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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING CELL MIGRATION

(57) Abstract: A method of inducing cell migration is provided. The method comprising contacting the cell with: (a) an agent capable of upregulating activity and/or expression of cten; and (b) an agent capable of downregulating activity and/or expression of tensin-1 or tensin-3, thereby inducing cell migration.



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COMPOSITIONS AND METHODS FOR MODULATING CELL MIGRATION

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to novel compositions and methods for modulating cell migration.

Cell migration depends on the coordinated execution and integration of complex individual processes. In its simplest form, movement requires that a cell generates and maintains a state of asymmetry or polarity. Once polarized, a cell must execute a four-step cycle to migrate or translocate.

First, a cell must extend a process, known as the leading edge, in the direction of movement. During this step, increased actin polymerization is seen in the area of the leading edge. This increased polymerization arises from the creation of new barbed ends that are oriented towards the membrane, either by nucleation of new filaments from pools of G-actin or by severing or uncapping of existing filaments. Actin monomers are added onto barbed ends until they are capped. The combination of actin nucleation and filament elongation is thought to play a critical role in the protrusion of the leading edge.

Second, once a cell has extended a process, it must form semi-stable points of attachment with the underlying substratum to serve as anchor points. One class of attachment points, focal adhesions, contain aggregates of integrin receptors and a variety of cytosolic signaling and cytoskeletal proteins and serve as sites of bidirectional signaling between the extracellular matrix and the actin cytoskeleton. Although attachment of newly extended processes may be critical for cell translocation, process extension itself does not require adhesion.

Third, once a cell has extended and anchored a new process, it must slide the cell body forward by traction.

The fourth step is release of points of substratum attachment at the rear of the cell.

Cell migration plays a central role in a wide variety of biological phenomena including tumor metastasis. In this process, tumor cells migrate from the initial tumor mass throughout the whole body. Directed tumor cell motility by chemotaxis is the final step of tumor invasion, and the modulation, e.g., inhibition of this process has been a major focus of research.

Cancer progression is associated with changes in cell shape as well as cell migration. For example, well organized epithelial sheets lose their polarization and compact packing during the transition into invasive carcinoma. These changes are critically dependent on re-organization of the actin cytoskeleton.

EGFR is involved in regulation of various cellular processes, including proliferation and motility, and constitutive receptor activation contributes to transition from a localized primary tumor mass into invasive secondary metastases. Binding of EGF to its receptor results in receptor dimerization, activation of the tyrosine kinase activity, and phosphorylation of specific tyrosine residues on the EGFR cytoplasmic tail as well as its downstream substrates. The phosphorylated residues on EGFR in turn serve as docking sites for Src homology 2 (SH2) domain-containing proteins forming signaling complexes. Some substrates do not appear to interact with the receptor itself, but they are activated in a receptor-dependent manner.

One group of EGFR substrates is localized to focal adhesions, the transmembrane junctions between the extracellular matrix and the actin cytoskeleton. These focal adhesion molecules include focal adhesion kinase (Fak), paxillin, and p130Cas.

The tensins are a family of focal adhesion molecules, currently comprising four members - tensin1, tensin2, tensin3 and COOH-terminal tensin-like protein (cten) [Lo et al., Cancer Research 62, 4217-4221, August 1, 2002]. Tensin proteins are composed of three regions with different structural roles: the NH₂-terminal (which comprises the actin binding site) middle, and the COOH-terminal regions which comprises the SH2 and PTB domains. Through the PTB domain, tensins interact with the cytoplasmic tail of β integrin, a cell adhesion molecule, whereas the SH2 domain mediates interactions with tyrosine-phosphorylated proteins, which participate in adhesion complexes. The NH₂ and COOH termini are conserved between tensin1, tensin2 and tensin 3, whereas the middle regions are divergent. Cten does not contain the NH₂-terminal region and therefore does not bind to actin cytoskeleton.

Recent studies have shown that expression of tensin1 and tensin 2 promotes cell migration [Chen et al., Proc. Natl. Acad. Sci. USA, 99: 733-738, 2002]. Furthermore, analysis of tensin1 knockout mice has demonstrated a critical role of tensin1 in renal function and wound healing [Lo S. H., et al., J. Cell Biol., 136: 1349-1361, 1997; Ishii A., Lo S. H. Biochem. J., 356: 737-745, 2001]. Tensin 3 has been proposed to function as a platform for the disassembly of EGF-related signaling complexes at focal adhesions [Yumin Cui et al., Mol Cancer Res 2004;2(4):225-32].

Lo and Lo [Cancer Research, 2002;62 (15): 4217-21] teach cten as a negative marker for prostate cancer.

Sasaki et al [Lung Cancer (2003) 40: 151-5] teach cten as a positive marker for tumor progression in lung cancer.

U.S. Pat. Appl. No. 20060019256 teaches solid tumor stem cells cancer markers. Included in their list of markers is Cten.

Although U.S. Pat. Appl. No. 20060019256 suggests alterations of these markers for therapeutic targeting, there is no evidence that regulation of these markers may be used for treating cancer as there is no basis to elucidate whether such changes in gene expression are causative or resultant of the tumorigenic phenotype. Indeed no particular agents were proposed and therapeutic efficacy was not shown.

There is thus a widely recognized need for, and it would be highly advantageous to have, compositions and methods for modulating cell migration and as such can be used for treating cancer which are devoid of the above limitation.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of inducing cell migration, the comprising contacting the cell with: (a) an agent capable of upregulating activity and/or expression of cten; and (b) an agent capable of downregulating activity and/or expression of tensin-1 or tensin-3, thereby inducing cell migration.

According to another aspect of the present invention there is provided a method of inhibiting cell migration, the method comprising contacting the cell with: (a) an agent capable of downregulating activity and/or expression of cten; and (b) an agent capable of upregulating activity and/or expression of tensin-1 or tensin-3, thereby inhibiting cell migration.

According to yet another aspect of the present invention there is provided a method of treating an EGF-responsive tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of cten in cells of the EGF-responsive tumor, thereby treating the EGF-responsive tumor in the subject.

According to still another aspect of the present invention there is provided a method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of: (i) an agent capable of down-regulating activity and/or expression of cten in cells of the tumor; and (ii) an agent capable of up-regulating activity and/or expression of tensin-3 or tensin-1 in cells of the tumor, thereby treating the tumor in the subject.

According to an additional aspect of the present invention there is provided a method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a nucleic acid agent which comprises SEQ ID

NO: 14 or 15, the nucleic acid agent being capable of down regulating expression of cten in cells of the tumor, thereby treating the tumor in the subject.

According to yet an additional aspect of the present invention there is provided a method of staging a tumor in a subject in need thereof, the method comprising determining a level of cten and tensin-3 in cells of the tumor of the subject, wherein an upregulation of cten above a predetermined threshold and downregulation of tensin-3 below a predetermined threshold are indicative of the stage of the tumor.

According to still an additional aspect of the present invention there is provided a method of monitoring treatment of a tumor in a subject in need thereof, the method comprising determining a level of cten and tensin-3 in cells of the tumor of the subject, the subject being treated with an anticancer therapy, wherein downregulation of cten and upregulation of tensin-3 in the cells is indicative of responsiveness of the subject to the anticancer therapy.

According to a further aspect of the present invention there is provided a method of identifying an EGF-responsive tumor, the method comprising determining a level of cten and tensin-3 in cells of the tumor, wherein an upregulation of cten above a predetermined threshold and downregulation of tensin-3 below a predetermined threshold are indicative of an EGF-responsive tumor.

According to yet a further aspect of the present invention there is provided a method of determining an anti-cancer treatment regimen, the method comprising: (a) determining a level of cten and tensin-3 in cells of a tumor of the subject (b) selecting the anticancer treatment based on the level, thereby determining the anti-cancer treatment regimen.

According to still further features in the described preferred embodiments the anticancer therapy is EGFR-directed.

According to still a further aspect of the present invention there is provided a method of diagnosing an ovarian or breast cancer in a subject in need thereof, the method comprising determining a level of cten in cells of the subject, wherein an upregulation of cten above a predetermined threshold is indicative of an ovarian or breast cancer.

According to still further features in the described preferred embodiments the tumor is an EGF-responsive tumor.

According to still a further aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence as set forth in SEQ ID NO: 14.

According to still a further aspect of the present invention there is provided an isolated polynucleotide comprising a nucleic acid sequence as set forth in SEQ ID NO: 15.

According to still a further aspect of the present invention there is provided a pharmaceutical composition comprising as an active ingredient the isolated polynucleotide of claim 13 or 14.

According to still a further aspect of the present invention there is provided an isolated monoclonal antibody comprising an antigen binding domain capable of specifically binding cten and not tensin-3.

According to still further features in the described preferred embodiments the isolated antibody is humanized.

According to still further features in the described preferred embodiments, the method further comprising administering to the subject a therapeutically effective amount an agent capable of up-regulating activity and/or expression of tensin-3 in the cells.

According to still further features in the described preferred embodiments the EGF-responsive tumor is not breast cancer.

According to still further features in the described preferred embodiments the EGF-responsive tumor is selected from the group consisting of head and neck cancer, glioblastoma and lung cancer.

According to still further features in the described preferred embodiments the agent capable of down-regulating activity and/or expression of cten is a nucleic acid agent.

According to still further features in the described preferred embodiments the nucleic acid agent is selected from the group consisting of:

- (a) a single stranded polynucleotide for inhibiting the transcription of cten;
- (b) a single stranded polynucleotide for hybridizing to the cten mRNA thereby leading to a reduction in cten activity;
- (c) a double stranded polynucleotide, leading to degradation of cten mRNA;
- (d) a triplex forming polynucleotide for cleaving cten mRNA;
- (e) a catalytic polynucleotide for cleaving cten mRNA;
- (f) a single stranded polynucleotide for hybridizing to cten mRNA leading to enzymatic degradation thereof; and
- (g) nucleic acid sequences coding for any one of (a) to (f).

According to still further features in the described preferred embodiments the nucleic acid agent is selected from the group consisting of an siRNA, a ribozyme and a DNazyme.

According to still further features in the described preferred embodiments the nucleic acid agent is a siRNA.

According to still further features in the described preferred embodiments the nucleic acid agent comprises SEQ ID NO: 14 or 15.

According to still further features in the described preferred embodiments the agent capable of up-regulating activity and/or expression of tensin-3 is as set forth in SEQ ID NO: 3 or 4

According to still further features in the described preferred embodiments the agent capable of up-regulating activity and/or expression of cten is as set forth in SEQ ID NO: 1 or 2.

According to still further features in the described preferred embodiments the agent capable of downregulating activity and/or expression of tensin-3 is as set forth in SEQ ID NO: 16.

According to still further features in the described preferred embodiments the administering is effected in situ.

According to still further features in the described preferred embodiments the determining is effected ex vivo.

According to still further features in the described preferred embodiments the determining is effected in situ.

According to still a further aspect of the present invention there is provided a method of identifying an agent capable of modulating cell migration, the method comprising: (a) treating a plurality of cells with a plurality of agents; and (b) identifying at least one cell of the plurality of treated cells exhibiting an alteration in an expression ratio of cten to tensin 3 relatively to the ratio in untreated cells, the alteration being indicative of an agent capable of modulating cell migration.

According to a further aspect of the present invention there is provided a method of inducing cell migration, the comprising contacting the cell with: an agent capable of downregulating activity and/or expression of tensin-1 or tensin-3,

thereby inducing cell migration.

According to a further aspect of the present invention there is provided a method of inhibiting cell migration, the method comprising contacting the cell with: an agent capable of upregulating activity and/or expression of tensin-1 or tensin-3,

thereby inhibiting cell migration.

According to a further aspect of the present invention there is provided a method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of: an agent capable of up-regulating activity and/or expression of tensin-3 or tensin-1 in cells of the tumor,

thereby treating the tumor in the subject.

The present invention successfully addresses the shortcomings of the presently known configurations by providing compositions and methods for modulating cell migration.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-e show EGF-dependent downregulation of tensin3, along with up-regulation and reorganization of cten. Figure 1a - HeLa cells were stimulated with EGF (20 ng/ml) for the indicated time intervals and RNA analyzed using oligonucleotide arrays. MAS5 expression values of the four tensin family members, at the indicated time points, are presented. Figure 1b - Serum-starved MCF10A cells were treated without or with EGF for the indicated time intervals. Cells were then lysed and subjected to immunoblot analysis. Figure 1c - Serum-starved MCF10A cells were incubated for 12 hours with EGF (10 ng/ml) containing minimal medium supplemented without or with the following inhibitors: U0126 (5 μ M; an Mek inhibitor), GF109203 (3 μ M; a protein kinase C inhibitor) and SB202190 (10 μ M; a p38 inhibitor). For control, cells were incubated without the addition of EGF (-). The effect of the different inhibitors on EGF dependent cten expression is shown. Figure 1d - MCF10A cells were treated as in Figure 1b. An anti-tensin3, or an anti-Erk antibody were used for immunoblotting. Figure 1e - Serum-starved MCF10A cells, were incubated without

(*Control*) or with EGF for 24 hours. Thereafter, immunofluorescent analysis was performed using a rabbit anti-cten antibody (green), TRITC-conjugated phalloidin (red) and a mouse anti-paxillin monoclonal antibody (blue). An overlay of the three fluorescent signals, generating white color in areas of co-localization, is shown in the right column. Enlargement of plasma membrane regions are presented in the inner boxes. *Bar*, 10 μ m.

FIGs. 2a-d illustrate an EGF effect on transcription of migration-related genes, including cten. Figure 2a-b - HeLa cells were stimulated with EGF (20 ng/ml) for the indicated time intervals and RNA analyzed using oligonucleotide arrays. Array values were standardized by subtracting the mean expression of each gene from its MAS5 expression values at the indicated time points, and the result divided by its standard deviation. Shown are standardized expression patterns of a selected group of motility related genes (presented as gene symbols). Both up- (Figure 2a) and down- (Figure 2b) regulated genes are presented. Figure 2c - HeLa cells were serum starved for 24 hours, and then incubated without or with EGF (20 ng/ml) for the indicated times. Cells were then lysed and subjected to immunoblot analysis using a mouse anti-cten monoclonal antibody, or an anti-Erk2 antibody, as a protein loading control. Figure 2d - Serum-starved HeLa cells were trypsinized and re-plated in minimal medium, without or with an inhibitory anti-EGFR antibody (40 μ g/ml), for the indicated time intervals. For control, cells were incubated without or with EGF (20ng/ml), in the presence or absence of the inhibitory antibody, for 24 hours. Whole cell extracts were subjected to immunoblotting (IB) with the indicated rabbit antibodies.

FIGs. 3a-c illustrate EGF-induced MCF10A cell migration and stress fiber disassembly. Figure 3a - MCF10A cell migration was analyzed by using the Transwell migration assay following incubation with either serum containing full medium without EGF, EGF- and serum-containing full medium, or with minimal medium (*control*). Representative photomicrographs and quantification of migrating cells are presented. Figure 3b - Serum-starved MCF10A cells were stimulated with EGF (10 ng/ml) for the indicated time intervals. Thereafter, cells were lysed using Triton X-100-based solubilization buffer and the supernatant was collected (*TX-100 Soluble*). Triton X-100 insoluble cell-pellets were dissolved by boiling the samples for 5 minutes in a buffer containing 1% SDS (*TX-100 Insoluble*). Cell lysates were further subjected to immunoblot analysis with the indicated antibodies. Figure 3c - Serum-starved MCF10A cells, were incubated without (*Control*) or with EGF for 24 hours. Thereafter, immunofluorescent analysis was performed using a rabbit anti-tensin3 antibody (green), TRITC-conjugated phalloidin (red) and a mouse anti-paxillin monoclonal antibody (blue). An overlay of the three fluorescence signals, generating white color in areas of co-localization, is shown in the right column. Enlargement

of plasma membrane regions of cells stained for tensin3 and paxillin are presented (inserts).

Bar, 10 μ m.

FIGs. 4a-f shows inhibition of mammary cell migration upon knockdown of cten and increase of same following tensin3 knockdown. Figure 4a - MCF10A cells were transfected with an siRNA oligonucleotide directed to cten (siCTEN), a control siRNA oligonucleotide (siCont), or siRNA oligonucleotides directed to tensin3 (siTNS3), and incubated in serum-containing full medium, with (left panel) or without EGF (right panel). Thereafter, whole cell extracts were prepared and subjected to immunoblot analysis. Figure 4b - MCF10A cells were transfected with the indicated siRNA oligonucleotides and then incubated for 48 hours in serum-containing medium, without or with EGF. Images were obtained using a digital camera. *Bar*, 500 μ m. Figure 4c - MCF10A cells were transfected with the indicated siRNA oligonucleotides and grown in full medium with EGF (top two rows) or without EGF (bottom row), prior to immunofluorescence analysis. The fluorescent signals of cten (green) and actin (red) are shown, along with an overlay (right column). *Bar*, 10 μ m. Figure 4d - MCF10A cells transfected with the indicated siRNA oligonucleotides were incubated in Transwell chambers in serum-containing medium, without or with EGF, and their migration measured and normalized to cells transfected with control siRNA. Shown are average values ($\pm$ s.d.; bars). Figure 4e - MCF10A cells were infected with shRNA-tensin3 or with shRNA-control lentiviruses. Thereafter, cells that stably incorporated the shRNA were selected with puromycin, and grown in serum-containing medium, without or with EGF, prior to fixation and staining with TRITC-phalloidin. *Bar*, 10 μ m. Figure 4f - MCF10A cells transfected with the indicated siRNA oligonucleotides were plated in Transwell plates containing full medium without or with EGF. Cell migration was quantified 24 hours later as in Figure 4d. Shown are average values ($\pm$ s.d.; asterisks indicate $p < 0.005$, Student t-test).

FIGs. 5a-e illustrate that Cten expression disrupts actin stress fiber organization. Figure 5a - A schematic representation of GFP fusion constructs of cten, Δ PTB-cten, tensin1 and tensin1's carboxyl-terminus. ABD, actin-binding domain. Figures 5b-e - MCF10A cells expressing the indicated GFP fusion proteins were immunostained with TRITC-phalloidin. The fluorescent signals of GFP (green) and actin (red) are shown, along with a merge column (right). *Bar*, 10 μ m.

FIGs. 6a-d show that integrin beta-1 is essential for EGF-induced motility. Figure 6a - MCF10A cells were plated in a Transwell chamber in full medium supplemented with the following antibodies (each at 10 μ g/ml): Inhibitory anti-integrin β 1 (P5D2; R&D Systems), inhibitory anti-integrin β 4 (ASC-3; Chemicon), and normal mouse IgG for control. Migration

was measured and normalized to cells treated with a control antibody (left panel). MCF10A cells were plated in a 96-well plate in full medium supplemented with the above indicated antibodies (10 μ g/ml). Two hours later, cells were washed, adherent cells were stained with crystal violet and lysed in 10% SDS. Purple color representing the amount of adherent cells was determined using a spectrophotometer, and signals normalized to cells treated with a control antibody. *Bar*, $\pm$ s.d. Figure 6b - The migration (left) or adhesion (right) of MCF10A cells transfected with either control or integrin β 8 siRNA-oligonucleotide, were performed as in Figure 6a. *Bar*, $\pm$ s.d. Figure 6c - MCF10A cells transfected with the indicated siRNA oligonucleotides were grown in serum-containing medium, without EGF, prior to fixation and staining with TRITC-phalloidin. *Bar*, 10 μ m. Figure 6d - Serum-starved MCF10A cells were incubated without EGF for 24 hours. Thereafter, immunofluorescent analysis was performed using a rabbit anti-cten antibody (green), TRITC-conjugated phalloidin (red) or a mouse anti-integrin β 1 monoclonal antibody (blue). An overlay of the three fluorescence signals, generating white color in areas of co-localization, is shown in the right column. Enlargement of plasma membrane regions of cells stained for cten and integrin β 1 are presented in the inner boxes. *Bar*, 10 μ m.

FIGs. 7a-e show that Cten displaces tensin3 from the tail of integrin β 1. Figure 7a - MCF10A cells transfected with the indicated siRNA oligonucleotides were grown in serum-containing medium, without EGF, prior to fixation and staining with a rabbit anti-cten antibody (green) and TRITC-phalloidin (red). An overlay of the two fluorescence signals is shown in the right column. *Bar*, 10 μ m. Figure 7b - Lysates of HEK-293T cells exogenously expressing a GFP protein fused to full-length cten were subjected to a pull-down assay using immobilized forms of integrin α II and β 1 carboxyl-terminal tails. Bound proteins, as well as input mixtures (5%), were resolved by electrophoresis and detected using an anti-GFP antibody. *Ni* indicates proteins adsorbed to naked nickel beads. Figure 7c - Lysates of HEK-293T cells exogenously expressing a GFP protein fused to tensin3 lacking the amino-terminal actin-binding domain (Δ N-tensin3) were treated as in Figure 7b. Figure 7d - An immobilized carboxyl-terminal tail of integrin β 1 was incubated with a mixture of lysates derived from HEK-293T cells transiently expressing either GFP- Δ N-tensin3 or GFP-cten (increasing amounts). Tightly associated proteins were resolved by electrophoresis and detected using an anti-GFP antibody. An asterisk indicates a non-specific protein band. Figure 7e - MCF10A cells transfected with the indicated siRNA oligonucleotides, either alone or in combination, were grown in serum-containing medium with EGF, prior to fixation and staining with TRITC-phalloidin. *Bar*, 10 μ m. The lower row presents schematics showing actin

redistribution in response to knock-down (represented by X) of integrin $\beta 1$ (green), cten (purple), or both.

FIGs. 8a-e show Cten expression in mammary tumors associates with EGFR activation, and undergoes down-regulation in patients treated with an EGFR-specific kinase inhibitor. Figure 8a - Thin sections of a paraffin-embedded inflammatory breast carcinoma (IBC) tumor were immunostained using an anti-cten monoclonal antibody (left panel), an anti-EGFR antibody (middle panel), or an anti-phosphorylated EGFR (tyrosine-1068) antibody (right panel). Regions positive for cten, EGFR or phospho-EGFR appear in brown, whereas nuclei stained with Methyl Green appear in dark green. Figure 8b - AU-565 human mammary cancer cells were pre-treated with or without a tyrosine kinase inhibitor (TKI; GW-2974, 25 μ M) for 1 hour followed by stimulation with EGF (100 ng/ml) for 15 minutes. Alternatively, cells were treated with EGF (10 ng/ml) for 24 hours in the absence or presence of GW-2974 (1 μ M). Thereafter, cell extracts were subjected to immunoblotting with the indicated antibodies. Figure 8c - Immunohistochemical analysis of cten in an inflammatory breast tumor obtained from a patient before and after a three week-long treatment with lapatinib (Tykerb). Note tumor emboli in the lymphovascular area. Figure 8d - Statistical analysis of cten expression levels in patients scored (0-3) by immunohistochemistry before and after TKI treatment ($n=11$, $p=0.002$, paired t-test). Figure 8e - A model representing the functions of cten and tensin3 in promoting EGF-induced cell migration. Resting cells, which are packed in an epithelial layer, contain stress fibers anchored to focal adhesion sites through tensin3, or other actin binders, which simultaneously binds actin filaments and integrin (through a PTB domain). Following EGF stimulation, tensin3 expression is down-regulated and cten is up-regulated, to a level sufficient for displacement of tensin3 from integrin. Consequently, tensin3 dissociates from focal adhesion sites, leading to the breakdown of actin stress fibers, and initiation of cell migration.

FIGs. 9a-e illustrate the effect of tensin3 on the malignancy of mammary xenografts. Figure 9a - Human breast cancer cells, RFP-MDA-MB231, that stably express either GFP (control) or GFP-tensin3 (tensin3) proteins, were allowed to migrate through a Transwell chamber in full growth medium for 16 hours, then migrating cells were fixed, stained with methyl violet and lysed using 10% acetic-acid. Purple color representing the amount of migrated cells was determined by spectrophotometer (bars represent S.D. values). Figure 9b - Control RFP-MDA-MB231 (black diamonds) or tensin3 RFP-MDA-MB231 (open squares) cells were incubated for up to 120 hours in a 96-well plate. Thereafter, cell proliferation was monitored by in sixplicates using MTT. Growth induction relative to the zero time point is presented and S.D. values indicated (bars). Figure 9c - The volume of tumors originated by

inoculation of either control RFP-MDA-MB231 cells (black diamonds; n=8 animals) or tensin3 RFP-MDA-MB231 cells (open squares; n=9 animals) into the mammary fat pads of 6-week-old female SCID mice, was measured. Shown are averages $\pm$ S.E. (Anova analysis, $p=0.04$ student t-test). Figure 9d - In vivo whole-body stereomicroscope fluorescence imaging of micrometastases in the lymph nodes of mice bearing control or tensin-3-overexpressing tumor xenografts. The percentage of mice in which metastases were detected at the ipsilateral lymph nodes or in the contralateral nodes at different time intervals is presented. Figure 9e - Pictures of lymph node metastases of control (top) or tensin-3-expressing (bottom) cells at the indicated times after implantation into SCID mice. Arrows indicate lymph nodes.

FIGs. 10a-b show the expression of cten in human tumors. Figure 10a - Level of cten expression in ovarian tumors in comparison with the surrounding benign tissue ($p<0.03$). Figure 10b - A group of 92 ovarian cancer patients was used to analyze the association between expression levels and patient survival time. The data are presented as Kaplan-Meier survival curves for high (red) and low (blue) expressors.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of compositions and methods for modulating cell migration. Specifically, the present invention can be used for treating cancer.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Cell migration plays a central role in a wide variety of biological phenomena including embryonic development, angiogenesis, wound healing, immunity and inflammation. In the adult organism, cell migration remains prominent in both physiological and pathological conditions. For example, migration of fibroblasts and vascular endothelial cells is essential for wound healing. In metastasis, tumor cells migrate from the initial tumor mass throughout the whole body. Directed tumor cell motility by chemotaxis is the final step of tumor invasion, and the modulation of this process has been a major focus of research.

In view of the significant involvement of cell migration in such basic physiological processes as well as in pathological states, a growing need for elucidating the molecular process that govern cell migration is becoming increasingly apparent.

While reducing the present invention to practice the present inventors have uncovered a molecular switch that governs cell migration, essentially, a coordinated down-regulation of tensin 3 (TNS3); and upregulation of cten (TNS4), a tensin family member lacking the actin-binding domain promotes cell migration.

As is illustrated hereinbelow and in the Examples section which follows, the present inventors have shown that EGF-induced signaling results in reduction in the levels of tensin3, and concomitant up-regulation of cten (see Figures 1a, b, d, 2c 3b). cten upregulation was shown to be ERK dependent (see Figures 1c). The effect of EGF on cten expression was shown to take place at the level of transcription (see Figures 2-b). Down-regulation of cten or tensin3 was effected to determine the direct involvement of these two factors in cell migration. As shown in Figures 4a-f, RNA interference-mediated down-regulation of cten, or tensin3, resulted in impaired, or enhanced, cell migration, respectively, along with reciprocal changes in stress fiber organization. The underlying mechanism involves competition of cten with tensin for binding to integrin $\beta 1$, driving breakdown of actin stress fibers, as elucidated from the structural functional analysis shown in Figures 6a-d and 7a-e. Consistent with this notion, cten expression levels and EGF-receptor (EGFR) activation are strongly associated in a cohort of invasive human mammary tumors (Figure 8a). Finally, statistically significant down-regulation of cten expression was exhibited in breast cancer patients undergoing treatment with a dual kinase inhibitor of EGFR and ErbB2, consistent with a role for the unraveled tensin3-cten switch in cancer progression (Figures 8b-d). The anti malignant effect of tensin 3 was further established in vivo, where overexpression of tensin 3 inhibited tumor development (Figures 9a-e). Finally, the expression of cten in various ovarian cancer biopsies was correlated with patients prognosis (see Figures 10a-b).

These findings are of imperative clinical implications to the design of novel therapeutic modalities based on the co-modulation of these central players. In addition, cten and tensin3 may be used as a marker for EGFR-driven tumors and as a component of a protein signature capable of predicting patient response to EGFR-targeted therapy.

Thus, according to one aspect of the present invention there is provided a method of inhibiting cell migration, the method comprising contacting the cell with:

- (a) an agent capable of at least partially downregulating activity and/or expression of cten; and/or

(b) an agent capable of at least partially upregulating activity and/or expression of tensin-3, thereby inhibiting cell migration.

According to another aspect of the present invention there is provided a method of inducing cell migration, the method comprising contacting the cell with:

- (a) an agent capable of upregulating activity and/or expression of cten; and
- (b) an agent capable of downregulating activity and/or expression of tensin-3, thereby inducing cell migration.

As used herein the phrase "cell migration" refers to the directed movement of cells from one location to another. In accordance with the teachings of the present invention, the cells are pathogenic or non-pathogenic (e.g., cancerous) eukaryotic cells which preferably express epidermal growth factor receptor (EGFR) GeneBank Accession No. NP_005219.2, NP_958439.1, NP_958440.1 or NP_958441.1.

As used herein the phrase "inhibiting cell migration" refers to at least partially inhibiting cell migration.

As used herein the phrase "inducing cell migration" refers to at least partially promoting cell migration.

As used herein the term "cten" refers to at least an active portion (e.g., capable of inducing cell migration e.g., SH2 and PTB domain corresponding to amino acids SH2: 449-555, PTB: 580-703 of SEQ ID NO: 2) of the C-terminal tensin protein, also known as tensin 4 and FLJ14950 such as set forth in GeneBank Accession Nos. NM_032865 and NP_116254. Also envisaged within the scope of the present invention are human and non-human homologues of cten e.g., XM_548132.2, XP_548132.2, XM_239343.3, XP_239343.3, NM_172564.1, NP_766152.1.

As used herein the term "tensin 3" refers to at least an active portion (e.g., capable of inhibiting cell-migration e.g., all three designated domains as follows, actin-binding domain ABD: 17-435, SH2: 1170-1273, PTB: 1306-1445 of NP_073585) of the tensin 3 protein such as set forth in GeneBank Accession Nos. NM_022748 and NP_073585. Also envisaged within the scope of the present invention are human and non-human homologues of tensin 3 e.g., XM_843449.1, XP_848542.1, XM_341256.2, XP_341257.2, XM_109868.7, XP_109868.5, XM_418886.1, XP_418886.1.

It will be appreciated that the present findings portray a similar role for tensin 1 (e.g., NM_022648 and NP_072174.3) in inhibiting EGF-induced cell migration (see Figures 5a-e). Thus, although the present application generally relates to tensin 3, tensin 1 may be interchangeably used in accordance with this aspect of the present invention.

Agents which are capable of modulating gene expression are described infra.

Upregulation of *ctn* and/or *tensin 3* can be effected at the genomic level, at the transcript, or at the protein level.

Following is a non-comprehensive list of agents capable of upregulating the expression level and/or activity of *tensin 3* or *ctn*.

An agent capable of upregulating expression of a *tensin 3* or *ctn* may be an exogenous polynucleotide sequence designed and constructed to express at least a functional portion of *tensin 3* or *ctn* (as described hereinabove). Accordingly, the exogenous polynucleotide sequence may be a DNA or RNA sequence encoding a *tensin 3* or *ctn* molecule, capable of mediating the desired activity (i.e., cell migration or inhibition of cell migration).

To express exogenous polynucleotides in mammalian cells, a polynucleotide sequence encoding *ctn* or *tensin 3* is preferably ligated into a nucleic acid construct suitable for mammalian cell expression. Such a nucleic acid construct includes a promoter sequence for directing transcription of the polynucleotide sequence in the cell in a constitutive or inducible manner.

Constitutive promoters suitable for use with the present invention are promoter sequences that are active under most environmental conditions and most types of cells, such as the cytomegalovirus (CMV) and Rous sarcoma virus (RSV). Inducible promoters suitable for use with the present invention include for example the tetracycline-inducible promoter (Zabala, M. et al. (2004). Optimization of the Tet-on system to regulate interleukin 12 expression in the liver for the treatment of hepatic tumors. *Cancer Res* 2004, 64(8), 2799-2804).

The nucleic acid construct (also referred to herein as an "expression vector") of the present invention includes additional sequences that render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, a typical cloning vector may also contain transcription and translation initiation sequences, transcription and translation terminators, and a polyadenylation signal.

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Enhancer elements can stimulate transcription up to 1,000-fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses

have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus or human or murine cytomegalovirus (CMV) and the long tandem repeats (LTRs) from various retroviruses, such as murine leukemia virus, murine or Rous sarcoma virus, and HIV. See Gluzman, Y. and Shenk, T., eds. (1983). *Enhancers and Eukaryotic Gene Expression*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference.

In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

Polyadenylation sequences can also be added to the expression vector in order to increase the efficiency of cten or tensin 3 mRNA translation. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU- or U-rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, namely AAUAAA, located 11-30 nucleotides upstream of the site. Termination and polyadenylation signals suitable for the present invention include those derived from SV40.

In addition to the embodiments already described, the expression vector of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote extra-chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The expression vector of the present invention may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, the vector is capable of amplification in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

The expression vector of the present invention may further comprise polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA, such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

Examples for mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1(+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, and pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV, which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2, for instance. Vectors derived from bovine papilloma virus include pBV-1MTHA, and vectors derived from Epstein-Barr virus include pHEBO and p2O5. Other exemplary vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by the present invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinarily skilled artisan and as such, no general description of selection considerations is provided herein. For example, bone marrow cells can be targeted using the human T-cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), as described by Liang, C. Y. et al. (2004). High efficiency gene transfer into mammalian kidney cells using baculovirus vectors. *Arch Virol* 149, 51-60.

Recombinant viral vectors are useful for in vivo expression of tensin 3 or cten since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of retrovirus, for example, and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is the rapid infection of a large area of cells, most of which were not initially infected by the original viral particles. This is in contrast to vertical-type infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are

unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

The present invention also envisages upregulation of tensin 3 or cten at the protein level, essentially by administering the polypeptide itself. However, in this case measures should be taken to administer the polypeptide into the cell without subjecting same to degradation. The use of appropriate carriers which are well known in the art is thus recommended.

Downregulation of cten or tensin 3 can be effected on the genomic and/or the transcript level using a variety of molecules that interfere with transcription and/or translation (e.g., antisense, siRNA, Ribozyme, or DNAzyme), or on the protein level using, e.g., antibodies, antagonists, enzymes that cleave the polypeptide, and the like.

Following is a non-comprehensive list of agents capable of downregulating expression level and/or activity of tensin 3 or cten.

One example of an agent capable of downregulating the activity of cten or tensin 3 is an antibody or antibody fragment capable of specifically binding cten but not tensin 3 and vice versa e.g., an antibody directed to bind an epitope within amino acid coordinates 181-353 of NP_116254 is expected to bind cten but not tensin 3. Preferably, the antibody specifically binds at least one epitope of the polypeptide. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Antibodies that bind cten are described in the General Materials and Methods section of the instant application. Other antibodies are available from Abnova Corp. H00084951-M01; and were described by Su Hao Lo and Tung Bin Lo 2002 Cancer Res. 62:4217-21.

Antibodies to tensin 3 are available from BD Biosciences Pharmingen.

Since both cten and tensin 3 proteins are cytoplasmic, preferably used are small antibody fragments which may be introduced into the cell via known methods (such as liposomes). Preparation of antibody fragments is described at length hereinbelow.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes any intact molecules and functional fragments thereof, such as Fab, F(ab')₂, and Fv, that are capable of binding to macrophages. The functional antibody fragments are defined as follows: (1) Fab, the fragment

which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of a heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')₂, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single-chain Antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single-chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art. (See, for example, Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, incorporated herein by reference.)

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g., by Chinese hamster ovary (CHO) cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')₂. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin directly produces two monovalent Fab' fragments and an Fc (constant region) fragment. Such methods are described, for example, by Goldenberg in U.S. Pat. Nos. 4,036,945 and 4,331,647, and in references contained therein, the patents being hereby incorporated by reference in their entirety. (See also Porter, R. R. (1959). The hydrolysis of rabbit gammaglobulins and antibodies by crystalline papain. *Biochem J* 73, 119-126.) Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques, may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

F_v fragments comprise an association of variable heavy (VH) and variable light (VL) chains. This association may be noncovalent, as described in Inbar, D. et al. (1972). Localization of Antibody-Combining Sites within the Variable Portions of Heavy and Light Chains. Proc Natl Acad Sci USA 69, 2659-2662. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the F_v fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cell synthesizes a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, in: Whitlow, M. and Filpula, D. (1991). Single-chain Fv proteins and their fusion proteins. METHODS: A Companion to Methods in Enzymology 2(2), 97-105; Bird, R. E. et al. (1988). Single-chain antigen-binding proteins. Science 242, 423-426; Pack, P. et al. (1993). Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of *Escherichia coli*. Biotechnology (N.Y.) 11(11), 1271-1277; and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick, J. W. and Fry, K. E. (1991). PCR Amplification of Antibody Genes. METHODS: A Companion to Methods in Enzymology 2(2), 106-110.

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) that contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins in which residues forming a CDR of the recipient (recipient antibody) are replaced by residues from a CDR of a non-human species (donor antibody), such as mouse, rat, or rabbit, with the desired specificity, affinity, and capacity. In some instances, the Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the

humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR corresponds to that of a non-human immunoglobulin, and all or substantially all of the framework region (FR) is that of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones, P. T. et al. (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321, 522-525; Riechmann, L. et al. (1988). Reshaping human antibodies for therapy. *Nature* 332, 323-327; Presta, L. G. (1992b). *Curr Opin Struct Biol* 2, 593-596; and Presta, L. G. (1992a). Antibody engineering. *Curr Opin Biotechnol* 3(4), 394-398).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues from a non-human source introduced into it. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers (see Jones et al. (1986); Riechmann et al. (1988); and Verhoeven, M. et al. (1988). Reshaping human antibodies: grafting an antilysozyme activity. *Science* 239, 1534-1536), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom, H. R. and Winter, G. (1991). By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *J Mol Biol* 227, 381-388). The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al. (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96; and Boerner, P. et al. (1991). Production of antigen-specific human monoclonal antibodies from in vitro-primed human splenocytes. *J Immunol* 147, 86-95). Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice, in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed to closely resemble that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach

is described, for example, in U.S. Pat. Nos.: 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; and in the following scientific publications: Marks, J. D. et al. (1992). By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology (N.Y.)* 10(7), 779-783; Lonberg et al., 1994. *Nature* 368:856-859; Morrison, S. L. (1994). News and View: Success in Specification. *Nature* 368, 812-813; Fishwild, D. M. et al. (1996). High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotechnol* 14, 845-851; Neuberger, M. (1996). Generating high-avidity human Mabs in mice. *Nat Biotechnol* 14, 826; and Lonberg, N. and Huszar, D. (1995). Human antibodies from transgenic mice. *Int Rev Immunol* 13, 65-93.

Another agent capable of downregulating cten or tensin 3 is a small interfering RNA (siRNA) molecule in the process of RNA interference (RNAi). RNAi is a two-step process. In the first, the initiation step, input double-stranded (dsRNA) is digested into 21- to 23-nucleotide (nt) small interfering RNAs (siRNAs), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which processes (cleaves) dsRNA (introduced directly or by means of a transgene or a virus) in an ATP-dependent manner. Successive cleavage events degrade the RNA to 19- to 21-bp duplexes (the siRNA), each with 2-nucleotide 3' overhangs (Hutvagner, G. and Zamore, P. D. (2002). RNAi: Nature abhors a double-strand. *Curr Opin Gen Dev* 12, 225-232; and Bernstein, E. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363-366). Specific examples of siRNA molecules which downregulate the expression of cten or tensin 3 are described in the Examples section which follows.

In the second step, termed the effector step, the siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base-pairing interactions and cleaves the mRNA into 12-nucleotide fragments from the 3' terminus of the siRNA (Hutvagner and Zamore (2002); Hammond et al. (2001) *Nat. Rev. Gen.* 2:110-119 (2001); and Sharp, P. A. (2001). RNA interference. *Genes Dev* 15, 485-490). Although the mechanism of cleavage remains to be elucidated, research indicates that each RISC contains a single siRNA and an RNase (Hutvagner and Zamore (2002)).

Because of the remarkable potency of RNAi, an amplification step within the RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs to generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC (Hammond et al. (2001), Sharp (2001); and Hutvagner and Zamore (2002)). For more information on RNAi,

see the following reviews: Tuschl, T. (2001). RNA interference and small interfering RNAs. *ChemBioChem* 2, 239-245; Cullen, B. R. (2002). RNA interference: antiviral defense and genetic tool. *Nat Immunol* 3, 597-599; and Brantl, S. (2002). Antisense-RNA regulation and RNA interference. *Biochim Biophys Acta* 1575, 15-25.

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the *cten* or *tensin 3* mRNA sequence is scanned downstream of the AUG start codon for AA-dinucleotide sequences. Occurrence of each AA and the 19 3'-adjacent nucleotides is recorded as a potential siRNA target site. Preferably, siRNA target sites are selected from the open reading frame (ORF), as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex (Tuschl (2001)). It will be appreciated, however, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH, wherein siRNA directed at the 5' UTR mediated about a 90% decrease in cellular GAPDH mRNA and completely abolished protein levels (www.ambion.com/techlib/tn/91/912.html).

Examples of specific RNAi molecules used to downregulate expression of *cten* and *tensin 3* are provided in Example 3, Figures 4a-f.

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat, etc.) using any sequence alignment software, such as the BlastN software available from the NCBI server (www.ncbi.nlm.nih.gov/BLAST/). Putative target sites that exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as templates for siRNA synthesis. Preferred sequences are those including low G/C content, as these have proven to be more effective in mediating gene silencing as compared with sequences including G/C content higher than 55%. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative-control siRNAs preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

Another agent capable of downregulating *cten* or *tensin 3* is a DNAzyme molecule, which is capable of specifically cleaving an mRNA transcript or a DNA sequence of the *cten* or *tensin 3*. DNAzymes are single-stranded polynucleotides that are capable of cleaving both single- and double-stranded target sequences (Breaker, R. R. and Joyce, G. F. (1995). A DNA enzyme with Mg^{2+} -dependent RNA phosphoesterase activity. *Curr Biol* 2, 655-660; Santoro,

S. W. and Joyce, G. F. (1997). A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA* 94, 4262-4266). A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro and Joyce (1997)); for review of DNAzymes, see: Khachigian, L. M. (2002). DNAzymes: cutting a path to a new class of therapeutics. *Curr Opin Mol Ther* 4, 119-121.

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single- and double-stranded target cleavage sites are disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh, T. et al., Abstract 409, American Society of Gene Therapy 5th Annual Meeting (www.asgt.org), June 5-9, 2002, Boston, Mass. USA.). In another application, DNAzymes complementary to bcr-abl oncogenes were successful in inhibiting the oncogene's expression in leukemia cells, and in reducing relapse rates in autologous bone marrow transplants in cases of Chronic Myelogenous Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL).

Downregulation of cten or tensin 3 can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the cten or tensin 3.

Design of antisense molecules that can be used to efficiently downregulate cten or tensin 3 must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide that specifically binds the designated mRNA within cells in a manner inhibiting the translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types (see, for example: Luft, F. C. (1998). Making sense out of antisense oligodeoxynucleotide delivery: getting there is half the fun. *J Mol Med* 76(2), 75-76 (1998); Kronenwett et al. (1998). Oligodeoxyribonucleotide uptake in primary human hematopoietic cells is enhanced by cationic lipids and depends on the hematopoietic cell subset. *Blood* 91, 852-862; Rajur, S. B. et al. (1997). Covalent protein-oligonucleotide conjugates for efficient delivery of antisense molecules. *Bioconj Chem* 8, 935-940; Lavigne et al. *Biochem Biophys Res Commun* 237: 566-71 (1997); and Aoki, M. et

al. (1997). In vivo transfer efficiency of antisense oligonucleotides into the myocardium using HVJ-liposome method. *Biochem Biophys Res Commun* 231, 540-545).

In addition, also available are algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide (see, for example, Walton, S. P. et al. (1999). Prediction of antisense oligonucleotide binding affinity to a structured RNA target. *Biotechnol Bioeng* 65, 1-9).

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF-alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiencies of specific oligonucleotides using an in vitro system were also published (Matveeva, O. et al. (1998). Prediction of antisense oligonucleotide efficacy by in vitro methods. *Nature Biotechnology* 16, 1374-1375).

Several clinical trials have demonstrated the safety, feasibility, and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully utilized (Holmund, B. P. et al. (1999). Toward antisense oligonucleotide therapy for cancer: ISIS compounds in clinical development. *Curr Opin Mol Ther* 1, 372-385), while treatment of hematological malignancies via antisense oligonucleotides targeting c-myc gene, p53, and Bcl-2 entered clinical trials and was shown to be tolerated by patients (Gewirtz, A. M. (1999). Oligonucleotide therapeutics: clothing the emperor. *Curr Opin Mol Ther* 1, 297-306).

More recently, antisense-mediated suppression of human heparanase gene expression was reported to inhibit pleural dissemination of human cancer cells in a mouse model (Uno, F. et al. (2001). Antisense-mediated suppression of human heparanase gene expression inhibits pleural dissemination of human cancer cells. *Cancer Res* 61, 7855-7860).

Thus, the current consensus is that recent developments in the field of antisense technology, which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily

skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating cten or tensin 3 is a ribozyme molecule capable of specifically cleaving the mRNA transcript. Ribozymes increasingly are being used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest (Welch, P. J. et al. (1998). Expression of ribozymes in gene transfer systems to modulate target RNA levels. *Curr Opin Biotechnol* 9, 486-496). The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers, and specific somatic mutations in genetic disorders (Welch, P. J. et al. (1998). Ribozyme gene therapy for hepatitis C virus infection. *Clin Diagn Virol* 10, 163-171). Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase I trials. More recently, ribozymes have been used for transgenic animal research, gene target validation, and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME™ was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGFR (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms, has demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME™, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Inc., Boulder, Colorado, USA (www.rpi.com)).

It will be appreciated that a non-functional analogue of the polypeptides of the present invention may be used for down regulating tensin 3 or cten, such as by way of competition (see e.g., a tensin sequence which is devoid of the actin binding domain or alternatively a cten mutant devoid of the PTB domain, see Figures 5a-e).

Other agents which modulate (i.e., upregulate or downregulate) cell migration can be identified as follows:

- (a) treating a plurality of cells with a plurality of agents (e.g., biomolecules, small synthetic or non-synthetic molecules, radiation and the like), ; and
- (b) identifying at least one cell of the plurality of treated cells exhibiting an alteration in an expression ratio of cten to tensin 3 relatively to the ratio in untreated cells, the alteration being indicative of an agent capable or modulating cell migration.

Any of the above-described agents may be qualified by monitoring an effect of same on cell migration.

Assaying cell migration may be effected by monitoring cell movement *per se*. Alternatively or additionally, molecular or structural (e.g., pseudopodia formation) determinants which are known to be associated with cell migration may be assayed.

The following summarizes a number of cell migration assays which may be used in accordance with the present invention (see also "GENERAL MATERIALS AND METHODS" section of the Examples section).

Naturally, live cell data based on time-lapse movies can be most informative for the quantification of dynamic events in individual cells or cell populations (Dai et al., 2005, Exp Cell Res 311, 272-80). Another common migration assay; namely, the trans-well migration system (Mastyugin et al., 2004, J Biomol Screen 9, 712-8), provides general information about the migratory potential of the cell population at large. In the *in vitro* "wound closure" assay into a wound introduced into a confluent cell culture is measured (Yarrow et al., 2004, BMC Biotechnol 4, 21).

Other common approaches for quantifying migratory parameters recording of migration are based on such assays as phagokinetic track (PKT) formation on flat surfaces (Albrecht-Buehler, 1977, Cell 11, 395-404; Kawa et al., 1997, FEBS Lett 420, 196-200; Lin et al., 2005, Mol Cancer 4, 21; Scott et al., 2000, Anal Biochem 287, 343-4; Zetter, 1980, Nature 285, 41-3).

As shown in Figures 4a-d, down-regulation of cten expression reduced cell migration in a statistically significant manner.

The ability to inhibit cell migration may be beneficially harnessed for the treatment of a myriad of medical conditions which are associated with cell migration. Essentially medical conditions in which cell migration contributes to disease onset or progression.

Thus, according to another aspect of the present invention there is provided a method of treating a medical condition associated with cell migration in a subject in need thereof.

The method according to this aspect of the present invention comprises administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of cten (such as described above) in migratory pathogenic cells, thereby treating the medical condition associated with cell migration.

Examples of medical conditions which are associated with cell migration include, but are not limited to, inflammatory disorders, cancer (e.g. cancer invasiveness) especially cancer metastasis (e.g. breast cancer metastasis).

As used herein the phrase "inflammatory disorders" includes but is not limited to chronic inflammatory diseases and acute inflammatory diseases. Examples of such diseases and conditions are summarized infra.

Inflammatory diseases associated with hypersensitivity

Examples of hypersensitivity include, but are not limited to, Type I hypersensitivity, Type II hypersensitivity, Type III hypersensitivity, Type IV hypersensitivity, immediate hypersensitivity, antibody mediated hypersensitivity, immune complex mediated hypersensitivity, T lymphocyte mediated hypersensitivity and DTH.

Type I or immediate hypersensitivity, such as asthma.

Type II hypersensitivity include, but are not limited to, rheumatoid diseases, rheumatoid autoimmune diseases, rheumatoid arthritis (Krenn V. *et al.*, *Histol Histopathol* 2000 Jul;15 (3):791), spondylitis, ankylosing spondylitis (Jan Voswinkel *et al.*, *Arthritis Res* 2001; 3 (3): 189), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Erikson J. *et al.*, *Immunol Res* 1998;17 (1-2):49), sclerosis, systemic sclerosis (Renaudineau Y. *et al.*, *Clin Diagn Lab Immunol.* 1999 Mar;6 (2):156); Chan OT. *et al.*, *Immunol Rev* 1999 Jun;169:107), glandular diseases, glandular autoimmune diseases, pancreatic autoimmune diseases, diabetes, Type I diabetes (Zimmet P. *Diabetes Res Clin Pract* 1996 Oct;34 Suppl:S125), thyroid diseases, autoimmune thyroid diseases, Graves' disease (Orgiazzi J. *Endocrinol Metab Clin North Am* 2000 Jun;29 (2):339), thyroiditis, spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, *J Immunol* 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, *Nippon Rinsho* 1999 Aug;57 (8):1810), myxedema, idiopathic myxedema (Mitsuma T. *Nippon Rinsho.* 1999 Aug;57 (8):1759); autoimmune reproductive diseases, ovarian diseases, ovarian autoimmunity (Garza KM. *et al.*, *J Reprod Immunol* 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, *Am J Reprod Immunol.* 2000 Mar;43 (3):134), repeated fetal loss (Tincani A. *et al.*, *Lupus* 1998;7 Suppl 2:S107-9), neurodegenerative diseases, neurological diseases, neurological autoimmune diseases, multiple sclerosis (Cross AH. *et al.*, *J Neuroimmunol* 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.*, *J Neural Transm Suppl.* 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, *Int Rev Immunol* 1999;18 (1-2):83), motor neuropathies (Kornberg AJ. *J Clin Neurosci.* 2000 May;7 (3):191), Guillain-Barre syndrome, neuropathies and autoimmune neuropathies (Kusunoki S. *Am J Med Sci.* 2000 Apr;319 (4):234), myasthenic diseases, Lambert-Eaton myasthenic syndrome (Takamori M. *Am J Med Sci.* 2000 Apr;319 (4):204), paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy, non-paraneoplastic stiff man syndrome, cerebellar atrophies, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette

syndrome, polyendocrinopathies, autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. *Rev Neurol (Paris)* 2000 Jan;156 (1):23); neuropathies, dysimmune neuropathies (Nobile-Orazio E. *et al.*, *Electroencephalogr Clin Neurophysiol Suppl* 1999;50:419); neuromyotonia, acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.*, *Ann N Y Acad Sci.* 1998 May 13;841:482), cardiovascular diseases, cardiovascular autoimmune diseases, atherosclerosis (Matsuura E. *et al.*, *Lupus.* 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. *Lupus.* 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, *Lupus* 1998;7 Suppl 2:S107-9), granulomatosis, Wegener's granulomatosis, arteritis, Takayasu's arteritis and Kawasaki syndrome (Praprotnik S. *et al.*, *Wien Klin Wochenschr* 2000 Aug 25;112 (15-16):660); anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, *Semin Thromb Hemost.* 2000;26 (2):157); vasculitises, necrotizing small vessel vasculitises, microscopic polyangiitis, Churg and Strauss syndrome, glomerulonephritis, pauci-immune focal necrotizing glomerulonephritis, crescentic glomerulonephritis (Noel LH. *Ann Med Interne (Paris).* 2000 May;151 (3):178); antiphospholipid syndrome (Flamholz R. *et al.*, *J Clin Apheresis* 1999;14 (4):171); heart failure, agonist-like beta-adrenoceptor antibodies in heart failure (Wallukat G. *et al.*, *Am J Cardiol.* 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. *Ann Ital Med Int.* 1999 Apr-Jun;14 (2):114); hemolytic anemia, autoimmune hemolytic anemia (Efremov DG. *et al.*, *Leuk Lymphoma* 1998 Jan;28 (3-4):285), gastrointestinal diseases, autoimmune diseases of the gastrointestinal tract, intestinal diseases, chronic inflammatory intestinal disease (Garcia Herola A. *et al.*, *Gastroenterol Hepatol.* 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. *Harefuah* 2000 Jan 16;138 (2):122), autoimmune diseases of the musculature, myositis, autoimmune myositis, Sjogren's syndrome (Feist E. *et al.*, *Int Arch Allergy Immunol* 2000 Sep;123 (1):92); smooth muscle autoimmune disease (Zauli D. *et al.*, *Biomed Pharmacother* 1999 Jun;53 (5-6):234), hepatic diseases, hepatic autoimmune diseases, autoimmune hepatitis (Manns MP. *J Hepatol* 2000 Aug;33 (2):326) and primary biliary cirrhosis (Strassburg CP. *et al.*, *Eur J Gastroenterol Hepatol.* 1999 Jun;11 (6):595).

Type IV or T cell mediated hypersensitivity, include, but are not limited to, rheumatoid diseases, rheumatoid arthritis (Tisch R, McDevitt HO. *Proc Natl Acad Sci U S A* 1994 Jan 18;91 (2):437), systemic diseases, systemic autoimmune diseases, systemic lupus erythematosus (Datta SK., *Lupus* 1998;7 (9):591), glandular diseases, glandular autoimmune diseases, pancreatic diseases, pancreatic autoimmune diseases, Type 1 diabetes (Castano L. and Eisenbarth GS. *Ann. Rev. Immunol.* 8:647); thyroid diseases, autoimmune thyroid diseases, Graves' disease (Sakata S. *et al.*, *Mol Cell Endocrinol* 1993 Mar;92 (1):77); ovarian diseases (Garza KM. *et al.*, *J Reprod Immunol* 1998 Feb;37 (2):87), prostatitis, autoimmune prostatitis (Alexander RB. *et al.*, *Urology* 1997 Dec;50 (6):893), polyglandular syndrome, autoimmune polyglandular syndrome, Type I

autoimmune polyglandular syndrome (Hara T. *et al.*, Blood. 1991 Mar 1;77 (5):1127), neurological diseases, autoimmune neurological diseases, multiple sclerosis, neuritis, optic neuritis (Soderstrom M. *et al.*, J Neurol Neurosurg Psychiatry 1994 May;57 (5):544), myasthenia gravis (Oshima M. *et al.*, Eur J Immunol 1990 Dec;20 (12):2563), stiff-man syndrome (Hiemstra HS. *et al.*, Proc Natl Acad Sci U S A 2001 Mar 27;98 (7):3988), cardiovascular diseases, cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.*, J Clin Invest 1996 Oct 15;98 (8):1709), autoimmune thrombocytopenic purpura (Semple JW. *et al.*, Blood 1996 May 15;87 (10):4245), anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, Viral Immunol 1998;11 (1):9), hemolytic anemia (Sallah S. *et al.*, Ann Hematol 1997 Mar;74 (3):139), hepatic diseases, hepatic autoimmune diseases, hepatitis, chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), biliary cirrhosis, primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551), nephric diseases, nephric autoimmune diseases, nephritis, interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140), connective tissue diseases, ear diseases, autoimmune connective tissue diseases, autoimmune ear disease (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249), disease of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci 1997 Dec 29;830:266), skin diseases, cutaneous diseases, dermal diseases, bullous skin diseases, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of delayed type hypersensitivity include, but are not limited to, contact dermatitis and drug eruption.

Examples of types of T lymphocyte mediating hypersensitivity include, but are not limited to, helper T lymphocytes and cytotoxic T lymphocytes.

Examples of helper T lymphocyte-mediated hypersensitivity include, but are not limited to, T_H1 lymphocyte mediated hypersensitivity and T_H2 lymphocyte mediated hypersensitivity.

Autoimmune diseases

Include, but are not limited to, cardiovascular diseases, rheumatoid diseases, glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.

Examples of autoimmune cardiovascular diseases include, but are not limited to atherosclerosis (Matsuura E. *et al.*, Lupus. 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9), Wegener's granulomatosis, Takayasu's arteritis, Kawasaki syndrome (Praprotnik S. *et al.*, Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660), anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, Semin Thromb Hemost.2000;26 (2):157), necrotizing small vessel vasculitis,

microscopic polyangiitis, Churg and Strauss syndrome, pauci-immune focal necrotizing and crescentic glomerulonephritis (Noel LH. *Ann Med Interne* (Paris). 2000 May;151 (3):178), antiphospholipid syndrome (Flamholz R. *et al.*, *J Clin Apheresis* 1999;14 (4):171), antibody-induced heart failure (Wallukat G. *et al.*, *Am J Cardiol.* 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. *Ann Ital Med Int.* 1999 Apr-Jun;14 (2):114; Semple JW. *et al.*, *Blood* 1996 May 15;87 (10):4245), autoimmune hemolytic anemia (Efremov DG. *et al.*, *Leuk Lymphoma* 1998 Jan;28 (3-4):285; Sallah S. *et al.*, *Ann Hematol* 1997 Mar;74 (3):139), cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.*, *J Clin Invest* 1996 Oct 15;98 (8):1709) and anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, *Viral Immunol* 1998;11 (1):9).

Examples of autoimmune rheumatoid diseases include, but are not limited to rheumatoid arthritis (Krenn V. *et al.*, *Histol Histopathol* 2000 Jul;15 (3):791; Tisch R, McDevitt HO. *Proc Natl Acad Sci units S A* 1994 Jan 18;91 (2):437) and ankylosing spondylitis (Jan Voswinkel *et al.*, *Arthritis Res* 2001; 3 (3): 189).

Examples of autoimmune glandular diseases include, but are not limited to, pancreatic disease, Type I diabetes, thyroid disease, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, idiopathic myxedema, ovarian autoimmunity, autoimmune anti-sperm infertility, autoimmune prostatitis and Type I autoimmune polyglandular syndrome. diseases include, but are not limited to autoimmune diseases of the pancreas, Type 1 diabetes (Castano L. and Eisenbarth GS. *Ann. Rev. Immunol.* 8:647; Zimmet P. *Diabetes Res Clin Pract* 1996 Oct;34 Suppl:S125), autoimmune thyroid diseases, Graves' disease (Orgiazzi J. *Endocrinol Metab Clin North Am* 2000 Jun;29 (2):339; Sakata S. *et al.*, *Mol Cell Endocrinol* 1993 Mar;92 (1):77), spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, *J Immunol* 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, *Nippon Rinsho* 1999 Aug;57 (8):1810), idiopathic myxedema (Mitsuma T. *Nippon Rinsho.* 1999 Aug;57 (8):1759), ovarian autoimmunity (Garza KM. *et al.*, *J Reprod Immunol* 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, *Am J Reprod Immunol.* 2000 Mar;43 (3):134), autoimmune prostatitis (Alexander RB. *et al.*, *Urology* 1997 Dec;50 (6):893) and Type I autoimmune polyglandular syndrome (Hara T. *et al.*, *Blood.* 1991 Mar 1;77 (5):1127).

Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.*, *Gastroenterol Hepatol.* 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. *Harefuah* 2000 Jan 16;138 (2):122), colitis, ileitis and Crohn's disease.

Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid and pemphigus foliaceus.

Examples of autoimmune hepatic diseases include, but are not limited to, hepatitis, autoimmune chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551; Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595) and autoimmune hepatitis (Manns MP. J Hepatol 2000 Aug;33 (2):326).

Examples of autoimmune neurological diseases include, but are not limited to, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.*, J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83; Oshima M. *et al.*, Eur J Immunol 1990 Dec;20 (12):2563), neuropathies, motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000 May;7 (3):191); Guillain-Barre syndrome and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenia, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319 (4):204); paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy and stiff-man syndrome (Hiemstra HS. *et al.*, Proc Natl Acad Sci units S A 2001 Mar 27;98 (7):3988); non-paraneoplastic stiff man syndrome, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome and autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); dysimmune neuropathies (Nobile-Orazio E. *et al.*, Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.*, Ann N Y Acad Sci. 1998 May 13;841:482), neuritis, optic neuritis (Soderstrom M. *et al.*, J Neurol Neurosurg Psychiatry 1994 May;57 (5):544) and neurodegenerative diseases.

Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. *et al.*, Int Arch Allergy Immunol 2000 Sep;123 (1):92) and smooth muscle autoimmune disease (Zauli D. *et al.*, Biomed Pharmacother 1999 Jun;53 (5-6):234).

Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9).

Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. *et al.*, Cell Immunol 1994 Aug;157 (1):249) and

autoimmune diseases of the inner ear (Gloddek B. *et al.*, Ann N Y Acad Sci 1997 Dec 29;830:266).

Examples of autoimmune systemic diseases include, but are not limited to, systemic lupus erythematosus (Erikson J. *et al.*, Immunol Res 1998;17 (1-2):49) and systemic sclerosis (Renaudineau Y. *et al.*, Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.*, Immunol Rev 1999 Jun;169:107).

Infectious diseases

Examples of infectious diseases include, but are not limited to, chronic infectious diseases, subacute infectious diseases, acute infectious diseases, viral diseases, bacterial diseases, protozoan diseases, parasitic diseases, fungal diseases, mycoplasma diseases and prion diseases.

Graft rejection diseases

Examples of diseases associated with transplantation of a graft include, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft versus host disease.

Allergic diseases

Examples of allergic diseases include, but are not limited to, asthma, hives, urticaria, pollen allergy, dust mite allergy, venom allergy, cosmetics allergy, latex allergy, chemical allergy, drug allergy, insect bite allergy, animal dander allergy, stinging plant allergy, poison ivy allergy and food allergy.

Cancerous diseases (including cancer metastases)

Examples of cancer include but are not limited to carcinoma, lymphoma, blastoma, sarcoma, and leukemia. Particular examples of cancerous diseases include but are not limited to: Myeloid leukemia such as Chronic myelogenous leukemia. Acute myelogenous leukemia with maturation. Acute promyelocytic leukemia, Acute nonlymphocytic leukemia with increased basophils, Acute monocytic leukemia. Acute myelomonocytic leukemia with eosinophilia; Malignant lymphoma, such as Birkitt's Non-Hodgkin's; Lymphocytic leukemia, such as Acute lymphoblastic leukemia. Chronic lymphocytic leukemia; Myeloproliferative diseases, such as Solid tumors Benign Meningioma, Mixed tumors of salivary gland, Colonic adenomas; Adenocarcinomas, such as Small cell lung cancer, Kidney, Uterus, Prostate, Bladder, Ovary, Colon, Sarcomas, Liposarcoma, myxoid, Synovial sarcoma, Rhabdomyosarcoma (alveolar), Extraskeletal myxoid chondrosarcoma, Ewing's tumor; other include Testicular and ovarian dysgerminoma, Retinoblastoma, Wilms' tumor, Neuroblastoma, Malignant melanoma, Mesothelioma, breast, skin, prostate, and ovarian.

Since the present inventors have shown that the molecular switch of the present invention (upregulation of cten and downregulation of TNS3) is particularly active in cells treated with EGF, EGF-responsive tumors are preferably included under the scope of the present invention.

As used herein an "EGF-responsive tumor" refers to a tumor which expresses EGFR and which cell proliferation is EGF-dependent.

Examples of EGF-responsive tumors include, but are not limited to, head and neck cancer, glioblastoma and lung cancer. It will be appreciated that the present invention also envisages identification or detection of EGF-responsive tumors by way of detecting an increase in cten activity or expression and a decrease in tensin 3 activity or expression in response to EGF.

As used herein, the term "treating" or "treatment" refers to preventing, alleviating or diminishing a symptom associated with a disease. Preferably, treating cures, e.g., substantially eliminates, the symptoms associated with the disease.

As used herein the term "subject" refers to any (e.g., mammalian) subject, preferably a human subject.

According to a preferred embodiment of this aspect of the present invention the method further comprises administering to the subject a therapeutically effective amount of an agent capable of upregulating activity and/or expression of tensin-3. Such agents are described in length hereinabove.

As shown in Figure 4f down-regulation of tensin 3 expression induced cell migration in a statistically significant manner. Likewise treatment of cells with a deletion mutant of tensin 1, GFP-CT-tensin, lacking the actin-binding domain (Figure 5a; GFP-CT-tensin), resulted in disassembly of the actin stress fibers, a critical event in cell migration.

The ability to induce cell migration may be beneficially harnessed for the treatment of a myriad of medical conditions which may be treated by inducing cell migration in accordance with the teachings of the present invention (e.g., downregulation of tensin 3 and/or upregulation of cten).

Examples of medical conditions which would benefit from induction of cell migration include any conditions associated with tissue damage such as wound healing, a spinal cord injury, brain injury, brain trauma, a neuronal disease or disorder and inflammatory disorders.

The above-described agents are administered to the subject according to the intended use. Thus, for example, migrating pathogenic cells (e.g., cells of an EGF-responsive tumor) are administered with appropriate agents which inhibit cell migration in accordance with the present invention. Alternatively, when the medical condition may be alleviated by the activity

of migrating cells such as those capable of promoting wound healing, the agents are administered to healthy cells such as of the immune system (e.g., infiltrating macrophages and lymphocytes).

The agents of the present invention can be provided to the individual *per se*, or as part of a pharmaceutical composition where it is mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the polypeptide or polynucleotide preparation, which is accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier," which may be used interchangeably, refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein, the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils, and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in the latest edition of "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, which is herein fully incorporated by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal, or parenteral delivery, including intramuscular, subcutaneous, and intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the present invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable

carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries as desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, and sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof, such as sodium alginate, may be added.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane, or carbon dioxide. In the case of a pressurized aerosol, the dosage may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base, such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with, optionally, an added preservative. The compositions may be suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing, and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water-based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters such as ethyl oleate, triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the active ingredients, to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., a sterile, pyrogen-free, water-based solution, before use.

The pharmaceutical composition of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, for example, conventional suppository bases such as cocoa butter or other glycerides.

For ex-vivo and in vivo gene therapy (using the above-mentioned exogenous polynucleotide sequences) the appropriate carriers are used. Gene therapy as used herein refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition or phenotype. The genetic material of interest encodes a product (e.g. a protein, polypeptide, peptide, functional RNA, antisense)

whose production *in vivo* is desired. For review see, in general, the text "Gene Therapy" (Advanced in Pharmacology 40, Academic Press, 1997).

Two basic approaches to gene therapy have evolved: (1) *ex vivo* and (2) *in vivo* gene therapy. In *ex vivo* gene therapy cells are removed from a patient, and while being cultured are treated *in vitro*. Generally, a functional replacement gene is introduced into the cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the host/patient. These genetically reimplanted cells have been shown to express the transfected genetic material *in situ*. The cells may be autologous or non-autologous to the subject. Since non-autologous cells are likely to induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation.

In *in vivo* gene therapy, target cells are not removed from the subject rather the genetic material to be transferred is introduced into the cells of the recipient organism *in situ*, that is within the recipient. In an alternative embodiment, if the host gene is defective, the gene is repaired *in situ* (Culver, 1998. (Abstract) Antisense DNA & RNA based therapeutics, February 1998, Coronado, CA).

These genetically altered cells have been shown to express the transfected genetic material *in situ*.

Pharmaceutical compositions suitable for use in the context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a "therapeutically effective amount" means an amount of active ingredients (e.g., a nucleic acid construct) effective to prevent, alleviate, or ameliorate symptoms of a disorder (e.g., ischemia) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the dosage or the therapeutically effective amount can be estimated initially from *in vitro* and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage can be chosen by the individual physician in view of the patient's condition. (See, e.g., Fingl, E. et al. (1975), "The Pharmacological Basis of Therapeutics," Ch. 1, p.1.)

Dosage amount and administration intervals may be adjusted individually to provide sufficient plasma or brain levels of the active ingredient to induce or suppress the biological effect (i.e., minimally effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks, or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA-approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser device may also be accompanied by a notice in a form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions for human or veterinary administration. Such notice, for example, may include labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a pharmaceutically acceptable carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as further detailed above.

In addition to therapeutic advances pioneered by the present invention, the newly revealed molecular switch may also be used for diagnosing cancer, especially EGF-responsive tumors.

Thus, according to yet another aspect of the present invention there is provided a method of diagnosing (e.g., staging) a tumor in a subject in need thereof.

The method is effected by determining a level of cten and tensin 3 in suspected tumor cells, wherein an upregulation of cten above a predetermined threshold and downregulation of tensin 3 below a predetermined threshold are indicative of the tumor (e.g., invasive tumor).

It will be appreciated that the present invention also envisages diagnosing ovarian cancer or breast cancer by determining a level of cten in cells of a biological sample (see Example 7), wherein a level above a predetermined threshold is indicative of the cancer. Such diagnosis may be further substantiated by the use of traditional markers, pathology or imaging which are well known in the art.

As used herein the term "diagnosing" refers to classifying a disease or a symptom as cancer, determining a severity of the cancer (i.e., staging), monitoring cancer progression, forecasting an outcome of a disease and/or prospects of recovery.

As used herein, the term "level" refers to expression levels of RNA and/or protein of cten or tensin 3 in accordance with this aspect of the present invention.

A level correlatable with presence, absence or stage of the cancer can be a level of cten in the cancerous sample which is higher from the level of cten in a normal healthy cells obtained from a similar tissue or cellular origin. In addition, a level correlatable with presence, absence or stage of the cancer can be a level of tensin 3 in the cancerous sample which is lower from the level of tensin 3 in a normal healthy sample obtained from a similar tissue or cellular origin. In another embodiment the level may refer to a ration of cten/tensin 3 which is higher then that found in normal healthy cells obtained from a similar tissue or cellular origin.

As used herein "biological sample" refers to a cellular sample of the suspected tissue. Numerous well known tissue collection methods can be utilized to collect the biological sample from the subject in order to determine the level of DNA, RNA and/or polypeptide of cten or tensin 3 in the subject.

Examples include, but are not limited to, fine needle biopsy, needle biopsy, core needle biopsy and surgical biopsy (e.g., breast biopsy).

For example, a surgical biopsy procedure may be used to obtain a cellular sample of a tissue. The surgical biopsy procedure includes any biopsy procedure presently known in the

art. It is preferable to use an imaging technique in conjunction with the surgical biopsy so as to improve its accuracy.

The tissue may be frozen. Methods of freezing tissues are well known in the art. Alternatively, or additionally, cells may be cultured. Standard culturing techniques are also well known in the art. Cells may also be fixed on slides using standard procedures for ex vivo analysis. Fixing acts to preserve cells in a reproducible and life-like manner, following their removal in a surgical biopsy procedure. Fixation methods are well known in the art. These methods typically rely on either crosslinking agents, such as paraformaldehyde, or rapidly dehydrating agents, such as methanol.

Regardless of the procedure employed, once a biopsy is obtained the level of cten and tensin 3 can be determined and a diagnosis can thus be made.

Determining a level of cten or tensin 3 can be effected using various biochemical and molecular approaches used in the art for determining expression.

Determining the level of cten or tensin 3 of the present invention in normal tissues of the same origin is preferably effected alongside to detect alterations in gene expression.

Typically, detection of a nucleic acid of interest in a biological sample is effected by hybridization-based assays using an oligonucleotide probe. Measures are taken to employ probes which differentiate between cten and tensin 3 (e.g., cten: ACCCTAACCATGGGCCAACCCAGAACACCCCACTCTCCACCACTGGCCA, SEQ ID NO: 11; tensin3: GCCGACAATGGCTTCCTGTCCCACAACCTTCTCACGGTGGCGCCTGGA; SEQ ID NO: 12). If such are not used, the hybridization product is preferably size analyzed to determine the presence or level of cten or tensin 3 based on the difference in molecular weight.

Hybridization based assays rely on the use of oligonucleotide which can be 10, 15, 20, or 30 to 100 nucleotides long preferably from 10 to 50, more preferably from 40 to 50 nucleotides.

The detection of hybrid duplexes can be carried out by a number of methods. Typically, hybridization duplexes are separated from unhybridized nucleic acids and the labels bound to the duplexes are then detected. Such labels refer to radioactive, fluorescent, biological or enzymatic tags or labels of standard use in the art. A label can be conjugated to either the oligonucleotide probes or the nucleic acids derived from the biological sample (target).

For example, oligonucleotides of the present invention can be labeled subsequent to synthesis, by incorporating biotinylated dNTPs or rNTP, or some similar means (e.g., photo-

cross-linking a psoralen derivative of biotin to RNAs), followed by addition of labeled streptavidin (e.g., phycoerythrin-conjugated streptavidin) or the equivalent. Alternatively, when fluorescently-labeled oligonucleotide probes are used, fluorescein, lissamine, phycoerythrin, rhodamine (Perkin Elmer Cetus), Cy2, Cy3, Cy3.5, Cy5, Cy5.5, Cy7, FluorX (Amersham) and others [e.g., Kricka et al. (1992), Academic Press San Diego, Calif] can be attached to the oligonucleotides.

Traditional hybridization assays include PCR, RT-PCR, RNase protection, in-situ hybridization, primer extension, Southern blot, Northern Blot and dot blot analysis.

Those skilled in the art will appreciate that wash steps may be employed to wash away excess target DNA or probe as well as unbound conjugate. Further, standard heterogeneous assay formats are suitable for detecting the hybrids using the labels present on the oligonucleotide primers and probes.

It will be appreciated that a variety of controls may be usefully employed to improve accuracy of hybridization assays. For instance, samples may be hybridized to an irrelevant probe and treated with RNase A prior to hybridization, to assess false hybridization.

It will be appreciated that antisense oligonucleotides may be employed to quantify expression of a splice isoform of interest. Such detection is effected at the pre-mRNA level. Essentially the ability to quantitate transcription from a splice site of interest can be effected based on splice site accessibility. Oligonucleotides may compete with splicing factors for the splice site sequences. Thus, low activity of the antisense oligonucleotide is indicative of splicing activity [see Sazani and Kole (2003), supra].

Polymerase chain reaction (PCR)-based methods may be used to identify the presence of mRNA of cten or tensin 3. For PCR-based methods a pair of oligonucleotides is used, which is specifically hybridizable with the polynucleotide sequences described hereinabove in an opposite orientation so as to direct exponential amplification of a portion thereof (including the hereinabove described sequence alteration) in a nucleic acid amplification reaction.

The polymerase chain reaction and other nucleic acid amplification reactions are well known in the art and require no further description herein. The pair of oligonucleotides according to this aspect of the present invention are preferably selected to have compatible melting temperatures (T_m), e.g., melting temperatures which differ by less than that 7 °C, preferably less than 5 °C, more preferably less than 4 °C, most preferably less than 3 °C, ideally between 3 °C and 0 °C.

Hybridization to oligonucleotide arrays may be also used to determine expression of cten or tensin 3 (see Example 1 of the Examples section which follows). Such screening has been undertaken in the BRCA1 gene and in the protease gene of HIV-1 virus [see Hacia et al.,

(1996) Nat Genet 1996;14(4):441-447; Shoemaker et al., (1996) Nat Genet 1996;14(4):450-456; Kozal et al., (1996) Nat Med 1996;2(7):753-759].

The nucleic acid sample which includes the candidate region to be analyzed is isolated, amplified and labeled with a reporter group. This reporter group can be a fluorescent group such as phycoerythrin. The labeled nucleic acid is then incubated with the probes immobilized on the chip using a fluidics station. For example, Manz et al. (1993) Adv in Chromatogr 1993; 33:1-66 describe the fabrication of fluidics devices and particularly microcapillary devices, in silicon and glass substrates.

Once the reaction is completed, the chip is inserted into a scanner and patterns of hybridization are detected. The hybridization data is collected, as a signal emitted from the reporter groups already incorporated into the nucleic acid, which is now bound to the probes attached to the chip. Since the sequence and position of each probe immobilized on the chip is known, the identity of the nucleic acid hybridized to a given probe can be determined.

It will be appreciated that when utilized along with automated equipment, the above described detection methods can be used to screen multiple samples for hormonal dependent prostate cancer both rapidly and easily.

The presence of cten or tensin 3 may also be detected at the protein level. Numerous protein detection assays are known in the art, examples include, but are not limited to, chromatography, electrophoresis, immunodetection assays such as ELISA and western blot analysis, immunohistochemistry and the like, which may be effected using antibodies specific to cten or tensin 3 (measures are taken to use antibodies which will not cross react between cten and tensin 3). Such antibodies are directed, for example, to cten's amino acid coordinates 181-353 of NP_116254; tensin3 amino acid coordinates 730-1160 of NP_073585. Alternatively, detection may be effected by analyzing the molecular size of the immunostained band.

In doing the above, additional information may be gleaned pertaining to the determination of treatment regimen and responsiveness to treatment course such as with EGFR-directed therapy [e.g., the EGFR tyrosine kinase inhibitors Gefitinib (Iressa®, AstraZeneca), Erlotinib (Tarceva®, OSI Pharma), Lapatinib (Tykerb, GW572016) and Pertuzumab (Omnitarg®); and monoclonal antibodies such as Erbitux (Cetuximab)].

Conceivably analysis of the levels of cten and tensin 3 and treatment steps may be repeated a number of times during the course of a treatment.

The diagnostic reagents described hereinabove can also be included in kits. For example a kit for determining the stage, or presence of an EGF-responsive tumor in a subject can include an antibody directed at the unique amino acid sequence of cten in a one container

and a solid phase for attaching multiple biological samples packaged in a second container with appropriate buffers and preservatives and used for diagnosis.

Diagnostics using the above-described methodology can be validated using other diagnostic methods which are well known in the art such as by imaging, molecular detection of known markers and the like.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for

example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND METHODS

Reagents and antibodies: Unless indicated, materials were purchased from Sigma (St. Louis, MO). The EGFR-kinase inhibitor AG1478, the Erk inhibitor U0126 and the p38 inhibitor SB202190 were purchased from Calbiochem (San Diego, CA). An anti-Cten monoclonal antibody was generated by immunizing mice with a GST-cten fusion protein (SEQ ID NO: 17). A polyclonal antibody to cten and a polyclonal antibody to tensin3 have been described^{6,28}. Other rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to paxillin were from BD Bioscience (Heidelberg, Germany). A mouse monoclonal anti-EGFR was from Dako (Glostrup, Denmark). A rabbit polyclonal antibody directed to EGFR phosphorylated at tyrosine 1068 was purchased from Cell Signaling (Beverly, MA). All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). The following siRNA oligonucleotides directed to cten were obtained from Invitrogen (Groningen, the Netherlands): siCTEN-I UUCUCAUUGACAUGGUGCUCUGGGC (SEQ ID NO: 14); and siCTEN-II GGAGGAAUCUGAAGCCUUGGACAU (SEQ ID NO: 15). SMARTpool siRNA oligonucleotides directed at tensin3 were purchased from Dharmacon (Lafayette, CO, 01: CCACGAAGCUGCCCUGAGAUU, 02: GAAGGAGGAUCUGGACAAUUU, 03: GUACAAGGCGGAUUAUUUCAUU, 04: GCACAAAGGAGGACGUGAAUU, SEQ ID NOs: 18, 19, 20, 21.).

Expression vectors: Full-length cten cDNA was assembled in an expression vector using two-way PCR, where the 3' part of the coding region was amplified using as template a

cDNA clone from the RZPD library (RZPD, Berlin; clone no. IRAUp969B0180D), and the 5' part was amplified from a previously described cten plasmid⁶. Expression vectors encoding full-length tensin1 and CT-tensin1 were provided by Dr. Kenneth Yamada (National Institutes of Health, Bethesda, Maryland) and Dr. Shin Lin (University of California at Irvine, Irvine, CA). shRNA lentiviral plasmids directed at tensin3 were purchased from Sigma (St. Louis, MO). Bacterial expression vectors for His-tagged integrins were a generous gift from Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA).

Cell lines and transfection: MCF10A cell were grown in DMEM:F12 (1:1) medium supplemented with antibiotics, EGF (10 ng/ml), insulin (10 µg/ml), cholera toxin (1 µg/ml), hydrocortisone (1 µg/ml) and heat-inactivated horse serum (5%). Cells were transfected using jet-PEI (PolyPlus transfection; Illkirch, France). Alternatively, cells were transfected with siRNA oligonucleotides using Oligofectamine (Gibco BRL; Grand Island, NY). HeLa cells were transfected using jet-PEI (PolyPlus transfection; Illkirch, France), whereas HEK293T cells were transfected using the Ca²⁺-phosphate method.

Lysate preparation and immunoblotting analysis: Cells were washed briefly with ice-cold saline, and scrapped in buffered detergent solution [25mM HEPES (pH7.5), 150mM NaCl, 0.5% Na-deoxycholate, 1% NP-40, 0.1% Na-dodecylsulfate (SDS), 1mM EDTA, 1mM EGTA, 0.2mM Na₃VO₄ and a protease inhibitor cocktail diluted at 1:1000]. For equal gel loading, protein concentrations were determined by using the Bradford technique. Following gel electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked in TBST buffer [0.02 M Tris-Hcl (Ph 7.5), 0.15M NaCl, and 0.05 % Tween 20] containing 10 % low-fat milk, blotted with a primary antibody for 1h, washed with TBST and incubated for 30 minutes with a secondary antibody linked to horseradish peroxidase (HRP). Immuno-reactive bands were detected using chemiluminescence.

Immunofluorescence: Cells were treated and labeled for immunofluorescence as described²². Confocal microscopy was performed using a Nikon eclipse TE300 microscope (Oberkochen, Germany) with a 60XA/1.4 plan-Apochromat objective, attached to the Bio-Rad Radiance 2000 laser scanning system, operated by the LaserSharp software.

Transwell cell migration assay: MCF10A cells (4x10⁵ cells/well) were plated in the upper compartment of a 24-well Transwell tray (Corning, Acton, MA) and allowed to adhere for 16 hours at 37 °C in full medium lacking EGF. Thereafter, the medium in the lower compartment was replaced with the indicated medium, and cells were allowed to migrate through the intervening nitrocellulose membrane (8 µm pore size) during 16 hours of incubation at 37 °C. The filter was removed, fixed for 15 minutes in PBS containing paraformaldehyde (3 %), followed by cell permeabilization in Triton X-100 (0.05 %, in PBS),

and staining with methyl violet. Cells growing on the upper side of the filter were scraped using a cotton swab, whereas cells growing on the bottom side of the filter were photographed and counted. Alternatively, stained membranes were lysed in 10% acetic-acid, and their signal measured using a spectrophotometer.

RNA preparation and oligonucleotide microarray hybridization - Sub-confluent HeLa cells (3×10^6 per sample) were serum starved for 24 hours, followed by treatment with EGF (20 ng/ml) for various time intervals. Thereafter, cells were lysed, and RNA purified using a Versagene kit (Gentra Systems, Minneapolis, MN). RNA (10 μ g) was labeled, fragmented and hybridized to an Affymetrix HG-U133A oligonucleotide arrays (Affymetrix Incorporated, Santa Clara, CA). Arrays were scanned using a laser confocal scanner, and the expression value for each gene was calculated using Affymetrix Microarray Software v.5.0 (MAS5).

Integrin binding assay: Poly-histidine tagged carboxyl tails of integrins (α II, β 1) immobilized on Nickel beads have been described³⁰. Immobilized proteins were incubated for 1h at 4°C with cleared extracts of transfected HEK293T cells. The beads were then extensively washed with imidazole-containing washing solution [50mM NaH_2PO_4 (pH 8), 300mM NaCl, 20mM Imidazole, and 0.1% Triton X-100], and tightly bound proteins analyzed. For competition assays, beads coated with integrin β 1 were incubated with lysates of cells expressing GFP-tensin, along with increasing volumes of lysates of cells expressing GFP-cten. To equal the total amount of GFP fusion proteins in each reaction, lysates of GFP-expressing cells were respectively added to the reactions.

Immunohistochemical analysis: Fresh tumor biopsies were collected prior to initiating lapatinib therapy as part of a clinical trial in which patients with recurrent IBC were treated with lapatinib (1500 mg/day). The trial has been conducted in accordance with the 1996 version of the Declaration of Helsinki. The study protocol was approved by Institutional Review Boards and all patients provided a signed informed consent. Paraffin embedded tumor sections were deparaffinized and hydrated in water. Thereafter, immunostaining was performed by using a labeled streptavidin peroxidase technique. Slides for EGFR and cten were processed with antigen retrieval using 0.1 M citrate buffer, pH 6.0 (Dako, Glostrup, Denmark), and the sections incubated for 30 minutes with the primary antibodies at room temperature. Slides for p-EGFR were processed with antigen retrieval using 1 mM EDTA (pH 8.0) solution, and the sections incubated for an hour with the primary antibodies at room temperature. After immunostaining, all slides were counterstained with 4% methyl green. 3,3'-diaminobenzidine (DAB+; Dako, Glostrup, Denmark) was used as the chromogen to visualize the bound primary antibodies. Protein expressions on slides were independently scored by three pathologists.

Statistical Analysis: All analyses were performed using SPSS 13.0. First, means and standard deviations of the continuous variables and frequencies of the categorical variables were obtained. Then, the Chi-Square test and the Fishers Exact test were used to assess the relationships between the categorical variables (cten and EGFR or p-EGFR). Paired t-test was used to assess the difference from “before” to “after” treatment in the continuous variables (cten expression levels).

EXAMPLE 1

EGF alters transcription of migration-related genes, including tensin family members

RATIONALE and RESULTS

EGFR is involved in regulation of various cellular processes, including proliferation and motility, and constitutive receptor activation contributes to transition from a localized primary tumor mass into an invasive state leading to secondary metastases ^{1,7}. Several mechanisms have been identified, through which EGFR may regulate cell migration. These include transient activation of proteases, kinases, phospholipases and small GTPases ⁸. In addition to early signals that follow EGFR activation, cell migration is also regulated by changes in gene expression programs; the transcription factor complex AP-1 is required for EGF-induced cell migration ⁹, and inhibition of either transcription or translation impairs endothelial cell migration ^{3,4}. In invasive prostate cancer cells, EGFR modulates the expression of a plasminogen activator (uPAR) and of prostin-1 ¹⁰, but the identity of other genes underlying EGF-induced cell motility remains largely unknown.

To address the identity of EGF-induced genes potentially involved in cell migration, a previously established transcriptional profiling dataset derived from EGF-treated human HeLa cells was utilised ¹¹. Analysis of this dataset revealed that prolonged EGF treatment induced the expression of various genes involved in regulation of cell adhesion or organization of the actin cytoskeleton (see Table 1 below and Figure 2a). In addition, among other alterations, a reduction in transcripts that encode mediators of cell-to-cell interactions, as well as motility regulators was observed (see Table 1 below and Figure 2b).

Table 1

<i>Gene symbol</i>	<i>Gene name</i>	<i>Association to cell migration</i>	<i>Ref.</i>
<i>Up-regulated genes</i>			
ACTC	Actin α	Actin fiber formation, cell movement	A
SMTN	Smoothelin	Actin stress fiber	B

		binding protein 2	
COL4A1	Collagen IV α 1	Extracellular matrix protein	C
ITGB5	Integrin β 5	Involved in cell adhesion to the matrix protein vitronectin	D
PLAU	Plasminogen activator, urinary	Stimulates cell migration	E
Down-regulated genes			
CALD1	Caldesmon 1	Calmodulin and actin-binding protein involved in inhibition of cell migration	F
ADD3	Adducin 3	Membrane protein involved in the assembly of spectrin-actin networks at sites of cell-to-cell contact	G, H
ADIP	Afadin- and alphaactinin-binding protein	assembles intercellular adherence junctions	I
HMMR	Hyaluronan-mediated motility receptor	Promotes focal adhesion turnover and correlates with cellular locomotion	J
ARHGEF10L	Rho guanine nucleotide exchange factor (GEF) 10-like	Rho-specific GEF	K

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Notably, reciprocal alterations in the expression of two members of the tensin family were observed. This family comprises four proteins (tensin-1 through -3, and cten), which are involved in cell migration, and are localized to focal adhesion sites^{5,6}. All four tensin family members contain SH2 and PTB domains in their carboxyl-termini. Through the PTB domain, tensins interact with the cytoplasmic tail of β integrins, which are transmembrane cell adhesion molecules, whereas the SH2 domain mediates interactions with tyrosine-phosphorylated proteins that participate in adhesion complexes. While tensin-1 through -3 contain an actin-binding domain in their amino-termini, cten (C-terminal tensin-like protein) is a shorter protein lacking this domain⁶. According to the present analysis, following EGF-treatment, TNS3 (tensin3) mRNA undergoes down-regulation, while TNS4 (cten) expression exhibits remarkable up-regulation that initiates one hour after EGF stimulation and reaches a 25-fold induction after eight hours. Notably, expression of TNS1 (tensin1) is barely detectable, while the moderate expression of tensin2 is unchanged following EGF treatment (Figures 1a). Immunoblotting analysis confirmed induction of cten expression at the protein level (Figures 2c). In addition, cellular adhesion, a signal known to allow cellular migration¹², remarkably induced cten expression through a mechanism that does not involve autocrine EGFR activation (Figures 2d).

In conclusion, these results raise suggest a reciprocal regulation of cten and tensin3 by EGF plays general roles in cell adhesion and migration.

EXAMPLE 2

EGF-induced migration of mammary cells involves down-regulation of tensin3, along with up-regulation and reorganization of cten

RESULTS

The normal human mammary cell line, MCF10A, has been characterized as a cellular model for EGF-induced migration regulated by the Erk/MAPK cascade¹³. As shown in Figure 3a, migration of MCF10A cells through Transwell membranes is dependent upon the presence of EGF in the growth medium, while serum factors are insufficient for migration induction. However, proliferation of these cells is independent of EGF (data not shown). Similarly to the observations made in HeLa cells, treatment of MCF10A cells with EGF resulted in induction of cten and reduction in tensin3 levels (Figures 1b, 1d), but no expression of tensin1 or tensin2 was observed in these cells (data not shown). Because a Mek inhibitor (U0126) completely blocked cten induction, but inhibition of either protein kinase C (with GF109203) or p38 (using SB202190) was ineffective (Figures 1c), it was concluded that Erk activity is essential for the induction of cten by EGF. The connection between Erk activity and increased expression of cten suggests that the reported Erk/MAPK-driven migration of MCF10A cells¹³ may be dependent on induction of regulatory proteins like cten.

In agreement with previous immuno-localization studies⁶, cten was localized to paxillin-containing focal adhesion sites of un-stimulated cells (Figures 1e, upper row). Remarkably, along with EGF-induced cten expression (Figures 1e; induction is not apparent due to normalization of the immunofluorescence signal) and dissociation of cellular colonies, cten localization was no longer restricted to focal adhesion sites, but redistributed throughout the cytoplasm. It was assumed that cten's increased cytoplasmic localization overrides the staining signal of adhesion sites, and indeed biochemical analysis confirmed an EGF-induced enrichment of cten in both the detergent- soluble fraction and in the insoluble fraction, the latter is known to include actin-associated proteins (Figures 3b). In parallel, actin stress fibers, which are normally anchored at paxillin-containing focal adhesion sites, disintegrated upon EGF treatment and reorganized in cortical bundles expanding outward from focal adhesion sites (Figure 1e; lower row). Similar analyses indicated that tensin3 is predominantly localized in cell-matrix adhesions of untreated cells, and it largely insoluble and disappears following EGF-stimulation (Figures 3b, 3c).

These results indicate that EGF-induced up-regulation of cten and down-regulation of tensin3 correlate with actin fiber remodeling, which may assist cell spreading.

EXAMPLE 3***Mammary cell migration is inhibited upon knockdown of cten and increased following tensin3 knockdown*****RESULTS**

To address the dependence of EGF-induced cell migration on the levels of cten and tensin3, endogenous cten and tensin3 were depleted using pre-tested, specific short interfering RNA (siRNA; Figures 4a). In the absence of EGF, MCF10A cells form dense cell clusters, whereas EGF-treatment induces cell scattering (Figures 4b). In contrast, upon depletion of cten, EGF-stimulated cells presented large, densely packed colonies, which resembled the morphology of cells grown in the absence of EGF. Fluorescent labeling revealed that depletion of cten resulted in restoration of a dense network of actin fibers, despite the presence of EGF in the growth medium (Figure 4c), similar to the pattern observed in untreated cells (Figure 4c, lower row). On the other hand, stress fibers were observed neither in neighboring cells that retained cten expression (cells labeled with asterisks in Figure 4c), nor in EGF-treated cells transfected with control oligonucleotides (siCont; Figure 4c). Moreover, depletion of cten caused a 70 % reduction in EGF-driven cell migration (Figure 4d).

In conclusion, EGF-driven cell movement is dependent upon induction of cten, and associates with reorganization of the actin cytoskeleton.

Apart from inducing expression of cten, EGF drives a reduction in the expression of tensin3 (Figs. 1a, 1d). Cells in which tensin3 was stably depleted displayed colony scattering and actin stress fiber disintegration, even in the absence of EGF (Figures 4e). In addition, under these conditions tensin3 depletion was sufficient to promote partial cell migration (23% of that promoted by EGF; Figures 4f). Conceivably, down-regulation of tensin3 complements EGF-induced up-regulation of cten, and together they instigate reorganization of the actin cytoskeleton and cell motility.

EXAMPLE 4***Cten expression disrupts actin stress fiber organization*****RESULTS**

To resolve the relationships between cten expression and actin stress fiber reorganization, protein chimeras which comprise a green fluorescent protein (GFP) fused to the amino-terminus of full-length cten were used (Figures 5a). Ectopic expression of GFP-cten in un-stimulated MCF10A cells caused extensive disassembly of stress fibers in 60 % of GFP-cten expressing cells (n=28; Figures 5b). Importantly, neighboring cells that expressed no GFP-cten retained intact networks of actin stress fibers. Thus, enhanced expression of cten

is sufficient for induction of one aspect of the multi-faceted EGF-induced motility of MCF10A cells, namely: disassembly of actin stress fibers.

Actin stress fibers are stabilized by interactions with integrin-centered focal adhesion sites ¹⁴, a process in which tensins have been implicated ⁵. Indeed, up-regulation of tensin induces formation of stress fibers ¹⁵, whereas cleavage of tensin by caspase-3, induces disintegration of stress fibers ¹⁶. Structurally, cten resembles a truncated form of tensin, lacking the actin-binding domain, but sharing the integrin-binding domain (PTB) with other tensins ⁶. Hence, it was speculated that cten acts as a competitive inhibitor, which blocks binding of PTB-containing proteins to integrins. In line with this proposition, overexpression of a cten mutant lacking the PTB domain (Figures 5a; GFP- Δ PTB-cten) did not significantly affect the organization of actin stress fibers (Figures 5c). Moreover, in similarity to the effect of cten, ectopic expression of a truncated mutant form of tensin1 lacking the actin-binding domain (Figures 5a; GFP-CT-tensin1), resulted in disassembly of the actin stress fibers in 75% of GFP-CT-tensin1 expressing cells (n=24; Figures 5d), while overexpression of full-length tensin1 exerted no significant effect (Figures 5e).

In conclusion, the ability of cten to disassemble actin stress fibers is mediated by the integrin-binding, PTB domain.

EXAMPLE 5

Integrin beta-1 is essential for EGF-induced motility and cten competes with tensins on binding to Integrin beta-1

RESULTS

The above results indicate that cten regulates the integrity of stress fibers, as well as cell migration, by binding to integrins. Integrins β 1, 4, and 8 are highly expressed in MCF10A cells (data not shown), but analyses using blocking antibodies or siRNAs identified integrin β 1 as the subtype most relevant to both adhesion and migration of MCF10A cells (Figs. 6a, 6b). Indeed, depletion of integrin β 1 resulted in complete loss of stress fibers (Figures 6c). Conceivably, mutually competitive binding of tensin3 and cten to integrin β 1 may determine the pattern of stress fibers. In line with this model, cten co-localizes with integrin β 1 in cell-matrix adhesions (Figures 6d), but cten dissociates from these sites when integrin β 1 is depleted (Figures 7a; note the punctate distribution of cten in control cells). Moreover, *in vitro* analyses not only verified PTB-mediated interactions between the cytoplasmic tail of integrin β 1 and both cten and a soluble form of tensin3 (Δ N-tensin3; Figures 7b), but also established competitive relationships (Figures 7d) that presumably enable

cten to displace tensin3 from the tail of integrin $\beta 1$ in intact cells. In experiments which are not presented, displacement of tensin3 from adhesion sites were observed in living MCF10A cells, upon forced enhancement of cten expression.

Retention of stress fibers by cten-depleted EGF-stimulated cells (Figures 4c) may reflect recruitment of PTB-containing, actin-binding proteins (e.g., tensins) to the tail of integrin $\beta 1$, as it is no longer capped by cten. To examine this model, and also confirm that cten is positioned downstream to integrin $\beta 1$, cten and integrin $\beta 1$ were simultaneously knocked-down in MCF10A cells, which were then stimulated with EGF. The observed double knock-down phenotype was strikingly similar to that of integrin $\beta 1$ depletion (cell scattering and filopodia-like structures, along with disappearance of stress fibers and almost no cortical actin), and remarkably different from that of cells deficient only in cten (Figures 7e). Thus, the phenotypic dominance of integrin $\beta 1$ depletion strongly identifies this adhesion molecule as the target of cten, in line with a 'tensin-cten switch' model.

EXAMPLE 6

Cten expression in mammary tumors associates with EGFR activation, and undergoes down-regulation in patients treated with an EGFR-specific kinase inhibitor

Aberrant expression and activation of EGFR are richly implicated in breast cancer progression, and are associated with formation of metastases ^{1,7}. Inflammatory breast cancer (IBC) is a highly aggressive form of mammary cancer ¹⁷, that often overexpresses ErbB2 and manifests a large degree of lymphovascular invasion ¹⁸. Using a monoclonal antibody generated against cten and a cohort of ErbB2-positive IBC patients (n=18), cten expression was detected in 56% of tumor biopsies (Figure 8a). Remarkably, a strong association was observed between EGFR activation, as determined by using antibodies to phosphorylated EGFR (pEGFR), and cten expression (p<0.0001, Table 2 below).

Table 2

	<i>Patient Number</i>	<i>Cten Positive (N=10) Patient Number Association with Cten positivity</i>	
	Total = 18	(%)	p-value
ErbB-2 positive	18	56	
EGFR positive	14	71	0.023
Phospho-EGFR positive	11	91	<0.0001



This association was studied *in vitro* by treating cultured IBC cells, AU-565, with a selective dual EGFR/ErbB2 tyrosine kinase inhibitor (TKI; GW-2974). In these cells low basal expression of cten was observed that was highly induced following incubation with EGF. Yet, upon TKI treatment a reduction in the basal expression of cten was observed, as well as inhibition of EGF-mediated cten induction (Figures 8b). The potency of this TKI is apparent from the complete inhibition of EGFR phosphorylation following treatment with EGF in the presence of GW-2974 (Figures 8b). Furthermore, because a structurally related dual specificity TKI, lapatinib (Tykerb), is currently tested in clinical trials, the effect on cten in sequential IBC biopsies obtained prior to and during lapatinib monotherapy was tested. Representative immunohistochemical analyses of tumor biopsies from the same patient, before and after a 21-day long treatment with the drug, are presented in Figure 8c. Similar analyses performed on biopsies obtained from 11 phospho-EGFR positive inflammatory breast tumors revealed statistically significant down-regulation of cten expression in most tumors (Figures 8d; $p=0.002$). These observations are in line with the previously reported reduction in pEGFR, pErbB2 and pErk following lapatinib (Tykerb) treatment^{19,20}.

It may thus be concluded that cten expression in breast cancer is highly dependent on EGFR activation, a conclusion that may relate to other tumors. Indeed, Figure 10b shows that high cten expression can predict poor prognosis of ovarian cancer patients (see Example 7 below).

The transition of an epithelial sheet into motile cells is a complex process, involving disassembly of actin stress fibers and cell-cell adhesion sites, while cortical actin bundles and new focal adhesion sites form at the leading edge of the cell^{2,21}. The present findings propose that EGF regulates actin remodeling by the following mechanism (see Figures 8e). In resting mammary epithelial tissues, the polarized cell structure is maintained by actin stress fibers that are stabilized by focal adhesion sites¹⁴. This stabilization is mediated by tensin3, which can interact with actin fibers (through its amino-terminus), as well as with the cytoplasmic tail of beta 1 integrin⁵. Accordingly, dissociation of the integrin-tensin-actin complex would disintegrate stress fibers. To ensure effective dissociation, a pre-requisite for cell migration, EGF activates two complementary processes: tensin3 levels are reduced, while concomitantly cten levels are up-regulated, thereby capping the tail of integrin (Figures 1).

Actin stress fibers within the epithelial sheet are stabilized by both cell-matrix and cell-cell adhesion sites. It is likely that under physiological conditions cell migration will result from alterations in both adhesion sites. In analogy to the 'tensin-cten switch' reported herein, a molecular switch has been described within cadherin-based cell-cell adhesion sites,

whereupon during cell migration the epithelial cadherin (E-cadherin) is replaced by the mesenchymal cadherin (N-cadherin)²⁵. As tensin3 depletion results in cell migration of lower magnitude than that promoted by EGF treatment, this suggests that the full EGF-induced motile response comprises alteration of both cell-cell and cell-matrix interactions.

The transition from well-organized epithelial sheets into invasive carcinoma critically depends on re-organization of the actin cytoskeleton²⁶. The present results raise the possibility that the observed up-regulation of cten contributes to actin reorganization and, eventually, to mammary tumor invasion. In line with this proposition, preliminary examination of human mammary xenografts, whose tensin3 levels have been elevated, revealed reduced ability to invade into nearby lymph nodes (Figures 9a-c). Moreover, dynamic changes in cten expression may not be restricted to mammary carcinoma progression; cten is up-regulated also in thymomas and in lung cancers^{27,28}, but in prostate cancers it is down-regulated⁶. The association in mammary tumors between cten expression and active EGFR (Figures 8a), as well as the observed reduction in cten expression in lapatinib-treated patients (Figures 8c, d), suggest that the 'tensin-cten switch' uncovered herein may serve as a marker for EGFR-driven tumors, as well as a component of a protein signature capable of predicting patient response to EGFR-targeted therapy.

EXAMPLE 7

Over-expression of tensin 3 delays primary tumor development and lymph node metastasis

To address the effects of cten and tensin3 in an animal model, the highly invasive MDA-MB231 mammary cancer cell model was used. Since these cells express relatively low levels of cten, and knockdown of tensin3 exerts no effect on cell migration in vitro (data not shown), the effect of a stably overexpressed ectopic tensin3 was tested. The results presented in Figures 9a-b show that tensin3 inhibits migration of MDA-MB231 cells in a Transwell assay, while moderately enhances the rate of cell growth.

To allow in vivo imaging, red fluorescence protein (RFP) was co-expressed with tensin3 or alone in MDA-MB231 cells. Cells were inoculated into the mammary fat pads of 6-week-old female CB-17 severe combined immunodeficient (SCID) mice [see M. Dadiani et al., (2006) Cancer Research 60:8037-41, herein incorporated by reference]. Primary tumor growth (Figure 9c) and metastasis to ipsilateral and contralateral inguinal lymph nodes (Figure 9d) were monitored for 45 days. As shown, although tensin3 overexpression enhanced growth rates in vitro, the corresponding cells formed smaller primary tumors and their migration to ipsilateral and contraateral lymph nodes was delayed (see Figure 9e).

Thus, a mouse xenograft model of breast cancer confirms the ability of tensin3 to retard cellular invasion. Interestingly, tensin3 also inhibits tumor growth, probably because the orthotopic mammary model used herein involves invasion of the primary tumor into surrounding tissues.

EXAMPLE 8

Cten expression correlates with cancer malignancy

Affymetrix arrays (Human Genome U133 plus 2) in which *cten* is represented by two probe sets: 222265_at; 230398_at) and *tensin3* is represented by four probe sets: 217853_at; 234257_at; 236167_at; 237902_at were used to uncover association between gene expression and cancer stage. As shown in Figure 10a, a significant association between *cten* expression and malignancy in ovarian tumors was demonstrated.

The anti-*cten* mouse monoclonal antibody generated according to the teachings of the present invention (see above experimental procedures), was used to analyze a cohort of 356 breast cancer patients. Statistically significant association was found between *cten* expression and histological grade ($p=0.002$), HER2 presence ($p=0.0001$), EGFR presence ($p=0.0004$), and estrogen receptor absence ($p=0.0001$). Interestingly, no association with tumor size has been found. Initial analysis of 92 ovarian cancer patients, showed weak association of *cten* with shorter patient survival (Figure 10b).

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference

in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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WHAT IS CLAIMED IS:

1. A method of inducing cell migration, the comprising contacting the cell with:
 - (a) an agent capable of upregulating activity and/or expression of cten; and
 - (b) an agent capable of downregulating activity and/or expression of tensin-1 or tensin-3,thereby inducing cell migration.
2. A method of inhibiting cell migration, the method comprising contacting the cell with:
 - (a) an agent capable of downregulating activity and/or expression of cten; and
 - (b) an agent capable of upregulating activity and/or expression of tensin-1 or tensin-3,thereby inhibiting cell migration.
3. A method of treating an EGF-responsive tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent capable of down-regulating activity and/or expression of cten in cells of the EGF-responsive tumor, thereby treating the EGF-responsive tumor in the subject.
4. A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of:
 - (i) an agent capable of down-regulating activity and/or expression of cten in cells of the tumor; and
 - (ii) an agent capable of up-regulating activity and/or expression of tensin-3 or tensin-1 in cells of the tumor,thereby treating the tumor in the subject.
5. A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a nucleic acid agent which comprises SEQ ID NO: 14 or 15, said nucleic acid agent being capable of down regulating expression of cten in cells of the tumor, thereby treating the tumor in the subject.
6. A method of staging a tumor in a subject in need thereof, the method comprising determining a level of cten and tensin-3 in cells of the tumor of the subject,

wherein an upregulation of cten above a predetermined threshold and downregulation of tensin-3 below a predetermined threshold are indicative of the stage of the tumor.

7. A method of monitoring treatment of a tumor in a subject in need thereof, the method comprising determining a level of cten and tensin-3 in cells of the tumor of the subject, said subject being treated with an anticancer therapy, wherein downregulation of cten and upregulation of tensin-3 in said cells is indicative of responsiveness of the subject to the anticancer therapy.

8. A method of identifying an EGF-responsive tumor, the method comprising determining a level of cten and tensin-3 in cells of the tumor, wherein an upregulation of cten above a predetermined threshold and downregulation of tensin-3 below a predetermined threshold are indicative of an EGF-responsive tumor.

9. A method of determining an anti-cancer treatment regimen, the method comprising:

- (a) determining a level of cten and tensin-3 in cells of a tumor of the subject
- (b) selecting the anticancer treatment based on said level, thereby determining the anti-cancer treatment regimen.

10. The method of claim 7, wherein said anticancer therapy is EGFR-directed.

11. A method of diagnosing an ovarian or breast cancer in a subject in need thereof, the method comprising determining a level of cten in cells of the subject, wherein an upregulation of cten above a predetermined threshold is indicative of an ovarian or breast cancer.

12. The method of claim 4, 5, 6 or 7, wherein the tumor is an EGF-responsive tumor.

13. An isolated polynucleotide comprising a nucleic acid sequence as set forth in SEQ ID NO: 14.

14. An isolated polynucleotide comprising a nucleic acid sequence as set forth in SEQ ID NO: 15.

15. A pharmaceutical composition comprising as an active ingredient the isolated polynucleotide of claim 13 or 14.

16. An isolated monoclonal antibody comprising an antigen binding domain capable of specifically binding cten and not tensin-3.

17. The isolated antibody of claim 16, is humanized.

18. The method of claim 3 or 5, further comprising administering to the subject a therapeutically effective amount an agent capable of up-regulating activity and/or expression of tensin-3 in said cells.

19. The method of claim 3, wherein the EGF-responsive tumor is not breast cancer.

20. The method of claim 3, wherein the EGF-responsive tumor is selected from the group consisting of head and neck cancer, glioblastoma and lung cancer.

21. The method of claim 2, 3 or 4, wherein said agent capable of down-regulating activity and/or expression of cten is a nucleic acid agent.

22. The method of claim 5 or 21, wherein said nucleic acid agent is selected from the group consisting of:

- (a) a single stranded polynucleotide for inhibiting the transcription of cten;
- (b) a single stranded polynucleotide for hybridizing to the cten mRNA thereby leading to a reduction in cten activity;
- (c) a double stranded polynucleotide, leading to degradation of cten mRNA;
- (d) a triplex forming polynucleotide for cleaving cten mRNA;
- (e) a catalytic polynucleotide for cleaving cten mRNA;
- (f) a single stranded polynucleotide for hybridizing to cten mRNA leading to enzymatic degradation thereof; and
- (g) nucleic acid sequences coding for any one of (a) to (f).

23. The method of claim 22, wherein said nucleic acid agent is selected from the group consisting of an siRNA, a ribozyme and a DNAzyme.

24. The method of claim 5 or 21, wherein said nucleic acid agent is a siRNA.
25. The method of claim 5, 21 or 24, wherein said nucleic acid agent comprises SEQ ID NO: 14 or 15.
26. The method of claim 2, 4 or 18, wherein said agent capable of up-regulating activity and/or expression of tensin-3 is as set forth in SEQ ID NO: 3 or 4
27. The method of claim 1, wherein said agent capable of up-regulating activity and/or expression of cten is as set forth in SEQ ID NO: 1 or 2.
28. The method of claim 1, wherein said agent capable of downregulating activity and/or expression of tensin-3 is as set forth in SEQ ID NO: 16.
29. The method of claim 3, 4 or 5, wherein said administering is effected in situ.
30. The method of claim 6 or 7, wherein said determining is effected ex vivo.
31. The method of claim 6 or 7, wherein said determining is effected in situ.
32. A method of identifying an agent capable or modulating cell migration, the method comprising
- (a) treating a plurality of cells with a plurality of agents; and
 - (b) identifying at least one cell of said plurality of treated cells exhibiting an alteration in an expression ratio of cten to tensin 3 relatively to said ratio in untreated cells, said alteration being indicative of an agent capable or modulating cell migration.
33. A method of inducing cell migration, the comprising contacting the cell with: an agent capable of downregulating activity and/or expression of tensin-1 or tensin-3, thereby inducing cell migration.
34. A method of inhibiting cell migration, the method comprising contacting the cell with: an agent capable of upregulating activity and/or expression of tensin-1 or tensin-3, thereby inhibiting cell migration.

35. A method of treating a tumor in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of: an agent capable of up-regulating activity and/or expression of tensin-3 or tensin-1 in cells of the tumor, thereby treating the tumor in the subject.

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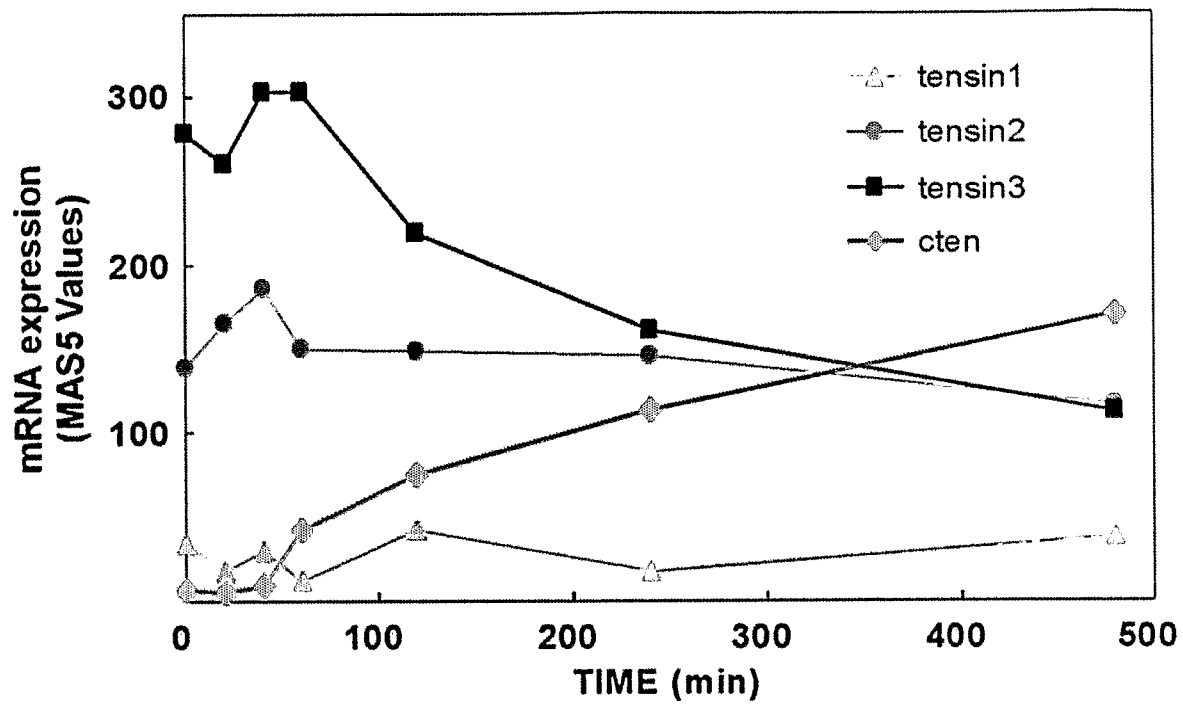


Fig. 1a

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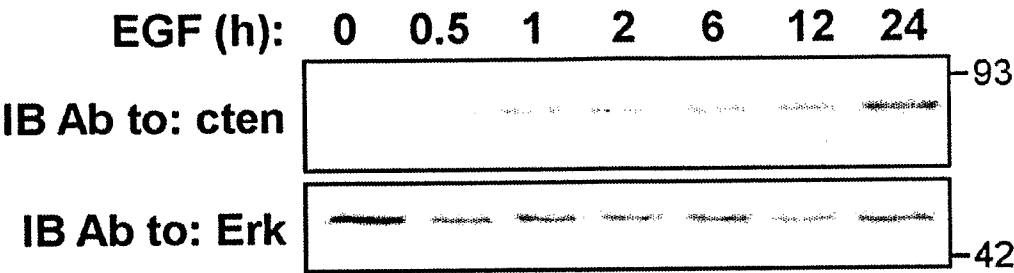


Fig. 1b

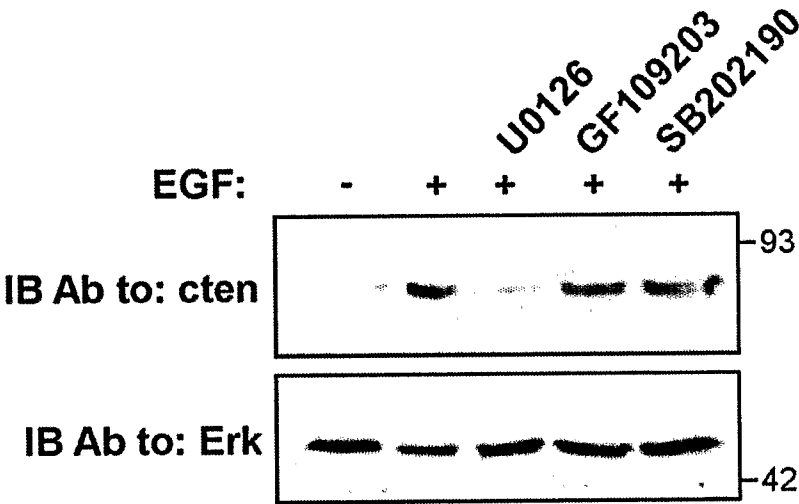


Fig. 1c

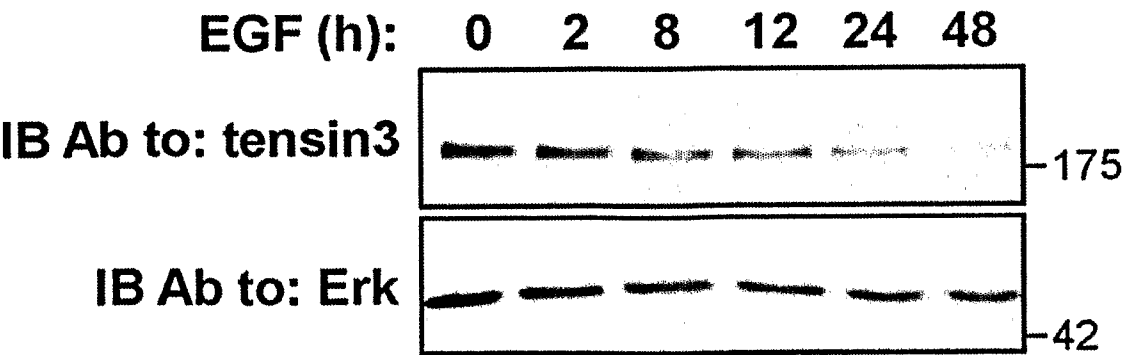


Fig. 1d

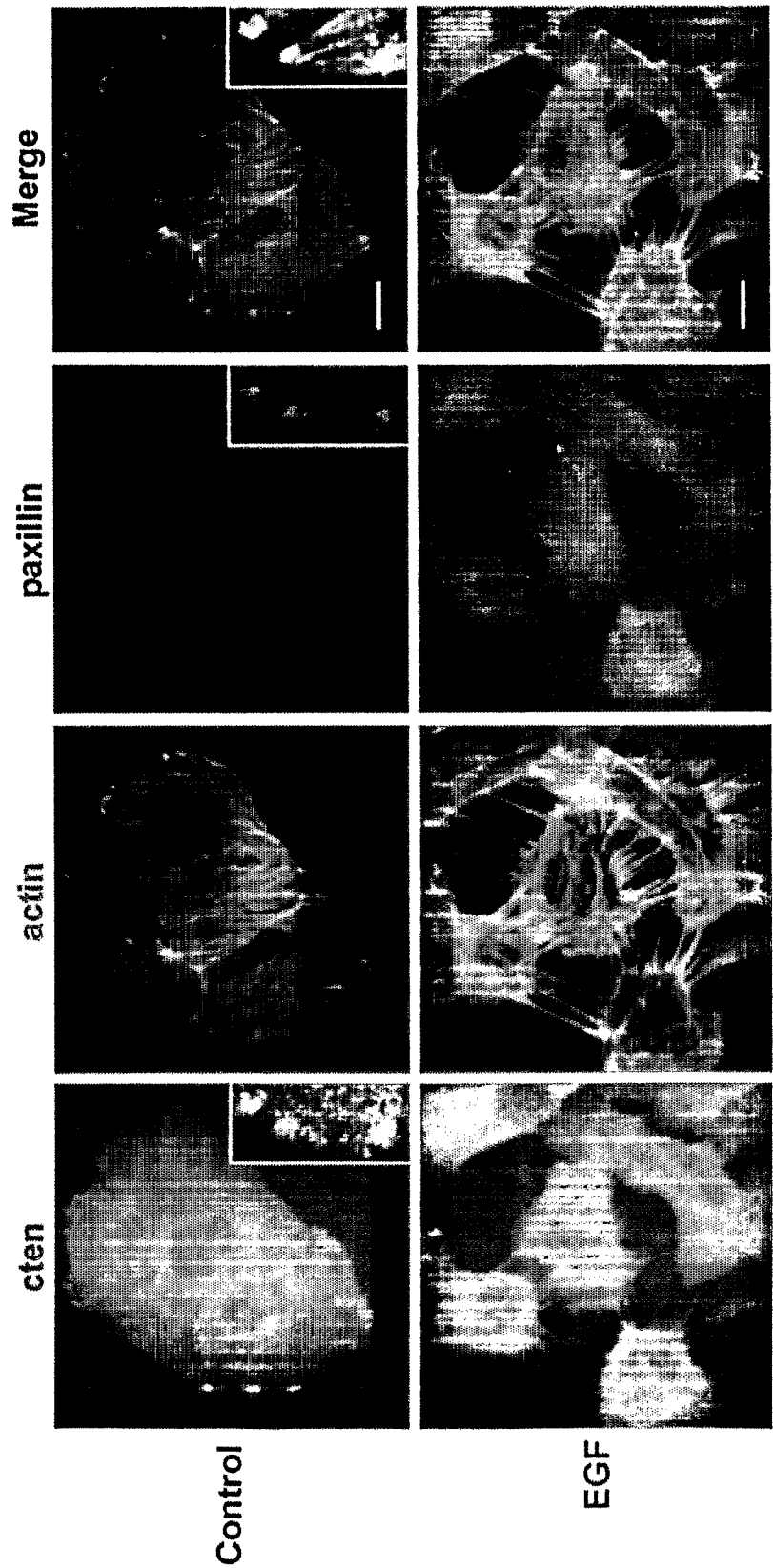


Fig. 1e

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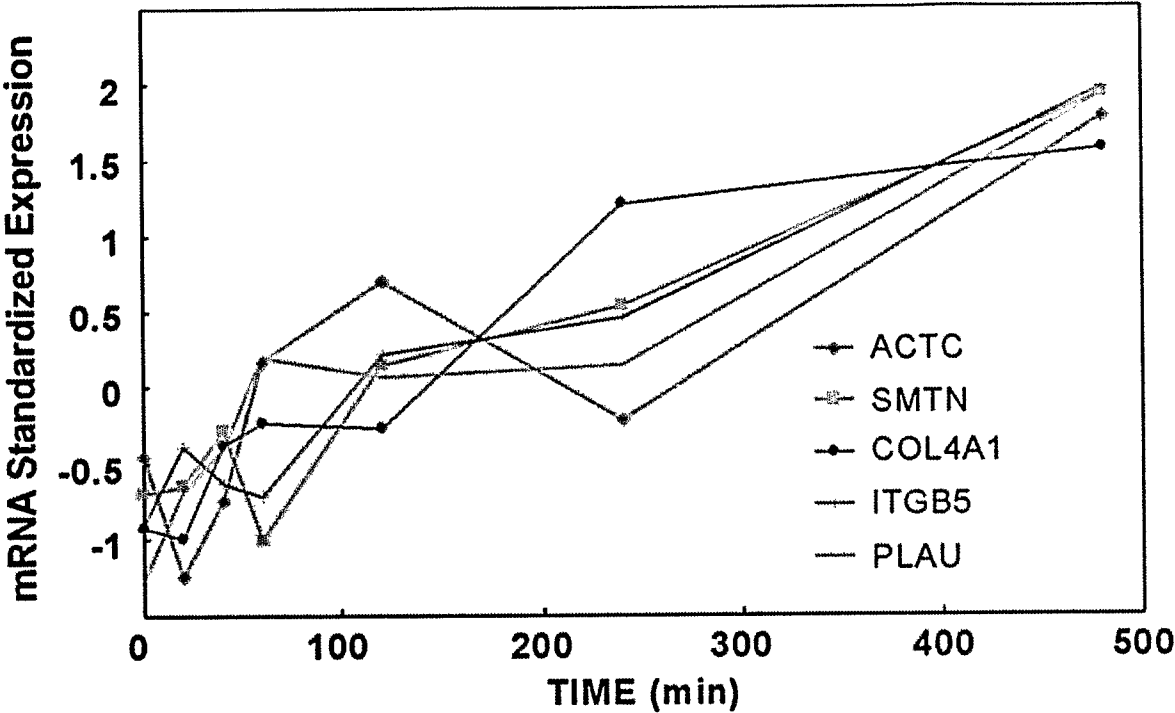


Fig. 2a

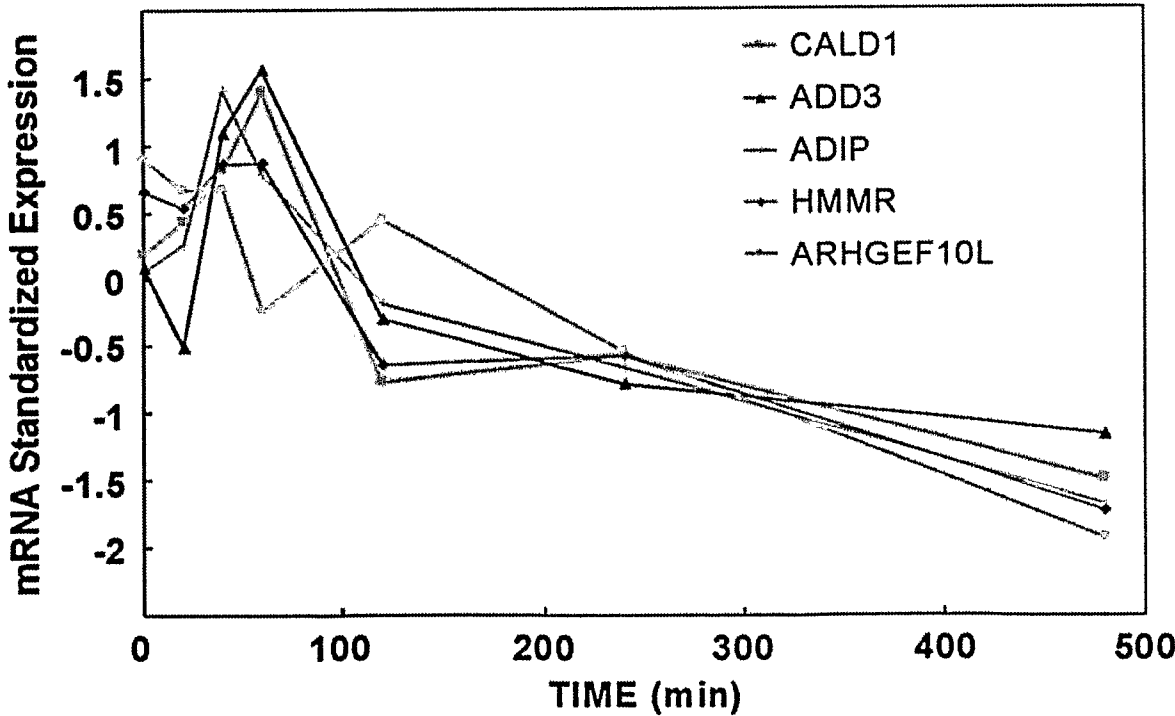


Fig. 2b

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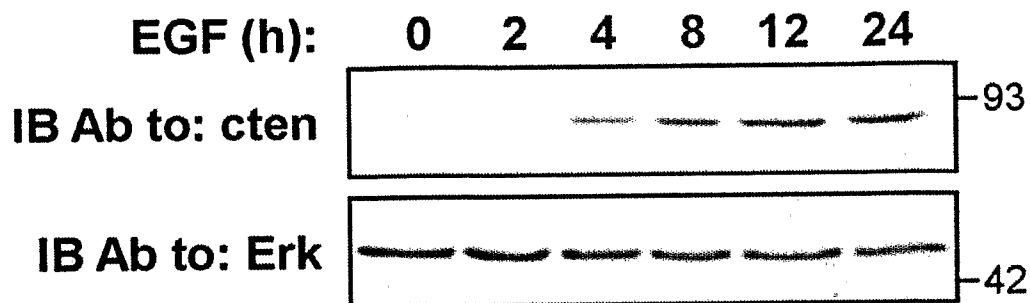


Fig. 2c

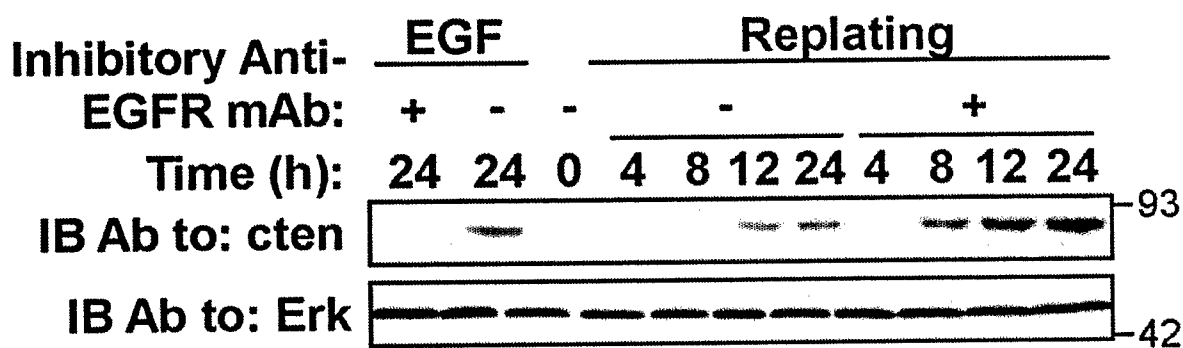


Fig. 2d

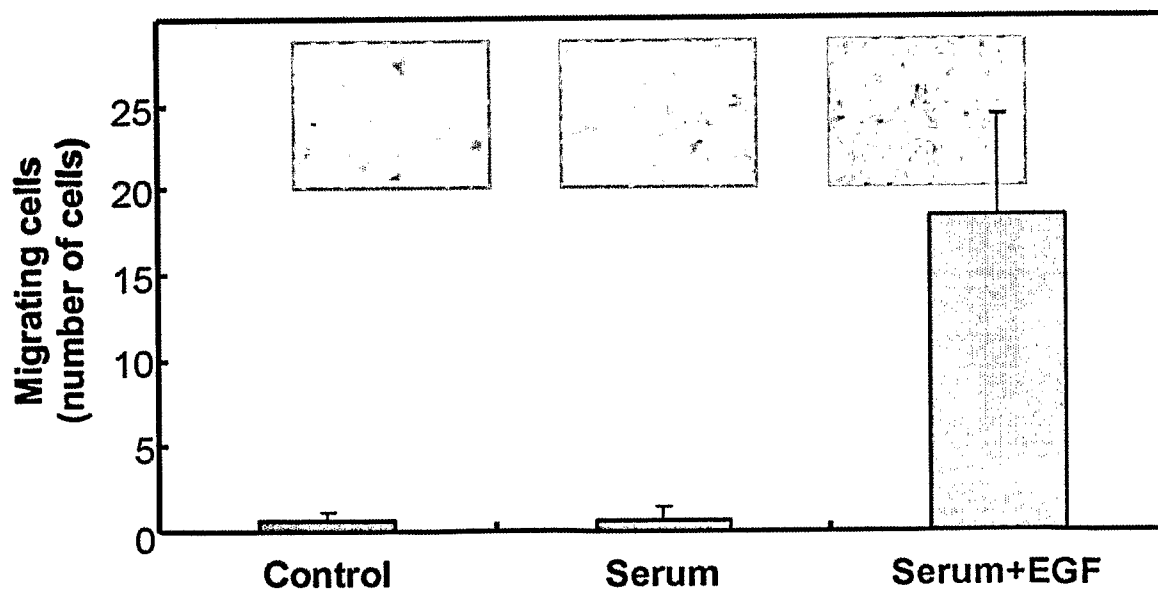
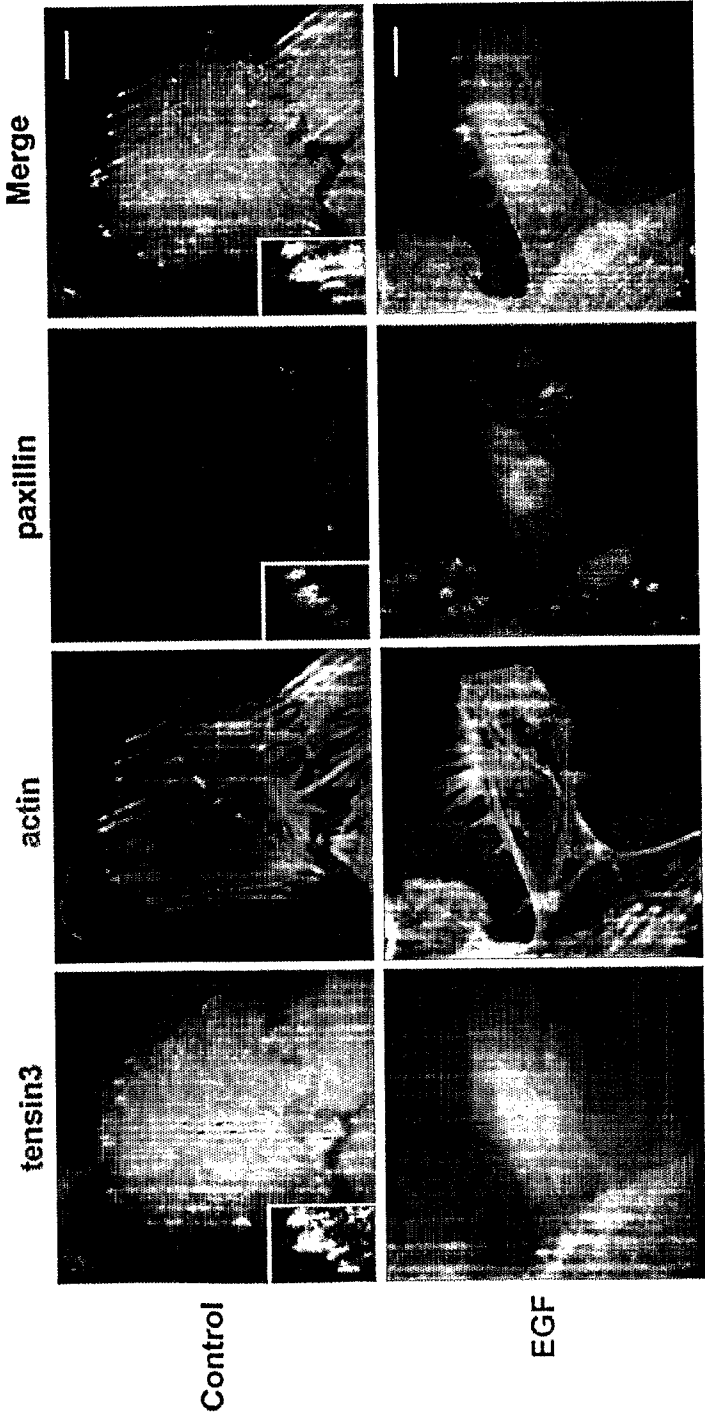
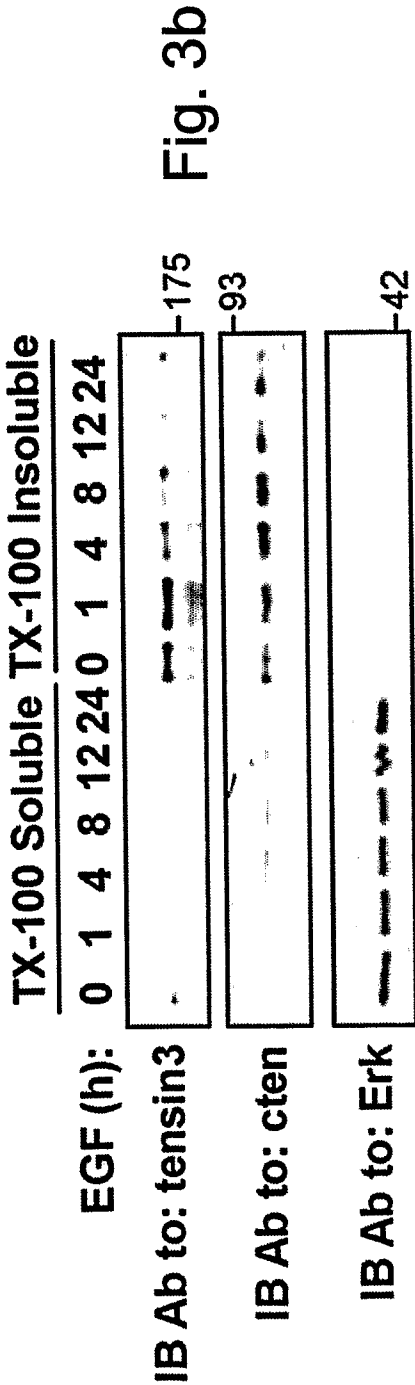


Fig. 3a

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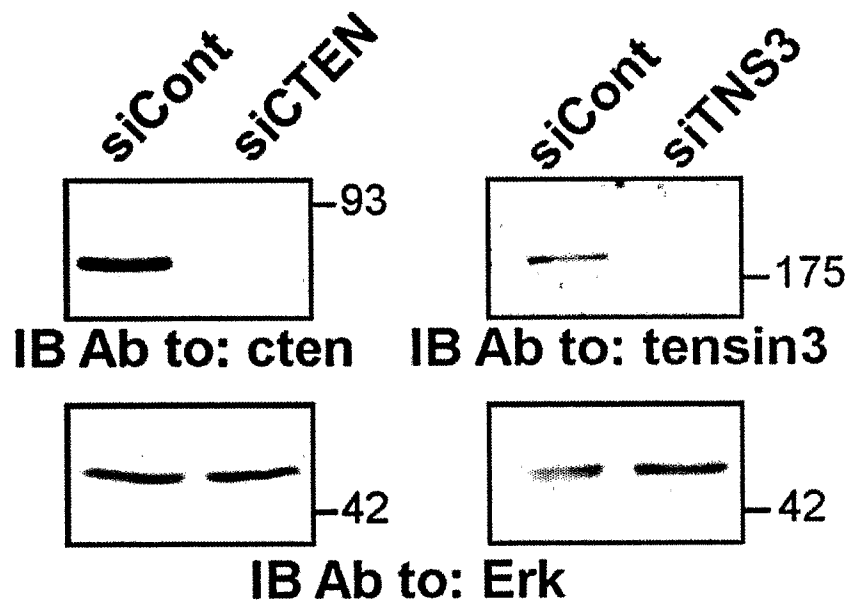


Fig. 4a

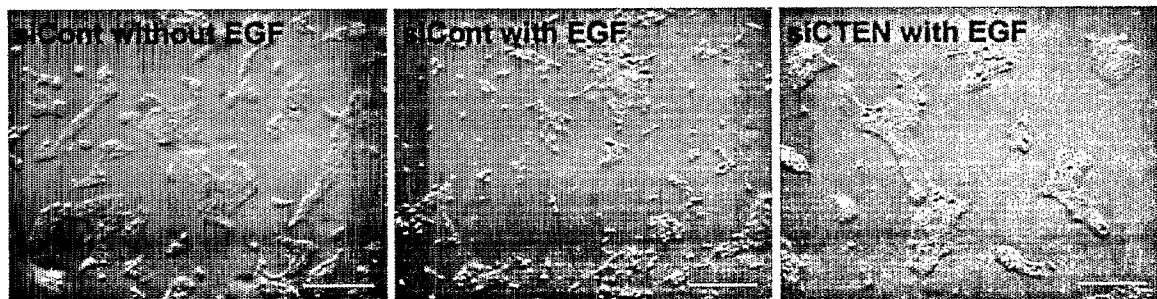


Fig. 4b

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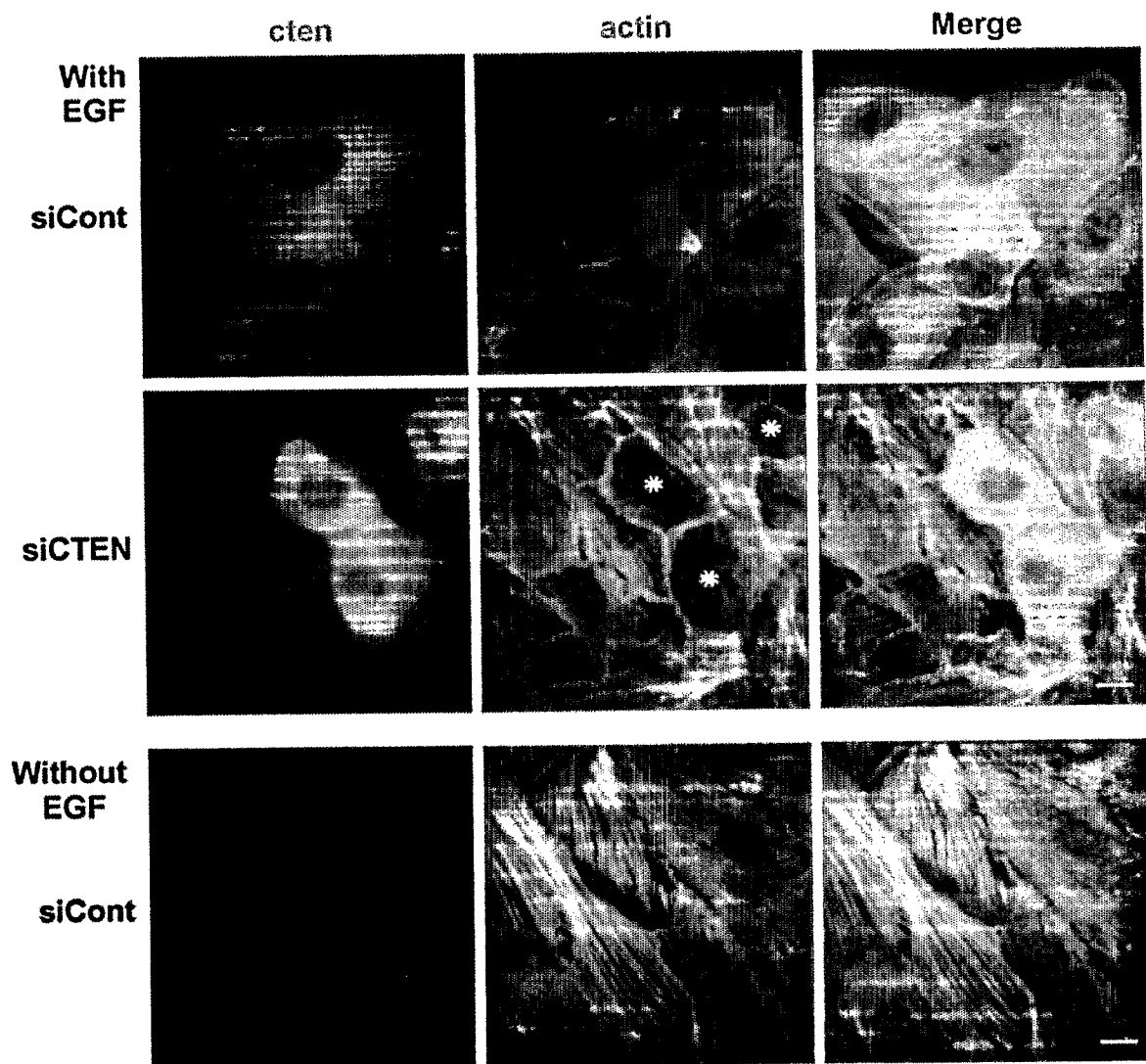


Fig. 4c

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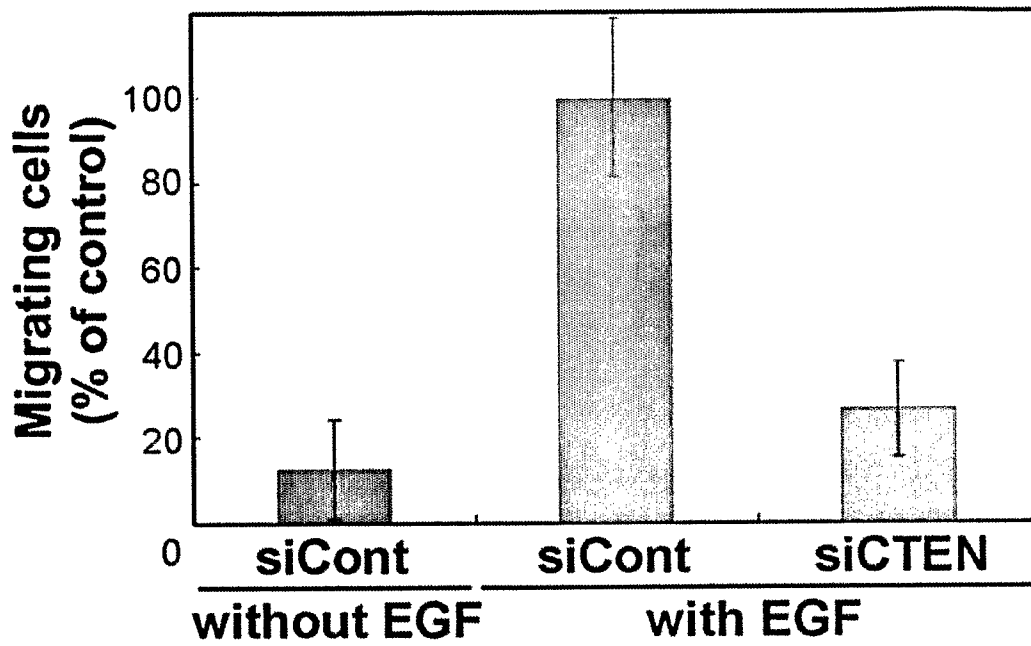


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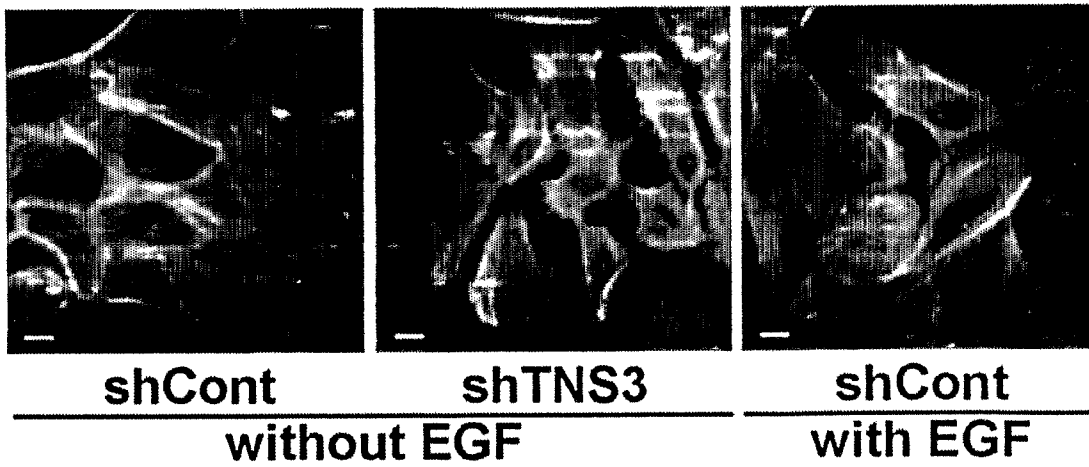


Fig. 4e

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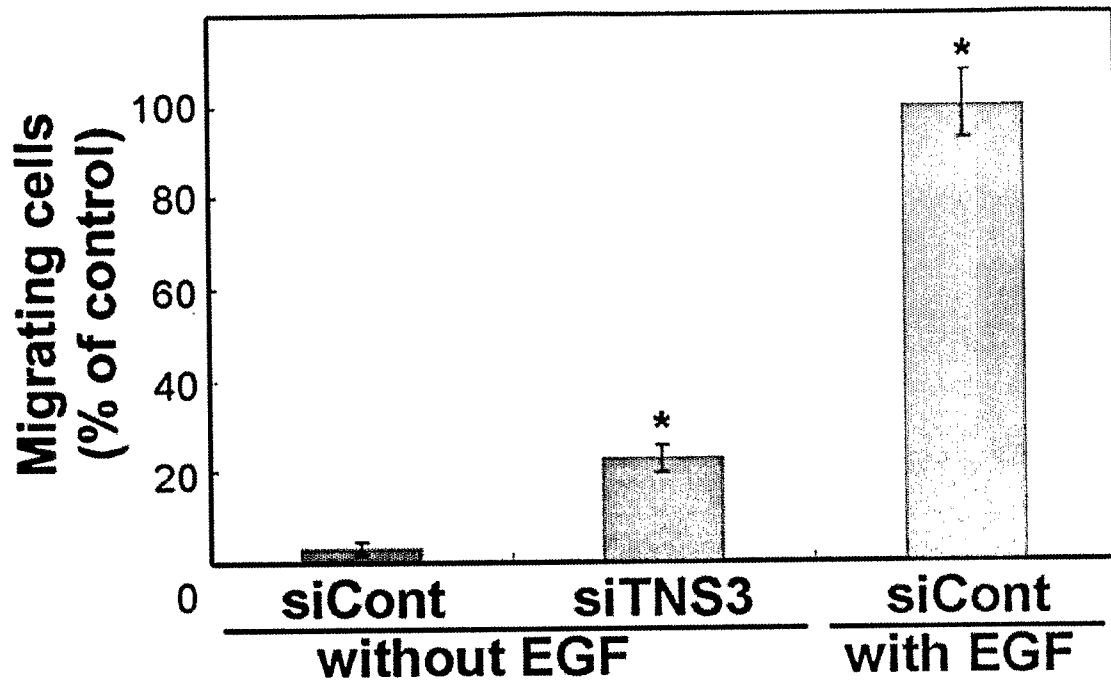


Fig. 4f

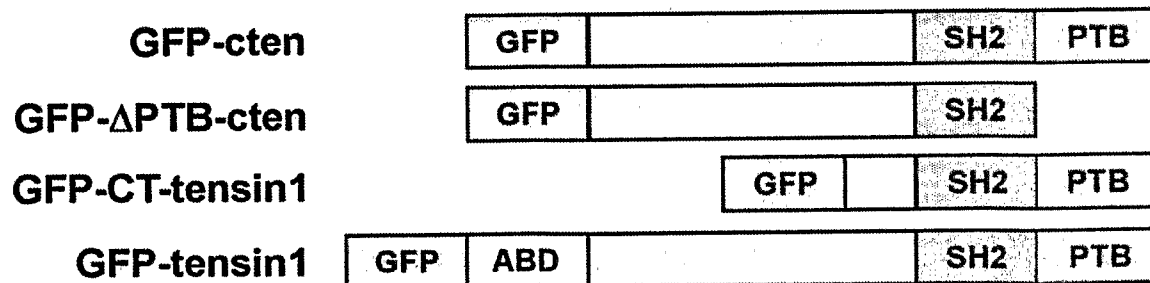


Fig. 5a

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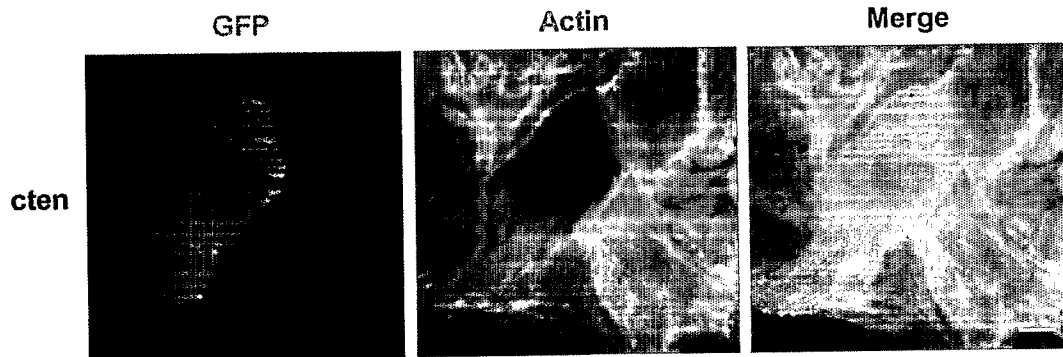


Fig. 5b

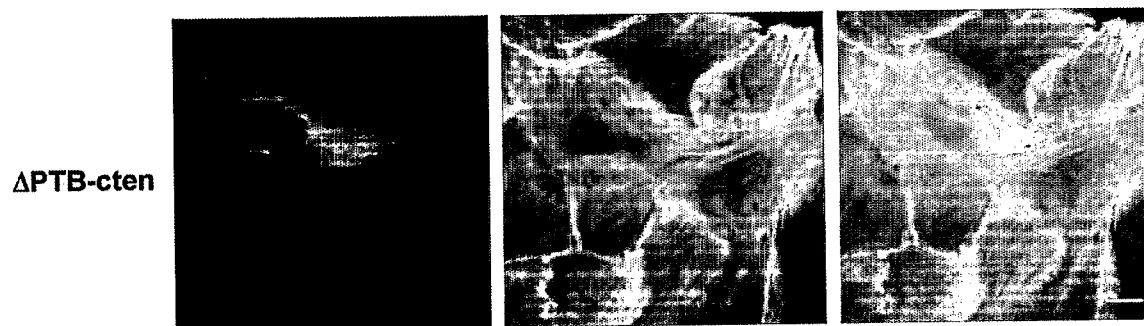


Fig. 5c



Fig. 5d

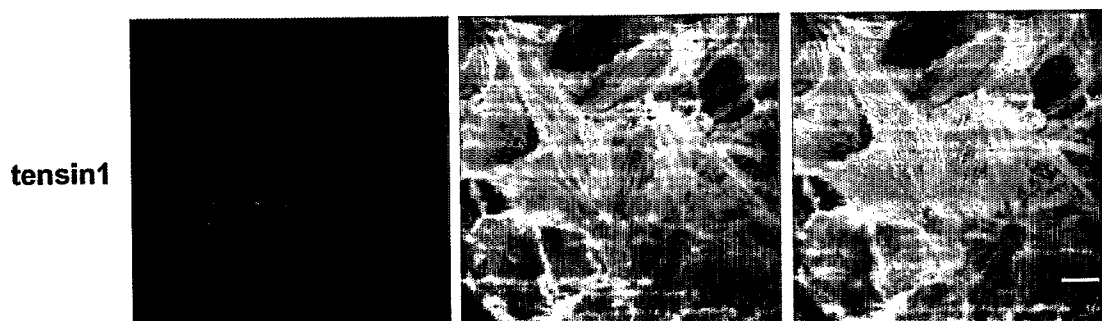


Fig. 5e

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Fig. 6a

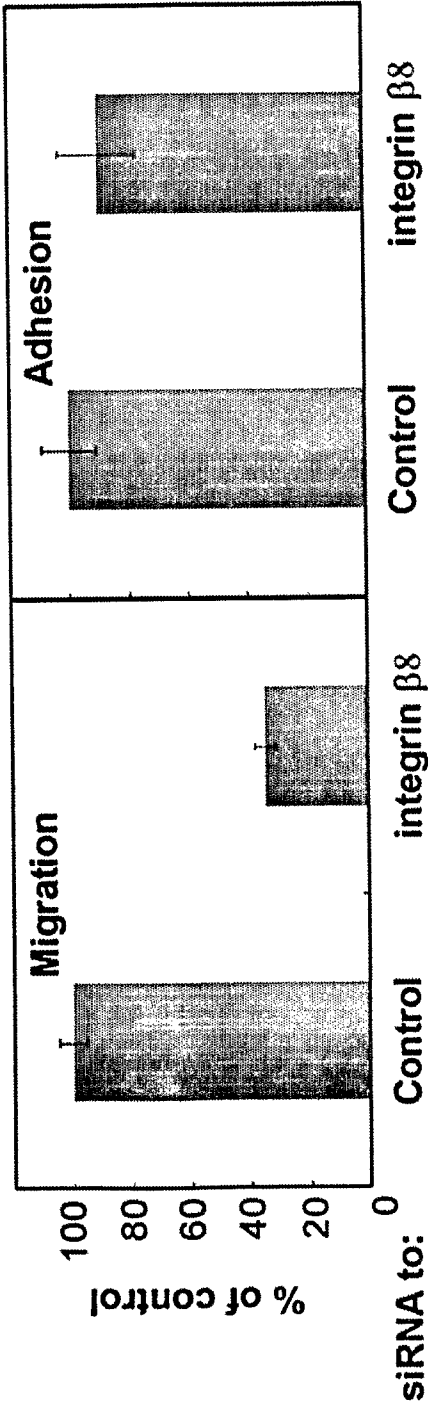
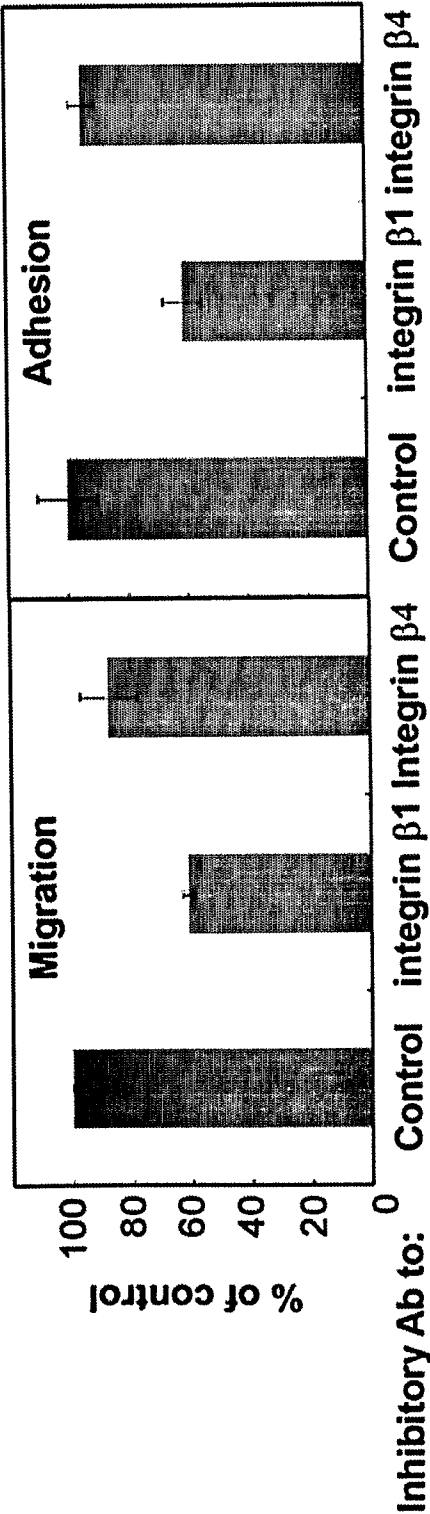


Fig. 6b

Fig. 6c

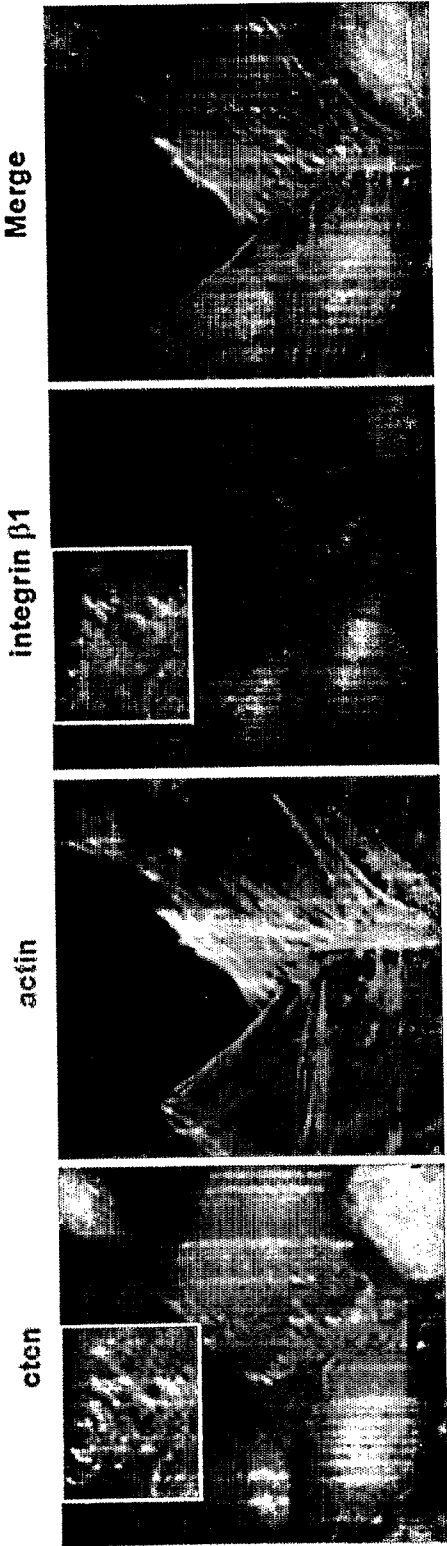
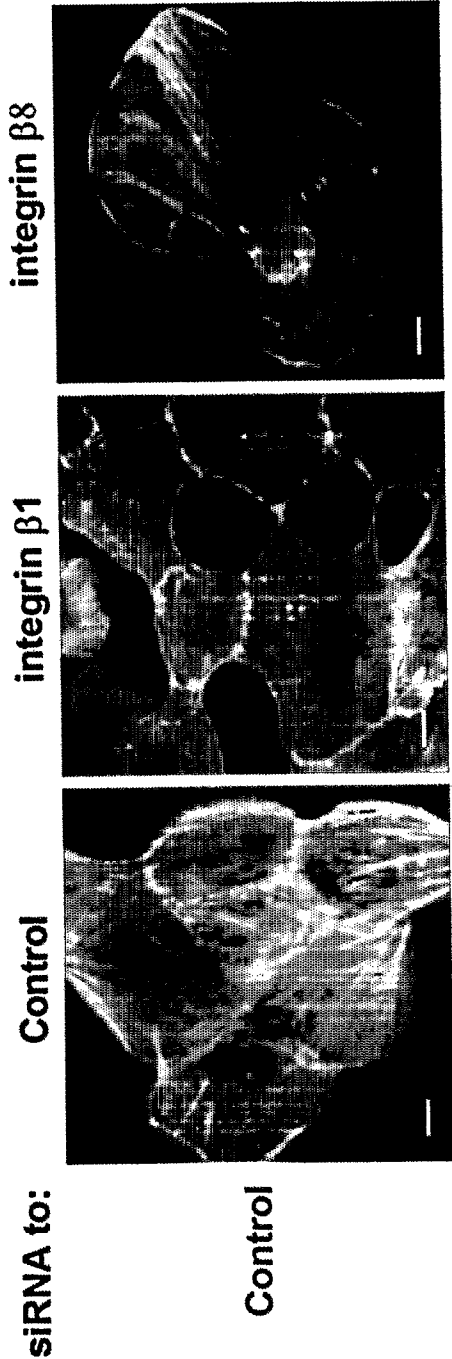


Fig. 6d

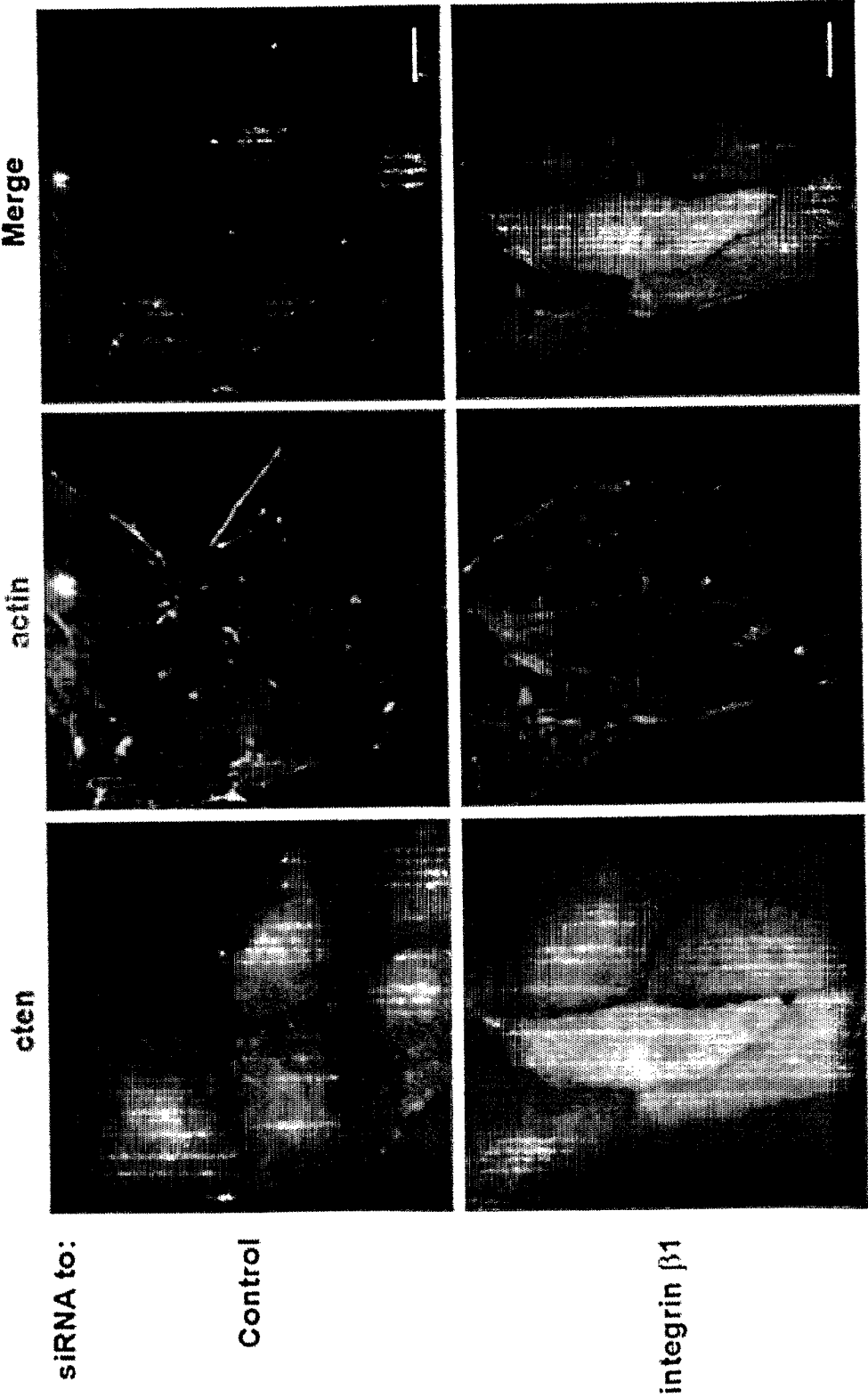


Fig. 7a

Fig. 7b

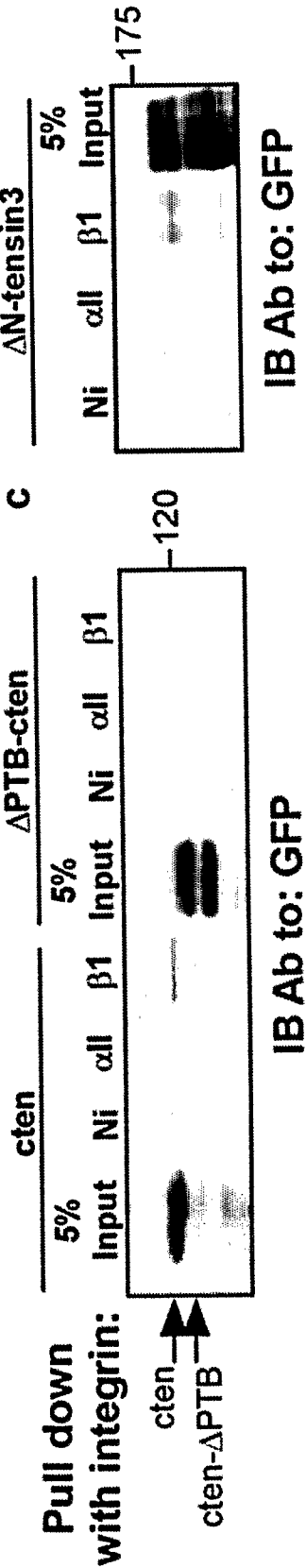


Fig. 7c

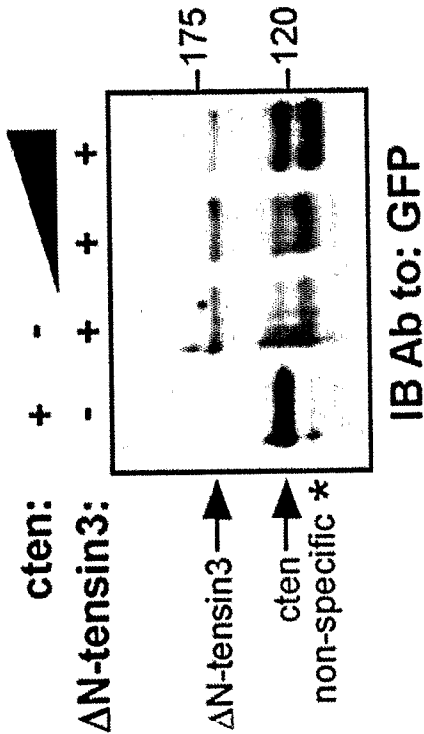


Fig. 7d

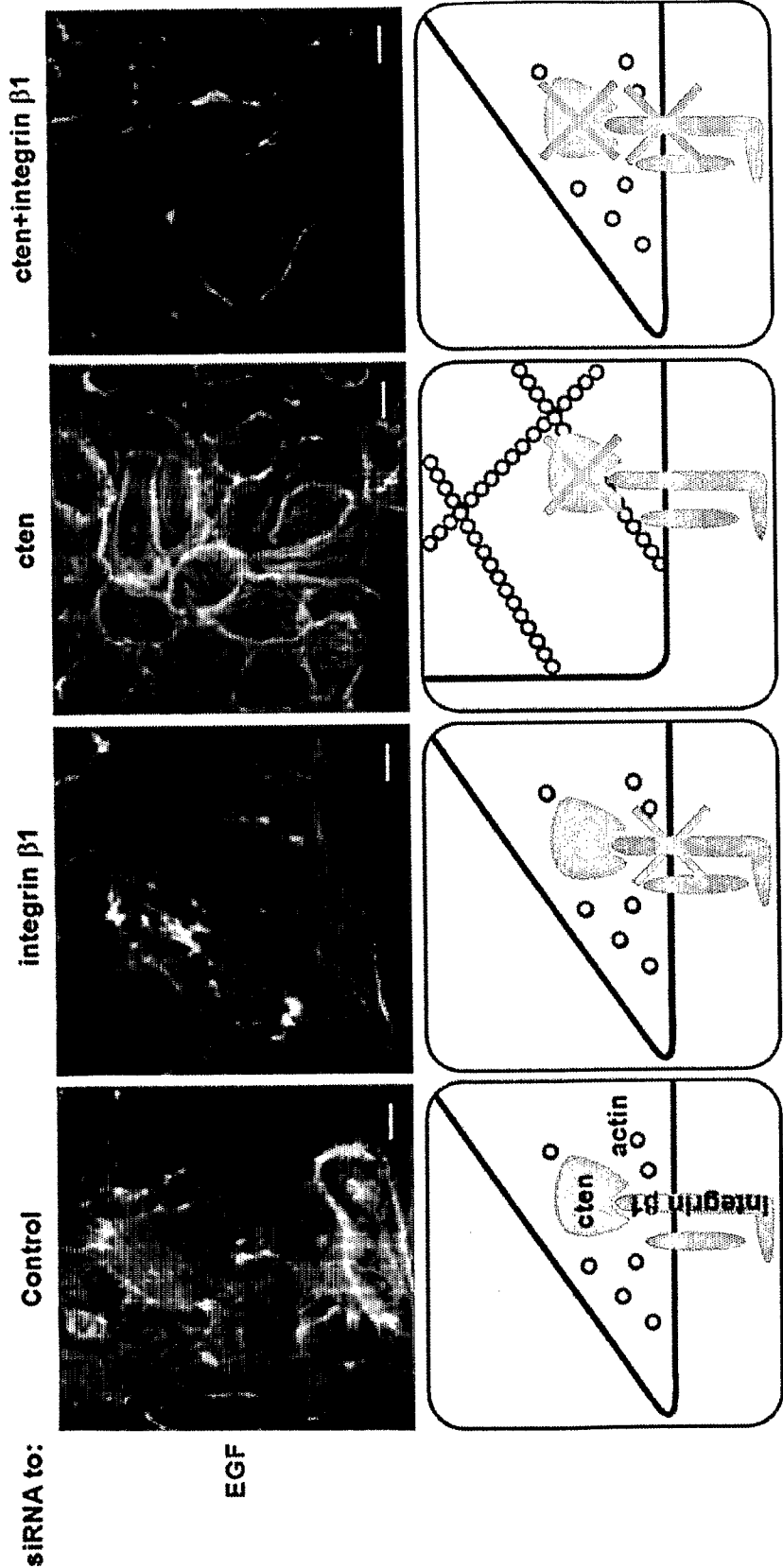


Fig. 7e



Fig. 8a

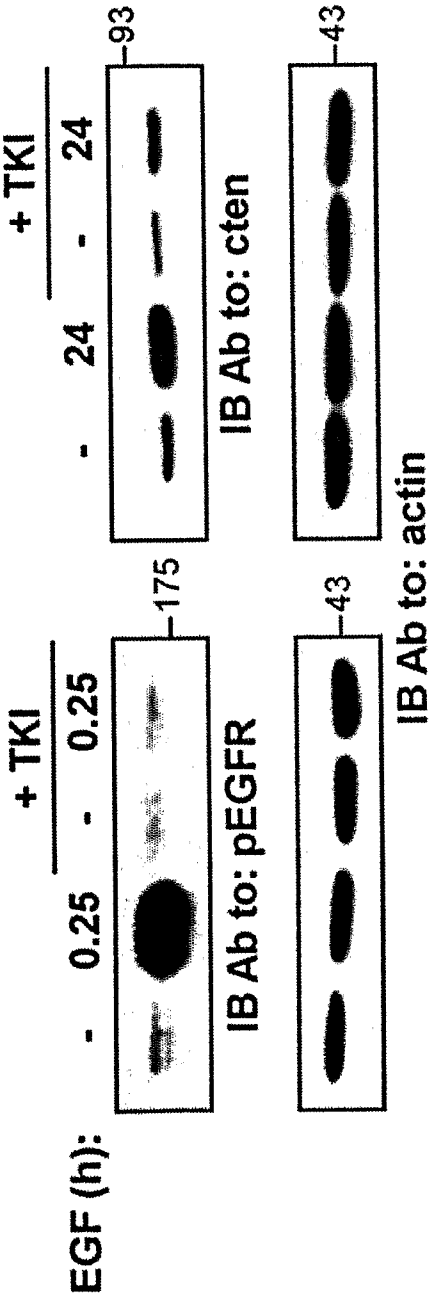


Fig. 8b

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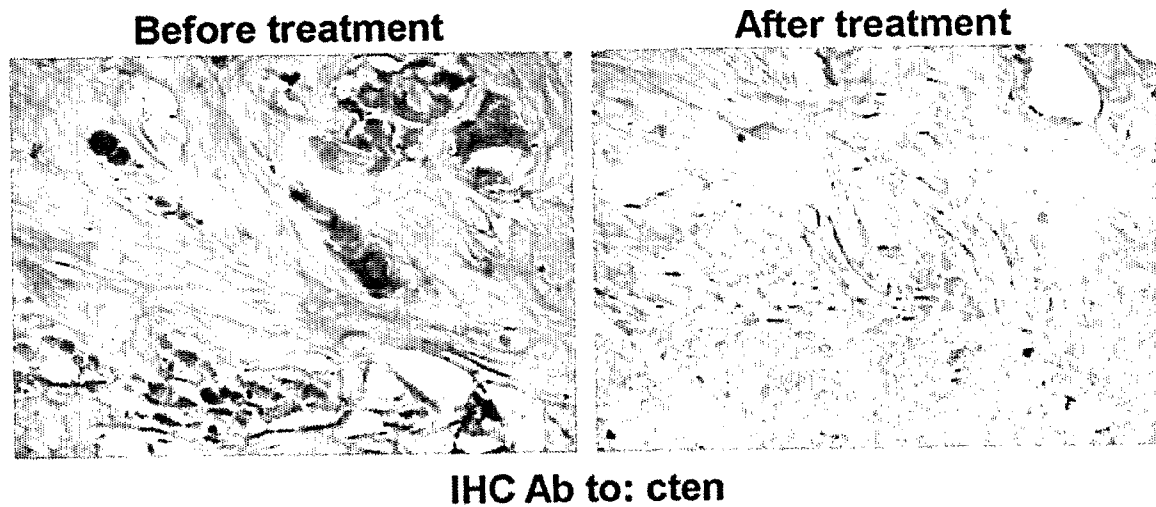


Fig. 8c

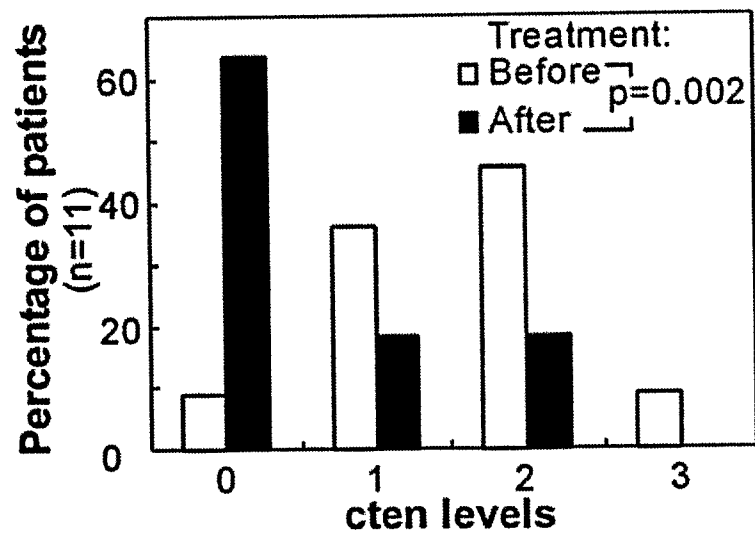


Fig. 8d

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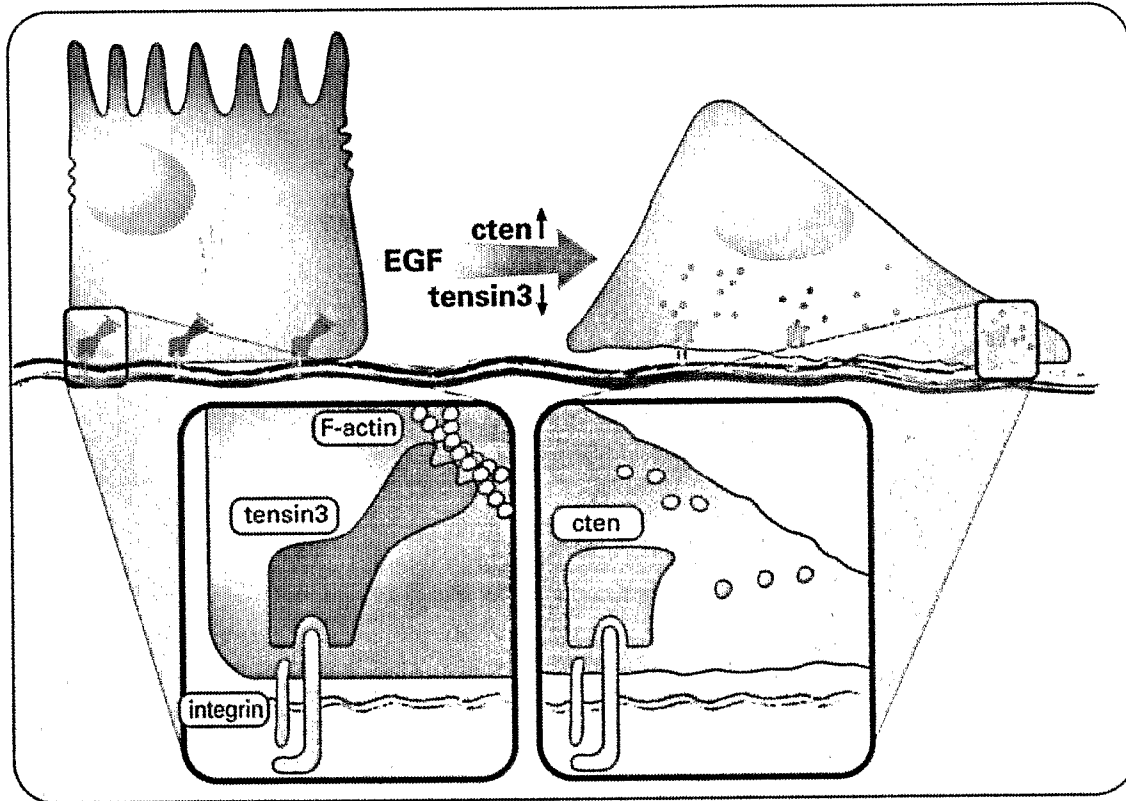


Fig. 8e

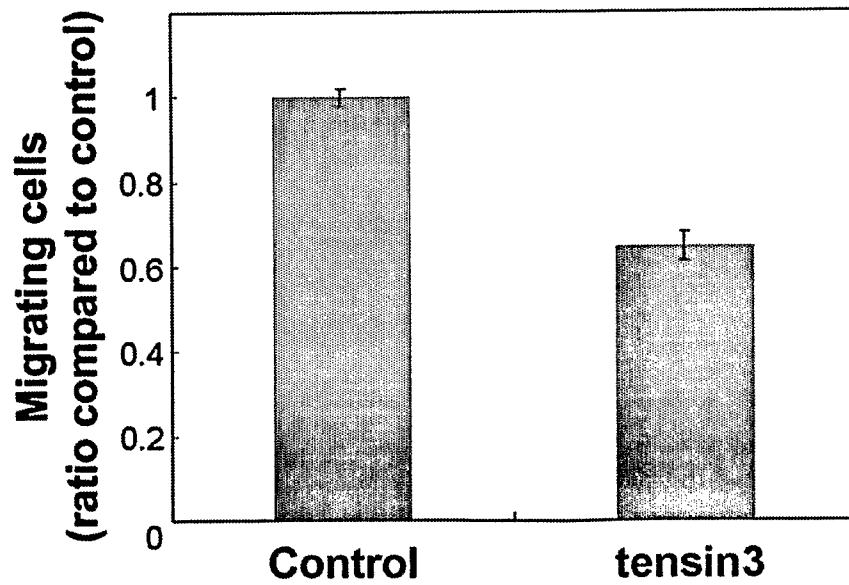


Fig. 9a

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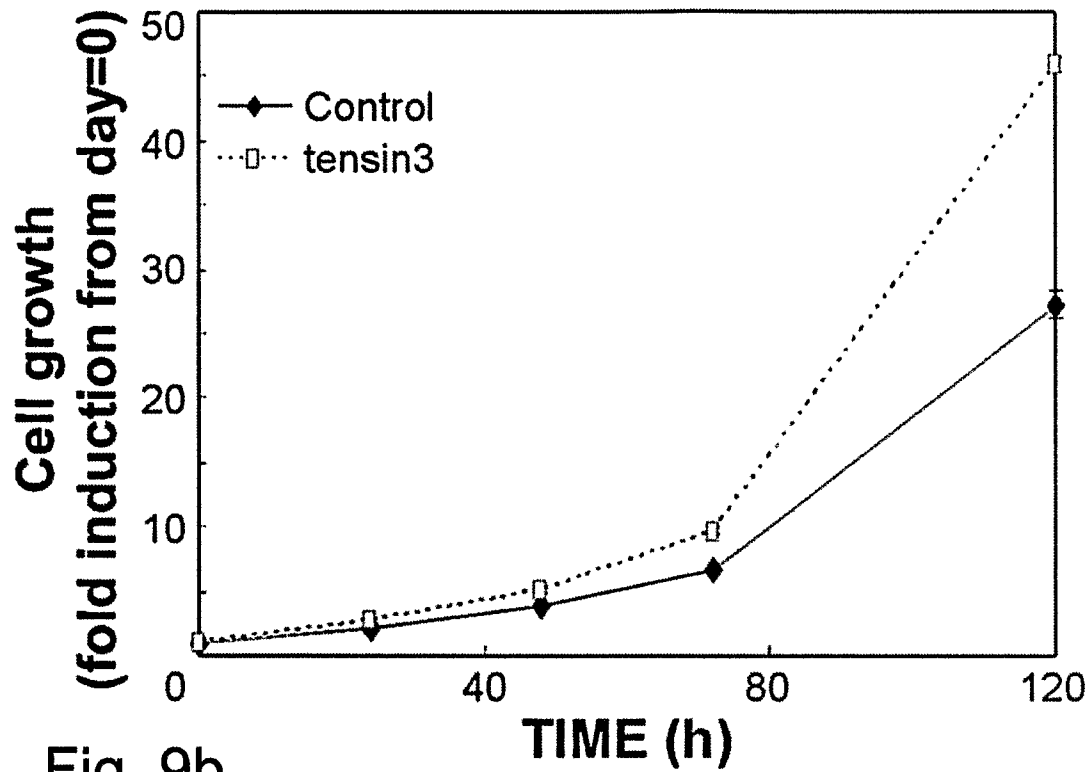


Fig. 9b

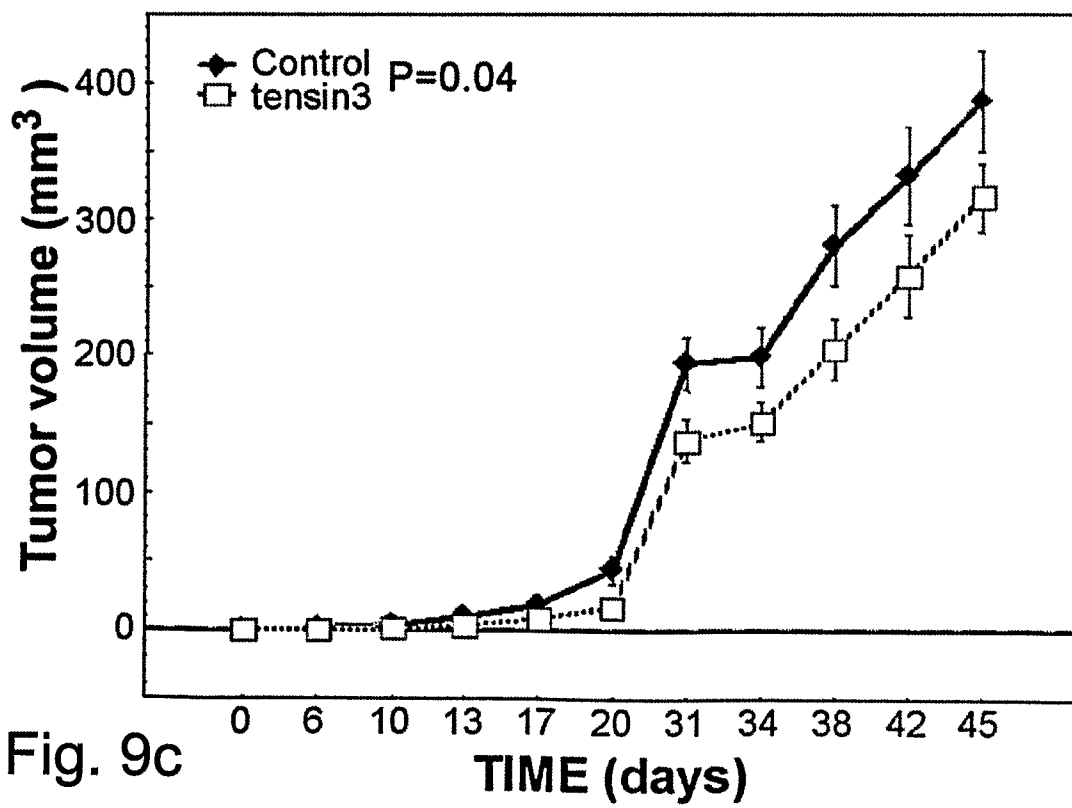


Fig. 9c

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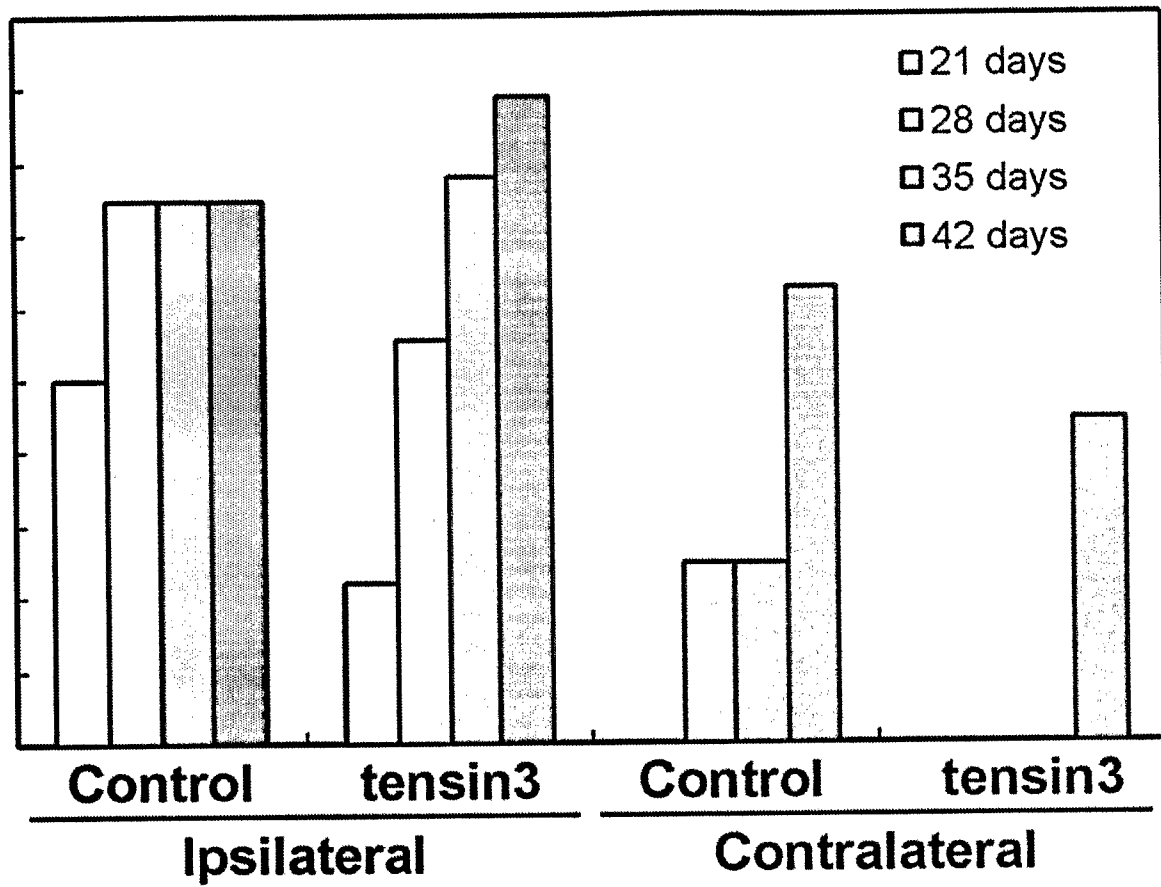


Fig. 9d

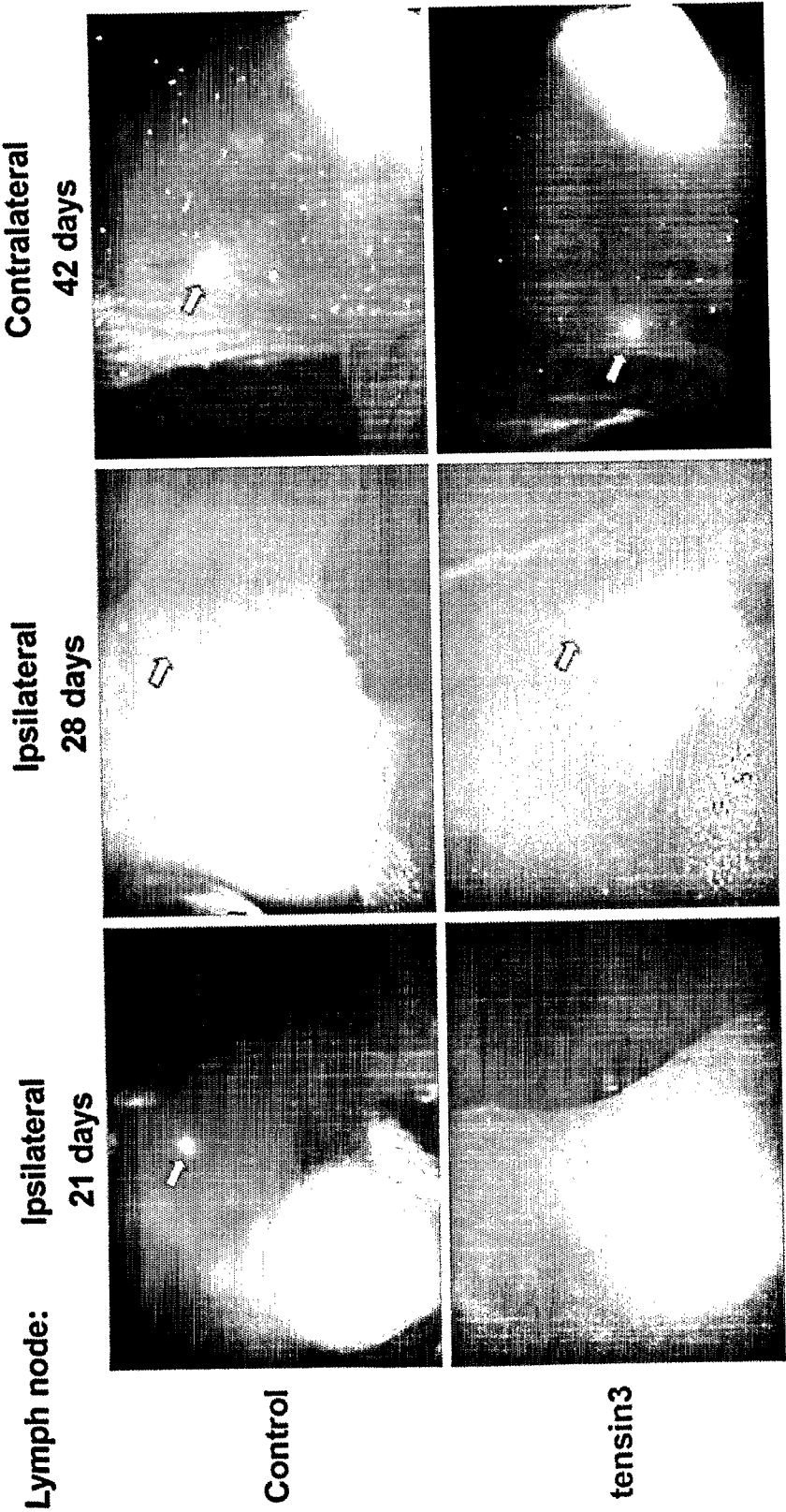
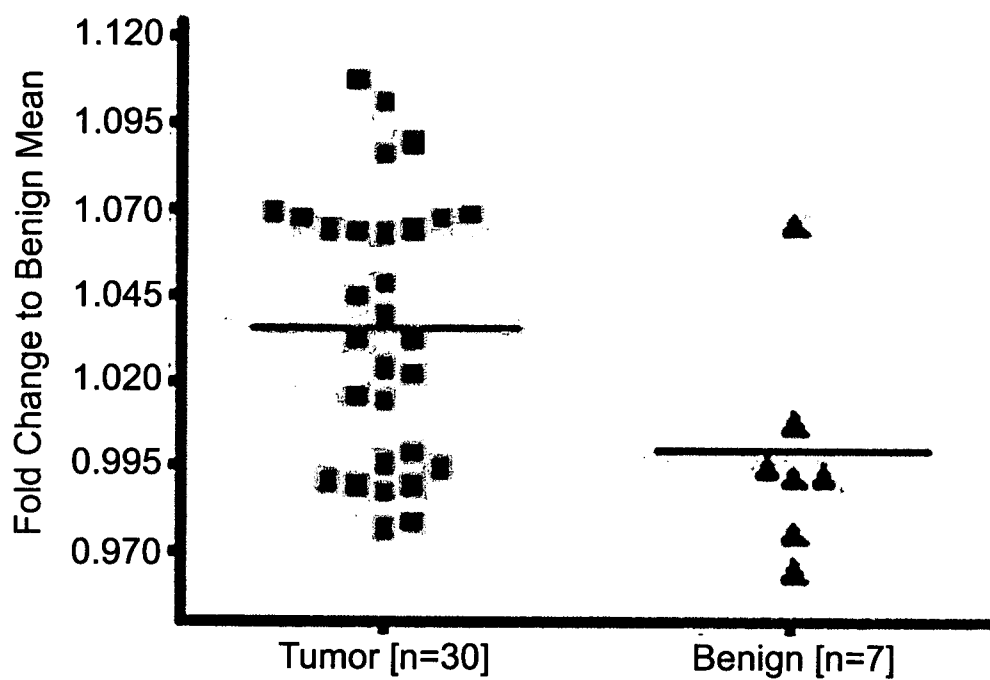


Fig. 9e

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P<0.03

Fig. 10a

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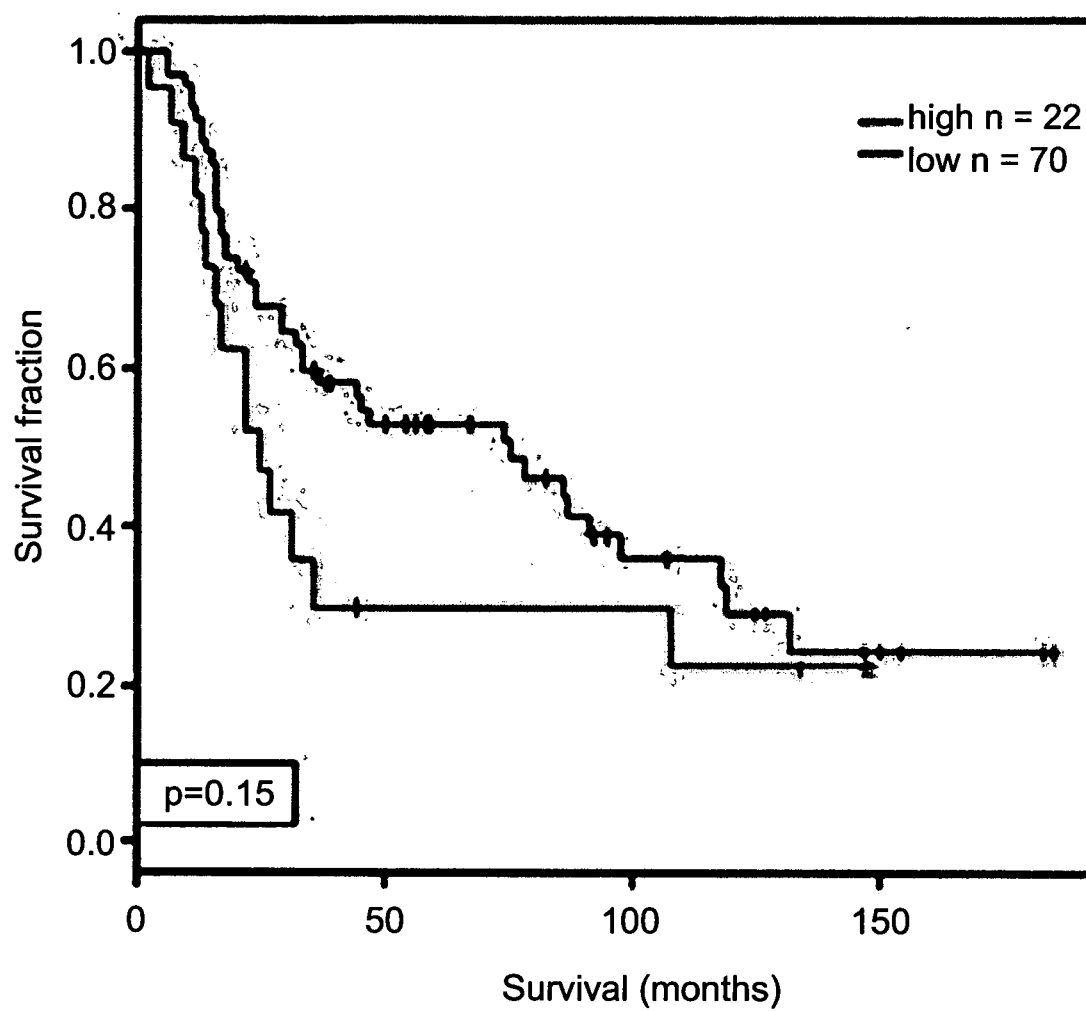


Fig. 10b