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(54) Title: FAT1 GENE IN CANCER AND INFLAMMATION

(57) Abstract: The present invention demonstrates for the first time that FAT1 plays an important role in modulating PDCD4 expression, which in turn regulates AP-1 dependent transcription, controls processes crucial for migration and invasion in cancer cells, controls induction of a pro-inflammatory micro environment in cancer cells. The study illustrates a link between inflammation and cancer in cells or in a subject. This work highlights the importance of FAT1 in the induction of the cellular pathways of migration and invasion, proteolysis of the ECM and the expression of pro-inflammatory molecules leading to a favorable micro environment for tumor and cancer progression. The present invention also provides the use of FAT1 in regulating neoplastic phenotypes and genotypes including invasiveness and inflammatory micro environment of the cancer cells by acting as a novel apical regulator of a signaling pathway by affecting the AP1 transcriptional activity, affecting the property of both cell migration and invasion and at the same time affecting the expression of inflammatory modulators. The present invention also identifies a novel regulatory pathway for inflammatory mediators and inflammatory cellular responses via PDCD4 and AP1. The invention describes a pathway for the upregulation of inflammatory processes as such, and hence a means of regulating inflammatory pathologies in general.



“*FAT1* GENE IN CANCER AND INFLAMMATION”

The following specification particularly describes the invention and the manner in
5 which it is to be performed.

FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology. More
specifically, the invention relates to the field of cancer biology and inflammation.

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BACKGROUND & PRIOR ART OF THE INVENTION

Cancer and inflammatory diseases are multifactorial diseases and different mode of
therapies are being exploited and attempted. Glioblastomas (GBM) are the most
frequent and most malignant form of brain tumors. Tumor angiogenesis, invasiveness
15 and rapid growth go in concert in GBM. The tumor microenvironment exhibits
expression of pro-inflammatory molecules that promote migration and invasion of
tumor cells. There is increasing evidence of the role of the pro-inflammatory
molecules in making glioma and other tumors more aggressive and resistant to
chemo- and/or radio-therapy.

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Common pathways may promote tumor invasiveness and expression of pro-
inflammatory molecules in GBM. Of these COX-2 and cytokines like IL1 β , IL6 are
the known mediators of both the processes. However the therapeutic translation of this
knowledge has been limited.

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FAT1, a member of the cadherin gene family, is the human homologue of the
Drosophila fat. Fat in *D. melanogaster* is a known tumor suppressor gene acting via
the Salvador-Warts-Hippo (SWH) signaling pathway, and is essential for controlling
cell proliferation during development and regulating planar cell, and any defect in the
30 expression of fat would lead to tumor development. In *Drosophila*, fat is an upstream
regulator of the SWH pathway. The signaling molecules of SWH pathway are
conserved in human but the role of *FAT1* as an apical regulator has not yet been
established. Disruption of fat causes imaginal disc tumors in *Drosophila*. The fat gene

was first discovered in *Drosophila* followed by the identification of its orthologue in man, rat, mouse and zebrafish etc.

The expression profile of *FAT1* has been studied in human fetal and adult tissues.

5 *FAT1* in human is reported to be localized to region of chromosome 4q35. The expression of *FAT1* peaks in embryonic stages and diminishes later in adult life. High levels of *FAT1* transcripts were found in kidney, lungs, and eye epithelia, and were down regulated in the corresponding adult tissues, indicating the role of *FAT1* in organ development. *FAT1* is also known to have a role in cell polarity and migration.

10 *FAT1* is found to be involved in the glomeruli as a slit junction adhesion molecule and have been found to be interacting with atropine in regulating cell orientation and migration. *FAT1* is up-regulated in cell migration, induces cellular process formation when overexpressed, and is necessary for efficient wound healing. *FAT1* was found to be localized at filipodial tips, lamellipodial edges, and cell-cell boundaries,

15 overlapping with dynamic actin structures.

Very few reports are available on the role of *FAT1* in human cancer. A study from the same group of inventors has shown low expression of and loss of heterozygosity (LOH) at *FAT1* locus in the gliomas. Studies have shown the altered expression of

20 *FAT1* in different cancers but the exact role of *FAT1* has not been fully elucidated, even the mechanism of action of *FAT1* in mammalian epithelial cell migration is not fully understood. The signaling cascades and cellular processes through which *FAT1* acts in different contexts are still being elucidated and very few functional studies are available on the role of *FAT1* in human cancers, including GBM. However the main

25 focus of *FAT1* so far in literature has been as a tumor suppressor gene.

There are studies showing LOH and/or deletion of the chromosome 4q34-35 region in many tumors including gliomas but the gene itself has not been analyzed and implicated. LOH/alterations in the chromosomal 4q34-35 region was found in grade

30 IV gliomas using microsatellite markers, Small Cell Lung Carcinoma, hepatocellular carcinoma and cervical carcinoma etc. In all these LOH studies, a significant association of 4q34-q35 region with increased risk of progression to higher grade or with the malignancy of the tumors was suggested. Since the *FAT* gene is located in

this region it may have an important role to play in the development and progression of these tumors.

5 A positional cloning strategy, combined with association analysis has provided evidence that *FAT1* confers susceptibility to bipolar disorder.

It is important to note that all the previous literature has concentrated on *FAT1* as a tumor suppressor gene and there is no literature on *FAT1* promoting either tumorigenesis or inflammation or the link between the two.

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The signaling cascade(s) being regulated by *FAT1* gene and the function of *FAT1* gene in human cancers including GBM is not known. Therefore, proper functional assays need to be done to support and to identify the precise role of *FAT1* gene in inflammation and tumors including GBM.

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Till now, targeting pro-inflammatory molecules or related pathways as therapeutic strategies to prevent or treat cancers has had a limited success and there is considerable interest in focusing on such molecules. The transmembrane molecule, *FAT1* thus promises to be one such novel target for therapeutic intervention in cancer and inflammation as well as the link between the two processes.

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SUMMARY OF THE INVENTION

25 The present disclosure demonstrates the role of *FAT1*, a transmembrane protein, in linking the neoplastic phenotype and inflammatory mediators in cancer cells.

The present disclosure further demonstrates that *FAT1* can upregulate some features of tumorigenesis and can function as an oncogene under certain conditions and can also influence the expression of mediators of inflammation.

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The present invention demonstrates for the first time that *FAT1* plays an important role in modulating PDCD4 expression, which in turn regulates API dependent transcription, controls processes crucial for migration and invasion in cancer cells, controls induction of a pro-inflammatory microenvironment in cancer cells. The study

illustrates a link between inflammation and cancer in cells or in a subject. This work highlights the importance of *FATI* in the induction of the cellular pathways of migration and invasion, proteolysis of the ECM and the expression of pro-inflammatory molecules leading to a favorable microenvironment for tumor and cancer progression.

The present invention also provides the use of *FATI* in regulating neoplastic phenotypes and genotypes including invasiveness and inflammatory microenvironment of the cancer cells by acting as a novel apical regulator of a signaling pathway by affecting the API transcriptional activity, affecting the property of both cell migration and invasion and at the same time affecting the expression of inflammatory modulators.

The present invention by establishing a novel cell signaling pathway that regulate PDCD4 and API mediated transcription - thereby regulating the general inflammatory mediators, IL1 β , COX-2, IL6 and prostaglandins - also provides a way of beneficially intervening in pathologies that are intrinsically inflammatory and may not have any link with cancer.

This novel work has not only elucidated the role of *FATI* in regulating some the key invasive properties of a cancer cell and the expression of inflammatory molecules in the cell by being a novel mediator of cell signaling, but this also points to the utilization of the *FATI* modulation as a therapeutic targets in both cancer and inflammatory diseases. In addition, the present elucidation of the link between cancer and inflammation provides insights on how the inflammatory processes are controlled. Hence they provide a basis for the control of intrinsic inflammatory pathology as well.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings illustrate embodiments of the invention and, together with the description, serve to explain the invention. These drawings are offered by way of illustration and not by way of limitation.

Fig 1: Knockdown of *FAT1* inhibited glioma cell migration and invasion;

(a) *FAT1* expression was checked in six glioma cell lines by q-PCR, out of which grade IV glioma cell lines U87MG, A172, U373MG and T98G were found to have high *FAT1* expression while grade III glioma cell lines GOS3 and SW1088 had low *FAT1* expression. 18s was used as internal control. (b) Knockdown of *FAT1* in U87MG and A172 cell lines (72 hrs post transfection) was confirmed by q-PCR using *FAT1* specific primers. $\geq 90\%$ down-regulation of *FAT1* mRNA in *siFAT1* cells was observed as compared to siControl treated cells. 18S was used as internal control; all experiments were done in triplicates and repeated thrice. (c) Distinct phenotypic changes (72 hrs post transfection) were observed in U87MGsiFAT1 and A172siFAT1 cells as compared to siControl cells. *siFAT1* treated cells were spindle shaped or rounded, scattered and had reduced cell-cell interaction. (d,e) *FAT1* knockdown inhibited cell migration and invasion in U87MGsiFAT1 as well as A172siFAT1 cells as compared to siControl cells. Modified Boyden chamber assay/matrigel assay was performed for 24 hrs (72 hrs post-transfection) to assess cell migration and invasion. Cells migrated across membrane or invaded through matrigel were fixed, stained and were counted in five different fields and means were calculated. The number of cells that migrated or invaded in *siFAT1* cells were normalized against number of cells that migrated in siControl cells. Each value is expressed as mean $\pm$ standard deviation. Each experiment was done in triplicate and repeated twice. Significant differences were observed in *siFAT1* treated cells as compared to siControl cells as determined by Student's *t* test. Mock: lipofectamin treated cells; siControl: Invitrogen universal medium GC control siRNA treated cells and *siFAT1*: *FAT1* specific siRNA (Invitrogen) treated cells.

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Fig 2: Knockdown of *FAT1* upregulated PDCD4 expression;

(a) PDCD4 mRNA expression as assessed by q-PCR (72 hrs post siRNA transfection) was found to be increased by more than two fold in all the GBM cell lines (U87MG, A172, U373 and T98) analysed after *FAT1* knockdown as compared to their respective siControl cells. 18s was used as internal control (b) Similarly PDCD4 protein level was also found to be increased in the above GBM cell lines after *FAT1* knockdown as compared to respective siControl cells as assessed by Western blot analysis. β -actin was used as loading control. (c) GOS3 (WHO grade III glioma) cell line with low endogenous

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FAT1 was found to have high PDCD4 mRNA expression which was comparable to U87MGsi/vi77 treated cells.

Fig 3: Inhibition of AP-1 transcriptional activity after *FAT1* knockdown

- 5 (a) *FAT1* knockdown in U87MG cells up-regulated PDCD4 expression which in turn inhibits phosphorylation of c-jun. Western blot was performed on cell lysates from U87MGsi*FAT1* and siControl cells with phospho-c-jun (Ser63) and c-jun antibodies. There was significant reduction in the p-c-jun level. β -actin antibody as loading control. (b) The mRNA level of c-jun was found to be reduced by two folds after
- 10 *FAT1* knockdown. A reduction in the total c-jun protein observed after *FAT1* knockdown could be due to decreased positive autoregulation of c-jun expression by AP-1 as well as the ubiquitination and rapid degradation of the unphosphorylated c-jun. (c,d) AP-1 luciferase assay was done to assess AP-1 transcriptional activity. U87MG cells were independently transfected with si*FAT1* or siPDCD4 and siControl.
- 15 After 24hrs, cells were transfected with 1 μ g of AP-1 reporter plasmid, along with 50 ng of pRL-TK (Renilla luciferase) control plasmid. The luciferase activity was measured after 48hrs. The luciferase activity with control siRNA is designated as 100% and the difference calculated, significant difference in si*FAT1* and siPDCD4 treated cells ($p < 0.01$ and $p < 0.05$ respectively) was observed compared with siControl
- 20 cells, as determined by Student's *t* test. The experiment was repeated thrice and results are expressed as mean $\pm$ standard deviation.

Fig 4: Decrease in COX-2 expression and cytokines synthesis after *FAT1* knockdown;

- 25 (a) Expression of COX-2 and cytokines were found to be decreased after *FAT1* knockdown. q-PCR was done to measure the mRNA expression of COX-2, IL6 and IL1 β and almost 10 fold reduction was observed in U87MGsi*FAT1* cells as compared to siControl cells. 18s was used as internal control. (b) *FAT1* knockdown was found to inhibit COX-2 protein expression. Western blot for indicated cells showed decreased COX-2 expression after *FAT1* knockdown. β -actin was used as
- 30 loading control. (c) PGE2 assay was done to quantify the production of PGE2 after *FAT1* knockdown. Synthesis of enzymatic product of COX-2, PGE2 was decreased significantly by >2 fold in U87MGsi*FAT1* cells as compared to siControl cells. Experiment was done in triplicate and repeated twice. Each value is expressed as mean $\pm$ standard deviation. Significant difference ($p < 0.01$) was observed as

determined by Student's *t* test. **(d)** COX-2 luciferase assay was done in U87MGsiControl and U87MGsiFAT1 cells after treatment with either 0.1% DMSO or 10 M SRI 1302. There was significant ($p < 0.05$) reduction in COX-2 luciferase activity after *FAT1* knockdown as well as *FAT1* knockdown with SRI 1302. **(e)** q-PCR was done to evaluate the mRNA expression of COX-2, IL6 and IL1 β after treatment with 10 M SRI 1302. There was >2 fold reduction in the mRNA expression of COX-2, IL6 and IL1 β after treatment with SRI 1302 as compared to DMSO control.

Fig 5: Simultaneous knockdown of *FAT1* and PDCD4 reverses the effects of *FAT1* knockdown; **(a)** *FAT1* and PDCD4 mRNA expression was analyzed by q-PCR in U87MG cells treated with si*FAT1* and siPDCD4 alone as well as both the siRNAs treated simultaneously. Treatment with si*FAT1*+siControl was found to upregulate PDCD4 expression while treatment with si*FAT1*+siPDCD4 downregulated the PDCD4 expression to the level of siControl alone treated cells. **(b,c)** Simultaneous knockdown of *FAT1* and PDCD4 in U87MG cells restored their migratory and invasive properties comparable to that of siControl treated cells. There was significant increase in cell migration and invasion in U87MG cells treated with si*FAT1*+siPDCD4 as compared to cells treated with si*FAT1*+siControl. Cells were counted in five different fields. Each value is mean $\pm$ standard deviation. Experiment was put up in triplicate and repeated twice. **(d)** AP-1 luciferase activity significantly increased after PDCD4 knockdown in U87MGsi*FAT1* cells. The luciferase activity with siControl is designated as 100%. There was significant increase in AP-1 luciferase activity in U87MG cells treated with si*FAT1*+siPDCD4 as compared to si*FAT1*+siControl. And the luciferase activity in cells treated with si*FAT1*+siPDCD4 is comparable to siControl treated cells. The experiment was repeated thrice following each of three independent transfections and representative data are shown. Results are expressed as mean $\pm$ standard deviation. **(e)** The mRNA expression of AP-1 target genes increased in U87MG cells treated with si*FAT1*+siPDCD4 as compared to si*FAT1*+siControl. 18s was used as internal control and experiments were done in triplicate. **(f)** PDCD4 knockdown in U87MGsi*FAT1* cells revert back the protein expression of p-c-jun, VEGF-C and COX-2 comparable to siControl alone treated cells. Lysates from indicated cells were probed with respective antibodies. β -actin was used as control antibody.

Fig 6: Signaling pathway downstream of *FAT1* regulating AP-1-dependent transcription; Knockdown of *FAT1* expression releases its inhibitory effect on PDCCD4 and increase the expression of PDCCD4. Increased PDCCD4 expression in turn inhibits the phosphorylation of c-jun, thus decreasing phospho-c-jun levels. Since phospho-c-jun is required for AP-1 dependent transcription, there was inhibition of AP-1 transcriptional activity and down-regulation of target genes like COX-2, MMP3, VEGF-C, PLAUG, IL-6 and IL-1 β .

Fig 7: *FAT1* siRNA-I has maximum knockdown efficiency; Three *FAT1* siRNA [HSS 103567 (*FAT1* siRNA-I), HSS 103568 (*FAT1* siRNA-II) and HSS176716 (*FAT1* siRNA-III)] corresponding to different regions of *FAT1* transcript were screened for knockdown efficiency. *FAT1* siRNA-I was found to have maximum knockdown efficiency as assessed by q-PCR for *FAT1* in U87MG cell line. 18S was used as internal control.

Fig 8: (a) Knockdown of *FAT1* leading to high PDCCD4 expression does not alter cell cycle progression in U87MG cells; For cell cycle analysis, on day 0, 2×10^5 cells were plated in 25cm² flask. On day 1, siRNA transfection was carried out. 1ml of optimum, 50 picomoles of siRNA (siControl and si*FAT1*) and 3 μ l of lipofectamine 2000 was used per well. Cells were then analyzed by flow-cytometry after 72 hrs of transfection to assess the cell cycle distribution of individual cells. Cells were fixed in 70% ethanol overnight at -20 °C followed by propidium iodide (PI) staining. Fluorescence was measured on FACS (fluorescence activated cell sorter, BD Biosciences, Bedford, MA). No difference in the cell population was observed at different cell cycle stages in both si*FAT1* and siControl treated cells. **(b) *FAT1* knockdown does not alter cell proliferation as assessed by MTT assay;** On Day 0, four thousand cells were plated per well in 96 well plate in triplicate. On day 1, transfection was carried out. 50 μ l of optimum, 2 picomoles of siRNA (control and *FAT1* siRNA) and 0.5 μ l of lipofectamine 2000 was used per well. After 4 hrs incubation at 37 °C, 50 μ l 2x media was added per well. After every time point (24, 48, 72 and 96hrs of transfection), media was removed from wells and 100 μ l of MTT (0.2mg/ml) was added per well and incubated for 2-4 hrs. The formazan crystals formed were dissolved by addition of 100 μ l of DMSO and absorbance was measured

at 570nm. No significant difference was observed in U87MGsi/ $\frac{3}{4}$ *Tl* cells as compared to siControl cells. **(c,d) *FAT1* knockdown does not result in apoptosis;** Apoptosis induction after *FAT1* knockdown was assessed by Western blot analysis of caspase3 and by DAPI staining. No differences were observed between
5 U87MGsi/vi *Tl* and siControl cells.

Fig 9: *FAT1* knockdown results in increased p-akt level in U87MG cell line; Akt is known to negatively regulate PDCD4 expression; therefore, the effect of *FAT1* knockdown on the phosphorylation status of Akt in U87MG cells was checked by
10 Western blot analysis using β -actin as a loading control. An increase in phospho-Akt level was observed after *FAT1* knockdown indicating that the up-regulation of PDCD4 after *FAT1* knockdown is likely to be independent of Akt pathway in glioma cells.

Fig 10: *FAT1* knockdown inhibits ECM protease activity; (a) q-PCR analysis showed that AP-1 regulated ECM remodelling molecules like MMP3, VEGF-C and PLAUG were down-regulated more than 10-fold upon *FAT1* knockdown in U87MG cells. 18s was used as internal control and each experiment was put up in triplicate and repeated twice. (b) Inhibition of extra cellular protease activity after *FAT1*
20 knockdown was determined by gelatin zymography. Conditioned media from indicated cells were separated on a 0.1% gelatin acrylamide gel. The gel was then stained with Coomassie R-250 and photographed. The areas of protease activity appear white on blue background. There was reduced protease activity in *siFAT1* treated cells as compared to siControl cells.

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Fig 11: Dose response curve of SRI 1302; U87MG cells were treated with increasing concentration of SRI 1302 from 0.1 M to 10 M. Cells were assayed for COX-2 luciferase activity after 48 hrs and 90% decrease was observed in the COX-2 luciferase activity at SRI 1302 concentrations of 5 and 10 M. No toxicity to the cells
30 was observed at 10 M concentration and thus this concentration was used further in other experiments.

Fig 12: GOS3 (grade III glioma) cell line with low *FAT1* expression had low expression of AP-1 regulated transcripts;

GOS3 cell line was found to have low *FAT1* and high PDCD4 expression, thus the expression of AP-1 regulated transcripts in GOS3 cell line was checked by q-PCR. Expression of AP-1 regulated transcripts in GOS3 cell line was observed to be low as compared to U87siControl and U87MGsi*FAT1* cells. 18S was used as internal control.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is described with reference to the tables/ figures etc. and specific embodiments; this description is not meant to be construed in a limiting sense. Various alternate embodiments of the invention will become apparent to persons skilled in the art, upon reference to the description of the invention. It is therefore contemplated that such alternative embodiments form part of the present invention.

Unless defined otherwise, 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. Some of the terms are defined briefly herebelow; the definitions should not be construed in a limiting sense.

The term "cancer" is defined as a hyperproliferation of cells whose unique trait - loss of normal controls - results in unregulated growth, lack of differentiation, local tissue invasion, and metastasis. Examples include but are not limited to, melanoma, non-small cell lung, small-cell lung, lung, hepatocarcinoma, leukemia, retinoblastoma, astrocytoma, glioblastoma, gum, tongue, neuroblastoma, head, neck, breast, pancreatic, prostate, renal, bone, testicular, ovarian, mesothelioma, cervical, gastrointestinal, lymphoma, brain, colon, sarcoma or bladder.

The terms "cell," "cell line," and "cell culture" may be used interchangeably. All of these terms also include their progeny, which are any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations.

"Glioma" is a type of tumor that starts in the brain or spine. It is called a glioma because it arises from glial cells. The most common site of gliomas is the brain.

5 "GBM", Glioblastoma Multiforme is the most aggressive and the commonest primary brain tumor with a tendency for local invasiveness.

"Cancer/ Tumor microenvironment" denotes the normal cells, molecules, and blood vessels that surround and feed a cancer/ tumor cell. The tumor microenvironment plays a crucial role in cancer development and in its progression.

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"Cancer/Tumor aggressiveness": Tumor grades are commonly described by four degrees of severity: Grades 1, 2, 3, and 4. The cells of Grade 1 tumors resemble normal cells, and tend to grow and multiply slowly. Grade 1 tumors are generally considered the least aggressive in behavior. Conversely, the cells of Grade 3 or Grade
15 4 tumors do not look like normal cells of the same type. Grade 3 and 4 tumors are most aggressive, and tend to grow rapidly and spread faster than tumors with a lower grade.

"API", the activator protein 1 is a transcription factor which is a heterodimeric
20 protein composed of proteins belonging to the c-Fos, c-Jun, ATF and JDP families. It regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial and viral infections. AP-1 in turn controls a number of cellular processes including differentiation, proliferation, and apoptosis.

25 "*FAT1*" is a member of cadherin superfamily and was first identified as a tumor-suppressor in *D. melanogaster*, acting via the Salvador-Warts-Hippo (SWH) signaling pathway.

30 "*PDCD4*", Programmed Cell Death 4, is a known tumor suppressor gene and it plays an essential role in many biological processes like regulating cap-dependent translation, apoptosis, modulating various signal transduction pathways etc. The expression of *PDCD4* is often decreased in human glioma and many other progressive tumors like lung, breast etc., leading to increased invasiveness and metastasis. In addition, *PDCD4* is also reported to suppress induction of inflammatory mediators.

PDCD4 is known to inhibit AP-1-mediated transcription which has a central role in multiple processes involved in tumorigenesis including proliferation, migration and invasion. AP-1 inhibition has been shown to have anti-invasive and anti-growth effect.

"Small interfering RNA (siRNA)", sometimes known as short interfering RNA or silencing RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length. siRNA plays many roles, but its most notable is in the RNA interference (RNAi) pathway, where it interferes with the expression of specific genes with complementary nucleotide sequence. siRNAs are a potent new drug class that can silence a disease-causing gene.

The present disclosure demonstrates that *FAT1* can upregulate some features of tumorigenesis and can function as an oncogene under certain conditions and can also influence the expression of mediators of inflammation. The link between *FAT1* and *PDCD4* is a novel aspect of this invention.

In order to elucidate the functional role of *FAT1* in cancer its expression is studied in several cancer cell lines such as glioma cell lines followed by siRNA knockdown of *FAT1* in cells in GBM cell lines such as U87MG and A172 with high *FAT1* expression. High *FAT1* mRNA expression is observed in grade IV glioma cell lines like U87MG, A172, U373MG and T98G and decreased expression in grade III glioma cell lines (GOS3 and SW1088). Knockdown of *FAT1* in U87MG and A172 leads to marked reduction in migratory and invasive properties of the cells along with high expression of *PDCD4*. Increased *PDCD4* expression is also observed upon *FAT1* knockdown in U373MG and T98 cell lines. *PDCD4* inhibits migration and invasion in various cancers like breast, ovarian, colon etc. Loss of *PDCD4* expression is linked with cell proliferation and unfavorable prognosis in gliomas. Decreased migration and invasion observed in U87MG and A172 cell lines after *FAT1* knockdown in this invention might be attributed to increased *PDCD4* expression. High *PDCD4* expression after *FAT1* knockdown does not induce apoptosis or alter cell cycle distribution in both U87MG and A172 cell lines. It is observed that high *PDCD4* levels after *FAT1* knockdown mainly regulate migration and invasion in glioma cells. Decreased *PDCD4* plays an important role in tumor progression and in remodeling

ECM during invasion. To further confirm the link between *FAT1* and PDCD4, PDCD4 and *FAT1* expression is simultaneously knocked down and reversal of migratory and invasive properties of the cells is found, which further confirms that the observed effects of *FAT1* are mainly mediated by PDCD4.

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PDCD4 attenuates Activator Protein-1 (AP-1) mediated transcription by inhibiting c-jun phosphorylation. A further research by the present team of inventors to check the effect of *FAT1* knockdown on the AP-1 activity by luciferase assay shows diminished AP-1 transcriptional activity and decreased expression of AP-1 regulated transcripts like ECM remodeling molecules MMP3, VEGF-C, PLAU; inflammatory mediator COX-2 and cytokines IL1 β and IL6. The reduced AP-1 activity observed in glioma cell lines after *FAT1* knockdown is due to inhibition of c-jun transcription and c-jun phosphorylation by increased PDCD4 expression. These effects are further confirmed by dual knockdown *of FAT1* and PDCD4 in both U87MG and A172 cell lines. Also, the increased PDCD4 expression after *FAT1* knockdown in glioma cells is found to be independent of Akt pathway. The observation of *FAT1* in regulating PDCD4 expression and its downstream effect on tumor migration and invasion is a novel finding with implications for cancer and tumor cell biology such as glioma biology.

AP-1 binding to c-AMP response element in the COX-2 promoter regulates COX-2 expression. The present disclosure shows that inhibition of AP-1 by SR 11302 (a known inhibitor of API activity) leads to significant reduction in COX-2 promoter luciferase activity as well as mRNA expression of COX-2. This indicates that down regulation of *FAT1* has the same effect on the COX-2 promoter as the small molecule inhibitor of API activity (SR 11302), thus further illustrating the mode of action of *FAT1*. The down-regulation of COX-2 expression and pro-inflammatory cytokines via PDCD4 up-regulation after *FAT1* knockdown has considerable significance in the regulation of inflammatory responses in cancer and tumor cells. Decreased COX-2 expression after *FAT1* knockdown leads to reduced PGE2 production and decreased mRNA expression of IL6 and IL1 β , thus providing a crucial link between tumor progression and inflammation. PDCD4 has a role in down-regulating COX-2 and suppression of PDCD4 increases invasive activity of COX-2 in breast cancer cells. COX-2 over-expression is also reported in high grade gliomas and many other tumors with potential for targeting it as anti-cancer modality. The present disclosure of

reduced COX-2 expression and decreased synthesis of prostanoids (PGE₂) and cytokines, IL1 β and IL6, after *FAT1* knockdown indicates the role of *FAT1* in regulating oncogenic and inflammatory properties in cancer and tumor such as gliomas. COX2 is a molecule known to be involved in tumor and inflammation.

- 5 Regulation of COX2 expression by *FAT1* is a novel finding linking inflammation and tumorigenesis in cancer cells. The novel pathways indicate a mechanism of regulating inflammatory pathology as such, even without any link to cancer. Thus, targeting *FAT1* provides strong advantages for managing both tumorigenicity as well as the inflammatory micro environment of cancer cells.

10

To further validate the *in-vitro* findings about the relationship between *FAT1* and PDCD4, the expression of *FAT1* and PDCD4 in 35 primary GBM samples is studied by q-PCR and a statistically significant inverse correlation between the two is observed. Further, a positive correlation between *FAT1* and COX-2 expression and a
15 similar trend for IL6 expression in GBM samples studied is also observed. These findings further support the *in-vitro* results about the inverse relationship between *FAT1* and PDCD4 and their influence on the expression of AP-1 mediated transcripts like COX-2, IL6 etc.

- 20 The present disclosure demonstrates for the first time that *FAT1* plays an important role in modulating PDCD4 expression, which in turn regulates AP-1 dependent transcription, controls processes crucial for migration and invasion in cancer cells, controls induction of a pro-inflammatory micro environment in cancer cells. The study illustrates a link between inflammation and cancer in cells or in a subject. This work
25 highlights the importance of *FAT1* in the induction of the cellular pathways of migration and invasion, proteolysis of the ECM and the expression of pro-inflammatory molecules leading to a favorable micro environment for tumor and cancer progression.

- 30 The present invention shows that *FAT1* affects some of the key modulators of cancer phenotype (including invasion and metastasis) and genotypes like the up-regulation of targets of the transcription factor, API, down-regulation of the Tumour Suppressor Gene PDCD4, angiogenesis (VEGF). It also affects some of the key inflammatory

molecules like IL1 β , IL6 and COX2. One of the ways in which *FAT1* acts is through the key transcription factor API .

5 The finding of reduced COX-2 expression and decreased synthesis of prostanoids (PGE2) and cytokines, IL1 β and IL6, after *FAT1* knockdown points toward the role of *FAT1* in regulating oncogenic and inflammatory properties in cancer cells. Thus, targeting *FAT1* has strong advantages for managing both tumorigenicity as well as the inflammatory microenvironment of gliomas.

10 This novel work has not only elucidated the role of *FAT1* in regulating some the key invasive properties of a cancer cell and the expression of inflammatory molecules in the cell by being a novel mediator of cell signaling, but this also points to the utilization of the *FAT1* modulation as a therapeutic target in both cancer and inflammatory diseases.

15

The present disclosure demonstrates the role of *FAT1*, a transmembrane protein, in linking the neoplastic phenotype and inflammatory mediators in glial cells.

20 Down-regulation of *FAT1* and its action is a key point of therapeutic intervention. It is also used as a target, at the level of gene, RNA and proteins and derivatives thereof, for either cancer or inflammation or both. Naturally occurring as well as synthetic molecules that modulate *FAT1* expression and activity also may have a role in the amelioration of either or both the processes. *FAT1* (gene, RNA or protein or its derivatives) also serves as a biomarker for the two processes i.e. cancer and
25 inflammation.

This is the first report showing a novel role of *FAT1* in regulating both aggressiveness and inflammatory microenvironment of the cancer cells via a signaling pathway where *FAT1* is acting as an apical regulator.

30

Thus, the present disclosure provides down regulation of API by *FAT1*, in conditions where overexpression of API is causing adverse effect, such as cancer and inflammation.

Further, the present disclosure provides modulation of *FAT1* mRNA/protein as a method of abrogating cancer and/or inflammation,

The present disclosure further provides *FAT1* as a link between cancer and
5 inflammation and also as a biomarker for the same.

The present disclosure further provides *FAT1* as a biomarker for cancer and inflammation.

10 An embodiment of the invention provides a method for abrogating cancer and/or inflammation in a cell or cell line or tissue, said method comprising down-regulating *FAT1* gene expression, wherein the cell or cell line or tissue is adversely affected by over expression of *FAT1*.

15 In another embodiment, the down-regulation of *FAT1* gene results in:

- a. upregulation of PDCD4;
- b. inhibition of AP-1 transcriptional activity;
- c. decrease in expression of AP-1 target genes;
- d. decrease in expression of inflammatory modulators and cytokines; and
- 20 e. decrease in cell migration and invasion.

In yet another embodiment, the AP-1 target genes are selected from the group comprising MMP3, VEGF-C, PLA2 and COX-2.

25 In yet another embodiment, the inflammatory modulator is COX-2 and inflammatory cytokines are such as IL6 and IL1.

A further embodiment of the invention is a method of regulating aggressiveness and inflammatory microenvironment of a cell or cell line or tissue, wherein the method
30 comprises regulating the expression of *FAT1* gene.

In a specific embodiment, the regulation of expression of *FAT1* gene comprises down-regulation of the *FAT1* gene.

In yet another embodiment, the regulation of expression of *FAT1* gene affects a signaling pathway primarily involving PDCD4 and API.

One more embodiment of the invention is a method of regulating neoplastic phenotypes and genotypes in a cell or cell line or tissue, said method comprising down-regulating expression of the *FAT1* gene. In another embodiment, said down-regulation of *FAT1* gene results in:

- a. upregulation of PDCD4;
- b. inhibition of AP-1 transcriptional activity;
- c. decrease in expression of AP-1 target genes;
- d. decrease in expression of inflammatory modulators and cytokines; and
- e. decrease in cell migration and invasion.

In yet another embodiment the cell or cell line or tissue is selected from glioma cell line, cells showing inflammation, cells showing higher levels of expression of API. Specifically, the glioma is glioblastoma multiforme. More specifically, the cell line is selected from group comprising U87MG, A172, U373MG and T98G.

The method as discussed above is preferably performed in a cell or a cell line or tissue in laboratory under controlled conditions and is performed *in vitro*.

The method as discussed above can be easily performed under *in vivo* conditions also.

Another aspect of the invention is to provide novel use of *FAT1* gene.

In an embodiment, the invention provides use of *FAT1* gene and its products for targeting inflammation and/or cancer and the associated phenotypes and genotypes.

In a further embodiment, the invention provides use of *FAT1* gene and its products as a biomarker for detecting inflammation and/or cancer.

In yet another embodiment, the invention provides use of *FAT1* gene and its products as a target for treatment of inflammation and/or cancer. The products of *FAT1* comprise *FAT1* RNA, protein and its derivatives. Said use comprises regulating *FAT1*

gene expression. More specifically, regulating of **FAT1** gene expression comprises down-regulation of **FAT1** gene. The down-regulation of **FAT1** gene results in:

- a. upregulation of PDCD4;
- b. inhibition of AP-1 transcriptional activity;
- 5 c. decrease in expression of AP-1 target genes;
- d. decrease in expression of inflammatory modulators and cytokines; and
- e. decrease in cell migration and invasion.

10 In another aspect, the invention provides a method of detecting or diagnosing cancer and/or inflammation in a subject or in a cell or cell line or tissue by studying expression of **FAT1** gene expression. Over expression of **FAT1** gene in a cell or cell line or tissue or a subject indicates presence of cancer and/or inflammation.

15 In another aspect, the invention provides a method for abrogating cancer and/or inflammation in a subject or in a cell or cell line or tissue, wherein the method comprises down-regulating **FAT1** gene expression.

20 In a further aspect, the invention provides a method of regulating aggressiveness and inflammatory microenvironment of adversely affected cells in a subject or in a cell or cell line or tissue, wherein the method comprises regulating the expression of **FAT1** gene in the subject.

25 In yet another aspect, the invention provides a method of regulating neoplastic phenotypes and genotypes in a subject or in a cell or cell line or tissue, wherein the method comprises down-regulating expression of the **FAT1** gene in the subject.

In one more aspect, the invention provides a method of controlling API transcriptional activity in a subject or in a cell or cell line or tissue, said method comprising down-regulating expression **ofFAT1** gene.

30

Another aspect of the invention provides **FAT1** gene for use in a method of detecting or diagnosing cancer and/or inflammation in a subject or in a cell or cell line or tissue by studying expression **ofFAT1** gene expression.

Yet another aspect of the invention provides **FAT1** gene for use in a method for abrogating cancer and/or inflammation in a subject or in a cell or cell line or tissue, wherein the method comprises down-regulating **FAT1** gene expression.

- 5 One more aspect of the invention provides **FAT1** gene for use in a method of regulating aggressiveness and inflammatory microenvironment of adversely affected cells in a subject or in a cell or cell line or tissue, wherein the method comprises regulating the expression of **FAT1** gene in the subject.
- 10 A further aspect of the invention provides **FAT1** gene for use in a method of controlling **API** transcriptional activity in a cell or cell line or tissue or a subject, said method comprising down-regulating expression of **FAT1** gene.

- Yet another aspect of the invention provides a method for selecting a drug/active molecule for the treatment of a cancer and/or inflammation, the method comprising:
- 15 a. obtaining a cell or cell line or tissue adversely affected by over-expression of **FAT1**
- b. contacting said cell or cell line or tissue with drug/active molecule;
- c. determining the expression level of **FAT1** in the cells or cell line or tissue; and
- 20 d. selecting a suitable anticancer/anti-inflammatory drug based upon the expression level of **FAT1** determined in step (c)
- wherein down-regulation of **FAT1** is indicative of a potential drug/active molecule.

- Down regulation for the purpose of the invention can be performed by any method
- 25 conventionally known in the art or known to a person skilled in the art, including but not limited to gene deletion; mutagenesis including insertional mutagenesis, induction of heterochromatinization in a targeted manner, dominant negative mutants, silencing using siRNA, shRNA, ribozyme, antisense RNA, antibodies, small molecules that interfere with protein function, peptides, protein fragments that inhibit the function of
- 30 the native protein, interference with post translational modifications. Preferably, down-regulation comprises silencing using siRNA.

The present disclosure with reference to the accompanying examples describes the present invention. However, those of skill in the art should, in light of the present

disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention. It is understood that the examples are provided for the purpose of illustrating the invention only, and are not intended to limit the scope
5 of the invention in any way.

EXAMPLES

Example 1: Materials and methods

10 **Reagents and antibodies:** Set of three siRNAs for *FAT1* knockdowns, FAT Stealth RNAi siRNA-I (HSS 103567) FAT Stealth RNAi siRNA-II (HSS 103568) and FAT Stealth RNAi siRNA-III (HSS 176716)) and universal medium GC control siRNA (cat. No 12935-112) were purchased from Invitrogen Life technologies, USA (Grand Island, NY). For simultaneous knockdowns, on-target plus siRNA against *FAT1* (J-010513-07-0020) and PDCD4 (J-004438-08-0020) and on-target plus non-targeting pool (D-001810-10-20) as control siRNA from Dharmacon (Chicago, IL, USA). AP-1 luciferase reporter plasmid (AP-1 7x) was purchased from Stratagene, COX-2 promoter luciferase construct (a kind gift from Dr. Miguel A. Iniguez, Centro de Biologia Molecular Universidad Autonoma de Madrid, Spain). AP-1 inhibitor SR
15 11302 was purchased from Tocris Biosciences (Bristol, UK); Normal brain RNA from Ambion, Life Technologies and Clontech (Mountain view, CA, USA). Antibodies used for western blots, like PDCD4, COX2, p-c-jun, c-jun, and β -actin were from Cell signaling technology (Beverly, MA, USA), VEGF and Caspase-3 were from Abcam (Cambridge, UK). Primers were designed using Primer3 software
20 and ordered from MWG Biotech (Germany).

Cell Culture and siRNA transfection: The human glioma cell lines U87MG, U373, A172, T98G, GOS3 and SW1088 were obtained from ATCC; Rockville, MD, cultured in DMEM (Sigma- Aldrich, Switzerland) supplemented with 10%(v/v) FCS
30 (Sigma- Aldrich), 3.7g/l sodium bicarbonate (Sigma- Aldrich), Ciprofloxacin 10 g/ml and 5%CO₂ at 37°C. For transfection, 2×10^5 cells were seeded per 25cm² flask. After 24hrs, cells were transfected with *FAT1* siRNA and universal medium GC control siRNA (Invitrogen) according to the manufacturer's protocol with a final concentration of 20nM of siRNA using Lipofectamine 2000 (Invitrogen) and Opti-

Mem media (Invitrogen). After 4hrs of siRNA transfection, the media was supplemented with DMEM containing 2% FCS. The ability of each siRNA to mediate knockdown of *FAT1* mRNA was confirmed using qRT-PCR. Photomicrographs of the cells were taken using an inverted phase contrast microscope (Nikon TMS, Phase contrast microscope, Japan). For simultaneous knockdown of *FAT1* and PDCD4, siRNA from Dharmacon were used. Transfection was performed as earlier with 50nM *oiFAT1* siRNA along with 50nM of PDCD4 siRNA or 50nM of control siRNA.

Table 1: Sequences of the primers and the annealing temperature used for real-time PCR

Target genes	Primer sequences	Annealing temperature ($^{\circ}$ C)
18s	Forward 5'-GTAACCCGTTGAACCCATT-3' Reverse 5'-CCATCCAATCGGTAGTAGCG-3'	55-60
<i>FAT1</i>	Forward 5'-TTCAAAATAGGTGAAGAGACAGGTG-3' Reverse 5'-TTGTGATGAGACCTGTTTTAGGATG-3'	59
PDCD4	Forward 5'-CCTGCAGGGTATTTTCCCTAA-3' Reverse 5'-TGGTTGGCACAGTTAATCCA -3'	58
COX-2	Forward 5'-CTGCTCAACACCGGAATTTT 3' Reverse 5'-TTGAATCAGGAAGCTGCTTTT -3'	59
c-JUN	Forward 5'- GTGTCCCCCGCTTGCCACAG -3' Reverse 5'- TCGGCGTGGTGGTGATGTGC -3'	61
MMP3	Forward 5'-CAGGGATTAATGGAGATGCC -3' Reverse 5'-AGTCAGGGGGAGGTCCATAG-3'	57
VEGF-C	Forward 5'-TGAACACCAGCACGAGCTAC -3' Reverse 5'-GTTGAGTCATCTCCAGCATCC -3'	57
IL1 β	Forward 5'-GAGCACCTTCTTTCCCTTCA-3' Reverse 5'-TCATCTTTCAACACGCAGGA-3'	57
IL6	Forward 5'-ATGAGGAGACTTGCCTGGTG-3' Reverse 5'-GCATTTGTGGTTGGGTCAG-3'	57
PLAU	Forward 5'-AGCGACTCCAAAGGCAGCAATGA-3' Reverse 5'-CAGGGTCGCCTCCGGTTGTC-3'	58

cDNA synthesis and Quantitative PCR: Total RNA was isolated from cells at appropriate time point (vide supra) using TRIzol reagent (Invitrogen), quantified using a Nanodrop ND-1000 spectrophotometer. DNase (Ambion) treatment was given

and 1 µg of total RNA was used for cDNA synthesis, done by Fermentas RevertAid™ First Strand cDNA Synthesis Kit using random decamers. PCR reactions were carried in 10 L reaction volumes (2.5 L of 1:5 diluted cDNA, 0.5 L of primer mix (0.5pmol/ L as final concentration of each primer), 1 L of 10×Taq Buffer A, 0.5U
5 Taq polymerase (Bangalore Genei, Bangalore, India), 1 L Syto9 (Invitrogen), 0.25 L of 10 mM dNTPs (MBI Fermentas, Thermo Fisher Scientific, Rockford, IL USA), and 4.6 L of nuclease-free water (Ambion) on a RotorGene6000 Real Time PCR in triplicates. The detail of the primers used is given in Table 1. ACt values were defined as target gene Ct minus 18s RNA Ct and averaged for each triplicate sample.
10 Statistical significance was determined by Student's t-test analysis (P<0.05).

Western Blotting Analyses: Cells were lysed in triple detergent buffer containing protease and phosphatase inhibitors (Sigma Aldrich), lysates were quantified by BCA kit (Pierce, Thermo Fisher Scientific, Rockford, IL USA) and electrophoresed in 10%
15 SDS-PAGE and electroblotted on nitrocellulose membrane (Millipore, Billerica, MA, USA). Membrane was blocked with 5% bovine serum albumin (BSA) in IxTBS at 4°C for overnight. Primary and secondary antibody was diluted in 5%BSA and 0.1% tween-20 in IxTBS. Blot was incubated in primary antibody overnight at 4°C followed by secondary antibody incubation for 2hrs at room temperature. Blot was
20 developed by BCIP-NBT (Promega, Madison, WI, USA) and captured using Alpha Imager EP software.

Migration and Invasion Assays: The modified Boyden chamber/matrigel assay was performed according to the manufacturer's directions (BD Biosciences, Bedford,
25 MA). For in vitro migration assays, chambers with control inserts that lacked the matrigel coating were used. Transfections were carried out in U87MG and A172 cells and 48 hrs later 3×10^4 U87MG cells/well and 7×10^4 A172 cells/well were resuspended in serum-free DMEM and seeded in triplicate in matrigel-coated (1mg/ml; BD Biosciences) and uncoated control inserts (8-µm pore-size, BD
30 Bioscience, Bedford, MA). The remaining protocol was done as previously described.

AP-1 and COX-2 promoter Luciferase Assay: U87MG cells (40000cells/well) were plated in 6-well plates in triplicate on day zero. On day 1, cells were transfected with *siFAT1* and siControl. On day 2 cells were transiently transfected with 4xAP-l-

luciferase reporter plasmids as described previously. Cells were transfected with 1µg of AP-1 7× luciferase reporter plasmid, along with 50ng of pRL-TK (Renilla luciferase) control plasmid using lipofectamine2000 as the transfection reagent. After 48hrs, the cells were lysed by 1x passive-lysis-buffer (Promega, Madison, WI, USA) and assayed for luciferase activity as previously described. For COX-2 luciferase assay U87MG cells were transfected with *siFAT1* and siControl on day1 and after 4 hrs incubation were treated with 10 M SRI 1302. On day2 cells were transiently transfected with COX-2 reporter plasmid and again treated with 10 M SRI 1302. After 48hrs, the cells were assayed for luciferase activity.

Gelatin Zymography: U87MG cells (2.5×10^5 cells/flask) were plated into 25cm² flask. Transfection was performed on day 1 and after 72hrs, the conditioned medium was collected, clarified by centrifugation, concentrated by Centricon YM-30 (10:1 concentration; Millipore, Billerica, MA, USA), and separated in non-reducing polyacrylamide gels containing 0.1% (wt/vol) gelatin. The gel was incubated with IX Zymogram renaturing buffer for 30min at room temperature, IX Zymogram developing buffer for 30min at room temperature, and IX Zymogram developing buffer at 37°C for overnight. The gel was then stained for protein with 0.25% (w/v) Coomassie Blue R-250 and then destained with Coomassie R-250 destaining solution (Methanol: Acetic acid: Water (50:10:40). Proteolysis was detected as a white zone in a dark field.

PGE2 ELISA: U87MG cells (2.5×10^5 cells/flask) were plated into 25cm² flask. Transfection was performed on day 1 and 72hrs later, the conditioned medium was collected and concentrated using Centricon YM-30. The concentration of PGE2 secreted into the medium was measured with an EIA kit for human PGE2 (Cayman Chemical, Michigan, USA).

Human GBM Samples: Tumor tissues were obtained at the time of surgery from 35 GBM patients from Neuro-surgery Department, AIIMS with due consent from patients and ethical clearance obtained from the ethics committee of the institute. The tumor typing and grading was done by Prof Chitra Sarkar, Department of Pathology, AIIMS. Portions of the resected tumors were snap frozen in liquid nitrogen and stored

at -80°C until prior to RNA extraction. Of these 35 patients, 14 were female and 21 were male, age between 30-65 years with a mean age $\pm$ s.d. of 43 ± 12.2 years.

Statistical Analysis: All in-vitro experiments were performed in triplicate and repeated thrice. q-PCR for patient tumor samples were performed in triplicate. Differences were determined using Student's t-test, and $p < 0.05$ was considered significant. Data are shown as the mean $\pm$ s.d.

Example 2: *FAT1* knockdown reduces migration and invasion of glioma cells

FAT1 mRNA expression was checked in a panel of glioma cell lines and high *FAT1* expression was observed in grade-IV glioma (GBM) cell lines (U87MG, A172, U373MG and T98G) as compared to grade-III glioma cell lines (GOS3 and SW1088) (Fig1a). The cell lines U87MG and A172 were studied further to analyze the effect of *FAT1* knockdown. Knockdown efficiency of *FAT1* in cells was checked by using a set of three *FAT1* specific siRNA (details in materials and methods section) from Invitrogen and *FAT1* siRNA I (HSS176716) was found to have maximum knockdown efficiency (FigS1, Fig 7). *FAT1* siRNA I was used for further experiments. $\geq 90\%$ knockdown of *FAT1* mRNA expression was observed in *FAT1* siRNA treated (U87MGsi*FAT1* and A172si*FAT1*) cells as compared to control siRNA (Invitrogen) treated (siControl) cells, 72hrs post transfection (Fig1b). There were significant morphological alterations (cells were more spindly) in si*FAT1* treated cells (Fig1c).

Significant reduction in migration [>2 fold in U87MGsi/vi77 ($P < 0.01$) and >3 fold in A172si/vi77 cells ($P < 0.001$)] (Fig1d) as well as invasion [>2 fold in U87MGsi/vi77 and >1.5 fold in A172si/vi77, $P < 0.01$)] (Fig1e) was observed upon *FAT1* knockdown. There was no variation in the distribution of cell population in different phases of cell cycle, as assessed by FACS analysis (Fig 8a), as well as in cell viability as assessed by MTT (Fig 8b), after *FAT1* knockdown. Further, there was no DNA fragmentation (DAPI staining) and no cleaved caspase-3 (Western blot) after 72hrs of transfection (Fig 8c,d), indicating that *FAT1* knockdown did not affect cell viability and apoptosis.

Example 3: *FAT1* knockdown enhances PDCD4 expression

In initial screening by microarray for altered gene expression after *FAT1* knockdown in U87MG cell line, PDCD4 was identified as one of the up-regulated genes. This was further confirmed by q-PCR and Western blot analysis in 4 GBM cell lines, U87MG, A172, U373MG and T98G. There was 2.4, 2.8, 3.2 and 3.5 fold increase in PDCD4 mRNA expression (Fig2a) as well as increased PDCD4 protein level (Fig2b) in *siFAT1* treated U87MG, A172, U373MG, and T98G cells respectively as compared to their respective siControl treated cells. The grade III glioma cell line, GOS3, with low endogenous *FAT1* expression (Fig1a) had high PDCD4 mRNA expression (Fig2c), corroborating the inverse relationship between *FAT1* and PDCD4.

The prior art teaches that phospho-Akt and PDCD4 negatively regulate each others expression. However, to the contrary, in the present invention, increased phospho-Akt levels (Fig 9) were observed along with increased PDCD4 expression (Fig2a, 2b) after *FAT1* knockdown in U87MG cells, suggesting that increased PDCD4 expression after *FAT1* knockdown was independent of p-Akt pathway.

Example 4: *FAT1* knockdown diminishes AP-1 mediated transcription

PDCD4 is reported to inhibit AP-1 dependent transcription via suppression of c-jun phosphorylation. Since c-jun phosphorylation is required for AP-1 activity, the effect of *FAT1* knockdown on c-jun phosphorylation status was investigated by Western blotting and found to be significantly decreased along with reduction in the total c-jun protein level in U87MGsi*FAT1* cells as compared to siControl cells (Fig3a). The c-jun mRNA level, as checked by q-PCR, was also found to be decreased by two folds in U87MGsi/vi77 cells as compared to U87MGsiControl cells (Fig 3b). The reduction in the total c-jun protein level in U87MGsi*FAT1* cells is due to reduction in the c-Jun mRNA level as well as ubiquitination and fast degradation of unphosphorylated c-jun.

Further, AP-1 luciferase assay was performed to examine whether the up-regulation of PDCD4 and diminished c-jun phosphorylation in *siFAT1* cells has any effect on AP-1 activity. A 2-fold reduction ($p < 0.01$) in AP-1 luciferase activity in U87MGsi*FAT1* cells as compared to U87siControl cells (Fig3c) was observed. Moreover the mRNA expression of AP-1 target genes like MMP3, VEGF-C and PLAU (Urokinase) were found to be decreased by more than 10-fold (Fig 10a) as well

as decreased ECM protease activity (Fig 10b), due to decreased MMP3, in U87MGsi/vi77 cells.

To further corroborate that PDCD4 regulates the AP-1 dependent transcription, PDCD4 was knocked down by using siPDCD4 in U87MG cells and observed 1.5 fold increase ($p < 0.05$) in AP-1 luciferase activity (Fig3d) as well as increased expression of AP-1 target genes like COX-2, IL6 and IL1 β (Fig3e) to add. Thus, confirming that increased PDCD4 expression after *FAT1* knockdown attenuates AP-1 transcriptional activity via reduction in the level of phosphorylated c-jun.

Example 5: Knockdown of *FAT1* decreases the expression of COX-2 and other cytokines

COX-2 is known to be negatively regulated by PDCD4 and aberrant induction of COX-2 with upregulation of prostaglandin synthesis is reported to play a pivotal role in carcinogenesis. COX-2 expression at mRNA and protein level was analyzed by q-PCR and Western blot. Significant reduction of COX-2 expression at both mRNA (Fig4a) and at protein level (Fig4b) in U87MGsi*FAT1* cells was observed as compared to siControl cells. The enzymatic product of COX-2, prostaglandin E2 (PGE2) plays a key role in influencing tumor progression. The concentration of PGE2 secreted into the medium was found to be reduced by >2 fold in U87MGsi*FAT1* cells as compared to siControl cells ($p < 0.01$) (Fig4c). Thus this result depicts inhibition of COX-2 function leading to diminished PGE2 synthesis after *FAT1* knockdown.

COX-2 has also been reported to regulate endogenous cytokine production and its inhibition markedly reduces the release of pro-inflammatory cytokines like IL-1 β and IL-6. A significant reduction in mRNA expression of COX-2 was also observed along with IL-1 β and IL-6 after *FAT1* knockdown in U87MG cells (Fig4a), thus identifying a novel function of *FAT1* in regulating the expression of pro-inflammatory molecules.

AP-1 mediated regulation of COX-2 transcription has been reported. To investigate whether the regulation of COX-2 expression by *FAT1* in U87MG cells is mediated via AP-1, AP-1 activity was inhibited by treating cells to the specific small molecule inhibitor of AP-1, SR 11302. On treating U87MG cells to increasing concentration of SRI 1302 (0.1 M to 10 M), a dose dependent decrease in COX-2 luciferase activity

was observed, with a maximum inhibition of about 90% at the dose of 5 M and 10 M concentrations (Fig 11). An 1.8 fold decrease ($p<0.01$), 5 fold decrease ($p<0.05$) and 5.5 fold decrease ($p<0.01$) was observed in COX-2 luciferase activity after *siFAT1*, 48
 5 firs SRI 1302 and simultaneous *siFA77*+SRI 1302 traatment respectively as compared to siControl+DMSO treated U87MG cells (Fig4d). To further confirm that AP-1 activity was indeed inhibited by SRI 1302, the mRNA level of COX-2, IL1 β and IL6 was checked by q-PCR and found to be markedly reduced on treatment with 10 M of SRI 1302 (Fig4e). These results thus provide further supportive evidence that AP-1 is a key link in the regulation of expression of COX-2 and other cytokines.

10

Example 6: Inhibition of PDCD4 reverses the effects of *FAT1* knockdown in *FAT1* attenuated cells

To confirm that the demonstrated effects of *FAT1* knockdown were indeed due to the up-regulation of PDCD4, *FAT1* and PDCD4 siRNA were co-transfected in U87MG
 15 cells and could satisfactorily knockdown levels of both the genes simultaneously. Simultaneous knockdown of both the genes (Fig5a) restored migration and invasion of cells to that of the level of siControl (Fig5b,5c). Moreover, AP-1 luciferase activity as well as the expression of AP-1 target genes was significantly regained with simultaneous PDCD4 inhibition in U87MGsiF *AT7* cells (Fig5d,5e). At protein level,
 20 simultaneous knockdown of PDCD4 and *FAT1* in U87MG cell line resulted in decreased PDCD4 expression to the level in siControl cells, but the expression of p-c-jun, VEGF-C and COX-2 was regained (Fig5f). Interestingly, U87MG cells knocked down for PDCD4 alone showed increased migration and invasion (Fig5b,5c) and high AP-1 luciferase activity (Fig5d) as compared to siControl cells. Low expression of
 25 AP-1 regulated transcripts in GOS3 cell line (Fig 12) which has low *FAT1* and high PDCD4 expression (Fig2c) was also observed. These results confirm the role of PDCD4 in mediating the observed effects of *FAT1*.

Example 7: *FAT1* and PDCD4 expression is inversely correlated in primary GBM samples

PDCD4 expression is reported to be lost in human glioma and may contribute to the development of tumor. To study the relationship between *FAT1* and PDCD4 expression in tumors, the mRNA expression of both *FAT1* and PDCD4 was checked in 35 GBM samples by q-PCR (Table 2).

Table 2:**Expression analysis of *FAT1*, *PDCD4*, *COX-2* and *IL6* in human GBM samples**

5 **by q-PCR.** GBM samples were divided into four groups (group A, B, C and D) based on quartiles showing decreasing *FAT1* expression. The *PDCD4* expression (mean $\pm$ SD) in the group A and group D were calculated (0.586 ± 0.998 and 60.856 ± 50.209 respectively) and the difference in the two groups were found to be statistically significant ($p = 0.0145$). Similarly, on comparing the expression of *COX-2* in group A and group D, there was a significant positive correlation between *FAT1* and the *COX-2* expression, with the mean $\pm$ SD of Group A and D being 4.99 ± 4.074 and 0.248 ± 0.174 respectively ($p = 0.048$). For *IL6*, there was a similar positive trend between Group A and D however it was not statistically significant ($p = 0.146$).

Group	Samples	<i>FAT1</i> /ISs	<i>PDCD4</i> /18S	<i>COX-2</i> /18S	<i>IL6</i> /18S
Group A	GBM 10	70.560	3.160	1.765	80.171
	GBM35	34.844	0.774	6.821	18.189
	GBM 11	19.990	0.438	8.168	5.152
	GBM8	19.490	0.430	1.905	0.020
	GBM24	13.990	0.004	2.346	3.238
	GBM30	13.707	0.056	0.006	0.067
	GBM25	8.138	0.002	0.387	1.597
	GBM6	5.980	0.303	14.929	12.862
	GBM5	5.290	0.112	0.337	1.834
Group B	GBM 12	4.700	0.555	0.742	25.020
	GBM7	4.660	10.754	1.778	4.302
	GBM3 1	4.000	0.176	0.056	0.034
	GBM33	3.949	0.290	0.143	0.155
	GBM2	2.479	0.100	0.100	0.372
	GBM32	1.548	0.195	0.158	0.509
	GBM29	1.500	134.809	0.001	3.340
	GBM28	1.300	1.372	0.001	0.963
	GBM27	1.200	0.333	1.892	2.5 14
Group C	GBM1	0.034	0.000	0.014	1.279
	GBM23	0.007	0.003	0.002	0.003
	GBM21	0.006	0.001	0.001	74.028
	GBM34	0.006	0.002	0.010	73.262
	GBM4	0.003	0.000	0.007	3.494
	GBM 13	0.003	0.000	0.702	4.332
	GBM3	0.002	0.000	0.61 1	3.399
	GBM22	0.002	0.002	0.010	0.081
	GBM9	0.001	0.000	1.347	0.232
	GBM 14	0.001	0.000	0.030	8.545
	GBM 15	0.001	0.000	0.004	0.838
	GBM 16	0.001	43.633	0.120	0.041

Group D	GBM 17	0.001	40.81 8	0.058	0.007
	GBM 18	0.001	106.912	0.743	0.226
	GBM 19	0.001	82.124	0.009	0.004
	GBM20	0.001	69.487	0.140	0.009
	GBM26	0.001	143.998	0.288	0.017

The 35 GBM samples were arranged according to decreasing *FAT1* expression and divided into quartiles. The PDCD4 expression (mean value $\pm$ standard deviation) in the first quartile (group A) with highest *FAT1* expression (0.586 ± 0.998) was significantly less than in the fourth quartile (group D) with the lowest *FAT1* expression (54.108 ± 51.18), with $p=0.0145$. The inverse relationship observed between the expression of *FAT1* and PDCD4 in tumor samples supports the *in-vitro* results.

Further, the mRNA expression of COX-2 and IL6 in these GBM samples was also analyzed and positive correlation with *FAT1* expression observed. The COX-2 expression in group A (4.99 ± 4.074) was significantly high as compared to group D (0.248 ± 0.174) with $p=0.048$. The IL6 expression in group A (mean $\pm$ SD) and group D (Mean $\pm$ SD) were found to follow the similar trend, however it was not statistically significant ($p=0.146$).

The claim:

1. A method for abrogating cancer and/or inflammation in a cell or cell line or tissue,
5 said method comprising down-regulating *FAT I* gene expression,
wherein the cell or cell line or tissue is adversely affected by over expression of
FAT I.
2. The method as claimed in claim 1, wherein down-regulation of *FA TI* gene results
10 in:
 - a. upregulation of PDCD4;
 - b. inhibition of AP-1 transcriptional activity;
 - c. decrease in expression of AP-1 target genes;
 - d. decrease in expression of inflammatory modulators and cytokines; and
15 e. decrease in cell migration and invasion.
3. The method as claimed in claim 2, wherein the AP-1 target genes are selected
from the group comprising MMP3, VEGF-C, PLAUI and COX-2.
- 20 4. The method as claimed in claim 2, wherein the inflammatory modulator is COX-2
and inflammatory cytokines are such as IL6 and IL1p.
5. A method of regulating aggressiveness and inflammatory microenvironment of a
cell or cell line or tissue, wherein the method comprises regulating the expression
25 of *FAT 1* gene.
6. The method as claimed in claim 5, wherein regulation of expression of *FA TI* gene
comprises down-regulation of the *FAT I* gene.
- 30 7. The method as claimed in claim 5, wherein the regulation of expression of *FA TI*
gene affects a signaling pathway primarily involving PDCD4 and API .

8. A method of regulating neoplastic phenotypes and genotypes in a cell or cell line or tissue, said method comprising down-regulating expression of the *FAT1* gene.
9. The method as claimed in any of the claims 1, 5 and 8, wherein the cell or cell line
5 or tissue is selected from a glioma cell line, cells showing inflammation, cells showing higher levels of expression of *FAT1*.
10. The method as claimed in any of the claims 1, 5 and 8 wherein the glioma is glioblastoma multiforme.
10
11. The method as claimed in claim 9, wherein the cell line is selected from group comprising U87MG, A172, U373MG, T98G.
12. The method as claimed in any of the claims 1, 6 and 8, wherein down-regulation
15 comprises gene deletion; mutagenesis including insertional mutagenesis, induction of heterochromatinization in a targeted manner, dominant negative mutants, silencing using siRNA, shRNA, ribozyme, antisense RNA, antibodies, small molecules that interfere with protein function, peptides, protein fragments that inhibit the function of the native protein, interference with post translational
20 modifications.
13. The method as claimed in claim 12, wherein preferably down-regulation comprises silencing using siRNA.
- 25 14. Use of *FAT1* gene and its products for targeting inflammation and/or cancer and the associated phenotypes and genotypes.
15. Use of *FAT1* gene and its products as a target for treatment of inflammation and/or cancer.
30
16. Use of *FAT1* gene and its products as a biomarker for detecting inflammation and/or cancer.

17. Use as claimed in any of the claims 14-16, wherein the products comprise *FAT1* RNA, protein and its derivatives.
18. Use as claimed in any of the claims 14 and 15, wherein use comprises regulating *FAT1* gene expression.
19. Use as claimed in claim 18, wherein regulating *FAT1* gene expression comprises down-regulation of *FAT1* gene.
20. Use as claimed in claim 19, wherein down-regulation of *FAT1* gene results in:
- a. upregulation of PDCD4;
 - b. inhibition of AP-1 transcriptional activity;
 - c. decrease in expression of AP-1 target genes;
 - d. decrease in expression of inflammatory modulators and cytokines; and
 - e. decrease in cell migration and invasion.
21. Use as claimed in claim 19, wherein down-regulation comprises gene deletion; mutagenesis including insertional mutagenesis, induction of heterochromatinization in a targeted manner, dominant negative mutants, silencing using siRNA, shRNA, ribozyme, antisense RNA, antibodies, small molecules that interfere with protein function, peptides, protein fragments that inhibit the function of the native protein, interference with post translational modifications.
22. Use as claimed in claim 21, wherein preferably down-regulation comprises silencing using siRNA.
23. A method of detecting or diagnosing cancer and/or inflammation in a cell or cell line or tissue or a subject by studying expression of *FAT1* gene expression.
24. The method as claimed in claim 23, wherein over expression of *FAT1* gene indicates presence of cancer and/or inflammation.

25. A method for abrogating cancer and/or inflammation in a cell or cell line or tissue or a subject, wherein the method comprises down-regulating **FATI** gene expression.
- 5 26. A method of regulating aggressiveness and inflammatory microenvironment of adversely affected cells in a subject, wherein the method comprises regulating the expression of **FATI** gene in the subject.
- 10 27. A method of regulating neoplastic phenotypes and genotypes in a subject, wherein the method comprises down-regulating expression of the **FATI** gene in the subject.
- 15 28. A method of controlling API transcriptional activity in a cell or cell line or tissue or a subject, said method comprising down-regulating expression of **FATI** gene.
- 20 29. **FATI** gene for use in a method of detecting or diagnosing cancer and/or inflammation in a cell or cell line or tissue or a subject by studying expression of **FATI** gene expression.
- 30 30. **FATI** gene for use in a method for abrogating cancer and/or inflammation activity in a cell or cell line or tissue or a subject, wherein the method comprises down-regulating **FATI** gene expression.
- 25 31. **FATI** gene for use in a method of regulating aggressiveness and inflammatory microenvironment of adversely affected cells in a subject, wherein the method comprises regulating the expression of **FATI** gene in the subject.
- 30 32. **FATI** gene for use in a method of controlling API transcriptional activity in a cell or cell line or tissue or a subject, said method comprising down-regulating expression of **FATI** gene.
33. A method for selecting a drug/active molecule for the treatment of a cancer and/or inflammation, the method comprising:

- a. obtaining a cell or cell line or tissue adversely affected by over-expression of *FAT1*
 - b. contacting said cell or cell line or tissue with drug/active molecule;
 - c. determining the expression level of *FAT1* in the cells or cell line or tissue; and
 - 5 d. selecting a suitable anticancer/anti-inflammatory drug based upon the expression level of *FAT1* determined in step (c)
- wherein down-regulation of *FAT1* is indicative of a potential drug/active molecule.

Figure 1

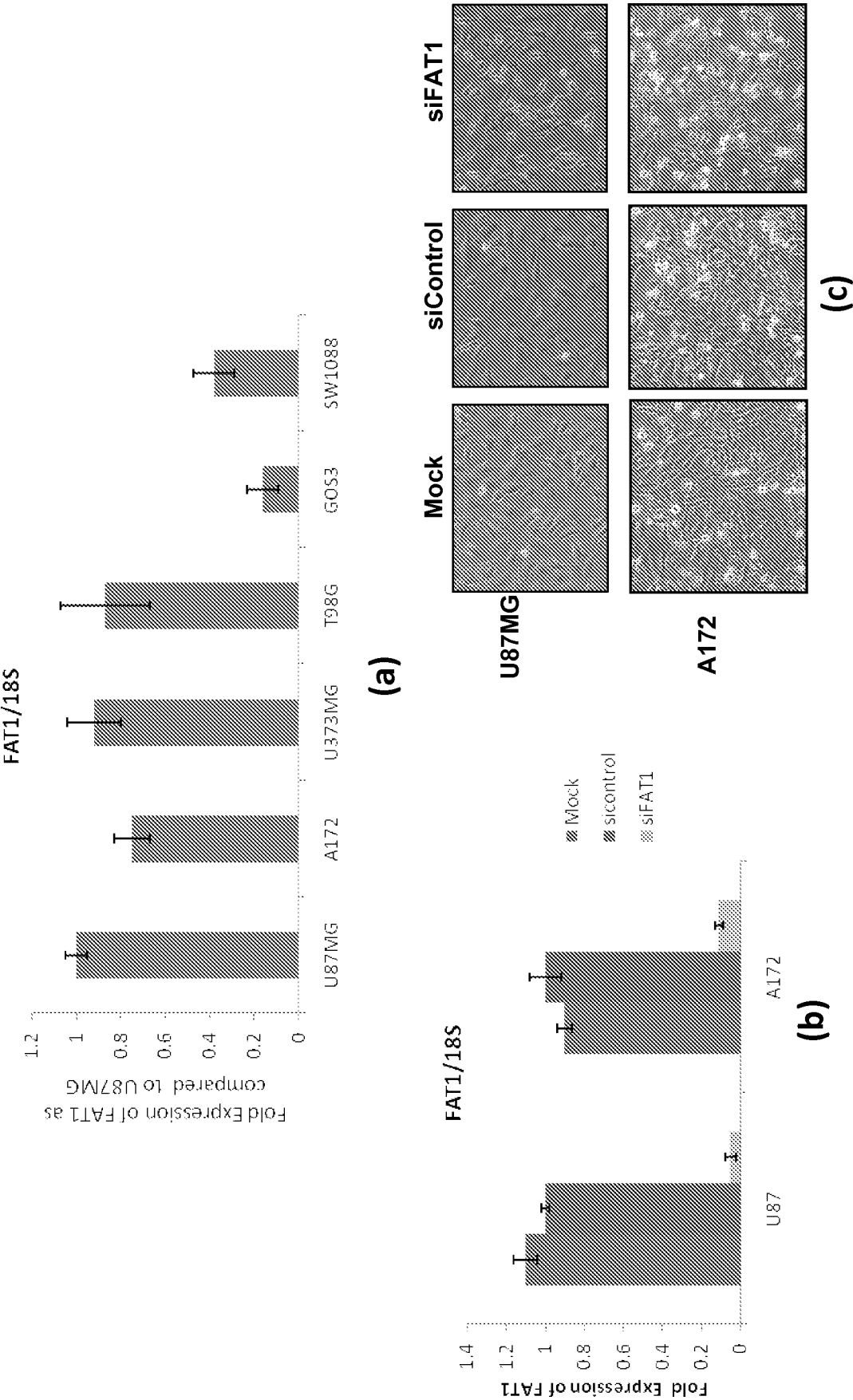


Figure 1

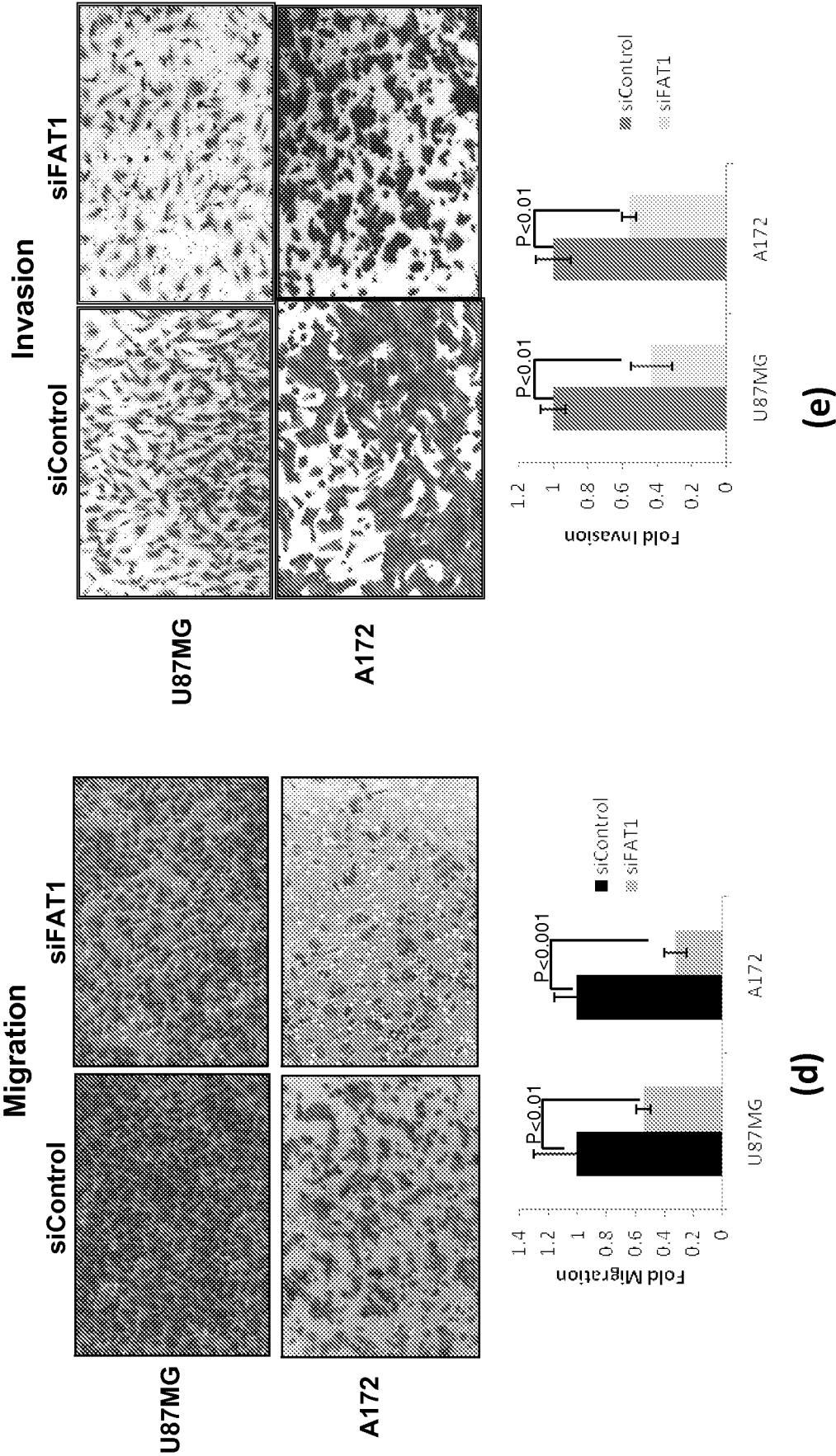
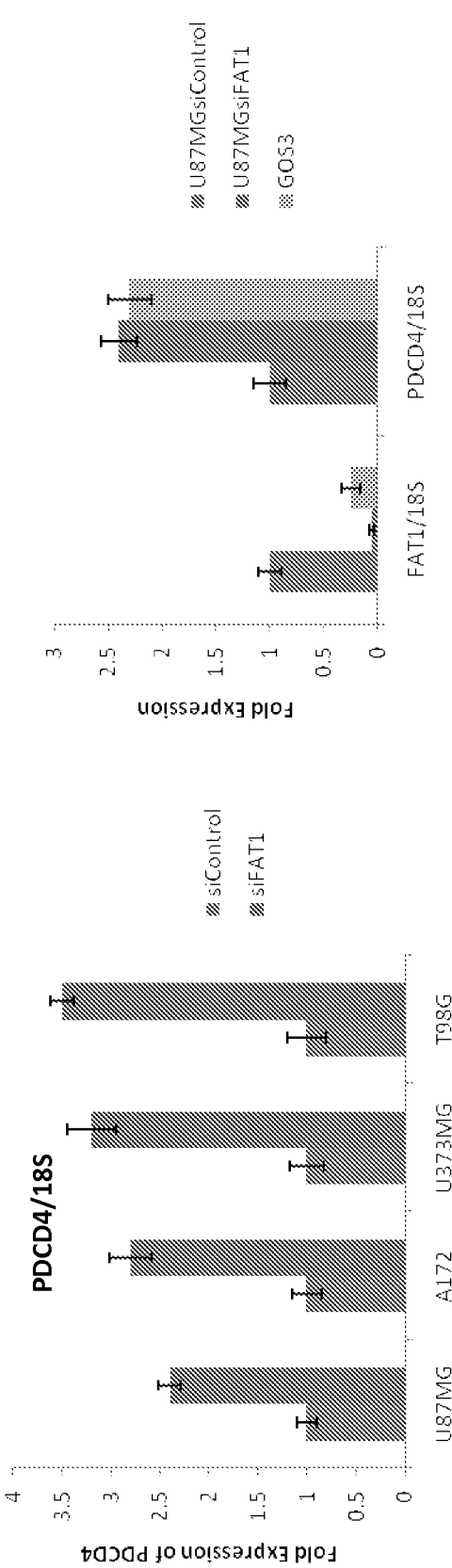
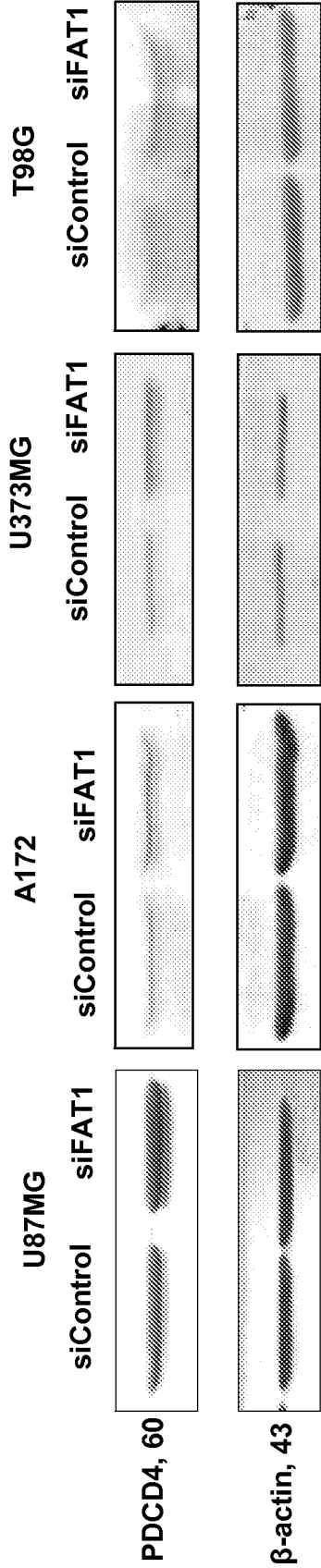


Figure 2



(a)

(c)



(b)

Figure 3

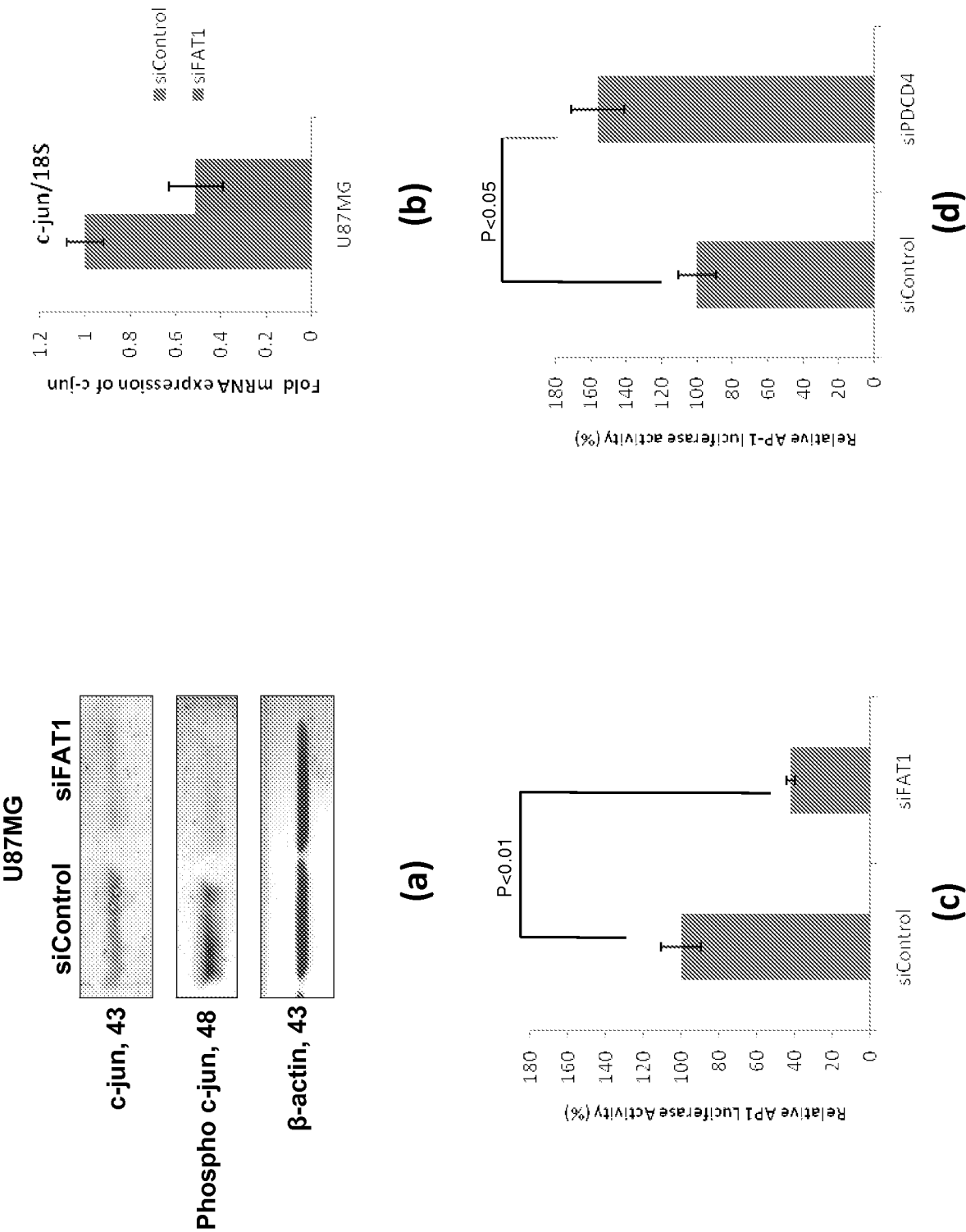


Figure 4

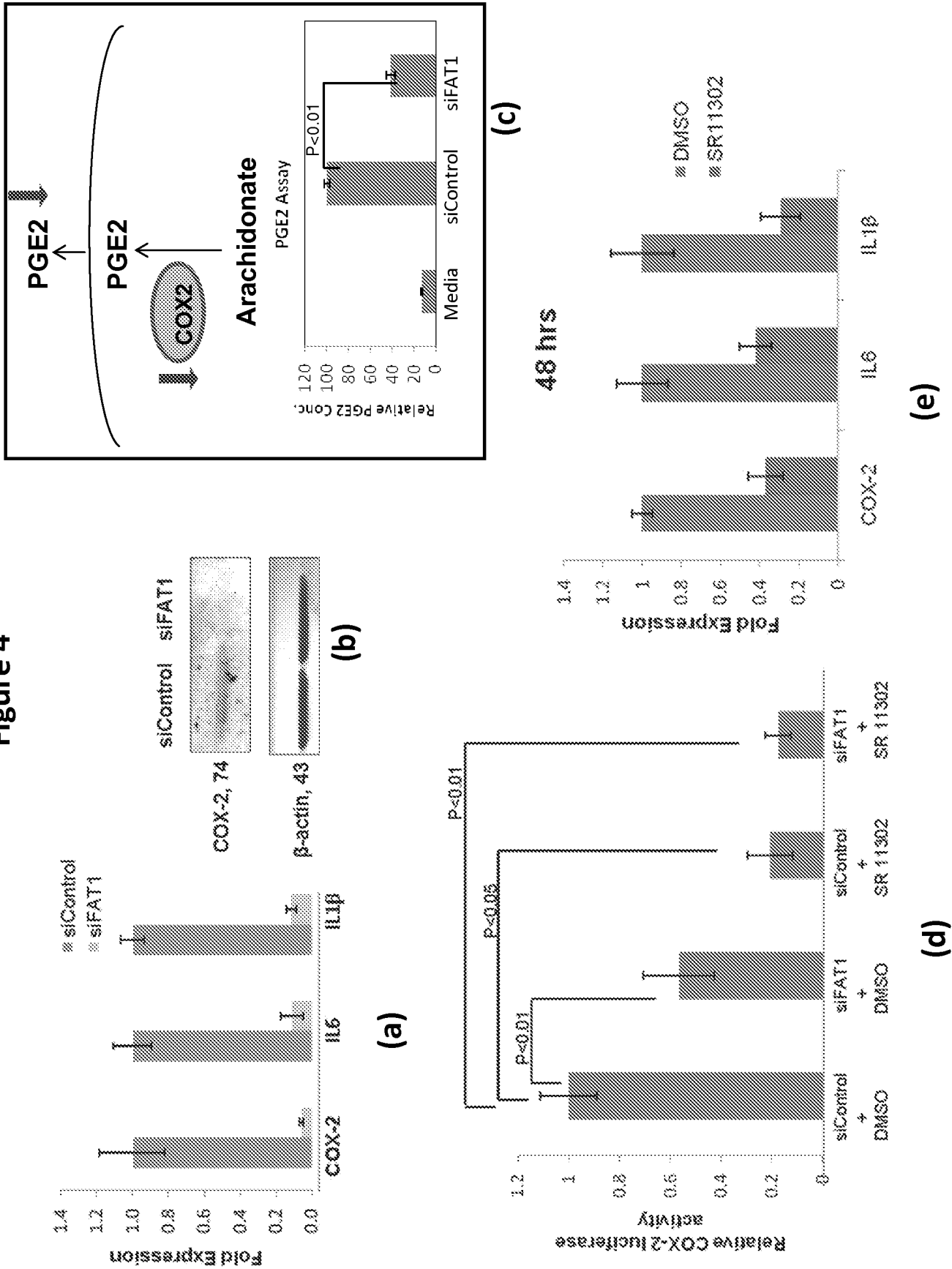


Figure 5

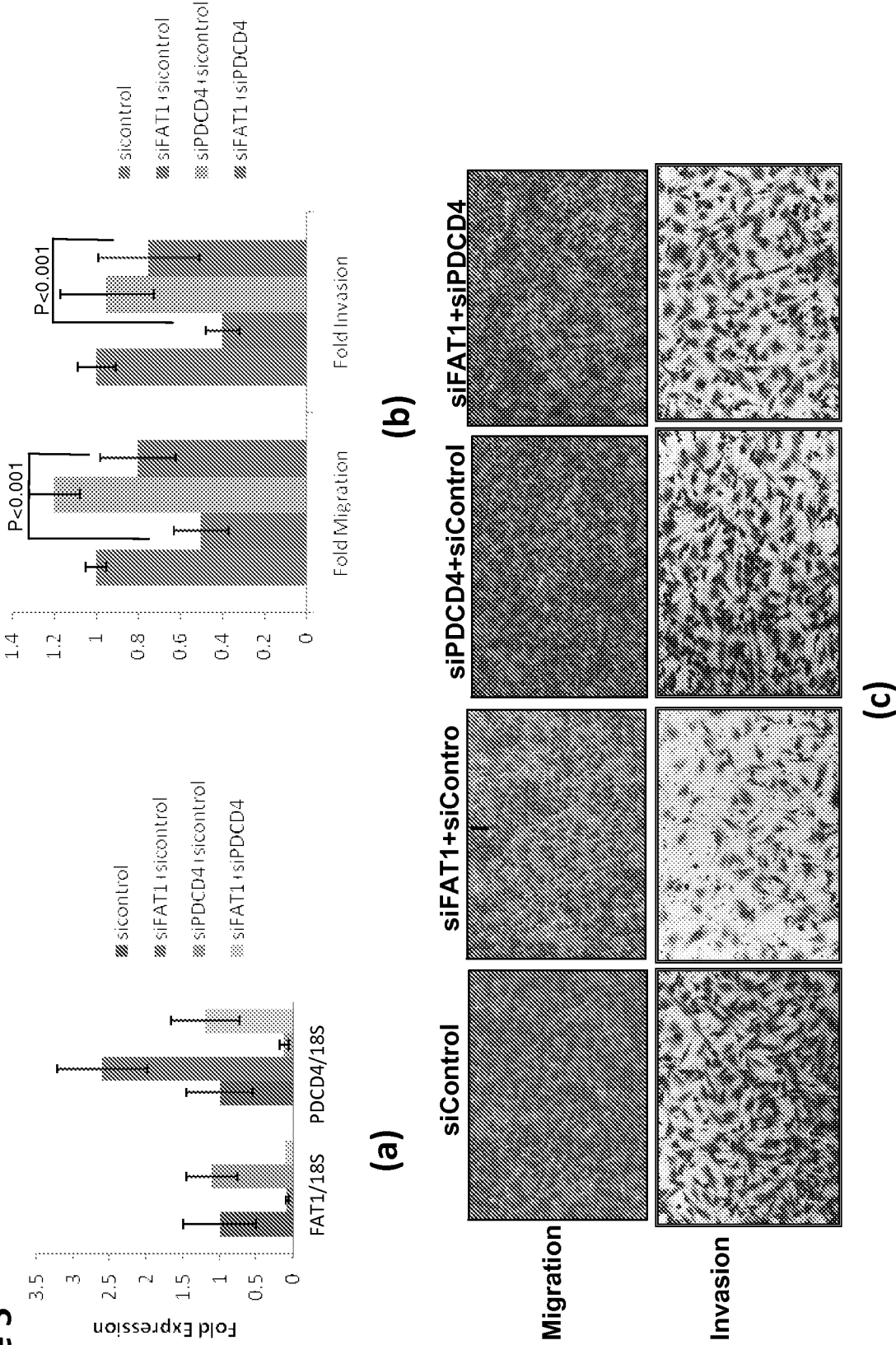


Figure 5

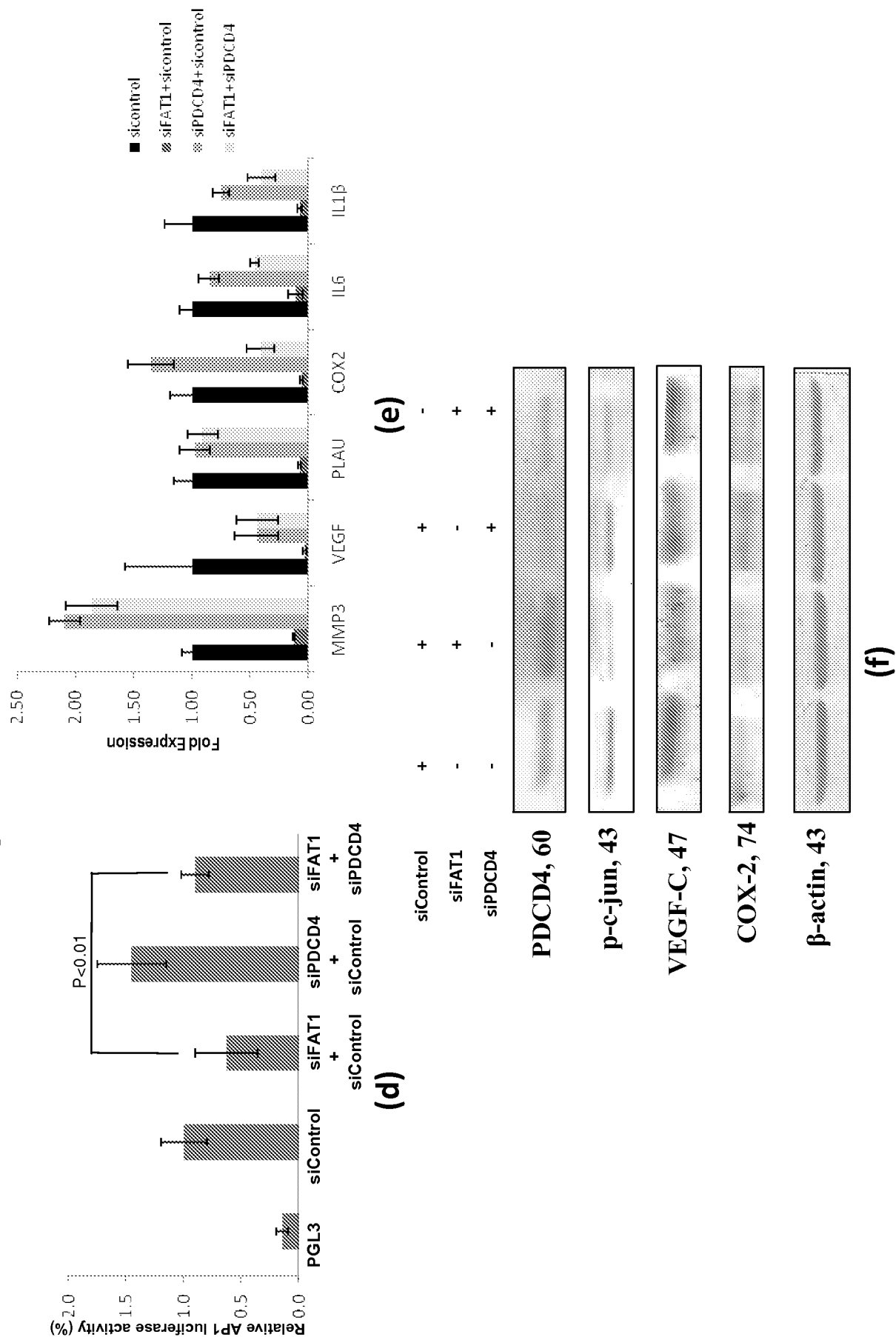


Figure 6

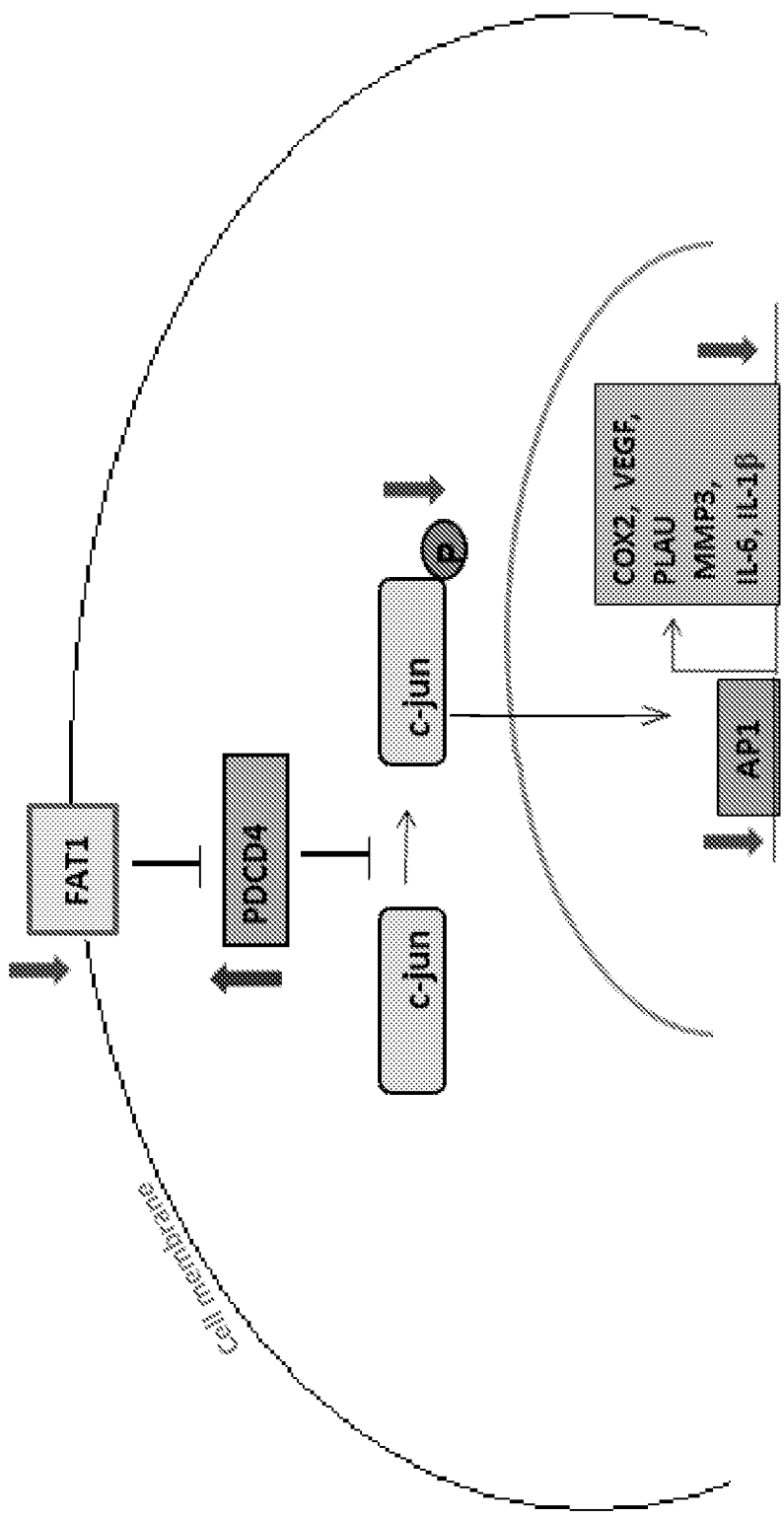


Figure 7

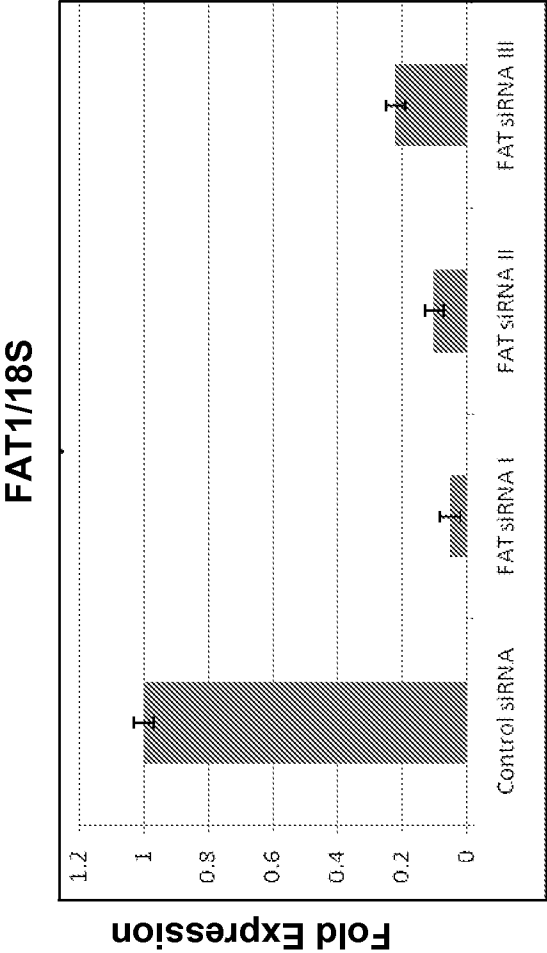
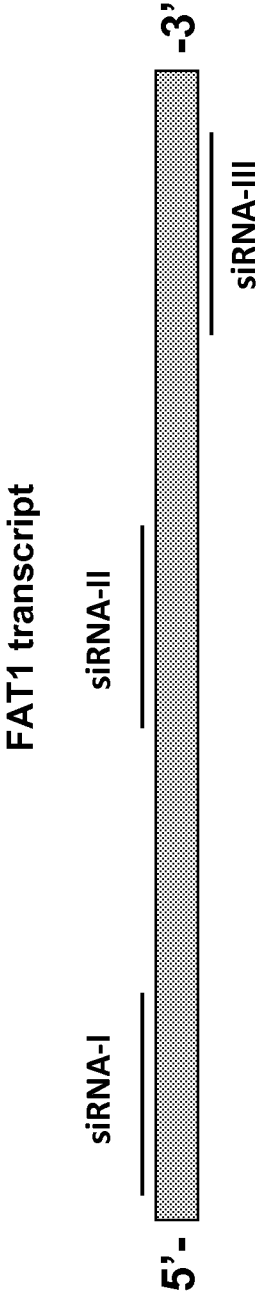


Figure 8

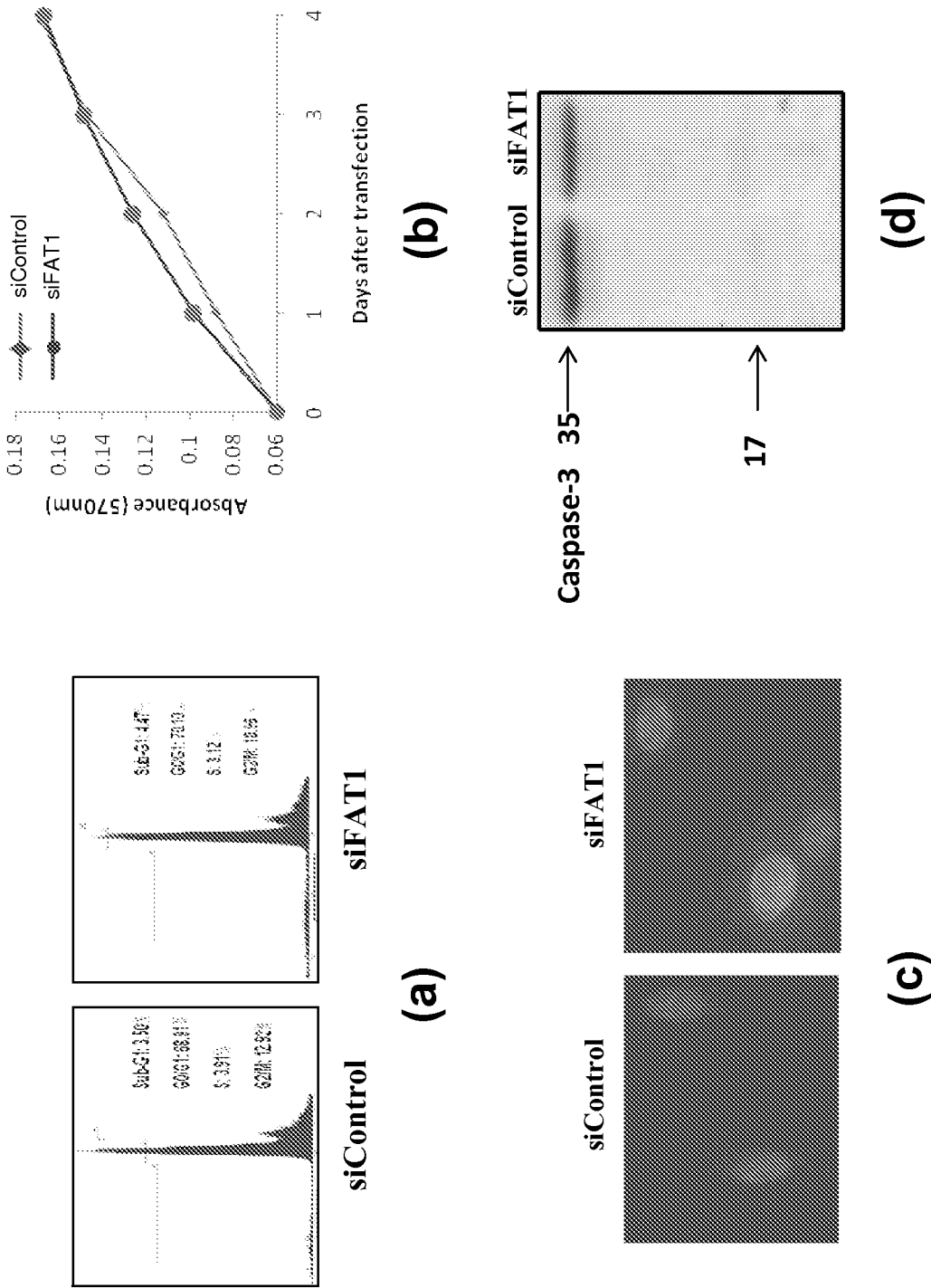


Figure 9

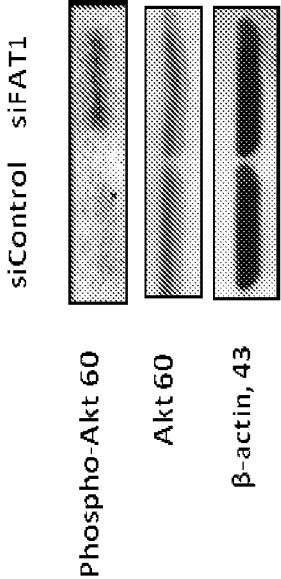
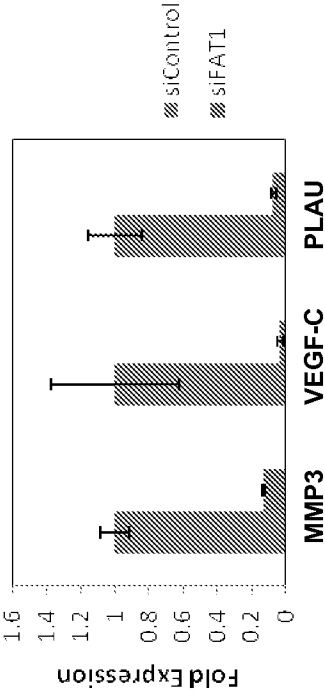
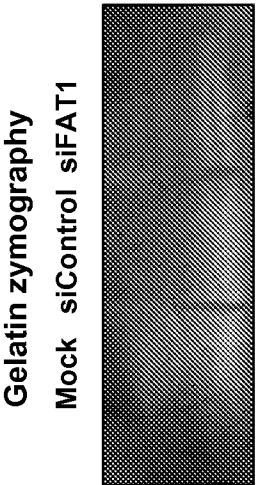


Figure 10



(a)



(b)

Figure 11

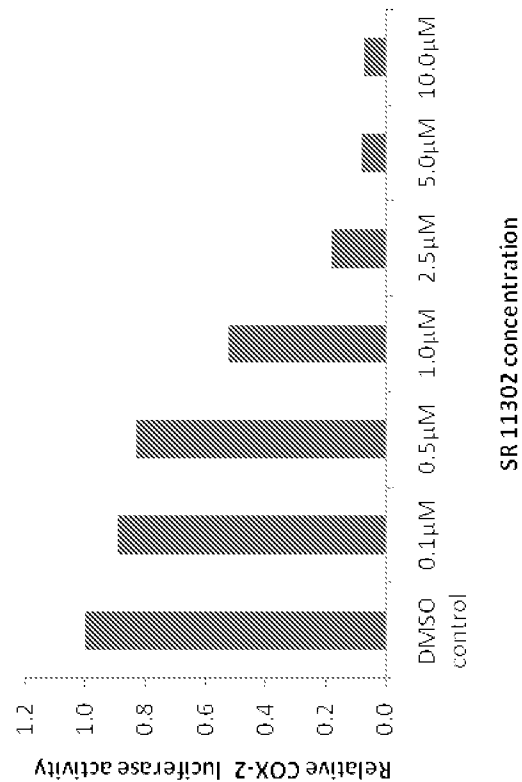


Figure 12

