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(54) Title: PIWIL2-LIKE (PL2L) PROTEINS-TARGETED CANCER DIAGNOSIS AND THERAPY

(57) Abstract: Described herein are tumor-specific antigens called Piwil2-like (PL2L) proteins, which are useful as biomarkers for cancer diagnostics, tumor vaccines; methods to deliver multi-valent PL2L tumor vaccines; and monoclonal antibodies (mAbs) to PL2L proteins that are effective for cancer therapy.



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TITLE

Piwil2-like (PL2L) Proteins-targeted Cancer Diagnosis and Therapy

Inventor: Jian-Xin Gao

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present invention claims the benefit of the provisional patent application Ser. No. 61/393,143 filed October 14, 2010.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was not made with any Government support and the Government has no rights in this invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted via EFS-web and is hereby incorporated by reference in its entirety. The ASCII copy, created on October 11, 2011, is named 604_52410_SEQ_LIST_OSURF-11026.txt, and is 33,091 bytes in size.

TECHNICAL FIELD AND INDUSTRIAL APPLICABILITY OF THE INVENTION

[0004] In one non-limiting aspect, this invention is directed to monoclonal antibodies (mAbs) to PL2L proteins. In another non-limiting aspect, the invention is also directed to tumor-specific antigens called Piwil2-like (PL2L) proteins.

BACKGROUND OF THE INVENTION

[0005] There is no admission that the background art disclosed in this section legally constitutes prior art.

[0006] A broad goal of cancer research is to identify and characterize a common developmental pathway for tumors, since such common pathway would be extremely beneficial for developing a safe and cost-effective therapeutic strategy for cancer.

[0007] Although cancer genes have been extensively investigated for decades, most cancer genes that have been discovered are cancer-contributing genes (including, for example, oncogenes (ONGs), tumor suppressor genes (TSGs) and stability genes (SGs)), rather than cancer-causing genes. These cancer-contributing genes are converged to several signaling pathways, which signaling pathways are also required for the normal control of cell survival and cell growth, and thus, are indispensable for tumor development.

[0008] Therefore, there is an urgent need to discover one or more "cancer causing" genes that are unique among genes, but are common for the development of various types of cancer.

[0009] A major obstacle for cure of cancer is that there is still no sufficient understanding of how a cancer is initiated and progressed, despite the revived cancer stem cell (CSC) hypothesis. There are several clonal precancerous stem cell (pCSC) lines (Lin⁻CD44⁺CD24⁻) from murine lymphoma, which have the potential of both benign and malignant differentiation, depending on environmental cues. The inventor herein now believes that the identification of pCSCs shows that pCSCs are a precursor of CSCs. Both pCSCs and CSCs, which are considered as representing two developing stages of tumor stem cells (TSCs), can serve as tumor vasculogenic progenitor cells (TVPCs). The existence of pCSCs has been functionally and clinically verified in the murine mammary carcinoma and in human leukemia, as well as in intestinal precancerous lesions.

[0010] The *PIWIL2* (piwi-like 2; alias *mili* in mice or *hili* in humans) is a member of PIWI/AGO gene family, located at chromosome 8 of humans and chromosome 14 of mice, with 23 exons coding 973 amino acids (110 kDa of MW), with about 88.77% homologous between humans and mice in gene sequence (genecards.org/cgi-bin/carddisp.pl?gene=PIWIL2). PIWI/AGO proteins contain Piwi and PAZ domains, having multiple biological functions (such as GSC self-renewal, cell cycling, RNA interference (RNAi), epigenetic modulation, and chromatin remodeling) in various organisms. Four members of PIWI or AGO subfamily have been identified in human genome (Piwi1, Piwi2, Piwi3 and Piwi4; and AGO1, AGO2, AGO3 and AGO4). All the members of PIWI subfamily are mainly expressed in the testis or embryonic tissues, and are essential for stem cell self-renewal such as in *Drosophila* and gametogenesis in mammals. The AGO subfamily is ubiquitously expressed in the embryonic and adult tissues, mediating RNAi via forming two types of RNAi complex: RNA-induced gene silencing complex (RISC), and RNA-induced initiation of transcriptional gene silencing (RITS) complex. The former (RISC) mediates posttranscriptional gene silencing through activating RNase activity and cleaving the RNA, and the latter (RITS) is required for transcriptional gene silencing and/or chromatin remodeling.

[0011] The *PIWIL2* is silenced in adult somatic and stem cells, but is widely expressed in various types of cancers, including hematopoietic, cervical and breast cancers. Especially, it is stably expressed in pCSCs, suggesting that it might play an important role in the tumor initiation and progression. Other members of PIWI subfamily have not been detected in cancer, except for *PIWIL1* (*HIWI* in humans), which was exclusively detected in human seminomas.

[0012] Recently, Piwi2 has been found to bind a novel class of small (26-30 nt) RNA, which is named as piwi-interacting RNA (piRNA) or repeat-associated small interfering RNAs (rasiRNAs), in mammal testis. It may silence selfish genetic elements, such as retrotransposons, in the GSCs of testis. Moreover, Piwi proteins also mediate epigenetic activation through promoting euchromatin histone modifications and piRNA transcription in subtelomeric heterochromatin in *Drosophila*, suggesting that Piwi2 could regulate tumor development epigenetically.

[0013] The knockdown of "Piwi2 mRNAs" by Piwi2-specific small interference RNAs

(siRNAs) significantly reduced murine pCSC expansion *in vitro*. However, overexpression of *Piwil2* gene in BM cells cultured in the XLCM-conditioned medium induced proliferation of the stem/progenitor cells, changes in cell morphology, and formation of embryonic body (EB)-like colonies, followed by apoptosis. The inventor herein refers to this phenomenon as the proliferation- or transformation-associated cell death (PACD or TACD), characterized by a timing difference between cell proliferation and apoptosis. This delayed cell death induced by exogenous *Piwil2* is in contrast to the growth-promoting or anti-apoptotic role of “*Piwil2*” that is spontaneously expressed in pCSCs or transiently expressed in NIH-3T3 cells. The inventor herein now believes that these contradictory observations show that *Piwil2* either plays a distinct role in pCSCs versus normal stem/progenitor cells or itself is expressed in a different form.

[0014] This is of importance since cancers are not simply genetic diseases, but are caused by both genetic mutations and immunocompromising of the patient's systems. Thus, immunoprevention and immunotherapy as adjuvant therapies are critical to prevent and/or treat cancer. Therefore, identification of common tumor specific antigens is a key toward the cure of cancers.

SUMMARY OF THE INVENTION

[0015] In a first broad aspect, there is provided herein an isolated monoclonal antibody which binds specifically to at least one of: a PIWIL2 peptide and a PL2L protein.

[0016] In certain embodiments, the PL2L protein comprises one of: PL2L80, PL2L60, PL2L50, PL2L42 and PL2L40.

[0017] In certain embodiments, the antibody specifically recognizes PIWIL2.

[0018] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US.

[0019] In certain embodiments, the antibody specifically recognizes PIWIL2 and PL2L proteins.

[0020] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA02, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.

[0021] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.

[0022] In another broad aspect, there is provided herein an isolated antibody which is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US

[0023] In another broad aspect, there is provided herein an isolated antibody which is produced by the hybridoma cell line MH-KA012, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.

- [0024] In another broad aspect, there is provided herein an isolated antibody which is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.
- [0025] In another broad aspect, there is provided herein a composition comprising the antibody as described herein and a pharmaceutically acceptable, sterile injection medium. In another broad aspect, there is provided herein a reagent comprising the antibody as described herein.
- [0026] In another broad aspect, there is provided herein a test kit comprising the antibody as described herein.
- [0027] In another broad aspect, there is provided herein an isolated cell which produces the antibody as described herein. In certain embodiments, the cell is an animal, plant, prokaryotic, or human cell.
- [0028] In another broad aspect, there is provided herein a hybridoma cell line which produces the isolated antibody as described herein.
- [0029] In another broad aspect, there is provided herein an isolated monoclonal antibody which binds specifically to an epitope comprising Pa [SEQ ID NO:26].
- [0030] In another broad aspect, there is provided herein an isolated monoclonal antibody which binds specifically to an epitope comprising Pb [SEQ ID NO:27].
- [0031] In another broad aspect, there is provided herein a method of assaying a biological specimen for a cancer, comprising: contacting a sample of the biological specimen with an isolated monoclonal antibody which binds specifically to at least one of: a PIWIL2 peptide and a PL2L protein and detecting PIWIL2 and or a PL2L protein by the monoclonal antibody.
- [0032] In certain embodiments, the PL2L protein comprises one of: PL2L80, PL2L60, PL2L50, PL2L42 and PL2L40.
- [0033] In certain embodiments, the antibody specifically recognizes PIWIL2.
- [0034] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US.
- [0035] In certain embodiments, the antibody specifically recognizes PIWIL2 and PL2L proteins.
- [0036] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA02, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.
- [0037] In certain embodiments, the antibody is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.
- [0038] In certain embodiments, the cancer is detected in an immunoassay.
- [0039] In certain embodiments, the immunoassay is selected from the group consisting of MIC (microparticle capture immunoassays, LAI (latex agglutination inhibition), IC (solid phase

chromatographic), RIA (radio immunoassays), ELISA (enzyme linked immunoabsorbent assay), BIA (enzyme linked assays), FIA (fluorescence linked assays), LIA (luminescence linked assays), CLA (chemiluminescence assays), OA (optical color label assays), EST (electrochemical signal transducers) and rare earth metals label assays.

[0040] In certain embodiments, the biological specimen is a tissue sample that expresses PL2L.

[0041] In certain embodiments, the biological specimen comprises one or more of: blood, bone marrow, brain, breast, cervix, colon, heart, intestines, kidney, liver, lung, lymph nodes, ovary, oviduct, pancreas, prostate, salivary, skin, soft tissues, spleen, stomach, testis, thymus and uterus.

[0042] In certain embodiments, the cancer is one or more of: leukemia, lymphoma, adenoma, adenocarcinoma, carcinoma, melanoma, sarcoma, neuroblastoma, hepatoma, insulinoma and endothelioma

[0043] In another broad aspect, there is provided herein a method of diagnosing a cancer, comprising: contacting a sample with an antibody as described herein; removing substances from the sample that are not bound by the antibody; and, measuring the concentration, amount, or presence of bound antibody in the sample via a label associated with the antibody.

[0044] In another broad aspect, there is provided herein a human antibody or an antigen-binding portion thereof that specifically binds to and activates a Piwil2-like (PL2L) protein.

[0045] In another broad aspect, there is provided herein a human antibody or an antigen-binding portion thereof that specifically binds to and activates a 15-mer fragment of a Piwil2-like (PL2L) protein.

[0046] In another broad aspect, there is provided herein a monoclonal antibody comprising mAb Kao1.

[0047] In another broad aspect, there is provided herein a monoclonal antibody comprising mAb Kao2/3.

[0048] In another broad aspect, there is provided herein a monoclonal antibody to human PL2L proteins which inhibits tumorigenesis of PL2L proteins-expressing tumor cells.

[0049] In another broad aspect, there is provided herein an isolated PL2L peptide Pa comprising a sequence having 21 aa: CFYPKRAMDQARELVNMLEKI [SEQ ID NO:26].

[0050] In another broad aspect, there is provided herein an isolated PL2L peptide Pb comprising a sequence having 15 aa: IPEKMKKDFRAMKDL [SEQ ID NO:27].

[0051] In another broad aspect, there is provided herein an isolated Piwil2 peptide comprising: 503 IPEKMKKDFRAMKDL 517 [SEQ ID NO:28].

In another broad aspect, there is provided herein a monoclonal antibody specifically immunoreactive with Piwil2 (Kao1).

- [0052] In another broad aspect, there is provided herein a monoclonal antibody specifically immunoreactive with both Piwil2 and PL2L proteins (Kao2 and Kao3).
- In another broad aspect, there is provided herein a monoclonal antibody specifically immunoreactive with Piwil2, PL2L80, PL2L60, PL2L50 and PL2L40.
- In another broad aspect, there is provided herein use of the antibody described herein, for detection of one or more of: primary and metastatic cancers.
- [0053] In another broad aspect, there is provided herein use of the antibody described herein, for inhibiting tumorigenesis of PL2L proteins-expressing tumor cells.
- [0054] In another broad aspect, there is provided herein an isolated protein comprising an amino acid sequence of human, PL2L60, starting from aa 444 to aa 973 of human Piwil2 **[SEQ ID No:23]**.
- [0055] In another broad aspect, there is provided herein an isolated PL2L protein having at least one tumor-specific antigen useful as a tumor vaccine.
- [0056] In another broad aspect, there is provided herein a PL2L peptide vaccine useful to induce effective anti-tumor immunity.
- [0057] In another broad aspect, there is provided herein use of the antibody described herein to induce apoptosis in PL2L60-expressing precancerous stem cells (pCSCs), thereby preventing their tumorigenesis.
- [0058] In another broad aspect, there is provided herein an isolated PL2L protein useful as a diagnostic biomarker for cancers.
- [0059] In another broad aspect, there is provided herein an isolated PL2L protein useful as a therapeutic biomarker for cancers.
- In another broad aspect, there is provided herein an isolated PL2L60 protein useful as a biomarker for one or more types of cancer.
- [0060] In another broad aspect, there is provided herein a PL2L peptide vaccine useful to induce effective anti-tumor immunity against tumors originated from various types of tumors.
- [0061] In another broad aspect, there is provided herein use of the antibody described herein as a medicament.
- [0062] In another broad aspect, there is provided herein use of the antibody described herein for the production of a medicament intended for the treatment of cancer.
- [0063] In another broad aspect, there is provided herein use of a monoclonal antibody directed against the human PL2L protein receptor for the production of a medicament intended for the treatment of a cancer.
- [0064] In another broad aspect, there is provided herein use of the antibody described herein, in combination with one or more other antibody(ies) directed against one or more other antigen(s) expressed on a cancer cell.

In another broad aspect, there is provided herein a pharmaceutical composition comprising at least one antibody described herein, and at least one excipient and/or at least one pharmaceutically acceptable vehicle.

[0065] In another broad aspect, there is provided herein a method of treating a subject having a malignancy which comprises a tumor, comprising administering to the subject a therapeutically effective amount of an anti-PL2L antibody or a fragment thereof in an amount sufficient to modulate the function of the malignant cells within the tumor that promote tumor growth and survival, to thereby elicit immunodulatory effects.

[0066] In certain embodiments, the anti-PL2L antibody or fragment thereof is a chimeric, humanized, or human antibody.

[0067] In certain embodiments, the anti-PL2L antibody or binding fragment thereof is selected from the group consisting of:

an anti-PL2L antibody or fragment thereof which binds a PL2L epitope bound by the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or fragment thereof which competes for binding to PL2L with the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises variable regions derived from variable regions of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises variable regions of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises complementarily determining regions (CDRs) of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855; and

an anti-PL2L antibody or a PL2L-binding fragment thereof which blocks PL2L signaling.

[0068] In certain embodiments, the anti-PL2L antibody is Kao1 or Kao2.

[0069] In certain embodiments, the method further comprises administering to the subject an anti-cancer agent, wherein the PL2L-targeted therapeutic and the anti-cancer agent are administered concurrently or consecutively in either order.

[0070] In another broad aspect, there is provided herein a method for enhancing an immune response against cancers, comprising administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855.

[0071] In another broad aspect, there is provided herein a method for treating a cancer-associated disease, comprising administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855.

[0072] In another broad aspect, there is provided herein a vaccine comprising an antibody as described herein.

[0073] In certain embodiments, the vaccine is for cancer.

[0074] In certain embodiments, PL2L is used as a specific tumor biomarker for detection and prognosis of cancer

[0075] In another broad aspect, there is provided herein use of a PL2L protein as a target to develop a tumor vaccine.

[0076] In another broad aspect, there is provided herein use of Piwil2 peptides homologous to PL2L proteins to develop a tumor vaccine.

[0077] In certain embodiments, the peptides are derived from exon 6 to 23 of Piwil2.

[0078] Various other objects and advantages of this invention will become apparent to those skilled in the art from the following detailed description of the preferred embodiment, when read in light of the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0079] The patent or application file may contain one or more drawings executed in color and/or one or more photographs. Copies of this patent or patent application publication with color drawing(s) and/or photograph(s) will be provided by the U.S. Patent and Trademark Office upon request and payment of the necessary fees.

[0080] **Figures 1A-1D:** pCSC lines do not express full length Piwil2 transcripts.

[0081] **Figure 1A:** pCSCs did not express full length Piwil2 (mili) transcripts. For RT-PCR

analysis, murine pCSC lines (2C4, 3B5C and 3B6C) and murine tumor cell line (EL-4) were harvested at log phase of growth; bone marrow-derived dendritic cells of mice (BMDC) were prepared and harvested at day 11 of culture; and murine testis was harvested from male B6 mice. Full length mili was amplified by primers P1-mili-forward and P4-mili-reverse (**Figure 13 - Table1 [SEQ ID Nos.:1-20]**). E18-21: transcripts of mili exons 18-21.

- [0082] **Figure 1B:** Diagram of the primers used for Piwil2-specific GEM RT-PCR.
- [0083] **Figure 1C:** Products of GEM RT-PCR. SPL: freshly isolated splenocytes from male B6 mice.
- [0084] **Figure 1D:** Diagram of the results in **Figure 1C**. Dotted lines indicate that the exons were not transcribed by corresponding primers. The star markers “*” indicates that transcripts E21-23 sometimes were detectable in 2C4, 3B5c, and 3B6C cells. P: primers; E: Exons. Pmili: the primers were used to detect mouse Piwil2 transcripts, which amplify transcripts E18-21.
- [0085] **Figures 2A-2C:** Diagram of *PIWIL2* and PL2L genes as well as their transcriptional and translational products.
- [0086] **Figure 2A:** Schematic depiction of the genomic structure of *PIWIL2* and PL2L genes. Five potential promoters inside the *PIWIL2* gene were identified. The promoter P_{Piwil2} is responsible for the transcription of Piwil2 mRNA in humans and mice, while other four promoters inside this gene, P_{PL2L60} , P_{PL2L50} , P_{PL2L42} and P_{PL2L40} , may initiate the transcription of *PL2L60*, *PL2L50*, *PL2L42* and *PL2L40* genes, respectively. Human PL2L60, PL2L42 and PL2L40 mRNAs, and mouse PL2L50 mRNA have been identified in GenBank.
- [0087] **Figure 2B:** Schematic presentation of mRNA structure of *PIWIL2* and PL2L genes. All variants are truncated at 5'-end but all contain Piwil2 exons 15 to 23, except for PL2L40 which contains exons 13 to 20 and another two exons within the intron between exons 20 and 21 (red area in **Figure 2A** and **Figure 2B**).
- [0088] **Figure 2C:** Schematic structures of Piwil2 and PL2L proteins. Compared to the full-length Piwil2 protein, all PL2L proteins are defective or absent of whole PAZ domain. Piwi domain is normal in PL2L60 but defective in PL2L50, PL2L40 and PL2L42.
- [0089] **Figures 3A-3C:** Identification and characterization of PL2L proteins of humans and mice.
- [0090] **Figures 3A-3B:** Western blot analysis of Piwil2 and PL2L proteins expressed in mouse testis (**Figure 3A**) and HeLa cells (**Figure 3B**), using rabbit polyclonal antibody to Piwil2 peptide.
- [0091] **Figure 3 A:** Testicular cell lysates of mili^{-/-} and mili^{+/+} mice.
- [0092] **Figure 3B:** HeLa cell lysate: Lane 1 & 5: 4 µg/mL antibody without peptide; Lane 2 & 6: 4 µg/mL antibody with 16 µg/mL Peptide; Lane 3: 2 µg/mL antibody alone; Lane 4: 2 µg/mL antibody with 8 µg/mL Peptide. P: Piwil2 peptide; Ab: antibody to Piwil2 peptide.

[0093] **Figure 3C:** GEM RT-PCR analysis of testicular tissues from wild-type (Lane 1) and *mili*^{-/-} mice (Lane 2). The primers specific for E1-7, E6-14, E13-21 and E21-23 of *Piwi2* were used for GEM RT-PCR analysis. Primers specific for E18-21 were used as a positive control. Lane 3: no cDNA control.

[0094] **Figures 4A-4C:** PL2L60, rather than *Piwi2*, is predominantly and stably expressed in pCSC lines.

[0095] **Figure 4A:** The cell lysates of 2C4, 3B5C, 3B6C, 326T and splenocytes freshly isolated from B6 mice were analyzed by Western blotting, with rabbit anti-*Piwi2* antibody.

[0096] **Figure 4B:** The ratio of PL2L60 to β -actin (AU) was determined using Image J software (NIH) and the folds of increased PL2L60 in each cell line relevant to normal splenocytes are shown in number at the top of each column. AU: arbitrary unit.

[0097] **Figure 4C:** Comparison of cell viability between 2C4 and 326T colonies in cultures. The 2C4 and 326T cells (1×10^4 /well) were cultured in 24-well plates, and the cell colonies were examined and taken as micrographs under Zeiss inverted microscope at day 5 of culture (original: $\times 200$). Note that apoptotic 326T cell in cultures were easily discerned under microscope compared to 2C4, 3B5C and 3B6C. Shown is one representative colony of 2C4 and 326T, respectively, with apoptosing and apoptotic cells [2C4: 4/32 (12.5%); 326T: 11/18 (61.1%)]. 3B5C and 3B6C cells had the viability similar to 2C4 (not shown). An arrow indicates a representative apoptosing cell with condensed nucleus in the 2C4 colony.

[0098] **Figures 5A-5F:** PL2L60 up-regulates *Stat-3* and *Bcl-2* gene expressions and promotes pCSC expansion *in vitro*. The pCSCs (clone 2C4) were transfected with *Piwi2* exon 11-specific siRNA or scrambled siRNA (scRNA), mock transfected (Mock), or untransfected (Untr), and examined for PL2L60 expression and cell expansion.

[0099] **Figure 5A:** Western blotting of PL2L60 in pCSCs.

[0100] **Figure 5B:** Quantitation of PL2L60 proteins (AU) in Figure 5A.

[0101] **Figure 5C:** RT-PCR analysis of *Piwi2* transcripts in pCSCs using primers specific for E6-14 or E18-21.

[0102] **Figure 5D:** pCSC expansion *in vitro* after siRNA transfection. Cell counts are shown as mean $\pm$ SD in triplicate. **, $p < 0.01$; as compared to the mock or scRNA transfected groups.

[0103] **Figure 5E:** RT-PCR analysis of *Stat-3*, *Bcl-2* and *Bcl-XL* transcripts in pCSCs. H₂O indicates that cDNA was omitted.

[0104] **Figure 5F:** Quantitation of *Stat3*, *Bcl-2* and *Bcl-XL* transcripts (AU) in **Figure 5E**. For Western blot (**Figure 5A** and **Figure 5B**) and RT-PCR (**Figure 5C**, **Figure 5E** and **Figure 5F**) analysis, the cells were harvested at 36-48 h after transfection; for cell expansion, the cells were counted at various time points post transfection as indicated. The cells were seeded in

triplicate. AU (Arbitrary Units) was determined by the ratio of a factor tested to β -actin. The data shown are a representative from at least 3 experiments.

- [00105] **Figures 6A-6D:** PL2L60 is predominantly expressed in human tumor cells and promotes their expansion *in vitro*.
- [00106] **Figures 6A-6B:** PL2L60 expression in human breast and cervical cancer cell lines. Breast (MDA-MB-231, MDA-MB-468, and MCF-7) and cervical (HeLa) cancer cell lines and primary human dermal fibroblasts (HDFs) were cultured in 6-well plates ($2 - 3 \times 10^5$ /well), harvested when they grew confluent, and examined for PL2L60 expression by Western blotting, using rabbit pAb to Piwil2 (**Figure 6A**). The PL2L60 in **Figure 6A** was normalized to β -actin and quantified (**Figure 6B**). Shown is a representative of three experiments. Numbers at the top of each column in (**Figure 6B**) indicate the increased folds of PL2L60 in breast and cervical cancer cell lines, as compared to HDFs.
- [00107] **Figures 6C-6D:** PL2L60 promotes human cancer cell expansion *in vitro*. MDA-MB-231 cells were transfected in triplicate in 6-well plates with human Piwil2 exon 21-specific dicer substrate RNA duplexes (siRNA) or scrambled RNA duplexes (scRNA), or mock transfected for 48 h, and the cells were harvested, counted and analyzed by RT-PCR for Piwil2 E18-21 mRNA expression. **Figure 6C:** Results of RT-PCR; **Figure 6D:** Cell numbers (mean + SD in triplicate). The data shown are a representative from three experiments.
- [00108] **Figures 7A-7E:** PL2L60 promoting tumorigenesis of human cancer cells is associated with the enhanced transition of $G_{0/1}$ to S phase of cell cycle and increased nuclear NF- κ B expression. Breast cancer cell lines (MDA-MB-231) were stably transduced with Lenti-PL2L60 or Lenti-GFP viral vectors, cloned and analyzed for their cell cycling, expansion and tumorigenic capacity in CB17 SCID mice.
- [00109] **Figures 7A-7B:** PL2L60 promotes the transition from $G_{0/1} \rightarrow$ S-phase of cell cycle and cell expansion. PL2L60-transduced and GFP-transduced stable cell lines were cloned and analysis for cell cycle (**Figure 7A**) and cell expansion (**Figure 7B**). The data shown in **Figure 7A** are derived from a representative clone of PL2L60-transduced (Clone C5) and GFP-transduced breast cancer cell lines (clone C6C2); and % of the cells entering S-phase of three experiments was summarized in **Figure 7B**. The number of cells cultured in 6-well plates (5×10^4 /well) was enumerated at day 5 of culture (**Figure 7B**). **, $p < 0.01$; as compared between the PL2L60-transduced and GFP-transduced cell lines.
- [00110] **Figure 7C:** NF- κ B (RelA/p65) expression in PL2L60-transduced (231-PL2L60) and GFP-transduced breast cancer cells (231-GFP), as revealed by ICC staining (original magnification: x 400).
- [00111] **Figures 7D-7E:** Enhanced tumorigenic capacity of 231-PL2L60 cells at the initial stage or latent phase of xenograft tumor formation. **Figure 7D:** Shown are the tumor growth

curves of 231-PL2L60 and 231-GFP cells (n=3/group). **Figure 7E:** Living images taken at the time as indicated by an arrow in **Figure 7D**. An arrow indicates weak GFP signal at the site injected with GFP-231. The data shown are representative of two reproducible experiments. GFP: 231-GFP; PL2L60: 231-PL2L60. **, $p < 0.01$, as compared between two groups.

[00112] **Figures 8A-8E:** PL2L proteins rather than Piwil2 are predominantly expressed in human primary and metastatic cancers in association with NF- κ B

[00113] **Figure 8A:** Characterization of mouse mAbs to Piwil2-peptide shared by Piwil2 and PL2L proteins of humans and mice. The supernatants of mAbs (clone Kao1, Kao2, and Kao3; 1:10 dilution) were used to Western-blot mouse testicular lysates and human colon cancer cell line SW 480 lysates. Kao1 specifically reacted with Piwil2 (~110 kDa) but not with PL2L proteins in the testis. The faint bands detected by mAb Kao1 in SW 480 reflected non-specific binding of secondary antibody, because (a) similar bands were observed in the absence of primary antibody (no mAb control); (b) no corresponding bands were observed in the testicular lysates; and (c) they were not matched with known PL2L protein bands blotted by Kao2 and Kao3 mAbs. Note that Kao2 and Kao3 reacted with Piwil2, PL2L80, PL2L60, PL2L50 and PL2L40, and the different intensity between them reflects their variable amounts in the relevant samples.

[00114] **Figure 8B:** PL2L proteins, but not Piwil2, are predominantly expressed in primary breast and cervical cancers. TMAs of breast cancer (n = 300) and cervical cancer (n = 100) or regular sections (breast cancer: n = 3; cervical cancer: n = 5) were stained with mAb to Piwil2 (Kao1) or PL2L proteins (Kao2 or Kao3). Shown are representative micrographs of Piwil2 (B1 & B3) and PL2L proteins expression (B2 & B4) in the primary breast (B1 & B2) and cervical cancers (B3 & B4). Arrows in B1 and B3 indicate the Piwil2-expressing (mAb Kao1⁺) apoptotic or apoptosing cells enlarged in the insets. The inset in B2 shows a tumor cell enriched with heterochromatin were faintly stained by mAb Kao2, while other two cells enriched with euchromatin were strongly stained by mAb Kao2 in cytoplasm and faintly in nuclei. The tumor cells in the inset were enlarged from those indicated by a red arrow in B2. In addition, green arrows in B2 indicate the C-N pattern of mAb Kao2⁺ cells. Magnification of the micrographs: B1 and B4: x 75; B3, x150, and B2, x300.

[00115] **Figure 8C:** PL2L proteins, but not Piwil2, are expressed in all metastatic tumor cells. Shown are representative micrographs of Piwil2 (C1 & C3) and PL2L proteins expression (C2 & C4) in the metastatic cancer cells in tissue stroma (C1 & C2) and in lymph nodes (C3 & C4). Arrows in C1 indicate Piwil2 (mAb Kao1)-negative metastatic tumor cells in the stroma; and arrows in C3 indicate apoptosing tumor cells expressing Piwil2 (mAb Kao1) in the lymph node. Magnification of micrographs: C1, x150; C2, C3 and C4: x400.

[00116] **Figure 8D:** Co-expression of PL2L proteins (mAb Kao2) with NF- κ B in tumor cells. Shown are representative micrographs of single and double color IHC staining of serial sections of

breast cancer. The serial sections were prepared from an invasive ductal carcinoma infiltrated by inflammatory cells. The serial sections were stained by mouse mAb Kao2 alone (D1: brown; x150), rabbit mAb to p65 alone (D2: pink; x150) or mAb Kao2 followed by mAb to p65 (D3: brown and pink; 150). Note that the infiltrated inflammatory cells are negative for PL2L proteins in D1 and positive for p65 in D2. The box in D3 is projected to D4 (x600). Arrows in D4 indicate the tumor cells doubly stained by mAb Kao2 and mAb to p65 in the nuclei.

[00117] **Figure 8E:** Summary of IHC staining of the TMAs of breast cancer and cervical cancer. Shown are percentages of breast (n=300) and cervical cancers (n=100), respectively, expressing Piwil2 or PL2L proteins.

[00118] **Figure 9:** The mechanisms underlying PL2L60 promoting tumorigenesis. PL2L60 can promote tumor cell growth through interaction with NF- κ B. Migration of PL2L60 from cytoplasm to nucleus may promote nuclear localization of NF- κ B. In the nucleus, PL2L60 may promote the transcriptional activity of NF- κ B through remodeling chromatin structure, resulting in enhanced transcription of cell survival and cell cycling genes.

[00119] **Figures 10A-10B:** *Piwil2* and PL2L genes are not expressed in normal tissues of mice.

[00120] **Figure 10A:** *Piwil2* and PL2L proteins expression: Mouse tissues (C57BL/6) of lung, heart, kidney, liver, testis, spleen, thymus and pancreas were analyzed by Western blotting for the expressions of *Piwil2* and PL2L proteins, using polyclonal rabbit anti-*Piwil2* peptide antibody (RB9926). The testicular lysates were used as a positive control. A band in kidney is non-specific (>50 kDa).

[00121] **Figure 10B:** *Piwil2* and PL2L mRNA expression: Mouse tissues of brain, heart, salivary, kidney, lung, stomach, liver, pancreas, colon, intestines, thymus, spleen, lymph nodes, ovary, bone marrow, skin, uterus, oviduct, and testis were analyzed by RT-PCR for *Piwil2* expression, using primers of E18-21, which can amplify the transcripts of both *Piwil2* and PL2L genes.

[00122] **Figure 11:** PL2L60 was predominantly expressed in various types of tumor cell lines and some immortalized cell lines of humans and mice. The tumor cell lines and immortalized cell lysates were analyzed by Western-blotting for *Piwil2* and PL2L proteins expression with polyclonal rabbit anti-*Piwil2* peptide antibody (RB9926). The origins of tissue and tumor type of the cell lines are shown in **Figure 13 (Table 1)**. All the cell lines examined predominantly expressed PL2L60 proteins except for immortalized HLMVEC-Sv and HLVEC-Pri, which expressed no or little PL2L60. Note that cell line H9 did not express β -actin.

[00123] **Figure 12:** Generation of stable breast cancer cell lines overexpressing PL2L60.

[00124] **Figure 12A:** Protein sequence of human *Piwil2* (973 aa) [SEQ ID No:21] and murine *Piwil2* (971 aa) [SEQ ID No:22]. The sequence of human PL2L60 (60 kDa) is started from aa

444 (indicated by an arrow head) to aa 973 of human Piwil2 [SEQ ID No:23]. aa: amino acid. The purple and green fonts indicate the sequences of PAZ [SEQ ID No:24] and PIWI [SEQ ID No:25] domains of Piwil2, respectively.

[00125] **Figure 12B:** Map of pLenti6-ZsGreen-PL2L60 plasmid (Lenti-PL2L60).

[00126] **Figure 12C:** Flow cytometric analysis of PL2L60- and GFP-expressing tumor cells. 231-PL2L60: a representative clone of MDA-MB-231 breast cancer cell line overexpressing enhanced green fluorescent proteins (GFP)-tagged human PL2L60; 231GFP: a representative clone of MDA-MB-231 breast cancer cell line overexpressing GFPs.

[00127] **Figure 13:** Table 1 showing the primer sequences for RT-PCT [SEQ ID NO:1 to SEQ ID No:20].

[00128] **Figure 14:** Table 2 showing tumor cell lines used in examples.

[00129] **Figures 15A-15D:** Failure of pCSC transplantation to develop into tumors in immunocompetent mice is associated with effective antitumor immunity induced by live pCSC. Mice were injected i.v. with 5×10^6 live pCSC or EL-4 cells (**Figure 15A** and **Figure 15B**), and then challenged with 2×10^6 EL-4 cells after 7 months of vaccination with CSCs (**Figure 15C** and **Figure 15D**). The data shown are derived from two experiments ($n=5/\text{group/expt}$), indicating that pCSCs can induce effective anti-tumor Immunity. $P < 0.01$, compared to control groups (EL-4 or vehicles).

[00130] **Figures 16A-16B:** Anti-tumor immunity induced by pCSCs is unique and dose-dependent.

[00131] **Figure 16A:** The lysates of pCSCs induces anti-tumor immunity as strong as live pCSCs did. 5×10^6 live 3B5C cells, cell lysates and vehicle (PBS) were injected s.c. into the left flank of B6 mice, respectively. 8 wk later, 7×10^5 B16 cells were injected into the right flank. Tumor growth rate was monitored by palpation. The data shown are from a representative experiment.

[00132] **Figure 16B:** pCSC-induced anti-tumor immunity is unique and dose-dependent. The cell lysates were prepared from various dose of pCSCs (3B5C) and tumor cell lines (EL-4), and injected into syngenic B6 mice. Control group received vehicles (PBS). The mice were challenged with 7×10^5 B16 melanoma cells per mouse 8 wk post vaccination, and tumor growth rate was monitored as above. The data shown are from a representative experiment ($n=6$). High dose of pCSC lysates (4.2×10^6 and 8.4×10^6) induced stronger anti-tumor immunity than low dose of pCSC lysates (2.5×10^6) ($p < 0.01$). In contrast, EL-4 cell lysates did not induce significant anti-tumor immunity compared to the group vaccinated with vehicles (PBS).

[00133] **Figures 17A-17B:** The pCSC-induced anti-tumor immunity is not tissue-specific B6 mice were injected s.c. with syngenic tumor cell lysates of pCSCs (3B5C), EL-4, B16, and vehicle ($n=8/\text{group}$), and were challenged s.c. with B16 tumor cell lines. Tumor incidence and tumor size

were monitored every other day.

[00134] **Figure 17A:** Tumor-free curve analyzed by Kaplan-Meier method. $p < 0.01$ when pCSC group was compared to control groups.

[00135] **Figure 17B:** Tumor growth kinetics: note that the tumor size in the pCSC-vaccinated group was significantly smaller than control groups (PBS, EL4 and B16).

[00136] **Figures 18A-18B:** Dendritic cell-based PL2L peptide vaccine can induce effective antitumor immunity. Two PL2L peptides (Pa and Pb) with shared by human and mouse PL2L proteins were used to immunize B6 mice ($n=3$).

[00137] Pa is a sequence having 21 aa: CFYPKRAMDQARELVNMLEKI [SEQ ID NO:26].

[00138] Pb is a sequence having 15 aa: IPEKMKKDFRAMKDL [SEQ ID NO:27].

[00139] Bone-marrow derived dendritic cells (BMDCs) were pulsed with or without PL2L peptides and injected s.c. into footpad of B6 mice. Three weeks later, the mice were challenged i.v. by NK cell-resistant melanoma cell line (MJ/V). The mice were sacrificed two weeks after challenging and microscopic tumors in the lung were counted and evaluated by pathologists. Five sections of each lung were examined. **Figure 18A** - show tumor incidence. **Figure 18B** shows tumor counts in the lungs. No DC: vehicle (PBS); DC alone: DCs not pulsed by peptides; DC+Pa: DCs pulsed by Pa; and DC+Pb: DCs pulsed by Pb. **, $p < 0.01$.

[00140] **Figures 19A-19B:** Inhibition of pCSC tumorigenesis in SCID mice by mAb to PL2L proteins pCSCs (clone 2C4G2) growing at log phase were harvested, washed and resuspended in the culture supernatant of Kao2 mAb to PL2L proteins ($5 \times 10^6/100\mu\text{l}$) or equivalent volume of culture medium. Then, the cells were injected s.c. into SCID CB17 mice ($5 \times 10^6/\text{mouse}$; $n = 3 \times 2$). The mice were monitored every other day after inoculation. **Figure 19A** shows tumor-free curves analyzed by analyzed by Kaplan-Meier method ($p < 0.01$). **Figure 19B** shows tumor growth kinetic curves ($p < 0.01$).

[00141] **Figures 20A-20D:** The mAb to PL2L proteins can lyze xenograft tumors expressing PL2L proteins. Breast cancer cell lines MDA-MB-231 (5×10^6), which expressed high level of membrane PL2L proteins (**Figure 20A**) were harvested at log phase of culture and injected s.c. into ICR/SCID mice ($n=3/\text{group}$). The culture supernatant of mAb Kao2 ($50 \mu\text{l}/\text{tumor}$) or control vehicles (culture medium) was injected into tumors for three consecutive days, when tumors grew up to about 1.0 cm in diameter. Twenty-four hours after last injection, the tumors were harvested, fixed in formalin and paraffin-embedded for histopathological examination.

[00142] **Figure 20A:** MDA-MB-231 cells expressed high level of membrane PL2L proteins before injection, as revealed by immunocytochemistry (ICC) staining with mAb Kao2. Two cells in the inset exhibited weak nuclear staining.

[00143] **Figure 20B:** Control ICC staining with rabbit mAb to HER2.

[00144] **Figure 20C:** Necrotic tumor tissues and neutrophil infiltration surrounding the

necrotic area: shown is a representative H & E micrograph of tumors injected with mAb Kao2.

[00145] **Figure 20D:** Appearance of tumor tissue injected with control vehicle: shown is a representative micrograph of H & E section exhibiting live tumor cells and little neutrophil infiltrates. Original magnification of the micrographs: x400.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

[00146] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

[00147] Definitions

[00148] The term "PL2L peptide" or "PL2L protein", as used herein, refers to a peptide which has an amino acid sequence which is the same as part of, but not all of, the amino acid sequence of the entire protein, and which retains at least one biological function or activity of the entire protein, for example, a fragment which retains an immunological activity of the full protein. It is to be understood that, in the present invention, most of them are the gene products of intragenic promoter activation.

[00149] The term "immunogenic peptide" or fragments thereof, as used herein, refers to amino acid sequences derived from the peptide which are capable of eliciting a cellular and/or humoral immune response when administered to a subject without MHC restriction.

[00150] The term "non-native" as used herein relative to an immunogenic peptide or epitope, means the amino acid sequence of the epitope differs by one or more amino acids from the amino acid sequence of the same immunogenic epitope as it is found in nature.

[00151] The "non-native" amino acid sequence may comprise an immunogenic epitope having a variant amino acid sequence which contains one or more of "conservative" or "non-conservative" amino acid substitutions, amino acid insertions or deletions, or in some cases may have additional amino acids at the N- or C-terminal end of the peptide. Such a "non-native" immunogenic peptide retains the biological activity (immunogenicity) of the native peptide from which it is derived.

[00152] The term "peptide carrier protein conjugate" is used interchangeably with the term "peptide derivatized to a carrier protein", and refers to an immunogenic peptide which has been linked to a carrier protein to enhance its immunogenicity.

[00153] The term "immunogenic peptide vaccine" or "peptide vaccine", as used herein refers to an immunogenic peptide derivatized to a suitable carrier molecule.

[00154] [0017] The term antibody means for the purposes of this invention not only complete antibodies but expressly also antibody fragments such as, for example, Fab, Fv, F(ab').sub.2, Fab'; and also chimeric, humanized, bi- or oligospecific, or single-chain antibodies; in addition

aggregates, polymers and conjugates of immunoglobulins and/or fragments thereof, as long as the properties of binding to the antigen or hapten are retained. Antibody fragments can be prepared for example by enzymatic cleavage of antibodies with enzymes such as pepsin or papain.

Antibody aggregates, polymers and conjugates can be generated by diverse methods, e.g. by thermal treatment, reaction with substances such as glutaraldehyde, reaction with immunoglobulin-binding molecules, biotinylation of antibodies and subsequent reaction with streptavidin or avidin, etc.

[00155] The term "vaccine", as used herein refers to an immunogenic peptide derivatized to a suitable carrier molecule and/or one or more passively administered anti-Piwil-2 human monoclonal antibodies.

[00156] The term "immune response" as used herein refers to a humoral immune response such as production of antibodies against an immunogenic epitope or a cellular immune response.

[00157] The term "immunocompetent subject", as used herein refers to a subject having immune response cells which upon exposure to an immunogenic epitope, is capable of mounting a cellular and/or humoral immune response against the immunogenic epitope.

[00158] The term immunogenic "epitope" or "antigenic determinant", as used herein relative to a peptide refers to a portion of the amino acid sequence which will generate an immune response against such peptide. It is preferred that the epitope be unique; that is, an immune response generated to the specific epitope shows little or no cross-reactivity with other antigens.

[00159] By "functional equivalent" is meant an amino acid sequence for an immunogenic peptide having equivalent biological activity to the native sequence immunogenic peptides described herein. The relevant biological activity is immunological activity, i.e. the ability to elicit an immune response in a subject to whom the immunogenic peptide vaccine is administered.

[00160] Amino acid residues are referred to herein by their standard single letter notations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

[00161] The term "active immunization", as used herein is meant the use of the peptide vaccines to induce an immune response by the immune response cells of the subject.

[00162] The term "administering", as used herein means bringing the immune response cells of the subject in contact with an immunogenic peptide vaccine. In most cases, such "administering", takes place either take by subcutaneous or intramuscular injection of the immunogenic peptide vaccine composition into the subject.

[00163] The term "passive immunization", as used herein is meant the direct administration of antibodies to a subject as an immunization approach.

[00164] The term "adjuvant" is used herein refers to a substance which can elevate the total

immune response of the subject to whom a vaccine is administered, i.e. by acting as a non-specific immunostimulator. An adjuvant can also be used to elicit an immune response more rapidly.

[00165] The term "successively spaced administrations" as used herein refers to treatment regimens of immunogenic peptides vaccines which comprise an initial administration, followed by one or more "booster" administrations at various time intervals following the initial administration. The "booster" administrations may or may not be given at regularly spaced intervals.

[00166] As used herein, the terms "solution suitable for injection in a human patient", "in a form suitable for injection in a human patient" and "pharmaceutically acceptable", may be used interchangeably and refer to composition comprising carriers, diluents and reagents, capable of administration to a human subject without the production of adverse physiological effects.

[00167] The terms "treating", "treatment" and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

[00168] The term "dose" as used herein refers to the amount of anti PL2L human monoclonal antibody or PL2L immunogenic peptide vaccine administered to a subject. The amount varies with the subject, the immunogen and the presence of adjuvant.

[00169] The term "treatment efficacy potential" as used herein relative to treatment of cancer refers to the potential effectiveness of a given treatment strategy as determined by evaluation of various clinical endpoints including the immune response to the immunogenic peptides, tumor size and subject survival time.

[00170] The term "body fluid" as used herein refers to a variety of sample types obtained from a subject including, urine, saliva, plasma, blood, spinal fluid, and other samples of biological origin.

[00171] As used herein, the term "analysis", relative to immunoassay of the immune response to the immunogenic peptides, may be an evaluation which is either qualitative (positive/negative) or quantitative.

[00172] *Piwil2 and Piwil2-Like PL2L) Proteins*

[00173] The present invention is based, at least in part, on the inventor's discovery that the Piwil2 and PL2L proteins have important, yet distinct roles, from the cancer-contributing genes (e.g., ONGs, TSGs and SGs) in tumor development. The inventor now shows that the transcripts of a germline stem cell (GSC) gene, *PIWIL2*, are constantly expressed in pCSC lines, but not in normal bone-marrow (BM)-derived stem/progenitor cells; further showing that *PIWIL2* plays an important role in TSC development.

[00174] The present invention is also based, at least in part, on the inventor's discovery that the "Piwil2" transcripts expressed in pCSCs represent the transcripts of Piwil2-like (PL2L) genes rather than Piwil2.

[00175] While not wishing to be bound by theory, the inventor herein now believes that a

cancer can develop from a lengthy process of benign proliferation → precancer → cancer, which is mediated by TISCs, pCSCs and CSCs, respectively; and that this process can be controlled and/or modulated by Piwil2 and PL2L proteins, such that functions of the Piwil2 and PL2L proteins can be used to override the functions of cancer-contributing genes.

[00176] Thus, in a first broad aspect, there are provided herein methods and compositions where the Piwil2 protein is useful as a tumor-barrier gene. For example, ectopic expression of Piwil2 can induced cell transformation (morphology) followed by apoptosis. Overexpression of Piwil2 in cancer cell lines can inhibit tumorigenesis of the lines. Knockout Piwil2 can accelerate tumor development in spontaneous tumor model of mice. Genotoxic agents can induce transient Piwil2 expression to facilitate DNA repair.

[00177] In another broad aspect, there are provided herein methods and compositions where the PL2L protein is useful as a tumor-initiating gene.

[00178] In another broad aspect, there are provided herein methods and compositions where the Piwil2 and PL2L proteins, are not only useful as a common biomarker for cancers, but are also useful as a target for the development of new anticancer drugs

[00179] The PL2L genes encode at least four proteins, including PL2L80, PL2L60, PL2L50 and PL2L40. Among them, PL2L60 is predominantly expressed in pCSCs and in various types of tumor cell lines, including those derived from the blood, skin, soft tissues, kidney, brain, breast, liver, pancreas, cervix, colon, ovarian, liver and lung of humans and mice. PL2L60 supports cell survival and proliferation through up-regulating Stat-3 and Bcl-2 genes, promoting transition of G0/1 to S-phase of cell cycle, and enhancing nuclear expression of RelA, a member of NF-κB (nuclear factor kappa-light-chain enhancer of activated B cells) family.

[00180] Overexpression of PL2L60 in human breast cancer cell lines promoted their tumorigenesis at the initial or latent stage of xenograft tumor formation. While PL2L proteins can be widely detected in the euchromatin-enriched proliferating tumor cells in primary and metastatic cancers, such as breast and cervical cancers, PIWIL2 was detected mainly in apoptotic or apoptosing cells. Moreover, PL2L proteins are always co-expressed with NF-κB/RelA in the cytoplasm or nucleus, showing that PL2L60, in cooperation with NF-κB, can play important, but opposite, roles to Piwil2 in tumor development.

[00181] Thus, the inventor's identification of PL2L proteins provides insight into the mechanisms of cancer development, and provides a novel target capable of bridging cancer diagnostics and anticancer drug development.

[00182] Precancerous stem cells do not express full length Piwil2 transcripts

[00183] In order to solve the contradictory function between the spontaneously expressed Piwil2 in pCSCs and transduced-Piwil2 in normal stem/progenitor cells, the inventor herein investigated whether the difference is caused by the dose of *Piwil2* gene or its variants in these

cells. The inventor herein designed a pair of primers to detect full length Piwil2 transcripts in pCSCs by RTPCR. The primers could amplify full length Piwil2 transcripts in the testis, but unexpectedly, failed to do so in the murine pCSC lines, including 2C4, 3B5C and 3B6C (**Figure 1A**). These results show, however, that a *Piwil2* gene variant(s) might be expressed in the pCSCs. To estimate the length of Piwil2 transcripts, the inventor performed GEM RT-PCR, which could estimate both full length Piwil2 transcripts and its potential variants.

[00184] The inventor herein then designed four pairs of Piwil2-specific primers which cover entire Piwil2 transcripts (23 exons). Each primer pair spans at least one intron (**Figure 1B** and **Figure 13 - Table 1**). The upstream of each pair of primers overlaps with the downstream of the previous primer. As shown in **Figure 1C**, exons 1-7 were amplified neither in 3 clonal pCSC lines (2C4, 3B5C and 3B6C) nor in a hematopoietic cancer stem cell (CSC) line (clone 326T). In addition, exons 21-23 were also not amplified in pCSCs (**Figure 1C**). This was not caused by the inability of these two primers, because each of the four pairs of primers amplified corresponding Piwil2 exons in the same sample of murine testis used in **Figure 1A** (**Figure 1C** and **Figure 1D**).

[00185] No Piwil2 transcripts were detected from the freshly isolated splenocytes (**Figure 1C**). The primer P_{mili} (which is specific for murine Piwil2 exons 18-21 (E18-21) and was used to detect “Piwil2” mRNA in pCSCs and other tumor cell lines) reproducibly amplified the “Piwil2” mRNA in the pCSC lines (**Figure 1C**). These results show that the gene products amplified by the primers P_{mili} may not necessarily represent full length Piwil2 transcripts and that “Piwil2” mRNAs detected by this primer pair in the pCSCs might represent a Piwil2 variant(s), likely truncated at the 5'-end (**Figure 1D**).

[00186] Identification of Piwil2-like (PL2L) genes and proteins in the testis and tumor cell lines but not in normal tissues

[00187] The data herein show that human *PIWIL2* gene containing 23 exons is significantly larger than an average human gene (**Figure 2A**). Differential exon utilization often leads to production of spliced mRNA variants, and differential promoter utilization inside a gene often results in the formation of related but functionally distinct proteins. These mechanisms greatly expand the coding capacity of a gene sequence.

[00188] The inventor then determined whether the *PIWIL2* gene uses these mechanisms to generate Piwil2 variants in particular conditions. To identify potential Piwil2 variants, the inventor first used the software Gene2Promoter from Genomatix Software Inc. (Ann Arbor, MI) to analyze human and mouse *PIWIL2* genes in order to find promoters inside these genes. The results showed that there are six promoters inside the *PIWIL2* gene of humans or mice (**Figure 2A**). Among these promoters, five promoters were identified as the transcriptional initiators of *PIWIL2*, *PL2L60*, *PL2L50*, *PL2L40* and *PL2L42*, respectively, in humans or mice (**Figure 2A**). The promoter of *PL2L60* is located in the region that starts from inside the intron 10 and

includes most sequence of Exon 11 (**Figure 2A**). This promoter initiates the transcription of PL2L60 mRNA that can be translated into 60 kDa protein PL2L60, in which PAZ domain is defective (**Figure 2B & Figure 2C**). The predicted PL2L60 mRNA is confirmed by transcribed sequence AK027497 from GenBank.

[00189] The promoter of *PL2L50* is located inside the intron 13 (**Figure 2A**), which transcribe PL2L50 mRNA covering exons 14 to 23 (**Figure 2B**) and its protein product contains Piwi domain truncated at N-terminus (**Figure 2C**). The predicted *PL2L50* is confirmed by transcribed sequence AK163647 (murine) from GenBank. The promoter of *PL2L42* is located at the region that starts from inside the intron 14 and includes entire Exon 15 (**Figure 2A**). This promoter initiates the transcription of PL2L42 mRNA that might be translated into 42 kDa protein PL2L42, which is only a part of Piwi domain truncated at N-terminus (**Figure 2B** and **Figure 2C**). The predicted PL2L42 mRNA is supported by transcribed sequence AK001213 from GenBank. The promoter of *PL2L40* is located inside the intron 12 (**Figure 2A**). It initiates the transcription of *PL2L40* gene, which contains exons from 13 to 20 and another two exons within the intron between exons 20 and 21. The PL2L40 contains a part of Piwi domain truncated at C-terminus (**Figure 2B** and **Figure 2C**). The predicted PL2L40 mRNA is confirmed by transcribed sequence XM_942053 in GenBank. These predicted mRNAs are all truncated at the 5'-end, resulting in a defective or absence of PAZ domain (**Figure 2C**). The mRNA sequences of human *PL2L60*, *PL2L50*, *PL2L40* and *PL2L60* and murine *PL2L50* can be found in GenBank.

[00190] To verify the protein expression of these PL2L genes in humans and mice, the inventor designed a C-terminal Piwil2 peptide with amino acid sequence (15-mers) between PAZ and Piwi domains, which is shared by Piwil2 and putative PL2L proteins as well as being homologous between humans and mice. The inventor herein immunized two rabbits with the peptide to generate a polyclonal antibody (pAb) specific for Piwil2 and PL2L proteins. The inventor herein obtained a high titer of peptide-specific rabbit pAb from one of the rabbits (RB9926), which was purified by the specific peptide-affinity chromatography and further characterized by Western-blot.

[00191] As shown in **Figure 3A**, the purified pAb can recognize a number of protein bands with estimated molecular weight (MW) of 110, 80, 60, and 50 kDa in both murine testicular cell lysates (**Figure 3A**) and human tumor cell lysates (HeLa) (**Figure 3B**). These proteins are Piwil2-peptide specific, because 110 and 50 kDa protein bands were not detected in the testicular cell lysates of *mili*^{-/-} mice and the 80 and 60 kDa protein bands were greatly reduced compared to wild-type (wt) mice (**Figure 3A**). Consistently, the same protein bands in the human tumor cell lysates were almost completely blocked by Piwil2 peptides, which were used as immunogens (**Figure 3B**). These results show that the Piwil2-specific polyclonal antibody at least recognizes three Piwil2-like proteins (PL2L80, PL2L60, and PL2L50). Among them, PL2L80 could not be

predicted from NCBI GeneBank. Incomplete disruption of PL2L80 and PL2L60 proteins in the *mili*^{-/-} testis appeared to be associated with the expression of transcripts covering *Piwil2* exons from 6 to 23, which were not abrogated at all in *mili*^{-/-} testis, as well as with the low level expression of *Piwil2* exons from 1 to 7 in *mili*^{-/-} mice, as demonstrated by *Piwil2*-specific GEM RTPCR (**Figure 3C**).

[00192] In the *mili*^{-/-} mice, *Piwil2* were disrupted via homologous recombination with the 5.2-kb BamHI fragment encompassing from exon 2 to exon 5 in the pPNT vector with a neomycin resistance cassette. Thus, the transcription of PL2L genes, which were defective at 5' end of *Piwil2* and controlled by other independent promoters, were not completely suppressed in *mili*^{-/-} testis (**Figure 2**). These results show that whole *Piwil2* protein, but not its variants such as PL2L80 and PL2L60, are completely disrupted in the *mili*^{-/-} mice.

[00193] The inventor herein has now successfully cloned *PL2L80*, which appears to utilize the same promoter as full length the *PIWIL2* (unpublished data).

[00194] *PL2L60 is predominantly expressed in precancerous stem cells*

[00195] To determine the expression profiles of *Piwil2* and PL2L proteins in pCSCs, the pCSC lines (clone 2C4, 3B5C and 3B6C) were analyzed by Western-blotting. As shown in **Figure 4A** and **Figure 4B**, each pCSC line expressed PL2L60 predominantly, but little PL2L50 and no *Piwil2*. As a negative control, no PL2L60 was detected in splenocytes (**Figure 4A** and **Figure 4B**).

[00196] Moreover, no *Piwil2* and PL2L proteins were detected in other tissues of normal mice, either by Western-blotting or RT-PCR (**Figure 10**). The level of PL2L60 protein in the pCSCs was 3.5 ~ 7.0 times more than that in 326T cells, a hematopoietic CSC line (unpublished data).

[00197] Compared to the background in normal splenocytes, the PL2L60 protein was dramatically increased by 56.6 to 120 times in the pCSCs but only by 17.2 times in 326T cells (**Figure 4B**). Interestingly, 326T cells were more susceptible to apoptosis compared to 2C4, 3B5C and 3B6C cells when they were cultured *in vitro* (**Figure 4C**), likely associated with the lower level of PL2L60. Consistently, the transcripts of *Piwil2* exons 18-21 were detected by primers P_{mili} in the pCSC lines more than in the 326T cells, but not in normal splenocytes (**Figure 1C**: P_{mili}: E18-21). These results implicated that “*Piwil2*” transcripts expressed in pCSCs *de facto* represent the transcripts of *PL2L60*.

[00198] *PL2L60 up-regulates Stat-3 and Bcl-2 genes and promotes pCSC expansion*

[00199] Since the pCSCs (2C4, 3B5C and 3B6C) predominantly expressed PL2L60 (**Figure 4**), the inventor herein now believes that PL2L60 is associated with pCSC expansion *in vitro*. To verify, the pCSCs (clone 2C4) were transfected with siRNAs targeting the exon 11 of *Piwil2* transcripts, and the scrambled siRNA (scRNA) transfected, mock transfected and un-transfected were set up as controls. As a result, PL2L60 expression was reduced at both transcriptional and translational levels in the siRNA-transfected cells, as compared to the controls (**Figure 5A**, **Figure**

5B and Figure 5C).

[00200] Correspondingly, pCSC expansion was also significantly suppressed in the Piwil2-specific siRNA-transfected pCSCs (**Figure 5D**). The reduced pCSC expansion was associated with down-regulation of the transcripts of *Stat-3* and *Bcl-2* genes but not *Bcl-XL* gene in the pCSCs (**Figure 5E** and **Figure 5F**).

[00201] The Piwil2 exon 11 is located within the putative open reading frame of *PL2L60* gene (**Figure 2A**). Consistently, the transcriptional products of both E6-14 and E18-21 were also significantly reduced in the pCSCs transfected with exon 11-targeting siRNA (**Figure 5C**). These results reveal that the “Piwil2” mRNAs (which were previously reported to be stably expressed in pCSC lines) are *de facto* represent the mRNAs of *PL2L60* gene, which can support pCSC expansion *in vitro* through promoting Stat-3/Bcl-2 pathway.

[00202] *PL2L60 is widely expressed in various types of human cancer cells*

[00203] To determine whether PL2L60 was also expressed in human tumors, the inventor herein examined PL2L60 expression in various types of human cancer cell lines. As shown in **Figure 6A**, PL2L60 is predominantly expressed in cervical (HeLa) and breast cancer cell lines (**Figure 6A**). Among the breast cancer lines, MCF7 lines that usually grow slower in culture than MDA-MB-231 and MDA-MB-468 (not shown) expressed lower level of PL2L60 proteins (**Figure 6B**).

[00204] In contrast, long-term cultured primary human dermal fibroblasts (HDF) expressed little PL2L60 proteins (**Figure 6A** and **Figure 6B**). In addition to cervical and breast cancer cell lines, PL2L60 proteins were also predominantly detected in other tumor cell lines derived from the tumors of blood, skin, soft tissues, kidney, brain, breast, liver, pancreas, cervix, colon, ovarian, liver and lung. The tumor types involved include leukemia, lymphoma, adenoma, adenocarcinoma, carcinoma, melanoma, sarcoma, neuroblastoma, hepatoma, insulinoma and endothelioma (**Figure 11** and **Figure 14 - Table 2**).

[00205] These results show that the PL2L60 protein is widely expressed in various types of cancers without restrictions of tissue origin and tumor types. In addition, Piwil2, PL2L80 and PL2L50 were also detected in low levels or not detected at all in these lines with great variation between lines as well as between individual experiments (**Figure 3** and **Figure 11**), showing that culture conditions might affect their expression *in vitro*, but PL2L60 expression is always predominant.

[00206] *PL2L60 promoting tumorigenesis is associated with increased nuclear NF- κ B expression*

[00207] Since PL2L60 can promote pCSC proliferation *in vitro* (**Figure 5**), the inventor herein examined whether PL2L60 also promoted human tumor cell proliferation *in vitro* by knocking down PL2L60 mRNAs in human breast cancer cell lines (MDA-MB-231) (**Figure 6C**).

As a result, the proliferation of the MDA-MB-231 cells was also significantly inhibited after knocking down PL2L60 mRNAs (**Figure 6D**), confirming that PL2L60, like in murine pCSCs, can also support cancer cell proliferation *in vitro*. To further confirm this function, the inventor herein generated stable PL2L60-expressing cancer cell lines by transducing human *PL2L60* gene into MDA-MB-231 cells (**Figure 7** and **Figure 12**). The PL2L60-transduced cells demonstrated significant increase in the entry of cycling cells from G_{0/1} into S-phase, as compared to GFP-transduced control cells; and this is consistent with increased cell expansion *in vitro* (**Figure 7A** and **Figure 7B**).

[00208] Moreover, the increased cell expansion was associated with enhanced expression of nuclear NF-κB (RelA/p65), a protein complex that controls the transcription of genes including those responsible for cancer cell survival and proliferation (**Figure 7C**). While NF-κB was strongly detected in the cytoplasm but weakly in the nucleus of the GFP-transduced cells, almost all of the PL2L60-transduced cells expressed a high level of NF-κB in both cytoplasm and nucleus. The cell size of PL2L60-transduced cells was greatly increased compared to that of GFP-transduced cells, thus showing an enhanced cell growth (**Figure 7C**). It should be noted that < 1% of cells expressed stronger cytoplasmic and nuclear NF-κB in both samples of PL2L60-transduced and GFP-transduced cells (**Figure 7C**: arrows).

[00209] To determine the tumorigenic capacity of the PL2L60-transduced tumor cell line, the inventor herein transplanted PL2L60- and GFP-transduced MDA-MB-231 cells into CB17 SCID mice, respectively. The tumor nodules formed by PL2L60-transduced cells were palpable as early as one week after transplantation, whereas GFP-transduced control cells did not form palpable tumor nodules at this time (**Figure 7D** and **Figure 7E**). Interestingly, the tumor nodules were regressed three weeks latter, but recurred with a tumor growth rate comparable to that of GFP-transduced cells (**Figure 7D** and **Figure 7E**). At the end of experiments, both PL2L60- and GFP-transduced tumors were GFP-positive as detected by live imaging (not shown). These results show that PL2L60 could promote tumorigenesis at the initiating stage of xenograft tumor formation.

[00210] Piwil2 and PL2L proteins are differentially expressed in human primary and metastatic cancers

[00211] To determine whether PL2L60 was also predominantly expressed in native cancers in addition to tumor cell lines, the inventor herein attempted to generate murine monoclonal antibody (mAb) to Piwil2, each PL2L protein or PL2L60. Because polyclonal Piwil2-peptide specific antibody recognizes both Piwil2 and PL2L proteins (**Figure 3A** and **Figures B**), the inventor used the same strategy to generate mAbs to Piwil2 and PL2L proteins. Although the inventor did not obtain a mAb exclusively recognizing PL2L60, the inventor has obtained one mAb specifically recognizing Piwil2 (mAb Kao1) and two mAbs recognizing both Piwil2 and PL2L proteins (Mabs

Kao2 and Kao3).

[00212] A mouse hybridoma, MH-KA01, is deposited under registration number PTA-11853 at the ATCC, Manassa, VA, USA.

[00213] A mouse hybridoma, MH-KA02, is deposited under registration number PTA-11854 at the ATCC, Manassa, VA, USA.

[00214] A mouse hybridoma, MH-KA03, is deposited under registration number PTA-11855 at the ATCC, Manassa, VA, USA.

[00215] As shown in **Figure 8A**, the mAb Kao1 reacted with ~ 110 kDa proteins in the murine testis, but not any proteins in the cancer cell lysates from human colorectal cancer cell line SW 480. In contrast, mAbs Kao2 and Kao3 reacted strongly with ~110 kDa and ~ 40 kDa proteins in the murine testis and ~ 60 kDa and ~ 50 kDa proteins in the cancer cell lysates. In addition, mAbs Kao2 and Kao3 react weakly with ~80 kDa, ~60 kDa and ~50 kDa proteins in the testis of mice, and ~110 kDa and ~80 kDa proteins of the cancer cell lysates (**Figure 8A**). The different strength of the proteins bands likely reflects different levels of the proteins in the murine testis and human cancer cell line. Thus, mAbs Kao2 and Kao3 can recognize Piwil2, PL2L80, PL2L60, PL2L50 and PL2L40, similarly to the proteins recognized by Piwil2 peptide-specific pAb, except for PL2L40 (**Figure 3B**). Again, PL2L60 was predominantly expressed in the colon cancer cell lines as detected by mAbs Kao2 and Kao3 (**Figure 8A**).

[00216] Then, the inventor used the mAbs Kao1 and Kao2/3 (thereafter referred to as Kao2) to examine the expression of Piwil2 and PL2L proteins in the tissue microarray (TMA) cores of human breast (n=300) and cervical cancers (n=100) or in some tissues sections of the cancers. As shown in **Figure 8E**, only 1.67% of breast cancer TMA cores (5/300) and 3% of cervical TMA cores (3/100) were stained by mAb Kao1 (**Figure 8E**), in which Piwil2 (Kao1)-expressing cells were usually low in frequency (**Figure 8B**); in contrast, all TMA cores of cervical and breast cancers were stained by mAb Kao2 (**Figure 8B**). However, the frequency of the positively stained cells varied between individuals (**Figure 8**). While Kao1⁺ cells display condensed nuclei, an apparent feature of apoptosing or apoptotic cells in morphology (**Figure 8B - 1 upper left**, and **Figure 8B - 3 lower left**), the Kao2⁺ cells showed large nuclei enriched with euchromatin, a feature of cell proliferation (**Figure 8B - 2 upper right**, and **Figure 8B - 4 lower right**). The tumor cells with a high level of heterochromatin were either negative or faintly positive for PL2L proteins (**Figure 8B - 2**, compare the nuclei between Kao2⁺ and Kao2⁻ cells in the inset).

[00217] In addition, PL2L proteins can be detected by Kao2 mAb mainly in cytoplasm (pattern: C) or in both cytoplasm and nucleus (pattern: C-N) (**Figure 8B - 2**, pattern C indicated by red arrow; and pattern C-N indicated by green arrows). In the metastatic cancer, all tumor cells were Kao2-positive, whereas only a few of apoptotic or apoptosing Kao1⁺ cells were occasionally detected regardless of the locations (lymph nodes or connective tissue) of tumor cells (**Figure 8C**).

These results show that Piwil2 is mainly expressed in apoptotic or apoptosing tumor cells, whereas PL2L proteins were expressed in euchromatin-enriched proliferating and metastatic cancer cells. Thus, Kao2 and Kao3 mAbs can be useful for detection of both primary and metastatic cancers.

[00218] Because PL2L60 could promote breast cancer cell proliferation *in vitro*, and overexpression of PL2L60 is associated with increased nuclear NF- κ B expression in tumor cell lines (**Figure 7**), the inventor herein further investigated whether the expression of PL2L60 or PL2L proteins were also associated with NF- κ B expression in primary and metastatic cancers. Breast cancer tissue sections were co-stained with murine mAb (Kao2) to PL2L proteins and rabbit mAb to RelA/p65, a subunit of NF- κ B. Both PL2L proteins and p65 were detected in the cytoplasm and nucleus of primary (**Figure 8D**) and metastatic cancers (not shown). Almost all the Kao2⁺ cells were co-stained by mAb to p65 regardless of C or C-N patterns (**Figure 8D - 3 lower left**); although the ratio of Kao2 (brown) to p65 (pink) was individually different (not shown). Importantly, nuclear NF- κ B expression appeared to be dependent on nuclear PL2L proteins expression, because p65 or PL2L proteins alone were not detected in the nucleus of tumor cells (**Figure 8D - 4 lower right**; yellow arrows indicate double stained nuclei), despite the fact that variable levels of nuclear p65 were detected in a few nuclei of infiltrated inflammatory cells (**Figure 8D - 4**; green arrows). Nuclear Kao2⁺p65⁺ cells were about 24 $\pm$ 10.58% among all Kao2⁺p65⁺ tumor cells (ranging from 9.09% to 42.42% per tumor nest; n=16). Given the roles of NF- κ B in tumorigenesis, these results show that nuclear Kao2⁺p65⁺ tumor cells most likely play a critical role in tumor development.

[00219] Discussion

[00220] The Piwil2 proteins or Piwil2 transcripts were detected in various types of cancers or cancer cell lines. While exploring the mechanism underlying Piwil2-mediated tumor development, the inventor herein found by GEM RT-PCR that the “Piwil2” transcripts expressed in the murine pCSCs were truncated at 5'-end and absent of first 6 exons of *Piwil2*. Through analysis of human and mouse *PIWIL2* gene, the inventor herein found several potential 5'-end truncated variants of *PIWIL2*, called herein Piwil2-like (PL2L) genes.

[00221] The inventor herein generated novel polyclonal and novel monoclonal antibodies to a peptide shared by Piwil2 and PL2L proteins of humans and mice. The inventor herein also identified four PL2L proteins, including PL2L80, PL2L60, PL2L50, and PL2L40, in the testis and cancer cell lines of humans and mice.

[00222] Interestingly, PL2L60 rather than Piwil2 and other PL2L proteins were predominantly expressed in pCSCs and various types of human and mouse tumor cell lines, including those of leukemia, lymphoma, adenoma, adenocarcinoma, carcinoma, melanoma, sarcoma, neuroblastoma, hepatoma, which were derived from various types of organs such as blood, skin, soft tissues, kidney, brain, breast, liver, pancreas, cervix, colon, ovarian, liver and/or lung.

[00223] The results herein show that PL2L60 is widely expressed in tumor cells regardless of tumor types and tissue origins. Consistent with these results from cancer cell lines, the PL2L proteins were also dominantly expressed in primary and metastatic cancers; whereas the Piwil2 proteins were essentially undetectable except in some apoptotic or apoptosing cells. Because PL2L60 is predominantly expressed in cancer cell lines, it is likely that PL2L proteins detected by the mAbs Kao2/3 in primary and metastatic cancers actually are PL2L60. These results reveal that PL2L60, rather than Piwil2, are widely and constitutively expressed in various types of cancers without the restriction of tissue origin. This is also supported by the inventor's discovery that Piwil2 transcripts were undetectable in the bladder cancers by real-time RT-PCR with the primers amplifying first 6 exons of *PIWIL*, which are absent in PL2L genes.

[00224] Therefore, the "Piwil2" transcripts amplified by primers within PL2L genes or Piwil2 proteins detected by mAbs Kao2/3 in cancer cell lines or in the tissues of primary and metastatic cancers *de facto* represent PL2L genes or PL2L proteins. In addition, the mAb Kao1 can be used to identify Piwil2 specifically and distinguish what detected by Kao2/3 from Piwil2.

[00225] The PL2L60 expression is not limited to pCSCs.

[00226] PL2L60 was also detected in various types of cancer cell lines. Knockdown of PL2L60 mRNAs in murine pCSCs or human breast cancer cells significantly suppressed their expansion *in vitro*. In agreement, over-expression of PL2L60 in the breast cancer cell lines led to their increased expansion *in vitro*. The mechanisms underlying the increased expansion appear to be mediated by reduced programmed cell death (PCD) because of enhanced expression of *Bcl-2* and *Stat3* genes and promoted $G_{0/1} \rightarrow S$ -phase in cell cycle by PL2L60. This is further supported by the increased expression of nuclear NF- κ B in the PL2L60-transduced cell lines. NF- κ B is a ubiquitous transcription factor that controls the expression of genes involved in immune response, cell survival, apoptosis, and cell cycle. Given the fact that NF- κ B is inactivated in cytosol complexed with the inhibitory protein I κ B α but activated by ubiquitination of INBD, shuttling to nucleus, only is nuclear NF- κ B considered to be functional. NF- κ B nuclear translocation may up-regulate pro-survival factor Bcl-2 in tumor cells, as observed in human hepatoma cells. Thus, the increased nuclear NF- κ B expression in the nuclei of PL2L60-transduced cells may lead to increased cell expansion through up-regulating cell survival genes *Stat-3* and *Bcl-2* and promoting cell cycling (**Figure 9**).

[00227] The *in vitro* findings are supported by the *in vivo* findings that PL2L60-transduced human breast cancer cells formed palpable tumor nodules rapidly within first few weeks after transplanted into CB17 SCID mice, while GFP-transduced cells were latent. Usually it takes at least two to three months (latent phase) to form palpable xenograft tumors, which are supposed to be mediated by rare numbers of CSCs in the context of freshly isolated tumor cells or tumor cell lines. The formation of tumor nodules by PL2L60-transduced cells at the latent phase shows that

they proliferated faster *in vivo* than GFP-transduced cells did. Moreover, in primary and metastatic cancers of humans, the exclusive expression of PL2L60 or PL2L proteins in the euchromatin-enriched tumor cells also show that the PL2L60-expressing cells are highly active or proliferative, which contributes to enhanced tumor growth. Thus, PL2L60 expression can promote the tumorigenic capacity of tumor cells through promoting cell survival and proliferation in cooperation with NF- κ B.

[00228] The Piwi proteins can promote euchromatin histone modifications and piRNA transcription in subtelomeric heterochromatin in *Drosophila*. The fact that co-expression of PL2L proteins with NF- κ B exclusively in the euchromatin-enriched tumor cells suggests that PL2L60 could promote the functions of NF- κ B through modulating chromatin structure or chromatin modifications (**Figure 9**).

[00229] Another discovery by the inventor herein is that, in primary and metastatic cancers, nuclear PL2L proteins-expressing tumor cells are always co-expressed with nuclear NF- κ B/p65 (nPL2L⁺p65⁺). Since nPL2L⁺p65⁺ cells were detected in various stages of breast cancers with individual variation in frequency (data not shown), quantitation of PL2L⁺p65⁺ cells in tumor tissues may predict the outcome of tumor progression or regression. NF- κ B can bind the promoters of progression-associated genes in melanoma; NF- κ B has been shown to play important roles in tumor stem cells; and nuclear localization of NF- κ B appears to be associated with the progression of prostate cancers. Therefore, while not wishing to be bound by theory, the inventor herein now believes that the nuclear PL2L⁺p65⁺ tumor cells represent tumor stem cells.

[00230] **EXAMPLES**

[00231] Certain embodiments of the present invention are defined in the Examples herein. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. All publications, including patents and non-patent literature, referred to in this specification are expressly incorporated by reference herein. Citation of the any of the documents recited herein is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicant and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00232] **Example 1**

[00233] **Materials and Methods**

[00234] **Mice, cell lines, and reagents**

[00235] Male C57BL/6 (B6) mice, CB17 SCID mice and mili^{-/-} mice with B6 background

were used at the age of 8 – 12 wk. The *mili*^{-/-} mice were bred and maintained in the animal pathogen-free facility at The Ohio State University Medical Center. The hematopoietic pCSC lines 2C4, 3B5C and 3B6C were cloned from a mouse with dendritic cell-like leukemia and characterized. The hematopoietic CSC line (clone 326T) was developed by the inventors. EL-4 thymoma cells are maintained in the inventor's laboratory. Primary human dermal fibroblasts (HDFs) that were obtained from foreskin were obtained. Cervical cancer cell line HeLa, breast cancer cell lines MD-MB-231, MD-MB-468, and MCF-7 were obtained. Other tumor or immortalized cell lines used are listed in **Figure 14 - Table 2**. All the hematopoietic tumor cell lines were maintained in R10F (RPMI 1640 plus 10% fetal calf serum supplemented with 5 mM glutamine, 50 PM 2-mecaptoethonal, 100 U/ml penicillin, and 100 Pg/ml streptomycin), and non-hematopoietic tumor cell lines were maintained in D10F [Dulbecco's Modified Eagle Medium (DMEM) with the same supplements as in R10F]. All cell lines were harvested at log phase of growth for RT-PCR or Western blot analysis.

[00236] RT-PCR

[00237] RT-PCR was performed and total RNA was extracted from cell lines or *de novo* isolated testicular cells and splenocytes. The cDNA was generated by reverse transcription using Superscript II (Invitrogen, CA) and oligo (dT) in a 20 µl reaction containing 1 µg of total RNA, which was pretreated with RNase-free DNase I (Invitrogen, CA) to eliminate contaminating genomic DNA. Briefly, an aliquot of 0.5 µl cDNA was used in each 20 µl PCR reaction, using PCR Master Mix (Promega, Ca). The following conditions were used: an initial denaturation at 95°C for 5 min followed by denaturation at 94°C for 30 seconds, annealing at 65°C for 1 min, touchdown -1°C per cycle, and extension at 72°C for 1 min for a total of 10 cycles. Then, the condition was fixed for 25 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel. The sequence of primers is listed in the **Figure 13 - Table 1**.

[00238] Gene-Exon-Mapping Reverse transcription polymerase chain reaction (GEM RT-PCR)

[00239] To identify Piwil2 variants, the inventor herein designed four pairs of murine Piwil2-specific primers and three pairs of human Piwil2-specific primers, which cover entire Piwil2 transcripts (23 exons) of mice or humans. Each primer spans at least one intron based on the data from GeneView (bibiserv.techfak.uni-bielefeld.de/GeneView/) and was designed using Ensembl (ensembl.org). The upstream of each pair of primers overlaps with the downstream of the previous one. The primers used for GEM RT-PCR are listed in **Figure 13 - Table 1**.

[00240] RNA interference (RNAi) Assay

[00241] RNAi assay was performed on murine pCSCs and human breast cancer cell lines. All siRNAs was purchased from the Integrated DNA Technologies (IDT, Coralville, IA).

[00242] The murine pCSC cells were transfected with murine Piwil2-specific small interference (si) RNA (UCGUACCUACCGAAUCGAU) [SEQ ID NO:29], which targets exon 11 of Piwil2 within the open reading frame of PL2L60,

[00243] or scrambled (sc) small RNA (scRNA) (CACGUGAGGAUCACCAUCA) [SEQ ID NO:30], using an siRNA transfection kit, as instructed by the manufacturer (Qiagen), and cultured in 24-well plates. For the effect of Piwil2 exon 11-specific siRNA on cell expansion, a low density of transfected cells (100/well) were seeded and counted at indicated times. For RT-PCR analysis of PL2L60, a high density of transfected cells (1×10^6 /well) were seeded and harvested at 36 ~ 48 h of culture, and were analyzed using primers specific for Piwil2 exon 6-14 (E6-14) and exon 18-21 (E18-21), respectively (Figure 13 - Table 1).

[00244] For RNAi experiments on human cancer cell lines, breast cancer cell lines MD-MB-231 were seeded overnight in triplicate at $2.5 - 3.0 \times 10^5$ /well in 24-well plates and transfected with serum-free DMEM medium mixed with transfection reagents and 100 pmol human Piwil2 exon 21-specific dicer substrate RNA duplexes of 25 nucleotide in length (sense strand: 5'CUAUGAGAUUCCUCAACUACAGAAG [SEQ ID NO:31]; antisense strand: 5'CUUCUGUAGUUGAGGAAUCUCAUAGUU [SEQ ID NO:32]), or universal negative control dicer substrate duplex (DS Scrambled Neg). Transfection reagents and RNA duplexes were mixed and incubated in serum-free DMEM medium for 60 min before adding into culture plates

[00245] Generation of rabbit polyclonal and murine monoclonal antibodies to Piwil2 and PL2L proteins

[00246] Polyclonal antibody to Piwil2 was generated in collaboration with Abgent Inc. Briefly, two New Zealand Rabbits were immunized each with 100 ~ 200 µg KLH-conjugated Piwil2 peptides (503 IPEKMKKDFRAMKDL 517) [SEQ ID NO:28] with a sequence common to humans and mice emulsified in the Complete Freund's Adjuvant (CFA) for twice in a two weeks interval, and then with 100 Pg peptide/rabbit in the Incomplete Freund's Adjuvant (IFA) at week 4. Starting from week 5, the rabbits were immunized with 100 Pg peptide/rabbit in PBS every week until week 9, and were bled for 20 ~ 30 ml every week until sacrifice at week 10. About 100 ml serum per rabbit was collected and rabbit IgG was purified by protein A/G chromatography followed by peptide-affinity chromatography. The titer and specificity of antibody was determined by competitive ELISA and Western-blot.

[00247] For generation of murine mAbs to Piwil2 and PL2L proteins, two BALB/c mice were immunized with the same peptide for rabbit polyclonal antibody in CFA, 100 µg per mouse, followed by boosting with 50Pg/mouse every week until the required titer is reached. Spleen cells from the mouse with best titer were fused with myeloma cell F0 by adding 1.5ml PEG 1500 per 3×10^8 mixed cells. Growing fused hybridoma clones were screened against antigen (such as free peptide) for test of their specificity and sensitivity. ELISA positive clones were tested by

appropriate applications such as Western-blotting. Selected clones from this test were subcloned at least two times.

[00248] Western blot

[00249] Cell lysates and tissue extracts were mixed with equal volume of SDS-sample buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 5% SDS, 20% glycerol, 10 mM DTT, 0.05% Bromophenol Blue) and heated at 95°C for 3 min. The proteins in samples were resolved on a 4 ~ 20% gradient Tris-glycine acrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% milk-TBS-TW 20, immunoblotting was performed using either rabbit polyclonal anti-Piwi2 peptide antibody (IgG) or murine monoclonal anti-Piwi2 peptide antibody (IgM), or rabbit-anti-E-actin antibody. Following development with the appropriate species-specific horseradish peroxidase-conjugated anti-immunoglobulin antibodies and SuperSignal chemiluminescence substrate, the bands were visualized on X-ray film.

[00250] Construction of lentiviral PL2L60 vector (pLenti6-ZsGreen-PL2L60)

[00251] To construct the lentiviral expression vector expressing PL2L60, a pair of primers, 5' -GCG GAT CCA CCA TGG CTG ATG GGA AAG AGA TCA CAT TCT-3' [SEQ ID NO:33] (sense) and 5'-GAG AAT TCA CAG GAA GAA CAG GTT CTC G-3' [SEQ ID NO:34] (antisense), were used to amplify the cDNA coding for PL2L60 gene by PCR. The full-length human Piwi2 cDNA in plasmid pBluescriptR (cDNA clone MGC:26732 IMAGE:4826162) purchased from ATCC (Manassas, VA) was used as the template. The synthesized PL2L60 cDNA from PCR, which has a BamHI site in 5' end and an EcoRI site in 3' end derived from primers, was cloned into the lentiviral vector pLenti6-ZsGreen, which is derived from a recombination of the Zoanthus sp. green fluorescent protein (ZsGreen) (Clontech) and pLenti6/V5-TOPO (Invitrogen), to generate pLenti6-ZsGreen-PL2L60. The insert of PL2L60 was verified by sequencing. Pseudotype pLenti6-ZsGreen-PL2L60 and pLenti6-ZsGreen viruses were produced by transfecting the 293FT cell line, as instructed by manufacturer (Invitrogen). The viral supernatants were harvested on 72 h post transfection.

[00252] Generation of stable cell lines overexpressing PL2L60

[00253] To generate cancer cell line overexpressing PL2L60, human breast cancer cell line MDAMB-231 were transduced with lentiviral pLenti6-ZsGreen-PL2L60 (Lenti-PL2L60) or pLenti6-ZsGreen viruses (Lenti-GFP). Briefly, cells were seeded (2×10^5 /well) in 24-well plate in complete culture medium (D10F) and incubated for overnight at 37°C in a humidified 5% CO₂ incubator. Culture medium was removed from the cells and then the medium containing viruses was added to the cells. After overnight incubation, the medium containing viruses was removed and replace with fresh D10F. After 24 h incubation, fresh D10F containing 10 µg/ml of Blasticidin was added to cells to select for stably transduced clones. Green fluorescent protein (GFP)-expressing cells (231-PL2L60 or 231-GFP) were sorted, cloned and maintained in D10F.

[00254] Cell cycle assay

[00255] Cell cycle analysis of tumor cell lines was performed with necessary modifications. Briefly, tumor cell lines were cultured in 3.0 ml D10F in the 6-well culture dishes to about 80% confluent, and washed with serum-free medium for twice. The cells were cultured in serum-free medium for 16 h and then D10F was added to recover them. Six hours later, the cells were harvested and washed in PBS containing 10 mg/ml RNase (Life Technologies), fixed (10^6 cells/ml) in 70% ethanol, and stored at 4°C until performing flow cytometric analysis. The cells were stained with propidium iodide (PI; 50 mg/ml) in PBS containing 1 mg/ml of glucose, incubated for 30 min at room temperature, and subjected to cell cycle analysis using flow cytometry. The acquired data were further analyzed for cell cycle parameters, using Modfit LT™ (Verity Software House).

[00256] Xenograft tumor transplantation

[00257] SCID CB17 mice were injected s.c., with 5×10^6 cloned 231-PL2L60 or 231-GFP cell lines. Tumor incidence and size were monitored once a week starting 1 wk after inoculation. Once tumors were palpable, tumor size was measured twice a week. The mice were sacrificed when one of the mice in any group developed a tumor about 15 ~ 20 mm in diameter. At the early stage of tumor formation and the end of experiments, living tumor images were taken using Macro Imaging System (LT-9MARCOIMSYS; Lighttools Research).

[00258] Immunohistochemical (IHC), and immunocytochemical (ICC) analysis

[00259] IHC analysis of human tumor specimens was performed. Tumor specimens of breast and cervical cancers were fixed in formalin and embedded in paraffin for pathological and immunohistochemical analysis. Tissue microarrays (TMAs) were built by the Histological Core Facility, Department of Pathology. Serial TMA or conventional tissue sections (4 ~ 5 μ m) were stained by H. & E. for pathological analysis, or a mouse mAb Kao1 (specific for Piwil2) or Kao2 (specific for Piwil2 and PL2L proteins) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody for IHC analysis. The immunostained sections were counterstained with hematoxylin. In some experiments, TMA cores or conventional sections were double-stained with mouse mAb Kao2 (supernatant; 1:3 dilution) and rabbit mAb to RelA/p65 (1:100 dilution; Epitomics Inc.) in sequence. PL2L proteins (brown) were developed by the detection kit Envision plus Dual Link (Dako) and DAB chromogen, and p65 (pink) was revealed by the detection kit Mach 4 (Biocare) and Fast Red chromogen. For the double IHC staining control, serial sections were stained with a single primary antibody of interest.

[00260] For ICC analysis, the tumor cells growing at log phase were harvested and washed twice in 2% FCS-PBS and resuspended in 1%-BSA-PBS (2×10^5 /ml). The single cell suspension (1×10^4 /200 μ l) was cytospun at 1000 rpm for 3 minutes, air-dried and stained with rabbit mAb to p65, as described for IHC staining.

[00261] Statistical analysis

[00262] Date of multiple group observations were statistically analyzed by the one-way analysis of variance (ANOVA), and two groups of observations were compared by student-T test. A value of $p < 0.05$ was considered significant. Data are represented as mean $\pm$ SD. *, $p < 0.05$; **, $p < 0.01$.

[00263] Example 2

[00264] PL2L protein targeted immunotherapy

[00265] Live precancerous stem cells can induce effective anti-tumor immunity to prevent challenge of tumor cells

[00266] Since precancerous stem cells (pCSCs) are not tumorigenic when transplanted into immunocompetent (IC) mice (1), the inventor herein now believes that live pCSCs can induce effective anti-tumor immunity to prevent the challenge of tumor cells. The inventor herein injected the live hematopoietic pCSCs (HpCSCs) (clone 2C4, 3B5C and 3B6C), control tumorigenic cell line EL-4 and vehicles subcutaneously (s.c.) into syngenic IC mice. These pCSC lines did not grow into tumors within 7 months of observation, while EL-4 cells grew out and formed palpable tumors as early as one week after inoculation (**Figure 15A** and **Figure 15B**). Then, the tumor free mice were challenged s.c. with EL-4 cells, which is derived from thymoma and different from pCSCs in origin. As shown in **Figure 15C**, the mice vaccinated with live pCSCs were resistant to tumor challenge. About 50% of the mice did not develop into palpable tumors, whereas all the control mice that received vehicles developed palpable tumors within one week post challenge. Moreover, the tumor growth in the pCSC-vaccinated mice was remarkably suppressed compared that in the control mice; and the tumor size in the pCSC-vaccinated mice was about 20 times smaller than in the control mice at the time of sacrifice (**Figure 15D**). These results confirm that self-renewing pCSC lines are not tumorigenic in IC mice (1) and reveal that live pCSCs can effectively induce anti-tumor immunity.

[00267] The lysates of pCSC but not classical tumor cell lines can induce effective anti-tumor immunity

[00268] To determine whether the anti-tumor immunity induced by live pCSCs was dependent on the live pCSCs in IC mice, the inventor herein examined the effects of pCSC lysates on the induction of anti-tumor immunity. The pCSC lysates were prepared from 3B5C pCSC clone, and used to immunize s.c. IC mice. The immunized mice were challenged with EL-4 tumor cells 8 weeks post immunization. Like live pCSCs, pCSC lysates also induced effective anti-tumor immunity comparable to that induced by live pCSCs (**Figure 16A**). The ability of pCSC lysates to induce antitumor immunity is dose-dependent (**Figure 16B**). More than 2.5×10^6 pCSCs were required for inducing maximal anti-tumor immunity in the immunized mice, showing that the quantity of antigens in the lysates may determine the capacity of anti-tumor immunity in the mice.

The anti-tumor immunity induced by pCSCs is unique because classical tumor cell lines EL-4 cell lysates did not induce significant anti-tumor immunity with the same immunization regimen. The results strongly show that pCSCs have distinct capacity from other tumor cell lines in the induction of anti-tumor immunity, and pCSCs are a more efficient target for the development of tumor vaccine.

[00269] *pCSC-induced anti-tumor immunity is not restricted by their tissue origin*

[00270] Most tumor associated antigens (TAAs) are expressed in limited types of tumors or tissue-specific, which induce narrow-spectrum of anti-tumor immunity. To determine whether the pCSC-induced anti-tumor immunity is tissue type-specific, the inventor immunized the mice with cell lysates of pCSCs (3B5C), EL-4 and B16 and then challenge them with B16 tumor cells. 3B5C, EL-4 and B16 were derived from lymphoma, thymoma and melanoma, respectively. As shown in **Figures 17A-17B**, hematopoietic pCSCs (HpCSCs)-induced anti-tumor immunity is able to suppress the challenge of melanoma B16. In contrast, the cell lysates of EL-4 and B16 induced weak but not significant anti-tumor immunity, further confirming that pCSC-induced anti-tumor immunity is stronger than general tumor cell lines and the activity is not tissue-specific. These results show that pCSCs can be used to develop broad-spectrum tumor vaccines.

[00271] *PL2L proteins have the usefulness to induce anti-tumor immunity*

[00272] To identify the proteins or antigens which are responsible for pCSC-induced anti-tumor immunity, the inventor herein examined the ability of peptides A and B (Pa and Pb) with 15 amino acid (aa) long to trigger anti-tumor immunity.

[00273] Pa is a sequence having 21 aa: CFYPKRAMDQARELVNMLEKI [**SEQ ID NO:26**].

[00274] Pb is a sequence having 15 aa: IPEKMKKDFRAMKDL [**SEQ ID NO:27**].

[00275] Pa and Pb are shared by both Piwil2 and PL2L proteins. Pb, but not Pa, can induce strong antibody response to Piwil2 and PL2L of humans and mouse (see **Example 1** herein).

[00276] To induce effective anti-tumor immunity, bone marrow-derived dendritic cells (DCs) were pulsed with Pa or Pb, and injected into the footpad of B6 mice. Control mice were injected with DC alone or vehicle. Three weeks later, the immunized mice were challenged i.v. with melanoma cell line (MJ/V), which could migrate to lungs. Two weeks later, microscopic tumors in the lungs were counted.

[00277] As shown in **Figures 18A-18B**, no metastatic tumors were in the lungs of all the mice immunized with Pb-pulsed DCs, but leukocytes-infiltrated residual tumors were detected in about 30% of the mice immunized with Pa-pulsed DCs. In contrast, 100% of the controlled mice (DCs alone and vehicle) developed metastatic tumors in the lungs (**Figure 18A**). Tumors detected in vehicle-treated mice were not infiltrated by leukocytes, while the tumors detected in DC-treated mice were infiltrated by leukocytes. Interestingly, inflammatory foci free of tumor cells were detected in all DC-treated mice but not in vehicle-treated mice (**Figure 18B**).

[00278] The mAbs to PL2L peptide can inhibit tumorigenesis of pCSCs in SCID mice and lyse human breast cancer xenografts

[00279] To further verify that PL2L proteins is a sensitive target of anti-tumor immunity induced by pCSCs, the inventor herein examined whether the mAb to Piwil2 and PL2 proteins (clone Kao2) can inhibit tumorigenesis of pCSCs in SCID mice.

[00280] As shown in **Figure 19A** and **Figure 19B**, treatment of pCSCs (clone 2C4G2) with mAb Kao2 (IgM) completely inhibited tumorigenesis of pCSCs; in contrast, all the mice inoculated with vehicle-treated 2C4G2 cells developed large tumors (**Figure 19A** and **figure 19B**). These results show that mAb Kao2 can kill PL2L60-expressing pCSCs, preventing their tumorigenesis.

[00281] Since mAb Kao2 can react with both human and mouse Piwil2 and PL2L proteins, the inventor herein also showed that mAb Kao2 can also kill human cancer cell lines. As show in **Figures 20A-20D**, human breast cancer cell line MBA-MD-231-GFP (231-GFP) expressed membrane PL2L proteins as detected by mAb Kao2 (**Figure 20A** and **Figure 20B**). The 231-GFP could develop into tumors in SCID mice. Intra-tumor injection of mAb into established 231-GFP tumors led to tumor lysis at the site of injection (**Figure 20C** and **Figure 20D**). Surrounding the necrotic area caused by mAb Kao2, a large number of polymorphonuclear leukocytes were observed (**Figure 20C**). No necrosis and significant infiltration was observed at the tumor site injected with vehicle. These results confirm that mAb Kao2 has the capacity to lyse both mouse and human tumor cells and also has usefulness in clinical applications.

[00282] **Example 3**

[00283] Therapeutic compositions comprising human monoclonal antibodies

[00284] The present invention therefore contemplates therapeutic compositions useful for practicing immunotherapy of cancer.

[00285] Therapeutic compositions containing one or more different human monoclonal antibodies specifically immunoreactive with an epitope of PL2L and the use thereof in the immunotherapy methods of the invention, are described herein. Preferred human monoclonal antibodies include mAb Kao1 and Kao2.

[00286] A therapeutically effective amount of a human monoclonal antibody is an amount calculated to achieve the desired effect, i.e., to neutralize the PL2L present in the tumor cells of the subject, and thereby decrease the amount of detectable PL2L or free PL2L in the tumor cells of the subject. Such a decrease is correlated with increased survival time for the subject.

[00287] The therapeutic compositions of the present invention contain a physiologically acceptable carrier, excipient, or diluent together with at least one species of human monoclonal antibody specifically immunoreactive with an epitope of PL2L, dissolved or dispersed therein as an active ingredient, formulated according to conventional practice.

[00288] Therapeutic compositions for injection or infusion may take such forms as suspensions, solutions or emulsions of the antibody in oily or aqueous vehicles, and, may contain components such as suspending, stabilizing and/or dispensing agents. Alternatively, the composition may be in a dry form, for reconstitution before use with an appropriate sterile liquid.

[00289] The human monoclonal antibodies of the invention can be administered parentally by injection or by gradual infusion over time. In most cases, therapeutic compositions comprising the human monoclonal antibodies of the invention are administered intravenously, however, other delivery means are contemplated. For example, human monoclonal antibodies of the invention can be injected intravenously, intraperitoneally, intramuscularly, subcutaneously, intratumorally; or administered transdermally or by peristaltic means.

[00290] Suitable regimens for antibody administration are variable, but are typified by an initial administration followed by repeated doses at one or more intervals by subsequent administration. In the treatment methods of the invention, the anti-PL2L antibodies may be administered together with or sequentially to, PL2L immunogenic peptide vaccines.

[00291] A therapeutically effective amount of an antibody for use in the methods of this invention is typically an amount of antibody in a pharmaceutically acceptable composition that is sufficient to decrease the amount of detectable PL2L in the subject.

[00292] The dosage can vary from about 25 mg to about 500 mg per subject per administration, wherein an average subject is 70 kg. In general, the anti-PL2L antibodies are administered 1 to 2 times per week for a period of 4 to 6 weeks. However, in some cases, such antibody administration may be continued for an indefinite time period. The duration of antibody treatment is evaluated in conjunction with PL2L immunogenic peptide vaccine administration based on the immune response of the subject to PL2L60.

[00293] It will be understood that when the immunotherapy methods of the invention comprise the administration of a human monoclonal antibody specifically immunoreactive with PL2L together with an PL2L immunogenic peptide vaccine, the dose of human monoclonal antibody may be adjusted based on the results of an immunoassay for PL2L immunogenic peptides, which is performed beginning approximately 9 to 15 weeks post initial PL2L vaccine administration. Preferably, the administration of an PL2L vaccine results in a level of detected antibodies

[00294] PL2L Immunogenic Peptides As Vaccines

[00295] A "PL2L immunogenic epitope" or "PL2L antigenic epitope" is any amino acid sequence, or combination of amino acid sequences which elicits an immune response against PL2L.

[00296] In one preferred embodiment, a PL2L vaccine is administered to a subject alone or in combination with one or more additional immunogenic PL2L peptides and/or together with passive administration of one or more anti-PL2L antibodies.

[00297] The PL2L vaccines described herein are representative of the types of PL2L vaccines that can be used in the methods of the current invention to generate an immune response. In general, the size of the peptide antigen must be at least large enough to encompass one or more immunogenic epitopes of PL2L. The smallest useful immunogenic epitope or fragment anticipated by the present disclosure would generally be on the order of about 8 contiguous amino acid residues in length, with sequences on the order of about 8 to about 40 or more of amino acids being preferred.

[00298] Follow-up or "booster" administrations of PL2L immunogenic peptide vaccines or passively administered anti-PL2L antibodies, can be provided to a cancer subject in successive, spaced administrations of one or more of the following: (i) a dose of the vaccine which is greater than that provided in the initial administration; (ii) a dose of the vaccine corresponding to less than 1.0 mg peptide per subject per administration; (iii) a promoter vaccine composed of the PL2L peptide derivatized to a suitable carrier, in combination with the PL2L vaccine; (iv) a promoter vaccine composed of the PL2L peptide, derivatized to a suitable carrier, followed by successive spaced administrations of the PL2L vaccine, at a dose of the latter vaccine of less than less than 1.0 mg peptide per subject per administration; (v) a peptide vaccine composed of PL2L, derivatized to a suitable carrier protein; (vi) passive administration of at least one dose every 1 to 2 weeks of a human monoclonal antibody specifically immunoreactive with a 15 mer fragment of PL2L; (vii) passive administration of at least one dose every 1 to 2 weeks of a human monoclonal antibody specifically immunoreactive with PL2L in combination with a human monoclonal antibody specifically immunoreactive with PL2L; and (viii) successive spaced administrations of a PL2L vaccine in combination with a human monoclonal antibody specifically immunoreactive with PL2L60.

In certain embodiments, the PL2L immunogenic peptide is conjugated to a carrier molecule, e.g., a protein. In general, each PL2L immunogenic peptide is separately conjugated to a carrier protein. When more than one conjugated PL2L immunogenic peptide is included in the treatment regimen for a given subject, the PL2L peptide vaccines may be administered to the subject at the same time, or at different times. In most cases, conjugated PL2L immunogenic peptides are administered together, in successively spaced administrations.

[00299] Biomarkers

[00300] In another aspect, there is provided herein the use of PL2L as a specific tumor biomarker for detection and prognosis of cancer. Also, gene exon screening (GEM) can be used for the detection of the gene products of intragenic promoter activation. Also, GEM can be used to detect potential gene production of intragenic promoter activation in cancer and other diseases.

[00301] In certain embodiments, GEM chips or GEM array can be developed for such screening.

[00302] In another aspect, there is provided herein use of the PL2L proteins as a target to develop a broad spectrum of tumor vaccines.

[00303] In certain embodiments, the Piwil2 peptides homologous to PL2L proteins are especially useful to develop tumor vaccines.

[00304] In one embodiment, the peptides can be derived from exon 6 to 23 of Piwil2.

[00305] In addition, the use of PL2L proteins and PL2L mRNA as a biomarker for detection of various types of cancer at various developing stages?

[00306] Variant or modified PL2L immunogenic peptides

[00307] The PL2L immunogenic peptides used in the methods of the invention have the same sequence as native PL2L immunogenic peptides. However, in some cases, PL2L immunogenic peptides are variant PL2L peptides. For example, some substitution of amino acids is possible without affecting the immunogenic character of the fragment.

[00308] Standard substitution classes are the six classes based on common side chain properties and highest frequency of substitution in homologous proteins in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix (Dayhoff). These classes are Class I: Cys; Class II: Ser, Thr, Pro, Hyp, Ala, and Gly, representing small aliphatic side chains and OH-group side chains; Class III: Asn, Asp, Glu, and Gln, representing neutral and negatively charged side chains capable of forming hydrogen bonds; Class IV: His, Arg, and Lys, representing basic polar side chains; Class V: Ile, Val, and Leu, representing branched aliphatic side chains, and Met; and Class VI: Phe, Tyr, and Trp, representing aromatic side chains. In addition, each group may include related amino acid analogs, such as ornithine, homoarginine, N-methyl lysine, dimethyl lysine, or trimethyl-lysine in class IV, and a halogenated tyrosine in Group VI. Further, the classes may include both L and D stereoisomers, although L-amino acids are preferred for substitutions.

[00309] Once an amino acid substitution or modification is made, the variant PL2L immunogenic peptide is screened immunogenicity, in an *in vitro* immunoassay.

[00310] The amino acid sequences of PL2L immunogenic peptides described herein may include additional residues, such as additional N- or C-terminal amino acids, and yet still be essentially the same as one of the sequences disclosed herein, so long as the peptide or polypeptide maintains the appropriate biological activity, e.g. immunogenicity.

[00311] The amino acids of a PL2L immunogenic peptide may be changed in a manner to create an equivalent, or even an improved molecule. It is the immunogenicity of the PL2L peptide which defines its biological activity. Certain amino acid sequence substitutions can be made in the amino acid sequence and the nucleic acid sequence encoding it, resulting in expression of a PL2L peptide with greater biological utility or activity (immunogenicity). In some case such changes may provide other benefits, e.g., stability or more desirable pharmacologic properties with maintenance of equivalent biological utility and activity.

[00312] In making such changes, the hydropathic index of amino acids may be considered. Certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity.

[00313] Variant PL2L immunogenic peptides for use in the methods of the invention may have improved properties for administration to subjects, however, such peptides retain the immunogenicity of the native sequence PL2L immunogenic peptides described herein.

[00314] *Treatment Regimens For Generating An Anti-PL2L Immune Response*

[00315] In vivo administration of PL2L vaccines may be achieved by inoculation in the presence of adjuvants or other substances that have the capability of recruiting immune system cells to the site of the inoculation.

[00316] In the methods of the invention, the immune response cells of a subject in whom protective immunization is desired, are exposed to a PL2L immunogenic peptide vaccine alone or in combination with one or more human monoclonal antibodies which are specifically immunoreactive with an epitope of PL2L. Protective immunization can be achieved following such exposure by effecting a reduction in the amount of circulating PL2L or tumor-associated PL2L in the subject. Both antibody- and cell-mediated immune responses may contribute to the anti-cancer activity of PL2L vaccines.

[00317] In the methods of the invention, a PL2L immunogenic peptide vaccine and/or human monoclonal antibody is administered in a manner compatible with the dosage formulation, and in an amount that is immunogenic and therapeutically effective. The quantity to be administered depends on the subject to be treated, including, e.g., the capacity of the individuals' immune system to respond to a peptide vaccine.

[00318] In general, the immunotherapeutic methods of the present invention are applicable to subject who already has cancer. This means the subject has been diagnosed as having cancer by one or more criteria employed by those of skill in the art to diagnose the relevant type of cancer. One such criteria is that the cancer is characterized by expression of PL2L by the cancer cells and in some cases further characterized by the presence of circulating PL2L in the plasma and/or other bodily fluids of the subject.

[00319] In some cases, the immunotherapeutic methods of the invention are applicable to a subject who is at high risk for developing cancer, or for whom one or more diagnostic criteria indicate that cancer may be developing.

[00320] Surgery, radiation therapy, and chemotherapy are currently the primary methods for

cancer treatment. It is contemplated that passive immunization with anti-PL2L human monoclonal antibodies and/or PL2L peptide vaccine-based treatment may be combined with such other cancer therapies. It will be appreciated that the methods described herein may interact in synergistic or additive ways with any one of surgery, radiation therapy, and chemotherapy, resulting in a greater therapeutic effect. In some cases, improved methods for treating cancer will combine conventional cancer treatments, e.g., chemotherapy or radiation therapy, together with passive immunization by administration of anti-PL2L human monoclonal antibodies and/or administration of PL2L peptide vaccines.

[00321] *PL2L Peptide Vaccine Compositions*

[00322] The invention provides a method of inducing an immune response against the PL2 peptide. This is accomplished by conjugating the peptide with a carrier molecule prior to administration to a subject.

[00323] In the methods of the invention, an immunologically effective amount of one or more PL2L immunogenic peptides derivatized to a suitable carrier molecule, e.g., a protein is administered to a cancer subject by successive, spaced administrations of a vaccine composed of an PL2L immunogenic peptide or peptides conjugated to a carrier molecule, in a manner effective to result in an improvement in the subject's condition.

[00324] In a preferred embodiment, PL2L immunogenic peptides are coupled to one of a number of carrier molecules, known to those of skill in the art. A carrier protein must be of sufficient size for the immune system of the subject to which it is administered to recognize its foreign nature and develop antibodies to it.

[00325] In certain embodiments, the carrier molecule is directly coupled to the mAb. In other cases, there is a linker molecule inserted between the carrier molecule and the mAb. The degree of conjugation must be adequate to induce an immune response by a cancer subject at a level sufficient to neutralize some or all of the effects of PL2L associated with the subject's condition. It will be appreciated that this will vary dependent upon the peptide, the carrier molecule and the subject.

[00326] The invention provides PL2L vaccine compositions as above-described wherein each PL2L immunogenic peptide is coupled to the same or a different carrier protein.

[00327] A PL2L vaccine composition may comprise single or multiple copies of the same or different PL2L immunogenic peptide, coupled to a selected carrier molecule. In one embodiment, the PL2L vaccine composition may contain different PL2L immunogenic peptides with or without flanking sequences, combined sequentially into a polypeptide and coupled to the same carrier. Alternatively, the PL2L immunogenic peptides, may be coupled individually as peptides to the same or a different carrier, and the resulting PL2L immunogenic peptide-carrier conjugates blended together to form a single composition, or administered individually at the same or

different times.

[00328] In general, derivatized PL2L peptide vaccine compositions are administered with a vehicle. The purpose of the vehicle is to emulsify the vaccine preparation. Numerous vehicles are known to those of skill in the art, and any

[00329] To further increase the magnitude of the immune response resulting from administration of the PL2L vaccine, an immunological adjuvant is included in the vaccine formulation. Exemplary adjuvants known to those of skill in the art include water/oil emulsions, non-ionic copolymer adjuvants, aluminum phosphate, aluminum hydroxide, aqueous suspensions of aluminum and magnesium hydroxides, bacterial endotoxins, polynucleotides, polyelectrolytes, lipophilic adjuvants and synthetic muramyl dipeptide (norMDP) analogs. In certain embodiments, the mass ratio of the adjuvant relative to the peptide conjugate is about 1:2 to 1:20. In a preferred embodiment, the mass ratio of the adjuvant relative to the peptide conjugate is about 1:10. It will be appreciated that the adjuvant component of the PL2L vaccine may be varied in order to optimize the immune response to the PL2L immunogenic epitopes therein.

[00330] Suitable pharmaceutically acceptable carriers for use in an immunogenic proteinaceous composition of the invention are well known to those of skill in the art. Such carriers include, for example, phosphate buffered saline, or any physiologically compatible medium, suitable for introducing the PL2L vaccine into a subject.

[00331] Numerous drug delivery mechanisms known to those of skill in the art may be employed to administer the PL2L immunogenic peptides and of the invention. Controlled release preparations may be achieved by the use of polymers to complex or absorb the peptides or antibodies in the methods of the present invention. Controlled delivery may be accomplished using macromolecules such as, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate, the concentration of which can alter the rate of release of the peptide vaccine.

[00332] In certain embodiments, the PL2L peptides may be incorporated into polymeric particles composed of e.g., polyesters, polyamino acids, hydrogels, polylactic acid, or ethylene vinylacetate copolymers. Alternatively, the PL2L peptide vaccine can be entrapped in microcapsules, liposomes, albumin microspheres, microemulsions, nanoparticles, nanocapsules, or macroemulsions, using methods generally known to those of skill in the art.

[00333] The present invention includes a diagnostic device for monitoring the immune response to the PL2L vaccine. The device includes a peptide having the amino acid sequence of the C-terminal fragment of PL2L, a peptide having the amino acid sequence of the N-terminal fragment of PL2L, and a reagent effective to label human antibodies, such as a reporter-labeled anti-human immunoglobulin antibody; in order to detect human antibodies that are immunoreactive with the peptides.

[00334] Such diagnostic devices are generally known to those of skill in the art of immunology and are routinely used to analyze antibodies in the body fluids of various subjects. In some cases, the device includes a solid-phase substrate to which the PL2L peptides are attached.

[00335] The invention further includes a method of monitoring the immune response to PL2L by carrying out the steps of reacting a body-fluid sample with a peptide having the amino acid sequence of PL2L60; and detecting antibodies in the sample that are immunoreactive with each peptide. It is preferred that the assay be quantitative and accordingly be used to compare the level of each antibody in order to determine the relative magnitude of the immune response to each peptide.

[00336] *Dendritic Cell Based Vaccines*

[00337] In certain embodiments, the vaccine can be a dendritic cell-based vaccine, which contains dendritic cells pulsed with any of the immunopeptides described herein. Methods for preparing dendritic cell-based vaccines are well known in the art. See, for example, Slingluff et al., Clin Cancer Res. 12:2342s-2345s, 2006; Buchsel et al. Clin J Oncol Nurs. 10:629-40, 2006; and Yamanaka et al., Expert Opin Biol Ther. 7:645-9, 2007.

[00338] In one embodiment, the immunotherapeutic vaccine can be administered to treat and/or prevent further progression of a disease in a subject already diagnosed with the disease. In certain embodiments, the vaccine can be formulated with autologous cells, i.e., cells that are an individual's own cells; while in other embodiments, the vaccine can be formulated with allogeneic cells, i.e., genetically different cells, but of the same species. It is to be understood that the vaccine compositions may be administered in a convenient manner, e.g., injection by a convenient and effective route. Non-limiting examples include "gene gun" delivery, subcutaneous, intravenous or intramuscular routes; oral administration, intrathecal, inhalation and transdermal application administration.

[00339] It is further to be understood that direct intratumoral injection can be used as administration route for an immunotherapeutic vaccine in the treatment of an existing tumor (which may not be completely resected or in which there is a recurrence). Depending on the route of administration, the composition may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation.

[00340] Also described herein is a method for enhancing an immune response against cancers, comprising administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an amino acid sequence of mAb Kao1 and/or mAb Kao2.

[00341] Also described herein is a method for treating a cancer-associated disease, comprising

administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an amino acid sequence of mAb Kao1 and/or mAb Kao2.

[00342] Also described herein is a dendritic cell-based vaccine comprising dendritic cells expressing composition having substantial sequence homology to mAb Kao1 and/or mAb Kao2.

[00343] Also described herein is a method for preparing a vaccine, comprising the step of preparing a construct having substantial sequence homology to mAb Kao1 and/or mAb Kao2 and ex vivo transducing dendritic cells with the construct.

[00344] Also described herein is a population of dendritic cells for use in vaccination of a subject produced by the process of: a) obtaining dendritic cells from the subject; b) causing the dendritic cells to express at least one antigen by either (a) exposing the dendritic cells to the antigen in culture under conditions promoting uptake and processing of the antigen; or (b) transfecting the dendritic cells with a gene encoding the antigen; c) activating the antigen-expressing dendritic cells, and d) treating the dendritic cells with an agent that inhibits Piwil2 expression.

[00345] Immunoassays

[00346] The methods of the invention are generally applicable to immunoassays, such as enzyme linked immunosorbent assay (ELISAs), radioimmunoassay (RIA), immunoprecipitation, Western blot, dot blotting, FACS analyses and other methods known in the art.

[00347] In one preferred embodiment, the immunoassay includes a PL2L peptide antigen immobilized on a solid support, e.g., an ELISA assay.

[00348] Kits

[00349] It will be appreciated that the immunoassay may be readily adapted to a kit format exemplified by a kit which comprises: (i) one or more peptides bound to a solid support; (ii) a means for collecting a sample from a subject; and (iii) a reaction vessel in which the assay is carried out. The kit may also comprise labeling means, indicator reaction enzymes and substrates, and any solutions, buffers or other ingredients necessary for the immunoassay.

[00350] Another aspect of the invention is a test kit which comprises one or more of the antibodies and/or peptides of the invention. It is to be understood that such kit normally comprises all or only some components of a test in packaged form. The antibodies and/or peptides of the invention may be associated for example with one or more solid phases and/or one or more components of a signal-generating system. The test kit may comprise for example standards; controls; and other reagents such as, for example, buffers, washing solutions, measured signal-inducing solutions and/or enzyme substrate; cuvettes; pipettes and/or test instructions.

[00351] The antibodies and peptides of the invention can also be used for affinity chromatography. The term "affinity chromatography" means a method for purifying and isolating

substances, in particular biopolymers, which is based on the fact that many substances are able to enter into a selective, noncovalent, reversible binding with binding partners which are specific for them. The principle of the method is that the specific binding partner is linked, usually covalently, to an insoluble matrix (e.g. porous glasses, gels based on agarose, cellulose, dextran, polymer and silica gel) and brought into contact with a sample containing the substance. The substance which is sought is immobilized and retained because of its specific interaction with the matrix-linked specific binding partner, whereas all the other substances contained in the sample are removed by elution. Subsequently, the substance which is sought is detached from the matrix using a suitable eluent which abolishes the noncovalent binding between substance and specific binding partner.

[00352] Another aspect of this invention includes antibodies of the invention or peptides of the invention in a pharmaceutically acceptable, sterile injection medium. A pharmaceutically acceptable, sterile injection medium means for example a sterile, pyrogen-free solution, e.g., saline or another electrolyte solution, as is normally used for intravenous, intramuscular, intraperitoneal or subcutaneous administration of medicaments, vaccines or contrast agents.

[00353] Another aspect of this invention is in turn the use of the antibodies of the invention as diagnostic aid or as constituent of a diagnostic aid.

[00354] Another aspect of this invention is a method for preparing an antibody of the invention, which comprises employing for the immunization one or more peptides. The peptides used as immunizing antigen may be unbound and/or carrier-bound when used for the immunization. Examples of typical carriers are proteins such as, for example, ovalbumin, albumin or hemocyanin, or polymers such as, for example, polyethylene glycol, poly-acrylamide or poly-D-glutamine-D-lysine. The peptides can be linked to these carriers for example with the aid of carbodiimide or glutaraldehyde, or else by means of a bifunctional reagent which may also act as spacer (for examples and coupling methods.

[00355] The immunizing antigen may be taken up for example in phosphate-buffered saline and be mixed with Freund's adjuvant. This emulsion can then be administered for example intradermally, intraperitoneally and/or subcutaneously to an animal, for example a rabbit, a mouse, a rat, a guinea pig, a horse, a sheep, a goat, a chicken, etc. Booster injections, for which the immunizing antigen can also be emulsified with incomplete Freund's adjuvant, may help to increase the immune response.

[00356] Depending on the desired purpose of use, it is advantageous to employ only parts of the antibodies, such as, for example, Fab, F(ab')₂ or Fab' fragments. These can be generated for example by enzymatic cleavage methods known to the skilled worker (see also, for example, Harlow & Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor). The antigen-binding sites of an antibody are located in the so-called variable domains which are encoded by the V genes. The known genetic engineering methods (see, for

example, Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, 2nd edition; McCafferty et al. (1990) *Nature* 348:552-554) can be used also to find the corresponding nucleic acid sequence of an antibody of the invention, and thus also the corresponding amino acid sequence, where this was not previously known from amino acid sequencing. It is possible with knowledge of the nucleic and/or amino acid sequence and with the aid of conventional methods of genetic manipulation and molecular biology then to prepare humanized, chimeric, bi- or oligospecific antibodies, and peptides derived from the complementarity-determining region (minimal recognition units), single-chain fragments, and/or functional fusion products, e.g. recombinantly prepared antibody-enzyme constructs, in particular to a peptide of the invention. It is possible with such peptides included by the term "antibodies" for example to reduce the immunogenicity and/or enhance the efficacy on administration as medicament or in vivo diagnostic aid, and/or advantages for use as or in an in vitro diagnostic aid emerge.

[00357] *Antibodies and uses Thereof*

[00358] Within the meaning of this invention, the term antibodies is to be understood as signifying not only complete antibodies but also, expressly, antibody fragments such as Fab, Fv, F(ab')₂ and Fab'; as well as chimeric, humanized, bispecific or oligospecific, or single-chain antibodies; and, in addition, also aggregates, polymers and conjugates of immunoglobulins and/or their fragments provided their properties with regard to binding to the antigen or hapten are retained. Antibody fragments can be prepared, for example, by cleaving antibodies enzymatically with enzymes such as pepsin or papain. Antibody aggregates, antibody polymers and antibody conjugates can be generated by many different methods, for example by heat treatment, by reaction with substances such as glutaraldehyde, by reaction with immunoglobulin-binding molecules, by biotinylation of antibodies and subsequent reaction with streptavidin or avidin, etc.

[00359] Another part of the subject matter of this invention relates to specific binding partners which bind to an epitope which is recognized by an antibody according to the invention.

[00360] A "specific binding partner" is to be understood as being a member of a specific binding pair. The members of a specific binding pair are two molecules which in each case possess at least one structure which is complementary to a structure of the other molecule, with the two molecules being able to bind to each other by means of the complementary structures binding. The term molecule also encompasses molecular complexes such as enzymes which consist of apoenzyme and coenzyme, proteins which consist of several subunits, lipoproteins which consist of protein and lipids, etc. Specific binding partners can be naturally occurring substances or else substances which are, for example, prepared by means of chemical synthesis, microbiological techniques and/or recombinant methods. As a result of providing the antibodies according to the invention, it is now possible for the skilled person, for example by means of competition

experiments to identify other specific binding partners, with antibodies being expressly included, which bind to the epitope of an antibody according to the invention.

[00361] *Solid Phase and/or Component of Signal-generating System*

[00362] This invention also relates to an antibody according to the invention which is linked to a solid phase and/or a component of a signal-generating system.

[00363] Within the meaning of this invention, the term "solid phase" means an object which consists of a porous and/or nonporous, as a rule water-insoluble, material and can have a very wide variety of forms, for example those of vessels, tubes, microtitration plates, spheres, microparticles, rods, strips, filter paper, chromatography paper, etc. As a rule, the surface of the solid phase is hydrophilic or can be made hydrophilic. The solid phase can consist of a very wide variety of materials, for example of inorganic and/or organic materials, of synthetic materials, of naturally occurring materials and/or of modified naturally occurring materials. Non-limiting examples of solid phase materials are polymers, such as cellulose, nitrocellulose, cellulose acetate, polyvinyl chloride, polyacrylamide, crosslinked dextran molecules, agarose, polystyrene, polyethylene, polypropylene, polymethacrylate or nylon; ceramic, glass or metals, in particular precious metals such as gold and silver; magnetite; mixtures or combinations thereof; etc. Cells, liposomes and phospholipid vesicles are also covered by the term solid phase.

[00364] The solid phase can also possess a coating consisting of one or more layers, for example of proteins, carbohydrates, lipophilic substances, biopolymers or organic polymers, or mixtures thereof, in order, for example, to suppress or prevent the nonspecific binding of sample constituents to the solid phase or in order, for example, to achieve improvements with regard to the suspension stability of particular solid phases, with regard to storage stability, with regard to dimensional stability or with regard to resistance to UV light, microbes or other agents having a destructive effect.

[00365] A "signal-generating system" can be one or more components with at least one of the components being a detectable label. A label is to be understood as being any molecule which itself produces a signal or which is able to induce the production of a signal, for example a fluorescent substance, a radioactive substance, an enzyme or a chemiluminescent substance. The signal can, for example, be detected or measured using the enzyme activity, the luminescence, the light absorption, the light scattering, the emitted electromagnetic or radioactive radiation or a chemical reaction.

[00366] A label may itself be able to generate a detectable signal, such that no further components are required. Many organic molecules absorb ultraviolet and visible light, resulting in these molecules being able to reach an excited energy state and to emit the absorbed energy in the form of light which is of a different wavelength from that of the incident light. Yet other labels can directly generate a detectable signal, for example radioactive isotopes or dyes.

[00367] Yet other labels require additional components for generating the signal, i.e., in such a case, the signal-producing system includes all the components, such as substrates, coenzymes, quenchers, accelerators, additional enzymes, substances which react with enzyme products, catalysts, activators, cofactors, inhibitors, ions, etc., which are required for producing the signal.

[00368] Examples of suitable labels are enzymes, including horseradish peroxidase, alkali phosphatase, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, glucose oxidase, .beta.-galactosidase, luciferase, urease and acetylcholine esterase; dyes; fluorescent substances, including fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanine, ethidium bromide, 5-dimethylamino-naphthalene-1-sulfonyl chloride and fluorescent chelates of rare earths; chemiluminescent substances including luminol, isoluminol, acridinium compounds, olefin, enolether, enamine, arylvinylether, dioxene, arylimidazole, lucigenin, luciferin and aequorin; sensitizers, including eosin, 9,10-dibromoanthracene, methylene blue, porphyrin, phthalocyanine, chlorophyll and Rose Bengal; coenzymes; enzyme substrates; radioactive isotopes, including magnetic particles or particles, preferably latex particles, which themselves can be labeled with, for example, dyes, sensitizers, fluorescent substances, chemiluminescent substances, isotopes or other detectable labels; sol particles including gold sols or silver sols; liposomes or cells which can themselves be labeled with detectable labels; etc.

[00369] Within the meaning of the invention, a "sample" is to be understood as signifying the material which is suspected of containing the substance to be detected. The term sample encompasses, for example, biological fluids or tissues from humans and animals, in particular, such as blood, plasma, serum, sputum, exudate, bronchoalveolar lavage, lymph fluid, synovial fluid, seminal fluid, vaginal mucus, feces, urine, cerebral spinal fluid, hair, skin and tissue samples/sections (including tumor tissues/sections). It also encompasses cell culture samples, plant liquids or tissues, forensic samples, water and effluent samples, nutrients and pharmaceuticals. Where appropriate, the samples have to be pretreated in order to make the analyte accessible to the detection method or in order to remove interfering sample constituents. Such a pretreatment of samples may involve separating off and/or lysing cells, precipitation, the hydrolysis or denaturation of sample constituents such as proteins, the centrifugation of samples, the treatment of the sample with organic solvents such as alcohols, in particular methanol; and the treatment of the sample with detergents. The sample is frequently transferred into another, usually aqueous, medium, which will, if at all possible, not interfere with the detection method.

[00370] In the case of a quantitative detection, the quantity, the concentration or the activity (e.g. enzyme activity) of the analyte in the sample is measured. The term "quantitative detection" also includes semi-quantitative methods which are only able to detect the approximate quantity, concentration or activity of the analyte in the sample or are only able to be used to give a relative indication of the quantity, concentration or activity. A qualitative detection is to be understood as

detecting whether the analyte is at all present in the sample or indicating that the concentration or activity of the analyte in the sample is below or above one particular threshold value or several particular threshold values.

[00371] Another part of the subject matter of this invention encompasses antibodies according to the invention or peptides according to the invention in a pharmaceutically tolerated, sterile injection medium. A pharmaceutically tolerated, sterile injection medium is to be understood, for example, as being an organism-free, pyrogen-free solution, for example saline or another electrolyte solution as is customarily used for the intravenous, intramuscular, intraperitoneal or subcutaneous administration of pharmaceuticals, vaccines or contrast agents.

[00372] Therapeutic Uses

[00373] Yet another part of the subject matter of this invention is the use of the antibodies according to the invention as a diagnostic agent or as a constituent of a diagnostic agent.

[00374] The peptides which are used as immunizing antigens can be used for the immunization in unbound form and/or in carrier-bound form. Examples of typical carriers are proteins, such as ovalbumin, albumin or keyhole limpet hemocyanin (KLH), or polymers, such as polyethylene glycol, polyacrylamide or poly-d-glutamine-d-lysine. The peptides can, for example, be bonded to these carriers using carbodiimide or glutaraldehyde or else using a heterobifunctional reagent, such as N-maleimidobutyryl-oxysuccinimide ester (GBMS), which can also act as a spacer.

[00375] While the invention has been described with reference to various and preferred embodiments, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted for elements thereof without departing from the essential scope of the invention. In addition, many modifications may be made to adapt a particular situation or material to the teachings of the invention without departing from the essential scope thereof.

[00376] Therefore, it is intended that the invention not be limited to the particular embodiment disclosed herein contemplated for carrying out this invention, but that the invention will include all embodiments falling within the scope of the claims.

CLAIMS

What is claimed is:

1. An isolated monoclonal antibody which binds specifically to at least one of: a PIWIL2 peptide and a PL2L protein.
2. The antibody of claim 1, wherein the PL2L protein comprises one of: PL2L80, PL2L60, PL2L50, PL2L42 and PL2L40.
3. The antibody of claim 1, wherein the antibody specifically recognizes PIWIL2.
4. The antibody of claim 1, wherein the antibody is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US.
5. The antibody of claim 1, wherein the antibody specifically recognizes PIWIL2 and PL2L proteins.
6. The antibody of claim 1, wherein the antibody is produced by the hybridoma cell line MH-KA02, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.
7. The antibody of claim 1, wherein the antibody is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.
8. An isolated antibody which is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US
9. An isolated antibody which is produced by the hybridoma cell line MH-KA012, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.
10. An isolated antibody which is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.
11. A composition comprising the antibody as claimed in claim 1 and a pharmaceutically acceptable, sterile injection medium.

12. A reagent comprising the antibody as claimed in claim 1.
13. A test kit comprising the reagent as claimed in claim 12.
14. An isolated cell which produces the antibody as claimed in claim 1.
15. The isolated cell as claimed in claim 14, wherein the cell is an animal, plant, prokaryotic, or human cell.
16. A hybridoma cell line which produces the isolated antibody as claimed in claim 1.
17. An isolated monoclonal antibody which binds specifically to an epitope comprising Pa [SEQ ID NO:26].
18. An isolated monoclonal antibody which binds specifically to an epitope comprising Pb [SEQ ID NO:27].
19. A method of assaying a biological specimen for a cancer, comprising: contacting a sample of the biological specimen with an isolated monoclonal antibody which binds specifically to at least one of: a PIWIL2 peptide and a PL2L protein and detecting PIWIL2 and or a PL2L protein by the monoclonal antibody.
20. The method of claim 19, wherein the PL2L protein comprises one of: PL2L80, PL2L60, PL2L50, PL2L42 and PL2L40.
21. The method of claim 19, wherein the antibody specifically recognizes PIWIL2.
22. The method of claim 19, wherein the antibody is produced by the hybridoma cell line MH-KA01, deposited under registration number PTA-11853 at the ATCC, Manassas VA, US.
23. The method of claim 1, wherein the antibody specifically recognizes PIWIL2 and PL2L proteins.
24. The method of claim 1, wherein the antibody is produced by the hybridoma cell line MH-KA02, deposited under registration number PTA-11854 at the ATCC, Manassas VA, US.

25. The method of claim 1, wherein the antibody is produced by the hybridoma cell line MH-KA03, deposited under registration number PTA-11855 at the ATCC, Manassas VA, US.

26. The method of claim 19, wherein the cancer is detected in an immunoassay.

27. The method of claim 26, wherein the immunoassay is selected from the group consisting of MIC (microparticle capture immunoassays), LAI (latex agglutination inhibition), IC (solid phase chromatographic), RIA (radio immunoassays), ELISA (enzyme linked immunoabsorbent assay), BIA (enzyme linked assays), FIA (fluorescence linked assays), LIA (luminescence linked assays), CLA (chemiluminescence assays), OA (optical color label assays), EST (electrochemical signal transducers) and rare earth metals label assays.

28. The method of claim 19, wherein the biological specimen is a tissue sample that expresses PL2L.

29. The method of claim 28, wherein the biological specimen comprises one or more of: blood, bone marrow, brain, breast, cervix, colon, heart, intestines, kidney, liver, lung, lymph nodes, ovary, oviduct, pancreas, prostate, salivary, skin, soft tissues, spleen, stomach, testis, thymus and uterus.

30. The method of claim 19, wherein the cancer is one or more of: leukemia, lymphoma, adenoma, adenocarcinoma, carcinoma, melanoma, sarcoma, neuroblastoma, hepatoma, insulinoma and endothelioma

31. A method of diagnosing a cancer, comprising: contacting a sample with an antibody of claim 1; removing substances from the sample that are not bound by the antibody; and, measuring the concentration, amount, or presence of bound antibody in the sample via a label associated with the antibody.

32. A human antibody or an antigen-binding portion thereof that specifically binds to and activates a Piwil2-like (PL2L) protein.

33. A human antibody or an antigen-binding portion thereof that specifically binds to and activates a 15-mer fragment of a Piwil2-like (PL2L) protein.

34. A monoclonal antibody comprising mAb Kao1.

35. A monoclonal antibody comprising mAb Kao2/3.
36. A monoclonal antibody to human PL2L proteins which inhibits tumorigenesis of PL2L proteins-expressing tumor cells.
37. An isolated PL2L peptide Pa comprising a sequence having 21 aa: CFYPKRAMDQARELVNMLEKI [SEQ ID NO:26].
38. An isolated PL2L peptide Pb comprising a sequence having 15 aa: IPEKMKKDFRAMKDL [SEQ ID NO:27].
39. An isolated Piwil2 peptide comprising: 503 IPEKMKKDFRAMKDL 517 [SEQ ID NO:28].
40. A monoclonal antibody specifically immunoreactive with Piwil2 (Kao1).
41. A monoclonal antibody specifically immunoreactive with both Piwil2 and PL2L proteins (Kao2 and Kao3).
42. A monoclonal antibody specifically immunoreactive with Piwil2, PL2L80, PL2L60, PL2L50 and PL2L40.
43. Use of the antibody of claim 1, for detection of one or more of: primary and metastatic cancers.
44. Use of the antibody of claim 1, for inhibiting tumorigenesis of PL2L proteins-expressing tumor cells.
45. An isolated protein comprising an amino acid sequence of human, PL2L60, starting from aa 444 to aa 973 of human Piwil2 [SEQ ID No:23].
46. An isolated PL2L protein having at least one tumor-specific antigen useful as a tumor vaccine.
47. A PL2L peptide vaccine useful to induce effective antitumor immunity.

48. Use of the antibody of claim 1 to induce apoptosis in PL2L60-expressing precancerous stem cells (pCSCs), thereby preventing their tumorigenesis.
49. An isolated PL2L protein useful as a diagnostic biomarker for cancers.
50. An isolated PL2L protein useful as a therapeutic biomarker for cancers.
51. An isolated PL2L60 protein useful as a biomarker for one or more types of cancer.
52. A PL2L peptide vaccine useful to induce effective anti-tumor immunity against tumors originated from various types of tumors.
53. Use of the antibody of claim 1, as a medicament.
54. Use of the antibody of claim 1, for the production of a medicament intended for the treatment of cancer.
55. Use of a monoclonal antibody directed against the human PL2L protein receptor for the production of a medicament intended for the treatment of a cancer.
56. Use of the antibody of claim 1, in combination with one or more other antibody(ies) directed against one or more other antigen(s) expressed on a cancer cell.
57. Pharmaceutical composition comprising at least one antibody of claim 1, and at least one excipient and/or at least one pharmaceutically acceptable vehicle.
58. A method of treating a subject having a malignancy which comprises a tumor, comprising administering to the subject a therapeutically effective amount of an anti-PL2L antibody or a fragment thereof in an amount sufficient to modulate the function of the malignant cells within the tumor that promote tumor growth and survival, to thereby elicit immunodulatory effects.
59. The method of claim 58, wherein the anti-PL2L antibody or fragment thereof is a chimeric, humanized, or human antibody.

60. The method of claim 59, wherein the anti-PL2L antibody or binding fragment thereof is selected from the group consisting of:

an anti-PL2L antibody or fragment thereof which binds a PL2L epitope bound by the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or fragment thereof which competes for binding to PL2L with the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises variable regions derived from variable regions of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises variable regions of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855;

an anti-PL2L antibody or a PL2L-binding fragment thereof which comprises complementarily determining regions (CDRs) of the antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855; and

an anti-PL2L antibody or a PL2L-binding fragment thereof which blocks PL2L signaling.

61. The method of claim 59, wherein the anti-PL2L antibody is Kao1 or Kao2.

62. The method of claim 59, further comprising administering to the subject an anti-cancer agent, wherein the PL2L-targeted therapeutic and the anti-cancer agent are administered concurrently or consecutively in either order.

63. A method for enhancing an immune response against cancers, comprising administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited

under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855.

64. A method for treating a cancer-associated disease, comprising administering to a subject in need thereof of an effective amount of a composition containing a carrier and an immunopeptide that includes an antibody produced one or more of hybridoma cell lines: MH-KA01, deposited under ATCC registration number PTA-11853; MH-KA012, deposited under ATCC registration number PTA-11854; and, MH-KA03, and deposited under ATCC registration number PTA-11855.

65. A vaccine comprising an antibody of claim 1.

66. The vaccine of claim 65, wherein the vaccine is for cancer.

67. PL2L as a specific tumor biomarker for detection and prognosis of cancer

68. Use of a PL2L protein as a target to develop a tumor vaccine.

69. Use of Piwil2 peptides homologous to PL2L proteins to develop a tumor vaccine.

70. Use of claim 69, wherein the peptides are derived from exon 6 to 23 of Piwil2.

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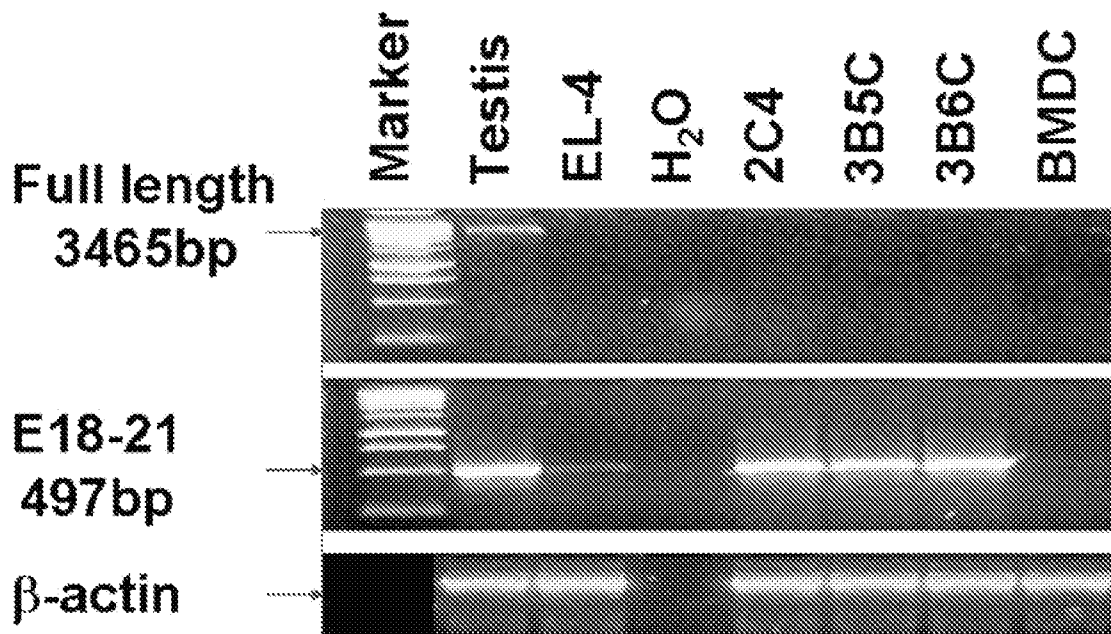


Figure 1A

Piwil2-specific primers for GEM RT-PCR
 (Piwil2 gene sequence: 4313 bp; 23 exons)

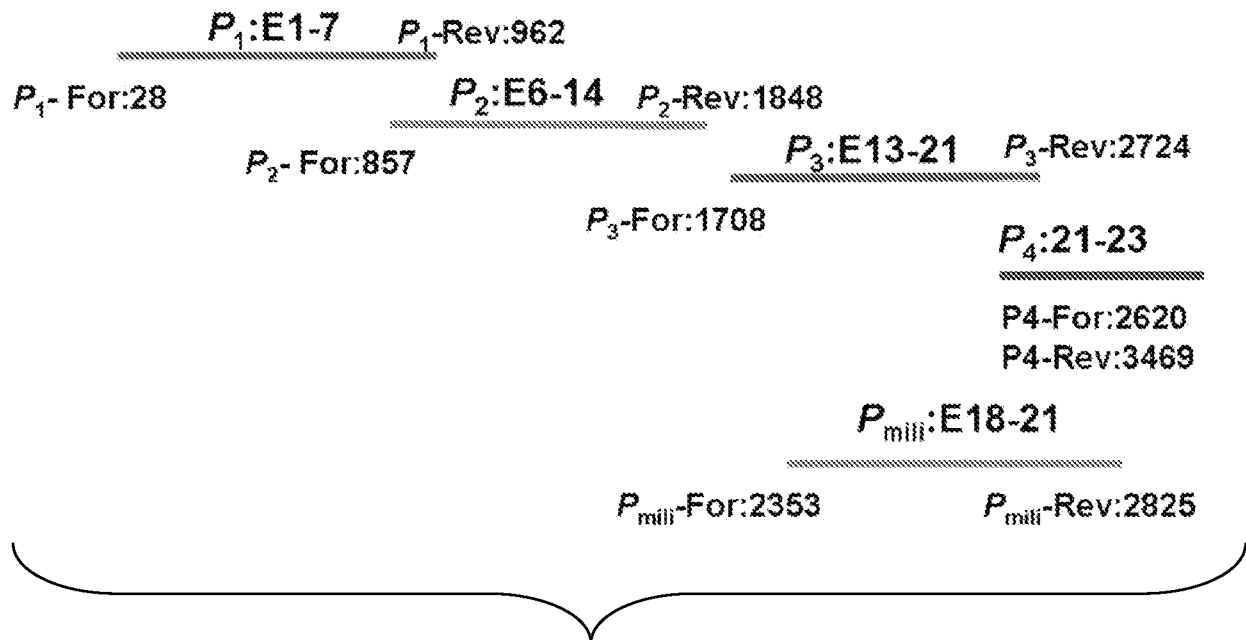


Figure 1B

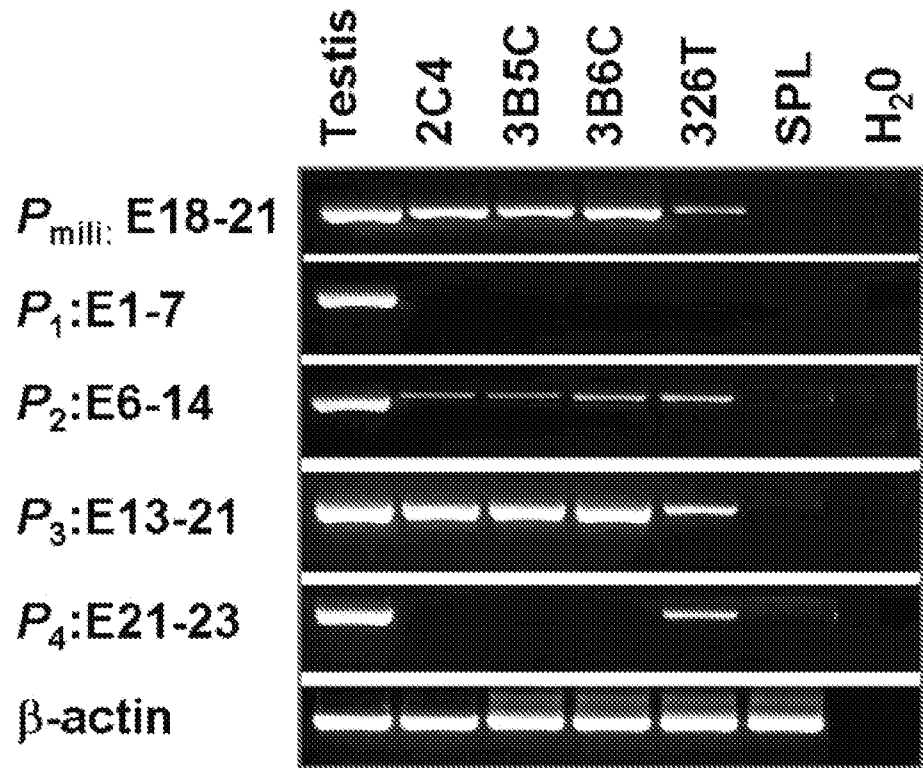


Figure 1C

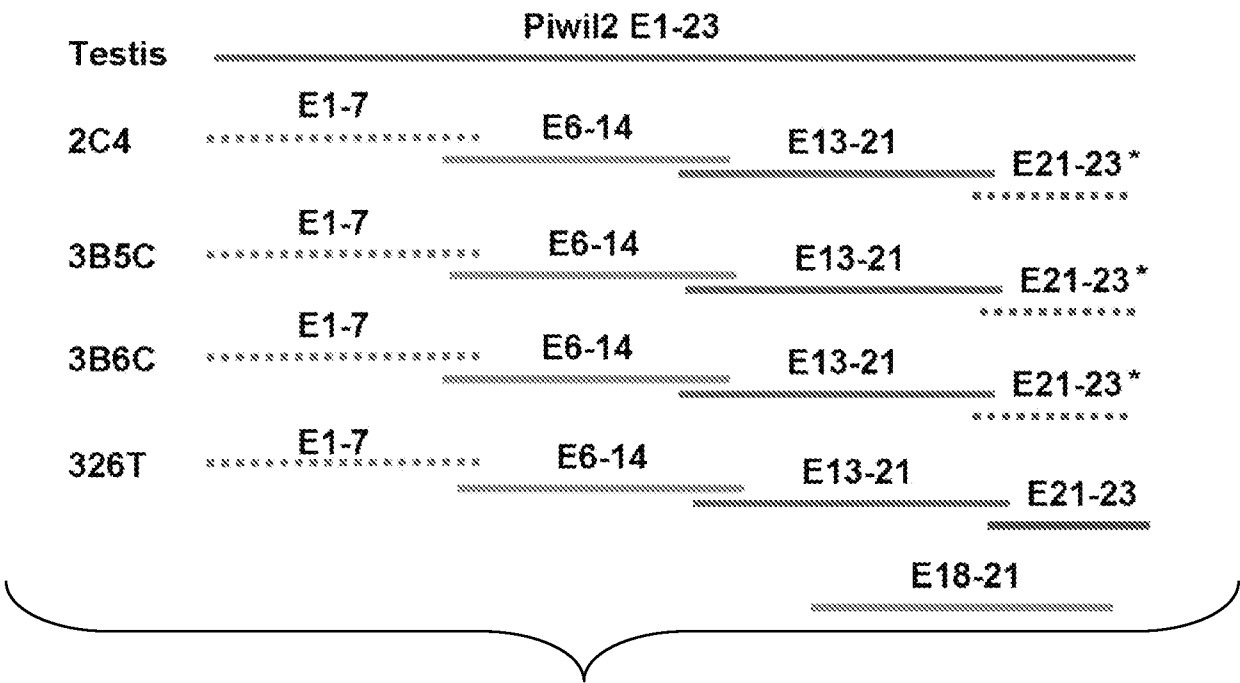


Figure 1D

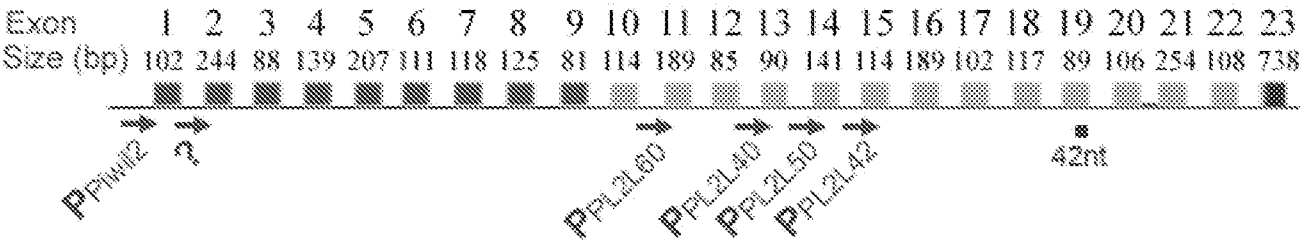


Figure 2A

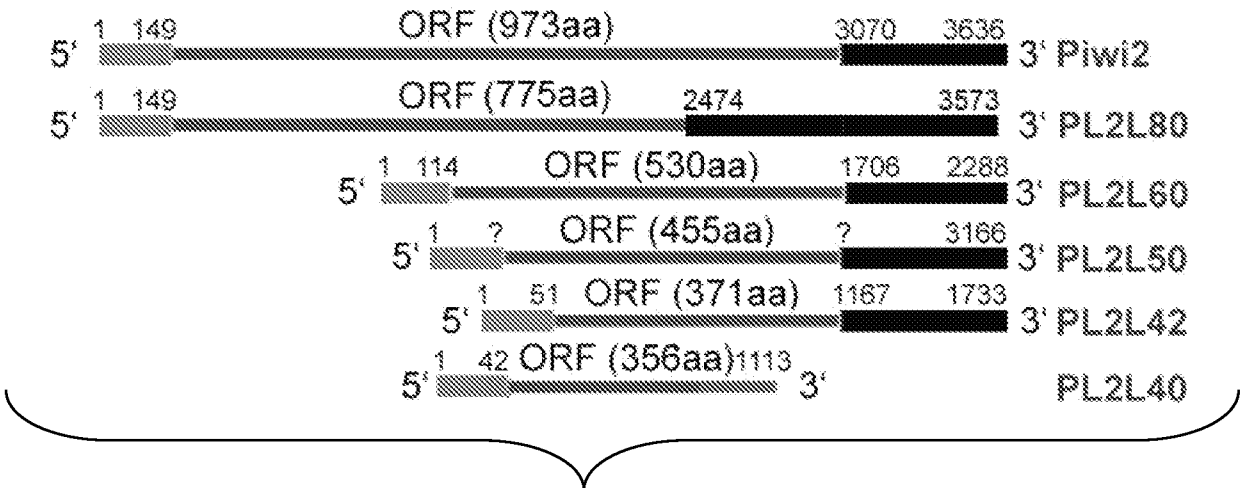


Figure 2B

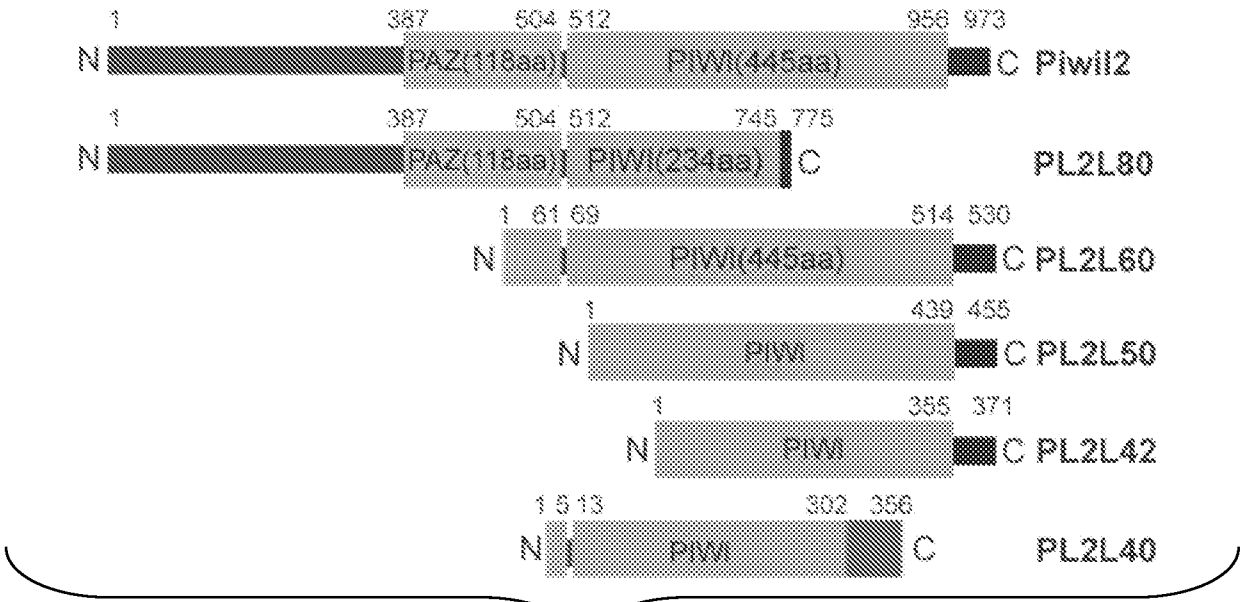


Figure 2C

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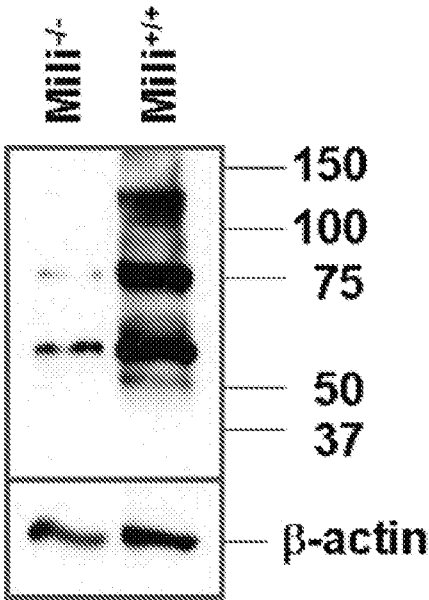


Figure 3A

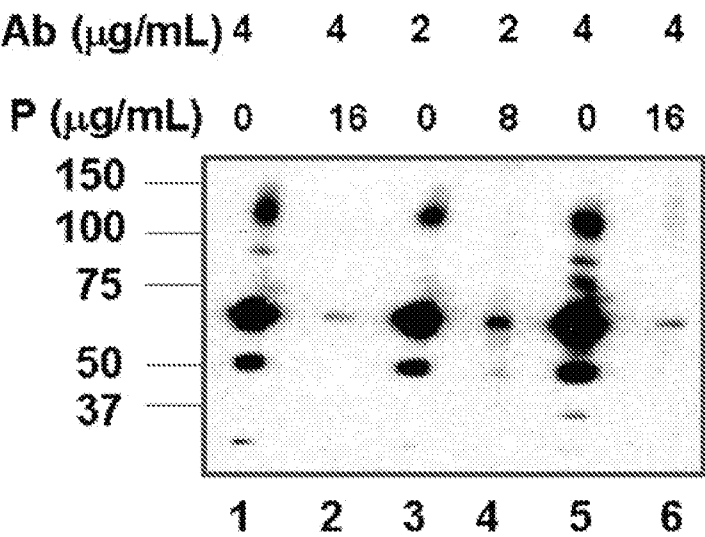


Figure 3B

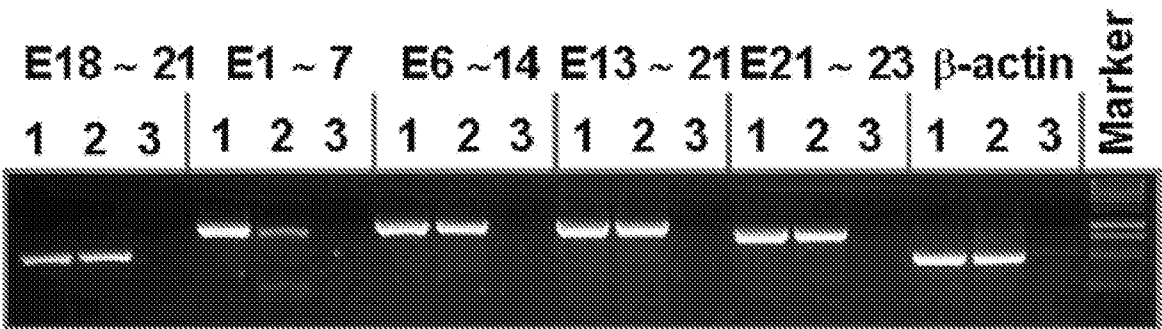


Figure 3C

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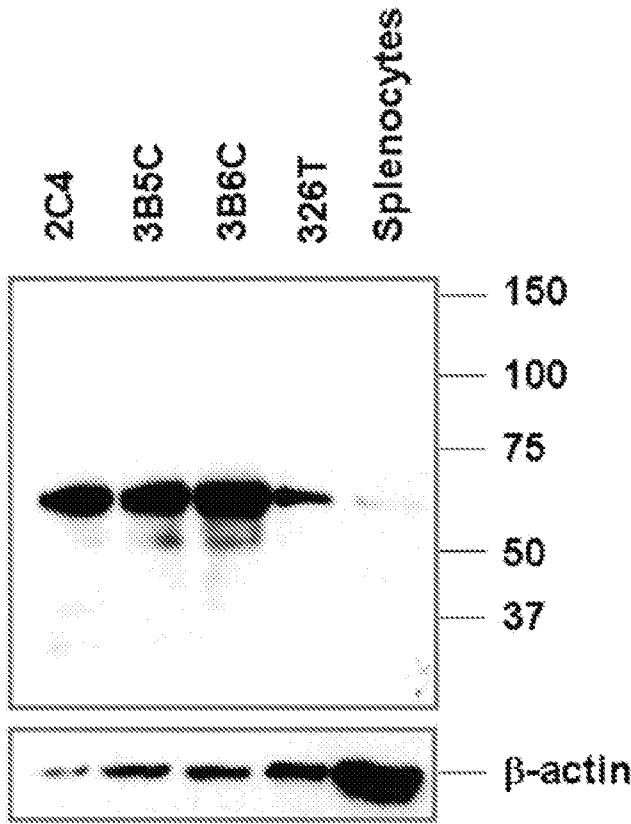


Figure 4A

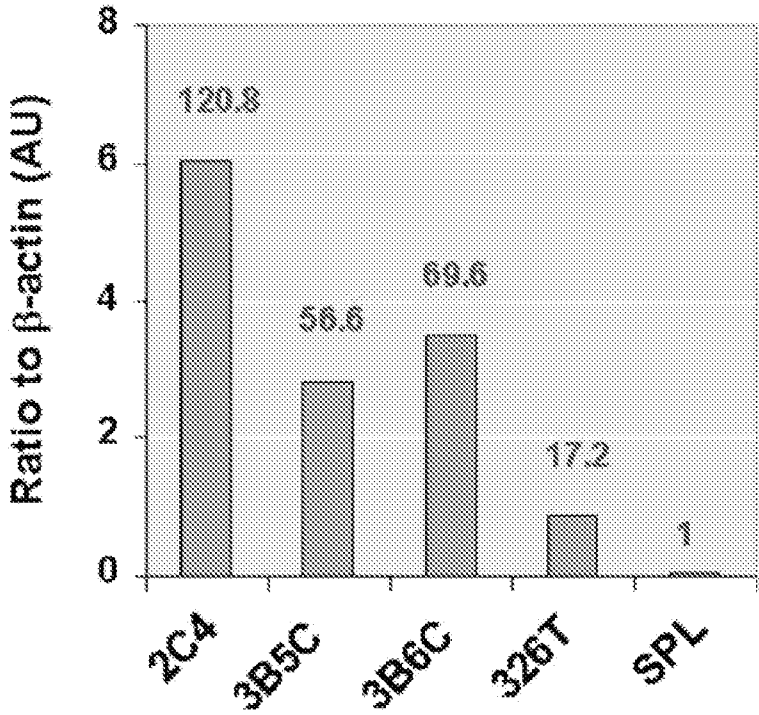


Figure 4B

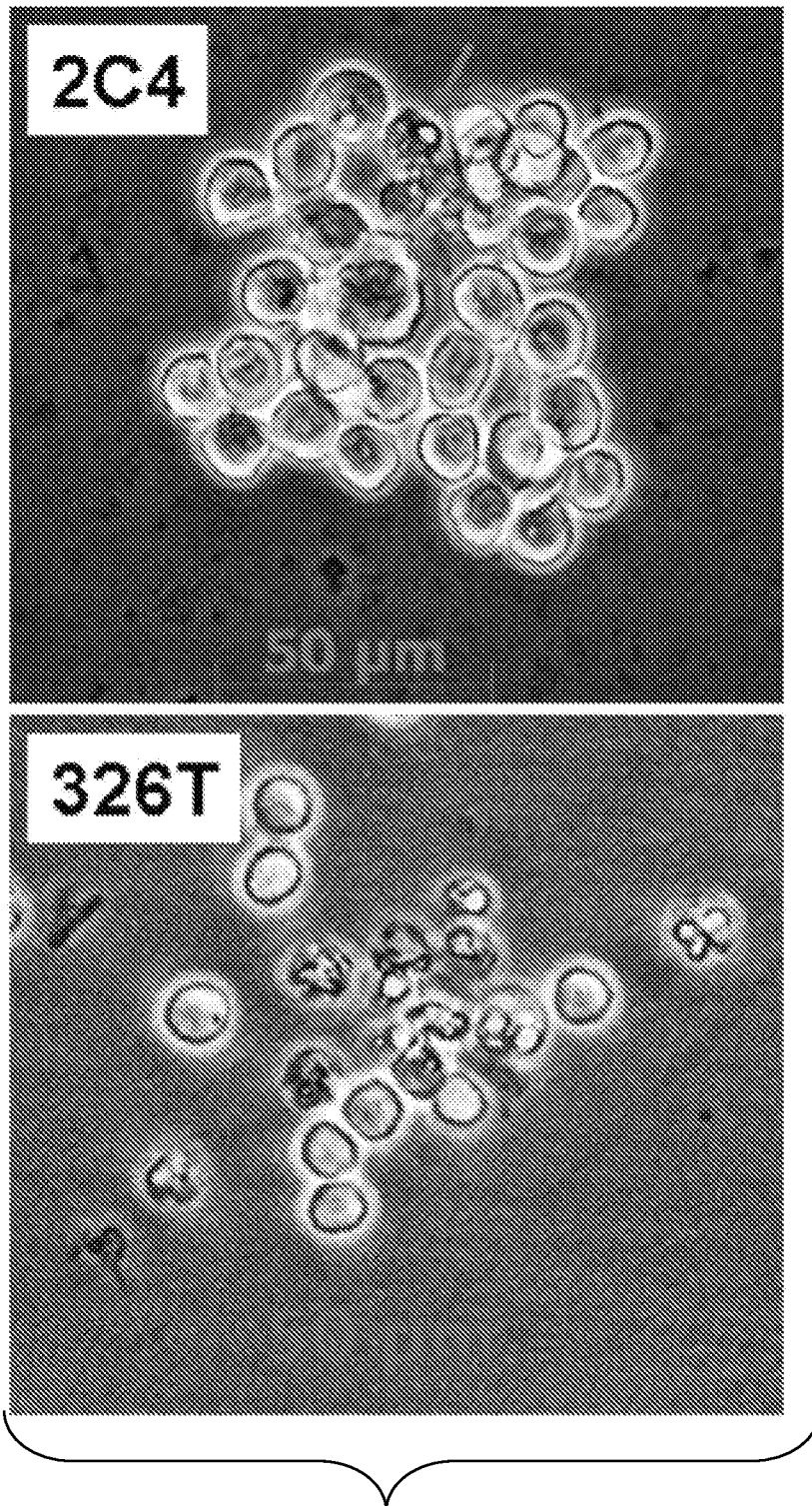


Figure 4C

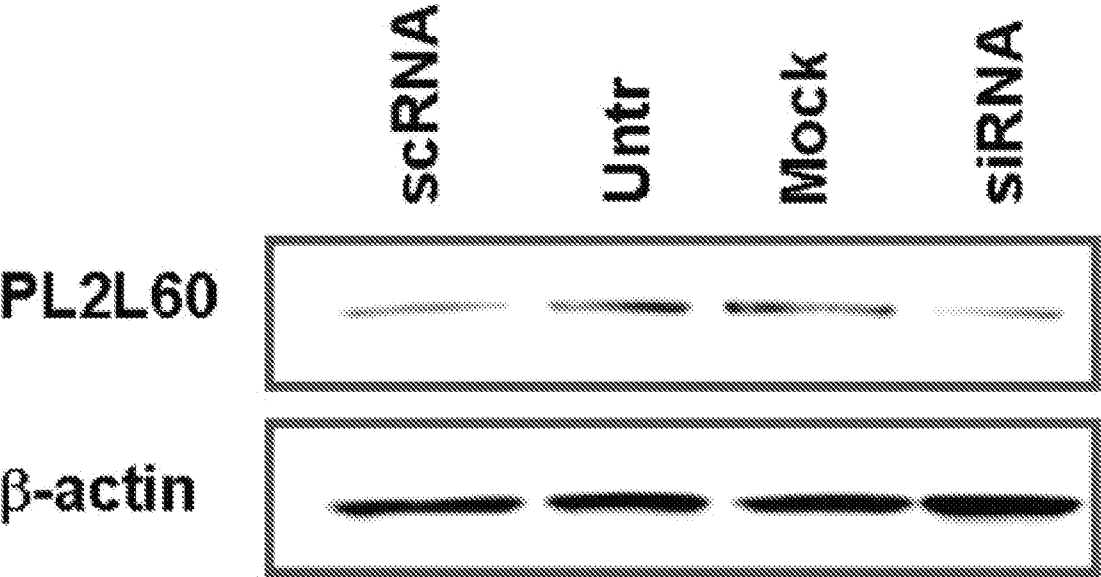


Figure 5A

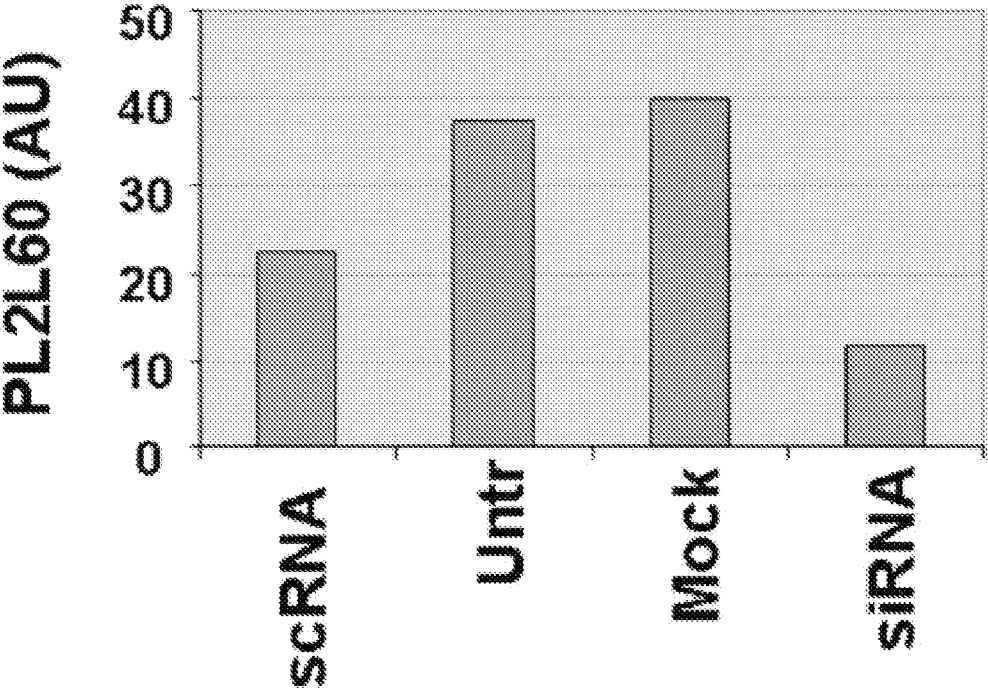


Figure 5B

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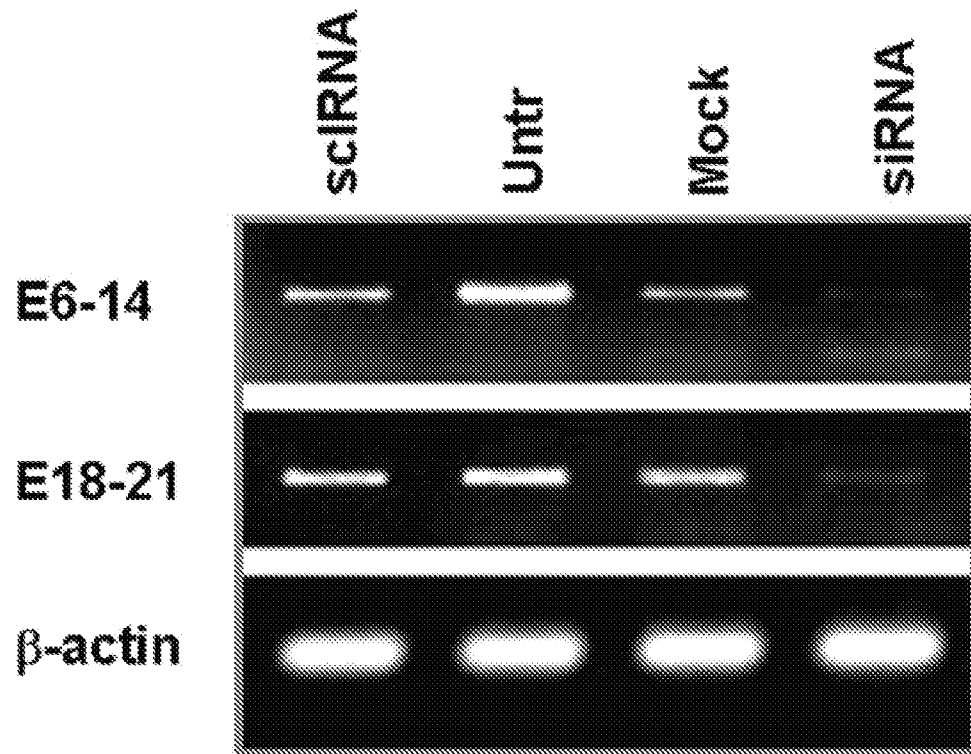


Figure 5C

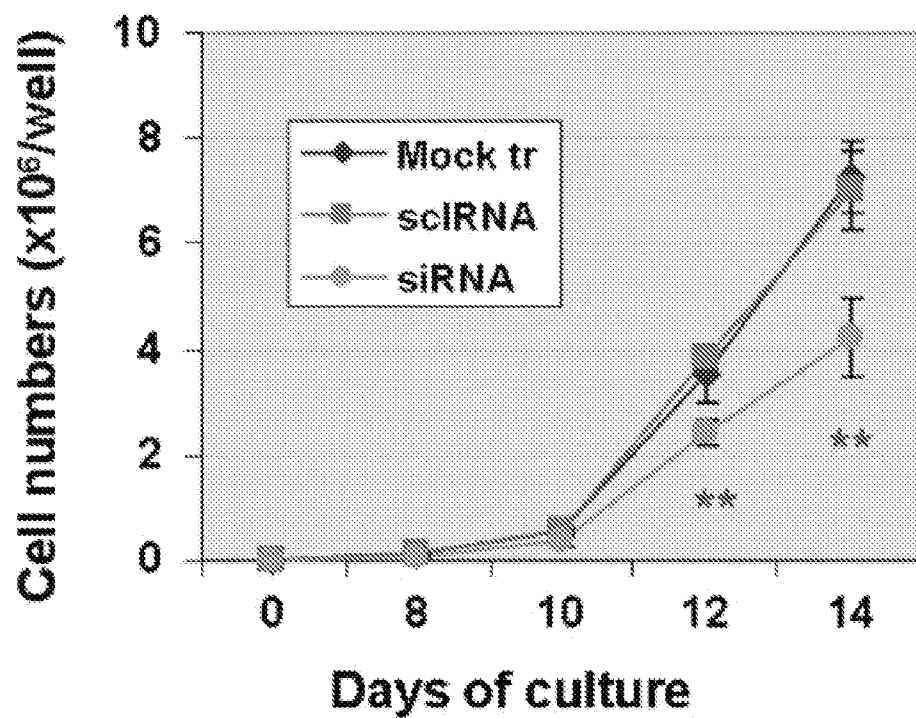


Figure 5D

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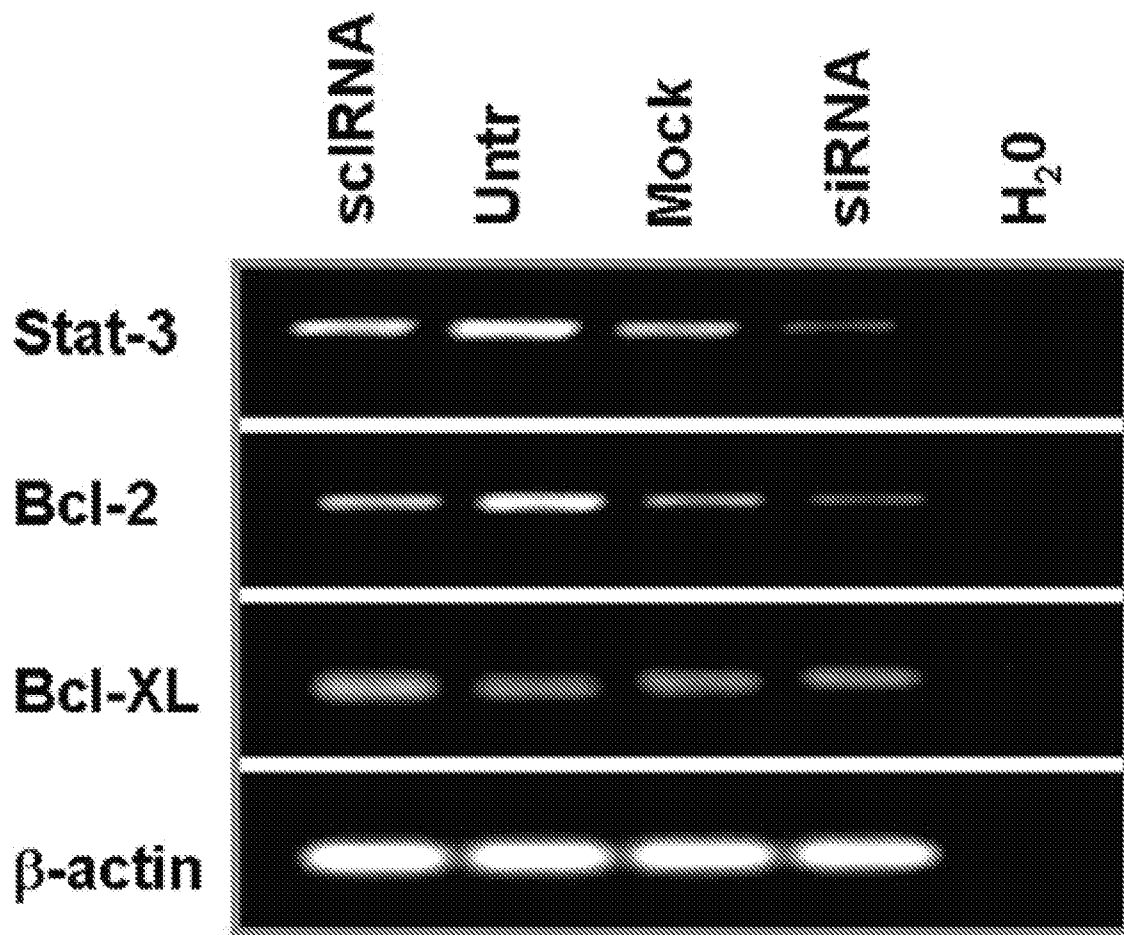


Figure 5E

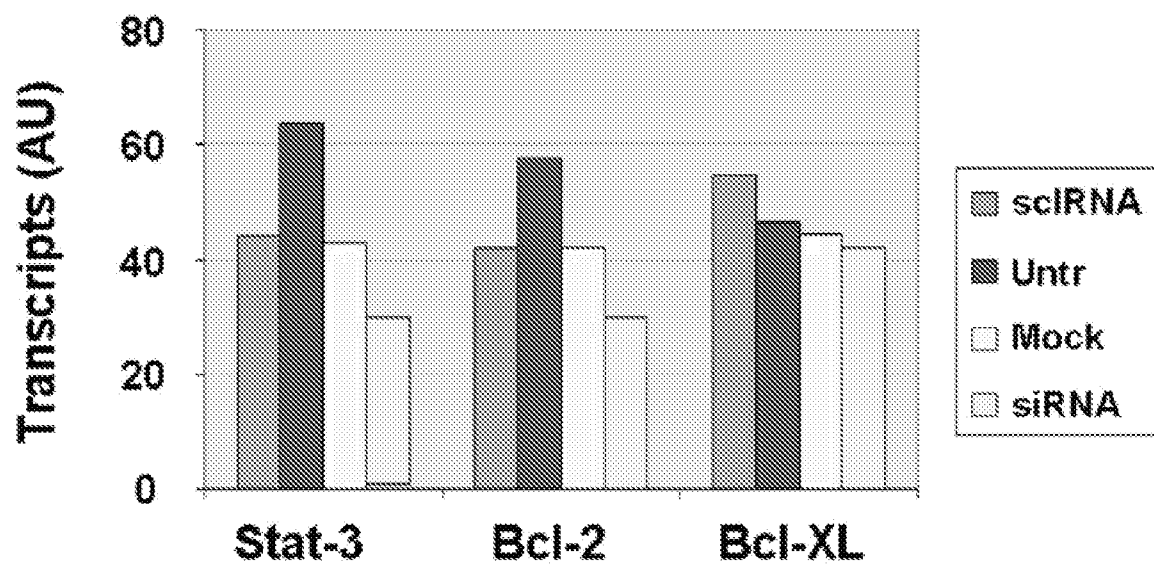


Figure 5F

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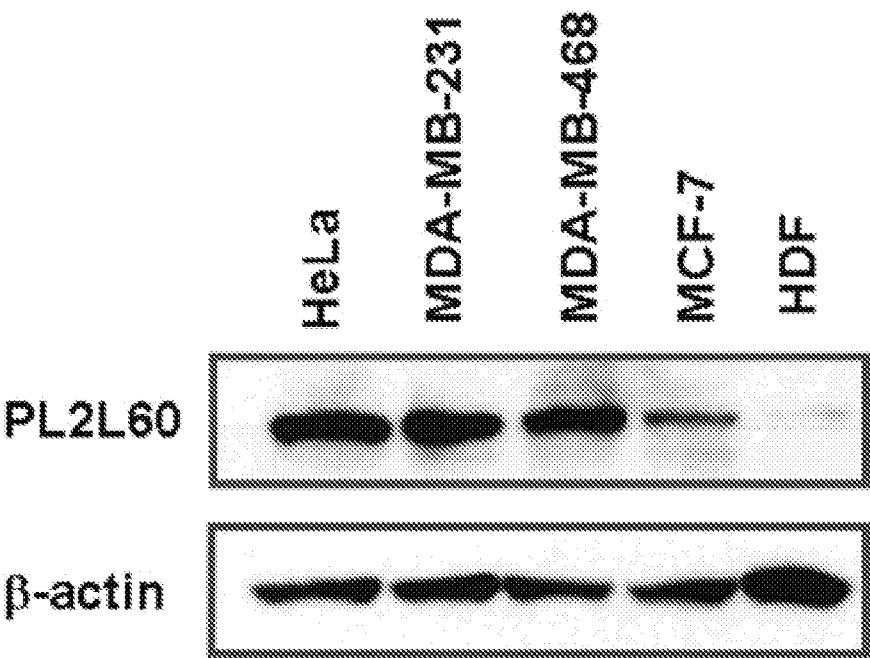


Figure 6A

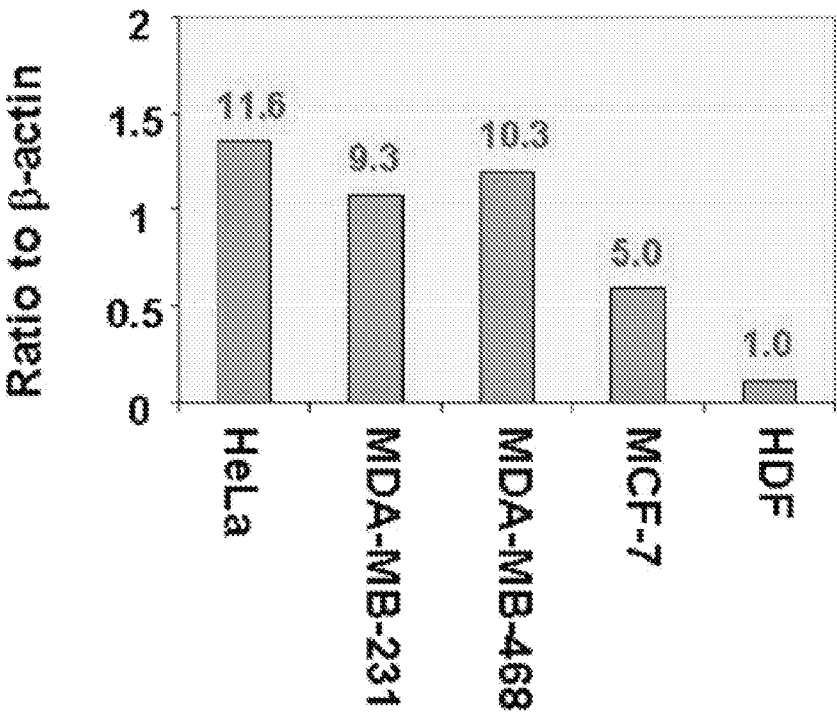


Figure 6B

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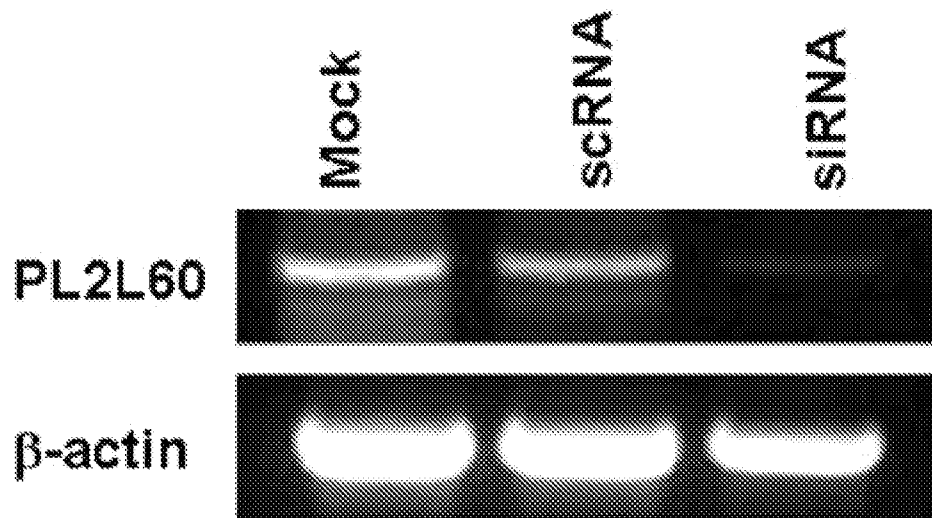


Figure 6C

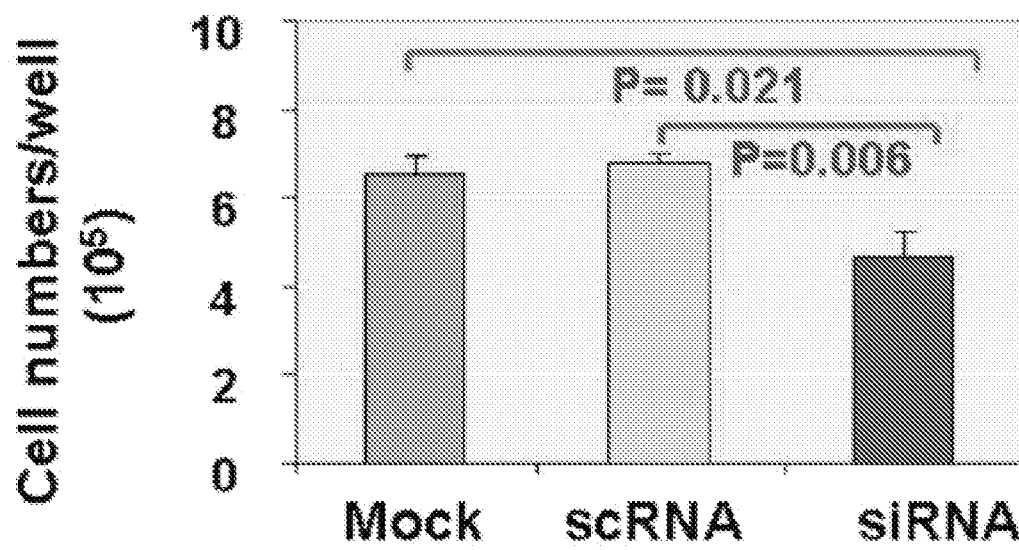


Figure 6D

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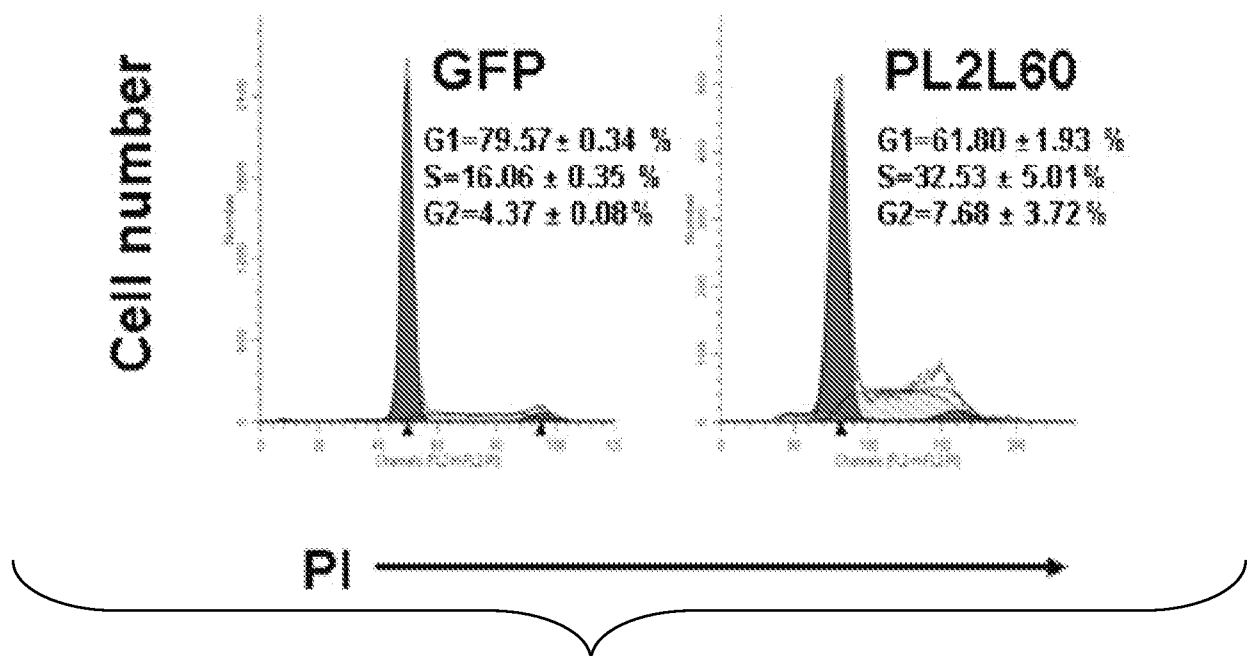


Figure 7A

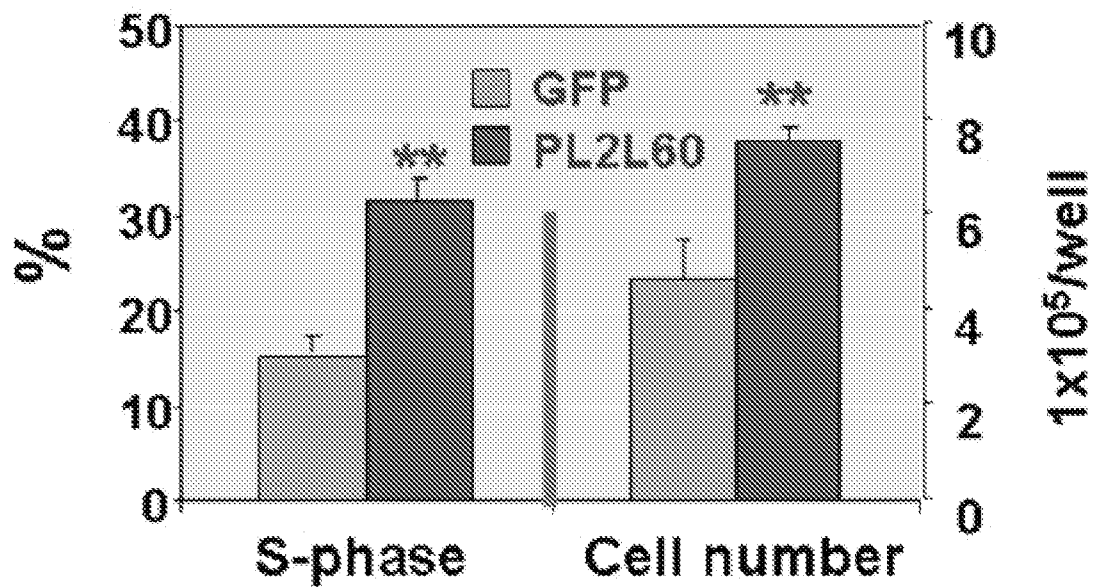


Figure 7B

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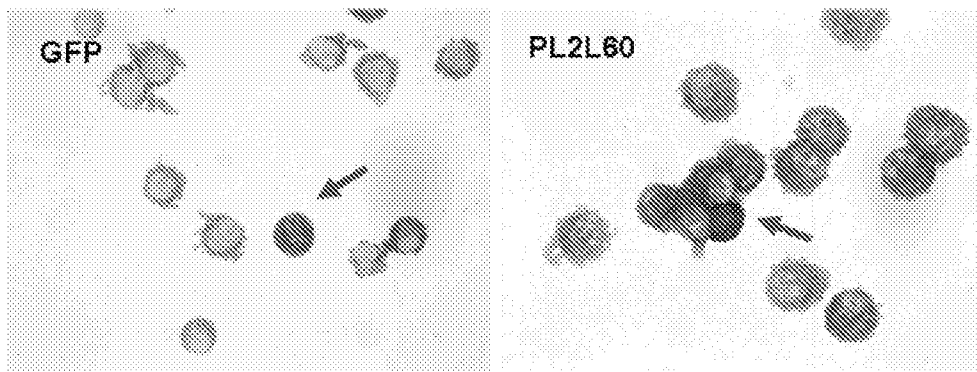


Figure 7C

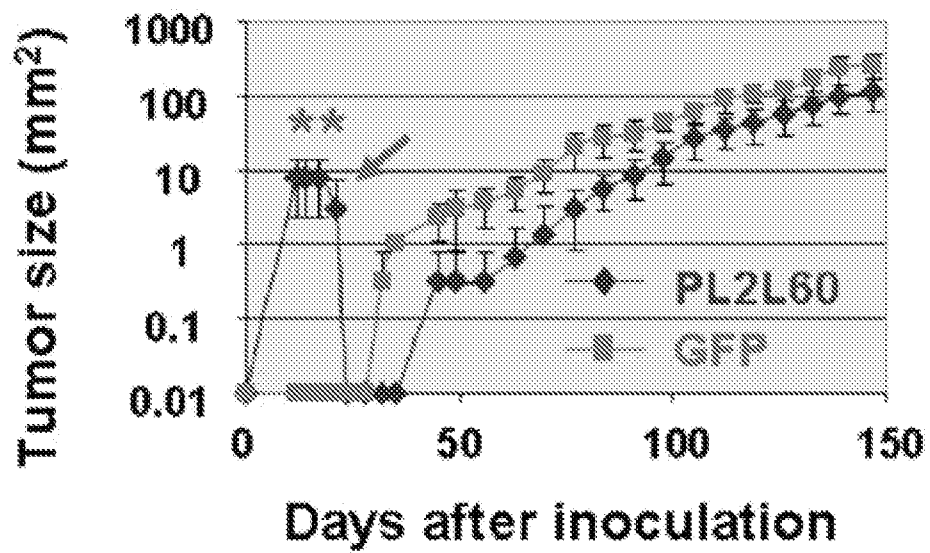


Figure 7D

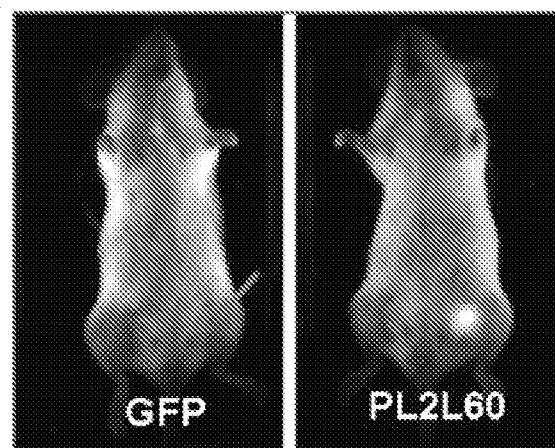


Figure 7E

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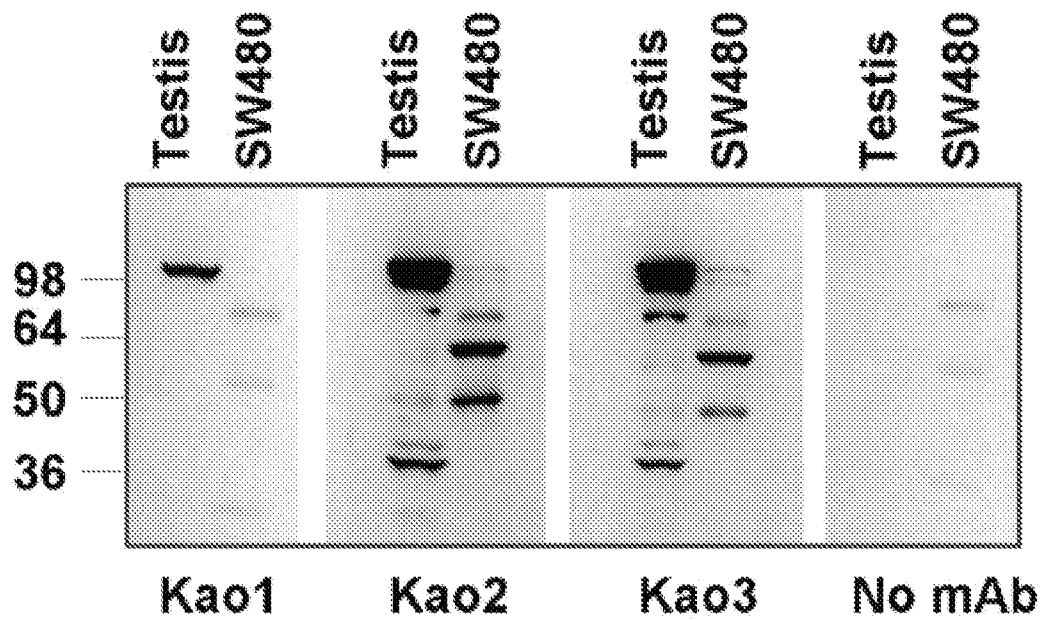
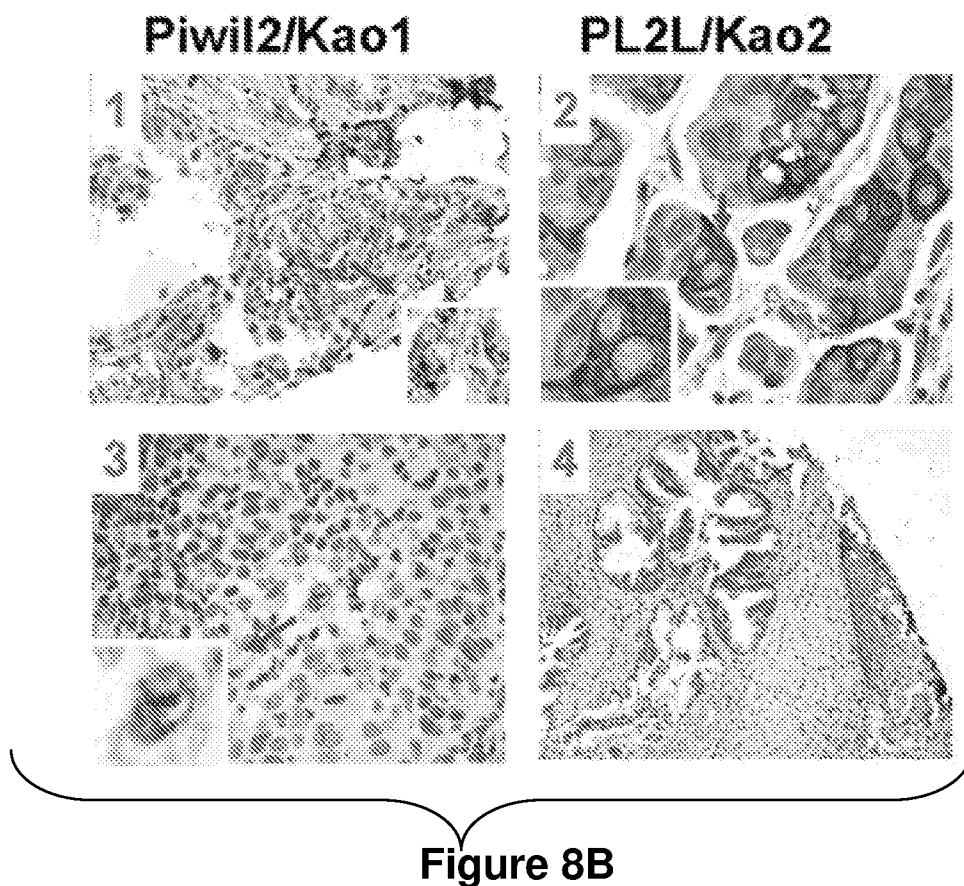


Figure 8A



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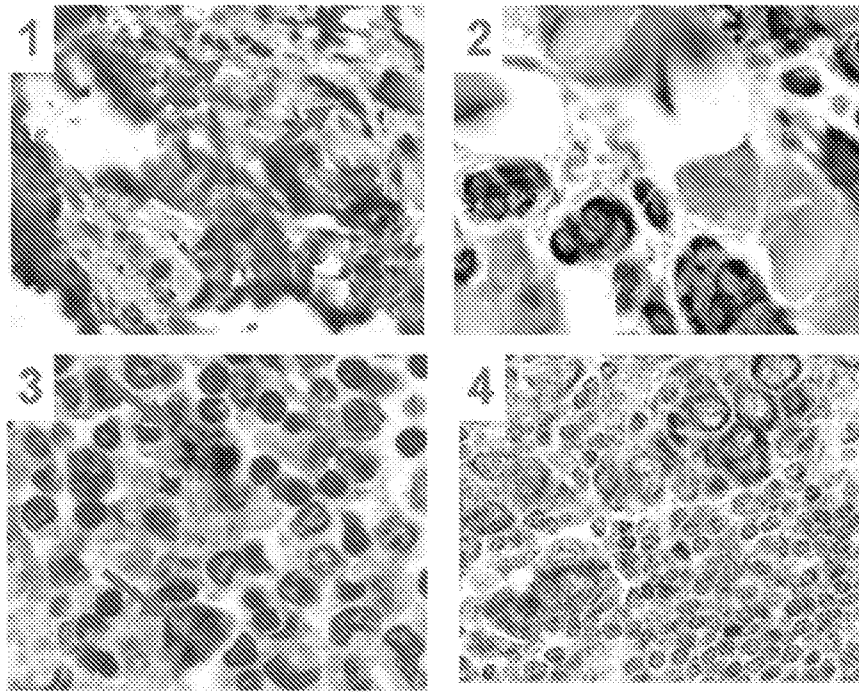


Figure 8C

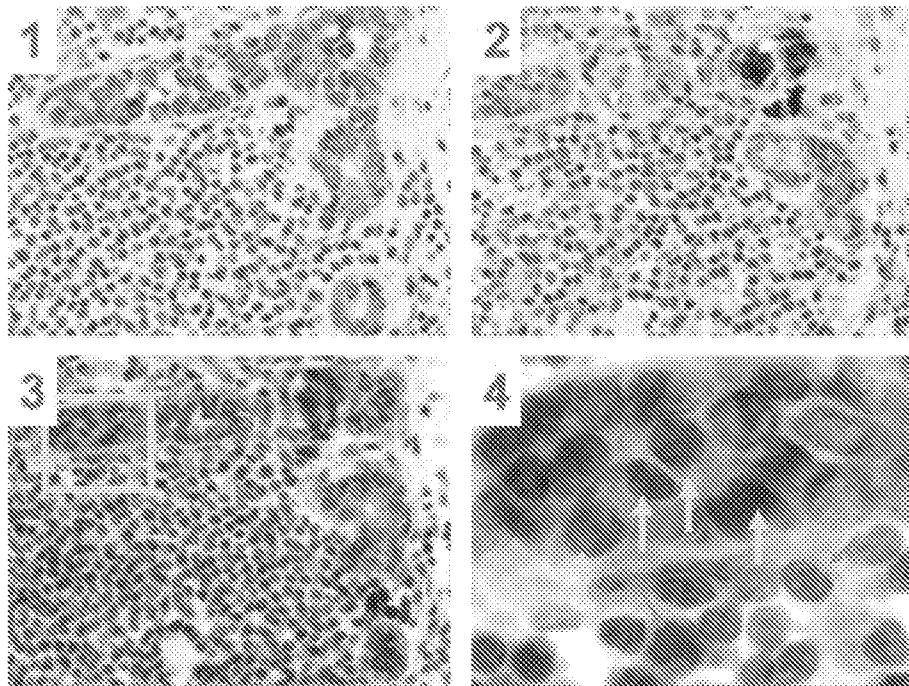


Figure 8D

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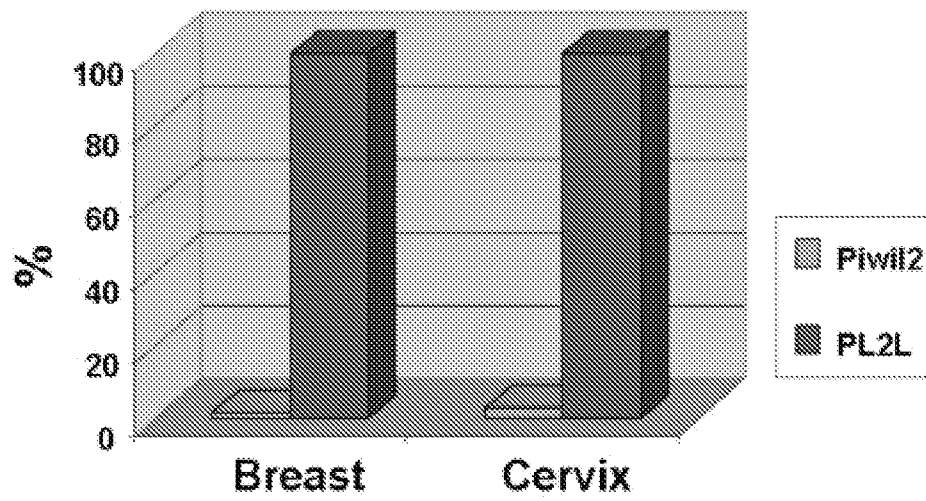


Figure 8E

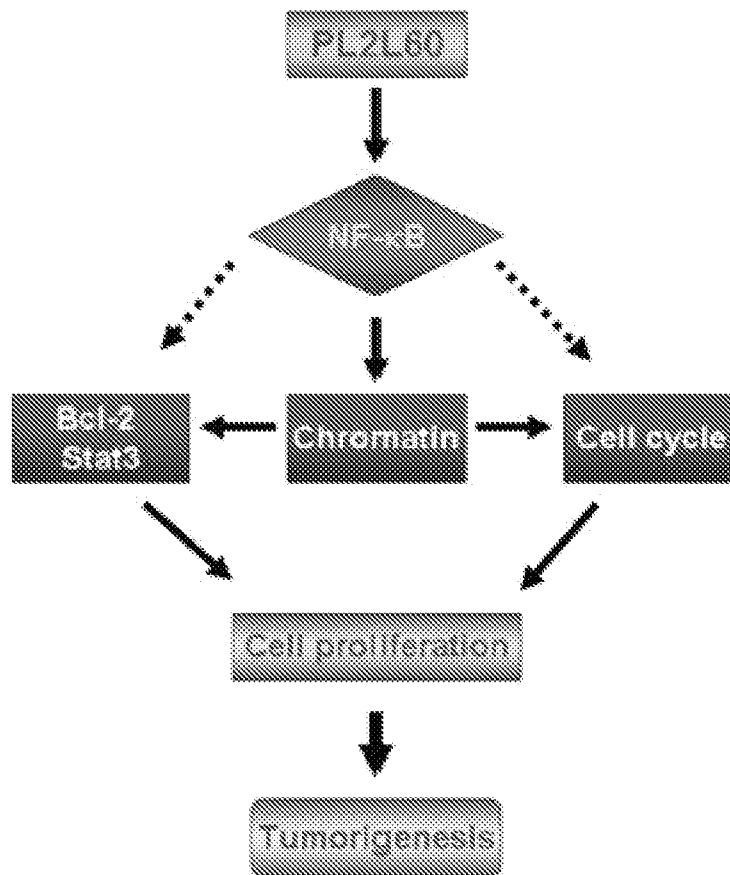


Figure 9

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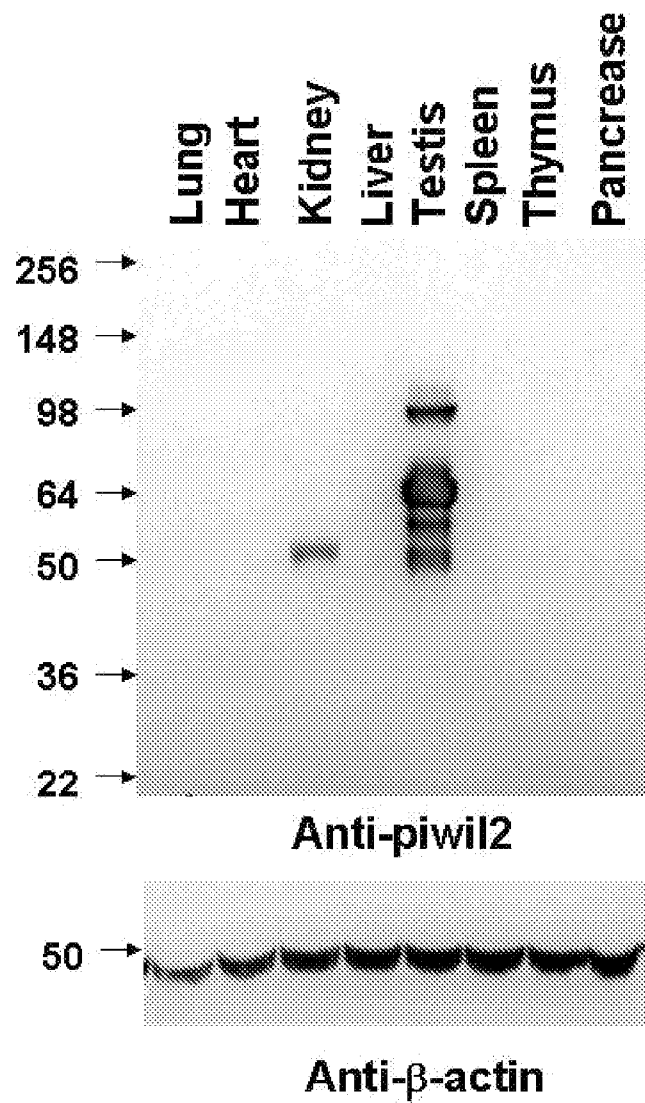


Figure 10A

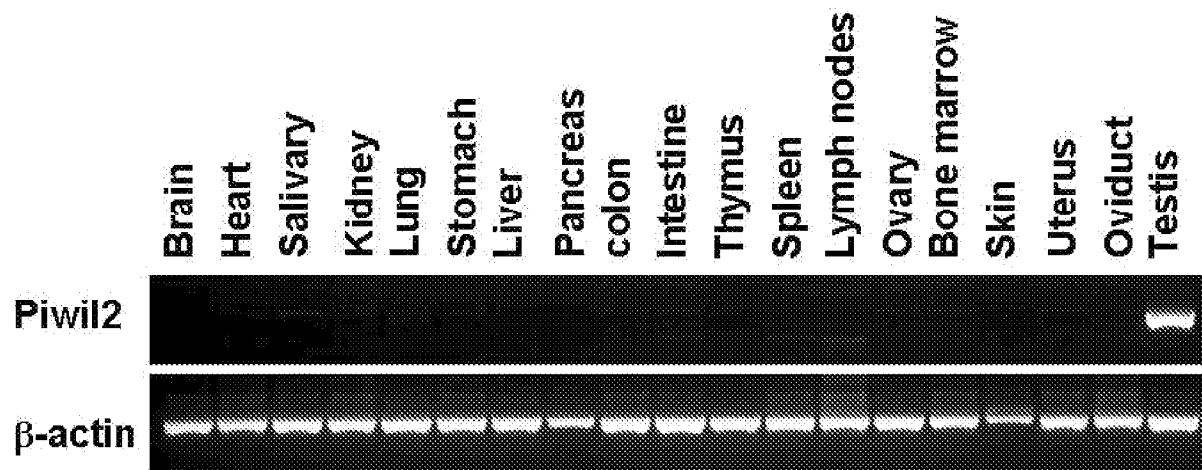


Figure 10B

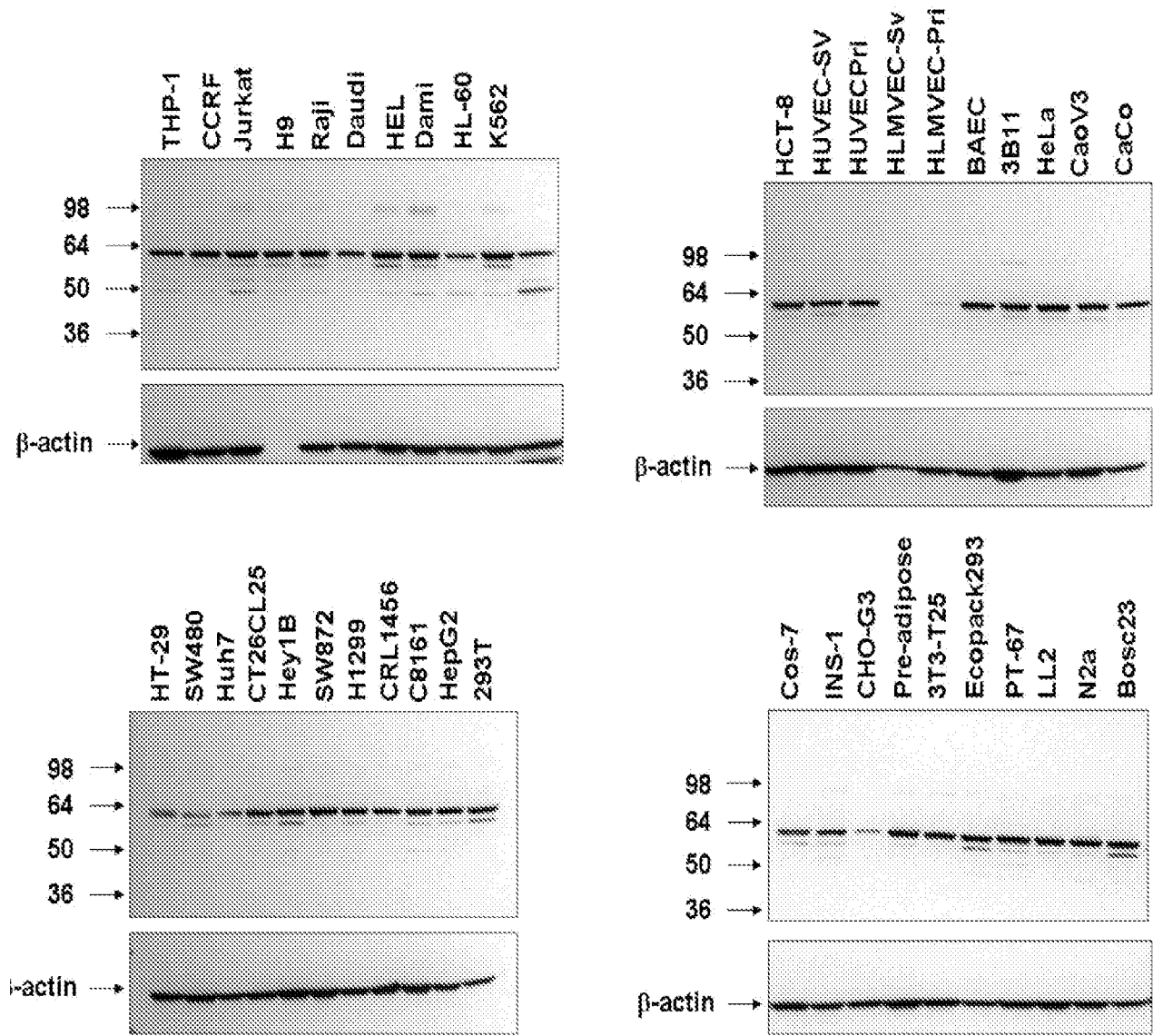


Figure 11

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Score = 1791 bits (4639), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 856/973 (87%), Positives = 905/973 (93%), Gaps = 2/973 (0%)

Human	1	MDFFRPSFRGQSPFHFSQCCQAVMFGCWPQASKFLDFALGRGAFAGRGRSVFSGKFEFPSTQ	60
Mouse	1	MDP RP FRG +P+HPSQC VRMPGCHPQA +PL+PA GR PARG VF KPE+ S	
Human	61	MDFFVRFLFRGPTFVHF3QC--VFMFGCWPQAPRFLEFANGRAGPAGRGLVTRKPEDSFP	58
Human	61	EGFAGRESVGLVSMFRGLGIETVSKTFLKREMLPSSRGILGRGLSANLVKRDREELSFTF	120
Mouse	59	F Q++SVGLVSMFRG+G++T + F KRE+ F GRG+LGRGLSAN+VRKDREE +	
Human	121	LQFVQKDSVGLVSMFRGMELDTAFRFPKKEVFPFLRGVYLGRLSANNVRKDRREEFRSGL	118
Human	121	WDFKVLAAQDSKMAETSVGWRSLRGSSDASLLFLGRRAAGGISREVDKFFCTFTSTPSRG	180
Mouse	119	DF VLAAGDSK+AE SVGWR LRGSS+ SLLFLGRRA I R +DRFF F +R	
Human	181	EDFVLAAGDSKLAEEASVGMWRMLRGSSSEVLLFLGRRAASSIGRGMKFFFAFOLTARD	178
Human	181	FFQLSSFFALPQSPLNSPDRPLVLTVEHKEKELIVQGSGKSTPQSLGLNLVXIQCNEAV	240
Mouse	179	FP+L FPAL + LHS D F VLT+E KEKEL+VRQSGKSTPQSLGLNL+XIQCNEAV	
Human	241	FFRLQFPFALSFTSLNSADFFVLTMERKEKELLVQGSGKSTPQSLGLNLIXIQCNEAV	238
Human	241	YQYHVTTFSPWVECKSMRFGMLKDHQAVTGNVTAFDGGSILYLFVKLQQVLELSQRKTDSA	300
Mouse	239	YQYHVTTFSP+VECKSMRFGMLKDHQ-VTGNVTAFDGGSILYLFVKLQQV+ELLSQRKT A	
Human	301	YQYHVTTFSPSVECKSMRFGMLKDHQSVTGNVTAFDGGSILYLFVKLQQVLELSQRKTDSA	298
Human	301	EISIKIQKTRILEPCSDLCIPFYVTVFRSVMKLLDMKLVGNFYDPTSAMVYQQHRLQIW	360
Mouse	299	EISIKIQ+TKILEPCSDLCIPFYVTVFRSVMKLLDMKLVGNFYDPTSAMVYQQHRLQIW	
Human	361	EISIKIQLTKILEPCSDLCIPFYVTVFRSVMKLLDMKLVGNFYDPTSAMVYQQHRLQIW	358
Human	361	FGYAASTRTDGGFLFLLADVSHKVIENDCVLDVMHAIYQQNKENPQDECTELLVGNVIT	420
Mouse	359	FGYAASTRTDGGFLFLLADVSHKVIEND VLDVMHAIYQQNKENPQDEC+KLLVG+IVIT	
Human	421	FGYAASTRTDGGFLFLLADVSHKVIENDSVLDVMHAIYQQNKENPQDECSKLLVGSIVIT	418
Human	421	RYNNRTYRIDVDWNSKTFPDSFTMSUGKEITFLEYYSKNYGITVKEEDQPLLIRPFSRQ	480
Mouse	419	RYNNRTYRIDVDWNSKTFPDSF MSUGKEITFLEYYSKNYGITVKE+DQPLLIRPFSRQ	
Human	481	RYNNRTYRIDVDWNSKTFPDSFVMSUGKEITFLEYYSKNYGITVKEEDQPLLIRPFSRQ	478
Human	481	NNHGMLLAGEITLLFELSPMTGIPFESKDKDFRAMEDLTQQINLSFQGHGALECLLQRIA	540
Mouse	479	+NNHGMLLAGEITLLFELSPMTGIPFESKDKDFRAMEL QQINLSFQGH GALECLLQRI+	
Human	541	NNHGMLLAGEITLLFELSPMTGIPFESKDKDFRAMEDLTQQINLSFQGHGALECLLQRTS	538
Human	541	KNEAATWELMHWGLAQFVVRHILGKVLMERINLANTSFTTSQELAWVVEVTRDFTILT	600
Mouse	539	+NE A+NE L KWL L EDVRIER+LMERINLANTSFTTSQELAWVVEVTRDFTILT	
Human	601	QNETASWELTWGLSLAKEDVRIERGLLWMERINLANTSFTTSQELAWVVEVTRDFTILT	598
Human	601	IPMFHWALPYPRANDQARELVMLEXIAQFPGMR+SPFPAWVELKDRITY+RTIQS L	660
Mouse	599	IPMFHWALPYPRANDQARELVMLEXIAQFPGMRISPFPAWVELKDRITYRTIQSLL	658
Human	661	CAEGRIGWVYCIHMGFRDLYGAIKKLCCVQSPVPSQVYVVTIGQPTLASVAQKILLQ	720
Mouse	659	G EGRIGWVYCIHMG FRDLYGAIKKLCCVQSPVPSQV+SVHTIGQPTLASVAQKILLQ	
Human	721	GVEGRIGWVYCIHMTSDLYGAIKKLCCVQSPVPSQVINVTIGQPTLASVAQKILLQ	718
Human	721	INCKLOGELWGVDFIPLAQLMVIGWYTHDPFRGMR+SVVGFVASINLTLETWYSRVVFQMP	780
Mouse	719	+NCKLOGELWGVDFIPLAQLMVIGWYTHDPFRGMR+SVVGFVASINLTLETWYSRVVFQMP	
Human	781	MNCKLOGELWGVDFIPLAQLMVIGWYTHDPFRGMR+SVVGFVASINLTLETWYSRVVFQMP	778
Human	781	HGEIVDSLKLCLVGSLEKXYEVHCLPEKIVVYRDSVSDGQLATVANYEIPQLQKCFEAF	840
Mouse	779	HGEIVDSLKLCLVGSLEK+YEVHCLPEKIVVYRDSVSDGQLATVANYEIPQLQKCFEAF	
Human	841	HGEIVDSLKLCLVGSLEKXYEVHCLPEKIVVYRDSVSDGQLATVANYEIPQLQKCFEAF	838
Human	841	ENYQPMWVTVVQKXISTNLXLAAPQNFVYTPSTTVVDMTITSCEWVDFYLLAHNVQGC	900
Mouse	839	+NY PMWVTVVQKXISTNLXLAAP +FVTP+PSTTVVDMTITSCEWVDFYLLAHNVQGC	
Human	901	DNYPMWVTVVQKXISTNLXLAAPNFVYTPSPSTTVVDMTITSCEWVDFYLLAHNVQGC	898
Human	901	GIFTHTYCVLNTANLSFDMQALTFKLCHMNMWPGTIRVPAPCKYANKLAFLSGILHH	960
Mouse	899	GIFTHTY+CVLNTANLSFDMQALTFKLCHMNMWPGTIRVPAPCKYANKLAFLSG ILHH	
Human	961	GIFTHTYCVLNTANLSFDMQALTFKLCHMNMWPGTIRVPAPCKYANKLAFLSGQILHH	958
Human	961	EPAIQLCENLFFL 973	
Mouse	959	EPAIQLC NLFPL	
Mouse	959	EPAIQLCGNLFFL 971	

Figure 12A

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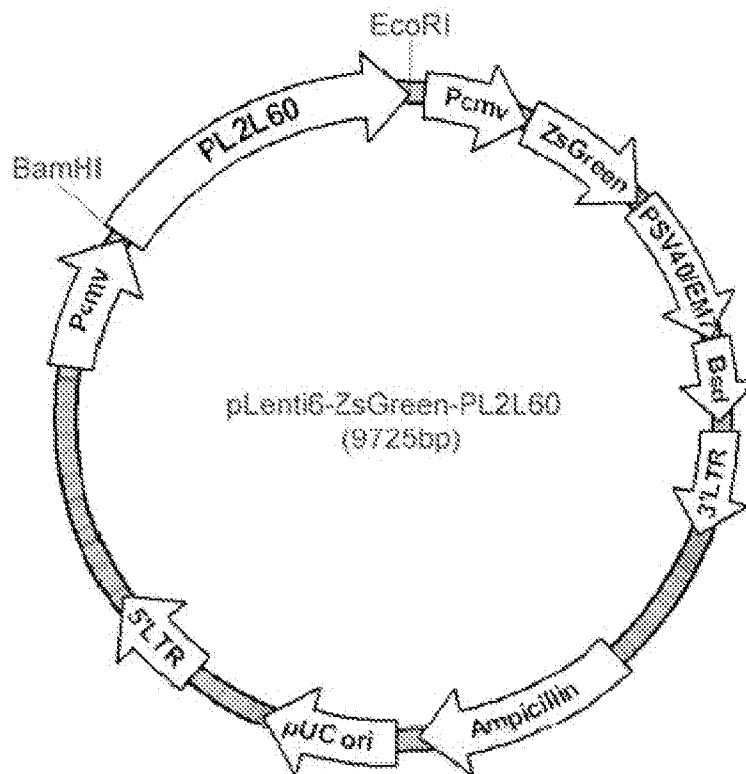


Figure 12B

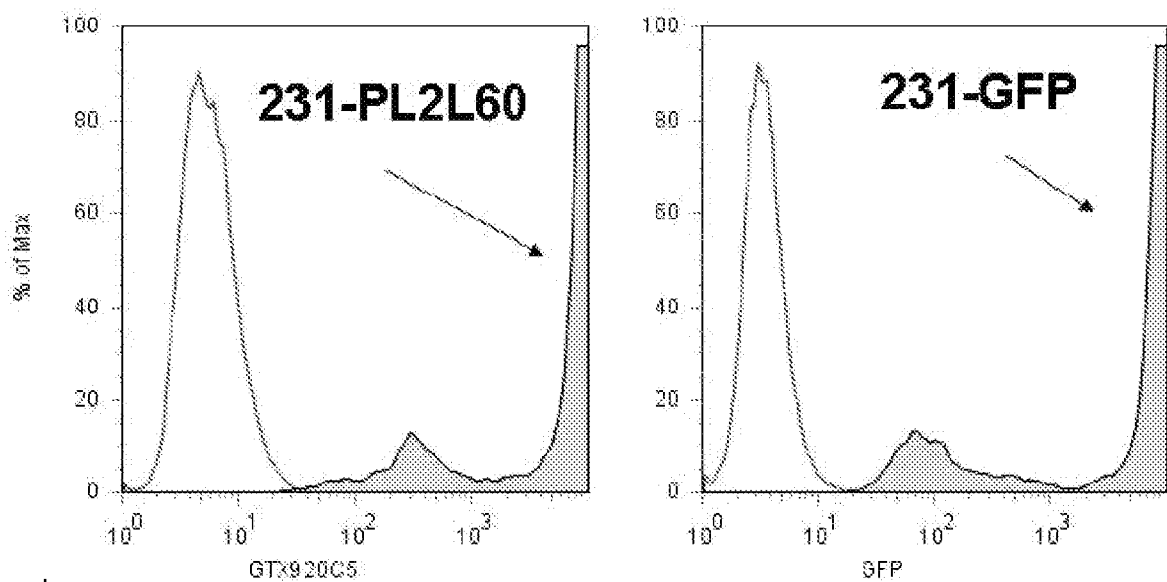


Figure 12C

In-house primer ID	GenBank ref. No.	Primer sequence	Size (bp)	exon location
p593-Stat3-For	NM_213659	5'-AGAGAAGCAGCAGATGTTGGAGCA-3'	148	5
p594-Stat3-Rev		5'-ATCCTGCATGTCTCCTTGGCTCTT-3'		6/7
p603-Bcl2-For	NM_009741	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'	185	2
p604-Bcl2-For		5'-AATCAAACAGAGGTCGCATGCTGG-3'		3
p609-Bcl-XL-For	NM_009743	5'-ACTGTGCGTGGAAAGCGTAGACAA-3'	152	2
p610-Bcl-XL-Rev		5'-CTGCATTGTTCCCGTAGAGATCCACA-3'		3
p547- β -actin-For	NM_007393	5'-TGAACCCCTAAGGCCAACCGTGAAA-3'	136	3
p548- β -actin-Rev		5'-GAGTCCATCACAATGCCTGTGGTA-3'		4
P241-P ₁ -Mitt-For	NM_021308 or AB032605	5'-AATAGGAGGGAAAGGAGGTGGCT-3'	958	1
P242-P ₁ -Mitt-Rev		5'-TGGTGGTCCTTCAACATGCCAAAC-3'		7
P243-P ₂ -Mitt-For	NM_021308 or AB032605	5'-TCC TCA GTC TTT GGG ACT GAA CCT-3'	1015	6
P244-P ₂ -Mitt-Rev		5'-GACATCTTTATGCAGACTGAGCCC-3'		14
P245-P ₃ -Mitt-For	NM_021308 or AB032605	5'-AGATGAAGAAGGACTTCAGGGCCA-3'	1041	13
P246-P ₃ -Mitt-Rev		5'-GAACTACAAACACCACCATCTTGGG-3'		21
P247-P ₄ -Mitt-For	NM_021308 or AB032605	5'-TTGTGGTGTACCGAGATGGAGTGT-3'	873	21
P248-P ₄ -Mitt-Rev		5'-CAG CGG CTA CCT ACA AAC TTG CTT-3'		23
p289-P _{Mitt} -for	NM_021308 or AB032605	5'-TGAAGTGTAACTGGGTGGTGAGC-3'	497	18
p290-P _{Mitt} -rev		5'-CACTCACAGCTGGTTATGGTATGA-3'		21
p023-P _{H8} -For	NM_018068	5'-TTCGGAGTGTGGCCAGAAAGATTT-3'	499	18
p024-P _{H8} -Rev		5'-ACAGTTCAGGAGTGGGAGTTACA-3'		21

Figure 13

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Cancer types	Organ or tissue origin	Cell lines	Specification
Leukemia	Blood	THP-1	Human acute monocytic leukemia cell line
	Blood	CCRF	Human T cell lymphoblast-like cell line
	Blood	Jurkat	T cell leukemia
	Blood	H9	Clonal derivative of the T-cell line HUT 78
	Blood	Raji	Human Burkitt's lymphoma cell line
	Blood	Daudi	Human Burkitt's lymphoma cell line
	Blood	HEL	Human erythroleukemia cell line
	Blood	Dami	Megakaryoblastic
	Blood	HL-60	Human promyelocytic leukemia cell line
	Blood	K562	Human chronic myeloid leukemia
	Blood	PBL985	Human myeloblastic leukemia cells
Adenocarcinoma	Intestine	HCT-8	Ileocecal colorectal adenocarcinoma
	Cervix	HeLa	Human cervical adenocarcinoma
	Ovary	CaoV3	Human ovarian adenocarcinoma
	Colorectum	CaCo-2	Human epithelial colorectal adenocarcinoma cells
	Colon	HT-29	Human colon adenocarcinoma cell line
	Colon	SW480	Human colon adenocarcinoma cell line
	Breast	MDA-MB-231	Human breast adenocarcinoma
	Breast	MDA-MB-2468	Human breast adenocarcinoma
	Breast	MCF-7	Human breast adenocarcinoma
Carcinoma	Ovary	HEY1B	Human ovarian carcinoma cell line
	Lung	H1299	Human lung carcinoma
	Liver	HepG2	Human hepatocellular liver carcinoma cell line
	Lung	LL2	Lewis lung carcinoma
	Colon	CT26CL25	Murine colon carcinoma
Tumor	brain	N2a	Murine neuroblastoma cell line
Melanoma	skin	C8161	Human melanoma cell line
Insulinoma	Pancreas	INS-1	A highly differentiated rat insulinoma cell line
Hepatoma	Liver	Huh7	Human hepatoma

Figure 14A

Sarcoma	adipose	SW872	Human liposarcoma cell line
		3B11(EL)	Mouse tumor endothelial cell line
Embryonic lines	Muscle	CRL1456	G-8 mouse myoblasts isolated from fetal Swiss-Webster mouse
	Kidney	293T	Human embryonic kidney (HEK) 293T cell line
	Kidney	Ecopack293	An ecotropic HEK 293-based packaging cell line
	Kidney	Cos-7	African Green Monkey SV40-transf'd kidney fibroblast cell line
	Skin	3T3	Mouse embryonic fibroblasts
	Skin	PT-67	An NIH/3T3-based packaging cell line
	Kidney	Bosc23	Human embryonic kidney cell line
Immortalized lines	Umbilical cord	HUVEL-SV	SV-40 transformed Human umbilical vein endothelial cells (SV-40)
	Umbilical cord	HUVEL-pri	Primary human umbilical vein endothelial cells
	Lung	HLMVEC-Sv	SV40-transformed human lung microvascular endothelial cells
	Lung	HLMVEC-Pri	Primary human lung microvascular endothelial cells
	Ovary	CHO-G3	CHO cells overexpressing human GLUT3; CHO: Chinese Hamster ovary cells
	Blood vessel	BAEC	Epithelial cells derived from bovine, aorta
	White adipose	Pre-adipose	Immortalized human pre-adipose cell lines

Figure 14B

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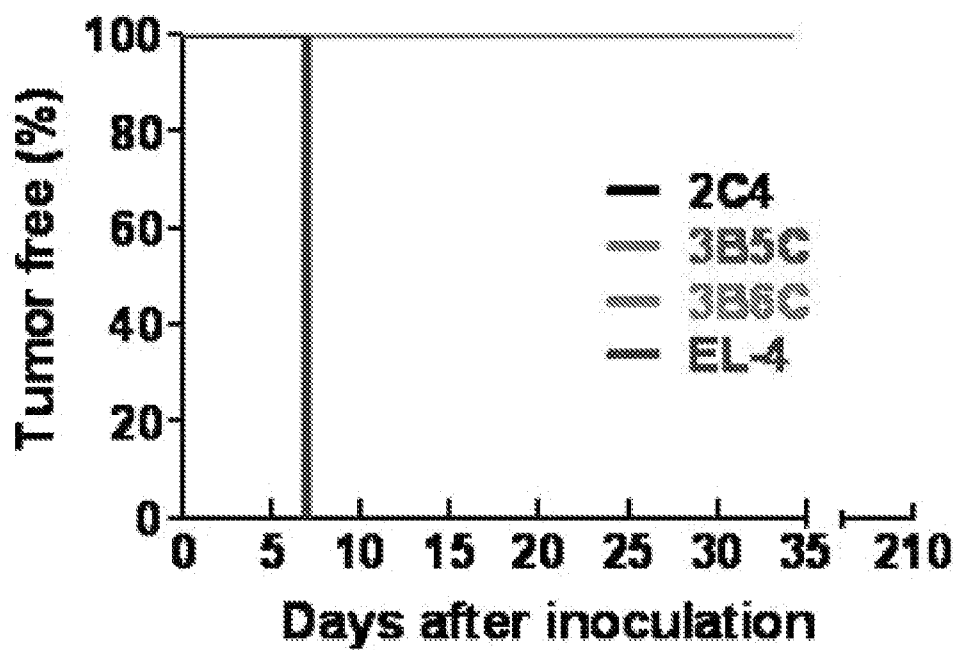


Figure 15A

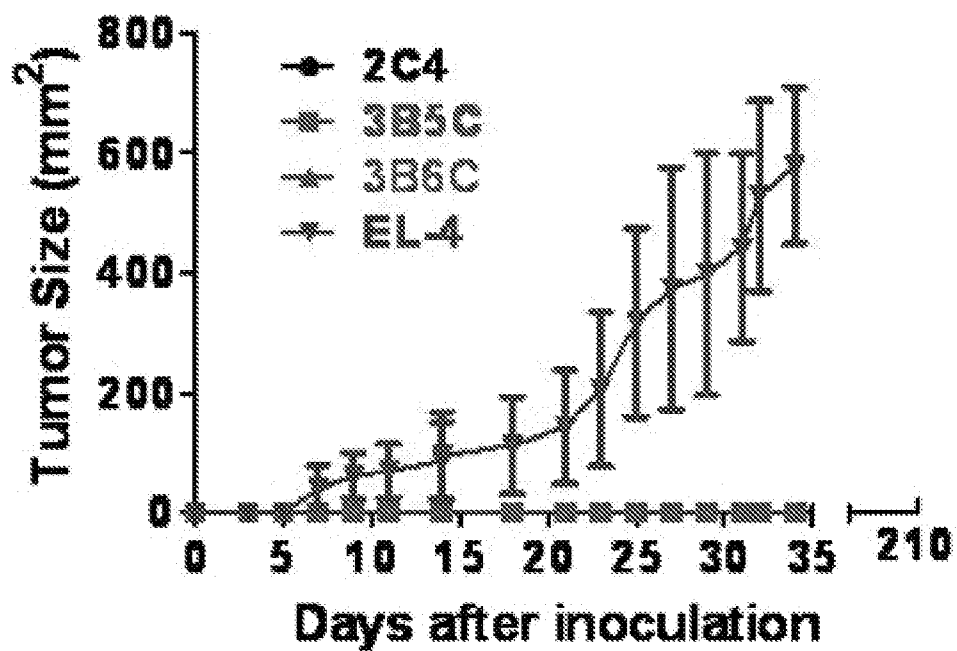


Figure 15B

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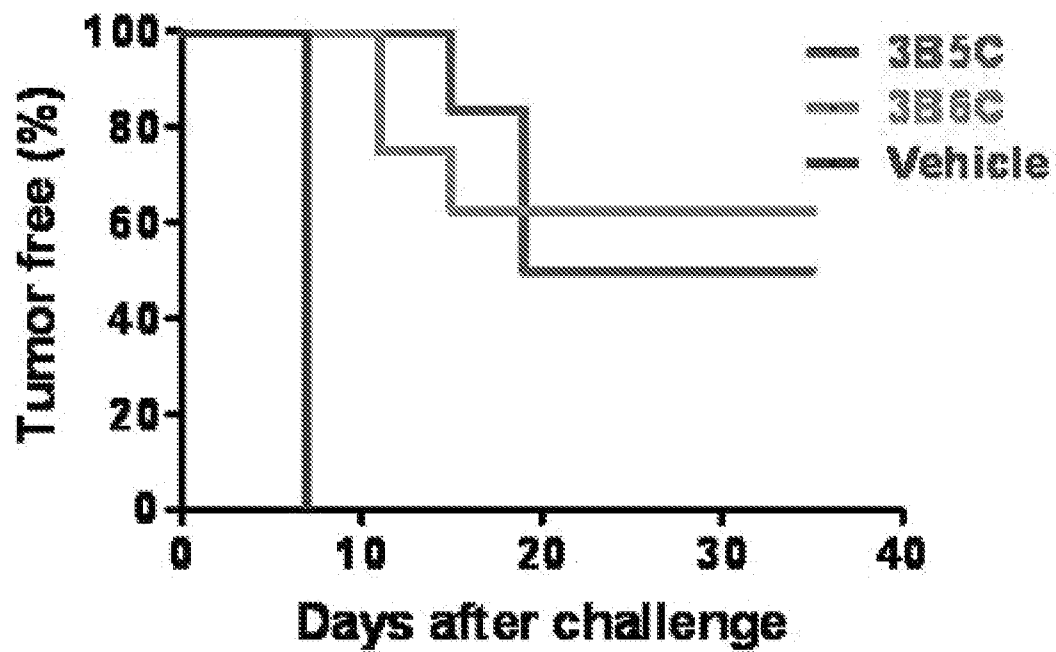


Figure 15C

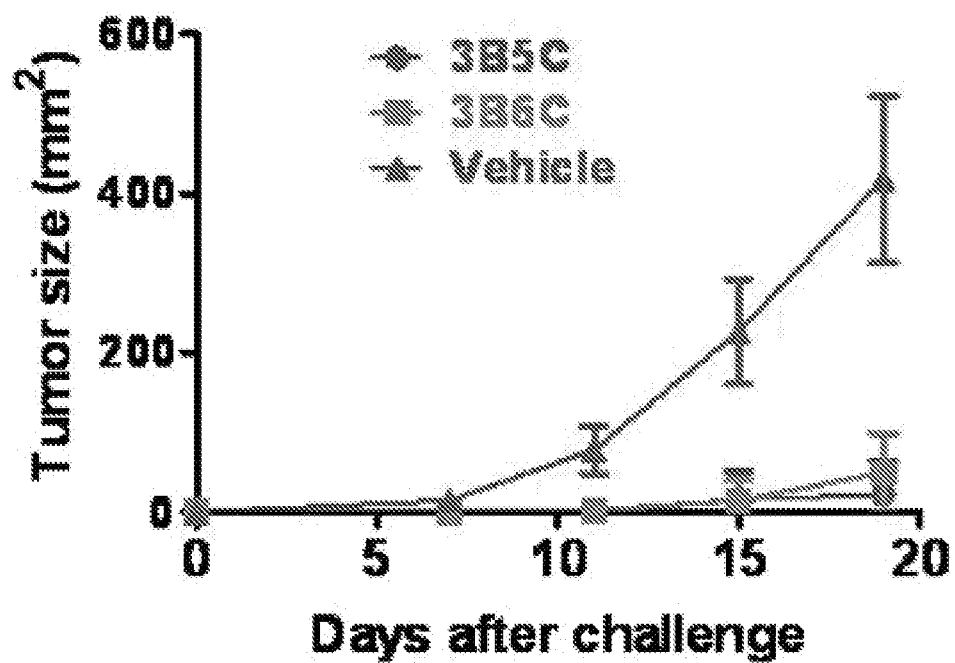


Figure 15D

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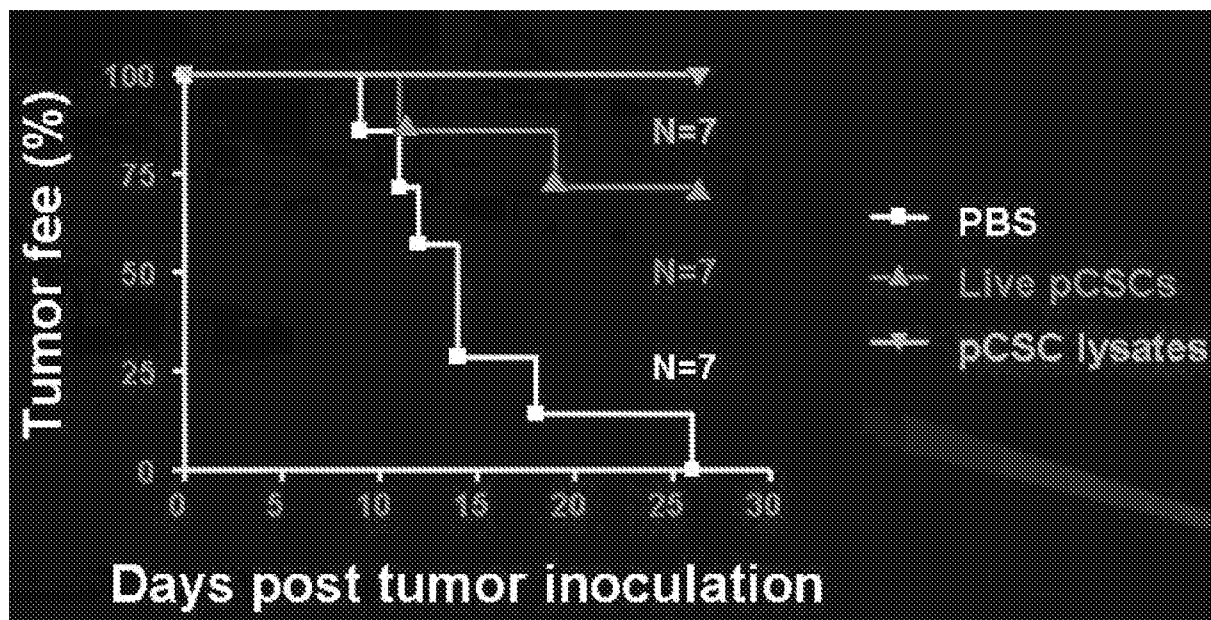


Figure 16A

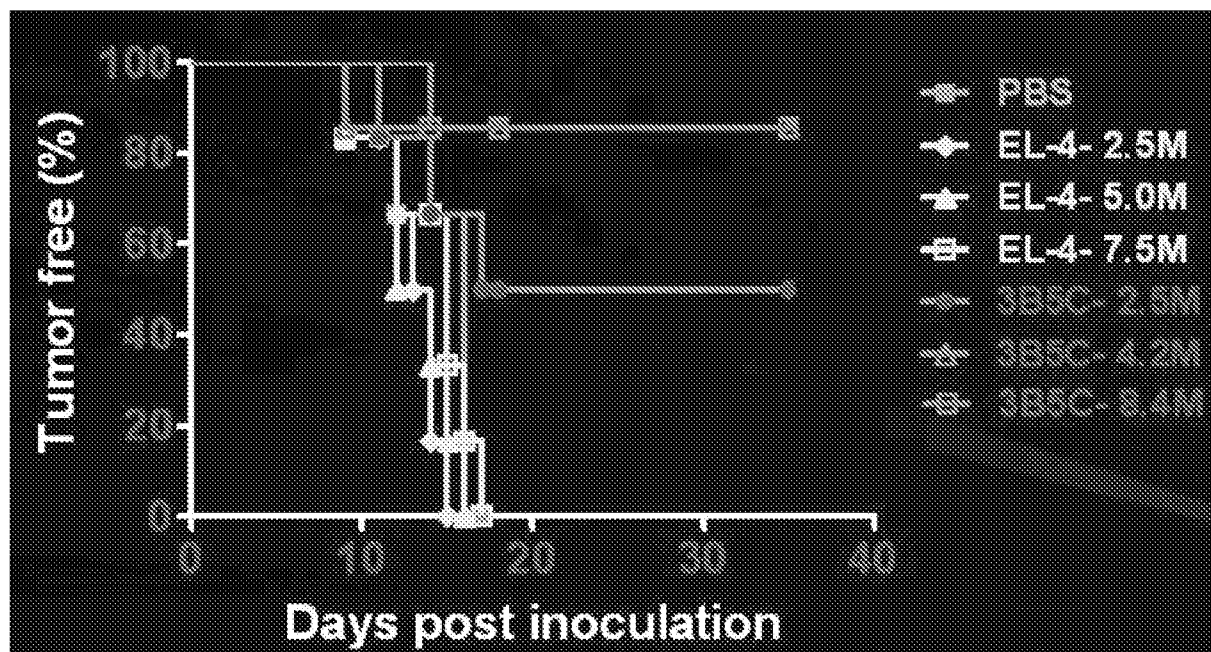


Figure 16B

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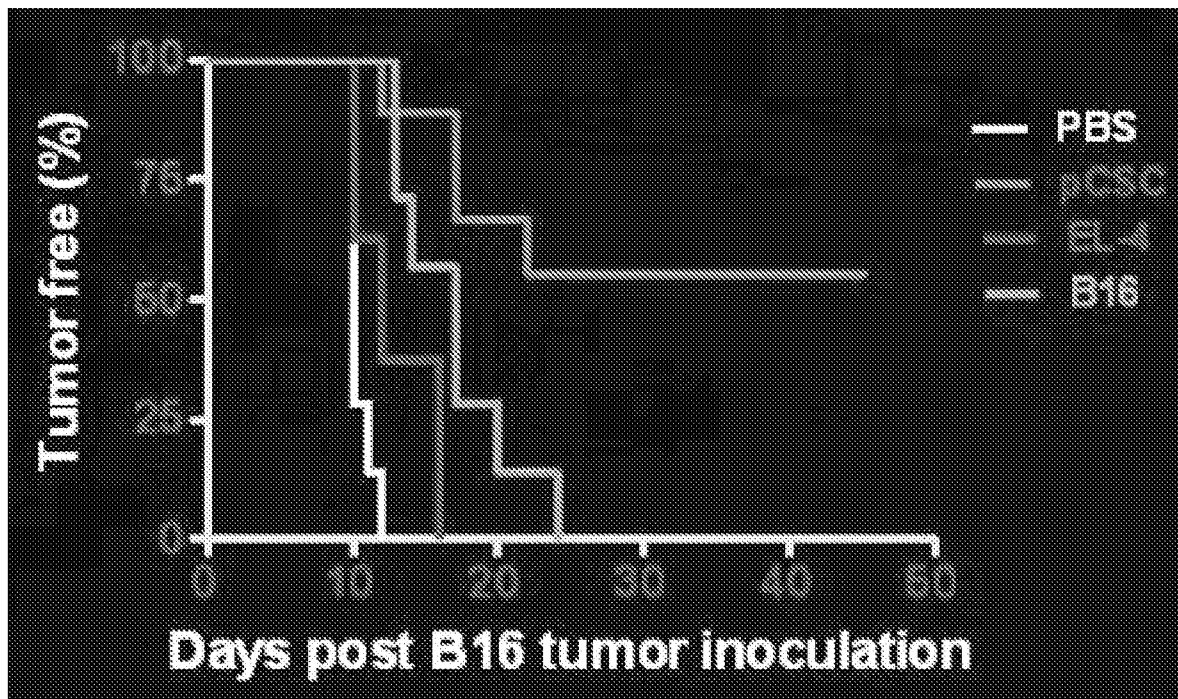


Figure 17A

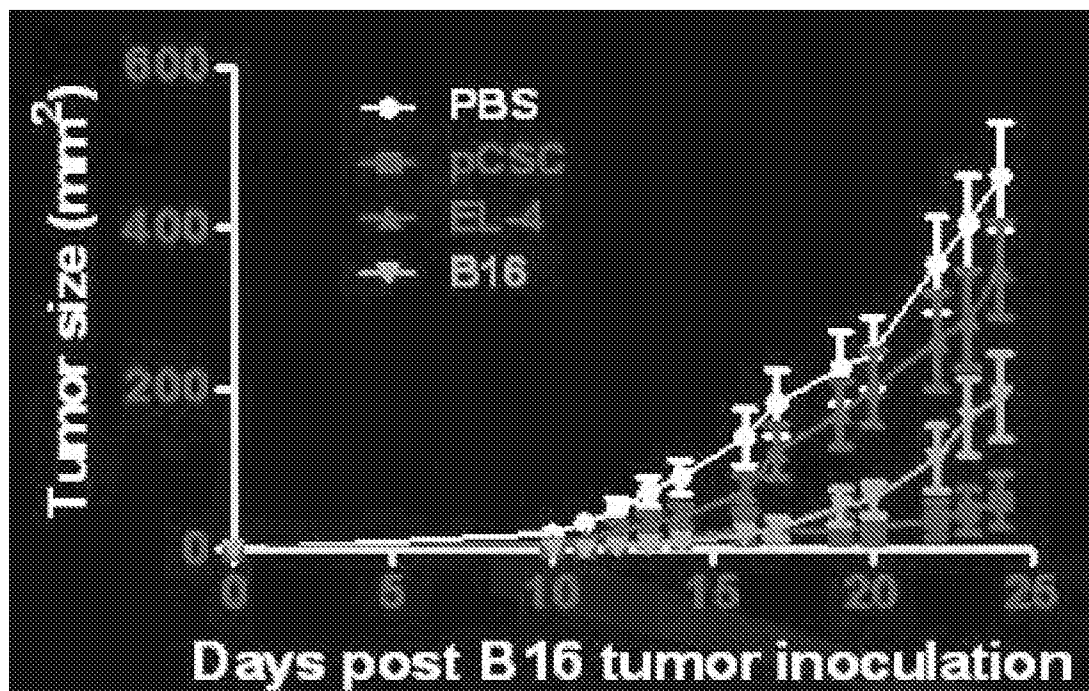


Figure 17B

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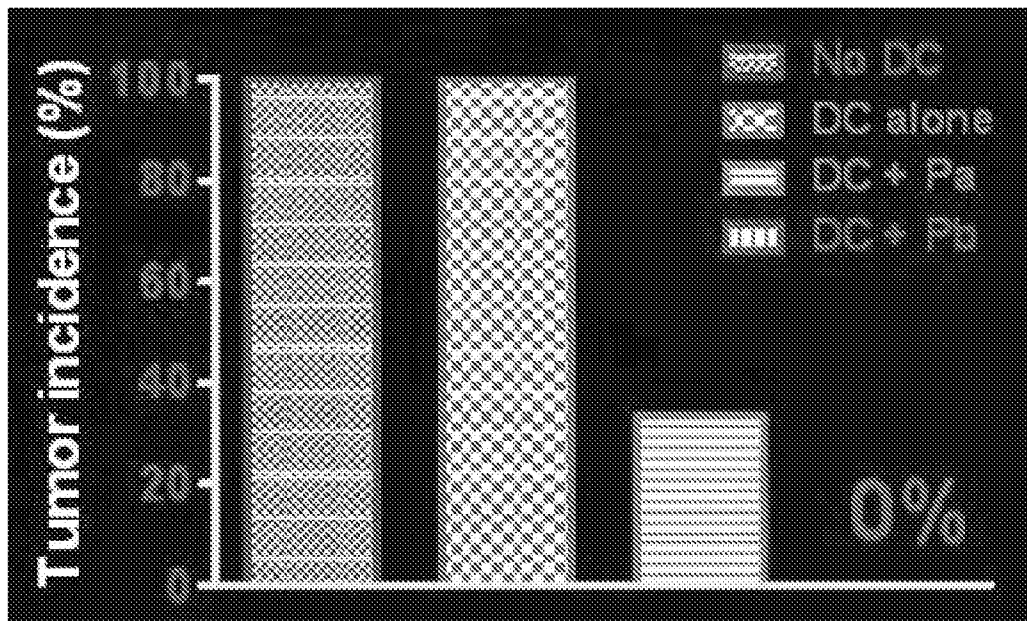


Figure 18A

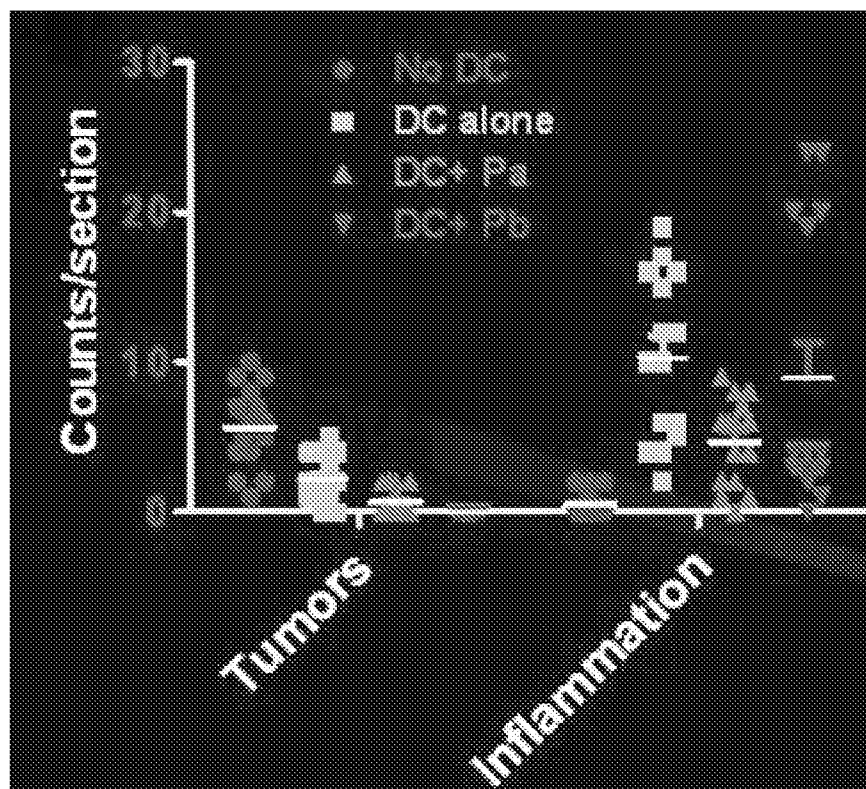


Figure 18B

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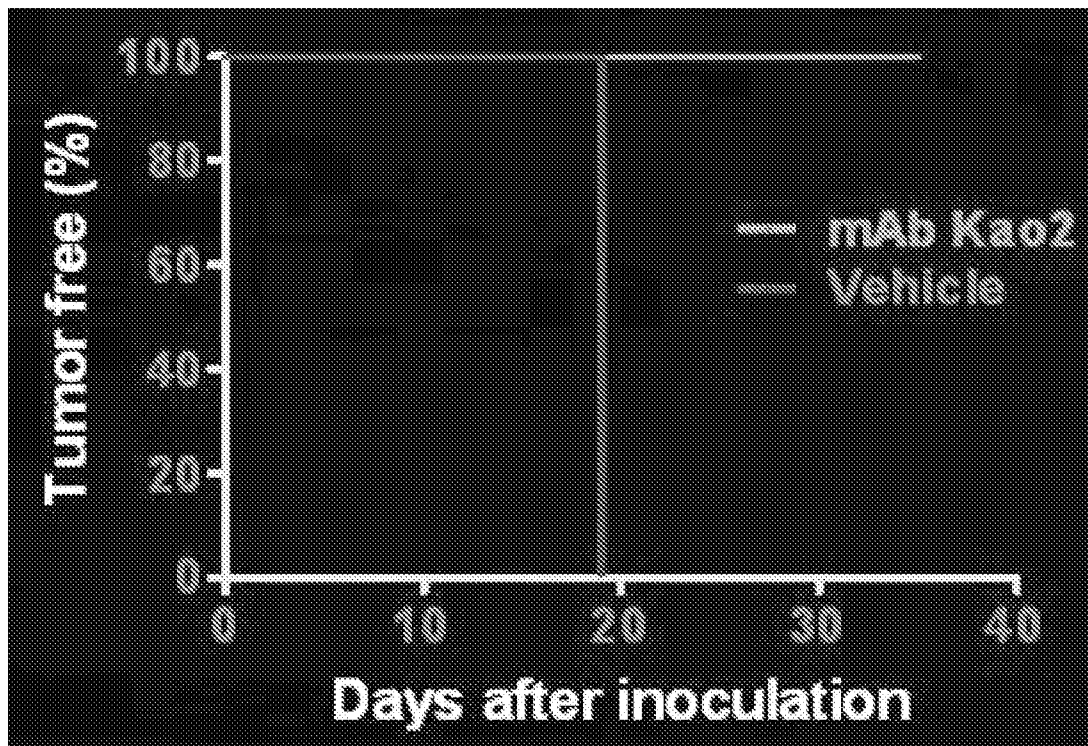


Figure 19A

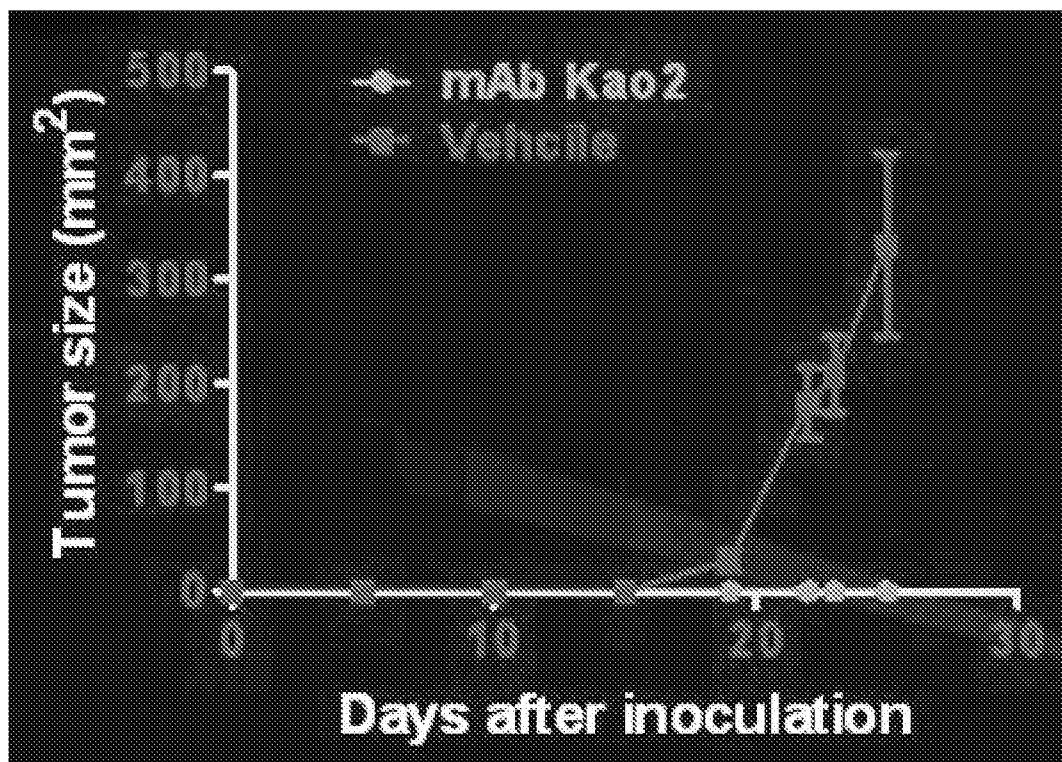


Figure 19B

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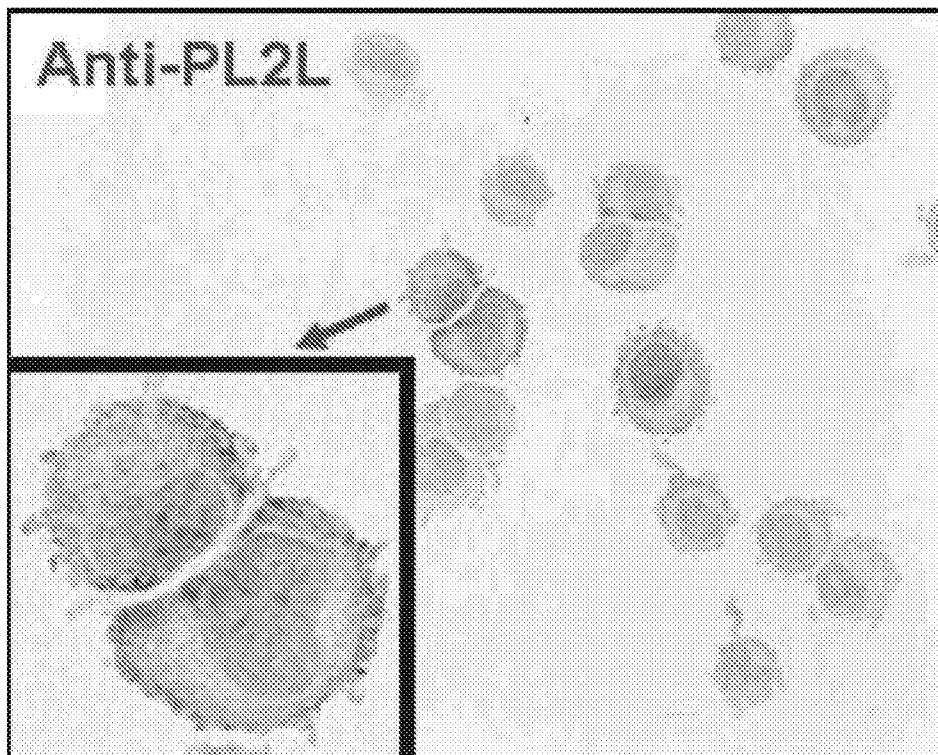


Figure 20A

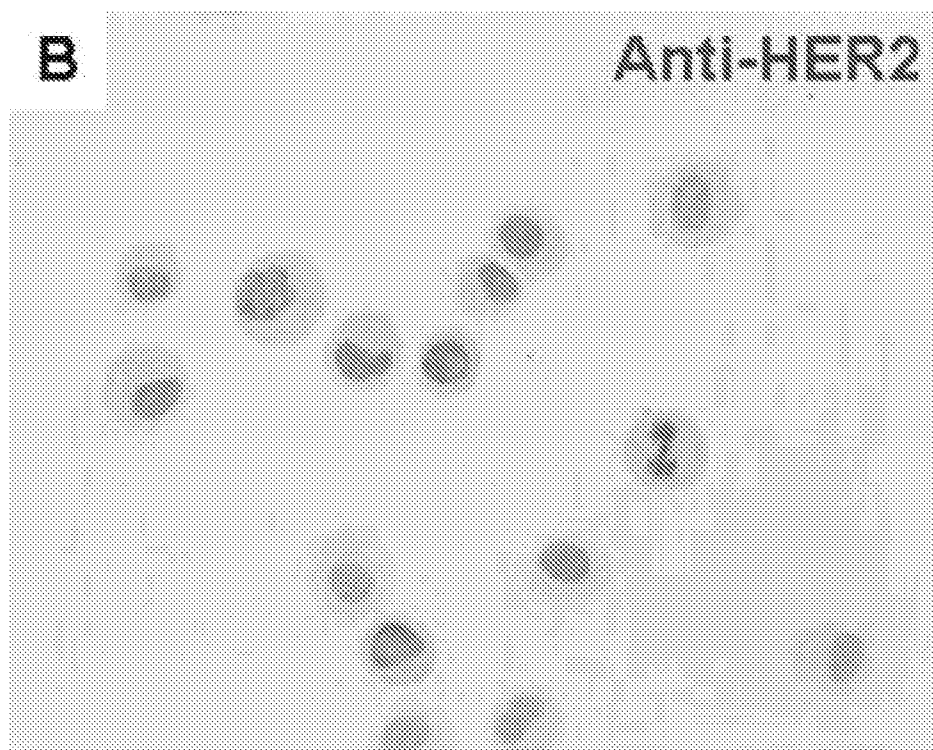


Figure 20B

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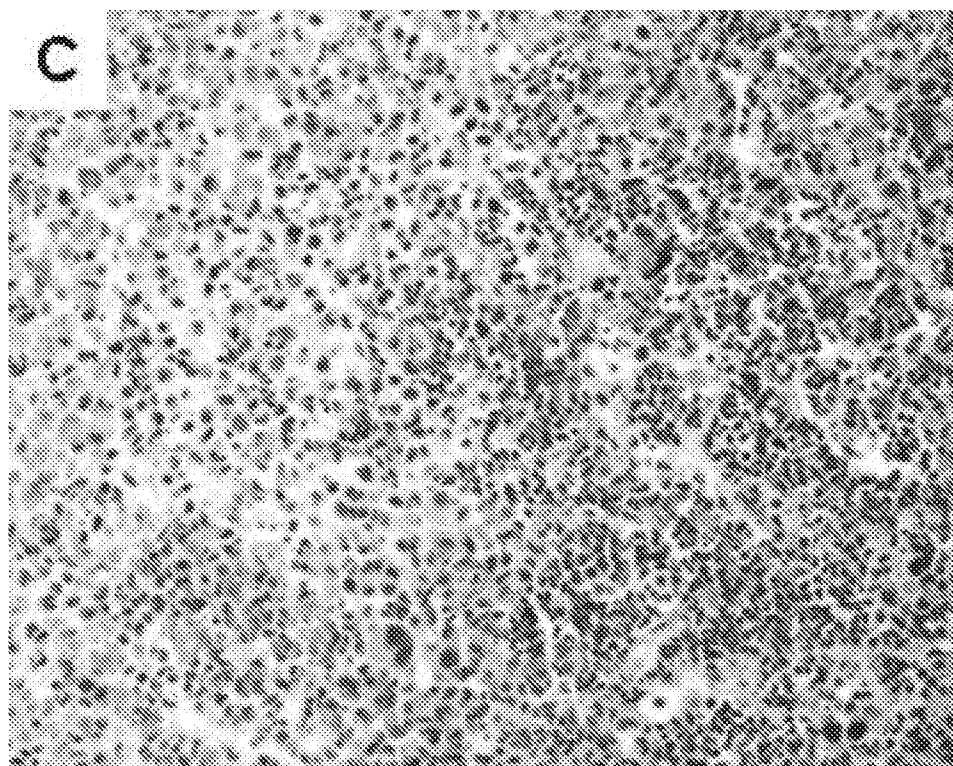


Figure 20C

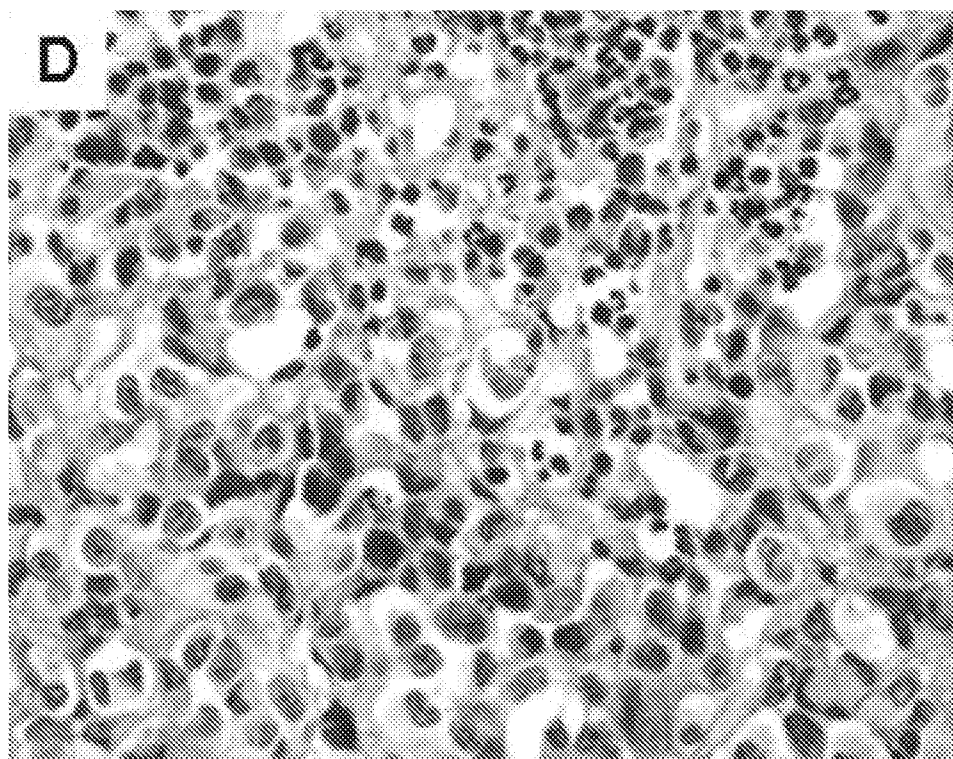


Figure 20D