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(54) **Title**: COMPOSITIONS AND METHODS FOR TREATING CANCER

(57) **Abstract**: A method of killing a cancer cell expressing NCAM and optionally FZD7 is provided. The method comprising contacting the cancer cell with a cytotoxic moiety and an NCAM targeting moiety, thereby killing the cancer cell.

COMPOSITIONS AND METHODS FOR TREATING CANCER

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to compositions and methods for treating cancer, specifically, pediatric cancers having embryonic stems cell origin, such as Wilm's tumor.

Wilms tumor (WT) is one of the most common solid tumors of childhood, occurring in 1 in 10,000 children and accounting for 8 % of childhood cancers. With improved multimodality therapy, WT survival rates have risen over the last 40 years to 85 % - 90 %; however, for those whose disease relapses or metastasizes, even intensive salvage regimens result in subsequent survival closer to 50 %. Moreover, survivors are at increased risk of a broad spectrum of adverse outcomes caused by chemotherapy and radiation therapy, such as late mortality and secondary cancers.

WT is believed to result from malignant transformation of abnormally persistent renal stem cells which retain embryonic differentiation potential. Indeed, recent molecular data obtained by microarrays demonstrate that WTs and WT-stem like xenografts systematically overexpress nephric-progenitor genes corresponding to the earliest stages of normal metanephric kidney development [Dekel et al., 2006a; Li et al., 2002; Metsuyanim et al., 2008)], connecting tumorigenesis and organogenesis in the kidney [Rivera & Haber, 2005]. In addition to genes that specify the renal lineage, Wnt pathway-related molecules including b-catenin (CTNNB1), frizzled7 (FZD7) and frizzled2 (FZD2), are concomitantly induced (Dekel et al., 2006a). In general, the binding of Wnt ligand to frizzled cell surface receptors normally leads to inhibition of a "destruction complex" consisting of APC/Axin/GSK-3β/Ck1/Dvl and other factors, with subsequent accumulation of dephosphorylated stabilized β-catenin, and transcription of its target genes. Interestingly, recent data have demonstrated an essential role for the Wnt–b-catenin signaling pathway in nephrogenesis (Park et al., 2007; Schedl, 2007; Schmidt-Ott & Barasch, 2008) and also show a striking link between b-catenin signaling and the development of Wilms tumor (Maiti *et al.*, 2000; Rivera *et al.*, 2007; Schedl, 2007). In the latter case, deregulated Wnt signaling with aberrant accumulation of β-catenin in the cytoplasm/nucleus plays an important role.

It is becoming clear that many, if not most, malignancies arise from a population of cells that exclusively maintain the ability to self-renew and sustain the tumor via the

expression of tumor-progenitor genes [Singh et al., 2003; Van der Kooy and Weiss, 2000]. These ''cancer stem cells'' are often biologically distinct from the differentiated cancer cells that comprise most of the tumor bulk. Because cancer stem cells are believed to be primarily responsible for tumor initiation as well as resistance to chemo- and radiotherapy, their persistence may account for relapsing disease in WT [Bao *et al.*, 2006; Clarke *et al.*, 2006; Galmozzi *et al.*, 2006; Reya *et al.*, 2001]. Very recently, following prospective isolation of primary WT cells according to cell surface markers, an $NCAM^+$ cell population was defined that is a strong candidate for the tumor stem/progenitor cell population (Pode Shakked et al., 2008). Attempts to sort a WT cell population according to the over-expressed Wnt receptor, FZD7, failed due to extensive cell death following application of an anti-FZD7 antibody required for the isolation process (Pode Shakked et al., 2008).

U.S. Application Number 20090111177 relates to methods for maintaining the undifferentiated state of embryonic stem cells without the use of a feeder layer by activating the Wnt signal transduction pathway or by inhibiting glycogen synthase kinase-3 activity by contacting the cell with, inter alia, 6-bromoindirubin-3'-oxime.

U.S. Application Number 20060019256 provides gene expression profiles associated with solid tumor stem cells, as well as novel stem cell cancer markers useful for the diagnosis, characterization, and treatment of solid tumor stem cells.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a cytotoxic moiety and an NCAM targeting moiety.

According to an aspect of some embodiments of the present invention there is provided a composition of matter comprising a cytotoxic moiety attached to an NCAM targeting moiety.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising as active ingredients a cytotoxic moiety and an NCAM targeting moiety and a pharmaceutically acceptable carrier or diluent.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway.

According to an aspect of some embodiments of the present invention there is provided a pharmaceutical composition comprising as active ingredients FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway and a pharmaceutically acceptable carrier or diluent.

According to an aspect of some embodiments of the present invention there is provided a method of killing a cancer cell expressing NCAM and optionally FZD7, the method comprising contacting the cancer cell with a cytotoxic moiety and an NCAM targeting moiety, thereby killing the cancer cell.

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a cytotoxic moiety and an NCAM targeting moiety, thereby treating the cancer.

According to an aspect of some embodiments of the present invention there is provided a use of a cytotoxic moiety and an NCAM targeting moiety for the treatment of cancer.

According to an aspect of some embodiments of the present invention there is provided a method of killing a cancer cell expressing FZD7 and optionally NCAM, the method comprising contacting the cancer cell with an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway, thereby killing the cancer cell.

According to an aspect of some embodiments of the present invention there is provided a method of treating cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway, thereby treating the cancer.

According to an aspect of some embodiments of the present invention there is provided a use of an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway for the treatment of cancer.

According to some embodiments of the invention, the method further comprises determining an expression level of FZD7 and additionally or alternatively NCAM in the cancer cell prior to the contacting.

According to some embodiments of the invention, the method further comprises determining an expression level of FZD7 and additionally or alternatively NCAM in cells of the cancer prior to the administering.

According to some embodiments of the invention, the cytotoxic moiety comprises an agent capable of inhibiting the Wnt pathway.

According to some embodiments of the invention, the agent comprises an FZD7 targeting moiety.

According to some embodiments of the invention, the FZD7 targeting moiety comprises an anti FZD7 antibody.

According to some embodiments of the invention, the cytotoxic moiety comprises a chemotherapy, a toxin, a cytokine, an antibody or a radiotherapy.

According to some embodiments of the invention, the agent comprises DKK1 and sFRP1.

According to some embodiments of the invention, the agent comprises a demethylating agent.

According to some embodiments of the invention, the cancer is a pediatric cancer having an embryonic stem cell origin.

According to some embodiments of the invention, the pediatric cancer having an embryonic stem cell origin comprises Wilm's tumor.

According to some embodiments of the invention, the cytotoxic moiety and the NCAM targeting moiety are covalently attached.

According to some embodiments of the invention, the cytotoxic moiety and the NCAM targeting moiety are non-covalently attached.

According to some embodiments of the invention, the cytotoxic moiety and the NCAM targeting moiety are not attached.

According to some embodiments of the invention, the the composition comprises a bispecific antibody.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIGs. 1A-S illustrate enhanced WT Cell death after exposure to anti FZD7 antibody in the FZD7-sensitive but not in the resistant WTs.

Flow cytometric analysis of WT cells incubated over night with (treated) or without (untreated) anti-FZD7 antibody. Cell number is plotted as a function of the intensity of staining for annexin V; cells stained positive with annexin V antibodies are apoptotic or necrotic. The percentages of dead cells are indicated (Figures 1B, C, E, F, H, I, K, L, N, O, Q and R) in correlation to the percentage of cells expressing FZD7 on their surface (Figures 1A, D, G, J, M, P). Quadrants were set according to isotype control staining.

(Figures 1A-I) FZD7-sensitive WTs (Figures 1A-C - WO10; Figures 1D-F - WOO5; Figures 1G-I - WOO6).

(Figures 1J-R) FZD7-resistant WTs (Figures 1J-L - WOO4; Figures 1M-O - WOO7; Figures 1P-R - WOO2). Data presented are representative of two independent experiments; FACS-based killing assay at different concentrations of anti FZD7 Ab was performed on FZD7-sensitive and resistant WTs.

(Figure 1S) Summary of FACS analysis results for 7-AAD staining (dead cells) in the sensitive and resistant tumors at 6 different anti-FZD7 concentrations showing an increase in the percentages of dead cells following elevation in Ab concentrations only in the FZD7-sensitive WT (red line), while no significant increase in dead cells was observed in the FZD7-resistant WT (blue line). Percentages of 7-AAD$^+$ cells were normalized to the untreated (solvent only) control for each anti-FZD7 concentration and there for, control=0. Data presented is the mean of at least two separate experiments.

FIGs. 2A-H are FACS plots of one experiment at 4 representative concentrations (from left to right in μg/ml – 0, 18.3, 75, 150) of anti-FZD7 Ab showing a significant increase in the percentage of 7-AAD$^+$ cells only in the FZD7-sensitive WT (upper panel);

FIGs. 2I-J are FACs plots illustrating the percentages of cells expressing FZD7 receptor on their surface (10.5%) correlates to the percentages of 7-AAD$^+$ cells (8.4%) only in the FZD7-sensitive WT (Sensitive - upper panel) while no correlation is seen for the FZD7-resistant WT (Resistant – lower panel).

FIGs. 3A-L are photographs illustrating Anti FZD7 Antibody-induced endocytosis. WOO4 and WO1O cells were grown on glass coverslips, incubated with monoclonal anti-FZD7 antibody for 60 minutes at 4 °C, washed and subsequently shifted to 37 °C for the indicated time periods. The reaction was stopped by placing the cells on ice. Cells were labeled with Alexafluor 488-conjugated secondary Ab. DAPI nucleic acid stain (blue), was used. Figures 3A-C represents FZD7-resistant WT while Figures 3D-F shows FZD7-sensitive WT. White arrows indicate accumulation of the Ab-FZD7 complex in a single perinuclear organelle for the FZD7-resistant WT and in multiple, dispersed organelles for the FZD7-sensitive WT; (Figures 3G-J) Nuclear fragmentation was detected after 30min (left panels) and 60min (right panels) incubation periods with the Ab in the FZD7-sensitive WT. White arrows indicate nuclear fragmentation; (Figures 3K-L) Right image is the Z section of the left image. Scale Bars dimensions are indicated in the images.

FIGs. 4A-N are graphs and photographs illustrating in vitro depletion of potential WT stem cell population following treatment with anti- FZD7 Ab. (A-D) Evaluation of sphere formation after treatment with anti FZD7 Ab for 3 time periods in FZD7-sensitive WT (A). Number of spheres formed after each incubation period is

shown. After 4h incubation with the Ab, no sphere formation was detected. (B-D) Phase-contrast images of spheres formed in untreated (solvent only) WT cells, after treatment for 2 hours and overnight; (E-I) Evaluation of sphere formation in either untreated or anti FZD7 Ab treated FZD7-sensitive and resistant WTs (E-H) Phase-contrast representative images images of spheres formed after 4h incubation in untreated (Solvent only) and treated WT cells in both FZD7-sensitive WT (sensitive) and FZD7-resistant WT (Resistant). (I) Comparison of the number of spheres formed (per 20,000 plated cells) between untreated and treated either sensitive or resistant WT showing a decrease in the number of spheres formed after treatment with the Ab only in the FZD7-sensitive WT. Data presented is the mean of two separate experiments. Comparison of the number of colonies formed (per 2,500 plated cells) between untreated and treated either sensitive or resistant WTs, showing a decrease in the number of colonies formed after treatment with the Ab only in the FZD7-sensitive WT. Data presented is the mean of two separated experiments. Magnifications are indicated on the images.

FIGs. 5A-G are photographs illustrating reduced survival/proliferation of anti-FZD7 Ab treated WT cells grafted to the chick embryo chorioallantoic membrane (CAM). (A) Tumor nodules formed by dissociated WT cells seven days after grafting to the chick. The nodules are fluorescently labeled with the vital dye CFSE. The arrow indicates a large blood vessel in the CAM. Illustration of WT cell transplantation to the CAM of the fertile chick egg; (B&C) FZD7 Ab treatment reduces size of tumors formed on the CAM. In (B) large masses of human proliferating cells, usually identifiable by their larger nuclei, are present, their boundaries are indicated by dotted lines. Many of the WT cells are labeled with the proliferation marker Ki67 (red). By contrast, in (C), few human cells and few $Ki67^+$ cells are present in the FZD7 Ab treated grafts. This field contains the most WT cells observed in serial sections through two Ab-treated grafts. Most other fields contained only individual cells. (D-G) NCAM is only observed on human WT cells in grafts not treated with FZD7 Ab. NCAM is a surface marker found on potential WT stem/progenitor cells. Confocal micrographs show that many cells in untreated control tumors (arrow in F) on the CAM are positive for NCAM (D&F green surface staining), while none of the WT cells treated with anti-FZD7 antibody are $NCAM^+$. This staining pattern was observed in all examined sections of

both untreated and treated grafts. Figures 5F and G show only the NCAM and nuclear staining for clarity and nuclei in B-G are stained with Hoechst (blue).

FIGs. 6A-N are graphs and photographs illustrating FZD7-resistant WTs show nuclear ß-catenin staining and very low expression levels of Wnt pathway inhibitors relative to the FZD7-sensitive WTs

(Figures 6A-I) Immunofluorescence staining to evaluate the expression of β – catenin in different cell types. A human epithelium cell line (A) was used as positive control, while a human fibroblast cell line (B) with or (C) without primary antibody was used as negative control. β – catenin immunostaining was performed on FZD7-resistant WTs (D) WOO2, (E) WOO4, (F) WOO7 - lower panels demonstrate nuclear staining of β – catenin in these tumors, as well as on FZD7-sensitive WTs (J) WOO5, (K) WOO6, and (L) WO1O showing very little to no staining. Cell nuclei were stained with Hoechst 33342 (Blue) in all samples tested. Magnification X63 was used. Bars are indicated in the images; qRT-PCR analysis for the relative expression of (Figure 6M) Wnt pathway inhibitors: sFRP1 and DKK1; (Figure 6N) Wnt pathway genes: FZD7, CTNNB1 in the 6 Wilms' tumors, show the FZD7-resistant WTs express very low levels of the Wnt pathway inhibitors in comparison to FZD7-sensitive WT, while FZD7 and CTNNB1 are expressed at similar levels (slightly higher in the FZD7-resistant WTs) in all tumors analyzed. The values for WOO2 were used for normalization and therefore (=1) other values were calculated with respect to them. Results are the mean ± S.E.M of three separate experiments.

FIGs. 7A-R are plots illustrating addition of the Wnt pathway inhibitors, sFRP1, DKK1 to WT cells treated with anti FZD7 antibody sensitizes the FZD7-resistant WT to anti-FZD7 antibody.

Flow cytometric analysis of WT cells incubated overnight with or without (untreated) anti-FZD7 antibody in the presence or absence of the secreted Wnt pathway inhibitors: sFRP1 and DKK1 in representative (Figures 7A-L) FZD7-resistant WT; (Figures 7M-R) FZD7-sensitive WT; Cell number is plotted as a function of the intensity of staining for annexin V; cells stained positive with annexin V antibodies are apoptotic or necrotic. FACS plots for the percentage of FZD7 receptor expressing cells in the representative tumors are on the right (Figures 7F, L and R). Data presented are representative of two independent experiments.

FIGs. 8A-H are graphs and plots illustrating that demethylating agents AzaC+TSA restore the Wnt inhibitors expression to the highest levels in the FZD7-resistant WTs in comparison to the FZD7-sensitive WTs.

qRT-PCR for the relative gene expression of the Wnt pathway inhibitors in the 6 Wilms' tumors either treated with AzaC+TSA or with their solvents. Expression of (A) sFRP1; (B) DKK1; (C) Wnt5A, between the treated and untreated samples. The value of the treated sample (light bars) was used to normalize (=1) and the value of the untreated sample (dark bars) was calculated with respect to it in each of the tumors. Results are the mean ± S.E.M of four separate experiments. Transcript levels were normalized to the expression of GAPDH; (D) FZD7-resistant WT (WOO4, WOO7) either untreated or treated with anti FZD7, AzaC+TSA or both, showing no change in cell survival following either treatment (WOO7). Results are presented as the mean absorbance at 450nm using the MTS survival assay ± S.E.M of at least 3 replicates. (E-H) No change in the amount of cells expressing annexinV following these treatments was observed (WOO4). Cell number is plotted as a function of the intensity of staining for annexin V.

FIGs. 9A-V are plots and graphs illustrating sorting of WT cells according to FZD7 surface expression is not reliable due both to receptor trafficking and its cellular localization.

(A-B) Sorting experiments of WTs according to FZD7 surface expression. Shown are: a highly enriched positive cell fraction (>90%) (Upper panels), and a highly pure negative fraction (<3%) (Lower panels). Tumor codes: WOO4 (left panel) and WOO7 (right panel). Data presented are representative of four independent experiments for each tumor; (C-D) qRT-PCR analysis for the expression of FZD7 in the sorted fractions. Open bars represent the FZD7(+) and gray bars represent the FZD7(-) cell fraction. The value for the FZD7(+) was used to normalize (=1) and all other values were calculated with respect to it. Data presented are of four independent experiments. Sep.=separation; (E-T) Receptor trafficking viewed by FACS at three sequential time points of incubation with the primary antibody – 1hour, 2hours and 5hours. (E-L) Incubation with anti-FZD7 antibody: upper and lower panels show opposite routes for FZD7 receptor trafficking, internalization – upper panel and re-expression – lower panel. (M-P) Incubation with anti-CD133 antibody, (Q-T) Incubation with anti-NCAM antibody,

showing no change in the percentage of cells expressing these antigens over the indicated time periods; (U-V) Confocal microscope images of immunofluorescent staining for detection of the FZD7 protein in WT cells showing FZD7 localization to the cell membrane, cytoplasm and nucleus.

FIGs. 10A-H are graphs and photographs illustrating that FZD7 is a putative marker for the malignant renal stem cell containing population. (A-B) Primary WTs and their derived xenografts were analyzed for the expression of FZD7 (A) and WT1 (Bt) showing both to be significantly overexpressed by the blastemal enriched WT xenorafts in comparison to their primary source. Results are expressed as the mean ± S.E.M of at least three different tumor sources and their xenografts; qRT-PCR for FZD7 expression (C) Three of the separations according to the surface expression of FZD7 in WOO4 correlated to FZD7 mRNA expression. The value for the FZD7(+) cells was used to normalize (therefore=1) and the value for FZD7(-) was calculated with respect to it. Results are presented as the mean ± S.E.M of three separate experiments, * $P<0.05$; (D-E) Phase-contrast images of clones from FZD7(+) and FZD7(-) WT cells; (F) clonogenic capacity of the FZD7(+) versus the FZD7(-) WT cells. Results are presented as the mean ± S.E.M of three separate experiments demonstrating the FZD7(+) to be highly clonogenic compared to the FZD7(-) WT cells, * $P<0.05$; (G) Proliferation capacity of FZD7(+) and FZD7(-) WT cells. 300 cells were plated in triplicates and the number of cells was counted by trypan blue staining on days 0, 3, 7, 14. Results are presented as the mean of at least two separate cell counts demonstrating the FZD7(+) to be highly proliferative compared to the FZD7(-) WT cells; (H) qRT-PCR analysis of the WT-stem signature genes (FZD7, NCAM, SIX2, SALL1, BMI1, OCT4) and the WT poor prognostis marker, TOP2A, demonstrate elevated mRNA levels of these genes in the FZD7(+) cell fraction compared to the negative one. The values for the FZD7(+) cells were used to normalize (therefore=1) and all other values were calculated with respect to them. Results are presented as the mean ± S.E.M of three separate experiments.

FIGs. 11A-M are graphs and photograhs illustrating that $NCAM^{+}FZD7^{+}$ cell fraction may define stem-like cells in WT tumor

(A-D) Immunohistochemical staining for (A-B) NCAM and (C-D) FZD7 in WT shows that both are present in the malignant metanephric blastema (A and C) - magnification

X10. In addition NCAM stains immature tubular structures (B) – magnification X20, and FZD7, mature tubules (D) - magnification X40. Abbreviations: B-Blastema; IT-Immature Tubules; MT-Mature Tubules; Separation of WOO4 cells according to NCAM and FZD7 surface expression; (E-F) a negative cell fraction (<9%) (left panel) and an enriched positive cell fraction (>70%) (right panel). (G) qRT-PCR analysis of NCAM expression in the $NCAM^{+}FZD7^{+}$ and $NCAM^{-}$ cell fractions showing the double-positive population to highly express NCAM in comparison to the $NCAM^{-}$ cells; (H-I) Colony forming (CFU) assay shows that (H) $NCAM^{+}FZD7^{+}$ form more colonies in comparison to the $NCAM^{-}$ cells (I). The colonies formed by $NCAM^{+}FZD7^{+}$ cells contain significantly higher cell numbers than those formed by the $NCAM^{-}$ cells, * $P<0.05$; (J-M) CFU images of (J-Ki) $NCAM^{+}FZD7^{+}$ WT cells (magnification: x4 (D), x10 (K)), (L-M) $NCAM^{-}$ (magnification: x4 (L) x10 (M)).

FIGs. 12A-B are cartoons illustrating the Wnt signaling pathway. In the absence of Wnt signaling (left image), a cytoplasmic degradation complex [consisting of at least axin, adenomatous polyposis coli (APC) protein, glycogen synthase kinase 3 (GSK-3), and ß-catenin] leads to the phosphorylation of APC, ß-catenin, and axin by GSK-3. This leads to the ubiquitination of ß-catenin and its degradation by the proteasome. Thus, at steady state in the absence of Wnt signaling, ß-catenin is rapidly degraded in the cytoplasm. In addition, nuclear levels of ß-catenin are kept low by its interaction with APC and axin, both of which exist in the nucleus and have a nuclear export activity that shuttles ß-catenin back to the cytoplasm. Activation of the pathway is caused (right image) by interaction of secreted Wnts with transmembrane receptors encoded by the Frizzled gene family and with coreceptors such as low-density lipoprotein receptor-related protein-5 and -6 (LRP5 and 6). The Wnt-Frizzled interaction is enhanced by some proteoglycans, such as the glypican-related protein Dally, and it is antagonized by several secreted proteins, including Dickkopf and secreted frizzled-related protein (sFRP) family members. Activation of Frizzled homologs by Wnt ligands leads to activation of the modular protein Dishevelled (Dvl). LRP5 or LRP6 may also activate the pathway in response to Wnts, although this mechanism is less clear. In response to activation of Frizzled, Dvl has been reported to interact directly with Frizzled and then function through binding components of the degradation complex to reduce the function of GSK-3. This in turn reduces the phosphorylation and degradation of b-catenin,

generally leading to its accumulation in the nucleus. In the nucleus, without Wnt signaling, LEF and TCF (known as TCF/LEF), often repress gene expression. Elevation of b-catenin levels by Wnt signaling leads to binding of ß-catenin to TCF /LEF, promoting changes in the transcriptional machinery that lead to activation of target genes often resulting in cell survival and proliferation. Inappropriate activation of the pathway in response to mutations is linked to a wide range of cancers, including Wilms' tumor, colorectal cancer and Melanoma (Moon RT: Wnt/beta-catenin pathway. *Sci STKE* 2005, 2005(271):cm1).

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to compositions and methods for treating cancer, specifically, pediatric cancers having embryonic stems cell origin, such as Wilm's tumor.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

Wilms tumor (WT), the most frequent solid tumor in children, has been linked to aberrant Wnt signaling. The present inventors have demonstrate that different WTs can be grouped according to either sensitivity or resistance to an antibody specific to frizzled7 (FZD7), a Wnt receptor. In the FZD7-sensitive WT phenotype, the Ab induced cell death of the FZD7(+) fraction (Figures 1A-S), which in turn depleted primary WT cultures of their clonogenic and sphere-forming cells (Figures 4E-N) and decreased in vivo proliferation/and survival upon xenografting to the chick chorio-allantoic-membrane (Figures 5A-G). In contrast, FZD7-resistant WT in which no cell death was induced showed a different intra-cellular route of the Ab-FZD7 complex compared to sensitive tumors and accumulation of β-catenin (Figures 3A-J). This coincided with a low sFRP1 and DKK1 (Wnt inhibitors) expression pattern, restored epigenetically with de-methylating agents, and lack of β-catenin or WTX mutations. The addition of exogenous DKK1 and sFRP1 to the tumor's cells enabled the sensitization of FZD7-resistant WT to the FZD7 Ab (Figures 7A-R). Although extremely difficult to achieve due to dynamic cellular localization of FZD7, sorting of FZD7(+) cells from resistant

WT, showed them to be highly clonogenic/proliferative, overexpressing WT 'stemness' genes (Figures 10A-G), emphasizing the importance of targeting this fraction.

Together the present results indicate that FZD7 antibody therapy alone or in combination with Wnt pathway antagonists may have a significant role in the treatment of WT via targeting of a tumor progenitor population.

While further reducing the present invention to practice, the present inventors have shown that treatment specificity may be further conferred by combined therapy directed at NCAM and the FZD7/Wnt pathway (Figures 11A-I).

Thus according to an aspect of the present invention there is provided a method of killing a cancer cell expressing NCAM and optionally FZD7, the method comprising contacting the cancer cell with a cytotoxic moiety and an NCAM targeting moiety (and optionally an FZD7 targeting moiety), thereby killing the cancer cell.

As used herein the term "cancer" refers to the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

According to a specific embodiment the cancer is a solid tumor (e.g., pediatric) having an embryonic stem cell origin.

Examples of such solid tumors include, but are not limited to, sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma,

bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma. _Examples of pediatric solid tumors include but are not limited to: Wilms' tumor/Nephroblastoma, rhabdomyosarcoma, Ewing's family of tumors/primitive neuroectodermal tumor, Osteosarcoma, peripheral neuroectodermal tumors, Childhood Germ Cell Tumor, Extragonadal Germ Cell Tumor, Kidney Cancer, Liver Cancer, Neuroblastoma, Ovarian Cancer, Retinoblastoma, Sarcoma, more specifically, Osteosarcoma, Rhabdomyosarcoma, Desmoplastic small round-cell tumor, Hepatoblastoma, Germ cell tumors, neuroblastoma and Medulloblastoma.

It will be appreciated that cancer cells which are contemplated for killing are those which express NCAM, FZD7 or both. Thus according to the present teachings, the method may also include determining the level of NCAM and/or FZD7 expression prior to treatment using methods which are well known in the art (such as described in the Examples section which follows).

As used herein the term "NCAM" refers to the gene product of the NCAM gene e.g., NM_181351, NM_000615, NM_001076682 (SEQ ID NOS: 1-3 respectively) NP_851996.2, NP_000606.3 AND NP_001070150.1 (SEQ ID NOS: 5-7 respectively).

As used herein the term "FZD7" refers to the gene product of the FZD7 gene e.g., NM_003507 (SEQ ID NO: 4), NP_003498.1 (SEQ ID NO: 8).

Alternatively or additionally the invention provides for a method of killing a cancer cell expressing FZD7 and optionally NCAM, the method comprising contacting the cancer cell with an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway, thereby killing the cancer cell.

According to another embodiment, the method is effected by contacting the cell with an NCAM targeting moiety and an agent capable of Wnt pathway inhibition.

According to a specific embodiment the method is effected by contacting the cell with an FZD7 targeting moiety, an NCAM targeting moiety and an agent capable of Wnt pathway inhibition.

As used herein the phrase "targeting moiety" refers to a molecule which specifically binds (i.e., sequence-specific binding or structure specific binding e.g., epitope dependent) a polypeptide or a nucleic acid sequence encoding same. The targeting moiety may be an antibody or a peptide.

An "FZD7-targeting moiety" refers to a targeting moiety specific to FZD7. It will be appreciated that the FZD7-targeting moiety may also be capable of downregulating activity or expression of FZD7, such as an antibody specific to FZD7. An FZD7 activity refers not exclusively to a Wnt signaling activity.

An "NCAM-targeting moiety" refers to a targeting moiety specific to NCAM.

An "agent capable of inhibiting the Wnt pathway" refers an antibody, a small molecule, a peptide, a polypeptide (e.g., antagonist such as DKK1 and sFRP1) or a nucleic acid agent which is capable or down regulating activity or expression of at least one component of the Wnt signaling pathway (cannonical and non-cannonical). A schematic illustration of the Wnt signaling pathway is provided in Figure 9. Each of the components illustrated (or those revealed during the term of this patent) is a putative target according to the present teachings.

As used herein the term "composition of matter" refers to a single component or a two-, three- or four- component compositions. Examples of components include an NCAM targeting moiety, an FZD7 targeting moiety, an agent capable of inhibiting the Wnt pathway, a cytotoxic moiety, a chemotherapeutic moiety and a radiotherapy as well as other active ingredients used as anti cancer treatments (e.g., antibody therapy, cytokine) or others such as Pseudomonas exotoxin, Diphteria exotoxin, interleukin 2, CD3, CD16, Interleukin 4, Interleukin 10, Ricin toxin and HLA-A2.

The composition of matter may be packed in a packaging material wherein the component(s) are packed in a single or in separate containers.

According to a specific embodiment of the present invention the composition of matter comprises a cytotoxic moiety and an NCAM targeting moiety.

According to a specific embodiment of the present invention the composition of matter comprises a FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway.

The above mentioned components may be bound to each other by covalent or non-covalent binding or can be unbound. Thus, for example the cytotoxic moiety

and/or the agent capable of inhibiting the Wnt pathway may be comprised in an encapsulating particle and the NCAM and/or FZD7 targeting moiety may be attached to the particle surface.

As used herein, the phrase "encapsulating particle" refers to an entity that is characterized by the presence of one or more walls or membranes formulated from lipids and/or fatty acids that form one or more internal voids. The walls or membranes may be concentric or otherwise. The walls or membranes of vesicles may be substantially solid (uniform), or referred to as, for example, liposomes, lipospheres, nanoliposomes, particles, micelles, bubbles, microbubbles, microspheres, nanospheres, nanostructures, microballoons, microcapsules, aerogels, clathrate bound vesicles, hexagonal/cubic/hexagonal II phase structures, and the like.

The lipid component included in the encapsulating particles could include either a lipid derivatized with the targeting molecule, or a lipid having a polar-head chemical group that can be derivatized with the targeting molecule in preformed particles, according to known methods.

Other methods for the covalent attachment of the targeting moiety to the encapsulating particles include the use of an amide, ester, or ether bond, streptavidin and biotin (see, for instance, U.S. Pat. No. 5,171,578), and activation of a polypeptide with carbodiimide followed by coupling to the activated carboxyl groups (U.S. Pat. No. 5,204,096)). Other examples of methods that can be used to covalently bind a polypeptide to a lipid are disclosed in U.S. Pat. No. 5,258,499.

Downregulation of FZD7 or Wnt can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription and/or translation (e.g., RNA silencing agents, Ribozyme, DNAzyme and antisense), or on the protein level using e.g., antagonists, enzymes that cleave the polypeptide and the like.

Following is a list of agents capable of downregulating expression level and/or activity of FZD7 or a Wnt pathway component.

One example, of an agent capable of downregulating FZD7 or Wnt is an antibody or antibody fragment capable of specifically binding FZD7 or a Wnt pathway component. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

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

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')2, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')2, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')2 is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

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

Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in E. coli or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')2. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from

cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar et al. [Proc. Nat'l Acad. Sci. USA 69:2659-62 (19720]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as E. coli. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and Filpula, Methods 2: 97-105 (1991); Bird et al., Science 242:423-426 (1988); Pack et al., Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

According to a specific embodiment the antibody is a bispecific antibody, an antibody molecule which is designed to bind to two different antigens (e.g., NCAM and FZD7). Bispecific antibodies and methods of generating same are known in the art see e.g., 7,537,931, which is hereby incorporated by reference.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction

to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice,

humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., Bio/Technology 10,: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild et al., Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Non-limiting examples of therapeutic moieties which can be conjugated to the antibody of embodiments of the present invention are provided in Table 1, hereinbelow.

Table 1

Therapeutic moiety	***Amino acid sequence (GenBank Accession No.)***	***Nucleic acid sequence (GenBank Accession No.)***
Pseudomonas exotoxin	ABU63124	EU090068
Diphtheria toxin	AAV70486	AY820132.1
interleukin 2	CAA00227	A02159
CD3	P07766	X03884
CD16	NP_000560.5	NM_000569.6
interleukin 4	NP_000580.1	NM_000589.2

Therapeutic moiety	*Amino acid sequence (GenBank Accession No.)*	*Nucleic acid sequence (GenBank Accession No.)*
HLA-A2	P01892	K02883
interleukin 10	P22301	M57627
Ricin toxin	EEF27734	EQ975183

According to some embodiments of the invention, the toxic moiety is PE38KDEL.

The therapeutic moiety may be attached or conjugated to the antibody of embodiments of the invention in various ways, depending on the context, application and purpose.

When the functional moiety is a polypeptide, the immunoconjugate may be produced by recombinant means. For example, the nucleic acid sequence encoding a toxin (e.g., PE38KDEL) and be expressed in a host cell to produce a recombinant conjugated antibody. Alternatively, the functional moiety may be chemically synthesized by, for example, the stepwise addition of one or more amino acid residues in defined order such as solid phase peptide synthetic techniques.

A therapeutic moiety may also be attached to the antibody of the invention using standard chemical synthesis techniques widely practiced in the art [see e.g., hypertexttransferprotocol://worldwideweb (dot) chemistry (dot) org/portal/Chemistry)], such as using any suitable chemical linkage, direct or indirect, as via a peptide bond (when the functional moiety is a polypeptide), or via covalent bonding to an intervening linker element, such as a linker peptide or other chemical moiety, such as an organic polymer. Chimeric peptides may be linked via bonding at the carboxy (C) or amino (N) termini of the peptides, or via bonding to internal chemical groups such as straight, branched or cyclic side chains, internal carbon or nitrogen atoms, and the like. Description of fluorescent labeling of antibodies is provided in details in U.S. Pat. Nos. 3,940,475, 4,289,747, and 4,376,110.

Exemplary methods for conjugating therapeutic peptide moieties to the antibody of the embodiments of this invention are described herein below:

SPDP conjugation – A non-limiting example of a method of SPDP conjugation is described in Cumber et al. (1985, Methods of Enzymology 112: 207-224). Briefly, a peptide, such as a detectable or therapeutic moiety (e.g., 1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol); the antibody is mixed with a 25-fold excess of SPDP in 20 mM sodium phosphate, 0.10 M NaCl pH 7.2 and each of the reactions is incubated for about 3 hours at room temperature. The reactions are then dialyzed against PBS. The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5 % sample/column volume) with 50 mM KH_2PO_4 pH 6.5. The reduced peptide is combined with the SPDP-antibody in a molar ratio of 1:10 antibody:peptide and incubated at 4 °C overnight to form a peptide-antibody conjugate.

Glutaraldehyde conjugation - A non-limiting example of a method of glutaraldehyde conjugation is described in G.T. Hermanson (1996, "Antibody Modification and Conjugation, in Bioconjugate Techniques, Academic Press, San Diego). Briefly, the antibody and the peptide (1.1 mg/ml) are mixed at a 10-fold excess with 0.05 % glutaraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After-the reaction, the excess glutaraldehyde is removed using a G-25 column equilibrated with PBS (10 % v/v sample/column volumes)

Carbodiimide conjugation - Conjugation of a peptide with an antibody can be accomplished using a dehydrating agent such as a carbodiimide, e.g., in the presence of 4-dimethyl aminopyridine. Carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of an antibody (resulting in the formation of an ester bond), or an amino group of an antibody (resulting in the formation of an amide bond) or a sulfhydryl group of an antibody (resulting in the formation of a thioester bond). Likewise, carbodiimide coupling can be used to form analogous covalent bonds between a carbon group of an antibody and an hydroxyl, amino or sulfhydryl group of the peptide [see, J. March, Advanced Organic Chemistry: Reaction's, Mechanism, and Structure, pp. 349-50 & 372-74 (3d ed.), 1985]. For example, the peptide can be conjugated to an antibody via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide [B. Neises et al. (1978), Angew

Chem., Int. Ed. Engl. 17:522; A. Hassner et al. (1978, Tetrahedron Lett. 4475); E.P. Boden et al. (1986, J. Org. Chem. 50:2394) and L.J. Mathias (1979, Synthesis 561)].

Downregulation of FZD7 or a Wnt pathway component (e.g., beta-catenin) can be also achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g, the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Accordingly, the present invention contemplates use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment, the dsRNA is greater than 30 bp. The use of long dsRNAs (i.e. dsRNA greater than 30 bp) has been very limited owing to the belief that these longer regions of double stranded RNA will result in the induction of the interferon and PKR response. However, the use of long dsRNAs can provide numerous advantages in that the cell can select the optimal silencing sequence alleviating the need to test numerous siRNAs; long dsRNAs will allow for silencing libraries to have less complexity than would be necessary for siRNAs; and, perhaps most importantly, long dsRNA could prevent viral escape mutations when used as therapeutics.

Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. Brain Res. Protoc. 2004;13:115–125; Diallo M., et al., Oligonucleotides. 2003;13:381–392; Paddison P.J., et al., Proc. Natl Acad. Sci. USA. 2002;99:1443–1448; Tran N., et al., FEBS Lett. 2004;573:127–134].

In particular, the present invention also contemplates introduction of long dsRNA (over 30 base transcripts) for gene silencing in cells where the interferon pathway is not activated (e.g. embryonic cells and oocytes) see for example Billy et al., PNAS 2001, Vol 98, pages 14428-14433. and Diallo et al, Oligonucleotides, October 1, 2003, 13(5): 381-392. doi:10.1089/154545703322617069.

The present invention also contemplates introduction of long dsRNA specifically designed not to induce the interferon and PKR pathways for down-regulating gene expression. For example, Shinagwa and Ishii [*Genes & Dev.* 17 (11): 1340-1345, 2003] have developed a vector, named pDECAP, to express long double-strand RNA from an RNA polymerase II (Pol II) promoter. Because the transcripts from pDECAP lack both the 5'-cap structure and the 3'-poly(A) tail that facilitate ds-RNA export to the cytoplasm, long ds-RNA from pDECAP does not induce the interferon response.

Another method of evading the interferon and PKR pathways in mammalian systems is by introduction of small inhibitory RNAs (siRNAs) either via transfection or endogenous expression.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned the RNA silencing agent of the present invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing

occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (Castanotto, D. et al. (2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

According to another embodiment the RNA silencing agent may be a miRNA. miRNAs are small RNAs made from genes encoding primary transcripts of various sizes. They have been identified in both animals and plants. The primary transcript (termed the "pri-miRNA") is processed through various nucleolytic steps to a shorter precursor miRNA, or "pre-miRNA." The pre-miRNA is present in a folded form so that the final (mature) miRNA is present in a duplex, the two strands being referred to as the miRNA (the strand that will eventually basepair with the target) The pre-miRNA is a substrate for a form of dicer that removes the miRNA duplex from the precursor, after which, similarly to siRNAs, the duplex can be taken into the RISC complex. It has been demonstrated that miRNAs can be transgenically expressed and be effective through expression of a precursor form, rather than the entire primary form (Parizotto et al. (2004) Genes & Development 18:2237-2242 and Guo et al. (2005) Plant Cell 17:1376-1386).

Unlike, siRNAs, miRNAs bind to transcript sequences with only partial complementarity (Zeng et al., 2002, Molec. Cell 9:1327-1333) and repress translation without affecting steady-state RNA levels (Lee et al., 1993, Cell 75:843-854; Wightman et al., 1993, Cell 75:855-862). Both miRNAs and siRNAs are processed by Dicer and associate with components of the RNA-induced silencing complex (Hutvagner et al., 2001, Science 293:834-838; Grishok et al., 2001, Cell 106: 23-34; Ketting et al., 2001, Genes Dev. 15:2654-2659; Williams et al., 2002, Proc. Natl. Acad. Sci. USA 99:6889-6894; Hammond et al., 2001, Science 293:1146-1150; Mourlatos et al., 2002, Genes

Dev. 16:720-728). A recent report (Hutvagner et al., 2002, Sciencexpress 297:2056-2060) hypothesizes that gene regulation through the miRNA pathway versus the siRNA pathway is determined solely by the degree of complementarity to the target transcript. It is speculated that siRNAs with only partial identity to the mRNA target will function in translational repression, similar to an miRNA, rather than triggering RNA degradation.

Synthesis of RNA silencing agents suitable for use with the present invention can be effected as follows. First, the FZD7 or Wnt pathway component (e.g., beta-catenin) mRNA sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level (www.ambion.com/techlib/tn/91/912.html).

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

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

It will be appreciated that the RNA silencing agent of the present invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-penetrating peptide used in the membrane-permeable complex of the present invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of the present invention preferably include, but are not limited to, penetratin, transportan, pIsl, TAT(48-60), pVEC, MTS, and MAP.

mRNAs to be targeted using RNA silencing agents include, but are not limited to, those whose expression is correlated with an undesired phenotypic trait. Exemplary mRNAs that may be targeted are those that encode truncated proteins i.e. comprise deletions. Accordingly the RNA silencing agent of the present invention may be targeted to a bridging region on either side of the deletion. Introduction of such RNA silencing agents into a cell would cause a down-regulation of the mutated protein while leaving the non-mutated protein unaffected.

Thus, genes relating to Wnt pathway (e.g., FZD7 and beta catenin) might be targeted. Cancer-related genes include oncogenes (e.g., K-ras, c-myc, bcr/abl, c-myb, c-fms, c-fos and cerb-B), growth factor genes (e.g., genes encoding epidermal growth factor and its receptor, fibroblast growth factor-binding protein), matrix metalloproteinase genes (e.g., the gene encoding MMP-9), adhesion-molecule genes (e.g., the gene encoding VLA-6 integrin), tumor suppressor genes (e.g., bcl-2 and bcl-X1), angiogenesis genes, and metastatic genes. Rheumatoid arthritis-related genes include, for example, genes encoding stromelysin and tumor necrosis factor. Viral genes

include human papilloma virus genes (related, for example, to cervical cancer), hepatitis B and C genes, and cytomegalovirus (CMV) genes (related, for example, to retinitis). Numerous other genes relating to these diseases or others might also be targeted.

Another agent capable of downregulating a wnt pwathway is a DNAzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the Wnt pathway member. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh et al , 20002, Abstract 409, Ann Meeting Am Soc Gen Ther www.asgt.org). In another application, DNAzymes complementary to bcr-ab1 oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Downregulation of a Wnt pathway (e.g., FZD7 or beta-catenin) can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding same.

Design of antisense molecules which can be used to efficiently downregulate a gene of interest must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which

specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett et al. Blood 91: 852-62 (1998); Rajur et al. Bioconjug Chem 8: 935-40 (1997); Lavigne et al. Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki et al. (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

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

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

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)].

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully used [Holmund et al., Curr Opin Mol Ther 1:372-85 (1999)], while treatment of hematological malignancies via antisense oligonucleotides targeting c-myb gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz Curr Opin Mol Ther 1:297-306 (1999)].

More recently, antisense-mediated suppression of human heparanase gene expression has been reported to inhibit pleural dissemination of human cancer cells in a mouse model [Uno et al., Cancer Res 61:7855-60 (2001)].

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating a Wnt pathway member is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding a the wnt gene. Ribozymes are being increasingly used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

Another agent capable of downregulating Wnt would be any molecule which upregulates endogenous expression of Wnt antegonists such as demethylating agents as described in the examples section. Alternatively the level of Wnt antagonists can be

elevated exogenously by the addition of those antagonists

Demethylating agents are compounds that can inhibit methylation, resulting in the expression of the previously hypermethylated silenced genes (see methylation: methylation and cancer for more detail). Cytidine analogs such as 5-azacytidine (azacitidine) and 5-azadeoxycytidine (decitabine) are the most commonly used demethylating agents. These compounds work by binding to the enzymes that catalyse the methylation reaction, DNA methyltransferases; and titrate out these enzymes. Both compounds have been approved for treatment by Food and Drug Administration (FDA) in United States. Azacitidine and decitabine are marketed as Vidaza® and Dacogen® respectively.

The ability of the compositions described herein to kill cancer cells prompts their use in treating cancer in a subject in need thereof.

The term "treating" refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein, the term "preventing" refers to keeping a disease, disorder or condition from occurring in a subject who may be at risk for the disease, but has not yet been diagnosed as having the disease.

As used herein, the term "subject" includes mammals, preferably human beings at any age which suffer from the pathology. Preferably, this term encompasses individuals who are at risk to develop the pathology.

Each of downregulating agents described hereinabove compositions can be administered to the individual per se or as part of a pharmaceutical composition which also includes a physiologically acceptable carrier. The purpose of a pharmaceutical composition is to facilitate administration of the active ingredient to an organism.

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

Herein the term "active ingredient" refers to the component (e.g., FZD7 targeting moiety, Wnt inhibitor, NKAM targeting moiety, cytotoxic moiety, radiotherapy) accountable for the biological effect.

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

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

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

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, inrtaperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion protein that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active

agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

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

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

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

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

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars,

including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

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

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

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

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

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuos infusion. Formulations

for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

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

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

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

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

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

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

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

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

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

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

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

compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to". This term encompasses the terms "consisting of" and "consisting essentially of".

The phrase "consisting essentially of" means that the composition or method may include additional ingredients and/or steps, but only if the additional ingredients and/or steps do not materially alter the basic and novel characteristics of the claimed composition or method.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners,

means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

The word "exemplary" is used herein to mean "serving as an example, instance or illustration". Any embodiment described as "exemplary" is not necessarily to be construed as preferred or advantageous over other embodiments and/or to exclude the incorporation of features from other embodiments.

The word "optionally" is used herein to mean "is provided in some embodiments and not provided in other embodiments". Any particular embodiment of the invention may include a plurality of "optional" features unless such features conflict.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

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

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John

Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Culture of Animal Cells - A Manual of Basic Technique" by Freshney, Wiley-Liss, N. Y. (1994), Third Edition; "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

MATETRIALS AND EXPERIMENTAL PROCEDURES

Primary Wilms' tumor tissues and cell cultures: Primary WT samples were retrieved from patients with WT within an hour after surgery. All studies were approved by the local ethical committee and informed consents were provided by the legal guardians of the patients involved in this research according to the declaration of

Helsinki. Cell suspension was achieved as previously described (Pope-Shakked et al, 2008). Briefly samples were minced in HBSS, soaked in a combination of dissociating enzymes for 2 hours and then cultured in IMDM medium supplemented with 10% FBS and growth factors: 50ng/ml of bFGF, 50ng/ml of EGF (R&D systems).

Sequencing of CTNNB1 and WTX genes: Genomic DNA was extracted from WT cells using Qiagen DNA Extraction Kit according to the manufacturer instructions. The entire coding regions and splice sites were PCR amplified using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) and 10pmol of primer in a total volume of 25µl. The amplification conditions included initial denaturation at 96 °C for 4 minutes, 35 cycles at 94 °C for 1 min, annealing at Tm temperature for 45 sec, and 72 °C for 1 min followed by a 10 min extension step at 72 °C. PCR Primers for the CTNNB1 gene were designed based on accession number NT 022517.

PCR products were purified using Geneclean® Spin Kit (Qbiogene) and automated sequencing was performed on an ABI PRISM 3100 Genetic Analyzer using BigDye Terminator Cycle Sequencing Kit according to the manufacturer's protocol (Applied Biosystems). CTNNB1 and WTX sequence analysis was done by comparing the sequence data obtained to the CTNNB1 gene sequence (accession number NT_022517 and NM_152424 respectively) using NCBI-BLAST (httpblastdotncbidotnlmdotnihdotgovBlastdotcgi).

Real Time PCR analysis Quantitative real time reverse transcription PCR (qPCR) reactions was carried out as previously described [Pode-Shakked et al., 2008], to determine fold changes in expression of the Wnt pathway inhibitors and Wnt related genes between the 6 different WT samples and the Wnt pathway inhibitors between cells treated with 5-Aza-'2-Deoxycytidine (AzaC) and Tricostatin A (TSA) and untreated controls. In addition fold changes in expression of a selection of genes for stemness and TOP2A, between FZD7+ and FZD7- WT cells was determined. RNA was extracted using the microRNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was carried out using the High capacity cDNA RT kit (Applied Biosystems, Foster City, California, USA). GAPDH was used as endogenous control throughout all experimental analyses. Gene expression analysis was performed using TaqMan Gene Expression Assays on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, California, USA).

AzaC and TSA treatment of primary WT cell cultures: Cells were seeded at a density of 10^5 cells/25T flask on day 0. 24h after seeding, the demethylating agents AzaC and the de-acetylase inhibitor (HDAC inhibitor) TSA (Sigma-Aldrich, Steinheim, Germany) were added to a final concentration of 50 μM and 250 nM in fresh medium, respectively. Control cells were incubated with the addition of the same volumes of the solvents used for the AzaC and TSA – ETOH and Acetic acid respectively in fresh medium. 24 hours later, cells were harvested from both treated and control flasks for RNA extraction and cDNA production.

Fluorescence-activated cell sorting (FACS) analysis: FACS analysis was performed as previously described (Pode-Shakked et al, 2008). For analysis of FZD7, CD133 and NCAM expression in different time points, cells were harvested using non enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis). Surface antigens were labeled by incubation with either fluorochrome conjugated – CD133:PE (Miltenyi biotech) and NCAM:APC (eBioscience, San Diego, CA) or biotin conjugated – anti FZD7 (R&D systems), primary antibodies, for 45 minutes in the dark at 4°C to prevent internalization of antibodies. After a washing step, the cells were incubated for 20–30 minutes with the strepavidin APC in addition to 7-amino-actinomycin-D (7AAD; eBioscience, San Diego, CA) for viable cell gating. Staining with strep-aviding APC (eBioscience, San Diego, CA) was then performed on additional samples after 2h and 5h to minimize signal downregulation due to bleaching. Staining was performed in FACS buffer consisting of 0.5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis) and 0.02% sodium azide in PBS and all washing steps were performed in buffer consisting of 0.1%BSA in PBS. Quantitative measurements were made from the cross point of the IgG isotype graph with the specific antibody graph. Data were additionally analyzed and presented using FlowJo software (Tree Star, Ashland, OR).

Cells were harvested as described above, filtered through a 30μm nylon mesh before final centrifugation, and then resuspended in flow cytometry buffer consisting of 0.1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS. Cells were labeled with anti FZD7 biotin conjugated antibody (R&D systems) and a strep-avidin:APC (eBioscience) was used for visualization of stained cells. Fluorescence-activated cell sorter FACSAria (BD Biosciences, San Jose, CA) was used in order to enrich for cells expressing FZD7.

Experiment conditions and analysis were performed as previously described (Pode-Shakked *et al.*, 2008)

Immunofluorescence staining for FZD7 receptor: WOO4 cells (10^5 cells/mL) were grown on 12-mm round glass coverslips. For immunofluorescence processing, cells were washed twice with PBS and fixed for 10 minutes at room temperature in 4 % paraformaldehyde/PBS. Cells were subsequently washed 3 times with PBS and permeabilized for 5 minutes with PBS, 1 % BSA and 0.5 % Triton X-100. After 3 washes with PBS, cells were incubated for 30 minutes at room temperature with PBS and 1 % BSA and were subsequently incubated for 60 min with anti FZD7 antibodies diluted in PBS/1 % BSA (4 μg/100 μl or 0.8 μg/coverslip), washed 3 times in PBS and incubated for 30 minutes in the dark with the appropriate secondary antibody (Alexa Fluor 488-conjugated gout anti–rat, at 1:500 dilution in PBS/1 % BSA). Coverslips were subsequently washed in PBS and mounted with Gel Mount medium containing DAPI nucleic acid stain. Samples were analyzed using Leica SP5 confocal microscope and Olympus IX81 microscope equipped with Orca-ER Hamamatsu camera.

***FZD7 Antibody uptake*:** – WOO4 and WO1O cells were incubated with monoclonal anti FZD7 antibodies (1.5 μg/100 μ l or coverslip) for 60 minutes on ice, washed 3 times in PBS and followed by incubation at 37 °C for the indicated time periods to allow internalization [Haberman et al, 2007]. Cells were washed in PBS, fixed for 10 minutes at room temperature in 4% paraformaldehyde/PBS and were subsequently processed as indicated above.

Assessment of cell death In order to evaluate the effect of anti FZD7 antibody on WT cells' survival, an Annexin-V staining kit (eBioscience, San Diego, CA) was used according to the manufacturer specifications. Early apoptotic cells can be stained by Annexin V, which binds to phosphotidyl-serines normally found in the cytoplasmic surface of the cell membrane but can be found on the outer aspect of the cell membrane in apoptotic cells. During necrosis and late apoptosis, membrane integrity is compromised, and cells are stained by both Annexin V and 7AAD; Cells were treated with 0.1 mg/ml of anti-FZD7 antibody with respect to untreated control. Both samples were kept at 4 °C for 12 h. After the indicated incubation time cells (1×10^6) were collected, washed and re-suspended in Annexin V binding buffer (eBiocience) for preparation of 100μl samples and appropriate controls. Subsequently, 5 μl of either

fluoroscein isothiocyanate (FITC) or Allophecoaritin (APC)-conjugated annexin V was added to the samples and/or controls followed by incubation for 15 min at room temperature in the dark. Annexin V binding was evaluated by using FACSria (Becton Dickinson) with CELLQUEST software (BD Biosciences). In order to determine the effect of exogenous addition of secreted Wnt inhibitors' - sFRP1 and DKK1 (R&D systems) or treatment with AzaC+TSA on FZD7 resistant, semi-resistant and sensitive-WT either treated with anti FZD7 or alone, cells were treated with 0.1μg/μl of anti-FZD7 antibody or with 0.02μg/μl and 0.005μg/μl of sFRP1 and DKK1 respectively or with all three, with respect to untreated control. Similarly, experiments were performed with AzaC+TSA and untreated cells. Samples were kept at 4 °C for 12 hour prior to Annexin V staining and FACS analysis as described above.

In vitro cytotoxicity assay (FACS-based killing assay)

In order to determine the dose of anti-FZD7 required to kill WT cells expressing FZD7 on their surface (as assessed previously by FACS analysis for the percentage of FZD7-expressing cells in a given tumor), a FACS-based killing assay was developed. FZD7- sensitive and -resistant WT cells were washed with PBS, resuspended at 1 × 10^6 cell/ml in PBS/BSA 0.1 %, and then either incubated with anti-FZD7 Ab or with the same amount of Ab diluent for 4 hours. Next, 10 μl 7-amino-actinomycin-D (7AAD; eBioscience, San Diego, CA) per 100 μl buffer was added to the treated and untreated cells. Following incubation in the dark for 10 minutes, cells were washed with 1 ml PBS, resuspended in 500 μl PBS/BSA 0.1 % and immediately analyzed using FACSAria with CELLQUEST software (BD Biosciences, San Jose, CA). Data were subsequently analyzed and presented using FlowJo software (Tree Star, Ashland, OR). 7-AAD was used which interacts with DNA in cells which have lost membrane integrity, as a marker for late cell death.

Cell survival assay Nephroblastoma cells were plated in 96 well plates at $5x10^3$ cells/well in culture medium and treated with 0.1μg/μl of anti FZD7 antibody or with 250μg/ml of AzaC or with a combination of both, in respect to untreated controls for 48h. After the indicated time, the viability was assessed by the addition of the novel tetrazolium compound (3-[4,5-dimethylthiazol-2-yl-5]-[3-carboxymethyoxyphenyl]-2-[4-sulfophenyl]-2H tetrazolium) MTS (Promega, Madison, WI) for 4 hours, followed by

measuring absorbance at 450 nm on an enzyme-linked immunosorbent assay (ELISA) microplate reader.

Immunofluorescence staining for β-catenin Cells were collected from 6 well plates after 5 minutes of Trypsin Digestion. Cells were washed and plated on glass slides for 5 hours or over night. Slides were fixed using cold acetone and then air dried. Cells were washed three times with PBS and blocked with 7 % horse serum. Primary anti Human β – catenin (Millipore corp.) was added at a 1:150 dilution for 60 min. Following staining the slides were washed three times with PBS and anti mouse alexafluor 568 (Molecular Probes INC. Invitrogen) was applied at 1:200 dilution for 30 min. After the final washes cover slips were mounted and put on the slide. Images were taken using the ZEISS ApoTome grid system. Breast epithelial cells and human fibroblasts were used as positive and negative control respectively.

Single-cell cloning by limiting dilution. Limiting dilution assay was performed as previously described [Pode-Shakked et al 2008; Tropepe et al., 1999]. WT tumor cells were sorted according to FZD7 expression and the sorted cell fractions were plated in 96-well micro well plates (Greiner Bio-One, Mediscan, Kremsmunster, Austria) in 150μl of culture media, at 0.3 or 1 cells per well dilution. The low cell concentration was achieved by serial dilutions reaching 1000 cells per ml. The number of colonized wells was recorded after 4 weeks.

Trypan blue proliferation assay: After sorting of FZD7-resistant WT cells according to FZD7 expression, positive and negative cells were seeded at the same density of 10^3 cells/well in 96-well plates in triplicates on day 0. On days 1, 3, 7, 14 cells from three wells were harvested and suspended in medium, separately for each well. Subsequently, an aliquot of cell suspension from each well was diluted with 0.4 % trypan blue (Sigma–Aldrich), pipetted onto a haemocytometer and counted under a microscope at 10× magnification. Live cells excluded the dye, whereas dead cells admitted the dye and consequently stained intensely with trypan blue. Counts were done in triplicates for each well and three wells for each time points were used. Proliferation was evaluated by comparing the number of viable FZD7+ with the number of viable FZD7- WT cells in 5 time points over 21 days.

Immunohistochemical staining of WT: Sections, 4-μm thick, were cut from whole blocks of human fetal kidney from 20 weeks of gestation, human adult kidney

and WT for immunohistochemistry. Immunostainings were performed as previously described [Dekel et al, 2006b;Pode Shakked et al, 2008]. In brief, the sections were processed within 1 week to avoid oxidation of antigens. Before immunostaining, sections were treated with 10mM citrate buffer, PH 6.0 for 10 min at 97°C in a microwave oven for antigen retrieval, followed by treatment of 3% H_2O_2 for 10 min. The slides were subsequently stained by the labeled strepavidin-biotin (LAB-SA) method using a Histostain plus kit (Zymed, San Francisco, CA, USA). Anti human FZD7 antibody (Novus biological), at a dilution of 1:50 were used. Controls were prepared by omitting the primary antibodies or by substituting the primary antibodies with goat IgG isotype. The immunoreaction was visualized by an HRP-based chromogen/substrate system, including DAB (brown) chromogen (liquid DAB substrate kit – Zymed, San Francisco, CA, USA).

Sphere forming assay: Cells from FZD7 resistant and sensitive WTs were either treated with 0.5mg/ml per 10^6 cells of anti FZD7 Ab or with the Ab solvent for 4h. Following incubation cells were plated in ultra low attachment six-well plates (Corning Life Sciences, Wilkes Barre, PA, USA) at 20,000 cells/well in serum-free DMEM-F12 (Invitrogen), supplemented with 10 ng/mL basic fibroblast growth factor (bFGF) and 20ng/mL Epidermal grot factor (EGF). Spheres were recorded and counted after incubation for 7 days at 37°C, 5% CO_2. In order to determine the ideal incubation duration for achieving the best Ab efficacy, incubation was conducted for 3 time periods (2h, 4h, 16h), and sphere formation was evaluated. We deduced that 4h are sufficient for achieving the maximal effect and all following procedures were performed in this incubation length.

Colony forming assay: FZD7 resistant and sensitive WT cells either treated or untreated with anti FZD7 Ab were plated in growth medium on matrigel coated 12 well plates at 2500 cells/well, in duplicates. $NCAM^+FZD7^+$ and $NCAM^-$ WT sorted cells were plated in growth medium on matrigel coated 12 well plates at 25000 cells/well dilution in duplicates. Medium was changed twice a week. Colonies were recorded after two weeks and both their number and the number of cells/colonies were counted. The mean colony number and the mean number of cells in each colony for each cell fraction were calculated from the sum of all triplicates.

Grafting of WT cells on the chick embryo chorioallantoic membrane (CAM): Fertile chicken eggs were obtained from a commercial supplier, and incubated at 37 ^{0}C at 60-70 % humidity in a forced-draft incubator. At 3 days of incubation, an artificial air sac was established dropping the CAM. A window was opened in the shell, and the CAM exposed on 9 or 10th day of incubation. WT cells derived from WT xenografts established in NOD/SCID mice were stained with CFSE as previously described (Ren et al., 2007) to allow detection of grafted cells. Following washing 3x, cells were treated with 0.5 mg/ml per 10^6 cells of anti FZD7 Ab or with its diluent (PBS/BSA 0.1 %) for 4 hours, were washed, suspended in 15 µl serum free medium and Matrigel (1:1 by volume) and pipetted into a plastic ring placed on the membrane. The egg was then sealed with adhesive tape and returned to the incubator. After one week, the graft was removed, paraffin embedded, and serially sectioned at 6 µm for histological and immunocytochemical analyses. Sample sections were stained with an anti-human lysosome antibody at intervals of 100-150 µm in order to find the grafted cells in the large mass of Matrigel (not shown). Double-immunocytochemistry for Ki67 (mammalian-specific monoclonal rabbit antibody, Lab Vision clone SP6) and NCAM (mouse monoclonal, Santa Cruz) was performed on the sections containing WT cells using microwave antigen retrieval. Detection was performed with Alexa-488 anti-mouse and Alexa-594 anti-rabbit antibodies (Molecular Probes), and slides were counterstained with Hoechst, and all serial sections were examined. Photomicrographs were made with digital cameras (CFW-1312M and CFW-1612C, Scion Corporation) on Olympus SZX12 and BX51 microscopes. Confocal images were made with an Olympus Fluoview 2000 using 3 lasers and sequential acquisition of fluorophores. A Z-series of 7 optical slices was made of a field, and the in-focus portions of each channel image combined into a single image using the "extended depth of field" plugin of ImageJ (httprsbwebdotnihdotgovij). All changes in the images (contrast, brightness, gamma, sharpening) were made evenly across the entire field, and no features were removed or added digitally

Statistical analysis Results are expressed as the mean values ± S.E.M of the mean, unless otherwise indicated. Statistical differences between WT cell populations were evaluated using the non parametric, one sided sign test. Statistical differences of

two group data were compared by Student's *t* test. For all statistical analysis, the level of significance was set as $P<0.05$

EXAMPLE 1

WT cell death following application of FZD7 antibody

Recently, failure to sort FZD7 expressing cells from primary WT cell cultures due to cell death following application of an FZD7 antibody was demonstrated (Pode Shakked et al., 2008). This was determined by trypan blue staining following cell sorting which showed only the FZD7 (+) cells to undergo extensive death while the FZD7(-) cells survived. As shown in Figures 1A-I, incubation of WT cells originating from 3 separated tumors (W005, W006, W010) with the FZD7 antibody resulted in increased percentage of cells expressing annexinV, indicative of apoptotic and dead cells (FZD7-sensitive WT). Elevation in the number of annexinV positive cells correlated with the size of FZD7 expressing population in a specific tumor, for example Figure 1B shows 34.9 % of cell death in the Ab treated vs. untreated cells in correlation to 37.6 % of the cells that express FZD7 receptor on their surface (Figure 1A). In contrast, examination of additional WTs enabled the collection of viable cells after cell sorting according to the FZD7. Incubation of cells retrieved from these tumors (W004, W007) with the FZD7 antibody did not have an effect on the amount of annexinV expressing cells (Figures 1J-1O) regardless of the percentage of cells expressing FZD7 (FZD7-resistent WT).

For example, Figures 1J-L shows the percentage of dead cells following treatment with the Ab is not elevated, and therefore there is no correlation with the 20.6 % of cells that express FZD7 receptor on their surface. In Figures 1P-R, W002, there was only a weak response to application of the anti-FZD7 antibody. The percentage of dead cells (Annexin V^+) was significantly lower (<3%) than the number of cells expressing FZD7 (>15%) in this tumor. A FACS-based killing assay on FZD7 sensitive and resistant WTs revealed that higher anti-FZD7 concentrations induced significant cell death exclusively in the sensitive tumors (Figures 1S and 2A-H) Moreover, percentage of cell death in the sensitive tumor at the highest anti FZD7 concentration (150μg/ml) correlated with the percentage of cells expressing FZD7 on their surface

(Figures 2I-J). Taken together these findings support both the anti-FZD7 Ab killing effect in a subset of WTs and its specificity for WT cells expressing the FZD7 receptor.

EXAMPLE 2

Intracellular trafficking of FZD7 in FZD7-sensitive and resistant WT

Having determined unique phenotypes of FZD7-sensitive and resistant WTs by FACS and killing assays, the present inventors next followed intracellular trafficking of FZD7 receptor in cells from these two types of tumor cells. Immunoflouresence and confocal microscopy showed that following conjugation of the receptor with the antibody, FZD7 takes different intracellular roots in FZD7-sensitive and resistant WTs. In the FZD7-sensitive WT the Ab-FZD7 complex accumulates in multiple organelles dispersed within the cell cytoplasm while in the FZD7-resistant WT cells, FZD7 traffics to a single perinuclear organelle and cells appear completely intact (Figures 3A-F). Additionally, after a 30 minute incubation period, initial nuclear fragmentation starts to appear only in the FZD7-sensitive WT cells (Figure 3G-J). Thus, following application of the FZD7 Ab, different intra-cellular pathways of the FZD7 receptor and cell outcome were noted, further verifying the FZD7 sensitive and resistant WT phenotypes.

EXAMPLE 3

Anti-FZD7 Ab depletes stem cell properties in FZD7-sensitive WT

FZD7(+) cells from FZD7-sensitive WT cannot be prospectively isolated in order to evaluate this cell fraction for stem/progenitor characteristics. The present inventors therefore asked whether application of the FZD7 Ab to FZD7-sensitive WT cultures and specific elimination of the FZD7-expressing cells might result in depletion of the stem/progenitor cell fraction assayed by sphere-formation and clonogenic ability ((Singh et al., 2004), (Fillmore & Kuperwasser, 2008; Somervaille & Cleary, 2006). Initially FZD7-sensitive WT were treated with anti-FZD7 for several time periods and the sphere-forming ability of the remaining WT cells at each time point was evaluated. After 4 hours of incubation with the Ab, no sphere formation was observed in the remaining cells, and therefore this duration of incubation was used in all following experiments (Figures 4A-D). As shown (Figure 4E-I), the number of spheres formed following treatment with the Ab was diminished only in the FZD7-sensitive tumor.

Additionally, CFU number originating from single cells was also significantly abrogated in the sensitive but not in the resistant WT (Figures 4J-N). Thus, the anti-FZD7 Ab is likely to target a potential tumor stem/progenitor cell population.

EXAMPLE 4

Anti-FZD7 Ab reduces proliferation/survival of FZD7-sensitive WT cells grafted to the chick embryo

Grafting of mammalian cancer cells to the chorioallantoic membrane (CAM) of the fertile chick egg is a well established xenograft system (Rashidi & Sottile, 2009; Taizi et al., 2006). The present inventors performed an experiment to see if this system could be used to distinguish the effects of the FZD7 Ab on growth and proliferation of FZD7-sensitive WT cells *in vivo (Deryugina & Quigley, 2008; Rashidi & Sottile, 2009)*. First, 3 million WT cells were vitally labeled with CFSE, and then grafted with Matrigel onto the CAM. Seven days after grafting, nodules of fluorescent cells were detected by macroscopic observation of CFSE fluorescence (Figure 5A). After determining feasibility, smaller numbers of WT cells that were incubated with FZD7 Ab or with buffer (untreated controls) were grafted. Serial sections through areas of the grafts containing human cells stained with a mammalian-specific Ki67 antibody for detection of proliferating human cells (n=2 treated and n=2 non-treated grafts), revealed that in grafts formed by control, untreated cells, many tumor foci labeled with the proliferation marker Ki67 were present (Figure 5B). In contrast, few, only single or very small groups of proliferating human cells were present in the FZD7-treated grafts; a field with the largest number of human cells found in the treated grafts is shown in Figure 5C. Thus, the anti-FZD7 Ab reduces the survival and/or proliferation of sensitive WT *in vivo*, in addition to in-vitro. Interestingly, NCAM expressing cells, recently proposed as putative WT stem/progenitor cells (Pode-Shakked et al., 2008), were also detected in the control grafts (Figure 5D and F- arrow) in tumor foci, but not in FZD7-treated WT grafts using confocal microscopy (Figure 5E and G).

EXAMPLE 5

ß-catenin localization and Wnt pathway gene expression in FZD7-sensitive and resistant WT.

Since constitutive Wnt/ß-catenin activation might explain resistance versus sensitivity to FZD7 Ab, the present inventors initially determined the expression and localization of ß-catenin in FZD7-resistant and sensitive WT's by immunofluorescence (Figures 6A-I). Nuclear positivity and accumulation of ß-catenin was found only in the resistant tumors, supporting constitutive activation of the Wnt pathway. Next, the WT cohort was screened for mutations in ß-catenin (15 % of WTs contain activating mutations Major et al., 2007; Rivera et al, 2007), a finding that can explain constitutive activation. Nevertheless, sequencing of all axons of both these gene revealed that none of the tumors contained mutations in ß-catenin or WTX. WTX polymorphism was found in one WT sample (rs34677493 that causes missense F159L). This polymorphism is common among the population from which he patient derives (Sub-Saharan African). To begin to elucidate additional mechanisms for the contrasting cell phenotypes, the mRNA expression of beta-catenin, FZD7, and the secreted Wnt inhibitors, sFRP1 and DKK1 was analyzed by qRT-PCR. In FZD7-resistant WT's both the Wnt inhibitors were found to show significantly low levels compared to FZD7-sensitive WT (Figure 5J), while FZD7 and beta-catenin did not show a specific trend between groups (Figure 5K), suggesting that constitutive Wnt/ß-catenin signaling might occur via silencing of the secreted Wnt inhibitors

EXAMPLE 6

Exogenous administration of the Wnt inhibitors can sensitize resistant cells to FZD7 Ab.

Having observed the reduced Wnt inhibitor levels in FZD7-resistant WTs, the effects of exogenous administration of sFRP1 and DKK1 were observed on these cells. Interestingly, significant elevation in annexinV expression relative to untreated or FZD7 alone treated cells was observed only when incubating resistant cells with sFRP1 and DKK1 in conjunction with FZD7 Ab, while the addition of Wnt inhibitors alone showed only mild elevation in apoptotic cells (Figures 7A-L). In contrast to FZD7-resistant WTs, the addition of sFRP1 and DKK1 to the FZD7 Ab in sensitive cells showed only a

minor effect on cell death in comparison to cells treated with FZD7 alone (Figure 7M-R). Thus, addition of the Wnt pathway antagonists sensitizes FZD7-resistant WT to the action of FZD7 Ab.

EXAMPLE 5

Epigenetic regulation of Wnt inhibitors in FZD7-sensitive and resistant WT.

Having demonstrated a functional role for the Wnt inhibitors in the FZD7 resistance mechanism, the present inventors investigated potential mechanisms regulating their expression. Both sFRP1 and DKK1 are known to be epigenetically modified in various types of cancer (Jost et al., 2008; Nojima et al, 2007; Suzuki et al, 2008). Nevertheless, epigenetic regulation of sFRP1 and DKK1 has yet to be demonstrated in WT. The present inventors therefore treated both FZD7-sensitive and resistant WT cells with the de-methylating agents AzaC and TSA and studied their effects on expression of the Wnt inhibitors. A striking difference between DKK1 and sFRP1 was found; while sFRP1 was especially re-induced in the FZD7- resistant WT cells (Figure 8A), DKK1 was re expressed after treatment with AzaC/TSA in all WT analyzed (Figure 8B). Changes in expression of the Wnt inhibitors following epigenetic modification were especially evident when compared to control molecules such as Wnt5A whose expression was hardly affected (Figure 8C). In contrast to exogenous administration of DKK1 and sFRP1, the elevation of DKK1 and sFRP1 after treatment with AzaC/TSA did not result in sensitization of resistant WT cells to the FZD7 antibody (Figure 8D). Since the addition of AzaC/TSA results in large-scale epigenetic alterations (including various constitutes of the Wnt pathway) the inability to acquire FZD7-sensitivity following their administration, emphasize the specific effect of the secreted Wnt inhibitors. Thus, while epigenetic silencing of the Wnt inhibitors may play a general role in WT tumorigenesis, this mechanism is likely to be of special importance in FZD7- resistant WT.

EXAMPLE 8

FZD7 receptor trafficking and localization hampers cell sorting in FZD7-resistant WT

The finding of FZD7-resistant WT and the fact that FZD7 is expressed on a fraction of cells, afforded the opportunity to examine WT cells sorted according to the FZD7 marker so as to determine the stem/progenitor capabilities. Four separations from each resistant tumor were performed after which sorted positive and negative FZD7 fractions were analyzed by FACS and qRT-PCR for sorting efficiency assessment. Interestingly, while FACS analysis showed highly enriched FZD7(+) cell fraction (Figures 9A-B), mRNA levels of FZD7 were mostly similar across samples and even elevated in the negative fraction (Figures 9C-D). These observations lead to the proposal that FZD7 might not be confined to the cell surface at a given timepoint. Indeed, FACS staining and analysis for FZD7 levels at sequential timepoints revealed decreased or increased expression with time (Figures 9E-L). Here the secondary Ab (strep-avidin APC) was added just prior to FACS acquisition in each timepoint. A similar phenomenon was not observed for NCAM1 or CD133 cell surface markers (Figures 9M-T). Accordingly, immunofluorescence staining for FZD7 showed it to localize in the cytoplasm and even nucleus in some of the WT cells (Figures 9U-V) including fast internalization of the receptor (see Figures 3A-L) supporting the notion that cells lacking surface expression of FZD7 (and therefore are labeled as "negative" during sorting) might actually express FZD7 in other cell compartments.

Thus, in most cases FZD7 is not a reliable surface marker for repeated cell sorting required to assess tumor-initiating capacity in vivo.

EXAMPLE 9

Cell sorting of FZD7(+) cells from FZD7- resistant WT

Independent verification by real-time PCR that FZD7 is highly overexpressed in WT-stem like xenografts [Dekel et al, 2006a] compared to their primary tumors (Figures 10A-B) further indicated the importance of analyzing an actual FZD7 enriched population from FZD7-resistant tumors. Thus, despite the understanding of the inherent limitations in FZD7 cell sorting and the fact that reliable cell sorting is difficult to achieve (see above), additional sorting attempts were made so as to collect several

separations in which both FACS and RQ-PCR analysis show significant enrichment for FZD7. Over all the present inventors were able to analyze sorted FZD7(+) and FZD7(-) cells from three separations in which such conditions were apparent (Figures 10C-D). In these cases, FZD7(+) cells were found to be both highly clonogenic, and proliferative compared to the negative fraction (Figures 10E-H). In addition, the FZD7(+) cell fraction was shown to overexpress WT 'stemness' genes, nephric-progenitor (Six2, Sall1), polycomb (Bmi-1), pluripotency and self renewal (Oct4). Analysis of TOP2A, an established poor prognostic marker in WT [Wittman et al, 2008] was highly elevated in the FZD7(+) population (Figure 10G). Altogether, these results indicate the importance of targeting the FZD7 expressing fraction in the FZD7-resistant WT.

The present inventors also tried to sort out cells expressing both FZD7 and NCAM on their surface ($NCAM^+FZD7^+$), as NCAM is a putative marker for the malignant renal progenitor population [Pode-Shaled et al, 2008] and immunostaining for NCAM and FZD7 in WT revealed both to mark blastemal cells in addition to other structures (Figures 11A-D). Due to inherent limitations (see above) this attempt yielded one successful sorting of $NCAM^+FZD7^+$ cells (Figures 11E-G), which when followed by CFU formation assay, showed higher colony formation ability and a significantly elevated cell number in each colony (Figures 11H-I). Since a negative $NCAM^-FZD7^-$ cell fraction could not be obtained; $NCAM^+FZD7^+$ cells were compared to the $NCAM^-$ fraction.

Thus the combination of FZD7 and NCAM is likely to indicate a stem/progenitor cells in WT.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or

identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

WHAT IS CLAIMED IS:

1. An article of manufacture comprising a cytotoxic moiety and an NCAM targeting moiety.

2. A composition of matter comprising a cytotoxic moiety attached to an NCAM targeting moiety.

3. A pharmaceutical composition comprising as active ingredients a cytotoxic moiety and an NCAM targeting moiety and a pharmaceutically acceptable carrier or diluent.

4. An article of manufacture comprising a FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway.

5. A pharmaceutical composition comprising as active ingredients FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway and a pharmaceutically acceptable carrier or diluent.

6. A method of killing a cancer cell expressing NCAM and optionally FZD7, the method comprising contacting the cancer cell with a cytotoxic moiety and an NCAM targeting moiety, thereby killing the cancer cell.

7. A method of treating cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a cytotoxic moiety and an NCAM targeting moiety, thereby treating the cancer.

8. Use of a cytotoxic moiety and an NCAM targeting moiety for the treatment of cancer.

9. A method of killing a cancer cell expressing FZD7 and optionally NCAM, the method comprising contacting the cancer cell with an FZD7 targeting

moiety and an agent capable of inhibiting the Wnt pathway, thereby killing the cancer cell.

10. A method of treating cancer in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway, thereby treating the cancer.

11. Use of an FZD7 targeting moiety and an agent capable of inhibiting the Wnt pathway for the treatment of cancer.

12. The method of claim 6 or 9, further comprising determining an expression level of FZD7 and additionally or alternatively NCAM in said cancer cell prior to said contacting.

13. The method of claim 7 or 10, further comprising determining an expression level of FZD7 and additionally or alternatively NCAM in cells of the cancer prior to said administering.

14. The method or use of any of claims 6-8, wherein said cytotoxic moiety comprises an agent capable of inhibiting the Wnt pathway.

15. The method or use of claim 14, wherein said agent comprises an FZD7 targeting moiety.

16. The method or use of any of claims 9, 10, 11 or 15, wherein said FZD7 targeting moiety comprises an anti FZD7 antibody.

17. The method or use of any of claims 6, 7 or 8, wherein said cytotoxic moiety comprises a chemotherapy, a toxin, a cytokine, an antibody or a radiotherapy.

18. The method or use of any of claims 9, 10, 11 or 14, wherein said agent comprises DKK1 and sFRP1.

19. The method or use of any of claims 9, 10, 11 or 14, wherein said agent comprises a demethylating agent.

20. The method or use of any of claims 6, 7, 8, 9, 10, 11 or 12, wherein said cancer is a pediatric cancer having an embryonic stem cell origin.

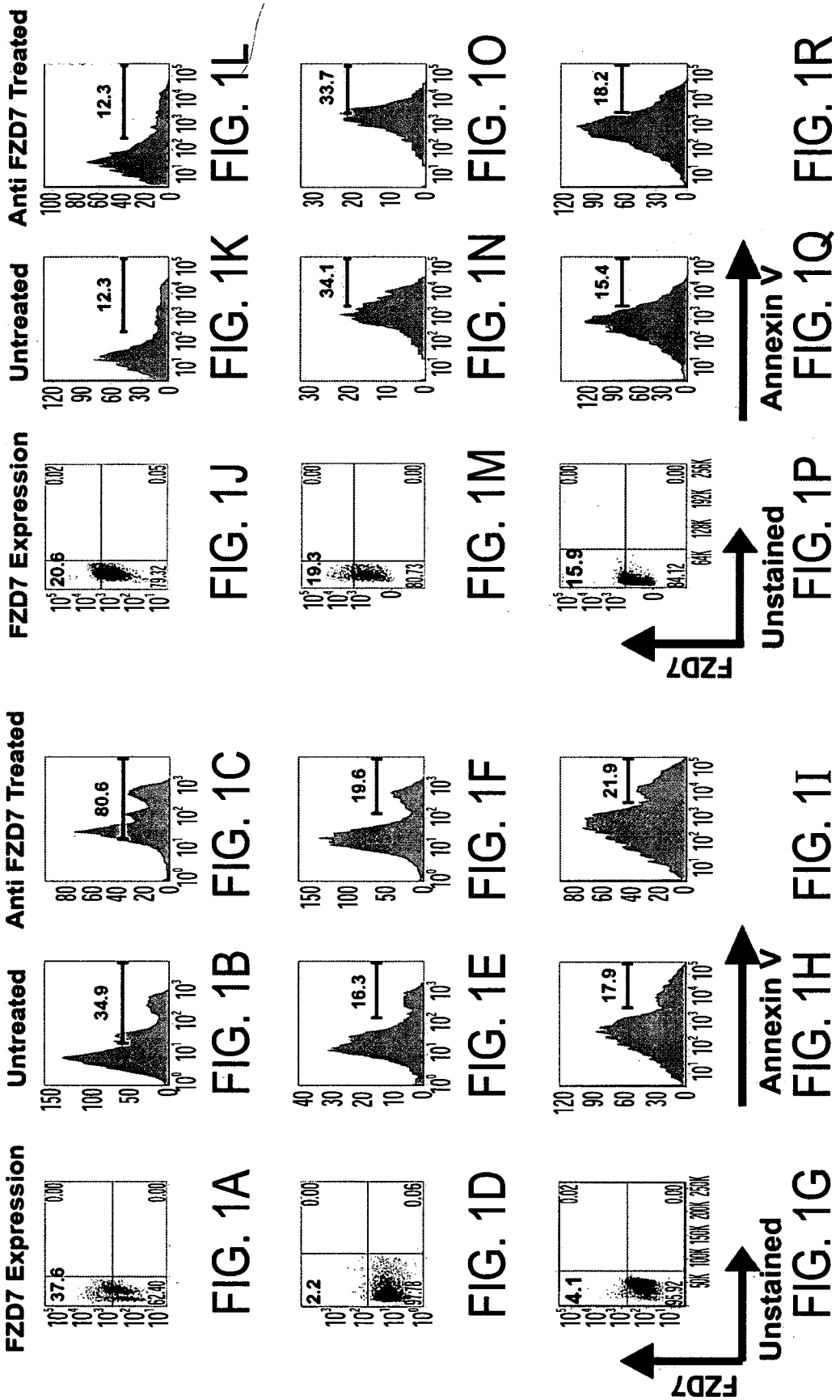
21. The method or use of claim 20, wherein said pediatric cancer having an embryonic stem cell origin comprises Wilm's tumor.

22. The composition of matter, pharmaceutical composition method or use of any of claims 2, 3, 6, 7 or 8, wherein said cytotoxic moiety and said NCAM targeting moiety are covalently attached.

23. The composition of matter, pharmaceutical composition, method or use of any of claims 2, 3, 6, 7 or 8, wherein said cytotoxic moiety and said NCAM targeting moiety are non-covalently attached.

24. The article of manufacture, pharmaceutical composition, method or use of any of claims 1, 3, 6, 7 or 8, wherein said cytotoxic moiety and said NCAM targeting moiety are not attached.

25. The method, composition of matter, pharmaceutical composition or use of claim 22, wherein the composition comprises a bispecific antibody.


FZD7 Expression
Untreated
Anti FZD7 Treated
37.6
34.9
80.6
FIG. 1A
FIG. 1B
FIG. 1C
2.2
16.3
19.6
FIG. 1D
FIG. 1E
FIG. 1F
4.1
17.9
21.9
FIG. 1G
FIG. 1H
FIG. 1I
20.6
12.3
12.3
FIG. 1J
FIG. 1K
FIG. 1L
19.3
34.1
33.7
FIG. 1M
FIG. 1N
FIG. 1O
15.9
15.4
18.2
FIG. 1P
FIG. 1Q
FIG. 1R
FZD7
Unstained
Annexin V

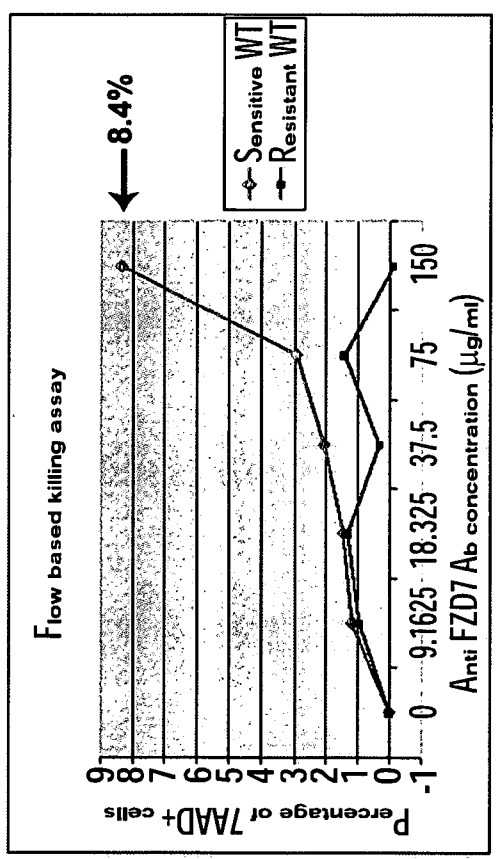


FIG. 1S

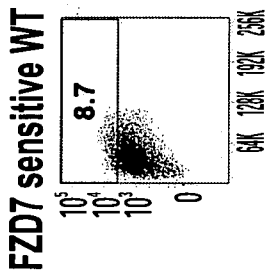


FIG. 2I

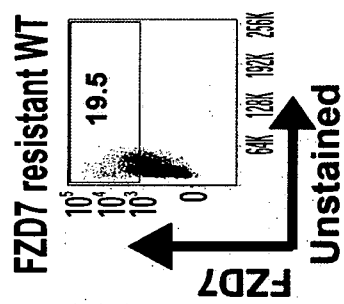


FIG. 2J

FIG. 3A FIG. 3B FIG. 3C

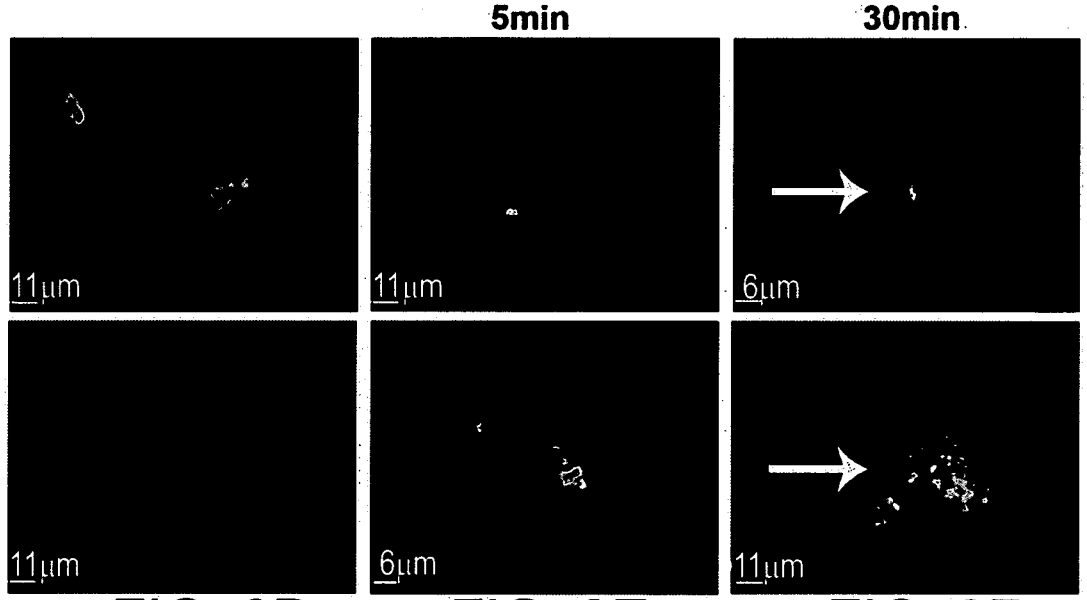


FIG. 3D FIG. 3E FIG. 3F

FIG. 3G

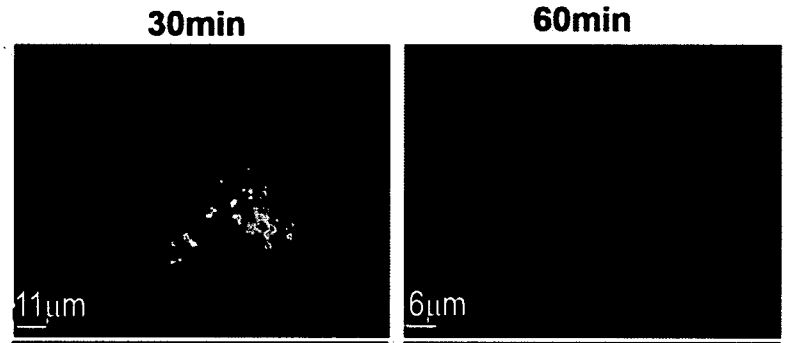


FIG. 3H

FIG. 3I

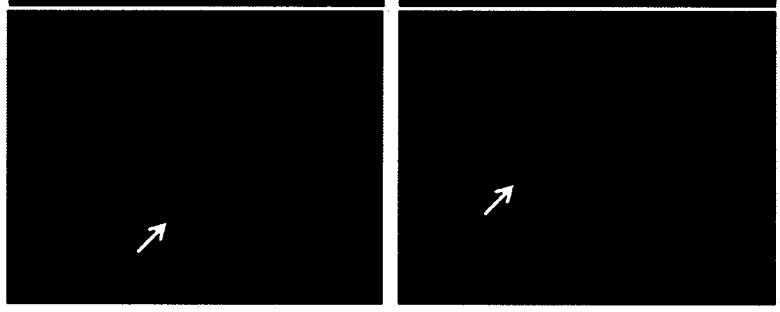


FIG. 3J

FIG. 3K

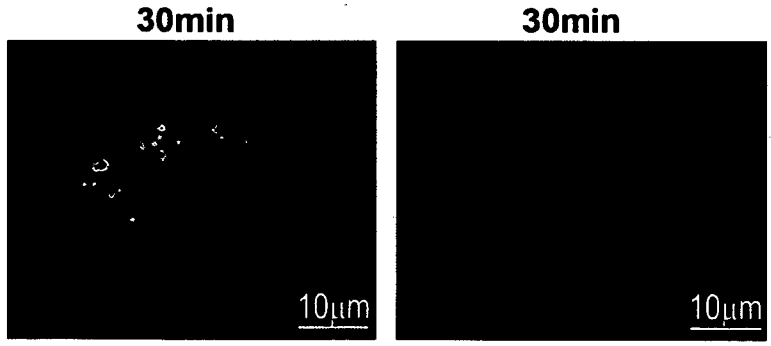


FIG. 3L

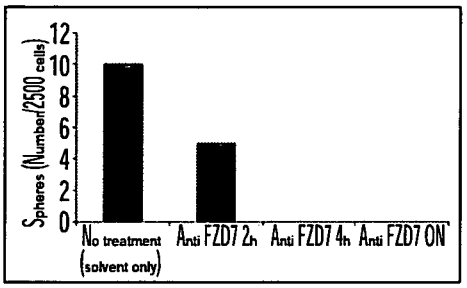


FIG. 4A

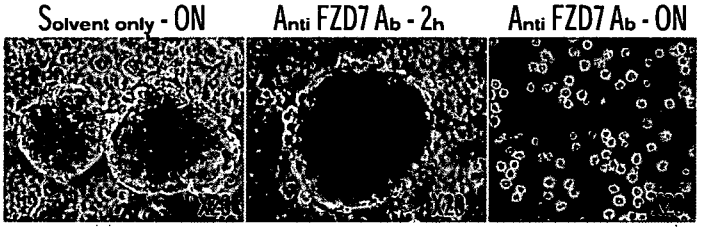


FIG. 4B FIG. 4C FIG. 4D

FIG. 4E FIG. 4F

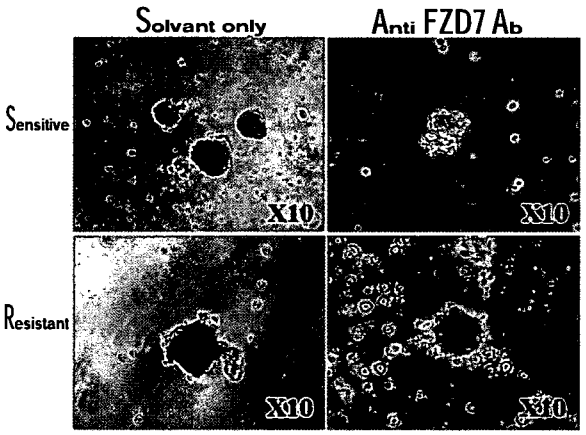


FIG. 4G FIG. 4H

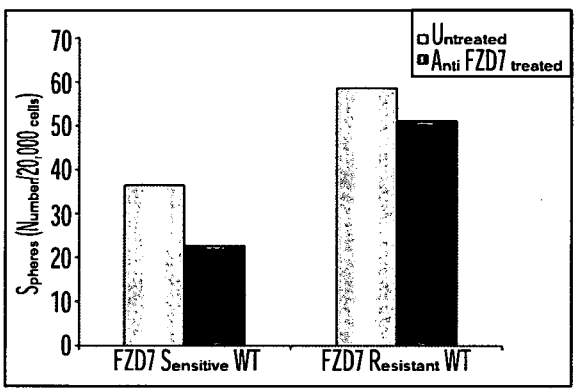


FIG. 4I

FIG. 4J FIG. 4K

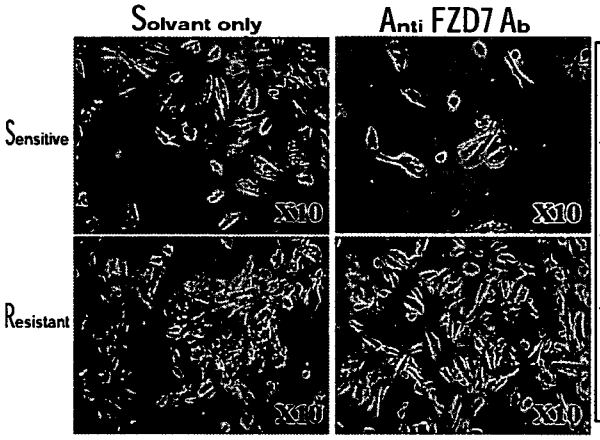


FIG. 4L FIG. 4M

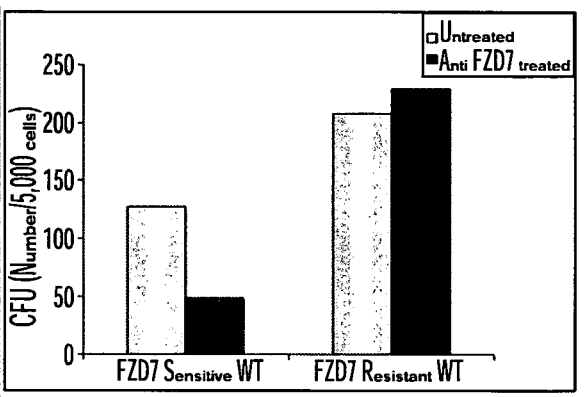
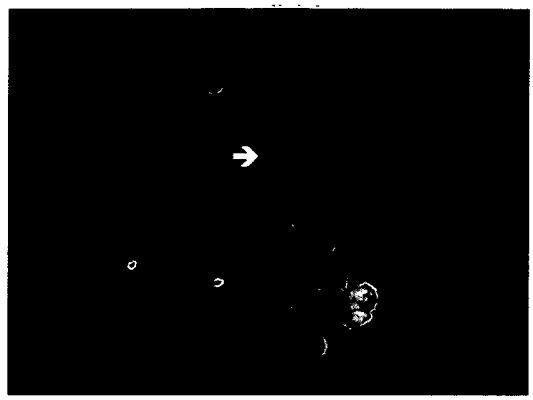


FIG. 4N

FIG. 5A



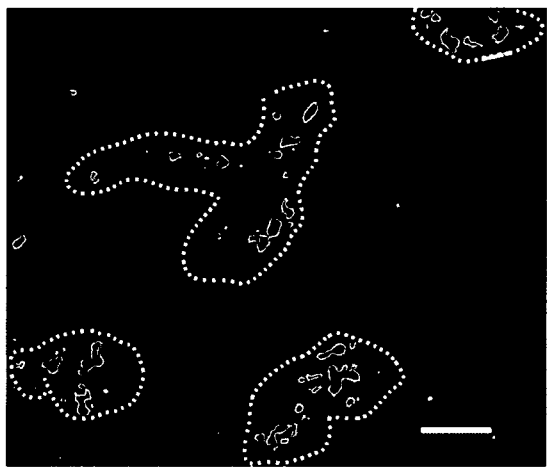


FIG. 5B

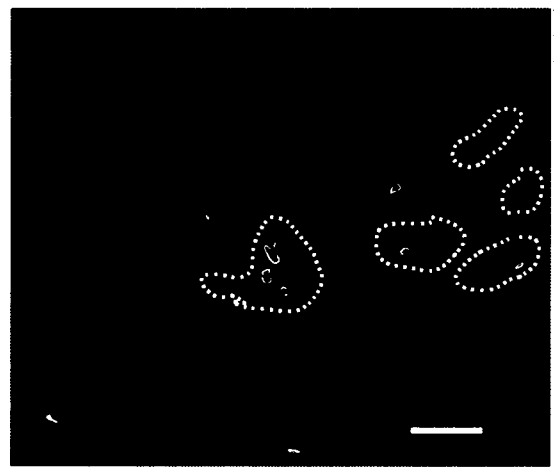


FIG. 5C

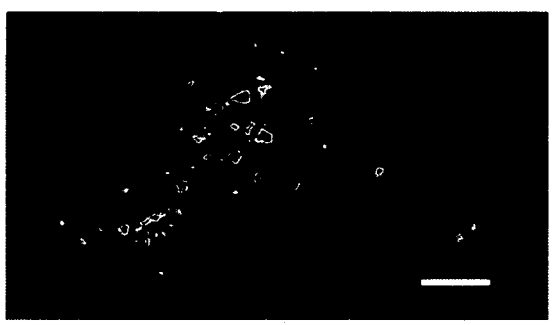


FIG. 5D

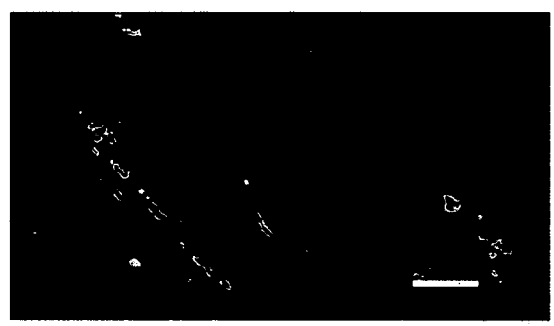


FIG. 5E

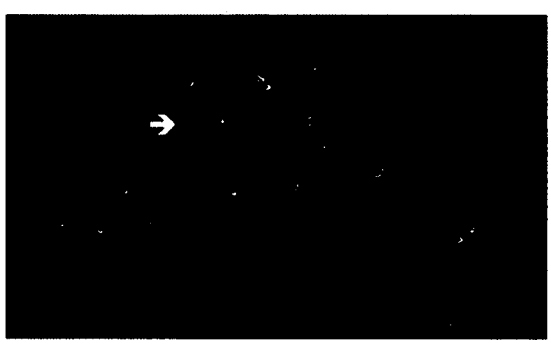


FIG. 5F

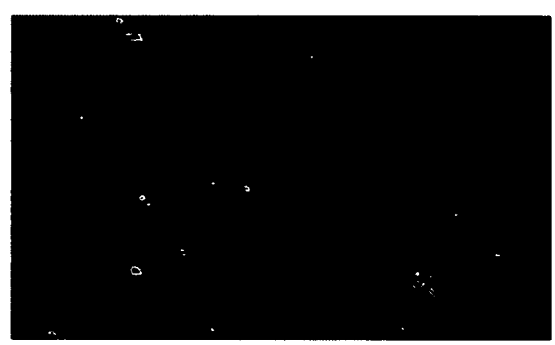


FIG. 5G

FIG. 6A

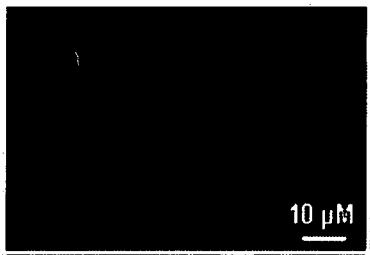


FIG. 6B

FIG. 6C

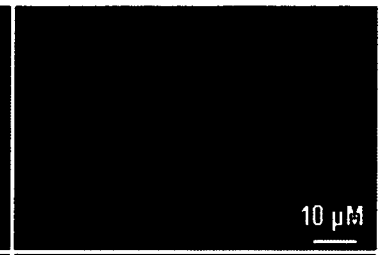


FIG. 6D

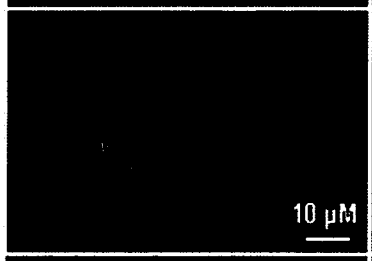


FIG. 6E

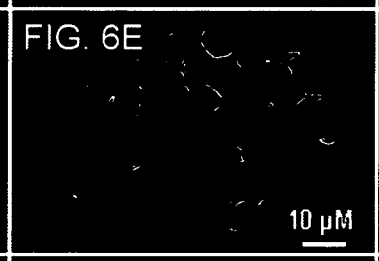


FIG. 6F

FIG. 6G

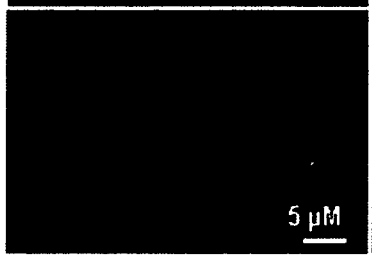


FIG. 6H

FIG. 6I

FIG. 6J

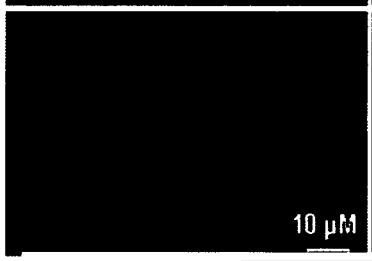


FIG. 6K

FIG. 6L

FIG. 6M

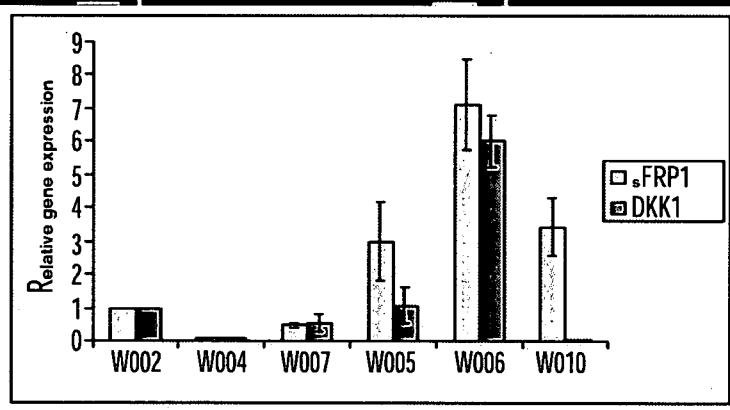
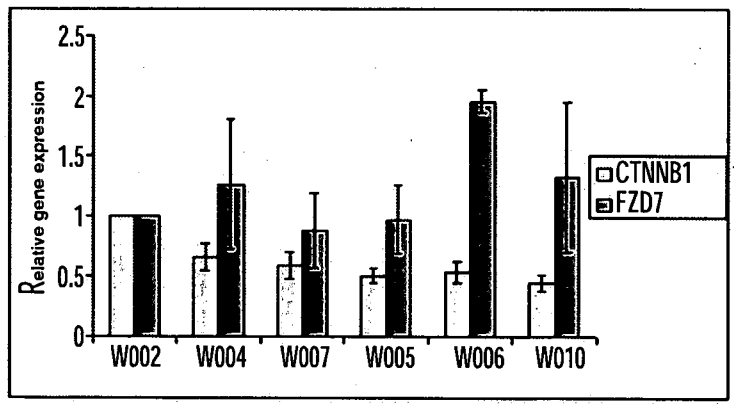


FIG. 6N



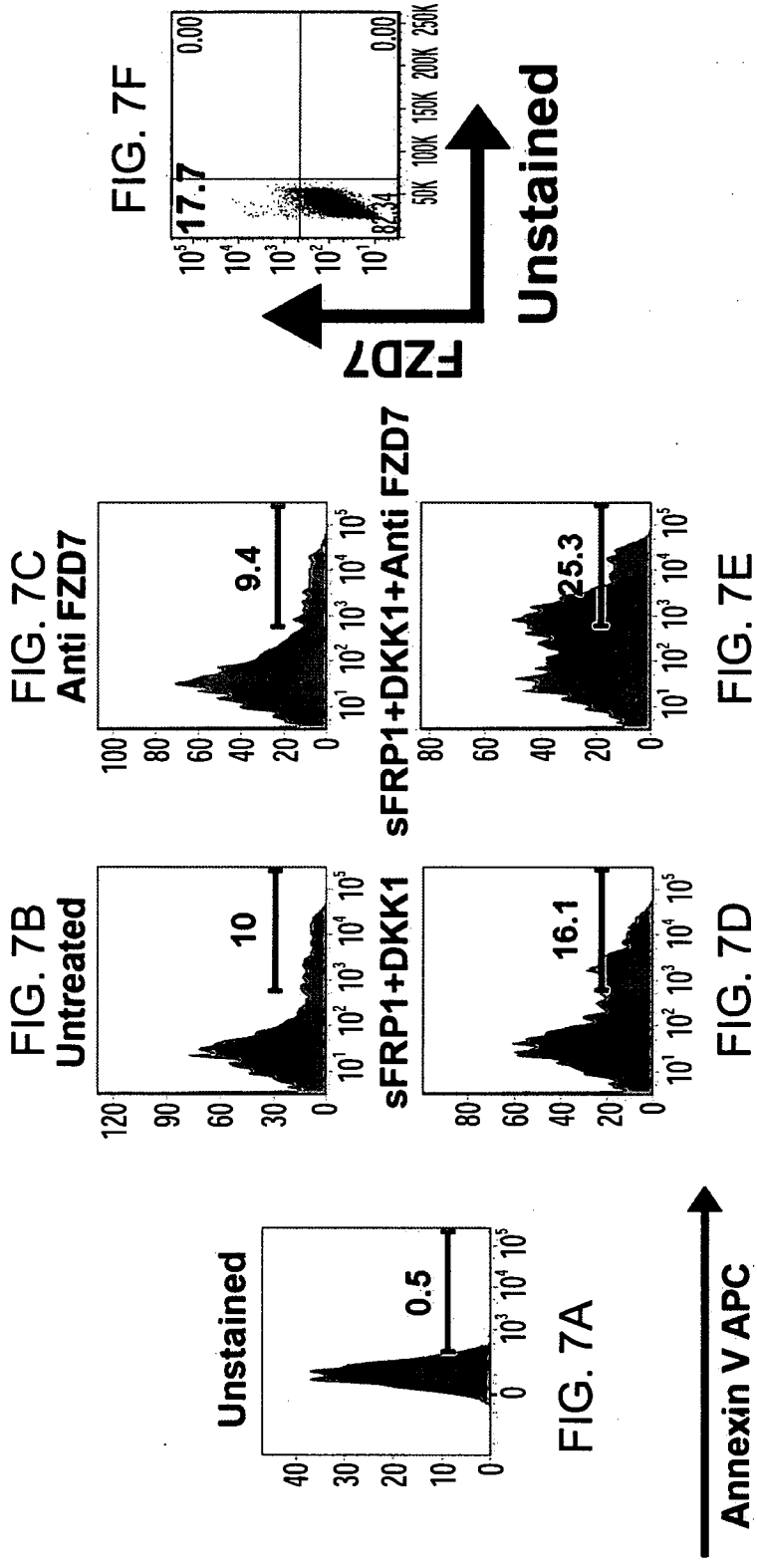


FIG. 7F
17.7
0.00
0.00
FZD7
Unstained
FIG. 7C
Anti FZD7
9.4
FIG. 7B
Untreated
10
sFRP1+DKK1+Anti FZD7
25.3
FIG. 7E
sFRP1+DKK1
16.1
FIG. 7D
Unstained
0.5
FIG. 7A
Annexin V APC

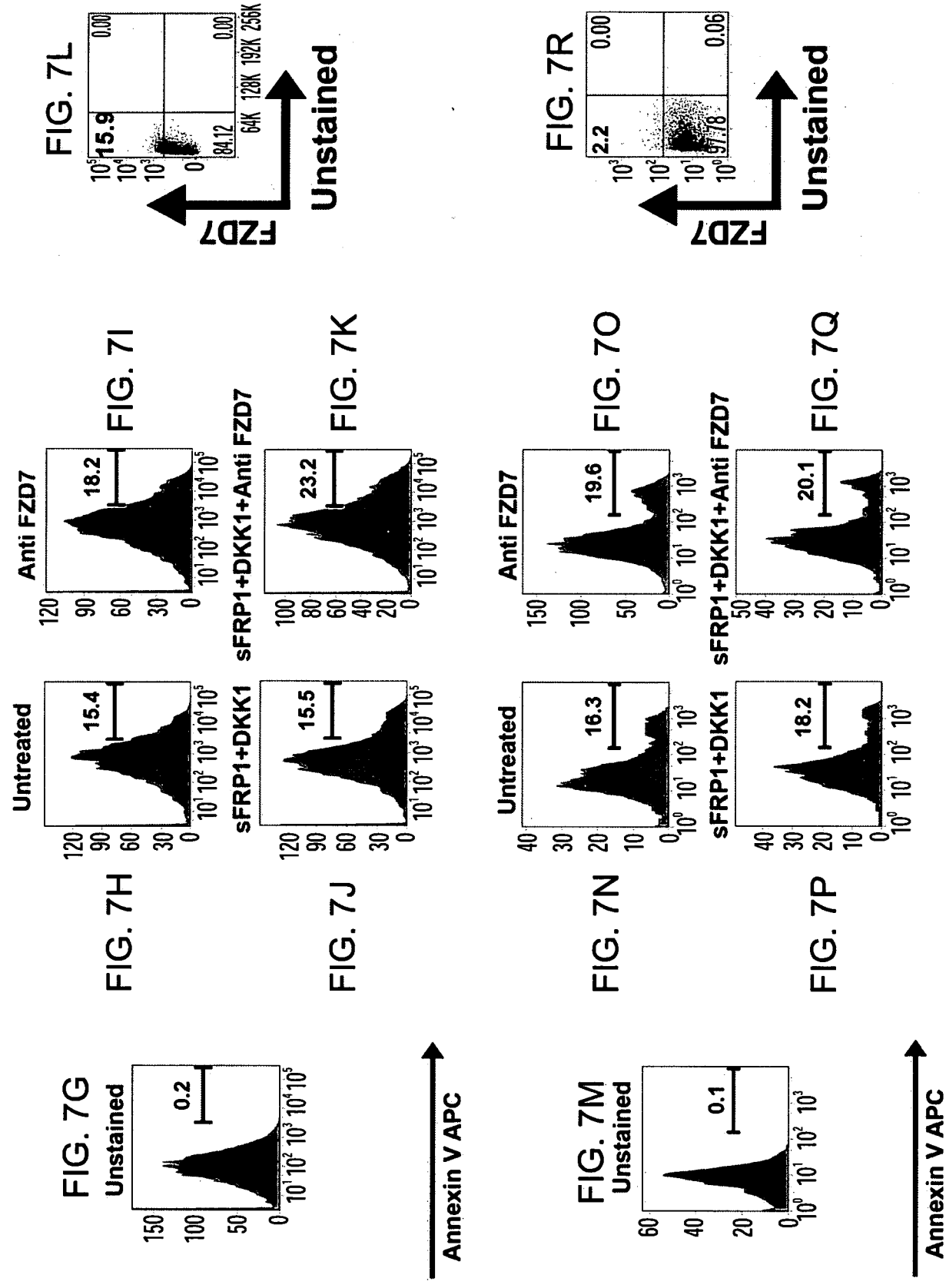


FIG. 7L
FZD7
Unstained
15.9
84.12
0.00
0.00
64K 128K 192K 256K
FIG. 7R
2.2
97.78
0.00
0.06
FIG. 7I
Anti FZD7
18.2
FIG. 7K
sFRP1+DKK1+Anti FZD7
23.2
FIG. 7H
Untreated
15.4
FIG. 7J
sFRP1+DKK1
15.5
FIG. 7O
Anti FZD7
19.6
FIG. 7Q
sFRP1+DKK1+Anti FZD7
20.1
FIG. 7N
Untreated
18.3
FIG. 7P
sFRP1+DKK1
18.2
FIG. 7G
Unstained
0.2
FIG. 7M
Unstained
0.1
Annexin V APC
Annexin V APC

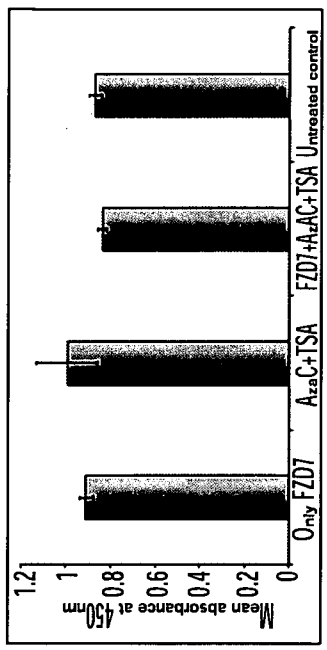


FIG. 8D

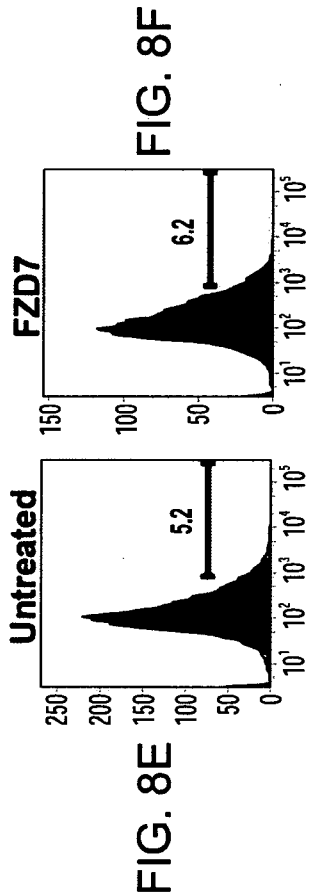


FIG. 8F

FIG. 8E

FIG. 8H

FIG. 8G

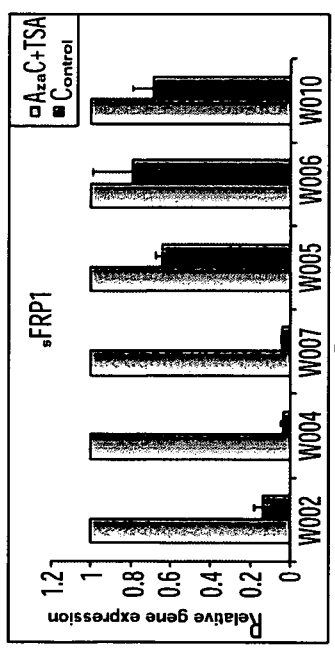


FIG. 8A

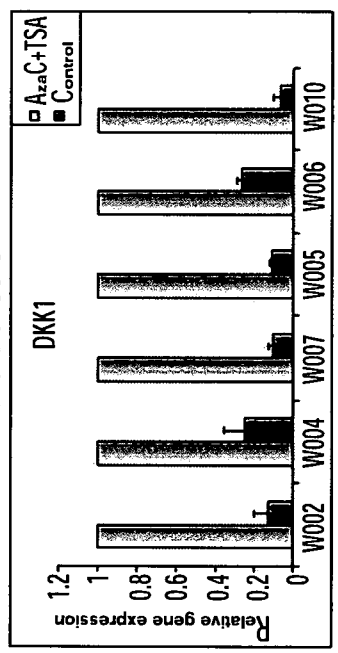


FIG. 8B

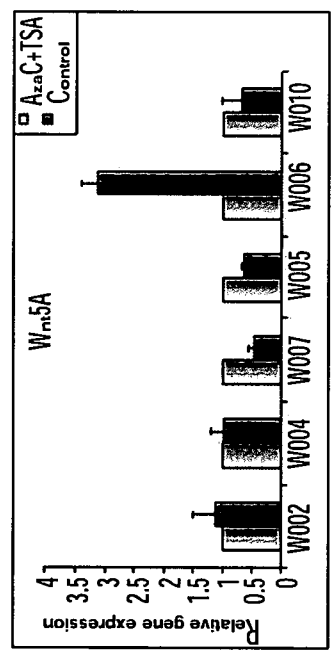


FIG. 8C

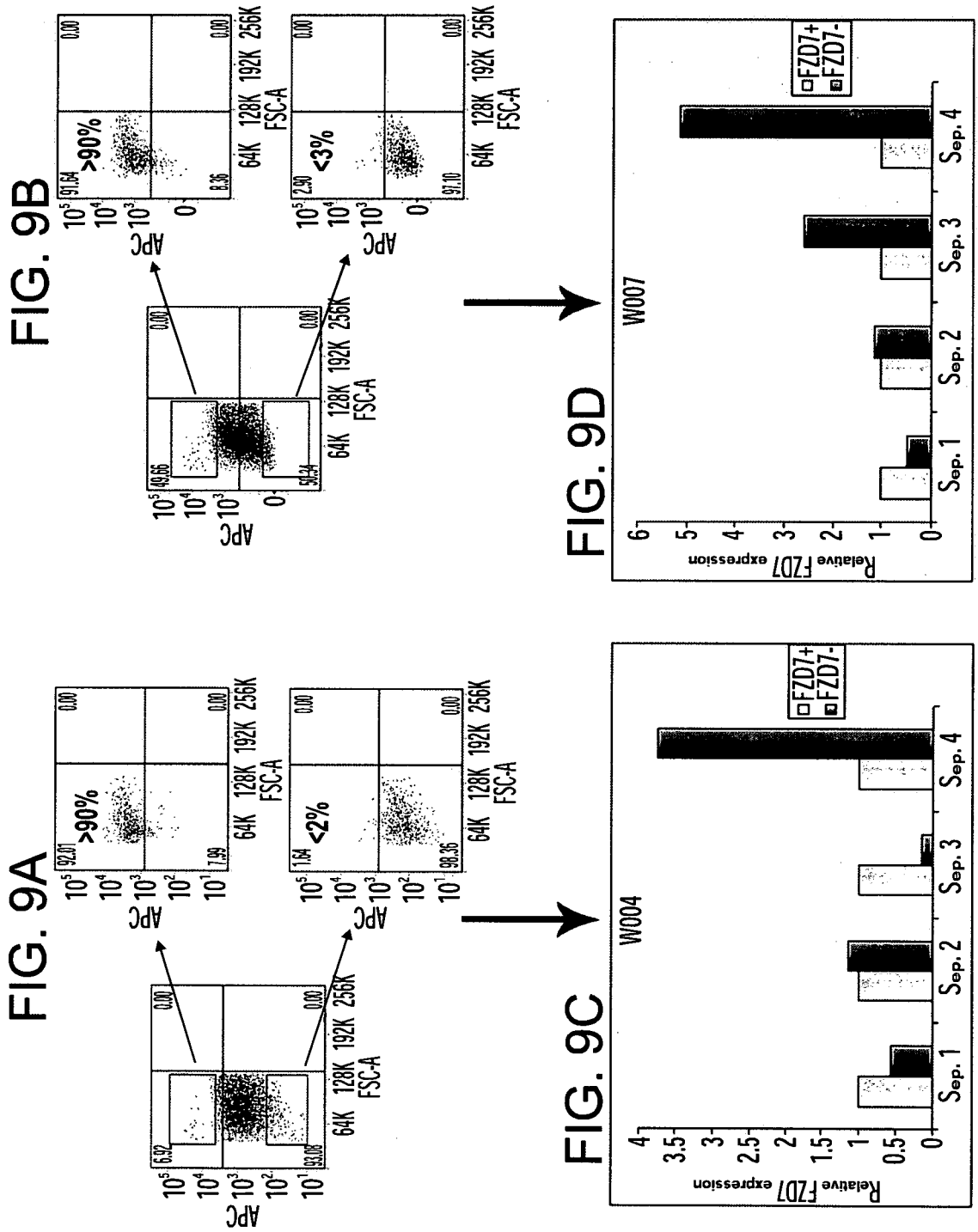


FIG. 9A
FIG. 9B
FIG. 9C
FIG. 9D
>90%
<2%
<3%
APC
FSC-A
64K 128K 192K 256K
W004
W007
FZD7+
FZD7-
Relative FZD7 expression
Sep. 1
Sep. 2
Sep. 3
Sep. 4

FIG. 9E

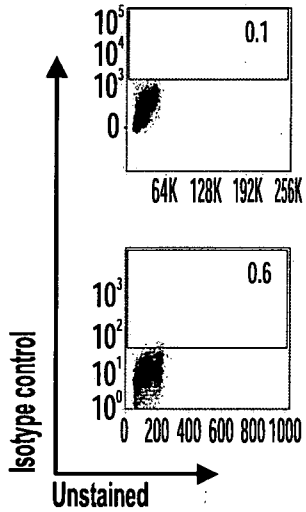


FIG. 9F

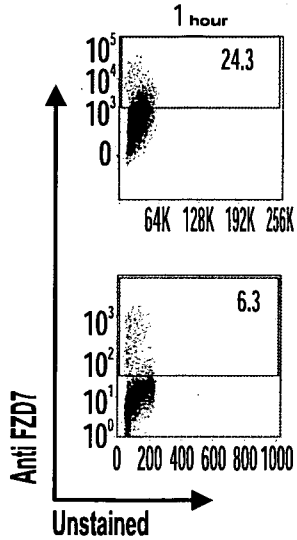


FIG. 9G

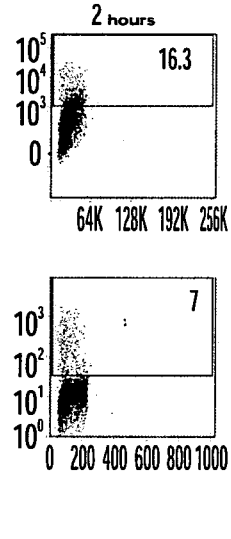


FIG. 9H

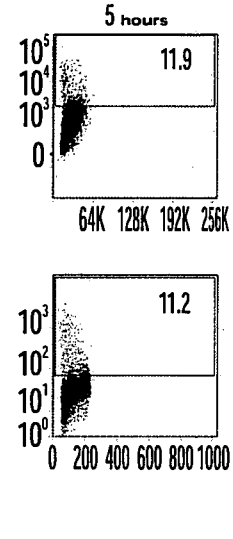


FIG. 9I

FIG. 9J

FIG. 9K

FIG. 9L

FIG. 9M

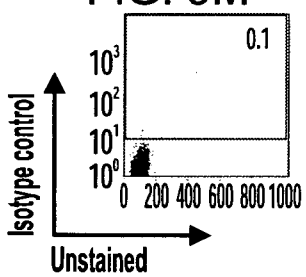


FIG. 9N

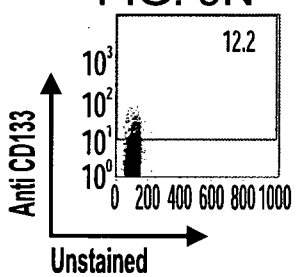


FIG. 9O

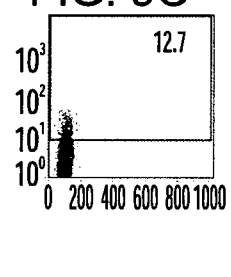
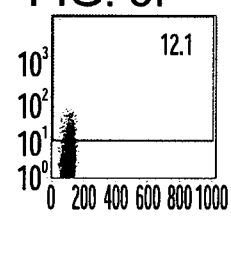
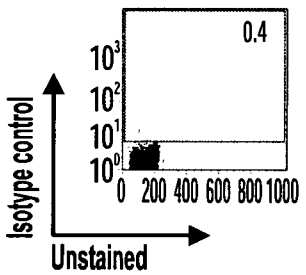
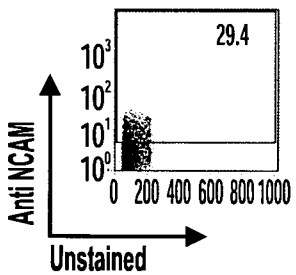
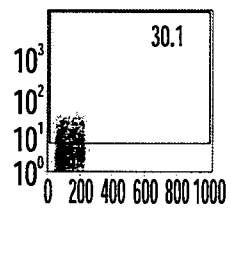


FIG. 9P









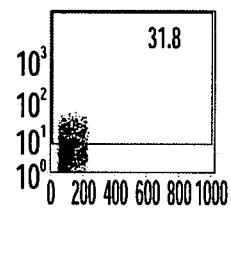


FIG. 9Q

FIG. 9R

FIG. 9S

FIG. 9T

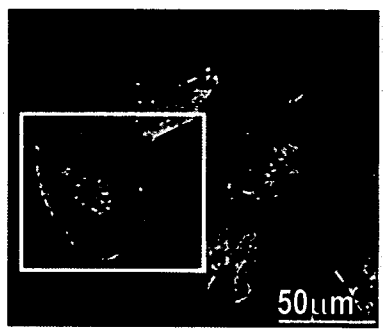


FIG. 9U

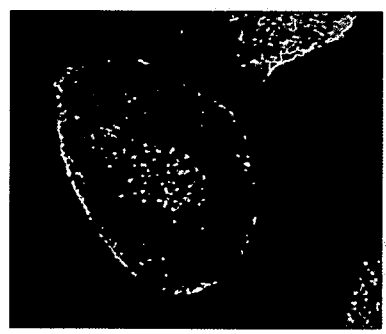


FIG. 9V

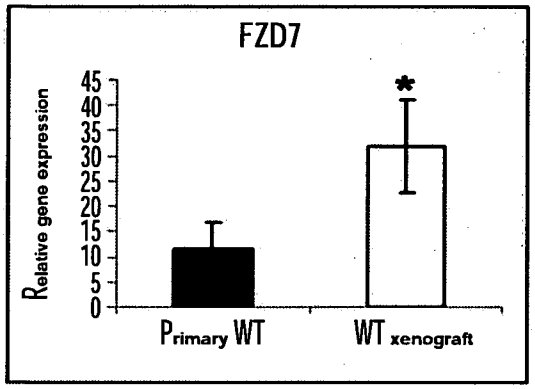


FIG. 10A

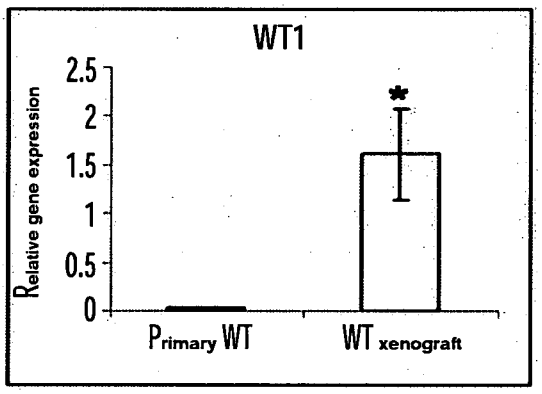


FIG. 10B

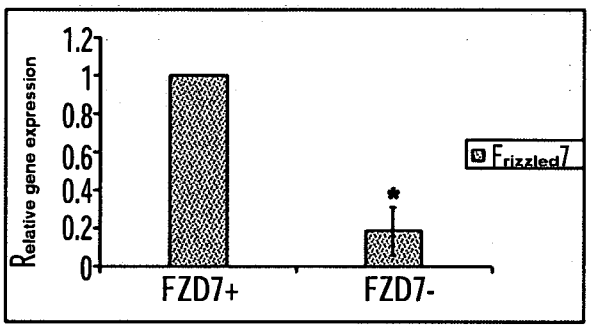


FIG. 10C

FIG. 10D

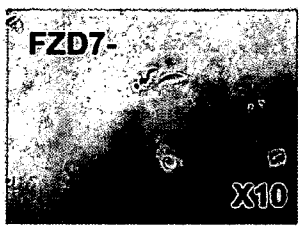


FIG. 10E

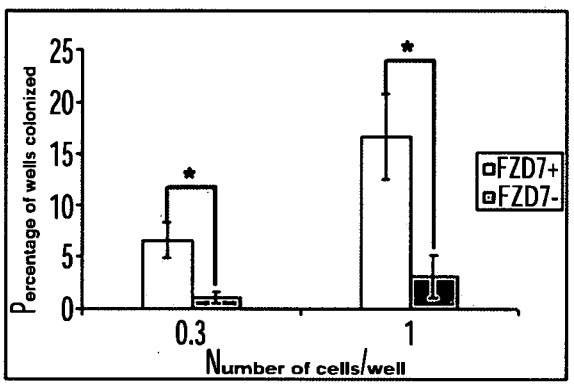


FIG. 10F

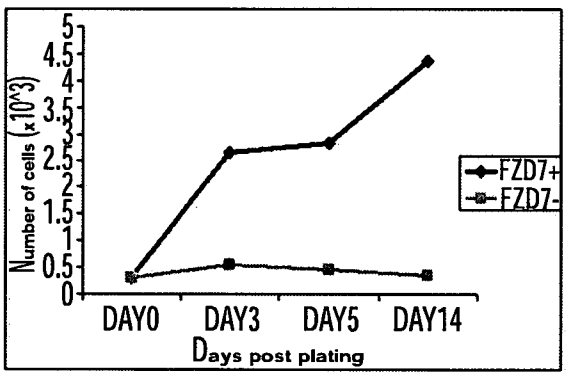


FIG. 10G

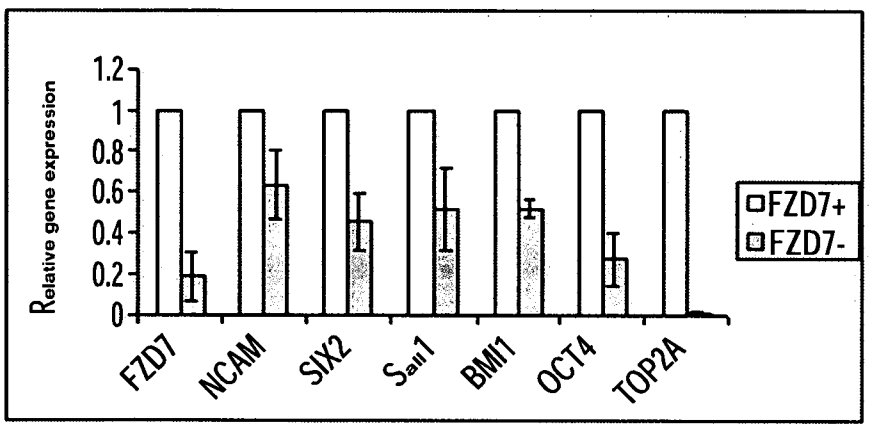


FIG. 10H

FIG. 11A

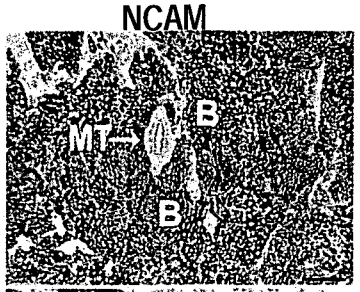


FIG. 11B

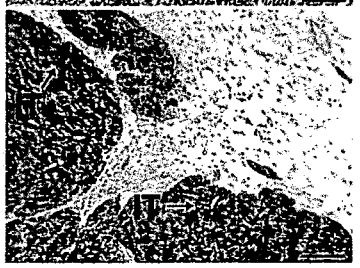


FIG. 11C

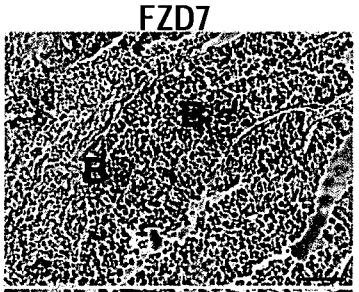


FIG. 11D

FIG. 11E

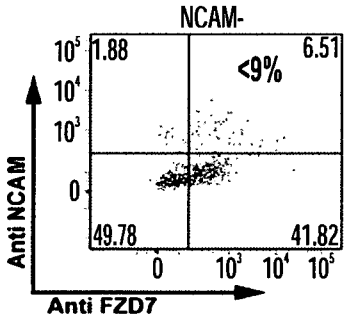


FIG. 11F

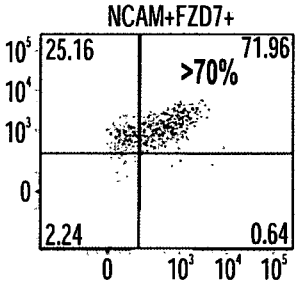


FIG. 11G

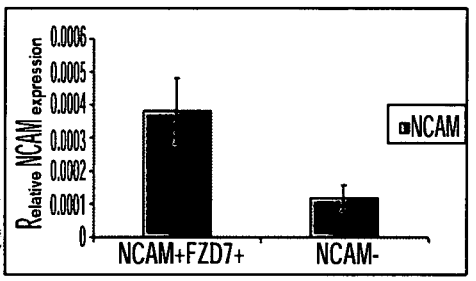


FIG. 11H

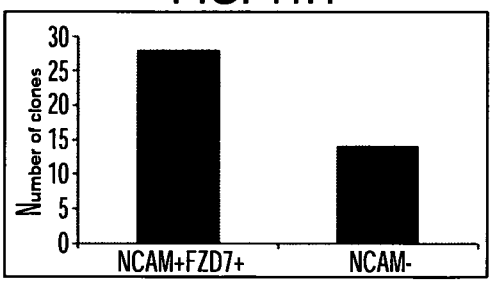


FIG. 11I

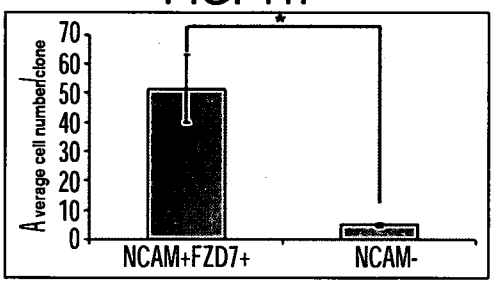


FIG. 11J

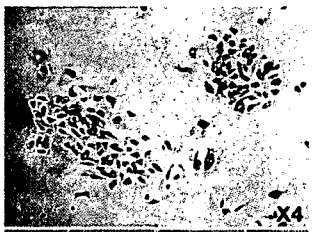


FIG. 11K

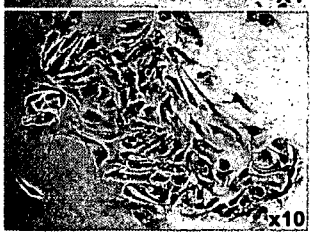


FIG. 11L

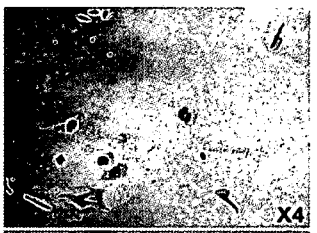


FIG. 11M

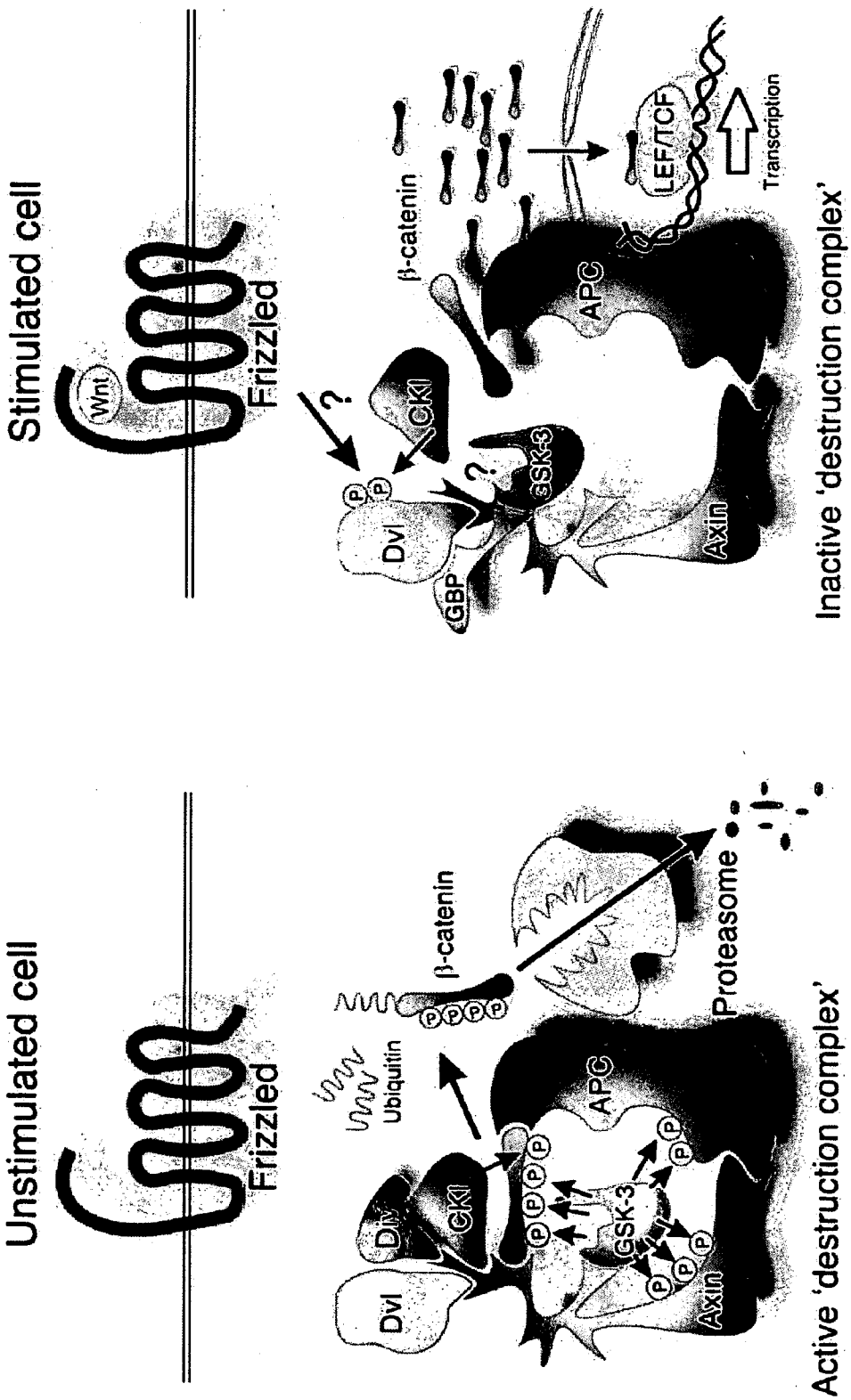


FIG. 12B

FIG. 12A

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2010/000554

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/48 A61K45/06 C07K16/30 A61P35/00 C07K16/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 832 605 A1 (UNIV ZU KOELN [DE]; HANNOVER MED HOCHSCHULE [DE]) 12 September 2007 (2007-09-12)	1-3,6-8, 12,13, 17,20-25
Y	paragraphs [0024], [0041], [0043], [0044], [0104] - [0111], [0123], [0124], [0130]	14-16, 18,19
X	JENSEN ET AL: "Targeting the neural cell adhesion molecule in cancer", CANCER LETTERS, NEW YORK, NY, US LNKD-DOI:10.1016/J.CANLET.2007.09.004, vol. 258, no. 1, 18 October 2007 (2007-10-18), pages 9-21, XP022310543, ISSN: 0304-3835	1-3,6-8, 12,13, 17,20-25
Y	the whole document	14-16, 18,19

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 2010	14/12/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bliem, Barbara

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2010/000554

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2003/082188 A1 (TSO J YUN [US] ET AL) 1 May 2003 (2003-05-01)	1-3,6-8, 12,13, 17,20-25
Y	paragraphs [0010], [0011], [0014], [0015], [0038] - [0042], [0066] - [0068]	14-16, 18,19
X	TANAKA J -I ET AL: "Preparation of a conjugate of mitomycin C and anti-neural cell adhesion molecule monoclonal antibody for specific chemotherapy against biliary tract carcinoma", SURGERY TODAY 1998 JP LNKD- DOI:10.1007/S005950050319, vol. 28, no. 11, 1998, pages 1217-1220, XP002600590, ISSN: 0941-1291	1-3,6-8, 12,13, 17,20-25
Y	* abstract	14-16, 18,19
X	WO 2007/053577 A2 (GURNEY AUSTIN [US]; LEWICKI JOHN [US]; SATYAL SANJEEV [US]; HOEY TIMOT) 10 May 2007 (2007-05-10)	4,5, 9-13,16, 20,21
Y	paragraphs [0064], [0068], [0112], [0147]	14-16, 18,19
Y	WO 2006/055635 A2 (ITY MOUNT SINAI SCHOOL OF MEDI [US]; BAFICO ANNA [US]; AARONSON STUART) 26 May 2006 (2006-05-26) claims 1,4-7 page 3, lines 11-19 page 10, lines 6-23	14-16,18
X,P	WO 2010/062143 A2 (KOREA RES INST OF BIOSCIENCE [KR]; HONG HYO JEONG [KR]; MIN JEONG-KI [) 3 June 2010 (2010-06-03) * abstract -& DATABASE WPI Week 201041 Thomson Scientific, London, GB; AN 2010-G09597 XP002600597, -& WO 2010/062143 A2 (KOREA RES INST BIOSCIENCE & BIOTECHNOLOG) 3 June 2010 (2010-06-03)	1-3,6-8, 17,24

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2010/000554

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PODE-SHAKKED NAOMI ET AL: "Developmental tumourigenesis: NCAM as a putative marker for the malignant renal stem/progenitor cell population", JOURNAL OF CELLULAR AND MOLECULAR MEDICINE, UNIVERSITY PRESS CAROL DAVILA, BUCHAREST, RO, vol. 13, no. 8B, 16 December 2008 (2008-12-16), pages 1792-1808, XP002581529, ISSN: 1582-1838, DOI: DOI:10.1111/J.1582-4934.2008.00607.X [retrieved on 2008-12-16] cited in the application	4,5, 9-13,16, 20,21
Y	* abstract page 1800, section "Exclusion of FZD7 and CD133 as markers for the isolation of WT stem/progenitor cells" page 1805, column 1, lines 1,2	14-16, 18,19
X	UENO K ET AL: "Frizzled-7 as a potential therapeutic target in colorectal cancer", NEOPLASIA 200807 US LNKD- DOI:10.1593/NEO.08320, vol. 10, no. 7, July 2008 (2008-07), pages 697-705, XP002612805, ISSN: 1522-8002	4,5, 9-13,16, 20,21
Y	* abstract figure 3	14-16, 18,19
X,P	WO 2010/037041 A2 (ONCOMED PHARMACEUTICALS INC [US]; GURNEY AUSTIN L [US]; SATO AARON KEN) 1 April 2010 (2010-04-01) paragraphs [0106], [0107], [0143] - [0147], [0251] - [0253], [0278] - [0284]; claims 27-32,49,58-84	4,5, 9-11,16
X,P	UENO K ET AL: "Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells", BRITISH JOURNAL OF CANCER 20091020 NATURE PUBLISHING GROUP GBR LNKD- DOI:10.1038/SJ.BJC.6605307, vol. 101, no. 8, 20 October 2009 (2009-10-20), pages 1374-1381, XP002612806, ISSN: 0007-0920 * abstract	4,9
Y	O'Neil M et al: "The Merck Index", 2006, Merck & Co., Inc., Whitehouse Station, NJ, USA, XP002612807, vol. 14 page 150	19

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2010/000554

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	O'Neil M et al: "The Merck Index", 2006, Merck & Co., Inc., Whitehouse Station, NJ, USA, XP002612808, vol. 14 page 482	19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2010/000554

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3, 6-8, 14, 15, 17, 22-25(completely); 12, 13, 16, 18-21(partially)

   Composition comprising cytotoxic moiety and an NCAM targeting moiety for the treatment of tumor, in particular for the treatment of Wilm's tumor.

   ---

2. claims: 4, 5, 9-11(completely); 12, 13, 16, 18-21(partially)

   Composition comprising FZD7 targeting moiety and an agent inhibiting the Wnt pathway for the treatment of tumor, in particular for the treatment of Wilm's tumor.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2010/000554

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 1832605 A1	12-09-2007	NONE	
US 2003082188 A1	01-05-2003	NONE	
WO 2007053577 A2	10-05-2007	AU 2006308870 A1	10-05-2007
		AU 2006344359 A1	13-12-2007
		CA 2628116 A1	13-12-2007
		CA 2628221 A1	10-05-2007
		EP 1961065 A2	27-08-2008
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		JP 2009513708 T	02-04-2009
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		US 2007116701 A1	24-05-2007
		WO 2007133250 A2	22-11-2007
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WO 2006055635 A2	26-05-2006	JP 2008520583 T	19-06-2008
		US 2009163407 A1	25-06-2009
WO 2010062143 A2	03-06-2010	KR 20100060351 A	07-06-2010
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