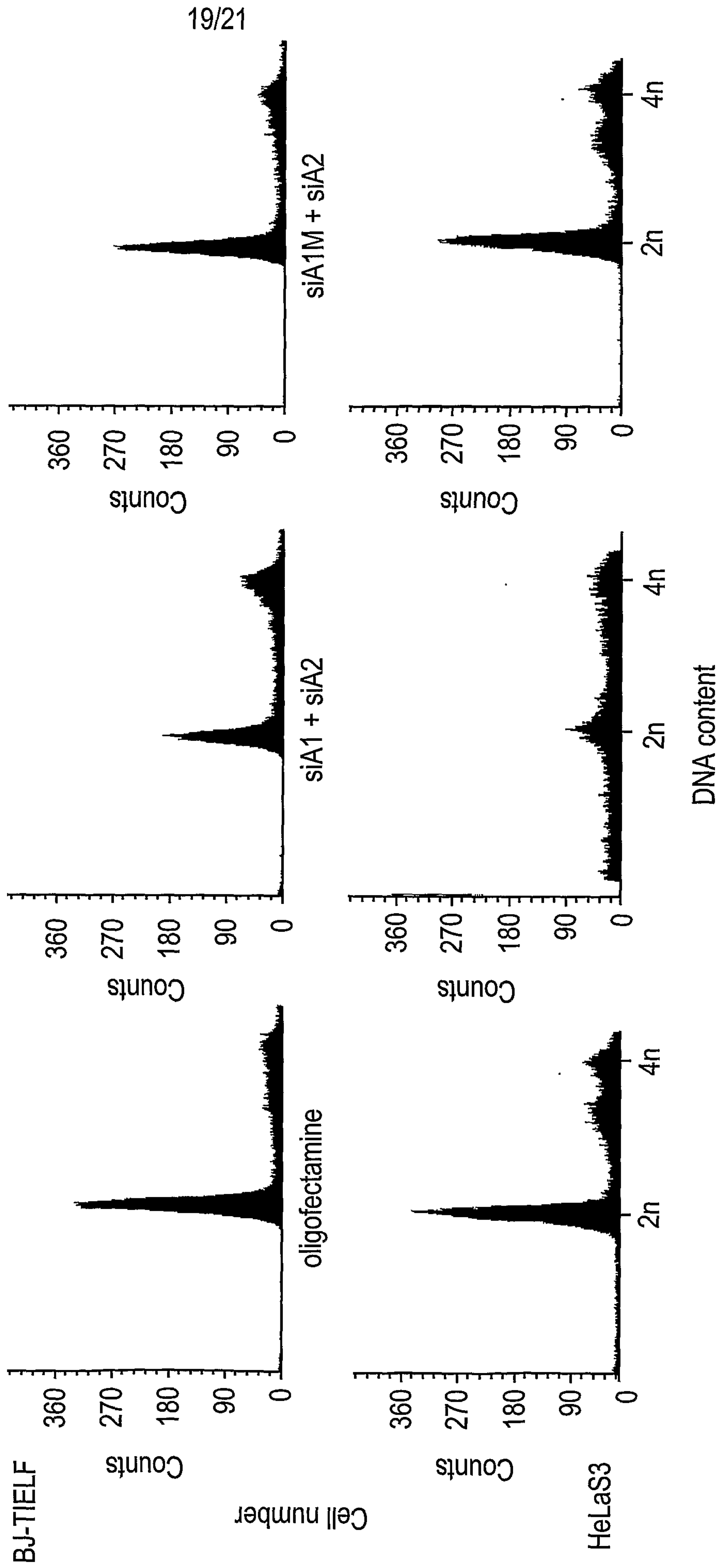


FIG. 18

Cell line	p53 status*	Effect of siA1 and siA2							
		A1/A2 expression	Cell growth and morphology	caspase-3	PARP	TUNEL	PKR activation	PI-DNA content cell cycle analysis	telomeres
HeLaS3 cervix adenocarcinoma	wild type	reduced	growth arrest dying cells	activated	cleaved	71% +	–	increase subG1 increase G2/M	loss of single-stranded overhang
HCT116 colorectal carcinoma	wild type	reduced	growth arrest dying cells	activated	cleaved	–	–	increase subG1 increase G2/M	–
MCF7 mammary adenocarcinoma	wild type	reduced	growth arrest dying cells	–	–	–	–	low increase subG1	–
HT1080 fibrosarcoma	wild type	reduced	growth arrest dying cells	–	–	–	–	increase subG1	–
HT-29 colorectal adenocarcinoma	mutant	reduced	growth arrest dying cells ?	–	–	–	–	–	–
PA-1 ovary metastatic teratocarcinoma	wild type	reduced	growth arrest dying cells	–	–	–	–	increase subG1 increase G2/M	–
SK-OV-3 ovary metastatic adenocarcinoma	null	reduced	growth arrest dying cells	–	–	–	–	increase subG1 increase G2/M	–
U-373 glioblastoma	mutant	reduced	growth arrest dying cells	–	–	–	–	increase subG1 increase G2/M	–
HIEC normal intestinal epithelial cells limited lifespan	wild type	reduced	cells normal morph.	–	–	–	–	no $subG_1$ no change in cell cycle	–
BJ normal foreskin fibroblasts limited lifespan	wild type	reduced	slower normal morph.	–	–	–	–	no $subG_1$ low increase G2/M	–
BJ-TIELF telomerase positive BJ cells unlimited lifespan	wild type	reduced	normal growth few rounded cells	–	–	–	–	no $subG_1$ low increase G2/M	–
CCD-18Co normal colon fibroblasts limited lifespan	wild type	reduced	cells normal morph.	–	–	–	–	no $subG_1$ no change in cell cycle	no change in single-stranded overhang

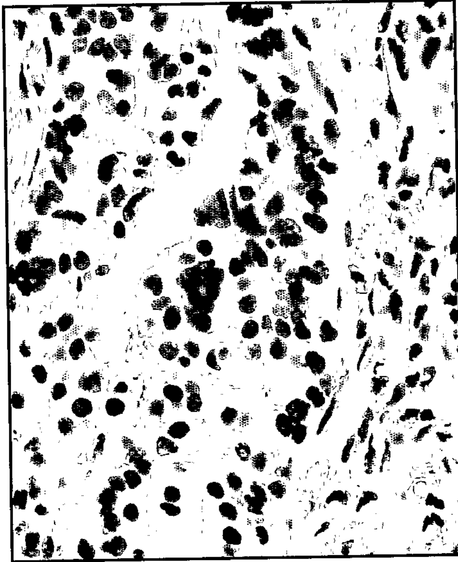


FIG. 19B

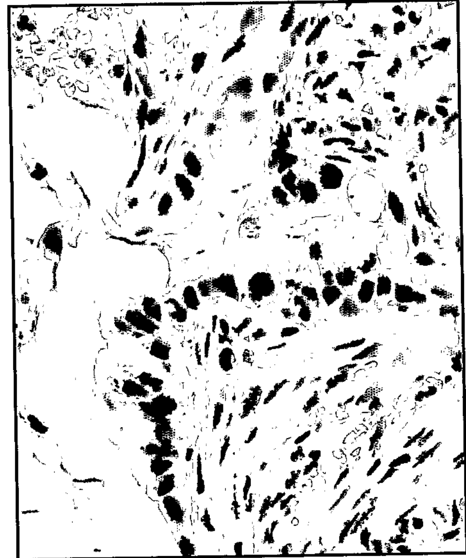


FIG. 19D

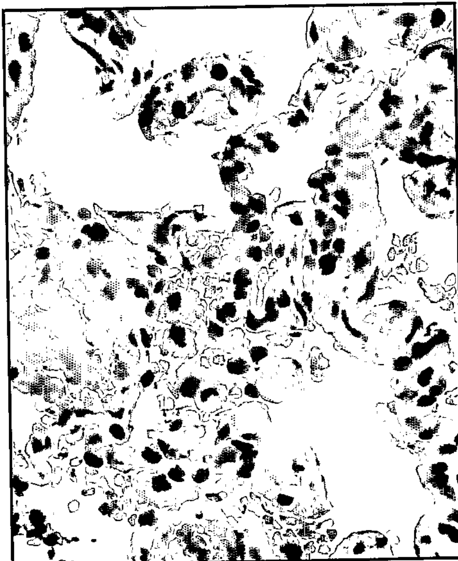


FIG. 19A

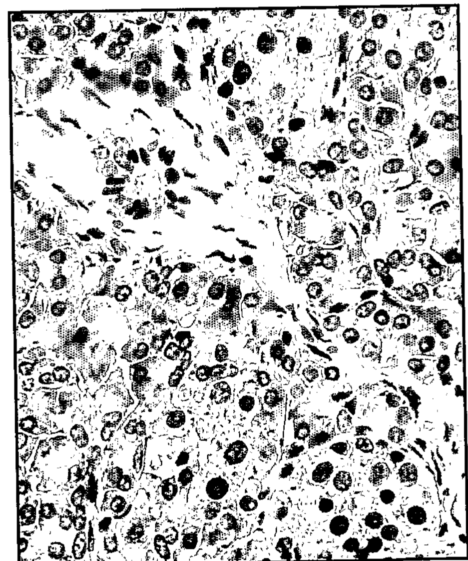


FIG. 19C

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patsnap

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申请号	EP2003727087	申请日	2003-05-30
[标]申请(专利权)人(译)	末端基因公司研究所的pharmacologie		
申请(专利权)人(译)	休止期INC.，药物研究所		
当前申请(专利权)人(译)	休止期INC.，药物研究所		
[标]发明人	CHABOT BENOIT BOUCHARD LOUISE LABRECQUE PASCALE PATRY CAROLINE WELLINGER RAYMUND		
发明人	CHABOT, BENOIT BOUCHARD, LOUISE LABRECQUE, PASCALE PATRY, CAROLINE WELLINGER, RAYMUND		
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优先权	60/384309 2002-05-30 US		
外部链接	Espacenet		

摘要(译)

本发明提供了与hnRNP A1和hnRNP A2核酸分子有关的瘤形成治疗的治疗和诊断方法。