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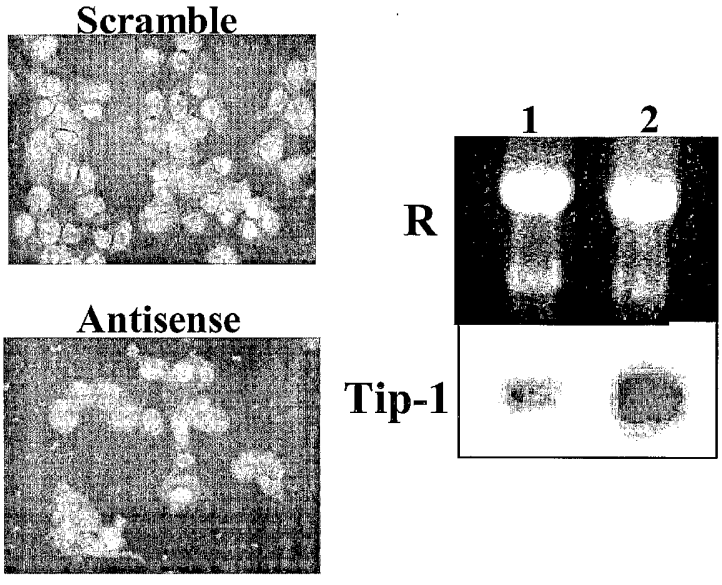
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(54) Title: TREATMENT OF AGE RELATED CONDITIONS



(57) Abstract: The present invention relates to the use of agents that reduce cellular levels of HTLV1 Tax Interacting Protein-1 (TIP-1) or act to inhibit or alter the functional effects of TIP-1 to treat, prevent or limit tissue damage. The agents are particularly useful for preventing damage that may occur as a result of the ageing process and in the treatment of cancer. The invention also concerns the diagnosis, and treatment (including prophylactic treatment) of, cancer and to a method for the identification of cancer targets involved in the process of tumour invasion.

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Treatment of Age related conditions

The present invention relates to the treatment, or limitation of tissue damage and especially such damage that may occur as a result of the ageing process. The invention also concerns the diagnosis, and treatment (including prophylactic treatment) of, cancer and to a method for the identification of cancer targets involved in the process of tumour invasion

Tissue damage may arise as a result of physical damage (e.g. wounding), chemical damage and viral damage. The damage can take a number of forms. For instance a consequence of hepatic damage can be the development of liver cirrhosis. One form of damage that may occur in individuals, and particularly aged individuals, is the development of genetic abnormalities that may be observed microscopically as polyploidy. Genetic damage, and polyploidy in particular, may lead to the development of a number of diseases including cancer.

Many different forms of cancer exist, and it is believed that there are many different causes of the disease. The incidence of cancer varies, but it represents the second highest cause of mortality, after heart disease, in most developed countries. Most striking is the observation that the incidence of cancer increases markedly with age. Current estimates suggest that one in three Americans alive at present will suffer from some form of cancer. Methods of treatment for cancer exist, although there is a well recognised need to develop new and improved techniques. Furthermore, there is also a requirement to develop chemopreventative agents that could be used to inhibit the development of cancer in the general population, susceptible high-risk individuals or as an agent to prevent re-occurrence of disease in individuals already affected. Since ageing is associated with increased risk of cancer it is likely that the increased understanding of the ageing process will provide novel chemotherapeutics aimed at inhibiting age related changes in physiology, which can lead to increased risk in developing cancer and other age related diseases.

Human tumour viruses are emerging as a major cause of human cancer and there is now a great deal of evidence which supports the contention that these viruses cause cancer by inducing genetic instability or damage in infected cells. Indeed both the human T-cell leukaemia virus type 1 (HTLV1) Tax and the human papilloma virus type 16 (HPV16) E6 oncoproteins are known to induce genetic instability producing abnormal numbers of centrosomes, multinucleation and nuclear atypia (1) (2) (3) although the mechanisms underlying this process are poorly understood. Therefore increased understanding of these mechanisms may lead to the development of drugs aimed at preventing these effects in normal, pre-malignant and malignant tissues.

In addition to new treatments there is also a need for new diagnostic tools able to detect cancers. In many cases the early detection of the disease is important in allowing effective treatment.

According to a first aspect of the present invention there is provided a use of an agent that reduces cellular levels of HTLV1 Tax Interacting Protein-1 (TIP-1) or acts to inhibit or alter the functional effects of TIP-1 in the manufacture of a medicament for the prevention or treatment of conditions characterised by tissue damage.

According to a second aspect of the invention there is provided a method for the prevention of and treatment of age or damage related conditions comprising administering to a patient in need of such treatment an agent which is effective to

(i) reduce cellular levels of TIP-1 protein or

(ii) acts to inhibit, or alter the functional effects of TIP-1.

By "conditions characterised by tissue damage" we mean tissue damage that arises as a result of physical damage (e.g. wounding), chemical damage or viral damage and in particular damage or degeneration that is associated with aging. Such damage, and particularly viral damage, may lead to the development of a number of diseases including cancer.

The invention has been based on the inventors' findings relating to the effects of the TIP-1 protein, (i.e.HTLV1 Tax interacting protein-1) on the development of genetically abnormal pre-cancerous cells and on cancer cell growth and motility. TIP-1 has the sequence as follows:

MSYIPGQPVT AVVQRVEIHK LRQGENLILG FSIGGGIDQD PSQNPFSEDK TDKGIYVTRV MSYIPGQPVT AVVQRVEIHK LRQGENLILG FSIGGGIDQD PSQNPFSEDK TDKGIYVTRV SMLS.

(Seq ID No.1)

By "TIP-1" we mean natural or synthetic homologues of the protein of the above-mentioned sequence, which have tumour-promoting activity *in vivo* and also active fragments thereof.

The inventors have found that the agents are particularly useful for preventing the development of cancers. Accordingly normal subjects (i.e. no detectable cancer) or subjects with pre malignant cells or particularly cancer prone subjects may be treated according to the invention.

Although we do not wish to be bound by any hypothesis, the utility of the agents according to the present invention was established following work performed by the inventors investigating the role of the HPV16 E6 protein in the development of cancer. A brief summary of their findings is given below and is also described in the Example and accompanying drawings.

The inventors have established, in cells expressing the HPV16 E6 protein that the PDZ domain TIP-1 protein is found associated with the virus E6 early protein (Figure 1 A, C & D). Surprisingly they have found, unlike many other E6 targeted proteins (e.g. p53), that this association of TIP-1 and HPV E6 does not cause degradation and hence reduction in the level of TIP-1 accumulated in the cell (Figure 1 E & F).

Furthermore they found that the expression of TIP-1 mRNA is altered in various tumours with expression usually being increased above expression levels found in corresponding normal tissues from the same patient (Figure 8).

TIP-1 is known to interact with the cellular protein rhotekin, which in turn interacts with constitutively GTP activated RhoA (4). Interaction of these three proteins has been shown to produce a strong activation of serum response factor (SRF) transcription as measured by an increase in the SRF component of the *c-fos* serum response element (SRE) transcription (4). Rhotekin has been previously shown to be a negative regulator of intrinsic RhoA GTPase activity (5, 6) which has the effect of stabilising GTP bound RhoA. The SRE activation reported by Reynaud *et al* (4) is dependent on the interaction of an exogenously introduced constitutively activated GTP bound RhoA mutant, with TIP-1 and rhotekin, although this work was carried out in HPV18 +ve Hela cells. The inventors realised that like type16 E6, HPV18 E6 also has a carboxyl terminal PDZ domain binding site consensus domain (see Figure 1B) which means that the *c-fos* SRE activation observed by Reynaud *et al* may be influenced by the interaction of type 18 E6 with TIP-1.

The inventors evaluated the effects of expressing TIP-1 in the presence and absence of type 16 E6 on SRE activation and found no difference between the negative control and the SRE test vector in E6/TIP-1 expressing cells (Figure 4A). These data indicate that E6 does not facilitate TIP-1 dependent activation of GTP bound RhoA signalling to the SRF binding site of the *c-fos* SRE..

However, the measurement of SRE activation is an indirect method of analysing RhoA activation status. In order to directly analyse the amount of activated GTP bound RhoA the inventors employed the EZ-Detect Rho Activation Kit (Pierce Biotechnology). In agreement with the SRE assays no difference was found between E6 expressing and non-E6 expressing cells. (Figure 4C). When the TIP-1 protein is present in the absence of E6, endogenous RhoA is readily de-activated to the GDP bound form by pre-treatment of cell lysates with GDP and is not activated to the GTP bound form by pre-treatment of lysates with GTP. However, when E6 is present GDP does not de-activate GTP RhoA and pre-treatment with GTP produces a strong

activation of RhoA to the GTP bound form. These data indicate that although steady state levels of activated RhoA may be similar between E6 and non-E6 expressing cells, RhoA is more readily activated and less readily deactivated in the presence of the HPV16 E6 protein and TIP-1.

Another consequence of GTP activation of RhoA is the activation of RhoA kinases (ROCK's) (7) which are known to regulate actin dynamics. ROCK's are known to deactivate myosin light chain (MLC) phosphatase and to directly phosphorylate MLC's (7) . Analysis of TIP-1 expression in the absence of E6 showed no detectable phospho-MLC expression whereas a signal was present in the presence of E6 (Figure 4B). These data are consistent with E6 activating RhoA dependent ROCK activity.

Although E6 does not activate SRE signalling from endogenous GTPRhoA, the inventors realised that E6 may be interacting with TIP-1 which in turn interacts with rhotekin to promote GTP RhoA dependent activation of ROCK.

Higher levels of GTP RhoA and thus activated ROCK's have been shown to be associated with the transition from an epithelial to mesenchymal cell phenotype that is associated with increased migration and is known to occur during tumour cell dissemination (metastases) (8). Accordingly agents used according to the first and second aspects of the invention have utility in treating cancer and most particularly the prevention of metastases.

Experiments conducted by the inventors to study the effects of TIP-1 modulation are summarised in Example 1.

The inventors have further established that TIP-1 interacts with the cellular protein guanidine triphosphate exchange factor 16 (ARHGEF16, Accession NM_014448), which belongs to the GEF family of proteins (Figure 1A & B). GEFs are positive regulators of GTP activation of RhoA and most are oncogenes for example the Vav family of proteins function as GEFs activating Rho GTP'ase (9).

Consistent with this finding the inventors have established that TIP-1 is capable of regulating both the activation status of endogenous RhoA and its downstream effects (ROCK activation). Thus it is significant that the inventors data provide a novel insight into the importance of TIP-1 as a crucial control point for the regulation of RhoA activity in cancer since the HPV E6 and HTLV1 Tax oncoproteins both induce genetic destabilisation characterised by polyploidy and both interact with TIP-1.

It has been previously shown that the ability of RhoA mutants to transform NIH3T3 cells does not correlate with their ability to transactivate *c-fos* SRE's but with their ability to bind with and activate the RhoA kinase ROCK(10). Thus although the work of Reynaud (4) indicated that TIP-1 could activate SRE transcription this study did not link this to transforming ability or to increased cell motility nor demonstrate the importance of TIP-1 in these physiological responses. Importantly the inventors have shown that transfection of tumour cells with HPV16 E6 produces increased cell motility and that this effect can be abrogated by antisense silencing of TIP-1 expression (See Figure 2). These data clearly demonstrate that the TIP-1 protein undergoes an E6 mediated gain of function and that this is involved in cell motility. Furthermore the inventors have also demonstrated that treatment of E6 expressing tumour cells with the RhoA kinase (ROCK) inhibitor Y27632 (10) reduces cell motility to that of non-E6 expressing cells (Figure 2). This is an effect consistent with blocking GTPRhoA dependent ROCK activity. Antisense silencing of TIP-1 produces an identical inhibitory effect on the motility of E6 expressing tumour cells indicating the importance of TIP-1 for ROCK activation.

The inventors have observed that the expression of TIP-1 is increased in many cancers (even those with a non-viral aetiology) (Figure 8). This indicates that TIP-1 activity may be regulated by alternative mechanisms other than interaction with viral oncoproteins in these cancers. For example up-regulation of expression, altered phosphorylation status, or interaction with other cellular proteins may play a role in controlling TIP-1 activity.

In support of this the inventors demonstrate that over expression of the TIP-1 protein is sufficient to completely abrogate cell-cell contact inhibition in NIH 3T3 cells (see Figure 5). This clearly shows that increased activity of TIP-1 in non-transformed cells produces an oncogenic effect in isolation from viral oncoproteins and provides evidence for a role in cancers with a non-viral aetiology. Interestingly the TIP-1 induced loss of contact inhibition in NIH3T3 cells was reversed by treatment with the ROCK inhibitor Y27632 which again supports the hypothesis that TIP-1 activates ROCK's through modulation of activated RhoA. Further evidence for this was the observation that TIP-1 expressing 3T3 cells had very high levels of phosphorylated MLC when compared to control cells.

Fluorescent activated cell sorter analysis of TIP-1 3T3 cells indicated that the ploidy of these cells had become unstable compared to control cells. Fluorescent microscopy visualisation of these cells with propidium iodide confirmed that many TIP-1 expressing cells had abnormal numbers and shapes of nuclei – an effect consistent with abnormal cytokinesis leading to polyploidy, genetic instability and eventually cancer. It is highly significant that increased numbers of cells with abnormal ploidy are found in many human tissues associated with the ageing process and that these cells are functionally compromised when compared to normal diploid cells (11). Indeed there are now *in vivo* models of pre-mature ageing which are characterised by the development of abnormal tissue ploidy (12). Furthermore abnormal ploidy occurs in response to many different types of tissue damage including that caused by wounding, chemicals or viruses (11). Therefore it was most significant that treatment of these abnormal cells with the ROCK inhibitor Y27632 suppressed this effect with very few abnormal cells being visible after 48 hours of treatment (Figure 7).

Based on these results the inventors thus claim that agents capable of inhibiting the oncogenic, polyploidy inducing, ROCK activating effects of TIP-1, for example Y27632, will inhibit the development of age or damage related abnormal polyploidy. This will in turn inhibit the development of many damage or age related diseases including cancer and thus form the basis of chemopreventative agents. Furthermore, since increased levels of polyploidy are known to be associated with

ageing and damage in many tissues (11) the inventors have established that agents such as Y27632 may inhibit or suppress these age or damage related changes.

The invention, to the extent that it is applicable to the prevention and treatment of cancer, may be applied to a wide range of cancers such as ovarian carcinoma, breast carcinoma, lung carcinoma, uterine carcinoma, cervical carcinoma and thyroid carcinoma. It may also be applicable to cancer prone conditions. The invention is applicable particularly, but by no means exclusively, to pre-cancerous conditions and cancers caused by oncogenic viruses, e.g. transforming human papilloma viruses (HPVs) or human T-cell leukaemia virus type-I (HTLV-I).

Several classes of compound, may be used as an agent according to the first or second aspects of the invention, to influence TIP-1 activity. These compounds include:

(i) compounds which reduce synthesis of TIP-1;

(ii) compounds which inactivate TIP-1 activity or increase the rate of metabolism or degradation of TIP-1

(iii) compounds which block the effects of TIP-1 (e.g. Y27632)

Agents according to the present invention may modulate the effects of TIP-1 on the activity and effects of GTP RhoA. Preferred agents for use according to the invention are inhibitors of GTPRhoA or RhoA Kinase (ROCK). Such agents are known to the art. For instance, preferred examples of RhoA Kinase inhibitors are disclosed in Japanese patent application No. 2001-266055.

A most preferred agent is Y27632. The inventors have found that this compound is not only useful for treating cancer but is also surprisingly useful for preventing age or damage related changes in cell ploidy (e.g. preventing the development of cancer). Accordingly Y27632 may be advantageously used as a prophylactic. For instance, it may be given to subjects with a genetic disposition to developing cancer or even those facing environmental risk (e.g. people exposed to carcinogens). It may also be given to modulate the effects of wounding and the

development of scarification and tissue fibrosis and wherever polyploidisation is a consequence of such damage.

Other preferred agents for use according to the first aspect of the invention include neutralising antibodies raised against TIP-1 (e.g. monoclonal or polyclonal); antibody fragments (for example Fab fragments or intrabodies); ribozymes; and in particular antisense oligonucleotides against TIP-1 (see the Example 1).

A preferred TIP-1 antisense oligonucleotide is:

5′ TGTAGGACATCTCGAC 3′ (Seq ID No.3)

Other antisense oligonucletides that may be used according to the invention are modulators of Rho family gene expression such as those disclosed in WO 00/17223 or US 6,410,323.

The compounds may be used to treat age related conditions such as cancer as a monotherapy (i.e. use of the compound alone or in combination with other compounds or treatments used in cancer therapy (e.g. chemotherapeutic agents, radiotherapy).

The medicaments used according to the invention may take a number of different forms depending, in particular on the manner in which the medicament is to be used. Thus, for example, the medicament may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micelle, liposome or any other suitable form that may be administered to a person or animal. It will be appreciated that the vehicle of the medicament of the invention should be one which is well tolerated by the subject to whom it is given and enables delivery of the agents to the effected site.

Therapy with the agents may be effected in a number of ways. For instance, systemic administration may be required in which case the agent may be contained within a medicament, which may, for example, be ingested orally in the form of a

tablet, capsule or liquid. Alternatively, the medicament may be administered by injection into the blood stream. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion). The agents may also be administered by inhalation (e.g. intranasally). Alternatively the agents may be administered transdermally by way of patches.

The agent may also be incorporated within a slow or delayed release device. Such devices may, for example, be inserted under the skin and the agent may be released over weeks or even months. The devices may be particularly advantageous when an agent is used which would normally require frequent administration (e.g. at least daily ingestion of a tablet or daily injection).

It will be appreciated that the amount of an agent required is determined by biological activity and bioavailability which in turn depends on the mode of administration, the physicochemical properties of the agent employed and whether the agent is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the abovementioned factors and particularly the half-life of the agent within the subject being treated.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials etc), may be used to establish specific formulations of medicaments and precise therapeutic regimes (such as daily doses of the agents and the frequency of administration).

Generally, a daily dose of between 0.01μg/kg of body weight and 1.0g/kg of body weight of an agent according to first or second aspects of the invention may be used for the treatment of cancer depending upon which specific agent is used. More preferably the daily dose is between 0.01mg/kg of body weight and 100mg/kg of body weight.

It will be appreciated that the required dose will be influenced by the route of administration. For instance when an agent is given intravenously the preferred dose may be lower than a suitable dose chosen for oral administration.

Daily doses may be given as a single administration (e.g. a daily tablet for oral consumption or as a single daily injection). Alternatively the agent used may require administration twice or more times during a day. A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3 or 4 hourly intervals thereafter. Alternatively a slow release device may be used to provide optimal doses to a patient without the need to administer repeated doses.

A potential means of using protein or peptide agents according to the invention is to deliver proteins or peptides to the tumour site by means of gene therapy. For instance, gene therapy may be used to modulate expression of TIP-1, by increased expression of enzyme(s) responsible for the degradation of TIP-1 or of a protein or peptide, which promotes the inactivation of TIP-1. Therefore according to a third aspect of the present invention there is provided a delivery system for use in a gene therapy technique, said delivery system comprising a DNA molecule encoding for a protein which directly or indirectly reduces TIP-1 activity, said DNA molecule being capable of being transcribed to allow the expression of said protein and thereby treat cancer.

The delivery systems according to the first and second aspects of the invention are highly suitable for achieving sustained levels of a protein, which directly or indirectly reduces TIP-1 activity over a longer period of time than is possible for most conventional therapeutic regimes. The delivery system may be used to induce continuous protein expression from cells of the tumour that have been transfected with the DNA molecule. Therefore, even if the protein has a very short half-life as an agent *in vivo*, therapeutically effective amounts of the protein may be continuously expressed from the treated tissue.

Furthermore, the delivery system of the invention may be used to provide the DNA molecule (and thereby the protein which is an active therapeutic agent) without the need to use conventional pharmaceutical vehicles such as those required in tablets, capsules or liquids.

The delivery system of the present invention is such that the DNA molecule is capable of being expressed (when the delivery system is administered to a patient) to produce a protein, which directly or indirectly has activity for reducing wild-type TIP-1 activity. By "directly" we mean that the product of gene expression *per se* has the required activity. By "indirectly" we mean that the product of gene expression undergoes or mediates (e.g. as an enzyme) at least one further reaction to provide an agent effective for reducing the capacity of TIP-1 to activate GTPRhoA dependent activation of ROCK's and thereby treating cancer.

The DNA molecule may encode for any of the compounds (i-ii) as defined above. A preferred DNA molecule encodes an intrabody against TIP-1.

The DNA molecule may encode for any of the compounds (i-ii) as defined above. The DNA molecule may be contained within a suitable vector to form a recombinant vector. The vector may for example be a plasmid, cosmid or phage. Such recombinant vectors are highly useful in the delivery systems of the invention for transforming cells with the DNA molecule.

Recombinant vectors may also include other functional elements. For instance, recombinant vectors can be designed such that the vector will autonomously replicate in the cell. In this case elements, which induce DNA replication may be required in the recombinant vector. Alternatively the recombinant vector may be designed such that the vector and recombinant DNA molecule integrates into the genome of a cell. In this case DNA sequences, which favour targeted integration (e.g. by homologous recombination) are desirable. Recombinant vectors may also have DNA coding for genes that may be used as selectable markers in the cloning process.

The recombinant vector may also further comprise a promoter or regulator to control expression of the gene as required.

The DNA molecule may (but not necessarily) be one, which becomes incorporated in the DNA of cells of the subject being treated. Undifferentiated cells may be stably transformed leading to the production of genetically modified daughter

cells (in which case regulation of expression in the subject may be required e.g. with specific transcription factors or gene activators). Alternatively, the delivery system may be designed to favour unstable or transient transformation of differentiated cells in the subject being treated. When this is the case, regulation of expression may be less important because expression of the DNA molecule will stop when the transformed cells die or stop expressing the protein (ideally when the cancer has been treated or prevented).

The delivery system may provide the DNA molecule to the subject without it being incorporated in a vector. For instance, the DNA molecule may be incorporated within a liposome or virus particle. Alternatively the "naked" DNA molecule may be inserted into a subject's cells by a suitable means e.g. direct endocytotic uptake.

The DNA molecule may be transferred to the cells of a subject to be treated by transfection, infection, microinjection, cell fusion, protoplast fusion or ballistic bombardment. For example, transfer may be by ballistic transfection with coated gold particles, liposomes containing the DNA molecule, viral vectors (e.g. adenovirus) and means of providing direct DNA uptake (e.g. endocytosis) by application of the DNA molecule directly to the cancer or prospective site of cancer, either topically or by injection.

The treatment according to the first, second or third aspects of the invention may be for the purposes of treating an existing cancer or may be a prophylactic treatment administered to a person believed to be at risk of developing such a cancer.

According to a fourth aspect of the present invention there is provided a method for the diagnosis of pre-cancer and cancer comprising analysing a cell sample from a patient for the presence of elevated levels of TIP-1 mRNA or protein or the presence of mRNA or protein for TIP-1 induced proteins.

By "TIP-1 induced proteins" we mean proteins that may be come inappropriately expressed as a consequence of TIP-1 expression. Examples of such

proteins are apparent from Figure 9 and include Rhotekin, GTPRhoA, ROCK, Myosin Light Chain (MLC) Kinase.

It is most preferred that the Tip-1 induced protein is phosphorylated MLC.

Diagnosis may be effected on a sample from a patient believed to be suffering from cancer, or alternatively to a patient believed to be at risk of developing cancer.

Diagnosis according to the invention may be effected in order to establish whether or not a patient is suffering from cancer and the metastatic potential of their disease. This diagnosis may be carried out in order to assess the suitability of a specified therapeutic regime for the treatment of a patient's disease.

The sample taken may, for instance, be a tissue biopsy, blood sample or swab.

Diagnosis of the cellular levels of TIP-1 or TIP-1 induced proteins may be carried out by assessing the level of expressed protein within the cell, or alternatively, by taking a measurement of the level of gene transcription. Protein may, for example, be assessed through the use of specific binding agents including polyclonal and monoclonal antibodies in techniques such as immuno-cytochemistry, immuno-precipitation or immuno-blotting (Western blotting).

According to a fifth aspect of the present invention there is provided a method of screening a compound to test whether or not the compound has efficacy for treating cancer, comprising:

(i) administering to living cells or a subject a test compound for a predetermined length of time;

(ii) detecting the activity or expression of TIP-1 or TIP-1 induced proteins from the cells or subject; and

(iii) comparing the activity or expression of TIP-1 or TIP-1 induced proteins in the cells or the subject treated with the compound relative to activity or expression found in control cells or a control subject that were not treated with the compound

wherein compounds with efficacy for treating cancer decrease activity or decrease expression of TIP-1 or Tip induced proteins relative to the controls.

It will be appreciated that the method according to the fifth aspect of the invention may be adapted such that it is used to test whether or not a compound causes cancer. Therefore according to a sixth aspect of the invention there is provided a method of screening a compound, to test whether or not the compound causes cancer, comprising:

(i) administering to living cells or a subject a test compound for a predetermined length of time;

(ii) detecting the activity or expression of TIP-1 or TIP-1 induced proteins from the cells or subject; and

(iii) comparing the activity or expression of TIP-1 or TIP-1 induced proteins tin the cells or the subject treated with the compound relative to activity or expression of TIP-1 in control cells or a control subject that were not treated with the compound

wherein compounds that cause cancer increase activity or increase expression relative to the controls.

The screening methods of the fifth and sixth aspects of the invention are based upon the inventors realisation that inappropriate TIP-1 expression and activity in defined cell types may be closely related to carcinogenesis and metastasis.

TIP-1 expression is increased in some cancerous tissues compared to normal tissues. Accordingly any compound, identified according to the fifth aspect of the invention, that decreases the expression or activity of TIP-1 or TIP-1 induced proteins, is a good candidate as an agent for treating cancer. It will be appreciated that the pharmaceutical industry will be able to use the method according to the fifth aspect of the invention in high throughput screens to identify candidate medicaments for use in the treatment of cancer. Therefore according an seventh aspect of the present invention there is provided an anti-cancer agent identified by the method according to the fifth aspect of the invention

The method according to the sixth aspect of the invention represents a good test for evaluating whether or not a test compound is carcinogenic. Accordingly any compound, identified according to the sixth aspect of the invention, that reduces the expression or activity of TIP-1 or TIP-1 induced proteins is less likely to be carcinogenic. The method may be used to screen compounds to assess whether or not they are safe to be used by the public. For instance cosmetics, foodstuffs, candidate therapeutic agents etc may all be tested to investigate whether or not they may cause cancer. The method according to the sixth aspect of the invention may also be used in an environmental setting. For instance, the test may be used to evaluate whether or not effluent from a factory may contain carcinogenic compounds.

The activity or expression of TIP-1 or TIP-1 induced proteins may be measured using a number of conventional techniques. For instance, labelled antibodies may be used in an immunoassay to evaluate protein levels in the cells or subject being tested. Alternatively a functional activity measuring TIP-1 activity may be employed.

Molecular biology techniques may be used to detect TIP-1 or the expression TIP-1 induced proteins according to the fifth or sixth aspects of the invention. For instance, cDNA may be generated from mRNA extracted from the tested cells or subject and primers designed to amplify test sequences used in a Polymerase Chain Reaction to amplify from cDNA.

It will be apparent from the Example that TIP-1 has a marked affect on the morphology of cells (e.g. See Figure 6). Although the inventors do not wish to be bound by any hypothesis, they believe the morphological changes may be caused by am alteration in the phosphorylation state of MLC or Actin. Therefore preferred methods according to the fourth, fifth or sixth aspects of the invention may involve monitoring the phosphorylation state of MLC or Actin.

Other preferred methods according to the fourth, fifth or sixth aspects of the invention may involve monitoring the morphological changes per se. These may be observable microscopically and/or histologically. Once such observable change is in

the ploidy of the cells (See Figure 6). Inappropriate expression of TIP-1 appears to increase disorganisation during mitosis and in particular causes cells to become polyploid. Therefore preferred methods can monitor morphological changes and particularly the proportion of polyploid cells in a tested cell sample. A most preferred way of monitoring polyploidy is be conducting a FACS analysis (see Figures 6 and 7). FACS analysis has the advantage that its is fast and automated and it is therefore easy to detect whether or not a screened compound increases or decreases polyploidy.

Another preferred way of conducting assays according to the fifth or sixth aspects of the invention is described in the Example and is based on a functional motility assay. The motility assay may involve comparison of the morphology of control and E6 expressing cells and is based upon the inventors' observation that E6 cells produce more projections and appear more motile in culture. The inventors adapted a method previously applied to endothelial cells (11) where cells are plated in oil, allowed to attach, the oil replaced with growth medium and the cells allowed to grow freely for approximately 18 hours. The inventors adapted the known method by combining it with engineered E6 expressing tumour cells. This motility assay represents a simple method for identifying protein targets or drugs that interfere with the process of cell movement since this is involved in the process of tumour invasion.

In a preferred embodiment of the motility assay, TIP-1 protein expression may be silenced by transfection with an antisense TIP-1 phosphorothioate oligonucleotide (e.g.5′ TGTAGGACATCTCGAC 3′ see -Seq ID No2 for location.) Transfected cells may be left for about 48 hours prior to carrying out the motility assay.

The motility assay represents an important aspect of the invention and according to an eight aspect of the invention there is provided a method of screening a compound to test whether or not the compound has efficacy for treating cancer, comprising:

(i) plating E6 expressing tumour cells in oil and allowing the cells to attach to the plate;

(ii) replacing the oil with growth medium and allowing the cells to grow freely for a defined period of time in the presence or absence of a test compound; and

(iii) evaluating the development of projections from the E6 expressing cells and motility of the cells;

wherein a decrease in the development of projections from the E6 expressing cells or a decrease in motility of the cells indicates the compound has efficacy for treating cancer.

According to a ninth aspect of the invention there is provided a method of screening a compound, to test whether or not the compound causes cancer, comprising:

(i) plating E6 expressing tumour cells in oil and allowing the cells to attach to the plate;

(ii) replacing the oil with growth medium and allowing the cells to grow freely for a defined period of time in the presence or absence of a test compound; and

(iii) evaluating the development of projections from the E6 expressing cells and motility of the cells;

wherein an increase in the development of projections from the E6 expressing cells or an increase in motility of the cells indicates the compound increases the migration and hence metastatic potential of cancer.

Cells used according to the fifth, sixth, eight or ninth aspects of the invention may be from a number of sources. The cells may be *ex vivo* samples but are preferably a cell-line that is either capable of expressing TIP-1 or has been engineered to do so. The cell-line is preferably eukaryotic and ideally a human cell line. However it will be appreciated that yeast may be used and even prokaryotic models that have been engineered to be capable of expressing TIP-1. The cells may be cultured with a test compound for a predetermined length of time and then tested for levels of TIP-1 or TIP-1 induced protein expression or activity.

When a subject is used (e.g. an animal model or preferably an animal model engineered to express human TIP-1), the test compound should be administered to the subject for a predetermined length of time and then a sample taken from the subject for testing TIP-1 or TIP-1 induced protein expression or activity or the consequences of abnormal Tip-1 expression such as abnormal ploidy. The sample may for instance be blood or biopsy tissue.

It will be appreciated that preferred methods according to the fifth, sixth, eight or ninth aspects of the invention may be adapted such that a cell or subject is first treated with TIP-1 and then the cells are subsequently treated with a screened compound to test its ability to augment or inhibit the activity of TIP-1. For instance, the Examples illustrate that TIP-1 treated cells have increased polyploidy. Therefore any test compound that has the ability to reduce TIP-1 induced polyploidy is likely to have general anti-ageing properties and will also act to decrease fibrosis and scarring that can occur during the repair of tissue damage. Such compounds will thus inhibit the development of cancer and many human diseases. Conversely any compound that has the ability to increase TIP-1 induced polyploidy is likely to accelerate age related tissue damage and is likely to be carcinogenic.

The invention will be further described, by way of Example and with reference to the following figures:-

Fig. 1 illustrates that TIP-1 Interacts With HPV16 E6 and GEF16 in:
(A) Mating assay between yeast strain RFY206 transformed with (1) pEG202 TIP-1 as bait; (2) pEG202; (3) pBAIT and EGY48 transformed with; (a) pJG4-5 GEF16; (b) pJG4-5 HPV16 E6; (c) pTARGET; (d) pJG4-5. Yeast strains were spotted on galactose/raffinose and glucose selection plates. Positive interactors turn blue on Gal/Raff plates only. (type 18 E6 not shown);
(B) Consensus PDZ binding sites of the three binding partners of TIP-1 identified in this study are illustrated in bold plus previously identified TIP-1 interactors;
(C) Northern blot of RNA isolated from; (1) Non transfected C33A-V cells; (2) TIP-1 transfected C33A-E6 cells and (3) TIP-1 Transfected C33A-V cells probed with labelled ^{32}P Actin and TIP-1. The endogenous TIP-1 transcript is expressed at the

same level in both cell types as is the ectopically expressed transcript indicating comparable transfection efficiencies;

(D) Western blot of anti V5 immunoprecipitated proteins isolated from; (1) C33A-E6 and (2) C33A-V cells transiently transfected with V5 tagged TIP-1 and immunoprobed with anti E6 antibody. E6 interacts with TIP-1 in C33A-E6 cells whereas no signal was seen in C33A-V cells;

(E) Western blot of proteins isolated from; (1) C33A-V cells; (2) C33A-V cells transiently transfected with V5 tagged TIP-1 immunoprobed with anti p53 and anti V5 antibodies. p53 protein was detected in lanes 1 and 2 whereas V5 tagged TIP1 was only present in lane 2; and

(F) Western blot of proteins isolated from; (1) C33A-E6; (2) C33A-E6 cells transiently transfected with V5 tagged TIP-1 immunoprobed with anti p53 and anti V5 antibodies. p53 protein was detected in lanes 1 and 2 whereas V5 tagged TIP1 was only present in lane 2. Comparison of (E) and (F) show no difference in the level of V5 TIP-1 whereas the p53 protein is clearly degraded in C33A-E6 cells.

Fig. 2. illustrates the results of Cell Motility Assays under the following conditions: (A) 40 x of C33A-V cells at time 0. No migration; (B) 40x of C33A-V cells at 18 h showing - Minimal migration; (C) 250x of C33A-V cells at 18 h - Compact cell morphology and maintenance of cell boundary; (D) 40x of C33A-E6 cells at time 0 - No migration; (E) 40x of C33A-E6 cells at 18 h - Extensive migration; (F) 400x of C33A-E6 cells at 18 h - Cell spreading and complete disruption of the cell boundary; (G) 40x of Y27632 treated C33A-E6 cells at 18 h - Minimal migration; (H) 400x of Y27632 treated C33A-E6 cells at 18 h - Reversal of cell spreading; (I) 250x of Y27632 treated C33A-E6 cells at 18 h - Maintenance of cell boundary; (J) 40x of scramble ODN treated C33AE6 cells at 18 hours - Massive dissemination of cells; (K) 40x of antisense TIP-1 ODN treated C33A-E6 cells at 18 h - Inhibition of migration; (L) 250x of antisense TIP-1 ODN treated C33A-E6 cells at 18 h - Maintenance of cell boundary and more compact cell morphology; (M) 40x of scramble ODN treated C33A-V cells at 18 h - No effect; (N) 40x of antisense TIP-1 ODN treated C33A-V cells at 18 h - No effect ;(O) 250x antisense TIP-1 ODN treated C33A-V cells 18 h - No effect.

Fig. 3 represents the effect of Antisense Inhibition of TIP-1 Expression in ploidy, the photographs represent data for: C33A-E6 cells transfected with fluorescein labelled scramble and antisense TIP-1 ODN's showing equal uptake of both ODN types; and Northern blot of RNA isolated from C33AE6 cells treated with (1) antisense TIP-1 ODN and (2) Scramble ODN probed with labelled TIP-1 cDNA. The ribosomal bands (R) are shown as a loading control. Antisense TIP-1 ODN induces a six-fold reduction in TIP-1 transcript expression compared to scramble ODN.

Fig. 4 illustrated analysis of SRF and RhoA Activation in C33A and C33AE6 cells in which:

(A) Secreted alkaline phosphatase assays on C33A-V cells and C33A-E6 cells transiently transfected with pTAL-SEAP –ve control; pSEAP-SRE vector and pTCF mutant pSEAP-SRE vector. High- level transcription of SEAP was detected from the pTAL-SEAP -ve control construct in C33AE6 cells, with no significant increase in TCF mutant or SRE directed SEAP transcription above the –ve control. All values were normalised using transfection with the pSEAP-Control vector. Thus there is no significant difference in the steady state levels of SRF or SRE mediated transcription between C33A-V and C33A-E6 cells;

(B) Western blot on proteins isolated from; (1) C33A-V and (2) C33A-E6 cells immunoprobed with anti phosphomyosin and anti actin antibodies. C33A-E6 cells express more phosphorylated myosin than C33A-V cells; and

(C) Western immunoblot showing levels of activated RhoA in; (1) GDP treated C33A-V cell lysate; (2) GTP treated C33A-V lysate; (3) GDP treated C33A-E6 lysate; (4) GTP treated C33A-E6 lysate; (5) C33A-V lysate; (6) C33A-E6 lysate; (7) Antisense TIP-1 ODN treated C33A-E6 lysate; (8) Scramble ODN treated C33A-E6 lysate. These data confirm that the level of activated RhoA is identical in C33A-V and C33A-E6 cells but show that the RhoA in C33A-E6 cells is more readily activated and less readily de-activated by treatment with either GTP or GDP.

Fig. 5 illustrates the effects of Constitutive Expression of TIP-1 in NIH/3T3 Cells in which:

(A) Contact growth inhibition of polyclonal NIH/3T3 cells transfected with TIP-1 or vector control and TIP-1 3T3 cells treated with Y27632. TIP-1 3T3 cells show loss of contact growth inhibition which can be reversed by treatment with Y27632;

(B) Growth assay of polyclonal vector and TiTIP-1 NIH/3T3 cells with and without Y27632 over 10 days. Y27632 restores contact inhibited growth to TIP-1 3T3 cells;

(C) Matrigel invasion assay of polyclonal vector and TIP-1 transfected NIH/3T3 cells with and without matrigel. Assay repeated 3 times in triplicate. TIP-1 3T3 cells are more capable of invading matrigel than vector transfected control cells;

(D) Western immunoblot of NIH/3T3 cell proteins isolated from; (1) Monoclonal TIP-1 transfected cells (TIP10); (2) Polyclonal TIP-1 transfected cells; (3) Vector transfected cells; (4) Parental cells immunoprobed with anti V5 and anti actin antibodies. The V5 tagged TIP-1 protein was detected in polyclonal and monoclonal TIP-1 transfected cells; and

(E) Western Immunoblot of proteins isolated from; (1) TIP10 3T3 cells; (2) Vector transfected 3T3 cells; (3) TIP-1 polyclonal 3T3 cells; (4) Parental 3T3 cells immunoprobed with anti phosphomyosin and anti actin antibodies. Tip1 expressing 3T3 cells have higher levels of phosphorylated MLC.

Fig 6. illustrates the effects of TIP-1 on the Ploidy of NIH/3T3 Cells in which:

(A) FACS analysis of propidium iodide stained monoclonal TIP10, vector transfected NIH/3T3 cells and C33A cells. Vector cells had the typical NIH/3T3 profile whereas TIP10 cells adopted a profile more characteristic of C33A tumour cells with far higher numbers of polyploid cells with abnormal nuclei.

(B-F) Fluorescence and phase microscopy of propidium iodide and toluidine blue stained; (B-D) Monoclonal TIP10 cells. Abnormal and multinucleated cells are arrowed which confirms the FACS results; (E) C33A cells. Multinucleated, abnormal cells are arrowed; (F) Fluorescence and phase microscopy of vector transfected monoclonal NIH/3T3 cells. Normal mononuclear cells shown. (B,C,E and F, 400x ; D, 1000x magnification)

Fig. 7 illustrates that Y27632 Inhibits the Ability of TIP-1 to Induce Abnormal Nuclei in TIP-10 3T3 cells that were seeded onto glass cover slips and grown in the presence and absence of 10μM Y27632 in which: (A) Shows the cells in the presence of Y27632; and (B) in the absence visualised by fluorescence microscopy analysis of Propidium iodide stained cells. The results clearly show that Y27632 had inhibited the development of polyploid TIP-10 3T3 cells.

Fig. 8 shows a TIP-1 Probe of Matched Pair Human Tumour cDNA Array in which:
(A) ^{32}P labelled TIP-1 probe of a tumour array showing cDNA from normal tissue on the left and tumour on the right from the same patient. of 250 matched pairs of normalised total cDNA's from normal (Left hand column dot in Figure 7 (A) and tumour tissue (Right hand column dot Figure 7 (A)) from the same individual. (Clontech). Figure 4 (B) represents the same blot probed with the housekeeping gene ubiquitin. This blot covers a wide range of human cancer types and the results indicated that the expression of TIP-1 was up-regulated approximately ten fold in; 28% of (n =14) ovarian carcinoma, 50% of (n = 50) breast carcinoma, 33% of (n = 21) lung carcinoma, 30% of (n = 42) in uterine carcinoma and 50% of (n = 6) in thyroid carcinoma. However, analysis of the histology from these various carcinomas indicated that different carcinomas, such as lung, had sub-classifications within the overall category. Out of 21 lung carcinomas, 5 were keratinising, of which, non showed any up-regulation of TIP-1. Out of the remaining 16 lung carcinomas, 8 (50%) showed extensive up-regulation of TIP-1 expression. The results are consistent with TIP-1 playing an oncogenic role in carcinogenesis.
(B) Tumour blot probed with labelled ^{32}P ubiquitin cDNA. Identical signal were detected in all samples.

Fig 9 is a scheme illustrating interactions of TIP-1 which identifies new binding partners of Tip-1 and how these may interact with RhoA and it's effectors.

EXAMPLE 1

Brief Summary of findings:- The Tip-1 protein interacts with the HPV16 E6 oncoprotein and the guanine exchange factor GEF16 (ARHGEF16, Accession NM_014448). Although many proteins targeted by E6 are subject to degradation (e.g. p53) TIP-1 is not degraded by its interaction with E6. Expression of the E6 protein in HPV negative cervical carcinoma cells induces increased levels of phosphorylated myosin light chains (MLC); increased capacity to GTP activate RhoA and a large increase in cell motility, which is prevented by the RhoA kinase (ROCK) inhibitor Y27632 and by antisense silencing of TIP-1 expression. These data suggest that E6 interacts with TIP-1 to inappropriately activate RhoA dependent ROCK. Over expression of TIP-1 in non-transformed NIH/3T3 cells induces gross nuclear atypia, increased phosphorylation of MLC and the growth of non-contact inhibited foci, which were all inhibited by treatment with Y27632. Since ROCK activity is tightly controlled during centriole migration and completion of cell division these data suggest a potential mechanism for E6 and TIP-1 in cancer induced genetic instability. They also indicate that over expression of TIP-1 can produce these effects in isolation and we demonstrate that TIP-1 mRNA is up-regulated in a variety of human tumours. Thus we propose that TIP-1 is a regulator of the effects of RhoA with a potential oncogenic, gain-of-function role in the control of cell growth, migration and cytokinesis in both viral and non-viral malignancies.

The published TIP-1 sequence (Accession No. AF028823) is as follows:-

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1    AGGGGCGCTC CGGCCAGTGA TTGGCTGGAG GTTTGTTAAC TATTCATGAG GGGGCGGGCC
61   GAGCGGGGCG GCCTTTGTTA AGCAGCGAGG GCGCGACCGC GGGTACTCTG CTGCCGGCTT
121  CTCGGAGCGG CGCTGGGCGA CCAGAGCAGG GTCGAGATGT CCTACATCCC GGGCCAGCCG
181  GTCACCGCCG TGGTGCAAAG AGTTGAAATT CACAAGCTGC GTCAAGGTGA GAACTTAATC
241  CTGGGTTTCA GCATTGGAGG TGGAATCGAC CAGGACCCTT CCCAGAATCC CTTCTCTGAA
301  GACAAGACGG ACAAGGGTAT TTATGTCACA CGGGTGTCTG AAGGAGGCCC TGCTGAAATC
361  GCTGGGCTGC AGATTGGAGA CAAGATCATG CAGGTGAACG GCTGGGACAT GACCATGGTC
421  ACACACGACC AGGCCCGCAA GCGGCTCACC AAGCGCTCGG AGGAGGTGGT GCGTCTGCTG
481  GTGACGCGGC AGTCGCTGCA GAAGGCCGTG CAGCAGTCCA TGCTGTCCTA GCAGCCACCA
541  CCATCTGCGA CTCCTGCCTG CCGCCTCTCT GTACAGTAAC GCCACTTCCA CACTCTGTCC
601  CCATCTGGCT TCTGCTGACC GCTGGGCCCC AGCTCAGAAG GGCTATAGCT GGTCCCAGAG
661  GCCTGGCCTG GCCTTCCTTC CCTTCTCCCA TCCCTGGCCT GGGGCCTCTG GGACCAGCTT
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721  TCTCTCCTGG ACACCGAGGA TTGGAAATAA GGGCCTGGAG CTGAGTAGTA GCCAGTCTGC
781  TGTGACCACA GGCTCAGGTC CGACCCTGCT GCTTGGCCAC AGCAGTGGCT GGGCAAGTGG
841  GAACCACTAT CTCTTGGGAG CCCCCAAAAG CTGGGAAATG CTGGAGGAAC CAGGCCTTTC
901  CCGCTTTTGC CTGGCTGCAG GGTTCGGCTC CGCCCCTGCC CCCCAGCCCT CGTGTGTCCA
961  CACCGCAGTG CCTCTGCCCC TCGGGGGACT GGACACACAT CCTGCCAGAG GCGCTACGAA
1021 GCTTTGCCCA GATGAAGCCA GGTGGGCTCC GCGTTCACTC CCACTCTCCC GAGGGGTGCT
1081 GGCCTCCCCA GGGTTTGCCT TCTTACGGAT TTAGACGAGG TTCGAGGCTC ACCTATCAGG
1141 GCAGCTCTCA GGATTGTCAT TTTCCTCTTT GCCTGTGGGT TTAACTTTTG TATTTTTTTA
1201 ATCACAAGTT TGATACAAAA TGTTTTTATC GTACTCTTTG GAGATGCCCA TTCTACTTTT
1261 GAATTTAGCT TTTACTAATT CGCATCTGGA AGCTCAGCAA GTGCACAAGC CTTACTTTGG
1321 TTACCGTGGA AACCACTGCC GCCCCTCCCC GATGTGGTGC GCTCAATAAA AATGCTGGAA
1381 TTCAAAAAAA
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(Seq ID No. 2)

HPV16 E6 Interacts with and Does Not Degrade TIP-1 and TIP-1 Binds to GEF16. Yeast two-hybrid screening with the HPV16 E6 protein as bait identified the HTLV1 Tax interacting protein TIP-1 (Accession No. NM_014604) as a binding partner. TIP-1 has a class 1 PDZ domain (12) and further screening using the TIP-1 protein as bait identified the guanine exchange factor 16 (GEF16, Accession No. BC051838)) as a TIP-1 binding protein. Fig. 1A shows yeast, mating assays carried out between E6 and TIP-1 and TIP-1 and GEF16, which confirm both of these interactions in yeast. Fig. 1B shows the C-terminal PDZ domain binding site consensus (13) for previously identified TIP-1 binding partners (4, 14-16) in addition to our observations with HPV16 E6, GEF16 and HPV18 E6 (13).

Fig. 1C shows a Northern blot of HPV negative human C33A cervical carcinoma cells stably transfected with the type 16 E6 protein and parent vector (C33A-E6 and C33A-Vector control cell lines (17)) before and after transient transfection with TIP-1 cDNA in pcDNA3.1V5His. This demonstrates that there is no difference in the level of endogenous TIP-1 RNA between C33A-V and C33A-E6 cells and that the transient transfection of TIP-1 produced equivalent levels of ectopically expressed TIP-1 RNA in both cell types. Identical transfected cultures were used for the co-immuneprecipitation of transiently expressed V5 tagged TIP-1 protein (Fig. 1C and 1D). This demonstrates that E6 and TIP-1 interact when co-

expressed in mammalian cells. It can be seen from Fig. 1E and 1F that the E6 protein produces a massive reduction in the level of the mutant p53 protein that is normally highly expressed in C33A cells (18). This clearly demonstrates that, unlike p53, transiently expressed, V5 tagged TIP-1 protein was not subject to E6 mediated degradation when expressed in C33AE6 cells

Expression of E6 Increases the Motility of C33A Tumour Cells. We developed a rapid means of assessing two dimensional cell migration based on the method of Cai et al (11) which has a significant advantage over scratch based assays in that it does not cause cell damage dependent activation. Briefly, cells were plated in a circle under oil, allowed to attach, the oil removed, growth medium applied and the cells allowed to migrate for 18 hours. Fig. 2A shows the C33A-V cells at the beginning and Fig. 2B and 2C after 18 hours. Fig. 2C is a 250x magnification of the cell boundary and demonstrates maintenance of a defined circular edge. Fig. 2D, 2E and 2F show the same experiment carried out with C33A-E6 cells. Clearly these data demonstrate that there is a massive difference in the migratory capability of these cells with C33A-E6 cells demonstrating a less cohesive, more motile phenotype than C33A-V control cells. Fig. 2F is a 400x magnification of the C33A-E6 cell boundary and demonstrates complete disruption of the circular edge. These observations are entirely independent of cell growth rates since both cell types have identical population doubling times (data not shown).

Increased Motility of C33AE6 Cells is Dependent on ROCK Activity. In order to ascertain if the increased motility of C33A-E6 cells was associated with ROCK activation, we carried out the same assay pre-treating cells with the specific ROCK inhibitor Y27632 (10) which completely abrogated the migration of C33A-E6 cells. Fig. 2G and 2I show the extent of migration obtained with Y27632 treated cells after 18 hours. Fig. 2I is a 250x magnification of the cell circle boundary and Fig. 2H is a 400x magnification of Y27632 treated C33A-E6 cells. Since Y27632 did not inhibit cell growth in our system, this indicates that the increased migratory capacity of C33AE6 cells is dependent upon ROCK activity. Interestingly, the Y27632 treated C33AE6 cells also assumed a more compact rounded morphology (Fig. 2H) similar to parental C33A cells (Fig. 2C).

C33AE6 cells have Higher Levels of phosphorylated MLC than C33A cells. Activated ROCK is known to directly phosphorylate myosin light chains (MLC) and to inactivate MLC phosphatase (7). Thus we compared the levels of phosphorylated MLC between C33A-V and C33A-E6 cells. These data (Fig. 4B) demonstrate that C33AE6 cells have higher levels of phosphorylated MLC than C33A-V cells, a result which is consistent with increased ROCK activity in these cells.

Increased Motility of C33AE6 Cells is Dependent on TIP-1 Expression. We carried out the cell motility assay on cells treated 48 hours previously with antisense TIP-1 phosphorothioate oligodeoxynucleotides (ODN's) and a scramble control ODN with the same GC/AT content. Uptake of fluorescence labelled ODN's was confirmed by fluorescence microscopy and Northern blot analysis (Fig. 3A) showed that treatment with antisense TIP-1 ODN reduced the level of TIP-1 transcript to one-sixth that of the scramble ODN treated cultures. Fig. 2J shows that scramble treated C33A-E6 cells were still highly motile whereas antisense TIP-1 treated cells were non-migratory (Fig. 2K). Fig. 2L is a 250x magnification of antisense TIP-1 C33A-E6 cells and demonstrates maintenance of the circle boundary and that the morphology now resembles that of C33A (Fig. 2C) and Y27632 treated C33A-E6 cells (Fig.'s 2G, 2H and 2I). Neither antisense TIP-1 nor scramble ODN's had any effect on the motility of C33A-V cells (Fig. 2 M, N and O).

Comparison of the Levels of SRF Activation in C33A-V and C33A-E6 Cells. In order to compare the level of activated RhoA in C33A-E6 and C33A-V cells we initially analysed the degree of *c-fos* SRE activation in these cells using transient transfection with the reporter vector pSEAP- SRE and a mutant pSEAP-SRE vector that is responsive to SRF and not TCF's (19). Fig. 4A shows the results of these experiments in 10% serum, indicating that similar steady state levels of both TCF and SRF activation were observed for both C33AE6 and C33A control cells. Repeat experiments in 0.5% serum followed by an eight-hour pulse in 10% serum did not alter these findings (data not shown). The high value obtained with the control negative pTAL-SEAP construct in C33AE6 cells is due to E6 directly activating the HSV thymidine kinase pTAL promoter independent of SRE enhancer activity (20).

These results demonstrate that the steady state levels of activated GTP RhoA dependent SRF stimulation are not significantly different between C33A-E6 and C33A-V control cells.

Comparison of the Levels of Activated RhoA in C33A and C33AE6 cells. The SRE SEAP based assay is an indirect means of assessing steady state levels of activated RhoA thus we used the EZ-Detect activated RhoA detection method to directly measure the levels of activated RhoA in C33A-E6 and C33A-V cells. Fig. 4C confirms the results of the SRE assay in that there is no difference in the steady state levels of activated RhoA in these cells. However, prior to isolating active RhoA, pre-treatment of cell lysates with an excess of GDP deactivates and an excess of GTP activates endogenous RhoA. Our data show a reduction in the level of activated RhoA in C33A-V cell lysates treated with GDP whereas no reduction in activated RhoA was observed in GDP treated C33A-E6 lysates. Interestingly no increase in activated RhoA was observed in GTP treated C33A-V lysate whereas GTP treatment of C33A-E6 lysate produced a strong activation of RhoA.

Ectopic Expression of TIP-1 Abrogates Contact Growth Inhibition in NIH/3T3 cells. We stably transfected TIP-1 cDNA into non-transformed NIH/3T3 cells with a proven ability to undergo contact inhibition. Fig. 5D shows a Western blot, which confirms expression of the V5 tagged TIP-1 protein in both polyclonal and monoclonal G418 selected NIH/3T3 cells. Polyclonal TIP-1 and vector transfected NIH/3T3's were inoculated at the same density, grown for nine days and the cells fixed and stained with toluidine blue. Fig. 5A shows the accumulation of large numbers of foci of non-contact inhibited cells in the TIP-1 transfected cells whereas vector control transfected cells had none visible. Surprisingly TIP-1 3T3 polyclonal cells did not form colonies in soft agar colony forming assays.

Y27632 Restores Contact Growth Inhibition to TIP-1 NIH/3T3 Cells. The above experiment was repeated in the presence of Y27632, which completely abrogated the growth of non-contact inhibited foci in TIP-1 expressing cells (Fig. 5A). In order to verify this effect, growth assays were carried out on TIP-1 transfected and vector transfected polyclonal cells plus and minus Y27632 (added at days 3, 6 and 8). After a

total of 10 days in culture TIP-1 transfected 3T3 cells grow to a higher density than vector transfected control cells - an effect which can be prevented by treatment with Y27632 (Fig. 5B). It can be seen that there is little change in the cell density of control vector cells between days 6 and 10 whereas TIP-1 transfected cells continued to expand over this period. Y27632 treatment of TIP-1 cells clearly restores contact growth inhibition to these cells.

Ectopic Expression of TIP-1 Increases the Invasive Potential of NIH/3T3 Cells. Matrigel invasion assays were carried out on TIP-1 transfected polyclonal cells, which clearly demonstrated that TIP-1 3T3's were approximately five times more invasive than control cells. These assays were carried out three times in triplicate and Fig. 5C represents a typical result of this comparison. It can be seen that even though greater numbers of vector control 3T3 cells were plated on the membrane minus matrigel, many more TIP-1 3T3's had migrated through the matrigel.

TIP-1 NIH/3T3 Cells Have Increased Levels of Phosphorylated MLC. A marked up-regulation in the level of phosphorylated MLC was observed in both polyclonal and monoclonal TIP-1 3T3 cells whereas this was undetectable in vector control and parental 3T3 cells (Fig. 5E).

Ectopic Expression of TIP-1 Increases Polyploidy in NIH/3T3 Cells. Several TIP-1 transfected and vector transfected monoclonal 3T3 cell lines were established. These were stained with propidium iodide and analysed by FACS. Fig. 6A shows two profiles of different 3T3 vector control monoclonal cell lines which both have the classical proportions of Go/G1, S and G2/M phase expected for normal NIH/3T3 cells. The TIP-1 clone (clone 10 referred to as TIP10 3T3 cells) shown is typical of other TIP-1 transfected monoclones and has a FACS profile indistinguishable from C33A tumour cells. Interestingly both of these latter profiles are consistent with increased numbers of polyploid cells indicating that constitutive expression of TIP-1 may be contributing to genetic instability in these cells. Fig. 6B, 6C and 6D are toluidine blue and propidium iodide stained TIP-1 3T3 cells viewed by fluorescence and phase microscopy which show gross nuclear abnormalities of number, size and shape (arrowed) compared to vector transfected cells (Fig. 6F). Fig. 6E shows C33A

tumour cells with abnormal nuclei clearly visible. Figure 7A & B shows that the development of abnormal nuclei in TIP10 3T3 cells is inhibited by treatment of cells with Y27632. The FACS profile (Figure 7C) demonstrates that Y27632 treatment of TIP10 3T3 cells produces a profile indistinguishable from normal 3T3 cells (Figure 6 A).

Expression of TIP-1 mRNA is Increased in a Variety of Human Tumours. We probed a matched pair human tumour array with labelled TIP-1 cDNA. Fig. 8A clearly shows that the expression of Tip-1 RNA is up-regulated in the majority of breast carcinomas and some from uterus, lung, ovarian and thyroid when compared to normal tissue from the same patient. Fig. 7B demonstrates the uniform signal obtained with a probe made from a selected housekeeping gene.

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CLAIMS

1. The use of an agent that reduces cellular levels of TIP-1 protein or acts to inhibit, either directly or indirectly, the function or effects of Tip-1 in the manufacture of a medicament for the treatment or prevention of conditions characterised by tissue damage.

2. The use according to claim 1 wherein the agent reduces synthesis of TIP-1.

3. The use according to claim 2 wherein the agent is a ribozyme or an antisense oligonucleotide against TIP-1.

4. The use according to claim 1 wherein the agent inactivates Tip-1 activity or increases the rate of metabolism or degradation of TIP-1.

5. The use according to claim 4 wherein the agent is a neutralising antibody raised against TIP-1, an antibody fragment, an intrabody or a small molecule antagonist of TIP-1.

6. The use according to claim 1wherein the agent is an inhibitor of GTP RhoA or RhoA Kinase.

7. The use according to claim 6 wherein the agent is Y27632.

8. The use according to any preceding claim wherein the condition is caused by physical, chemical or viral damage.

9. The use according to any one of claims 1 – 7 wherein the condition is associated with aging.

10. The use according to any preceding claim wherein the condition is characterised by polyploidy.

11. The use according to any preceding claim wherein the condition is cancer.

12. The use according to claim 11 wherein the cancer is ovarian carcinoma, breast carcinoma, lung carcinoma, uterine carcinoma, cervical carcinoma or thyroid carcinoma.

13. The use according to claim 11 wherein the cancer is metastatic cancer.

14. The use according to any one of claims 11 - 13 wherein the cancer is caused by a oncogenic virus.

15. The use according to claim 14 wherein the virus is a human papilloma virus (HPV) or human T-cell leukaemia virus type-I (HTLV-I).

16. A delivery system for use in a gene therapy technique, said delivery system comprising a DNA molecule encoding for a protein which directly or indirectly reduces the activity or effects of TIP-1, said DNA molecule being capable of being transcribed to allow the expression of said protein and thereby treat cancer.

17. A method for the diagnosis of pre-cancer and cancer comprising analysing a cell sample from a patient for the presence of elevated levels of TIP-1 mRNA or protein or the presence of mRNA or protein for TIP-1 induced proteins.

18. The method according to claim 17 wherein the TIP-1 induced protein is selected from Rhotekin, GTPRhoA, ROCK, Myosin Light Chain (MLC) Kinase.

19. The method according to claim 17 wherein the sample is assayed for over-expression of TIP-1 RNA or protein.

20. The method according to claim 17 wherein specific binding agents are used in a immuno-cytochemistry, immuno-precipitation or immuno-blotting (Western blotting) technique.

21. A method of screening a compound to test whether or not the compound has efficacy for treating cancer, comprising:

(i) administering to living cells or a subject a test compound for a predetermined length of time;

(ii) detecting the activity or expression of TIP-1 or TIP-1 induced proteins or mRNA from the cells or subject; and

(iii) comparing the activity or expression of TIP-1 or Tip-1 induced proteins or mRNA in the cells or the subject treated with the compound relative to activity or expression found in control cells or a control subject that were not treated with the compound;

wherein compounds with efficacy for treating cancer decrease activity or decrease expression of TIP-1 or TIP-1 induced proteins or mRNA relative to the controls.

22. The method according to claim 21 wherein activity of TIP-1 or Tip-1 induced proteins is measured and the activity is assayed by monitoring the phosphorylation state of Myosin Light Chains or Actin and decreased phosphorylation suggests the screened compound has efficacy for treating cancer.

23. The method according to claim 21 wherein activity of TIP-1 or Tip-1 induced proteins is measured and the activity is assayed by monitoring the proportion of polyploid cells and decreased polyploidy suggests the screened compound has efficacy for treating cancer.

24. The method according to claims 21 wherein activity of TIP-1 or TIP-1 induced proteins or mRNA is assessed and that activity is based upon an assessment of cell motility regulated by TIP-1.

25. The method according to claim 24 wherein E6 expressing tumour cells are used; the cells plated in oil and allowed to attach to the plate; the oil replaced with growth medium and the cells allowed to grow freely for a defined period of time in the presence or absence of the test compound wherein a reduction in the development

of projections from the E6 expressing cells or a reduction in motility of the cells indicates the compound has efficacy for treating cancer.

26. An anti-cancer agent or agent for preventing metastasis identified according to the method of any one of claim 21 - 25.

27. A method of screening a compound, to test whether or not the compound causes cancer, comprising:

(i) administering to living cells or a subject a test compound for a predetermined length of time;

(ii) detecting the activity or expression of TIP-1 or TIP-1 induced proteins or mRNA from the cells or subject; and

(iii) comparing the activity or expression of TIP-1 or TIP-1 induced proteins or mRNA's in the cells or the subject treated with the compound relative to activity or expression in control cells or a control subject that were not treated with the compound;

wherein compounds that cause cancer increase activity or increase expression relative to the controls.

28. The method according to claim 27 wherein activity of TIP-1 or Tip-1 induced proteins is measured and the activity is assayed by monitoring the phosphorylation state of Myosin Light Chains or Actin and increased phosphorylation suggests the screened compound causes cancer.

29. The method according to claim 27 wherein activity of TIP-1 or Tip-1 induced proteins is measured and the activity is assayed by monitoring the proportion of polyploid cells and increased polyploidy suggests the screened compound causes cancer.

30. The methods according to claims 27 wherein activity of TIP-1 is assessed and that activity is based upon an assessment of cell motility regulated by TIP-1.

31. The method according to claim 30 wherein non E6 expressing tumour cells are used; the cells plated in oil and allowed to attach to the plate; the oil replaced with growth medium and the cells allowed to grow freely for a defined period of time in the presence or absence of the test compound wherein an increase in the development of projections from non E6 expressing cells or an increase in motility of the cells that is consistent with that observed for E6 expressing cells indicates the compound is involved in the cause or progression of cancer.

32. A method according to any one of claims 16 – 31 adapted such that a compound is screened to evaluate its ability to modulate TIP-1 activity.

33. The method according to claim 32 wherein a compound that augments TIP-1 activity is identified as causing cancer.

34. The method according to claim 32 wherein a compound that inhibits TIP-1 activity is identified as being useful for preventing or treating cancer

35. A method of screening a compound to test whether or not the compound has efficacy for treating cancer, comprising:

(i) plating E6 expressing tumour cells in oil and allowing the cells to attach to the plate;

(ii) replacing the oil with growth medium and allowing the cells to grow freely for a defined period of time in the presence or absence of a test compound; and

(iii) evaluating the development of projections from the E6 expressing cells and motility of the cells;

wherein a decrease in the development of projections from the E6 expressing cells or a decrease in motility of the cells indicates the compound has efficacy for treating cancer.

36. A method of screening a compound, to test whether or not the compound causes cancer, comprising:

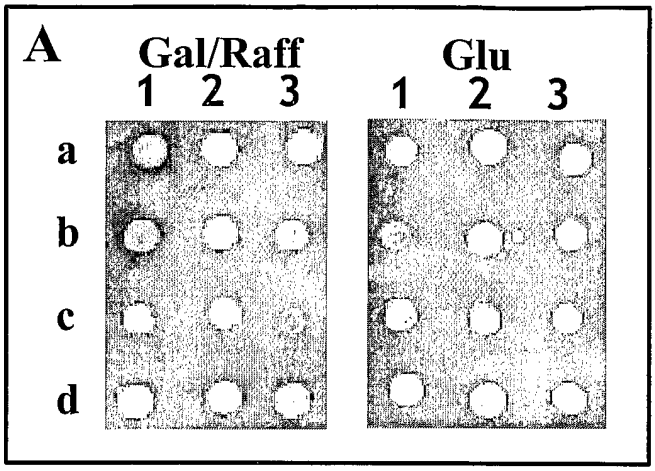
(i) plating non E6 expressing tumour cells in oil and allowing the cells to attach to the plate;

(ii) replacing the oil with growth medium and allowing the cells to grow freely for a defined period of time in the presence or absence of a test compound; and

(iii) evaluating the development of projections from the non E6 expressing cells and motility of the cells;

wherein an increase in the development of projections from the non E6 expressing cells or an increase in motility of such cells are consistent with that observed for E6 expressing cells, indicates the compound is involved in the cause or progression of cancer.

Fig. 1



B

Consensus PDZ				X	S/T	X	V/L
Gef16	L	R	V	E	T	D	V
HPV16 E6	T	R	R	E	T	Q	L
HPV18 E6	R	R	R	E	T	Q	V
HTLV1 Tax	H	F	R	E	T	E	V
L-Glutaminase	E	N	L	E	S	M	V
Rhotekin	T	W	L	Q	S	P	V
B-Catenin	A	W	F	D	T	D	L

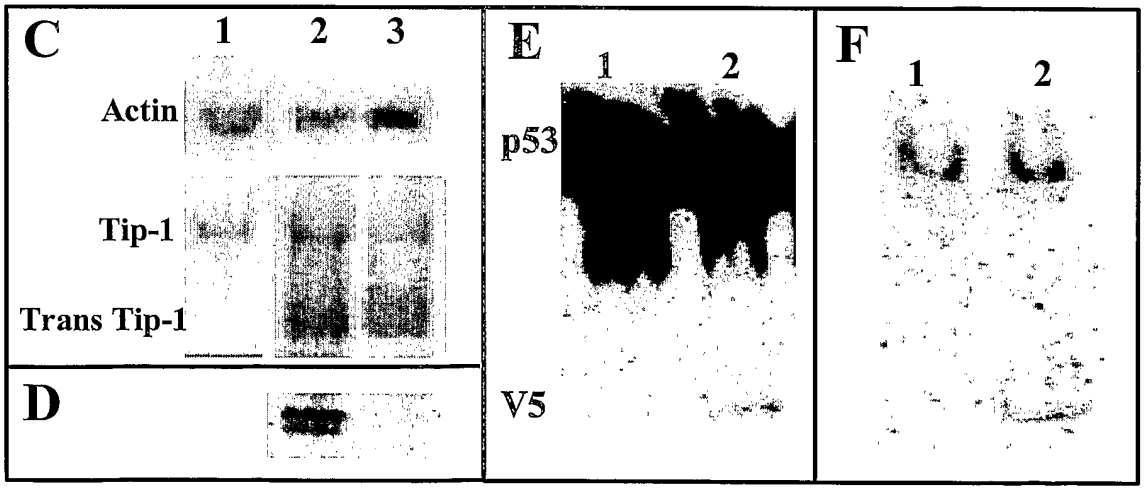


Fig. 2

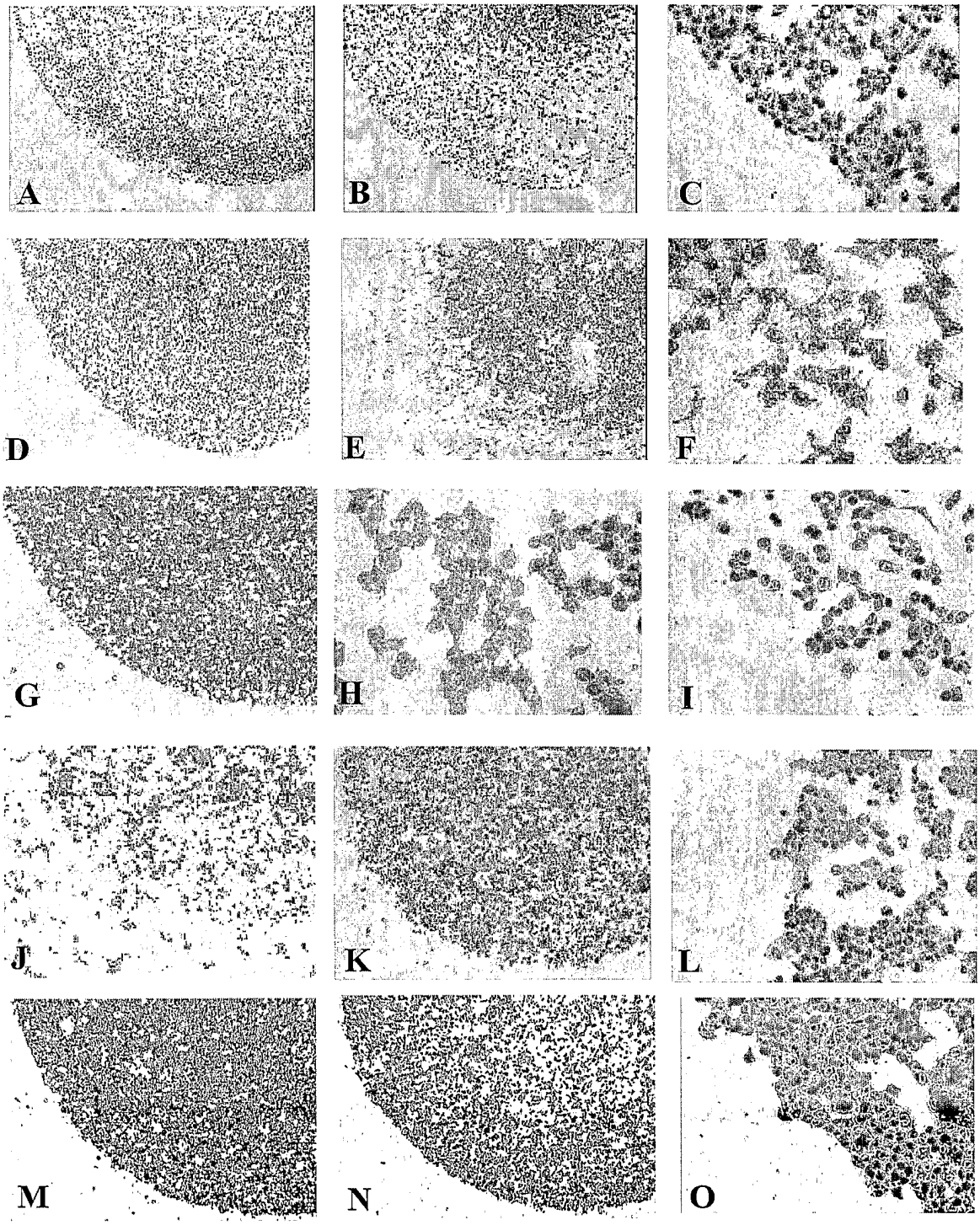


Fig. 3

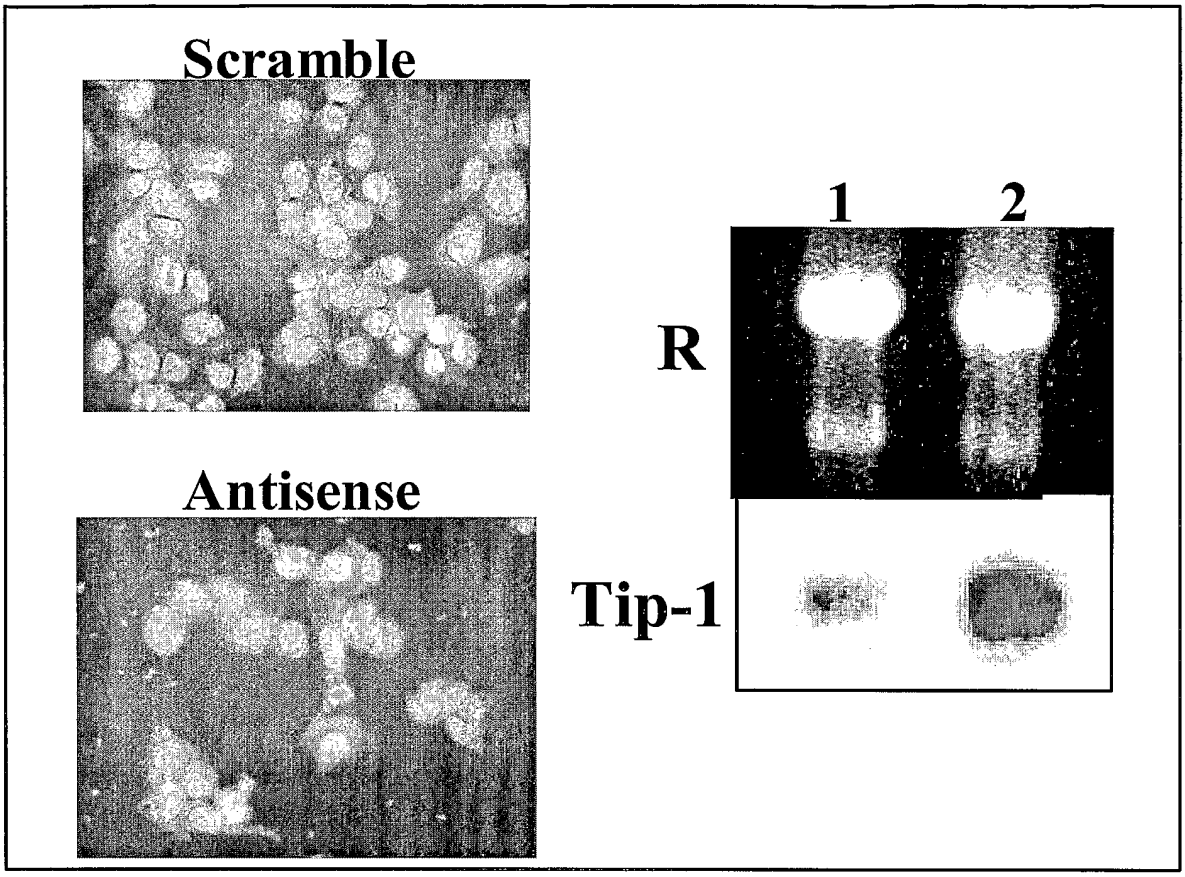


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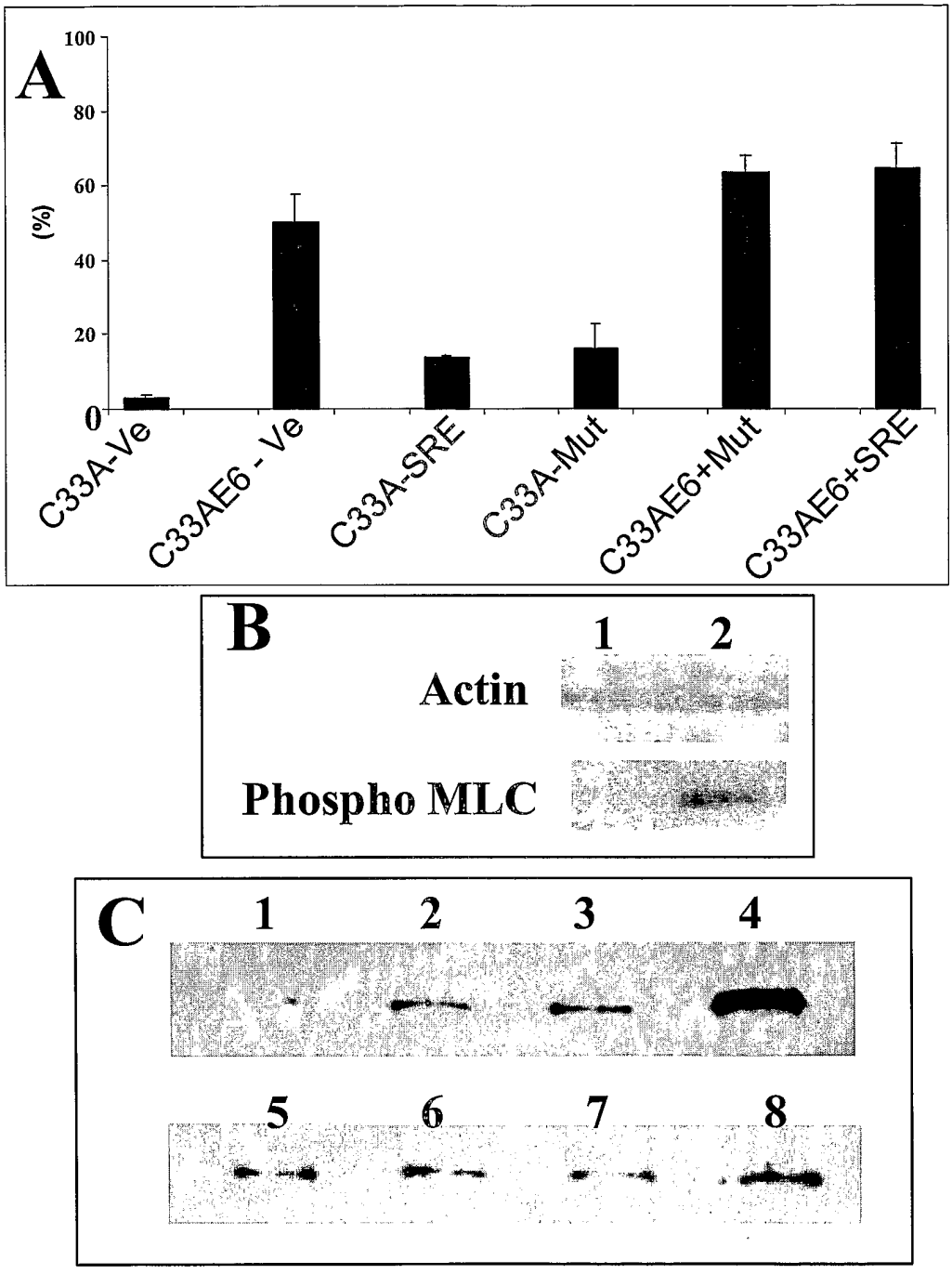

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60
40
20
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(%)
C33A-Ve
C33AE6 - Ve
C33A-SRE
C33A-Mut
C33AE6+Mut
C33AE6+SRE
B
1
2
Actin
Phospho MLC
C
1
2
3
4
5
6
7
8

Fig. 5

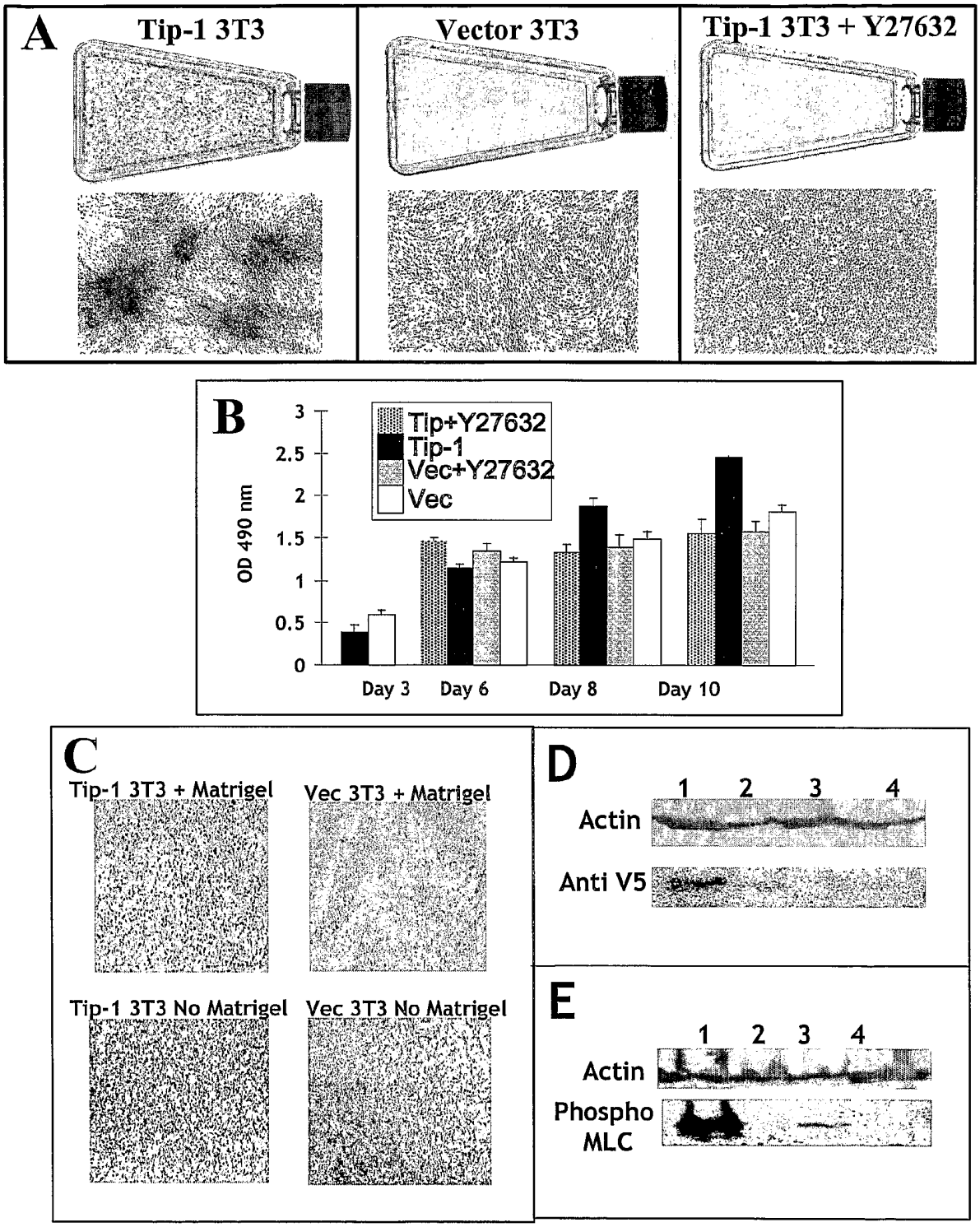

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Vector 3T3
Tip-1 3T3 + Y27632
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Tip+Y27632
Tip-1
Vec+Y27632
Vec
OD 490 nm
3
2.5
2
1.5
1
0.5
0
Day 3
Day 6
Day 8
Day 10
C
Tip-1 3T3 + Matrigel
Vec 3T3 + Matrigel
Tip-1 3T3 No Matrigel
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Actin
Anti V5
E
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Actin
Phospho MLC

Fig. 6

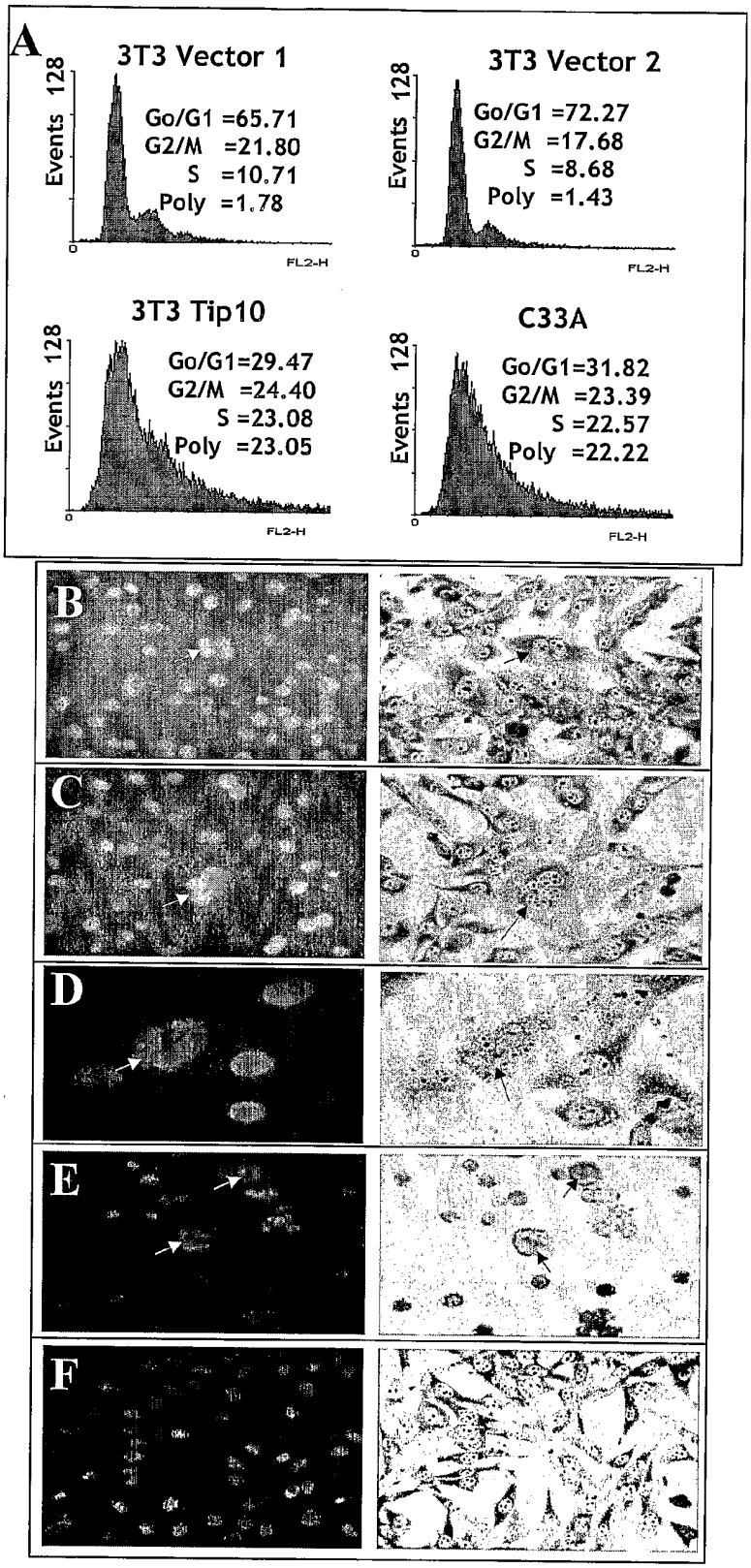

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S =10.71
Poly =1.78
0
FL2-H
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Events 128
Go/G1 =72.27
G2/M =17.68
S =8.68
Poly =1.43
0
FL2-H
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Poly =23.05
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Fig. 7

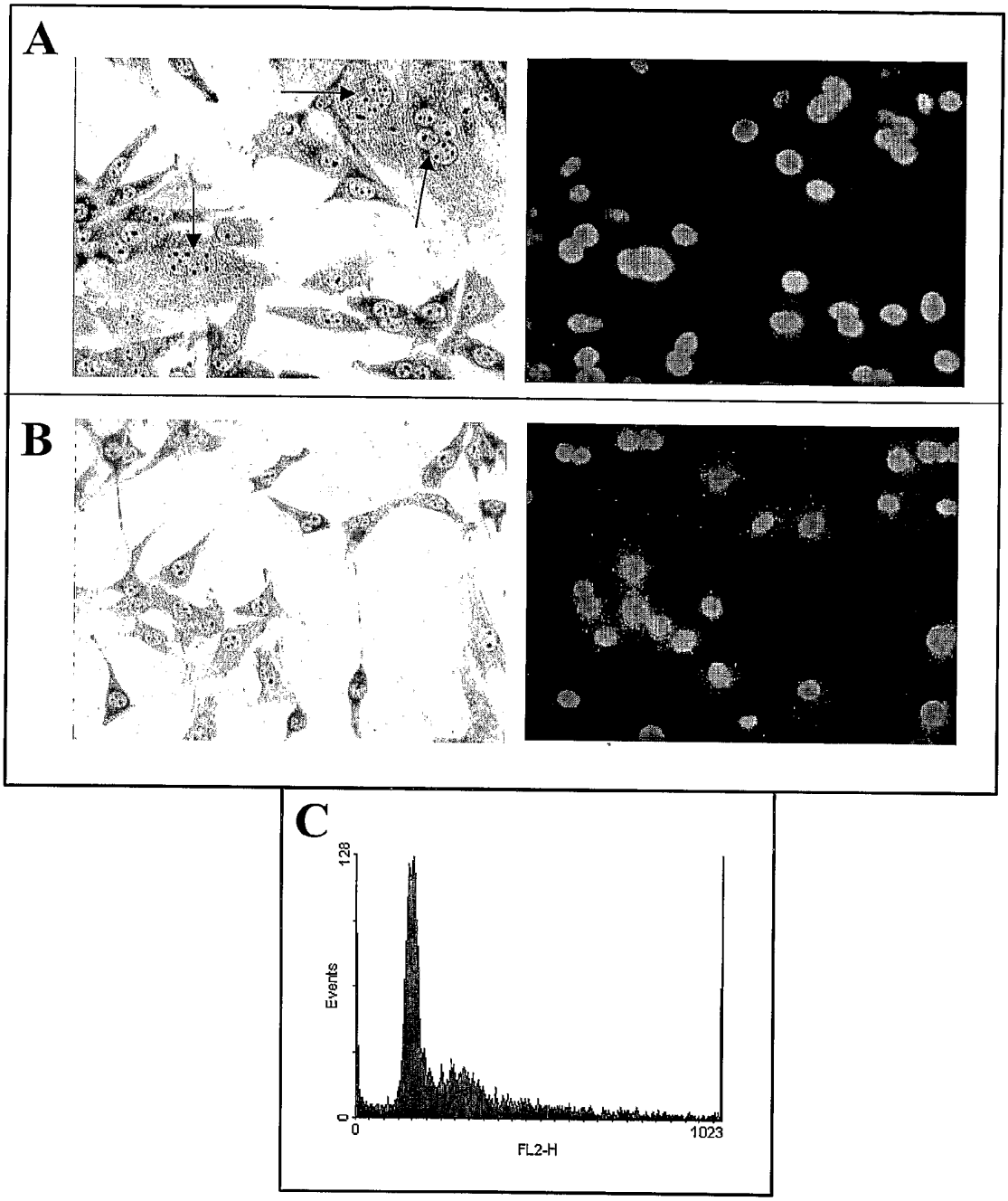


Fig. 8

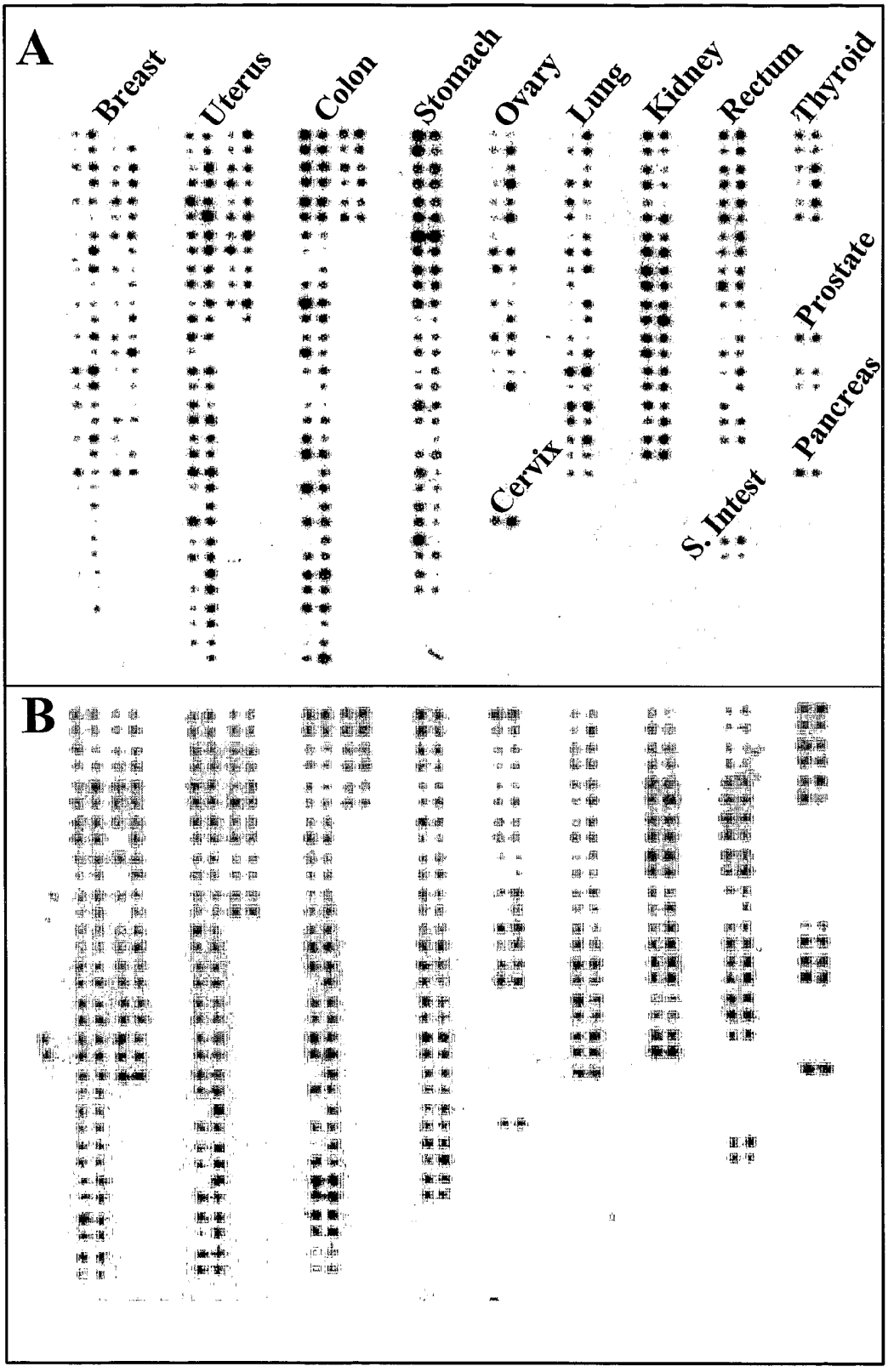
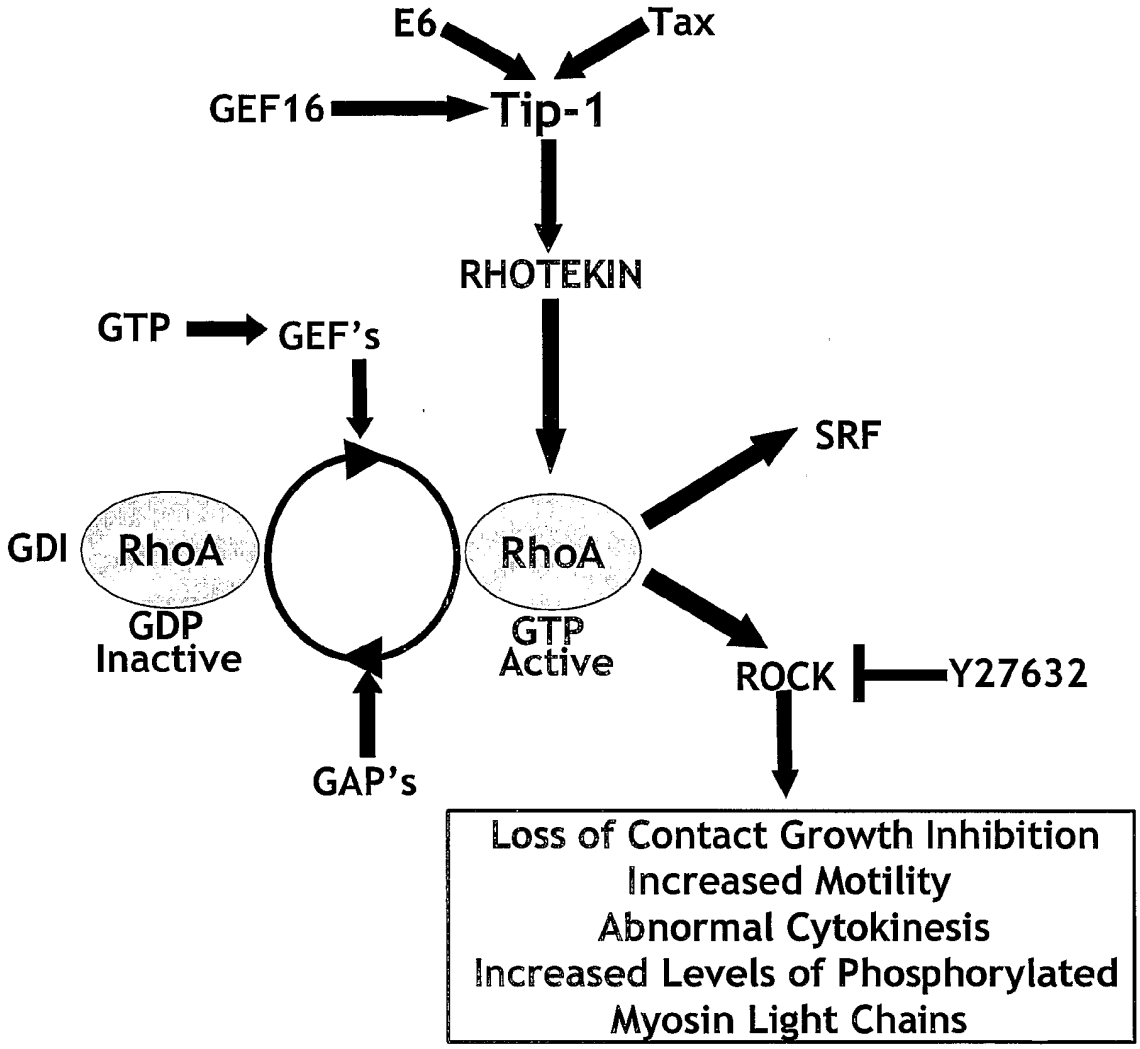

A
Breast
Uterus
Colon
Stomach
Ovary
Lung
Kidney
Rectum
Thyroid
Prostate
Pancreas
Cervix
S. Intest
B

Fig.9


E6
Tax
GEF16
Tip-1
RHOTEKIN
GTP
GEF's
SRF
GDI
RhoA
GDP
Inactive
RhoA
GTP
Active
ROCK
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GAP's
Loss of Contact Growth Inhibition
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89411.ST25
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