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(54) Titre : IDENTIFICATION D'ALTERATIONS EPIGENETIQUES DANS DE L'ADN ISOLE A PARTIR D'EXOSOMES
(54) Title: IDENTIFICATION OF EPIGENETIC ALTERATIONS IN DNA ISOLATED FROM EXOSOMES

(57) **Abrégé/Abstract:**

The present invention provides methods of diagnosing and prognosing disease by analyzing the methylation status of exosomal DNA. In particular, exosomal DNA, in particular exosomal DNA isolated from exosomes that originated from a diseased cell, is analyzed for either a global methylation fingerprint or for a particular methylation status at one or more genomic loci, thereby providing information regarding the disease-state of the originated cell.

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(54) Title: IDENTIFICATION OF EPIGENETIC ALTERATIONS IN DNA ISOLATED FROM EXOSOMES

(57) Abstract: The present invention provides methods of diagnosing and prognosing disease by analyzing the methylation status of exosomal DNA. In particular, exosomal DNA, in particular exosomal DNA isolated from exosomes that originated from a diseased cell, is analyzed for either a global methylation fingerprint or for a particular methylation status at one or more genomic loci, thereby providing information regarding the disease-state of the originated cell.



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DESCRIPTION

IDENTIFICATION OF EPIGENETIC ALTERATIONS IN DNA ISOLATED FROM EXOSOMES

REFERENCE TO RELATED APPLICATIONS

5 **[0001]** The present application claims the priority benefit of United States provisional application number 62/649,042, filed March 28, 2018, the entire contents of which is incorporated herein by reference.

BACKGROUND

1. Field

10 **[0002]** The present invention relates generally to the field of medicine and oncology. More particularly, it concerns the detection of methylation marks in exosomes DNA. Even more particularly, it concerns the detection of methylation marks in exosomes DNA in biomarker analysis and treatment of disease.

2. Description of Related Art

15 **[0003]** Epigenomics, i.e., the study of the complete set of epigenetic modifications on the genetic material of a cell, has revealed that epigenetic modifications (e.g., DNA methylation) can play a role in mammalian development and disease. For example, DNA methylation is implicated in embryonic development, genomic imprinting and X-chromosome inactivation through regulation of transcriptional activity, chromatin structure
20 and chromatin stability (Robertson, 2005). Increased DNA methylation (hypermethylation) at promoter regions of genes can be associated with transcriptional silencing, whereas decreased methylation (hypomethylation) at promoter regions can be associated with increased gene activity. Aberrant methylation patterns can be associated with various human pathologies, including tumor formation and progression (Feinberg and Fogelstein, 1983; Esteller, 2007;
25 Jones and Paylin, 2007). Therefore, analysis of DNA methylation status across the human genome can be of interest.

SUMMARY

[0004] Therefore, the present invention provides methods of analyzing and detecting epigenetic changes of genomic DNA found in exosomes isolated from human serum samples.

In one embodiment, methods are provided for diagnosing a disease in a patient, the method comprising (a) obtaining a body fluid sample from a patient; (b) isolating an exosomes fraction of the body fluid sample; and (c) determining a methylation or hydroxymethylation status of at least one DNA molecule or a modification of a histone present in the exosomes
5 fraction, wherein if the methylation status corresponds to the methylation or hydroxymethylation status of a disease state, then the patient is diagnosed as having the disease.

[0005] In some aspects, the methylation or hydroxymethylation status is the methylation or hydroxymethylation status at a single genomic locus. In some aspects, the
10 methylation or hydroxymethylation status is the methylation or hydroxymethylation status at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, or 50 genomic loci. In some aspects, the methylation or hydroxymethylation status is a global methylation or hydroxymethylation fingerprint. In some aspects, the methylation or hydroxymethylation status is hypermethylation. In some aspects, the methylation or hydroxymethylation status is
15 hypomethylation.

[0006] In some aspects, the determining step comprises performing reduced representation bisulfite sequencing. In some aspects, the determining step comprises comparing the methylation status to a methylation status known to be associated with the disease.

[0007] In some aspects, the patient is in remission and the method is a method of
20 detecting relapse. In some aspects, the body fluid sample is blood, lymph, saliva, sputum, urine, cerebrospinal fluid, bone marrow aspirates, eye exudate/tears, or serum.

[0008] In some aspects, the disease is a disease known to be associated with and/or caused by epigenetic changes. In some aspects, the disease is a cancer, a genetic imprinting
25 disorder, a neurological disorder, an autoimmune disease, or a metabolic disorder. In certain aspects, the disease is cancer, wherein the method further comprises isolating glypican 1-containing exosomes from the exosomes fraction. In certain aspects, the cancer is a breast cancer, lung cancer, head & neck cancer, prostate cancer, esophageal cancer, tracheal cancer, brain cancer, liver cancer, bladder cancer, stomach cancer, pancreatic cancer, ovarian cancer,
30 uterine cancer, cervical cancer, testicular cancer, colon cancer, rectal cancer or skin cancer. In some aspects, the genetic imprinting disorder is Angelman's syndrome, Prader-Willi

syndrome, or Beckwith-Wiedemann syndrome. In some aspects, the neurological disorder is Alzheimer's disease, autism, Fragile X syndrome, Huntington disease, Parkinson disease, schizophrenia, ATR-X syndrome, ICF syndrome, BWS, Rett syndrome, alpha-Thalassaemia, Rubinstein-Taybi syndrome, or Coffin-Lowry syndrome. In some aspects, the autoimmune
5 disease is systemic lupus erythematosus or rheumatoid arthritis. In some aspects, the metabolic disorder is type 2 diabetes.

[0009] In some aspects, the method further comprises administering to the patient a therapeutic agent. In certain aspects, the disease is cancer, wherein the therapeutic agent is an anti-cancer therapy. In some aspects, the patient is a human. In some aspects, the method
10 further comprises performing the method a second time. In certain aspects, the second time is at least one day, one week, or one month after the initial performance of the method.

[0010] In some aspects, the method further comprises reporting the diagnosis of the patient. In certain aspects, reporting comprises preparing a written or electronic report. In certain aspects, further comprising providing the report to the patient, a doctor, a hospital, or
15 an insurance company.

[0011] As used herein, "essentially free," in terms of a specified component, is used herein to mean that none of the specified component has been purposefully formulated into a composition and/or is present only as a contaminant or in trace amounts. The total amount of the specified component resulting from any unintended contamination of a composition is
20 therefore well below 0.01%. Most preferred is a composition in which no amount of the specified component can be detected with standard analytical methods.

[0012] As used herein the specification, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising," the words "a" or "an" may mean one or more than one.

25 [0013] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." As used herein "another" may mean at least a second or more.

[0014] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

[0015] Other objects, features and advantages of the present invention will become
5 apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

10 BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

15 [0017] **FIG. 1.** Reduced representation bisulfite sequencing analysis pipeline.

[0018] **FIG. 2.** Plan for isolating cellular and exosomal DNA from different cell types, having bisulfite conversion performed on the samples, and performing reduced representation bisulfite sequencing to determine the methylation patterns present on the DNA

[0019] **FIGS. 3A-B.** Detection of DNA methylation in exosomal DNA. FIG. 3A
20 provides a schematic representing the protocol used to prepare cellular and exosomal DNA for reduced representation bisulfite sequencing (RRBS). FIG. 3B provides an analysis of the resulting data. Panel panc1_C provides a histograms of %CpG methylation (frequency vs. % methylation per base) for both the cellular DNA. Panel panc1_E provides a histograms of %CpG methylation (frequency vs. % methylation per base) for both the exosomes DNA.
25 Sample correlation was determined and expressed with the depicted scatter plot (lower left panel) and correlation coefficient (0.94, Person correlation test).

[0020] **FIG. 4.** Alignment rates graphically represented using Samtools Flagstat.

[0021] **FIG. 5.** Reads base qualities graphically represented using FastQC.

[0022] FIG. 6. Sequence duplication levels graphically represented using FastQC.

[0023] FIG. 7. Analysis of Panc1 vs HPDE correlation among samples.

[0024] FIG. 8. Samples clustered by CpG methylation.

[0025] FIG. 9. Principle component analysis.

5 [0026] FIG. 10. Samples clustered using the distance method.

[0027] FIGS. 11A-H. Detection of methylation marks on all chromosomes. Each bar represents a chromosome, as shown in FIG. 11H. Each graph represents a sample. The Y-axes represent CpG_num. FIG. 11A. is BXPC3. FIG. 11B is Capan1. FIG. 11C is HPDE. FIG. 11D is HPNE. FIG. 11E is MiaPaCa2. FIG. 11F is Panc1. FIG. 11G is PSN1. FIG. 11H is T3M4.

DETAILED DESCRIPTION

[0028] Exosomal DNA preserves cellular DNA methylation marks, identified by DNA bisulfite sequencing, found in the cells shedding the exosomes. As such, the detection of differential methylation of exosomal DNA from liquid biopsies (blood, urine, saliva, sputum) offers novel precision in early disease detection, differential diagnosis, and disease monitoring and prognosis.

I. Exosomes

[0029] The terms “microvesicle” and “exosomes,” as used herein, refer to a membranous particle having a diameter (or largest dimension where the particles is not spheroid) of between about 10 nm to about 5000 nm, more typically between 30 nm and 1000 nm, and most typically between about 50 nm and 750 nm, wherein at least part of the membrane of the exosomes is directly obtained from a cell. Most commonly, exosomes will have a size (average diameter) that is up to 5% of the size of the donor cell. Therefore, especially contemplated exosomes include those that are shed from a cell.

[0030] Exosomes may be detected in or isolated from any suitable sample type, such as, for example, body fluids. As used herein, the term “isolated” refers to separation out of its natural environment and is meant to include at least partial purification and may include substantial purification. As used herein, the term “sample” refers to any sample suitable for

the methods provided by the present invention. The sample may be any sample that includes exosomes suitable for detection or isolation. Sources of samples include blood, bone marrow, pleural fluid, peritoneal fluid, cerebrospinal fluid, urine, saliva, amniotic fluid, malignant ascites, broncho-alveolar lavage fluid, synovial fluid, breast milk, sweat, tears, joint fluid, and bronchial washes. In one aspect, the sample is a blood sample, including, for example, whole blood or any fraction or component thereof. A blood sample suitable for use with the present invention may be extracted from any source known that includes blood cells or components thereof, such as venous, arterial, peripheral, tissue, cord, and the like. For example, a sample may be obtained and processed using well-known and routine clinical methods (*e.g.*, procedures for drawing and processing whole blood). In one aspect, an exemplary sample may be peripheral blood drawn from a subject with cancer.

[0031] Exosomes may also be isolated from tissue samples, such as surgical samples, biopsy samples, tissues, feces, and cultured cells. When isolating exosomes from tissue sources it may be necessary to homogenize the tissue in order to obtain a single cell suspension followed by lysis of the cells to release the exosomes. When isolating exosomes from tissue samples it is important to select homogenization and lysis procedures that do not result in disruption of the exosomes. Exosomes contemplated herein are preferably isolated from body fluid in a physiologically acceptable solution, for example, buffered saline, growth medium, various aqueous medium, *etc.*

[0032] Exosomes may be isolated from freshly collected samples or from samples that have been stored frozen or refrigerated. In some embodiments, exosomes may be isolated from cell culture medium. Although not necessary, higher purity exosomes may be obtained if fluid samples are clarified before precipitation with a volume-excluding polymer, to remove any debris from the sample. Methods of clarification include centrifugation, ultracentrifugation, filtration, or ultrafiltration. Most typically, exosomes can be isolated by numerous methods well-known in the art. One preferred method is differential centrifugation from body fluids or cell culture supernatants. Exemplary methods for isolation of exosomes are described in (Losche *et al.*, 2004; Mesri and Altieri, 1998; Morel *et al.*, 2004). Alternatively, exosomes may also be isolated via flow cytometry as described in (Combes *et al.*, 1997).

[0033] One accepted protocol for isolation of exosomes includes ultracentrifugation, often in combination with sucrose density gradients or sucrose cushions to float the relatively

low-density exosomes. Isolation of exosomes by sequential differential centrifugations is complicated by the possibility of overlapping size distributions with other microvesicles or macromolecular complexes. Furthermore, centrifugation may provide insufficient means to separate vesicles based on their sizes. However, sequential centrifugations, when combined
5 with sucrose gradient ultracentrifugation, can provide high enrichment of exosomes.

[0034] Isolation of exosomes based on size, using alternatives to the ultracentrifugation routes, is another option. Successful purification of exosomes using ultrafiltration procedures that are less time consuming than ultracentrifugation, and do not require use of special equipment have been reported. Similarly, a commercial kit is available
10 (EXOMIR™, Bioo Scientific) which allows removal of cells, platelets, and cellular debris on one microfilter and capturing of vesicles bigger than 30 nm on a second microfilter using positive pressure to drive the fluid. However, for this process, the exosomes are not recovered, their RNA content is directly extracted from the material caught on the second microfilter, which can then be used for PCR analysis. HPLC-based protocols could
15 potentially allow one to obtain highly pure exosomes, though these processes require dedicated equipment and are difficult to scale up. A significant problem is that both blood and cell culture media contain large numbers of nanoparticles (some non-vesicular) in the same size range as exosomes. For example, some miRNAs may be contained within extracellular protein complexes rather than exosomes; however, treatment with protease (*e.g.*,
20 proteinase K) can be performed to eliminate any possible contamination with “extraexosomal” protein.

[0035] In another embodiment, cancer cell-derived exosomes may be captured by techniques commonly used to enrich a sample for exosomes, such as those involving immunospecific interactions (*e.g.*, immunomagnetic capture). Immunomagnetic capture, also
25 known as immunomagnetic cell separation, typically involves attaching antibodies directed to proteins found on a particular cell type to small paramagnetic beads. When the antibody-coated beads are mixed with a sample, such as blood, they attach to and surround the particular cell. The sample is then placed in a strong magnetic field, causing the beads to pellet to one side. After removing the blood, captured cells are retained with the beads. Many
30 variations of this general method are well-known in the art and suitable for use to isolate exosomes. In one example, the exosomes may be attached to magnetic beads (*e.g.*, aldehyde/sulphate beads) and then an antibody is added to the mixture to recognize an

epitope on the surface of the exosomes that are attached to the beads. Exemplary proteins that are known to be found on cancer cell-derived exosomes include ATP-binding cassette sub-family A member 6 (ABCA6), tetraspanin-4 (TSPAN4), SLIT and NTRK-like protein 4 (SLITRK4), putative protocadherin beta-18 (PCDHB18), myeloid cell surface antigen CD33 (CD33), and glypican-1 (GPC1) (U.S. Pat. No. 9,921,223, which is incorporated herein by reference in its entirety). Cancer cell-derived exosomes may be isolated using, for example, antibodies or aptamers to one or more of these proteins.

[0036] As used herein, analysis includes any method that allows direct or indirect visualization of exosomes and may be *in vivo* or *ex vivo*. For example, analysis may include, but not limited to, *ex vivo* microscopic or cytometric detection and visualization of exosomes bound to a solid substrate, flow cytometry, fluorescent imaging, and the like. In an exemplary aspect, cancer cell-derived exosomes are detected using antibodies directed to one or more of ATP-binding cassette sub-family A member 6 (ABCA6), tetraspanin-4 (TSPAN4), SLIT and NTRK-like protein 4 (SLITRK4), putative protocadherin beta-18 (PCDHB18), myeloid cell surface antigen CD33 (CD33), glypican-1 (GPC1), Histone H2A type 2-A (HIST1H2AA), Histone H2A type 1-A (HIST1H1AA), Histone H3.3 (H3F3A), Histone H3.1 (HIST1H3A), Zinc finger protein 37 homolog (ZFP37), Laminin subunit beta-1 (LAMB1), Tubulointerstitial nephritis antigen-like (TINAGL1), Peroxiredoxin-4 (PRDX4), Collagen alpha-2(IV) chain (COL4A2), Putative protein C3P1 (C3P1), Hemicentin-1 (HMCN1), Putative rhophilin-2-like protein (RHPN2P1), Ankyrin repeat domain-containing protein 62 (ANKRD62), Tripartite motif-containing protein 42 (TRIM42), Junction plakoglobin (JUP), Tubulin beta-2B chain (TUBB2B), Endoribonuclease Dicer (DICER1), E3 ubiquitin-protein ligase TRIM71 (TRIM71), Katanin p60 ATPase-containing subunit A-like 2 (KATNAL2), Protein S100-A6 (S100A6), 5'-nucleotidase domain-containing protein 3 (NT5DC3), Valine-tRNA ligase (VARS), Kazrin (KAZN), ELAV-like protein 4 (ELAVL4), RING finger protein 166 (RNF166), FERM and PDZ domain-containing protein 1 (FRMPD1), 78 kDa glucose-regulated protein (HSPA5), Trafficking protein particle complex subunit 6A (TRAPPC6A), Squalene monooxygenase (SQLE), Tumor susceptibility gene 101 protein (TSG101), Vacuolar protein sorting 28 homolog (VPS28), Prostaglandin F2 receptor negative regulator (PTGFRN), Isobutyryl-CoA dehydrogenase, mitochondrial (ACAD8), 26S protease regulatory subunit 6B (PSMC4), Elongation factor 1-gamma (EEF1G), Titin (TTN), Tyrosine-protein phosphatase type 13 (PTPN13), Triosephosphate isomerase (TPI1), or

Carboxypeptidase E (CPE) and subsequently bound to a solid substrate and/or visualized using microscopic or cytometric detection.

[0037] It should be noted that not all proteins expressed in a cell are found in exosomes secreted by that cell. For example, calnexin, GM130, and LAMP-2 are all proteins expressed in MCF-7 cells but not found in exosomes secreted by MCF-7 cells (Baietti *et al.*, 2012). As another example, one study found that 190/190 pancreatic ductal adenocarcinoma patients had higher levels of GPC1+ exosomes than healthy controls (Melo *et al.*, 2015, which is incorporated herein by reference in its entirety). Notably, only 2.3% of healthy controls, on average, had GPC1+ exosomes.

A. Exemplary Protocol for Collecting Exosomes from Cell Culture

[0038] On Day 1, seed enough cells (*e.g.*, about five million cells) in T225 flasks in media containing 10% FBS so that the next day the cells will be about 70% confluent. On Day 2, aspirate the media on the cells, wash the cells twice with PBS, and then add 25-30 mL base media (*i.e.*, no PenStrep or FBS) to the cells. Incubate the cells for 24-48 hours. A 48 hour incubation is preferred, but some cells lines are more sensitive to serum-free media and so the incubation time should be reduced to 24 hours. Note that FBS contains exosomes that will heavily skew NanoSight results.

[0039] On Day 3/4, collect the media and centrifuge at room temperature for five minutes at 800 x g to pellet dead cells and large debris. Transfer the supernatant to new conical tubes and centrifuge the media again for 10 minutes at 2000 x g to remove other large debris and large vesicles. Pass the media through a 0.2 µm filter and then aliquot into ultracentrifuge tubes (*e.g.*, 25 x 89 mm Beckman Ultra-Clear) using 35 mL per tube. If the volume of media per tube is less than 35 mL, fill the remainder of the tube with PBS to reach 35 mL. Ultracentrifuge the media for 2-4 hours at 28,000 rpm at 4°C using a SW 32 Ti rotor (k-factor 266.7, RCF max 133,907). Carefully aspirate the supernatant until there is roughly 1-inch of liquid remaining. Tilt the tube and allow remaining media to slowly enter aspirator pipette. If desired, the exosomes pellet can be resuspended in PBS and the ultracentrifugation at 28,000 rpm repeated for 1-2 hours to further purify the population of exosomes.

[0040] Finally, resuspend the exosomes pellet in 210 µL PBS. If there are multiple ultracentrifuge tubes for each sample, use the same 210 µL PBS to serially resuspend each exosomes pellet. For each sample, take 10 µL and add to 990 µL H₂O to use for nanoparticle

tracking analysis. Use the remaining 200 μ L exosomes-containing suspension for downstream processes or immediately store at -80°C .

B. Exemplary Protocol for Extracting Exosomes from Serum Samples

[0041] First, allow serum (or other body fluid) samples to thaw on ice. Then, dilute 250 μ L of cell-free serum samples in 11 mL PBS; filter through a 0.2 μ m pore filter. Ultracentrifuge the diluted sample at 150,000 x g overnight at 4°C . The following day, carefully discard the supernatant and wash the exosomes pellet in 11 mL PBS. Perform a second round of ultracentrifugation at 150,000 x g at 4°C for 2 hours. Finally, carefully discard the supernatant and resuspend the exosomes pellet in 100 μ L PBS for analysis.

10 II. DNA Methylation

[0042] The term “CpG island,” as used herein, refers to any DNA region wherein the calculated CG % composition is over 50%.

[0043] The term “methylation biomarker,” “disease-specific methylated restriction site pattern,” or “methylation fingerprint,” as used herein, refers to any sequence of nucleotides, preferably CpG rich, where the 5' position of any cytosine base becomes methylated. These regions may be found in any nucleotide sequence including, but not limited to, promoters, regulatory elements, enhancers, and gene coding sequences. Changes in any methylation fingerprint may be an indicator of genome instability and may be useful in the diagnosis of disease. Without being bound by any theory, changes in a methylation fingerprint may alter the accessibility of the DNA binding proteins to bind to the DNA.

[0044] The term “hypomethylation,” as used herein, refers to the presence of any cytosine in a CG or CNG di- or tri-nucleotide site that does not contain a 5' methyl group.

[0045] The term “hypermethylation,” as used herein, refers to the presence any cytosine in a CG or CNG di- or tri-nucleotide site that does contain a 5' methyl group.

[0046] The term “global methylation,” as used herein, refers to genome-wide methylation events associated with all CG dinucleotides, all restriction enzyme cutting sites for specific methylation sensitive/insensitive enzyme(s), or all priming events with statistically designed primer set(s).

[0047] Epigenetics may be defined as a stable and potentially heritable form of cellular information that influences gene expression but does not involve a change in the DNA sequence (i.e., is non-mutagenic). This cellular information is in the form of covalent modifications applied to histones and nucleic acids. In histone proteins, one form of this cellular information may be exemplified by post-translation modifications including, but not limited to, phosphorylation, acetylation, methylation, poly-ADP ribosylation and ubiquitination. In nucleic acids, one form of this cellular information may be exemplified by nucleic acids comprising 5' methylated cytosines.

[0048] There are at least three inter-related types of epigenetic inheritance: i) nucleic acid methylation; ii) genomic imprinting; and iii) histone protein modification. Nucleic acid methylation occurs at CpG dinucleotides and plays a role in the regulation of gene expression as well as the silencing of repeat elements in the genome. Genomic imprinting comprises parent-of-origin-specific allele silencing mediated by differentially methylated regions within or near imprinted genes that may be normally reprogrammed in the germline. Histone modifications include, but are not limited to, methylation, acetylation, and phosphorylation are involved in transcriptional regulation wherein many histone modifications are stably maintained during cell division. Enzymes that mediate histone modifications are often associated within the same genomic complexes as those that regulate nucleic acid methylation (i.e., for example, CpG Islands).

[0049] Epigenetic regulation at the nucleic acid level has been reported to be mediated by covalent modifications (i.e., for example, 5' cytosine methylation). For example, a methyl group may be added to a cytosine carbon-5 position that is part of a symmetrical group of CpG dinucleotides. Many 5-methylcytosines have been found in retrotransposons, endogenous retroviruses, and repetitive sequences, which may have evolved as a host defense mechanism to prevent the mobilization of these elements and reduce the occurrence of chromosomal rearrangements.

[0050] Unmethylated CpG dinucleotides may be found in short CpG-rich sequences, commonly referred to as CpG islands. CpG islands have been observed to cluster in or around promoter regions of genes. Over 40% of protein encoding genes have at least one CpG island that is found within the vicinity of their promoters.

[0051] CpG island methylation may reduce the competency of expression of nearby genes. Further, if hypermethylation occurs, it is possible that the gene might become completely switched off. Consequently, epigenetic regulation may be able to respond to environmental influences by gradually changing a gene's methylation status. The removal of such environmental influences would then be expected to reverse a gene's methylation status and appropriately adjust the expression profile of that gene in the cell. Thus, epigenetic regulation may be elastic in nature and able to dynamically respond to fluctuations in the environment. It is not believed that a nucleic acid genotype is capable of such a control system.

[0052] Since epigenetic regulation may exert an influence on genotypic expression, this capability has the potential to activate an at-risk gene haplotype or a protective gene haplotype. Consequently, epigenetic modification of genotypic expression may help to explain the broad spectrum of phenotypes observed in patients affected with complex diseases (i.e., for example, cancer).

[0053] Data supporting a link between nucleic acid instability and disease is becoming increasingly stronger. For instance, epigenetic nucleic acid regulation plays a part in the physiologic and pathologic events associated with aging and cancer.

[0054] To maintain the stability of the genome, two major alterations in nucleic acid methylation have been observed: hypomethylation and hypermethylation. In some diseases, one or the other of these methylation states may be prevalent depending upon the gene locus. In neoplasia, for example, an overall genomic hypomethylation is present, in conjunction with a hypermethylation of promoter-associated CpG islands. The hypomethylated state is believed to silence some tumor-suppressive gene activity. Consequently, both hypomethylation and hypermethylation represent epigenetic dysregulation that is responsible for the development, expression and maintenance of cancer and/or tumors.

[0055] Genome-wide (global) loss of 5'-methyl cytosine is one of the earliest molecular abnormalities described in human neoplasia. Global demethylation has been shown to occur mostly outside of promoters in CpG-depleted areas as well as in repetitive elements and pericentric bulk DNA. Hypomethylation has mechanistic implications and can play a role in neoplasia through the activation and over-expression of growth promoting genes (i.e., for example, HRAS). Also, hypomethylation has been shown to play a role in the induction of

chromosomal instability leading to neoplasia. The pericentromeric satellite regions are vulnerable to hypomethylation causing unbalanced chromosomal translocations, which have been observed in ovarian and breast carcinomas. Hypomethylation of L1 retrotransposons has been shown to be correlated with chromosomal instability colorectal cancer cell lines.

5 **[0056]** Sixty percent of expressed genes have 5' regions and upstream promoters that are located in 0.5 to 3.0 Kb nucleic acid stretches unusually rich in CpG dinucleotides. These clusters of CpG sites are known as CpG Islands and are generally free of DNA methylation. De novo nucleic acid methylation of gene promoters has been a consistent abnormality in human neoplasia. Promoter methylation has been linked to the inactivation of tumor-
10 suppressor genes (i.e., for example, RB1, P16, BRCA1 and VHL), DNA repair genes (i.e., for example, hMLH1 and MGMT), angiogenesis inhibitors (i.e., for example, THBS1), and growth regulators (i.e., for example, ER and PGR).

[0057] For example, hypermethylated biomarkers to detect cancer can include: For acute lymphoblastic leukemia, DLC-1, PCDHGA12A, CDH1, HOXD10, RPIB9, CD44,
15 COX2, SOX2, KLF4, SLC26A, RECK, HOXA9, HOXD11, HOXA6, ADAM12, and HOXC4; for chronic lymphocytic leukemia, DLC-1, PCDHGA12A, HOXD10, CD44, COX2, HOXA9, HOXA4, HOXD11, and HOXA6; for follicular lymphoma, DLC-1, PCDHGA12A, CDH1, HOXD10, RPIB9, COX2, KLF4, HOXA9, HOXA6, HOXC4, and SLC26A4; for mantle cell lymphoma, DLC-1, PCDHGA12A, HOXD10, HOXA9, HOXD11,
20 and HOXA6; for Burkett lymphoma, DLC-1, PCDHGA12A, CDH1, HOXD10, RPIB9, CD44, COX2, KLF4, HOXA9, HOXD11, HOXA6, HOXC4, and SLC26A4; for diffuse large B-cell lymphoma, DLC-1, PCDHGA12A, CDH1, HOXD10, RPIB9, COX2, KLF4, HOXA6, and SLC26A4; for multiple myeloma, DLC-1, PCDHGA12A, CDH1, COX2, KLF4, HOXA9, HOXD11, HOXA6, HOXC4, HOXD10, and SLC26A; for acute myeloid
25 leukemia, PCDHGA12A, CDH1, HOXD10, CD44, CXCR1, KLF4, SLC26A, CDH13, HOXA9, HOXD11, HOXA6, HOXC4, ADAM12, and SLC26A4; for myelodysplastic syndrome, PCDHGA12A, SOCS-1, and HIN1; for breast cancer, DLC-1, PCDHGA12A, HOXD10, RPIB9, COX2, RECK, HOXA11, HOXA7, HOXA9, HOXD9, HOXD11, PCDHB15, PCDHA6, PCDHA13, PTPN6, HIC1, CDH13, GSTP1, ADAM12, p16,
30 GABRBA, and APC; for lung cancer, PCDHGA12A, HOXD10, HOXA7, HOXA6, HOXA9, PCDHB15, PCDHA6, PCDHA13, PTPN6, GSTP1, and HIC1; for colon cancer, DLC-1, PCDHGA12A, HOXD10, RPIB9, CD44, COX2, SOX2, CXCR1, SLC26A, RECK, HOXA7,

HOXA6, HOXA9, PCDHB15, PCDHA6, PCDHA13, PTPN6, ADAM12, p16, and HIC1; for ovarian cancer, PCDHGA12A, HOXD10, SLC26A, CDH13, and RECK; for prostate cancer, PCDHGA12A, HOXD10, COX2, HOXA7, HOXA6, HOXA9, HOXD11, HOXD9, PCDHB15, PCDHA6, PTPN6, HIC1, APC, CDH13, CDH5, HOXA11, GSTP1, p16, GABRBA, and HOXA7; and for melanoma, PCDHGA12A, HOXD10, KLF4, and COX2.

[0058] Additional diseases and disorders associated with known loci of methylation changes include genetic imprinting disorders, such as Prader-Willi syndrome (15q11–q13 region of human chromosome 15, small nuclear ribonuclearprotein polypeptide N (SNRPN)), Angelman syndrome (15q11–q13 region of human chromosome 15, small nuclear ribonuclearprotein polypeptide N (SNRPN)), Beckwith-Wiedemann syndrome (chromosome region 11p15.5, loss of methylation at DMR2), and Silver-Russell syndrome (chromosome region 11p15.5, loss of methylation at DMR2); and neurodevelopmental disorders, such as Autism spectrum disorder (proline-rich transmembrane protein 1 (PRRT1)).

[0059] In addition, methylation signatures for these and other disease or disorders can be identifying by examining the methylation marks in exosomes isolated from patients known to have a certain disease or disorder.

[0060] Modification of histones by methylation, acetylation and phosphorylation has been shown to play a role in maintaining the stability of the genome by silencing genes. However, silencing inappropriate genes by histone modification can have adverse effects. For example, the methylation of lysine 9 (Lys9) of histone H3 has been shown to silence the CDKN2A tumor suppressor gene in some cancer cells.

[0061] The machinery that is responsible for the modification of chromatin and nucleic acids work in a co-operative manner to silence genes in normal and malignant cells. There is evidence to indicate that there is cross-talk between histone and nucleic acid methylation machinery that makes this multi-protein complex a likely target for environmental carcinogens. (Feinberg A P. 2004. Seminars in Cancer Biology 14:427-432).

[0062] Methods to detect global methylation and methylation biomarkers include those provided in U.S. Pat. Appln. Publn. US2007/0292866, which is incorporated by reference herein in its entirety. Methods for measuring DNA methylation at specific genomic loci include, for example, immunoprecipitation of methylated DNA, methyl-binding protein enrichment of methylated fragments, digestion with methylation-insensitive restriction

enzymes, and bisulfite conversion followed by Sanger sequencing (reviewed in Laird, *Nat Review Genet* 11: 191-203, 2010). Bisulfite treatment can convert unmethylated cytosine residues into uracils (the readout of which can be thymine after amplification with a polymerase). Methylcytosines can be protected from conversion by bisulfite treatment to uracils. Following bisulfite treatment, methylation status of a given cytosine residue can be inferred by comparing the sequence to an unmodified reference sequence.

[0063] Techniques have been developed for profiling methylation status of the whole genome, i.e., the methylome, at a single-base resolution using high throughput sequencing technologies. Bisulfite conversion of genomic DNA combined with next generation sequencing (NGS), or BS-seq, is one strategy. Because of the high cost still associated with genome-wide methylation sequencing, variations of BS-seq technology that enable genome partitioning to enrich for regions of interest can be used. One such variation is reduced representation BS-seq (RRBS), which can involve digestion of a DNA sample with a methylation-insensitive restriction endonuclease that has CpG dinucleotide as a part of its recognition site, followed by bisulfite sequencing of the selected fragments (Meissner et al., *Nucleic Acids Res.* 33(18):5868-5877, 2005). RRBS provides less coverage than whole genome bisulfite sequencing, but allows the researcher to obtain quantitative DNA methylation information across many features with approximately 50-fold fewer sequencing reads, resulting in substantial sequencing cost reduction. Since the RRBS technique was first described (Meissner, et al. (2005) *Nucleic Acids Res* 33(18):5868, which is incorporated herein by reference in its entirety), there have been many enhancements and improvements (see for example, Boyle, et al. (2012) *Genome Biol* 13:R92). One approach utilizes the methylation insensitive restriction enzyme MspI, which recognizes CCGG. As a result of partial fragmentation during bisulfite conversion, PCR, and efficiency of cluster generation, only a subset of these fragments, typically under 300 bp in length, can be sequenced. However these smaller fragments are derived from genomic DNA that has a high frequency of MspI sites and therefore a high frequency of potential CpG methylation sites. See U.S. Pat. Appln. Publn. US2015/0284769, which is incorporated herein by reference in its entirety.

III. Diagnosis, Prognosis, and Treatment of Diseases

[0064] Detection, isolation, and characterization of exosomes, using the methods of the invention, is useful in assessing disease diagnosis and prognosis and in monitoring therapeutic efficacy for early detection of treatment failure that may lead to disease relapse.

In addition, exosomes analysis according to the invention enables the detection of early relapse in presymptomatic patients who have completed a course of therapy. This is possible because the presence of diseased cell-derived exosomes may be associated and/or correlated with disease progression, poor response to therapy, relapse of disease, and/or decreased survival over a period of time. Thus, enumeration and characterization of exosomes provides methods to stratify patients for baseline characteristics that predict initial risk and subsequent risk based upon response to therapy.

[0065] For example, cancer cell-derived exosomes isolated according to the methods disclosed above may be analyzed to diagnose or prognose cancer in the subject. As such, the methods of the present invention may be used, for example, to evaluate cancer patients and those at risk for cancer by comparing the methylation states of cancer cell-derived exosomes and exosomes originated from non-cancerous cells. In any of the methods of diagnosis or prognosis described herein, either the presence or the absence of one or more indicators of cancer, such as a cancer-specific methylation mark or signature, or of any other disorder, may be used to generate a diagnosis or prognosis.

[0066] In one aspect, a body fluid (e.g., blood, urine, saliva, etc.) sample is drawn from the patient and disease cell-derived exosomes are detected and/or isolated as described herein. For example, the exosomes may be labeled with one or more antibodies or aptamers that bind to ATP-binding cassette sub-family A member 6 (ABCA6), tetraspanin-4 (TSPAN4), SLIT and NTRK-like protein 4 (SLITRK4), putative protocadherin beta-18 (PCDHB18), myeloid cell surface antigen CD33 (CD33), and/or glypican-1 (GPC1), and the antibodies may have a covalently bound fluorescent label. Analysis may then be performed to determine the number and characterization of cancer cell-derived exosomes in the sample, and from this measurement, the number of cancer cell-derived exosomes present in the initial blood sample may be determined. Exosomes identified as cancer cell-derived exosomes may be verified as such through the detection of a second (or more) marker known to be found selectively or specifically in cancer cell-derived exosomes, such as, for example, Histone H2A type 2-A (HIST1H2AA), Histone H2A type 1-A (HIST1H1AA), Histone H3.3 (H3F3A), Histone H3.1 (HIST1H3A), Zinc finger protein 37 homolog (ZFP37), Laminin subunit beta-1 (LAMB1), Tubulointerstitial nephritis antigen-like (TINAGL1), Peroxiredoxin-4 (PRDX4), Collagen alpha-2(IV) chain (COL4A2), Putative protein C3P1 (C3P1), Hemicentin-1 (HMCN1), Putative raphilin-2-like protein (RHPN2P1), Ankyrin

repeat domain-containing protein 62 (ANKRD62), Tripartite motif-containing protein 42 (TRIM42), Junction plakoglobin (JUP), Tubulin beta-2B chain (TUBB2B), Endoribonuclease Dicer (DICER1), E3 ubiquitin-protein ligase TRIM71 (TRIM71), Katanin p60 ATPase-containing subunit A-like 2 (KATNAL2), Protein S100-A6 (S100A6), 5'-nucleotidase domain-containing protein 3 (NT5DC3), Valine-tRNA ligase (VARS), Kazrin (KAZN), ELAV-like protein 4 (ELAVL4), RING finger protein 166 (RNF166), FERM and PDZ domain-containing protein 1 (FRMPD1), 78 kDa glucose-regulated protein (HSPA5), Trafficking protein particle complex subunit 6A (TRAPPC6A), Squalene monooxygenase (SQLE), Tumor susceptibility gene 101 protein (TSG101), Vacuolar protein sorting 28 homolog (VPS28), Prostaglandin F2 receptor negative regulator (PTGFRN), Isobutyryl-CoA dehydrogenase, mitochondrial (ACAD8), 26S protease regulatory subunit 6B (PSMC4), Elongation factor 1-gamma (EEF1G), Titin (TTN), Tyrosine-protein phosphatase type 13 (PTPN13), Triosephosphate isomerase (TPI1), or Carboxypeptidase E (CPE). The number of cancer cell-derived exosomes may be determined by cytometric or microscopic techniques to visually quantify and characterize the exosomes. Cancer cell-derived exosomes may be detected and quantified by other methods known in the art (*e.g.*, ELISA).

[0067] In various aspects, analysis of a subject's cancer cell-derived exosomes number and methylation signature characterization may be made over a particular time course in various intervals to assess a subject's progression and pathology. For example, analysis may be performed at regular intervals such as one day, two days, three days, one week, two weeks, one month, two months, three months, six months, or one year, in order to track the level and characterization of cancer cell-derived exosomes as a function of time. In the case of existing cancer patients, this provides a useful indication of the progression of the disease and assists medical practitioners in making appropriate therapeutic choices based on the increase, decrease, or lack of change in cancer cell-derived exosomes. Any increase, be it 2-fold, 5-fold, 10-fold or higher, in cancer cell-derived exosomes over time decreases the patient's prognosis and is an early indicator that the patient should change therapy. Similarly, any increase, be it 2-fold, 5-fold, 10-fold or higher, indicates that a patient should undergo further testing such as imaging to further assess prognosis and response to therapy. Any decrease, be it 2-fold, 5-fold, 10-fold or higher, in cancer cell-derived exosomes over time shows disease stabilization and a patient's response to therapy, and is an indicator to not change therapy. For those at risk of cancer, a sudden increase in the number of cancer cell-derived exosomes detected may provide an early warning that the patient has developed a

tumor thus providing an early diagnosis. In one embodiment, the detection of cancer cell-derived exosomes increases with the staging of the cancer.

[0068] In any of the methods provided herein, additional analysis may also be performed to characterize cancer cell-derived exosomes to provide additional clinical
5 assessment. For example, in addition to image analysis and bulk number measurements, PCR techniques may be employed, such as multiplexing with primers specific for particular cancer markers to obtain information such as the type of tumor from which the cancer cell-derived exosomes originated, metastatic state, and degree of malignancy. Additionally, DNA or RNA analysis, proteome analysis, or metabolome analysis may be performed as a means of
10 assessing additional information regarding characterization of the patient's cancer.

[0069] For example, an epigenetic analysis may provide data sufficient to make determinations of responsiveness of a subject to a particular therapeutic regime, or for determining the effectiveness of a candidate agent in the treatment of cancer. Accordingly, the present invention provides a method of determining responsiveness of a subject to a
15 particular therapeutic regime or determining the effectiveness of a candidate agent in the treatment of cancer by detecting/isolating cancer cell-derived exosomes of the subject as described herein and analyzing said cancer cell-derived exosomes. For example, once a drug treatment is administered to a patient, it is possible to determine the efficacy of the drug treatment using the methods of the invention. For example, a sample taken from the patient
20 before the drug treatment, as well as one or more samples taken from the patient concurrently with or subsequent to the drug treatment, may be processed using the methods of the invention. By comparing the results of the analysis of each processed sample, one may determine the efficacy of the drug treatment or the responsiveness of the patient to the agent. In this manner, early identification may be made of failed compounds or early validation may
25 be made of promising compounds.

[0070] Certain aspects of the present invention can be used to prevent or treat a disease or disorder based on the presence of methylation marks found in genomic DNA isolated from exosomes. Certain aspects of the present invention provide for treating a patient with exosomes that express or comprise a therapeutic agent or a diagnostic agent. A
30 "therapeutic agent" as used herein is an atom, molecule, or compound that is useful in the treatment of cancer or other conditions. Examples of therapeutic agents include, but are not limited to, drugs, chemotherapeutic agents, therapeutic antibodies and antibody fragments,

toxins, radioisotopes, enzymes, nucleases, hormones, immunomodulators, antisense oligonucleotides, chelators, boron compounds, photoactive agents, and dyes. A “diagnostic agent” as used herein is an atom, molecule, or compound that is useful in diagnosing, detecting or visualizing a disease. According to the embodiments described herein, diagnostic agents may include, but are not limited to, radioactive substances (*e.g.*, radioisotopes, radionuclides, radiolabels or radiotracers), dyes, contrast agents, fluorescent compounds or molecules, bioluminescent compounds or molecules, enzymes and enhancing agents (*e.g.*, paramagnetic ions).

[0071] In some aspects, a therapeutic recombinant protein may be a protein having an activity that has been lost in a cell of the patient, a protein having a desired enzymatic activity, a protein having a desired inhibitory activity, *etc.* For example, the protein may be a transcription factor, an enzyme, a proteinaceous toxin, an antibody, a monoclonal antibody, *etc.* The monoclonal antibody may specifically or selectively bind to an intracellular antigen. The monoclonal antibody may inhibit the function of the intracellular antigen and/or disrupt a protein-protein interaction. Other aspects of the present invention provide for diagnosing a disease based on the presence of certain methylation marks found in the DNA of cancer cell-derived exosomes in a patient sample.

[0072] The term “subject” as used herein refers to any individual or patient to which the subject methods are performed. Generally the subject is human, although as will be appreciated by those in the art, the subject may be an animal. Thus other animals, including mammals, such as rodents (including mice, rats, hamsters, and guinea pigs), cats, dogs, rabbits, farm animals (including cows, horses, goats, sheep, pigs, *etc.*), and primates (including monkeys, chimpanzees, orangutans, and gorillas) are included within the definition of subject.

[0073] “Treatment” and “treating” refer to administration or application of a therapeutic agent to a subject or performance of a procedure or modality on a subject for the purpose of obtaining a therapeutic benefit of a disease or health-related condition. For example, a treatment may include administration of chemotherapy, immunotherapy, or radiotherapy, performance of surgery, or any combination thereof.

[0074] The term “therapeutic benefit” or “therapeutically effective” as used herein refers to anything that promotes or enhances the well-being of the subject with respect to the

medical treatment of this condition. This includes, but is not limited to, a reduction in the frequency or severity of the signs or symptoms of a disease. For example, treatment of cancer may involve, for example, a reduction in the invasiveness of a tumor, reduction in the growth rate of the cancer, or prevention of metastasis. Treatment of cancer may also refer to
5 prolonging survival of a subject with cancer.

[0075] It is also recognized that the present invention may also be used to diagnose a non-cancerous disease, and in particular to diagnose any disease known to be associated with alterations in DNA methylation. See Jin and Liu, *Genes & Diseases*, 5:1-8, 2018, which is incorporated herein by reference in its entirety, for a review of human diseases associated
10 with alterations in DNA methylation. For example, the present invention may be used to diagnose an autoimmune disease (e.g., rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis), a metabolic disorder (hyperglycemia, hyperlipidemia, cardiovascular disease, diabetes), a neurological disease (e.g., autism spectrum disorder, Rett syndrome, Parkinson's disease, schizophrenia), or a psychological disorder.

[0076] An effective response of a patient or a patient's "responsiveness" to treatment refers to the clinical or therapeutic benefit imparted to a patient at risk for, or suffering from, a disease or disorder. Such benefit may include cellular or biological responses, a complete response, a partial response, a stable disease (without progression or relapse), or a response with a later relapse. For example, an effective response can be reduced tumor size or
20 progression-free survival in a patient diagnosed with cancer.

[0077] Treatment outcomes can be predicted and monitored and/or patients benefiting from such treatments can be identified or selected via the methods described herein.

[0078] Regarding neoplastic condition treatment, depending on the stage of the neoplastic condition, neoplastic condition treatment involves one or a combination of the
25 following therapies: surgery to remove the neoplastic tissue, radiation therapy, and chemotherapy. Other therapeutic regimens may be combined with the administration of the anticancer agents, e.g., therapeutic compositions and chemotherapeutic agents. For example, the patient to be treated with such anti-cancer agents may also receive radiation therapy and/or may undergo surgery.

[0079] For the treatment of disease, the appropriate dosage of a therapeutic composition will depend on the type of disease to be treated, as defined above, the severity

and course of the disease, the patient's clinical history and response to the agent, and the discretion of the attending physician. The agent is suitably administered to the patient at one time or over a series of treatments.

[0080] Therapeutic and prophylactic methods and compositions can be provided in a combined amount effective to achieve the desired effect. A tissue, tumor, or cell can be contacted with one or more compositions or pharmacological formulation(s) comprising one or more of the agents, or by contacting the tissue, tumor, and/or cell with two or more distinct compositions or formulations. Also, it is contemplated that such a combination therapy can be used in conjunction with chemotherapy, radiotherapy, surgical therapy, or immunotherapy.

[0081] In some aspects, the disease is a cancer. The term "cancer," as used herein, may be used to describe a solid tumor, metastatic cancer, or non-metastatic cancer. In certain embodiments, the cancer may originate in the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, duodenum, small intestine, large intestine, colon, rectum, anus, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, pancreas, prostate, skin, stomach, testis, tongue, or uterus.

[0082] The cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma;

inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant;

5 paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; blue nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocyoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar

10 rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma,

15 malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma;

20 astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; hodgkin's disease; hodgkin's; paraganuloma; malignant

25 lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic

30 leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

[0083] In these aspects, therapy includes chemotherapy, radiation therapy, immunotherapy, and surgery. A wide variety of chemotherapeutic agents may be used in

accordance with the present embodiments. The term “chemotherapy” refers to the use of drugs to treat cancer. A “chemotherapeutic agent” is used to connote a compound or composition that is administered in the treatment of cancer. These agents or drugs are categorized by their mode of activity within a cell, for example, whether and at what stage they affect the cell cycle. Alternatively, an agent may be characterized based on its ability to directly cross-link DNA, to intercalate into DNA, or to induce chromosomal and mitotic aberrations by affecting nucleic acid synthesis.

[0084] Examples of chemotherapeutic agents include DNA damage checkpoint inhibitors, such as CHK inhibitors (AZD7762, Prexasertib, GDC-0575, CCT245737), ATR inhibitors (AZD6738, BAY1895344, M4344, Berzosertib), Wee1 inhibitors (AZD1775), DNA-PK inhibitors (M9831, Nedisertib, CC-115); alkylating agents, such as thiotepea and cyclophosphamide; alkyl sulfonates, such as busulfan, improsulfan, and piposulfan; aziridines, such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines, including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate, and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); temozolomide; bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratiastatin; a sarcodictyin; spongistatin; nitrogen mustards, such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, and uracil mustard; nitrosureas, such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics, such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authrarnycin, azaserine, bleomycins, cactinomycin, carabacin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, such as mitomycin C, mycophenolic acid, nogalarnycin, olivomycins,

peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, and zorubicin; anti-metabolites, such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues, such as denopterin, pteropterin, and trimetrexate; purine analogs, such as fludarabine, 6-mercaptopurine, thiamiprine, and thioguanine; 5 pyrimidine analogs, such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, and floxuridine; androgens, such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, and testolactone; anti-adrenals, such as mitotane and trilostane; folic acid replenisher, such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; 10 bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids, such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKpolysaccharide complex; razoxane; rhizoxin; 15 sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; taxoids, e.g., paclitaxel and docetaxel gemcitabine; 6-thioguanine; mercaptopurine; platinum coordination complexes, such as 20 cisplatin, oxaliplatin, and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (e.g., CPT-11); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids, such as retinoic acid; capecitabine; carboplatin, procarbazine, plicomycin, gemcitabine, navelbine, farnesyl-protein transferase 25 inhibitors, transplatinum, and pharmaceutically acceptable salts, acids, or derivatives of any of the above.

[0085] Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated, such as 30 microwaves, proton beam irradiation (U.S. Patents 5,760,395 and 4,870,287), and UV-irradiation. It is most likely that all of these factors affect a broad range of damage on DNA, on the precursors of DNA, on the replication and repair of DNA, and on the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200

roentgens for prolonged periods of time (3 to 4 wk), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

[0086] The skilled artisan will understand that additional immunotherapies may be used in combination or in conjunction with methods of the embodiments. In the context of cancer treatment, immunotherapeutics, generally, rely on the use of immune effector cells and molecules to target and destroy cancer cells. Rituximab (RITUXAN®) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either directly or indirectly, with a tumor cell target. Various effector cells include cytotoxic T cells and NK cells

[0087] Antibody-drug conjugates have emerged as a breakthrough approach to the development of cancer therapeutics. Cancer is one of the leading causes of deaths in the world. Antibody-drug conjugates (ADCs) comprise monoclonal antibodies (MAbs) that are covalently linked to cell-killing drugs. This approach combines the high specificity of MAbs against their antigen targets with highly potent cytotoxic drugs, resulting in “armed” MAbs that deliver the payload (drug) to tumor cells with enriched levels of the antigen (Carter *et al.*, 2008; Teicher 2014; Leal *et al.*, 2014). Targeted delivery of the drug also minimizes its exposure in normal tissues, resulting in decreased toxicity and improved therapeutic index. The approval of two ADC drugs, ADCETRIS® (brentuximab vedotin) in 2011 and KADCYLA® (trastuzumab emtansine or T-DM1) in 2013 by FDA validated the approach. There are currently more than 30 ADC drug candidates in various stages of clinical trials for cancer treatment (Leal *et al.*, 2014). As antibody engineering and linker-payload optimization are becoming more and more mature, the discovery and development of new ADCs are increasingly dependent on the identification and validation of new targets that are suitable to this approach (Teicher 2009) and the generation of targeting MAbs. Two criteria for ADC targets are upregulated/high levels of expression in tumor cells and robust internalization.

[0088] In one aspect of immunotherapy, the tumor cell must bear some marker that is amenable to targeting, i.e., is not present on the majority of other cells. Many tumor markers exist and any of these may be suitable for targeting in the context of the present embodiments. Common tumor markers include CD20, carcinoembryonic antigen, tyrosinase (p97), gp68, TAG-72, HMFG, Sialyl Lewis Antigen, MucA, MucB, PLAP, laminin receptor, erb B, and p155. An alternative aspect of immunotherapy is to combine anticancer effects with immune stimulatory effects. Immune stimulating molecules also exist including: cytokines, such as IL-2, IL-4, IL-12, GM-CSF, gamma-IFN, chemokines, such as MIP-1, MCP-1, IL-8, and growth factors, such as FLT3 ligand.

[0089] Examples of immunotherapies currently under investigation or in use are immune adjuvants, e.g., *Mycobacterium bovis*, *Plasmodium falciparum*, dinitrochlorobenzene, and aromatic compounds (U.S. Patents 5,801,005 and 5,739,169; Hui and Hashimoto, 1998; Christodoulides et al., 1998); cytokine therapy, e.g., interferons α , β , and γ , IL-1, GM-CSF, and TNF (Bukowski et al., 1998; Davidson et al., 1998; Hellstrand et al., 1998); gene therapy, e.g., TNF, IL-1, IL-2, and p53 (Qin et al., 1998; Austin-Ward and Villaseca, 1998; U.S. Patents 5,830,880 and 5,846,945); and monoclonal antibodies, e.g., anti-CD20, anti-ganglioside GM2, and anti-p185 (Hollander, 2012; Hanibuchi et al., 1998; U.S. Patent 5,824,311). It is contemplated that one or more anti-cancer therapies may be employed with the antibody therapies described herein.

[0090] In some embodiments, the immunotherapy may be an immune checkpoint inhibitor. Immune checkpoints are molecules in the immune system that either turn up a signal (e.g., co-stimulatory molecules) or turn down a signal. Inhibitory checkpoint molecules that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), programmed death ligand 1 (PD-L1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA). In particular, the immune checkpoint inhibitors target the PD-1 axis and/or CTLA-4.

[0091] The immune checkpoint inhibitors may be drugs such as small molecules, recombinant forms of ligand or receptors, or, in particular, are antibodies, such as human

antibodies (*e.g.*, International Patent Publication WO2015016718; Pardoll 2012; both incorporated herein by reference). Known inhibitors of the immune checkpoint proteins or analogs thereof may be used, in particular chimerized, humanized or human forms of antibodies may be used. As the skilled person will know, alternative and/or equivalent names
5 may be in use for certain antibodies mentioned in the present disclosure. Such alternative and/or equivalent names are interchangeable in the context of the present invention. For example it is known that lambrolizumab is also known under the alternative and equivalent names MK-3475 and pembrolizumab.

[0092] In some embodiments, the PD-1 binding antagonist is a molecule that inhibits
10 the binding of PD-1 to its ligand binding partners. In a specific aspect, the PD-1 ligand binding partners are PDL1 and/or PDL2. In another embodiment, a PDL1 binding antagonist is a molecule that inhibits the binding of PDL1 to its binding partners. In a specific aspect, PDL1 binding partners are PD-1 and/or B7-1. In another embodiment, the PDL2 binding antagonist is a molecule that inhibits the binding of PDL2 to its binding partners. In a specific
15 aspect, a PDL2 binding partner is PD-1. The antagonist may be an antibody, an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide. Exemplary antibodies are described in U.S. Patent Nos. 8,735,553, 8,354,509, and 8,008,449, all incorporated herein by reference. Other PD-1 axis antagonists for use in the methods provided herein are known in the art such as described in U.S. Patent Application No.
20 20140294898, 2014022021, and 20110008369, all incorporated herein by reference.

[0093] In some embodiments, the PD-1 binding antagonist is an anti-PD-1 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody). In some embodiments, the anti-PD-1 antibody is selected from the group consisting of nivolumab, pembrolizumab, and CT-011. In some embodiments, the PD-1 binding antagonist is an
25 immunoadhesin (*e.g.*, an immunoadhesin comprising an extracellular or PD-1 binding portion of PDL1 or PDL2 fused to a constant region (*e.g.*, an Fc region of an immunoglobulin sequence). In some embodiments, the PD-1 binding antagonist is PDR-001 (spartalizumab), AMP-224 or AMP-514. Nivolumab, also known as MDX-1106-04, MDX-1106, ONO-4538, BMS-936558, and OPDIVO®, is an anti-PD-1 antibody described in WO2006/121168.
30 Pembrolizumab, also known as MK-3475, Merck 3475, lambrolizumab, KEYTRUDA®, and SCH-900475, is an anti-PD-1 antibody described in WO2009/114335. CT-011, also known as hBAT or hBAT-1, is an anti-PD-1 antibody described in WO2009/101611. AMP-224, also

known as B7-DCIg, is a PDL2-Fc fusion soluble receptor described in WO2010/027827 and WO2011/066342. In some embodiments, the PD-1 axis inhibitor is an anti-PD-L1 antibody, such as atezolizumab, avelumab, durvalumab, BMS-936559, or CK-301.

[0094] Another immune checkpoint that can be targeted in the methods provided
5 herein is the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152. The complete cDNA sequence of human CTLA-4 has the Genbank accession number L15006. CTLA-4 is found on the surface of T cells and acts as an “off” switch when bound to CD80 or CD86 on the surface of antigen-presenting cells. CTLA4 is a member of the immunoglobulin superfamily that is expressed on the surface of Helper T cells and transmits
10 an inhibitory signal to T cells. CTLA4 is similar to the T-cell co-stimulatory protein, CD28, and both molecules bind to CD80 and CD86, also called B7-1 and B7-2 respectively, on antigen-presenting cells. CTLA4 transmits an inhibitory signal to T cells, whereas CD28 transmits a stimulatory signal. Intracellular CTLA4 is also found in regulatory T cells and may be important to their function. T cell activation through the T cell receptor and CD28
15 leads to increased expression of CTLA-4, an inhibitory receptor for B7 molecules.

[0095] In some embodiments, the immune checkpoint inhibitor is an anti-CTLA-4 antibody (*e.g.*, a human antibody, a humanized antibody, or a chimeric antibody), an antigen binding fragment thereof, an immunoadhesin, a fusion protein, or oligopeptide.

[0096] Anti-human-CTLA-4 antibodies (or VH and/or VL domains derived
20 therefrom) suitable for use in the present methods can be generated using methods well known in the art. Alternatively, art recognized anti-CTLA-4 antibodies can be used. For example, the anti-CTLA-4 antibodies disclosed in: US 8,119,129, WO 01/14424, WO 98/42752; WO 00/37504 (CP675,206, also known as tremelimumab; formerly ticilimumab), U.S. Patent No. 6,207,156; Hurwitz *et al.*, 1998; Camacho *et al.*, 2004; Mokyr *et al.*, 1998
25 can be used in the methods disclosed herein. The teachings of each of the aforementioned publications are hereby incorporated by reference. Antibodies that compete with any of these art-recognized antibodies for binding to CTLA-4 also can be used. For example, a humanized CTLA-4 antibody is described in International Patent Application No. WO2001014424, WO2000037504, and U.S. Patent No. 8,017,114; all incorporated herein by reference.

30 [0097] An exemplary anti-CTLA-4 antibody is ipilimumab (also known as 10D1, MDX- 010, MDX- 101, and Yervoy®) or antigen binding fragments and variants thereof

(see, *e.g.*, WOO 1/14424). In other embodiments, the antibody comprises the heavy and light chain CDRs or VRs of ipilimumab. Accordingly, in one embodiment, the antibody comprises the CDR1, CDR2, and CDR3 domains of the VH region of ipilimumab, and the CDR1, CDR2 and CDR3 domains of the VL region of ipilimumab. In another embodiment, the antibody competes for binding with and/or binds to the same epitope on CTLA-4 as the above- mentioned antibodies. In another embodiment, the antibody has at least about 90% variable region amino acid sequence identity with the above-mentioned antibodies (*e.g.*, at least about 90%, 95%, or 99% variable region identity with ipilimumab).

[0098] Other molecules for modulating CTLA-4 include CTLA-4 ligands and receptors such as described in U.S. Patent Nos. US5844905, US5885796 and International Patent Application Nos. WO1995001994 and WO1998042752; all incorporated herein by reference, and immunoadhesions such as described in U.S. Patent No. US8329867, incorporated herein by reference.

[0099] Approximately 60% of persons with cancer will undergo surgery of some type, which includes preventative, diagnostic or staging, curative, and palliative surgery. Curative surgery includes resection in which all or part of cancerous tissue is physically removed, excised, and/or destroyed and may be used in conjunction with other therapies, such as the treatment of the present embodiments, chemotherapy, radiotherapy, hormonal therapy, gene therapy, immunotherapy, and/or alternative therapies. Tumor resection refers to physical removal of at least part of a tumor. In addition to tumor resection, treatment by surgery includes laser surgery, cryosurgery, electrosurgery, and microscopically-controlled surgery (Mohs' surgery).

[00100] Upon excision of part or all of cancerous cells, tissue, or tumor, a cavity may be formed in the body. Treatment may be accomplished by perfusion, direct injection, or local application of the area with an additional anti-cancer therapy. Such treatment may be repeated, for example, every 1, 2, 3, 4, 5, 6, or 7 days, or every 1, 2, 3, 4, and 5 weeks or every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months. These treatments may be of varying dosages as well.

[00101] It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These additional agents include agents that affect the upregulation of cell surface receptors

and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring
5 hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further
10 contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

[00102] In some aspects, the disease is a genetic imprinting disorder, such as Angelman syndrome, Prader-Willi syndrome, or Beckwith-Wiedemann syndrome. In these
15 aspects, therapy includes anti-seizure medication (e.g., sodium valproate (Epilim), clonazepam (Rivotril), lamotrigine (Lamictal) and levetiracetam (Keppra)), indotecan, physical therapy, communication therapy, behavior therapy, piracetam (Nootropil), carbamazepine (Tegretol), a ketogenic diet, vagus nerve stimulation (VNS), human growth hormone replacement therapy, sex hormone replacement therapy, and surgery.

[00103] In some aspects, the disease is a neurological disorder, such as Alzheimer's disease, autism, Fragile X syndrome, Huntington disease, Parkinson disease, schizophrenia, ATR-X syndrome, ICF syndrome, BWS, Rett syndrome, alpha-Thalassaemia, Rubinstein-Taybi syndrome, or Coffin-Lowry syndrome. In these aspects, therapy includes
20 cholinesterase inhibitors (e.g., donepezil (Aricept), rivastigmine (Exelon), galantamine (Razadyne)), memantine, levodopa, carbidopa, and antipsychotics (e.g., Aripiprazole (Abilify), Asenapine (Saphris), Brexpiprazole (Rexulti), Cariprazine (Vraylar), Clozapine (Clozaril), Iloperidone (Fanapt), Lurasidone (Latuda), Olanzapine (Zyprexa), Paliperidone (Invega), Pimavanserin (Nuplazid), Quetiapine (Seroquel), Risperidone (Risperdal), Ziprasidone (Geodon)).

[00104] In some aspects, the disease is an autoimmune disease, such as systemic lupus erythematosus or rheumatoid arthritis. In these aspects, therapy includes
30 nonsteroidal anti-inflammatory drugs (e.g., ibuprofen, naproxen), antimalarial drugs (e.g.,

hydroxychloroquine), corticosteroids and immune suppressants (e.g., azathioprine, cyclophosphamide, cyclosporine), belimumab, antirheumatic drugs (e.g., methotrexate, leflunomide, sulfasalazine), batacept (Orencia), adalimumab (Humira), anakinra (Kineret), baricitinib (Olumiant), certolizumab (Cimzia), etanercept (Enbrel), golimumab (Simponi),
 5 infliximab (Remicade), rituximab (Rituxan), sarilumab (Kevzara), tocilizumab (Actemra), and tofacitinib (Xeljanz).

[00105] In some aspects, the disease is a metabolic disorder, such as type 2 diabetes. In these aspects, therapy includes metformin, sulfonylureas (e.g., glyburide, glipizide, glimepiride), meglitinides (e.g., repaglinide, nateglinide), thiazolidinediones (e.g.,
 10 rosiglitazone, pioglitazone), DPP-4 inhibitors (e.g., sitagliptin, saxagliptin, linagliptin), and GLP-1 receptor agonists (e.g., exenatide, liraglutide, semaglutide), SGLT2 inhibitors (canagliflozin, dapagliflozin, empagliflozin), and insulin.

IV. Kits and Diagnostics

[00106] In various aspects of the invention, a kit is envisioned containing the
 15 necessary components to purify exosomes from a body fluid or tissue culture medium. In yet other aspects, a kit is envisioned containing the necessary components to isolate exosomes and determine the presence of one or more disease cell-derived exosome-specific methylation mark(s) within the isolated exosomes.

[00107] The kit may comprise one or more sealed vials containing any of such
 20 components. In some embodiments, the kit may also comprise a suitable container means, which is a container that will not react with components of the kit, such as an eppendorf tube, an assay plate, a syringe, a bottle, or a tube. The container may be made from sterilizable materials such as plastic or glass. The kit may further include an instruction sheet that outlines the procedural steps of the methods set forth herein, and will follow substantially the
 25 same procedures as described herein or are known to those of ordinary skill. The instruction information may be in a computer readable media containing machine-readable instructions that, when executed using a computer, cause the display of a real or virtual procedure of purifying exosomes from a sample, and/or identifying one or more disease cell-derived methylation mark(s) therein.

V. Examples

[00108] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Detection of DNA Methylation in Exosomal DNA

[00109] The most widely used technique to investigate DNA methylation is Bisulfite Sequencing, which utilizes sodium bisulfite treatment to convert unmethylated cytosines to uracils, which are then converted to thymidines during PCR amplification. Methylated cytosines remain intact after bisulfite treatment; therefore, when compared back to the original genome for reference, methylated sites can be determined by observing where the sequence contains a “C” instead of a “T” (Meissner et al., 2005).

[00110] While the bisulfite technique can be used for genome-wide cytosine methylation analysis, CpG islands only reside in ~2% of the genome, making whole genome bisulfite sequencing extremely inefficient. Therefore, a common CpG enrichment strategy is reduced representation bisulfite sequencing, which targets CpG rich regions, capturing at least 70% of CpG islands and requiring only small quantities of input DNA, which is extremely useful for fragmented or low-concentration samples, such as exosomal DNA. After bisulfite conversion, a primer library is used that targets the CpG rich areas of the genome, generating site-specific fragments of DNA. During analysis, these fragments, or “reads” are mapped to specific regions of DNA using reference sequences. The number of C-to-T conversions is then counted for all of the mapped reads, generating a beta value for methylation at each site. Samples can then be compared in a site-specific manner between conditions, for example between exosomal and cellular DNA (FIG. 1).

[00111] For these experiments, the plan was to isolate cellular and exosomal DNA from different cell types, have bisulfite conversion performed on the samples, and perform reduced representation bisulfite sequencing to determine the methylation patterns

present on the DNA (FIG. 2). The first experiment aimed to determine if exosomal DNA can faithfully recapitulate the methyl landscape of the cell of origin, and whether this can be detected by sequencing. For the initial pilot experiment, DNA was isolated from non-tumorigenic HPDE cells and PDAC panc1 cells. DNA was then isolated from the exosomes of those same cells. The DNA was then subjected to bisulfite conversion, which changes unmethylated cytosines into uracils, which become thymines during PCR amplification, allowing them to be detected by sequencing. Methylated cytosines remain unchanged. The limitations of bisulfite—high temp and low pH required to deaminate unmethylated cytosines—can degrade DNA, which is a challenge with already fragmented exosomal DNA. As such, this method reduces read sequencing which can help maximize results from low quality input DNA.

[00112] In one experiment, cellular DNA was extracted from Panc-1 cells, and the culture supernatant from the cells was harvested and processed for exosomes enrichment (NanoSight™ analysis), after which exosomes DNA was extracted (FIG 3A). The samples (C, cells; and E, Exosomes) were processed for reduced representation bisulfite sequencing (RRBS), enabling the analysis of genome-wide DNA methylation profiles at a single nucleotide resolution level (FIG. 3A). The histograms of %CpG methylation (frequency vs. % methylation per base) for both the cellular and exosomes DNA (panels panc1_C and panc1_E, respectively), indicate a similar methylation pattern (FIG. 3B). Sample correlation was ascertained and expressed with the depicted scatter plot and correlation coefficient (0.94, Pearson correlation test, FIG. 3B). The results indicate a very high correlation, with strongly matching DNA methylation fingerprint patterns between the two samples (FIG. 3B). Detailed comparative analyses indicated N = 27 differential hypomethylated genes in exosomes vs. cells, and N = 3519 differential hypermethylated genes in exosomes vs. cells. The exosomes DNA is thus slightly enriched for hypermethylated DNA, possibly due to the protection of exosomes DNA from demethylating enzymes.

[00113] Next, eight different pancreatic cell lines (tumorigenic cell lines: Panc1, BXPC3, Capan1, T3M4, MiaPaCa2, and PSN1; non-tumorigenic cell lines: HPNE and HPDE) and their exosomes were tested in triplicate. First, quality control analyses were run on all of the samples. Alignment rates were high, providing good coverage for the site-specific analysis of CpG-rich regions (FIG. 4). Second, the read quality was determined to be high, meaning there were fewer sequencing errors and the reads could be accurately analyzed

(FIG. 5). Additionally, there was a high duplication level, which was expected with the reduced read sequencing since a primer library was used to amplify specific sites (FIG. 6). It is expected for an RRBS library to feature duplication levels this high since all fragments are expected to line up perfectly at exactly the same genomic location numerous times (e.g., because there are only so many MspI restriction recognition sites in a genome).

[00114] The specific analysis in FIG. 7 provides a comparison between the HPDE and Panc1 samples. As is outlined, the methylation profiles have a much higher correlation rate within each cell type, so the different methylation patterns can be distinguished from the cell lines using both cell and exosomal DNA, and the cell and exosomal DNA from the same cell line have highly correlated methylation patterns, supporting the hypothesis that exosomal DNA maintains the methylation patterns seen in the cells of origin.

[00115] FIG. 8 provides another way of visualizing the correlation, showing that the samples do segregate by cell type. Each batch of bisulfite conversion can be slightly different. As such, the Panc1 samples from the initial study (Panc1_C3 and Panc1_E3) are a bit different from the other Panc1 replicates, but they still cluster with the cell line.

[00116] A further example of the batch segregation is the principle component analysis provided in FIG. 9, which is used to visualize genetic distance and relatedness between populations. The HPDE samples all cluster very closely together.

[00117] The plot provided in FIG. 10 demonstrates that the samples from each cell line from all 8 lines tested cluster together, showing that the samples do segregate by cell type, regardless of whether the DNA is from cells or exosomes. Here again it can be seen that each batch of bisulfite conversion can be slightly different, so that the Panc1 samples from the pilot study are a bit different from the other Panc1 replicates, but they still cluster with the cell line. For each sample, the methylation patterns demonstrate coverage over every chromosome (FIGS. 11A-H).

* * *

[00118] All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be

apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the
5 agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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U.S. Patent 4,870,287

U.S. Patent 5,739,169

U.S. Patent 5,760,395

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CLAIMS

1. A method of diagnosing a disease in a patient, the method comprising:
 - 5 (a) obtaining a body fluid sample from a patient;
 - (b) isolating an exosomes fraction of the body fluid sample; and
 - (c) determining a methylation or hydroxymethylation status of at least one DNA molecule or a modification of a histone present in the exosomes fraction, wherein if the methylation status corresponds to the methylation status of a disease state, then the patient is
10 diagnosed as having the disease.
2. The method of claim 1, wherein the methylation or hydroxymethylation status is the methylation or hydroxymethylation status at a single genomic locus.
3. The method of claim 2, wherein the methylation or hydroxymethylation status is the methylation or hydroxymethylation status at at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,
15 15, 20, 25, 30, 35, 40, 45, or 50 genomic loci.
4. The method of claim 1, wherein the methylation or hydroxymethylation status is a global methylation or hydroxymethylation fingerprint.
5. The method of claim 1, wherein the methylation or hydroxymethylation status is hypermethylation.
- 20 6. The method of claim 1, wherein the methylation or hydroxymethylation status is hypomethylation.
7. The method of claim 1, wherein the histone modification is methylation, acetylation, or phosphorylation.
8. The method of claim 1, wherein the determining step comprises performing reduced
25 representation bisulfite sequencing.
9. The method of claim 1, wherein the determining step comprises comparing the methylation status to a methylation status known to be associated with the disease.

10. The method of claim 1, wherein the patient is in remission and the method is a method of detecting relapse.
11. The method of claim 1, wherein the body fluid sample is blood, lymph, saliva, sputum, urine, cerebrospinal fluid, bone marrow aspirates, eye exudate/tears, or serum.
- 5 12. The method of claim 1, wherein the disease is a cancer, a genetic imprinting disorder, a neurological disorder, an autoimmune disease, or a metabolic disorder.
13. The method of claim 1, wherein the disease is cancer, wherein the method further comprises isolating glypican 1-containing exosomes from the exosomes fraction.
14. The method of claim 13, wherein the cancer is a breast cancer, lung cancer, head &
10 neck cancer, prostate cancer, esophageal cancer, tracheal cancer, brain cancer, liver cancer, bladder cancer, stomach cancer, pancreatic cancer, ovarian cancer, uterine cancer, cervical cancer, testicular cancer, colon cancer, rectal cancer or skin cancer.
15. The method of claim 1, further comprising administering to the patient a therapeutically effective amount of a therapeutic agent.
- 15 16. The method of claim 15, wherein the disease is cancer, wherein the therapeutic agent is an anti-cancer therapy.
17. The method of claim 1, further comprising reporting the diagnosis of the patient.
18. The method of claim 17, wherein reporting comprises preparing a written or electronic report.
- 20 19. The method of claim 18, further comprising providing the report to the patient, a doctor, a hospital, or an insurance company.
20. The method of claim 1, wherein the patient is a human.
21. The method of claim 1, further comprising performing the method a second time.
22. The method of claim 21, wherein the second time is at least one day, one week, or one
25 month after the initial performance of the method.

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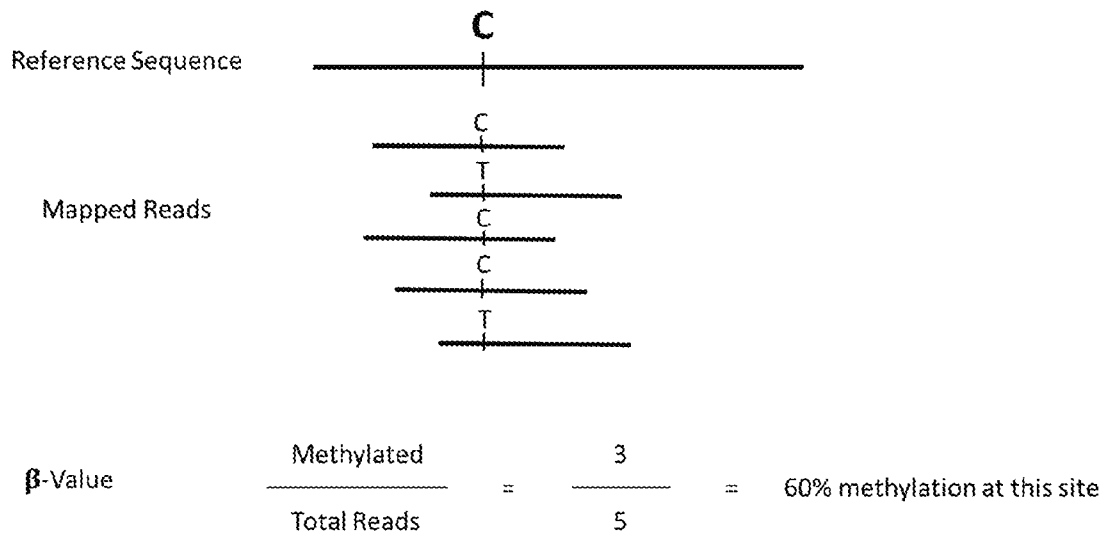


FIG. 1

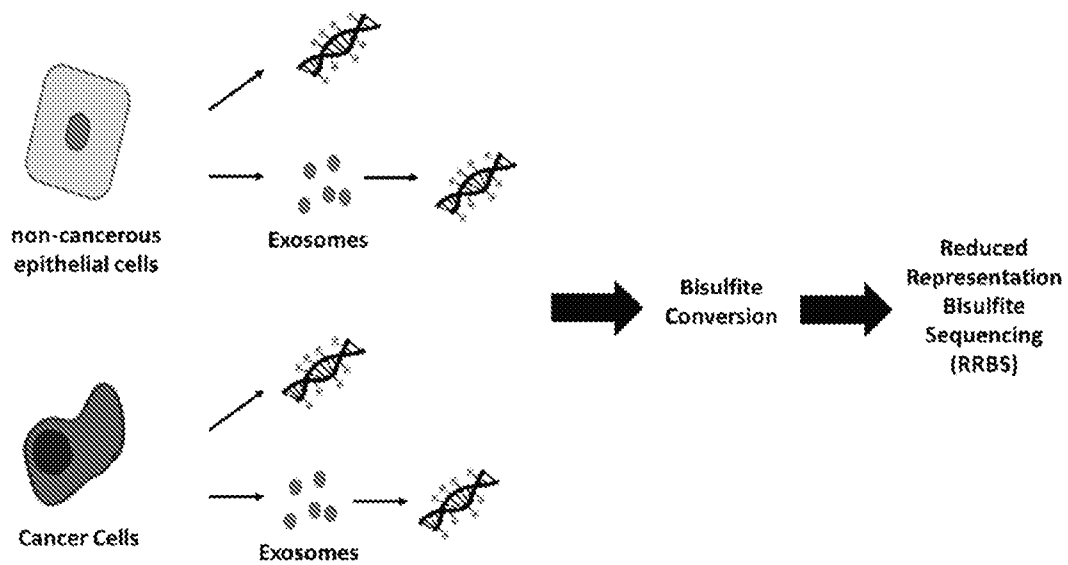
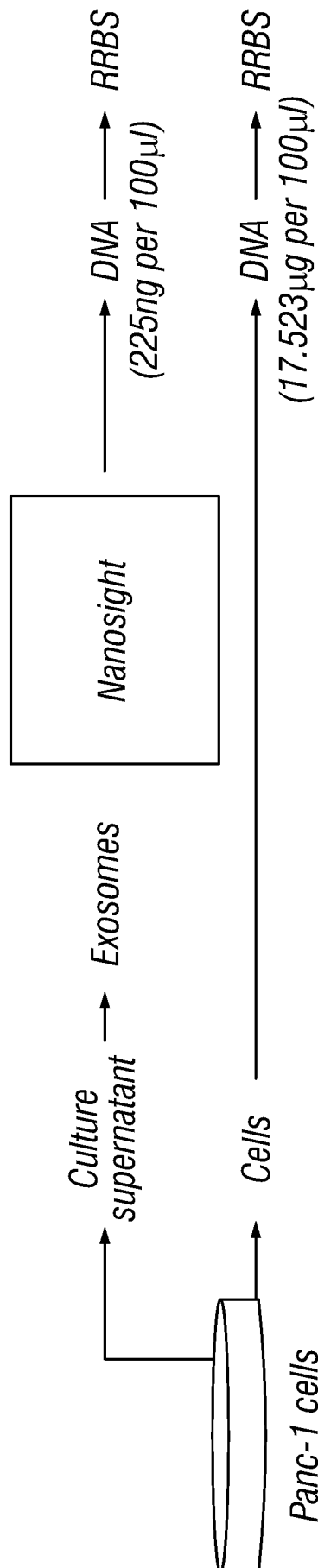


FIG. 2

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**FIG. 3A**

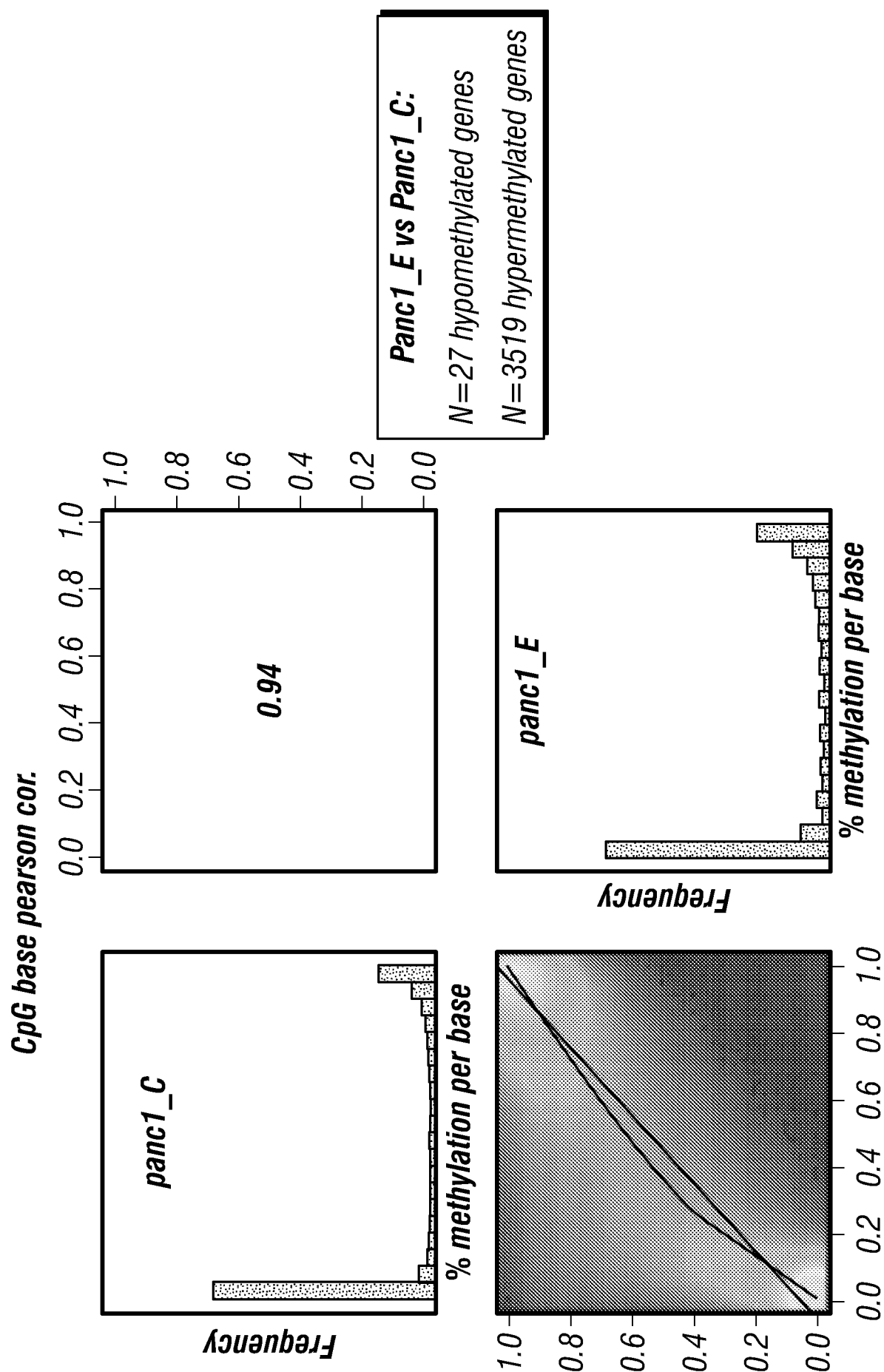
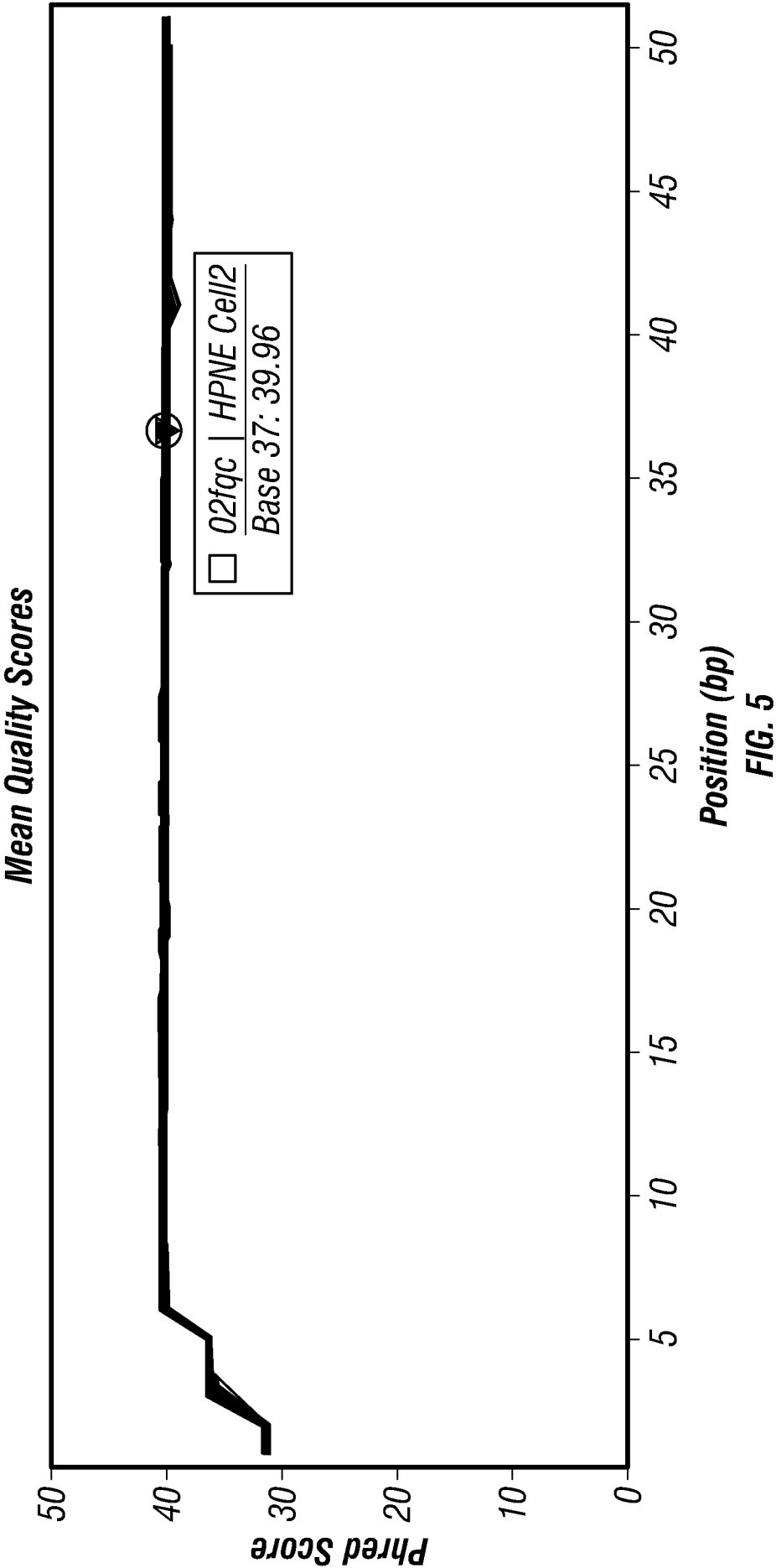


FIG. 4

FIG. 4



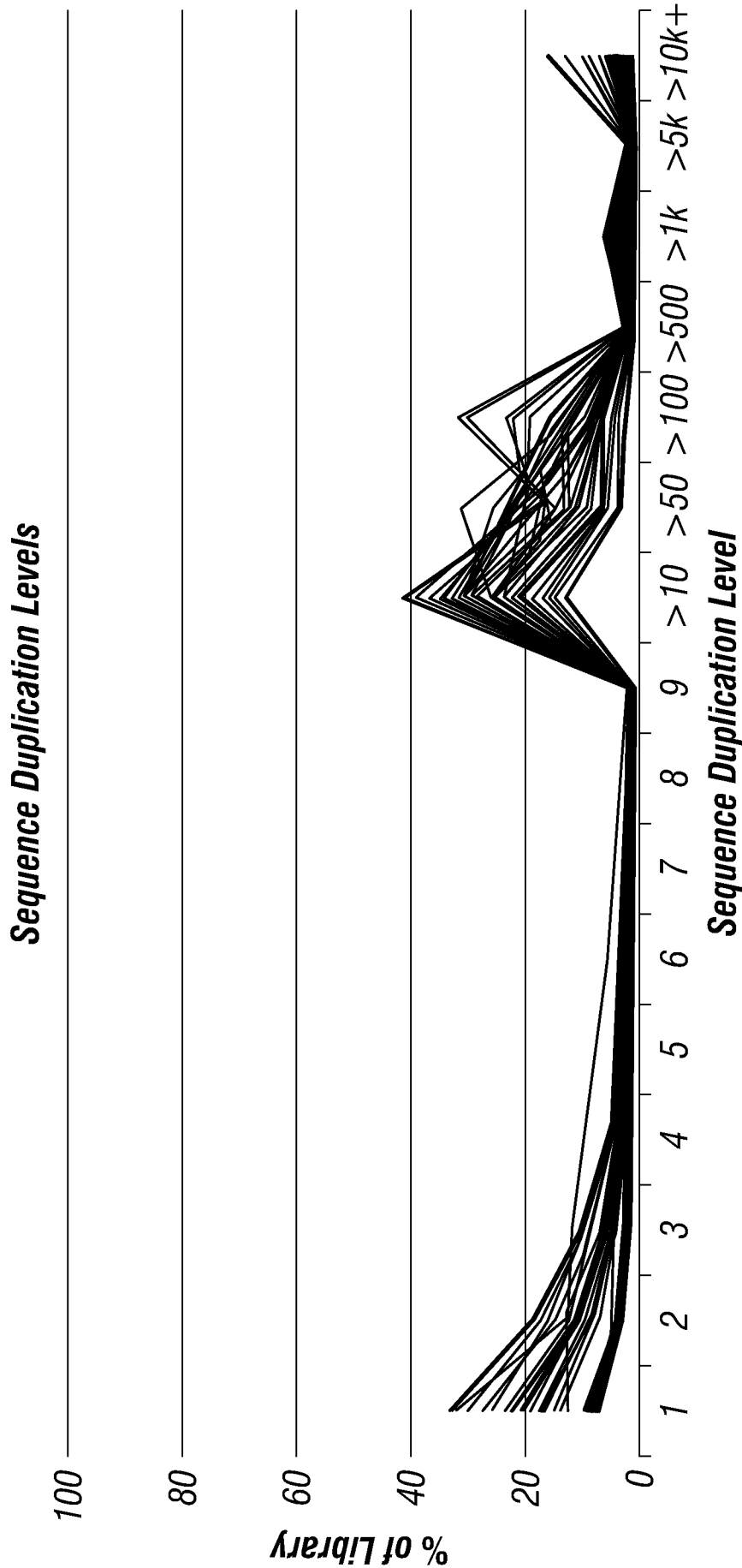
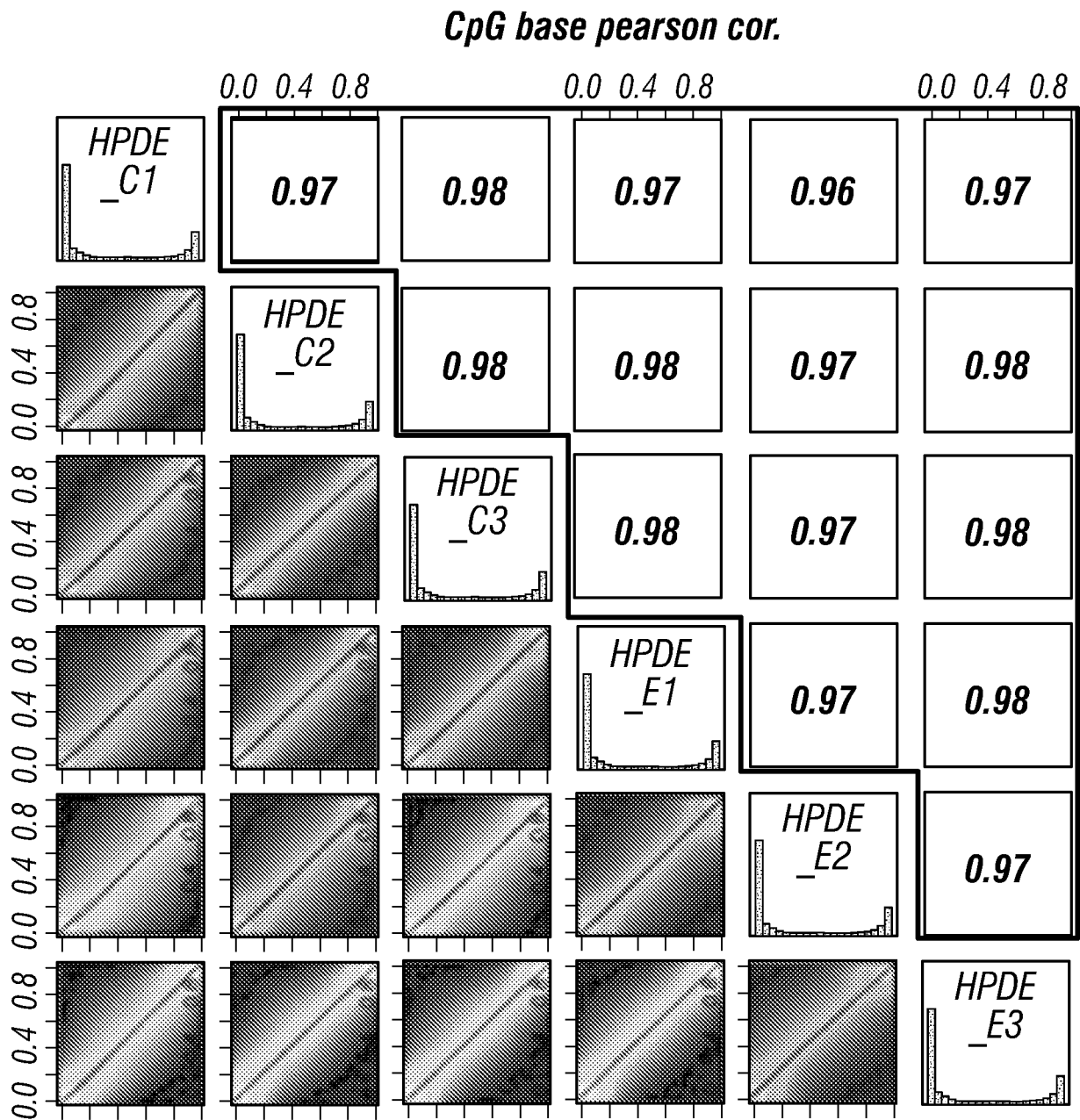


FIG. 6

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**FIG. 7**

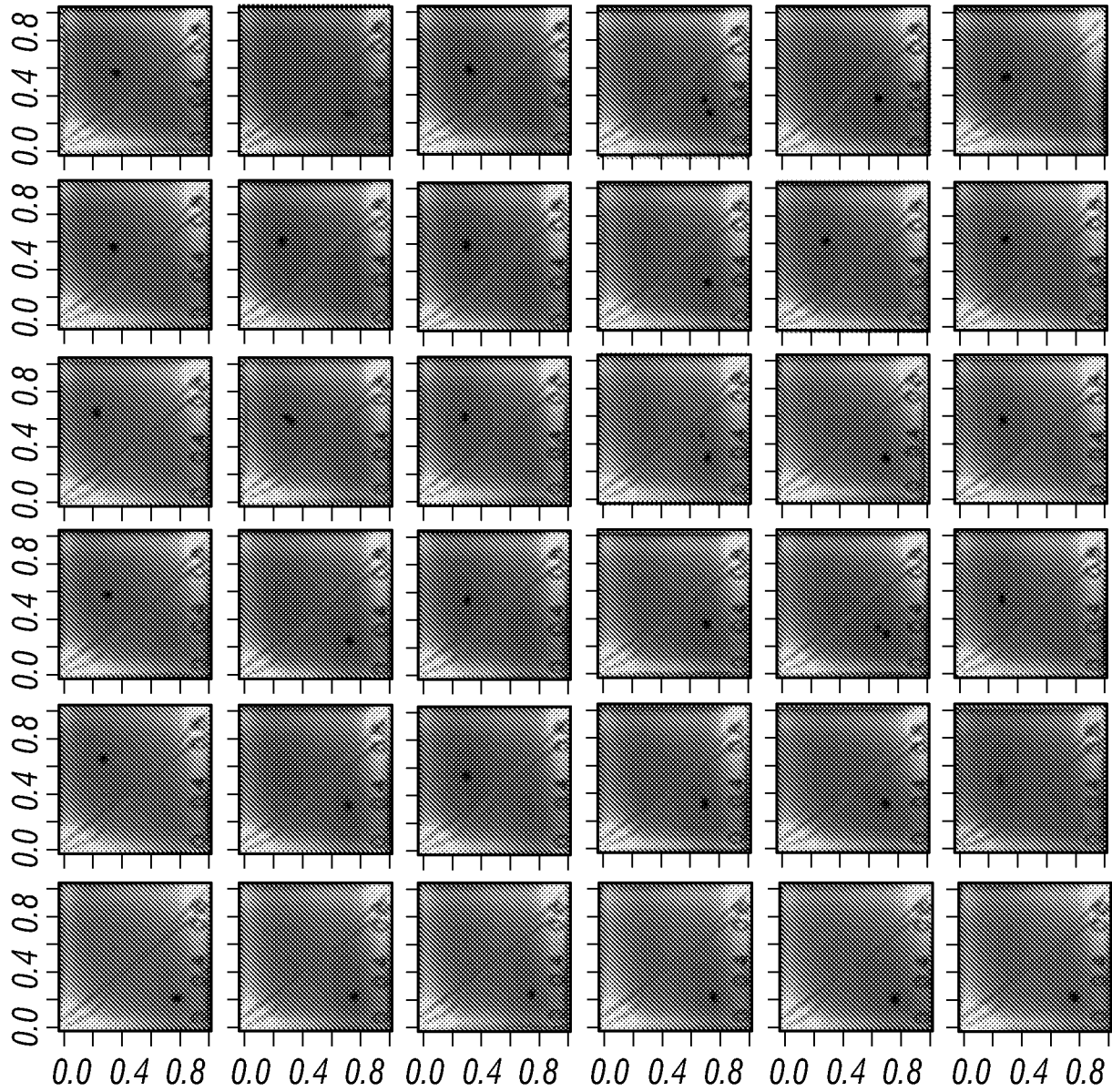


FIG. 7
(Cont'd)

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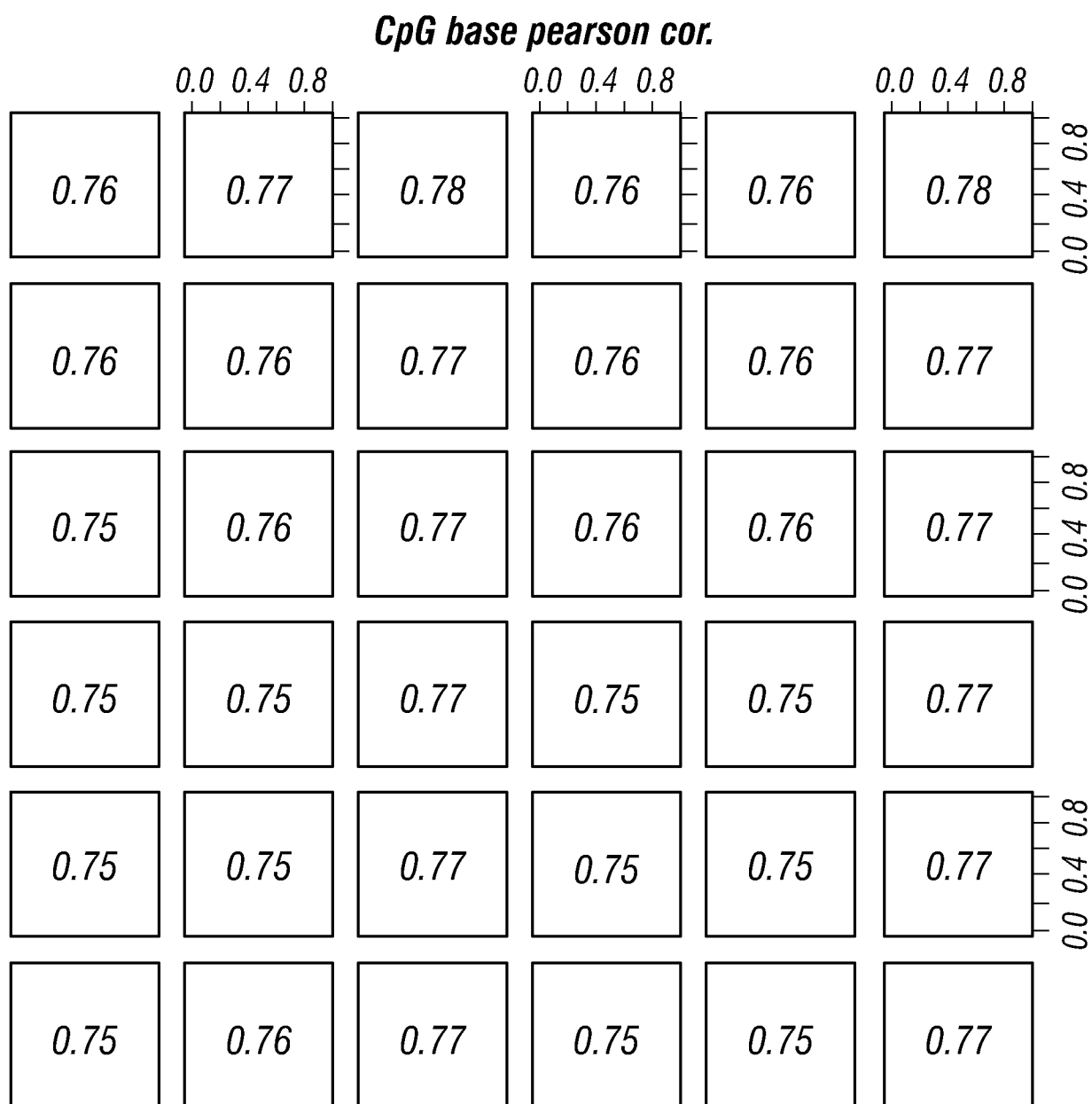


FIG. 7
(Cont'd)

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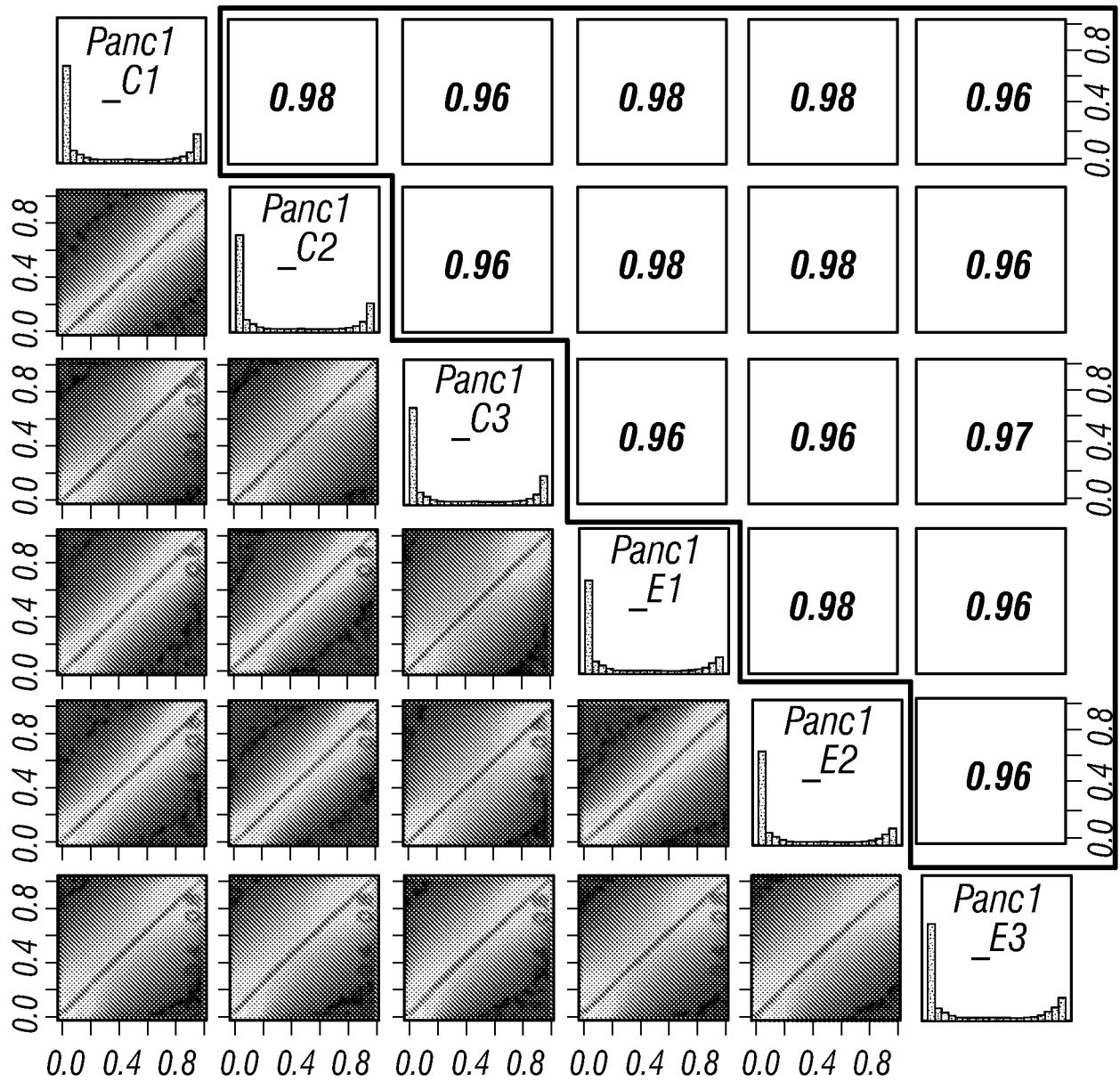
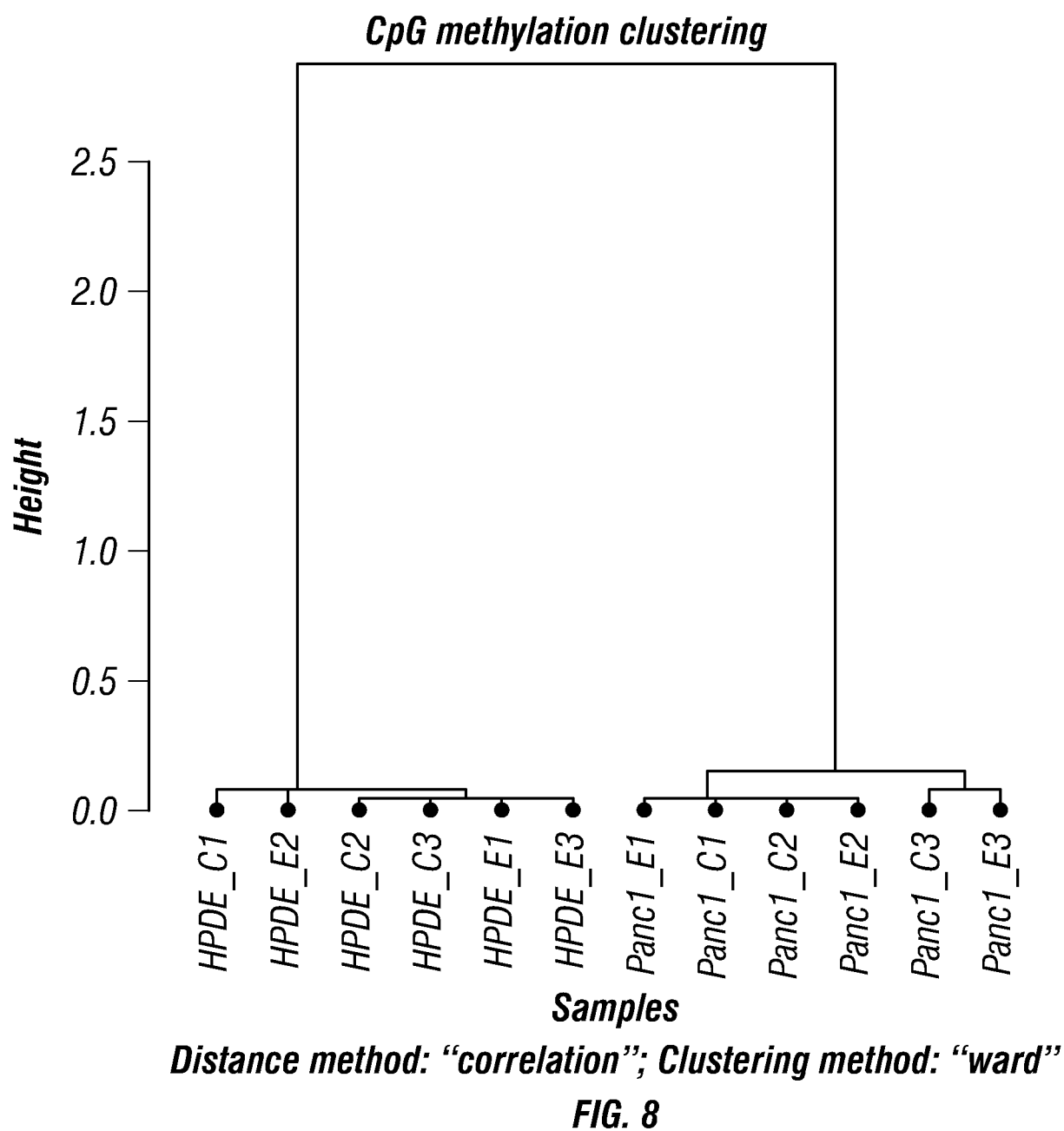


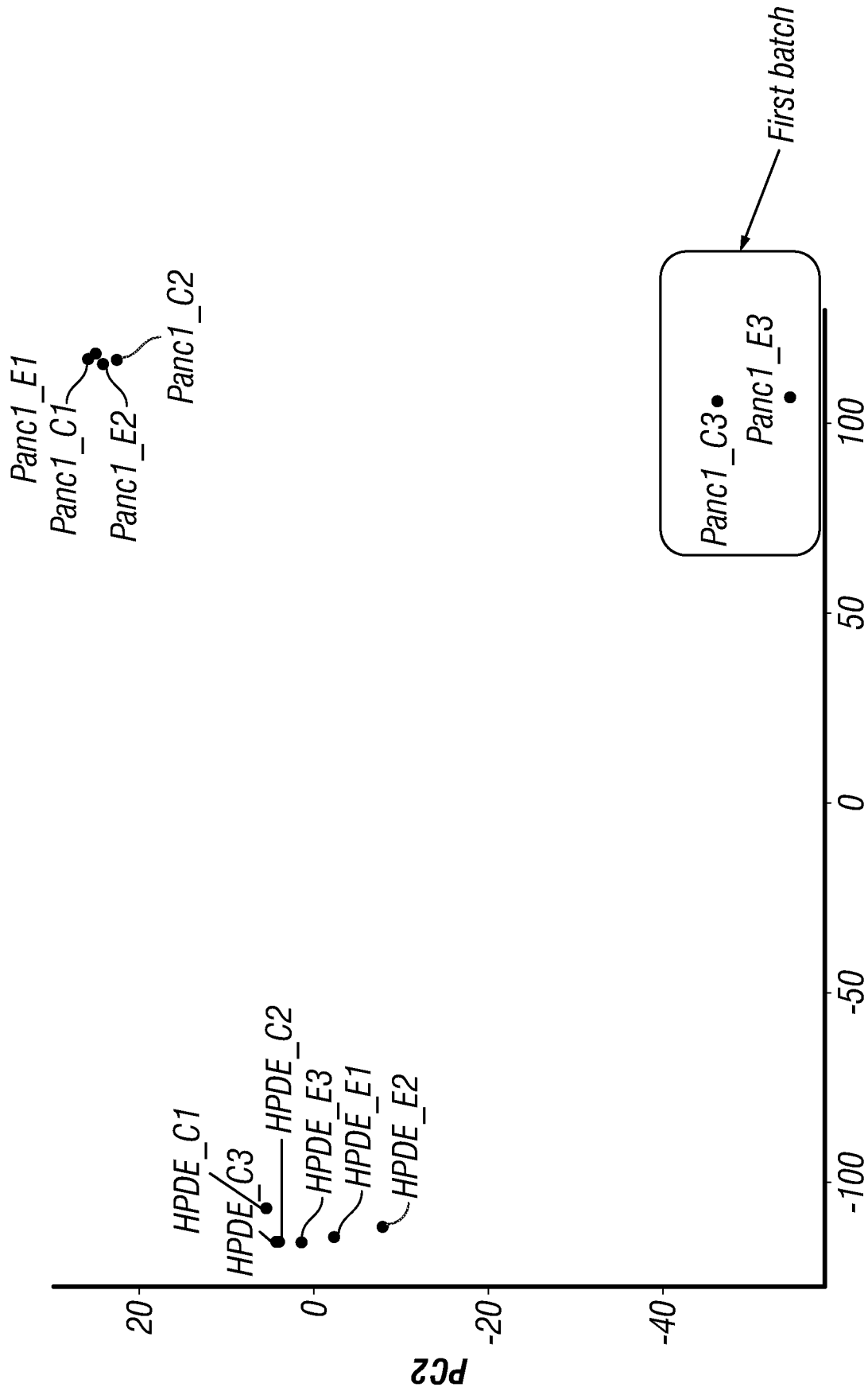
FIG. 7
(Cont'd)

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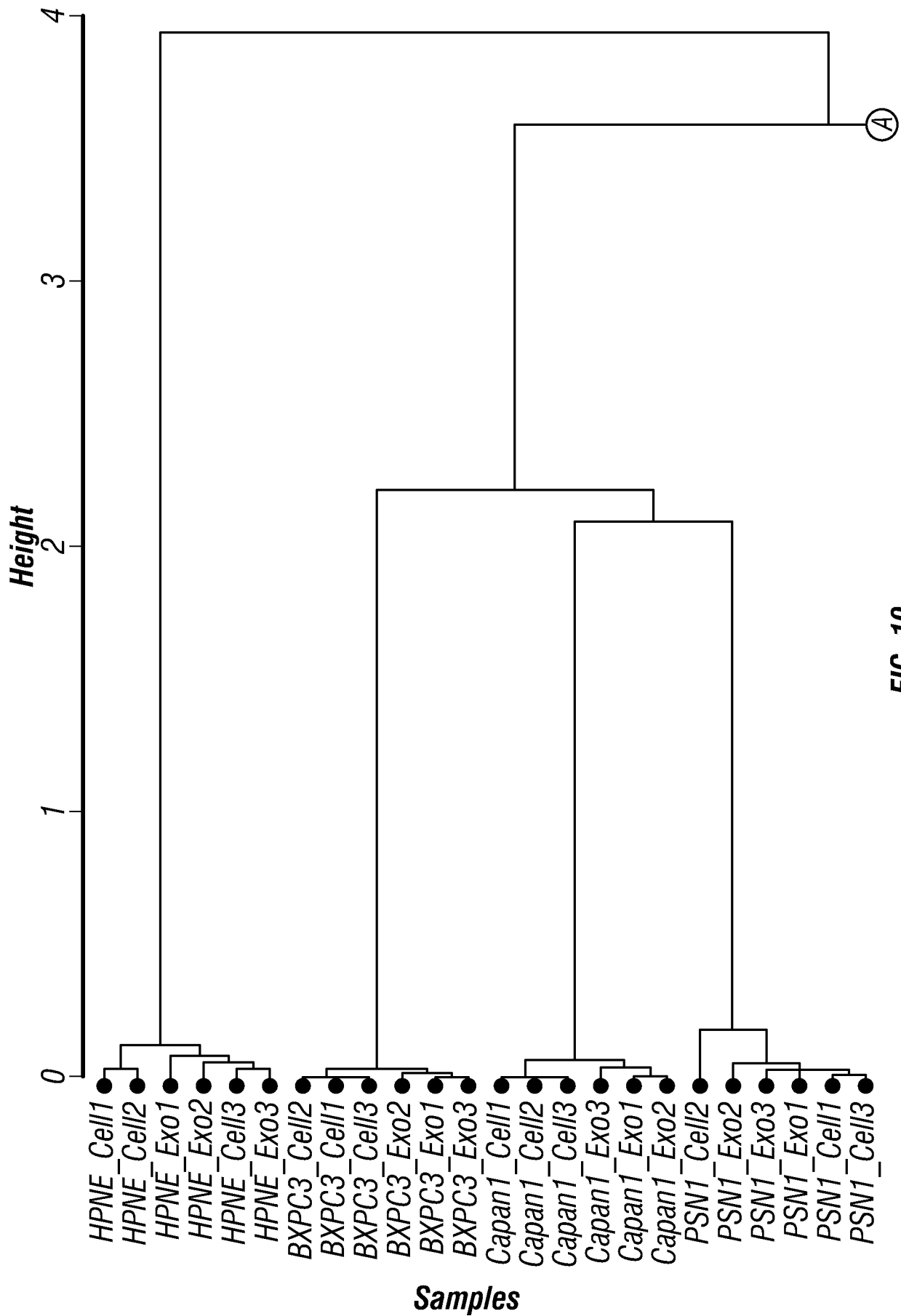


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PCA plot for HPDE and Panc1 cell/exosome RRBS



PC1
FIG. 9



Distance method: "correlation"; Clustering method: "ward"

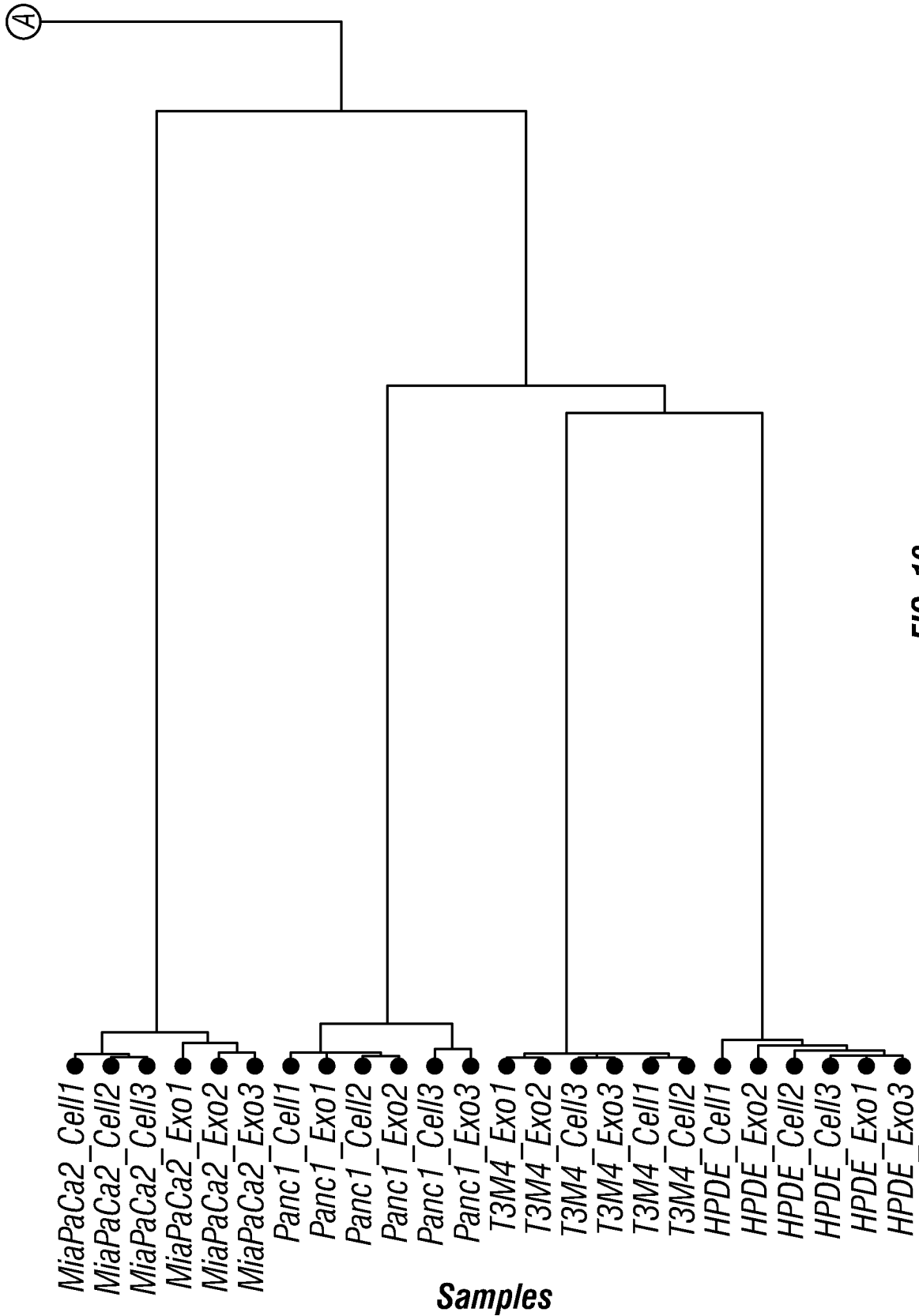


FIG. 10
(Cont'd)

Distance method: “correlation”; Clustering method: “ward”

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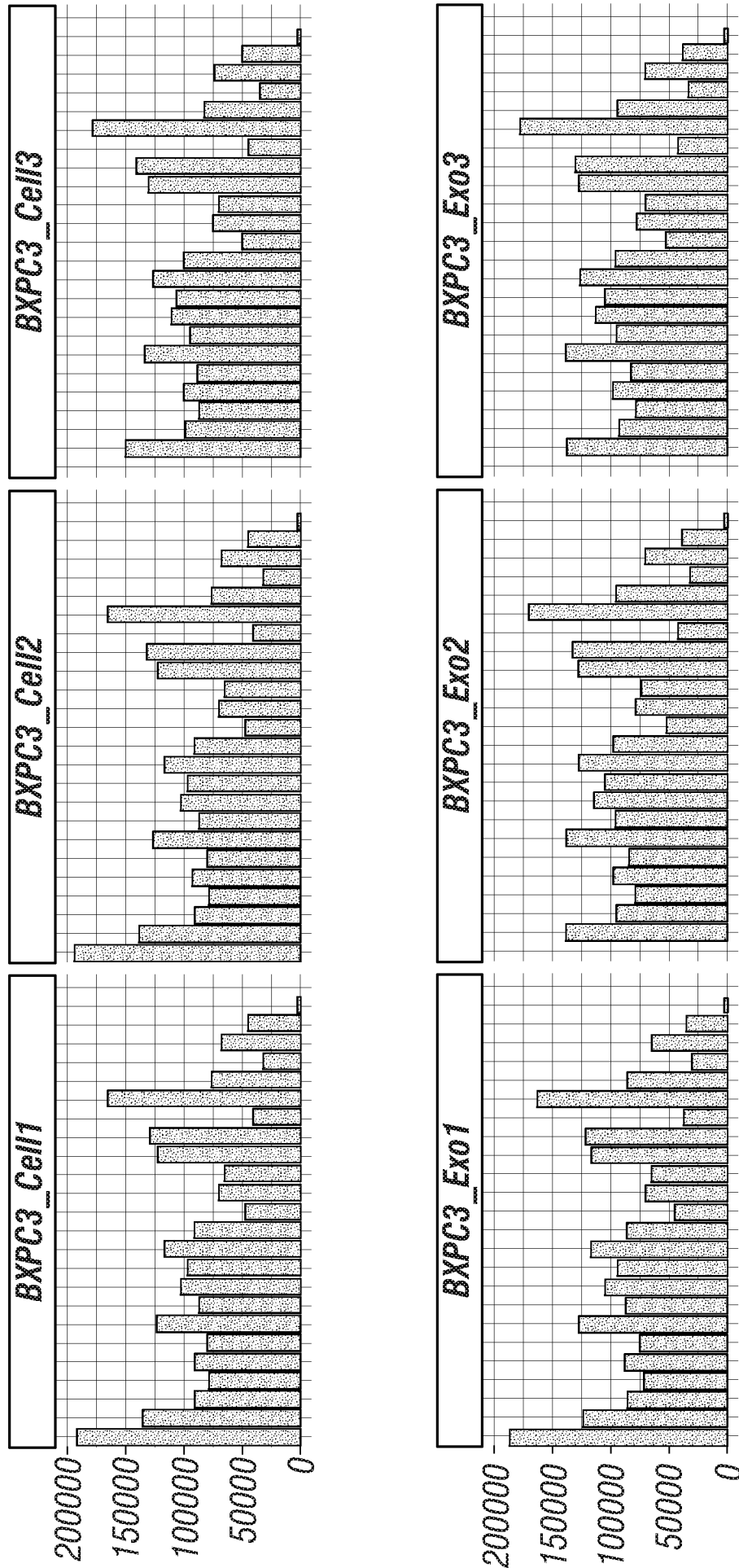


FIG. 11A

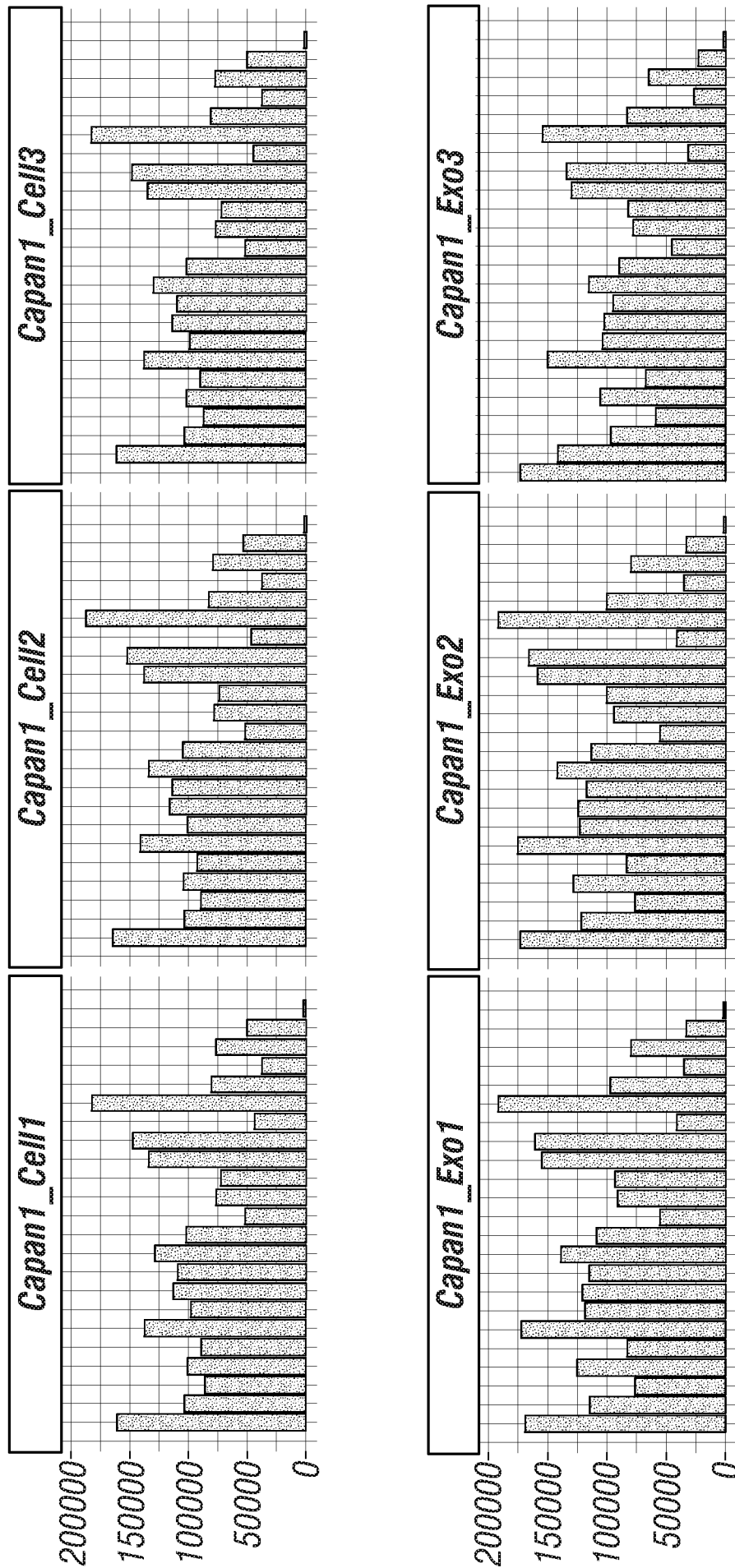


FIG. 11B

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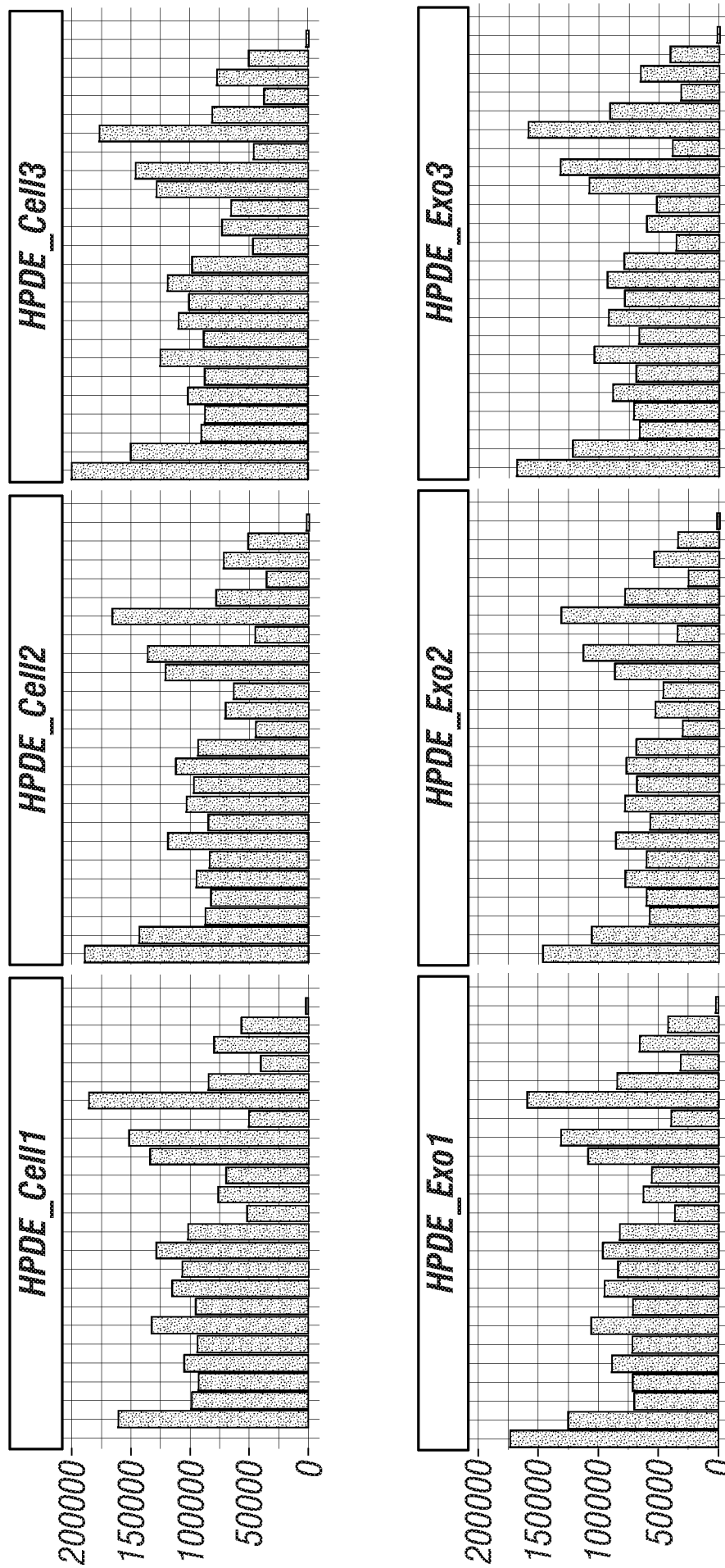


FIG. 11C

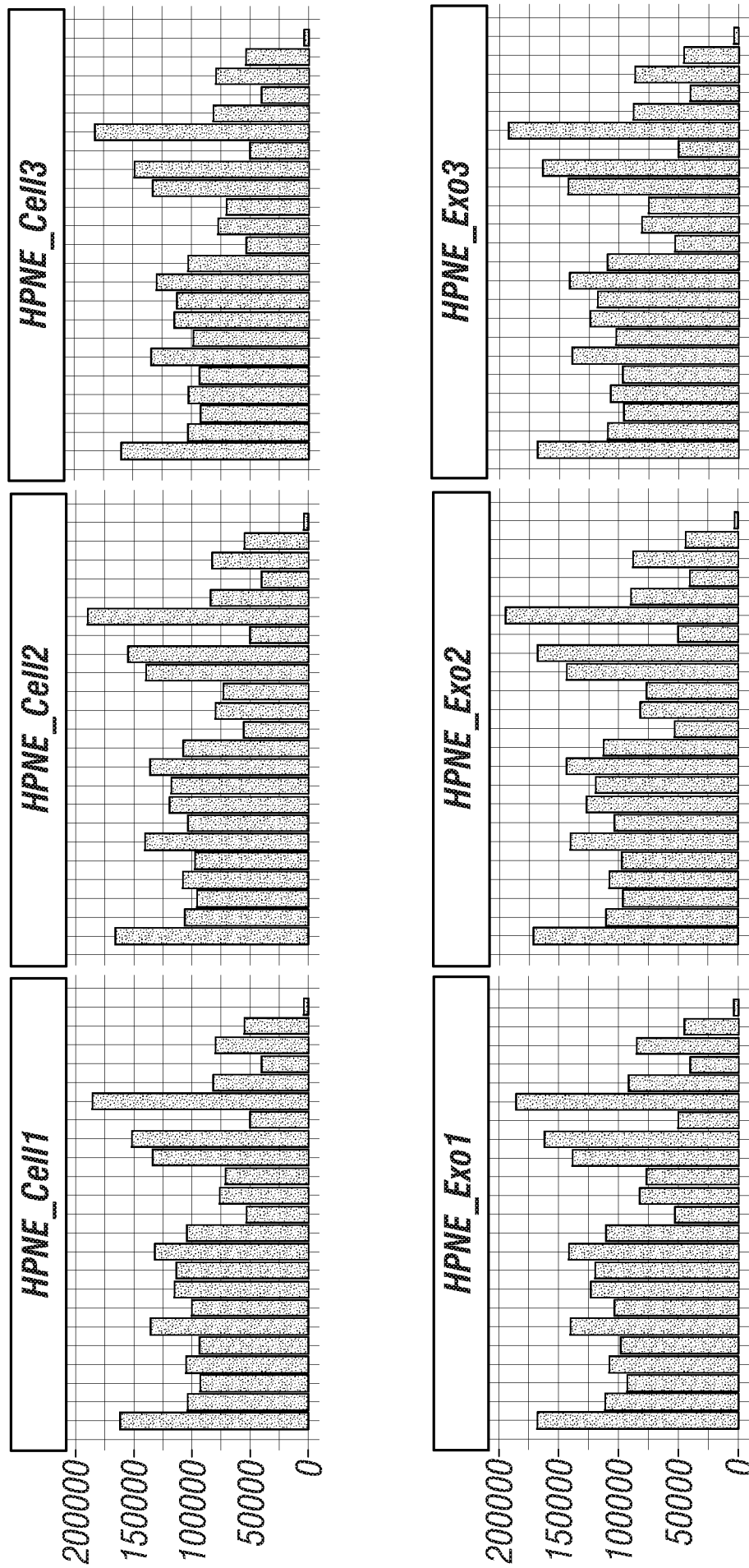


FIG. 11D

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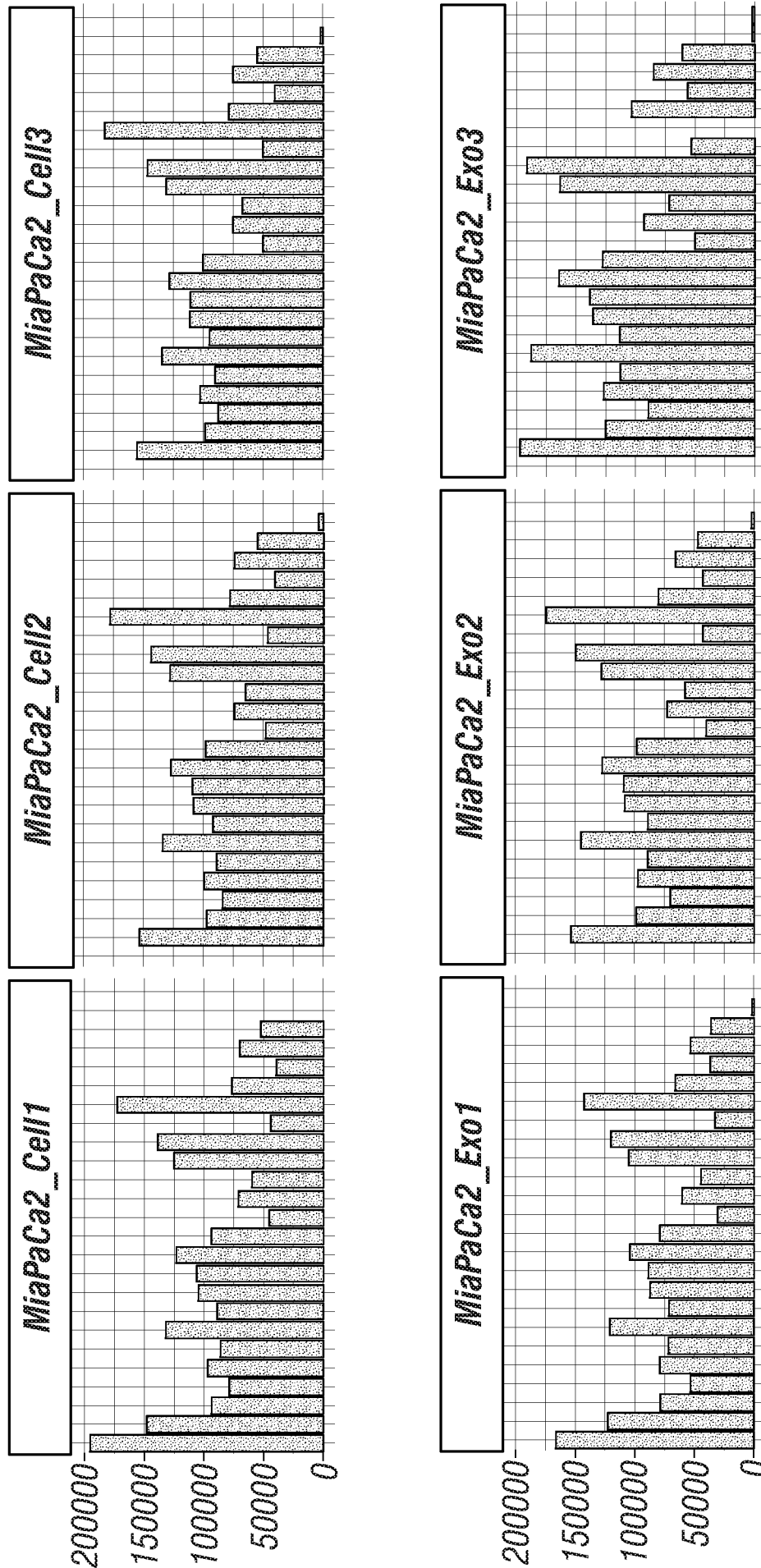


FIG. 11E

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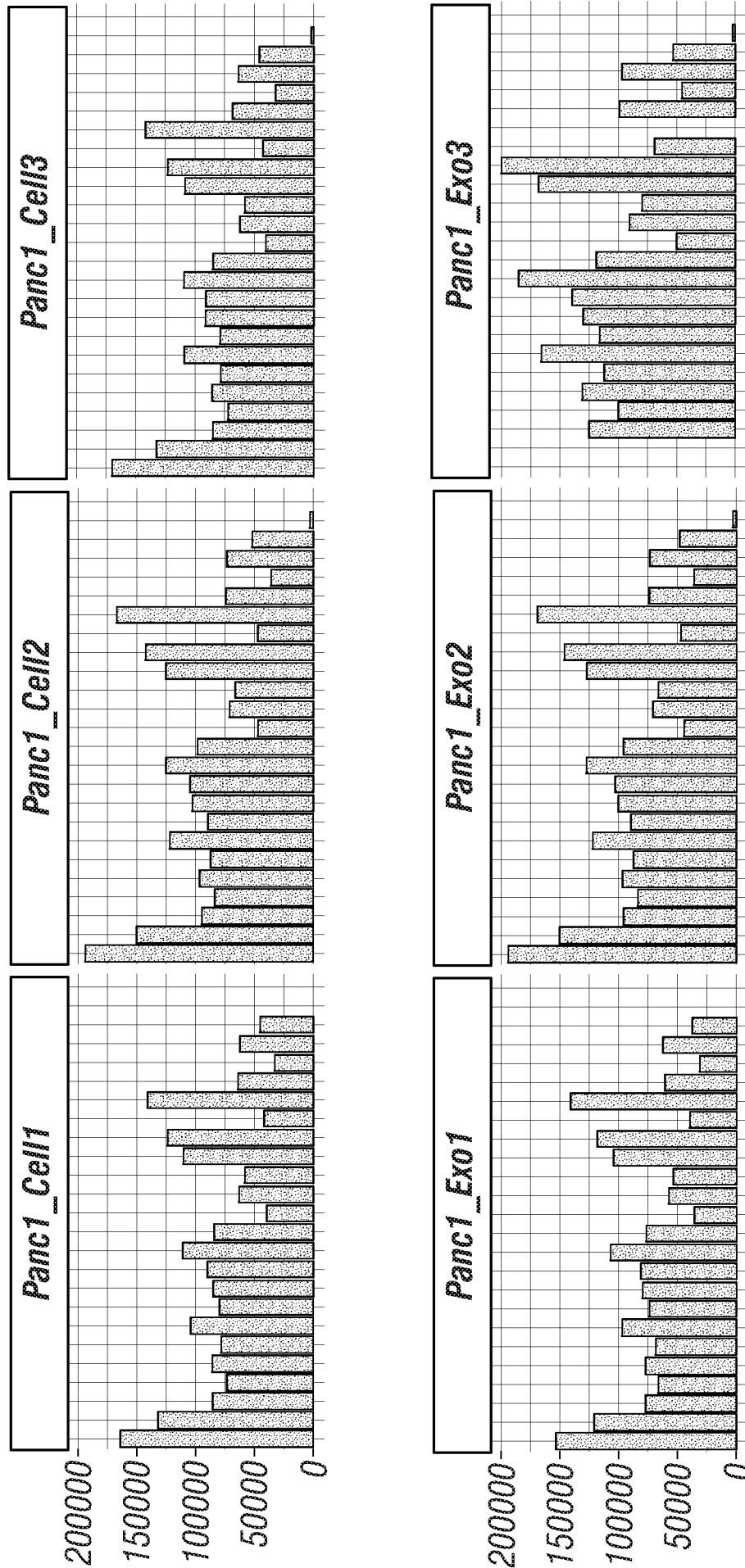


FIG. 11F

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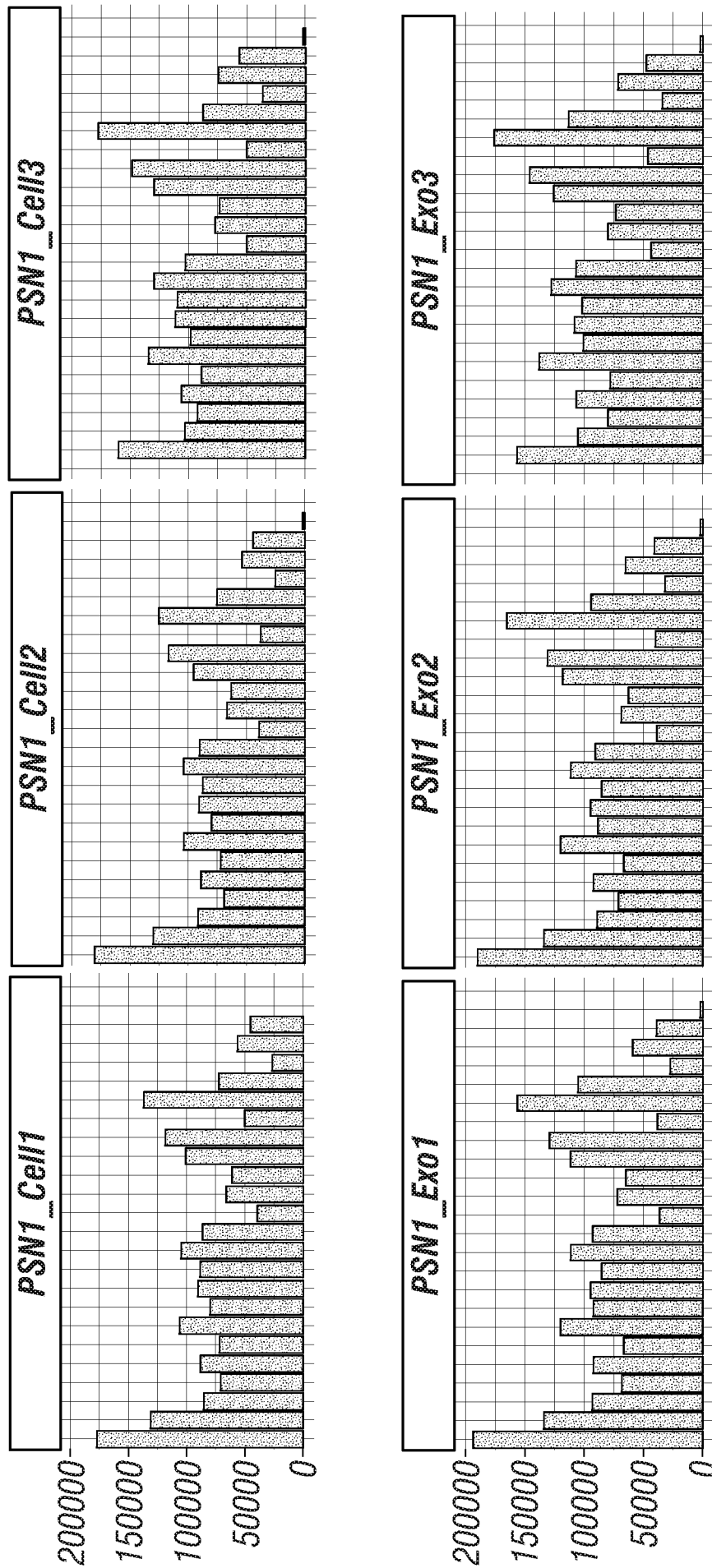
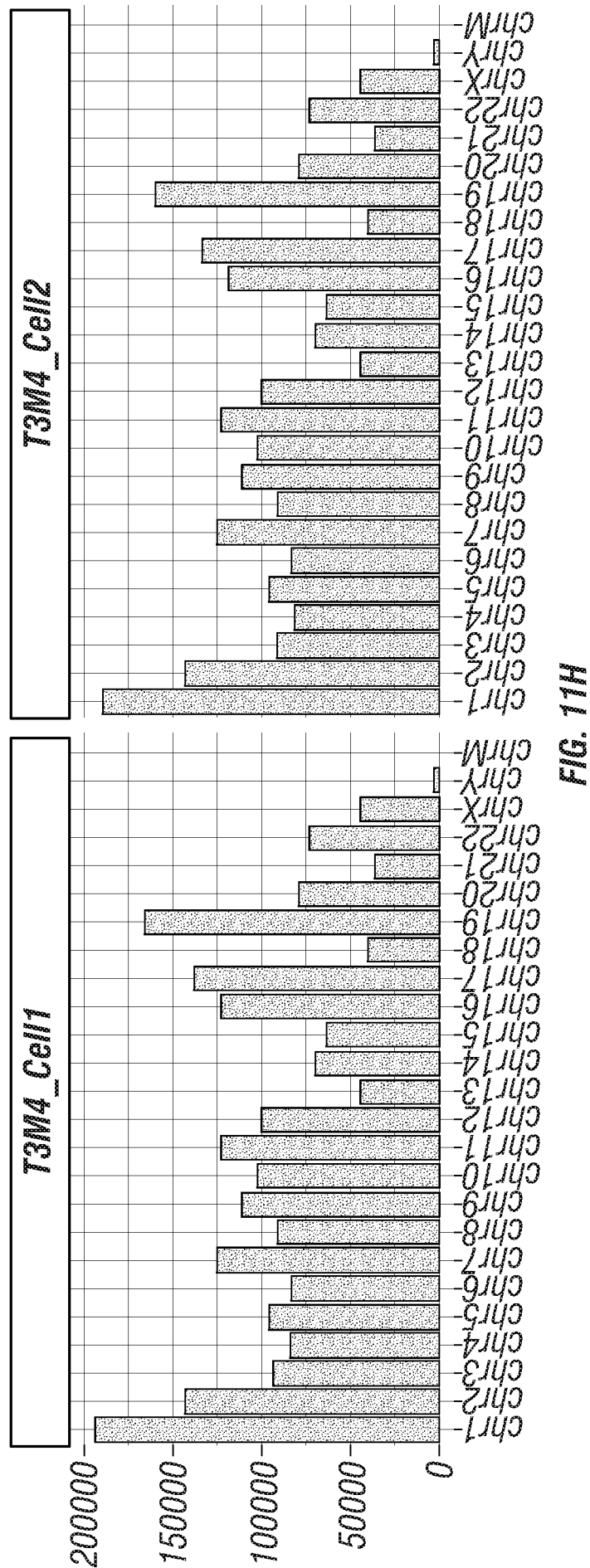


FIG. 11G

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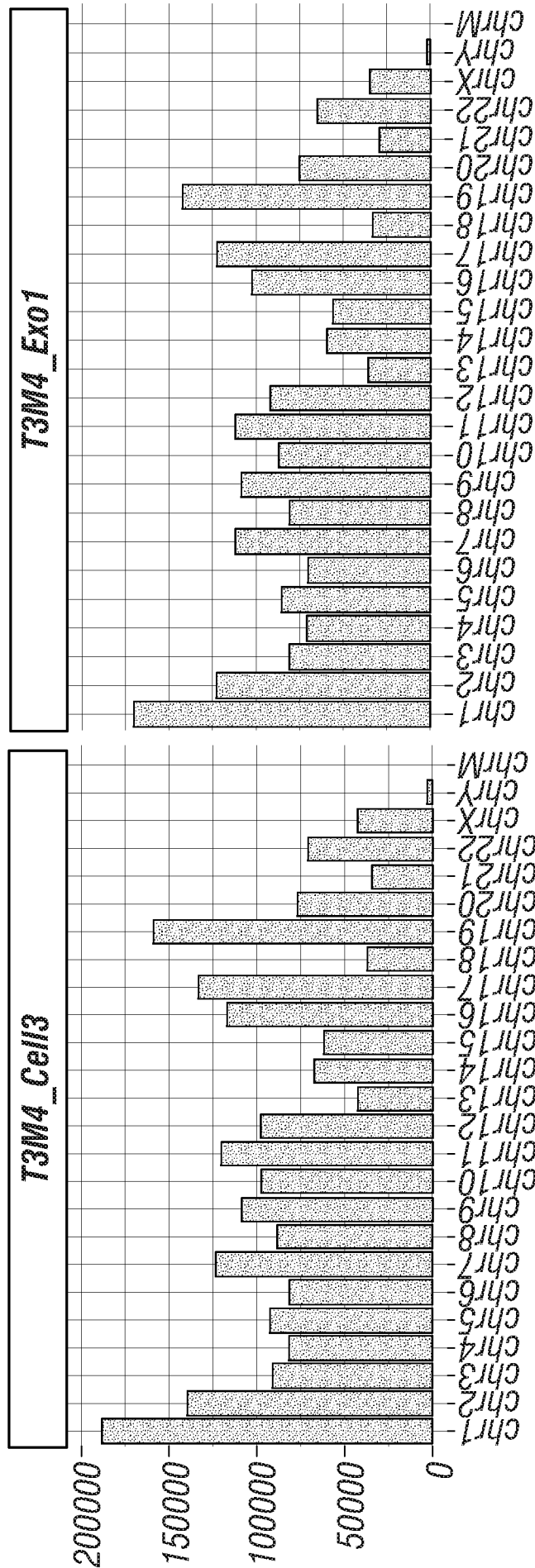


FIG. 11H
(Cont'd)

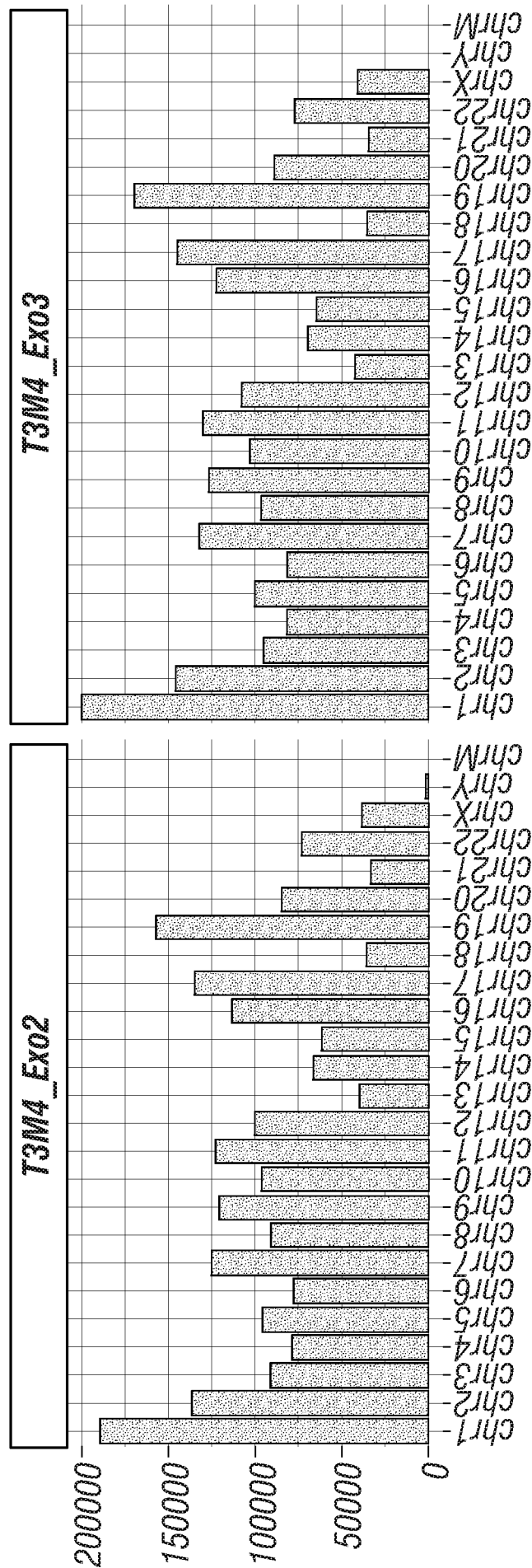


FIG. 11H
(Cont'd)