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(54) **Title**: NOVEL EFFECTORS AND METHODS OF USE

(57) **Abstract**: Novel effectors, including antibodies and derivatives thereof, and methods of using such effectors to ameliorate hyperproliferative disorders are provided.

NOVEL EFFECTORS AND METHODS OF USE

CROSS REFERENCED APPLICATIONS

This application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application Ser. No. 61/371,129 filed August 5th, 2010 which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This application generally relates to compositions and methods of their use in treating or ameliorating hyperproliferative disorders, expansion, recurrence, relapse or metastasis. In a broad aspect the present invention relates to the use of APCDD1 effectors, including APCDD1 antagonists, for the treatment or prophylaxis of neoplastic disorders. Particularly preferred embodiments the present invention provide for the use of anti-APCDD1 antibodies for the immunotherapeutic treatment of malignancies including, for example, KRAS and/or APC mutated colorectal cancer and KRAS mutated pancreatic cancers.

BACKGROUND OF THE INVENTION

Stem and progenitor cell differentiation and cell proliferation are normal ongoing processes that act in concert to support tissue growth during organogenesis, and cell replacement and repair of most tissues during the lifetime of all living organisms. Differentiation and proliferation decisions are often controlled by numerous factors and signals that are balanced to maintain cell fate decisions and tissue architecture. Normal tissue architecture is maintained as a result of cells responding to microenvironmental cues that regulate cell division and tissue maturation. Moreover, cell proliferation and differentiation normally occurs only as necessary for the replacement of damaged or dying cells. When normal cellular proliferation and differentiation are disturbed or somehow disrupted, the results are most commonly disease. Disruption of cell proliferation and/or differentiation can result from a myriad of factors including, for example, the under- or overabundance of various signaling chemicals, the presence of altered microenvironments, genetic mutations or some combination thereof.

Cancer or neoplasia generally refers to a disease caused by the uncontrolled, abnormal growth of cells in one organ or tissue type that can spread to adjoining tissues or other parts of the body. Cancer cells can form a solid tumor in which the cancer cells are massed together, or exist as dispersed cells as in lymphoma or leukemia. Often such cells are referred to as malignant or neoplastic because they divide irrespective of environmental cues that normally restrict proliferation, often leading to crowding out nearby cells and spreading to other parts of the body. The tendency of malignant cancer cells to spread from one organ to another or from one part of the body to another distinguishes them from benign tumor cells, which overgrow but do not spread to other organs or parts of the body. Malignant cancer cells typically metastasize and spread to other parts of the body via the bloodstream or lymphatic system, where they can multiply and form new tumors, making cancer a serious and often deadly disease.

Conventional treatments for cancer include chemotherapy, radiotherapy, surgery, immunotherapy (e.g., biological response modifiers, vaccines or targeted therapeutics) or combinations thereof. Unfortunately, far too many cancers are non-responsive or minimally responsive to such conventional treatments leaving few options for patients. Moreover, depending on the type of cancer some available treatments, such as surgery, may not be viable alternatives. The limitations of current standard of care therapeutics are particularly evident when attempting to care for patients who have undergone previous treatments and have subsequently relapsed. Likely as a result of prior toxic therapeutic regimens that have failed, refractory tumors often manifest themselves as a more aggressive disease than the original primary tumor and, in many cases, it is this refractory tumor that most often results in death. Although there have been great improvements in the diagnosis and treatment of cancer over the years, overall survival rates for many solid tumors have remained largely unchanged due to the failure of existing therapies to prevent tumor recurrence and metastases. Thus, it remains a challenge to develop more targeted and potent therapies.

While efforts to develop immunotherapeutics have proven successful in some cases, most notably with respect to hematologic malignancies, unanticipated complications with the drug products and deficiencies in the standard oncology paradigm have limited the diagnostic and therapeutic utility of some of the reagents thus far developed. This is particularly true with regard to solid tumors, which are rarely cured by standard of care regimens including resection, chemotherapy and/or biologics (e.g. Avastin®). For example, although standard of care chemotherapeutic regimens, often given in

combination with biological therapeutics, may result tumor regression and improve progression-free survival, both of which are commonly used clinical endpoints, overall survival changes very little. Moreover, some patient subpopulations exhibit gene mutations (e.g., KRAS,) that render them non-responsive despite the general effectiveness of certain targeted biological therapies.

One attempt at enhancing the effectiveness of such treatments involves the use of therapeutic antibodies to reduce undesirable cross-reactivity and increase tumor cell localization of one or more cytotoxic agents. The idea of recruiting antibodies to use in treating neoplastic disorders dates to at least 1953 when it was shown that antibodies could be used to specifically target tumor cells. However, it was the seminal work of Kohler and Milstein in hybridoma technology that allowed for a continuous supply of monoclonal antibodies (mAbs) that specifically target a defined antigen. By 1979 mAbs had been used to treat malignant disorders in human patients. Currently there are several therapeutic antibodies approved for the treatment of various neoplastic disorders with hundreds more at different stages of clinical or preclinical development. Moreover, with improvements in methods to stably conjugate antibodies to drugs, toxins, radionuclides or other cytotoxic agents, a substantial number of antibody-toxin conjugates are also presently in clinical trials or preclinical development.

One promising area of research involves the use of targeted therapeutics to go after the tumorigenic "seed" cells that appear to underlie many cancers. To that end most solid tissues are now known to contain adult, tissue-resident stem cell populations that generate differentiated cell types that comprise the majority of that tissue. Tumors arising in these tissues similarly consist of heterogeneous populations of cells that also arise from stem cells, but differ markedly in their overall proliferation and organization. While it is increasingly recognized that the majority of tumor cells have a limited ability to proliferate, a minority population of cancer cells (commonly known as cancer stem cells or CSC) have the exclusive ability to extensively self-renew thereby enabling them with tumor reinitiating capacity. More specifically, the cancer stem cell hypothesis proposes that there is a distinct subset of cells (i.e., CSC) within each tumor (approximately 0.1-10%) that is capable of indefinite self-renewal and of generating tumor cells progressively limited in their replication capacity as a result of their differentiation to tumor progenitor cells, and subsequently to terminally differentiated tumor cells.

In recent years it has become more evident these CSC (also known as tumor perpetuating cells or TPC) might be more resistant to traditional chemotherapeutic agents

or radiation and thus persist after standard of care clinical therapies to later fuel the growth of relapsing tumors, secondary tumors and metastases. Moreover, there is growing evidence suggests that pathways that regulate organogenesis and/or the self-renewal of normal tissue-resident stem cells are deregulated or altered in CSC, resulting in the continuous expansion of self-renewing cancer cells and tumor formation. See generally Al-Hajj et al., 2004, PMID: 15378087; and Dalerba et al., 2007, PMID: 17548814; each of which is incorporated herein by reference in its entirety. Thus, the effectiveness of traditional, as well as more recent targeted treatment methods, has apparently been limited by the existence and/or emergence of resistant cancer cells that are capable of perpetuating the cancer even in face of these diverse treatment methods. Huff et al., European Journal of Cancer 42: 1293-1297 (2006) and Zhou et al., Nature Reviews Drug Discovery 8: 806-823 (2009) each of which is incorporated herein by reference in its entirety. Such observations are confirmed by the consistent inability of traditional debulking agents to substantially increase patient survival, and through the development of an enhanced understanding as to how tumors grow, recur and metastasize. Accordingly, recent strategies for treating neoplastic disorders have recognized the importance of eliminating, silencing or promoting the differentiation of tumor perpetuating cells so as to diminish the possibility of tumor recurrence.

Recent work using non-traditional xenograft (NTX) models, wherein primary human solid tumor specimens are implanted and passaged exclusively in immune-compromised mice, strongly implicate the existence of a subpopulation of cells with the unique ability to fuel tumor growth. As previously hypothesized, work in NTX models has confirmed that the CSC subpopulation of tumor cells appears more resistant to debulking regimens such as chemotherapy and radiation, potentially explaining the disparity between clinical response rates and overall survival. By investigating the CSC paradigm through the use of NTX modeling, there has been a fundamental change in drug discovery and preclinical evaluation of drug candidates that will hopefully lead to CSC-targeted therapies having a major impact on tumor recurrence and metastasis, and thus improve overall survival.

The CSC paradigm as investigated using NTX models offers a fresh approach to better understanding cancer biology. However, inherent technical difficulties associated with handling primary and/or xenograft tumor tissue, along with a lack of experimental platforms to characterize CSC identity and differentiation potential, pose major challenges. Like their normal stem cell counterparts, CSC can best be defined by their inherent properties of self-renewal, multipotency and functional reconstitution capacity.

Because these classical characteristics of stem cells are most evidently exhibited *in vivo*, xenograft tumors are strongly preferred when examining CSC biology. While traditional human tumor cell lines are relatively easy to work with and can generate tumors upon implantation into immunocompromised mice, it is becoming increasingly clear that the biology of cells cultured *in vitro* do not accurately reflect those of cells growing in a physiologically appropriate setting. In particular, gene expression might irreversibly change with even brief periods of *in vitro* culture. Conversely, early passage NTX models generally have genetic abnormalities reflective of the patient tumors from which they were derived and appear to best maintain gene expression profiles, as well as the phenotypic and morphological diversity of tumors as they exist in patients (Daniel et al., Cancer Res 2009;69(8):3364–71). Although the work is often laborious, insight gained using these models is more likely than traditional models to reflect tumor biology as it exists *in vivo*. Moreover, the concern over translation of findings from NTX tumors to patients might arguably be less than insight gained from mouse models of human cancer where oncogene expression might not be physiological or proteins used to identify stem cells in mice do not have homologs in humans (*e.g.* Sca-1).

Despite these advancements in modeling and much effort over the past several years, CSC remain ill defined in most solid tumors. One marker phenotype that has been identified for colorectal CSC populations is the combined expression of epithelial-specific antigen (*i.e.* ESA or EpCAM), CD44 and CD166. When transplanted at low cell numbers or assessed *in vitro* using limiting dilution assays designed to read out colony-forming ability, it has previously been functionally demonstrated that tumorigenic cells appear to comprise roughly 1.25% of cells with this triple positive phenotype (Dalerba et al., supra). While such results suggest that $ESA^+CD44^+CD166^+$ cells might identify one tumorigenic subset of colorectal cancer, much more work remains to accurately identify CSC in this and various other solid tumor malignancies. More particularly, there remains a substantial need to identify effective CSC markers and marker phenotypes that can identify CSC with better accuracy such that they may be used to develop diagnostic, prophylactic or therapeutic compounds.

Further, as previously indicated, conventional anti-neoplastic therapies appear to have little effect on eliminating, impairing or otherwise modulating the effects of CSC. Based on research over the last several decades, this lack of activity by conventional agents is due, at least in part, to the fact that CSC are thought to be more quiescent and/or have relatively higher drug efflux ability than other cells that comprise the tumor bulk.

Given that conventional therapies and regimens have, in large part, been designed to attack rapidly proliferating cells and clinical responses have generally been measured using tumor regression endpoints, comparatively slow growing or quiescent, drug effluxing CSC may be more resistant to current therapeutic regimens and easily escape detection, only to refuel tumor recurrence and metastasis. CSC also express other features that make them relatively chemoresistant, such as an increased tolerance to harsh stimuli that normally induce apoptotic pathways and enhanced gene repair and maintenance capacities. Cumulatively, these properties likely constitute a key reason for the failure of standard oncology treatment regimens to ensure long-term benefit in most patients with advanced stage cancers--i.e. the failure to adequately target and eradicate, terminally differentiate or silence CSC Accordingly, deficiencies in current standards of care in oncology might be alleviated by diagnostic and therapeutic agents and methods that effectively identify, eliminate, reduce, impair or otherwise modulate the effects of tumor perpetuating cells (i.e., CSC).

The present invention addresses several of these issues and, by providing novel compounds, compositions, therapeutic regimens and methods, facilitates the treatment, prevention and/or management of hyperproliferative disorders.

SUMMARY OF THE INVENTION

These and other objectives are provided for by the present invention which, in a broad sense, is directed to methods, compounds, compositions and articles of manufacture that may be used in the treatment of APCDD1 associated disorders (e.g., hyperproliferative disorders or neoplastic disorders). To that end, the present invention provides novel APCDD1 effectors that effectively target cancer stem cells and may be used to treat patients suffering from a wide variety of malignancies. As will be discussed in more detail below, the disclosed APCDD1 effectors may comprise any compound that recognizes, competes, antagonizes, interacts, binds or associates with the APCDD1 polypeptide or its gene and modulates, adjusts, alters, changes or modifies the impact of the APCDD1 protein on one or more physiological pathways (e.g., the Wnt/beta-catenin pathway). In preferred embodiments the APCDD1 effector will reduce the frequency of tumor perpetuating cells. In selected embodiments of the invention APCDD1 effectors may comprise APCDD1 itself or fragments thereof, either in an isolated form or fused or associated with other moieties (e.g., Fc-APCDD1, PEG-APCDD1 or APCDD1 associated with a biological response modifier). In other selected embodiments the disclosed

APCDD1 effectors may comprise APCDD1 antagonists which, for the purposes of the instant application, shall be held to mean any molecule, construct or compound that recognizes, competes, interacts, binds or associates with APCDD1 and neutralizes, eliminates, reduces, sensitizes, reprograms, inhibits or controls the growth, expansion or recurrence of tumor initiating cells. In preferred embodiments the APCDD1 effectors of the instant invention comprise anti-APCDD1 antibodies, or immunoreactive fragments or derivatives thereof, that have unexpectedly been found to silence, neutralize, reduce, decrease, deplete, moderate, diminish, reprogram, eliminate, or otherwise inhibit the ability of tumor initiating cells to propagate, maintain, expand, proliferate or otherwise facilitate the survival, regeneration and/or metastasis of neoplastic cells.

In selected embodiments the APCDD1 effector may comprise a humanized antibody wherein said antibody comprises a heavy chain variable region amino acid sequence substantially similar to a sequence selected from the group consisting of SEQ ID NO: 90 and SEQ ID NO: 94 and a light chain variable region amino acid sequence substantially similar to a sequence selected from the group consisting of SEQ ID NO: 92 and SEQ ID NO: 96. In other preferred embodiments the invention will be in the form of a composition comprising a pharmaceutically acceptable carrier and an antibody selected from the group consisting of hSC7.13 and hSC7.48.

In certain other embodiments the invention will comprise an APCDD1 effector that reduces the frequency of tumor initiating cells upon administration to a subject. Preferably the reduction in frequency will be determined using *in vitro* or *in vivo* limiting dilution analysis. In particularly preferred embodiments such analysis may be conducted using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice. Alternatively, the limiting dilution analysis may be conducted using *in vitro* limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions. In either case, the analysis, calculation or quantification of the reduction in frequency will preferably comprise the use of Poisson distribution statistics to provide an accurate accounting. It will be appreciated that, while such quantification methods are preferred, other, less labor intensive methodology such as flow cytometry or immunohistochemistry may also be used to provide the desired values and, accordingly, are expressly contemplated as being within the scope of the instant invention.

As such, in another preferred embodiment of the instant invention comprises a method of treating an APCDD1 associated disorder comprising administering a

therapeutically effective amount of an APCDD1 effector to a subject in need thereof whereby the frequency of tumor initiating cells is reduced. Again, the reduction in the tumor initiating cell frequency will preferably be determined using *in vitro* or *in vivo* limiting dilution analysis.

In regard to the foregoing it will be appreciated that the present invention is based, at least in part, upon the discovery that the APCDD1 polypeptide is associated with tumor perpetuating cells (i.e., cancer stem cells) that are involved in the etiology of various neoplasia. More specifically, the instant application unexpectedly shows that the administration of various exemplary APCDD1 effectors can reduce, inhibit or eliminate tumorigenic signaling by tumor initiating cells (i.e. reduce the frequency of tumor initiating cells). This reduced signaling, whether by reduction, depletion or elimination of the tumor initiating cells or by modifying tumor cell morphology (e.g., induced differentiation, niche disruption) or otherwise interfering with their ability to exert affects on the tumor environment or other cells, in turn allows for the more effective treatment of APCDD1 associated disorders by inhibiting tumorigenesis, tumor maintenance and/or metastasis and recurrence. Further, as will be discussed in more detail below, APCDD1 polypeptide is intimately involved in the Wnt/beta-catenin oncogenic survival pathway. Intervention in this developmental signaling pathways, using the novel APCDD1 effectors described herein, may further ameliorate the disorder by more than one mechanism (i.e., tumor initiating cell reduction and disruption of developmental signaling) to provide an additive or synergistic effect.

Thus, another preferred embodiment of the invention comprises a method of treating an APCDD1 mediated disorder in a subject in need thereof comprising the step of administering an APCDD1 effector to said subject. In particularly preferred embodiments the APCDD1 effector will be associated (e.g., conjugated) with an anti-cancer agent. In addition, as seen in the Examples herein such disruption and collateral benefits may be achieved whether the subject tumor tissue exhibits elevated levels of APCDD1 or reduced or depressed levels of APCDD1 as compared with normal adjacent tissue.

Accordingly, in other particularly preferred embodiments the invention comprises a method of treating a subject suffering from neoplastic disorder comprising a solid tumor exhibiting a KRAS mutation, an APC mutation, a CTNNB1 mutation, or any mutation positively impacting the B-catenin/TCF signaling pathway, said method comprising the step of administering a therapeutically effective amount of at least one APCDD1 effector.

Similarly, as the compounds of the instant invention may exert therapeutic benefits through various physiological mechanisms, the present invention is also directed to selected effectors that are specifically fabricated to exploit certain cellular processes. For example, in certain embodiments the preferred effector may be engineered to associate with APCDD1 on the surface of the tumor initiating cell and stimulate the subject's immune response. In other embodiments the effector may comprise an antibody directed to an epitope that facilitates internalization which is then used to introduce a conjugated cytotoxic agent into the cell. As such, it is important to appreciate that the present invention is not limited to any particular mode of action but rather encompasses any method or APCDD1 effector that achieves the desired outcome.

Within such a framework it will be appreciated that preferred embodiments of the disclosed embodiments are directed to a method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one internalizing APCDD1 effector.

Other facets of the instant invention exploit the ability of the disclosed effectors to potentially disrupt multiple oncogenic survival pathways while simultaneously silencing tumor initiating cells. Such multi-active APCDD1 effectors (e.g. APCDD1 antagonists) may also prove to be particularly effective when used in combination with standard of care anti-cancer agents and/or debulking agents. In addition, two or more APCDD1 antagonists (e.g. antibodies that specifically bind to two discrete epitopes on APCDD1) may be used in combination in accordance with the present teachings. As discussed in some detail below, the APCDD1 effectors of the present invention may be used in a conjugated or unconjugated state and, optionally, as a sensitizing agent in combination with a variety chemical or biological anti-cancer agents.

Thus, another preferred embodiment comprises a method of sensitizing a tumor in a subject for treatment with an anti-cancer agent comprising the step of administering an APCDD1 effector to said subject. In a particularly preferred aspect of this facet of the invention the APCDD1 effector will specifically result in a reduction of tumor initiating cell frequency is as determined using *in vitro* or *in vivo* limiting dilution analysis.

In yet another embodiment the present invention provides methods of maintenance therapy wherein the disclosed effectors are administered over a period of time following an initial procedure (e.g., chemotherapeutic, radiation or surgery) designed to remove at least a portion of the tumor mass. Such therapeutic regimens may be administered over a

period of weeks, a period of months or even a period of years wherein the APCDD1 effectors may act prophylactically to inhibit metastasis and/or tumor recurrence.

Beyond the therapeutic uses discussed above it will also be appreciated that the effectors of the instant invention may be used to diagnose APCDD1 related disorders and, in particular, hyperproliferative disorders. As such, a preferred embodiment comprises a method of diagnosing a hyperproliferative disorder in a subject in need thereof comprising the steps of:

a. obtaining a tissue sample from said subject comprising tumorigenic cells;
b. contacting the tissue sample with at least one APCDD1 effector; and
c. detecting or quantifying an amelioration of the tumorigenic cells.

Such methods may be easily discerned in conjunction with the instant application and may be readily performed using generally available commercial technology such as automatic plate readers, dedicated reporter systems, etc. In preferred embodiments the amelioration of tumorigenic cells will comprise a reduction of tumor initiating cell frequency. Moreover, limiting dilution analysis may be conducted as previously alluded to above and will preferably employ the use of Poisson distribution statistics to provide an accurate accounting as to the reduction of frequency.

In a similar vein the present invention also provides kits that are useful in the diagnosis and monitoring of APCDD1 associated disorders such as cancer. To this end the present invention preferably provides an article of manufacture useful for diagnosing or treating APCDD1 associated disorders comprising a receptacle comprising an APCDD1 effector and instructional materials for using said APCDD1 effector to treat or diagnose the APCDD1 associated disorder through a reduction in frequency of tumor initiating cells.

Other preferred embodiments of the invention also exploit the properties of the disclosed effectors as an instrument useful for indentifying, isolating, sectioning or enriching populations or subpopulations of tumor initiating cells through methods such as fluorescent activated cell sorting (FACS) or laser mediated sectioning.

As such, another preferred embodiment of the instant invention is directed to a method of identifying, isolating, sectioning or enriching a population of tumor initiating cells comprising the step of contacting said tumor initiating cells with an APCDD1 effector.

The foregoing is a summary and thus contains, by necessity, simplifications, generalizations, and omissions of detail; consequently, those skilled in the art will

appreciate that the summary is illustrative only and is not intended to be in any way limiting. Other aspects, features, and advantages of the methods, compositions and/or devices and/or other subject matter described herein will become apparent in the teachings set forth herein. The summary is provided to introduce a selection of concepts in a simplified form that are further described below in the Detailed Description. This summary is not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A, 1B, 1C and 1D depict, respectively, nucleic acid sequences encoding human APCDD1 (SEQ ID NO: 1), the corresponding amino acid sequence of human APCDD1 precursor protein comprising an amino terminus signal sequence (SEQ ID NO: 2), the nucleic acid (SEQ ID NO: 3) and corresponding protein translation of his-tagged APCDD1-ECD and the nucleic acid (SEQ ID NO: 4) and corresponding protein translation of Fc-APCDD1-ECD;

FIG. 2 is a graphical representation depicting the gene expression levels of human APCDD1 obtained using whole transcriptome sequencing of highly enriched tumor progenitor cell (TProg) and tumor perpetuating cell (TPC) and non-tumorigenic cell (NTG) populations obtained from a subset of whole colorectal tumor specimens;

FIG. 3 is a graphical representation showing the relative gene expression levels of human APCDD1 in highly enriched tumor progenitor cell (TProg) and tumor perpetuating cell (TPC) populations obtained from untreated and irinotecan treated mice bearing one of three different non-traditional xenograft (NTX) colorectal tumor cell lines, and normalized against non-tumorigenic (NTG) enriched cell populations as measured using quantitative RT-PCR;

FIGS. 4A and 4B are graphical illustrations showing the relative gene expression levels of human APCDD1 in whole colorectal tumor specimens from patients with Stage I-IV disease, as normalized against the mean of expression in normal colon and rectum tissue and measured using quantitative RT-PCR;

FIGS. 5A and 5B are graphical representations showing the relative or absolute gene expression levels, respectively, of human APCDD1 in whole tumor specimens (grey dots) or matched normal adjacent tissue (white dots) from patients with one of eighteen different solid tumor types;

FIG. 6 depicts three FACs plots, a negative control and two exemplary antibody producing clones, illustrating the ability of effectors of the present invention to associate with APCDD1on a cell surface and enrich the subject cell population;

FIG. 7 graphically illustrates the ability of 165 individual effector secreting clones to inhibit proliferation of APCDD1 positive cells as normalized against a 20% inhibition of negative controls;

FIGS. 8A – 8C represent the ability of the APCDD1 effectors to internalize following association with APCDD1 on the surface of a cell with FIG. 8A providing FACs plots for two exemplary internalizing effectors and controls, FIG. 8B graphically illustrates that approximately half of the screened effectors internalized while FIG. 8C is indicative as to the amount of internalization for twenty selected effectors;

FIGS. 9A – 9M provide the nucleic acid and amino acid sequences of the heavy and light chain variable regions of thirteen discrete anti-APCDD1 effectors isolated and cloned as described in the Examples herein;

FIGS. 10A and 10B are tabular representations showing, respectively, the genetic arrangement and the heavy and light chain CDR sequences of twelve discrete APCDD1 effectors generated, isolated and cloned as described in the Examples herein;

FIG. 11 provides immunochemical characteristics of fourteen discrete APCDD1 effectors in a tabular format;

FIGS. 12A and 12B respectively illustrate the amino acid and nucleic acid sequences of the heavy (SEQ ID NO: 89 and SEQ ID NO: 90) and light chain (SEQ ID NO: 91 and SEQ ID NO: 92) variable regions of hSC7.13 (FIG. 12A) and the amino acid and nucleic acid sequences of the heavy (SEQ ID NO: 93 and SEQ ID NO: 94) and light chain (SEQ ID NO: 95 and SEQ ID NO: 96) variable regions of hSC7.48 wherein the CDR sequences as defined by Chothia et al. are underlined;

FIGS. 13A – 13C represent the measured affinity of murine antibody SC7.13 (FIG. 13A) and the humanized antibody derivative hSC7.13 (FIG. 13B) against three different concentrations of antigen, and provides a tabular summary including the measured values (FIG. 13C);

FIG. 14 graphically demonstrates that APCDD1, as recognized by the effectors of the present invention, is more highly expressed on tumorigenic cell populations that on non-tumorigenic cell populations;

FIGS. 15A - 15C demonstrate the ability of the disclosed effectors to selectively facilitate the association of an anti-cancer agent with APCDD1^{+} cells and thereby reduce

the target cell population where non-target cells are not eliminated (FIG. 15A) and non-specific effectors do not facilitate the association (FIGS. 15B and 15C);

FIG. 16 graphically illustrates the ability of the effectors of the present invention to facilitate the association of an anti-cancer agent with NTX breast tumorigenic cells thereby reducing the population of such cells; and

FIGS. 17A and 17B depict the capability of two different humanized effectors of the instant invention, hSC7.13 (FIG. 17A) and hSC7.48 (FIG. 17B) to facilitate the association of an anti-cancer agent with APCDD1+ cells and eliminate some portion thereof.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

In a broad sense, embodiments of the present invention are directed to novel APCDD1 effectors and their use in treating, managing, ameliorating or preventing the occurrence of hyperproliferative disorders including cancer. Without wishing to be bound by any particular theory, it has been discovered that the disclosed effectors are effective in reducing or retarding tumor growth and depleting, eliminating or neutralizing tumorigenic cells as well as altering the sensitivity of such cells to anti-cancer agents. Further, it has surprisingly been discovered that there is a heretofore unknown phenotypic association between selected tumor perpetuating cells (TPC) and the protein known as APCDD1. In this regard it has been found that selected TPCs (e.g., cancer stem cells associated with colorectal tumors), express elevated levels of APCDD1 when compared to normal tissue, tumor progenitor cells (TProg), and non-tumorigenic (NTG) cells that, together, comprise the majority of a solid tumor. In other exemplary tumor samples the levels of APCDD1 have actually been found to be repressed when compared to normal adjacent tissue. Given these differing expression levels, APCDD1 comprises a tumor associated marker (or antigen) that has been found to provide an effective agent for the detection and suppression of TPC and associated neoplasia due to altered levels of the protein in the tumor microenvironment. Significantly it has further been discovered that APCDD1 effectors, including immunoreactive antagonists and antibodies to the protein, effectively reduce the frequency of tumor initiating cells and are therefore be useful in depleting, sensitizing, eliminating, reducing, reprogramming, promoting the differentiation of, or otherwise precluding or limiting the ability of these tumor perpetuating cells to spread and/or continue to fuel tumor growth or recurrence in a patient.

In view of these discoveries those skilled in the art will appreciate that particularly preferred embodiments of the invention are largely directed to APCDD1 effectors and their use in reducing the frequency of tumor initiating cells. As will be discussed extensively herein, APCDD1 effectors compatible with instant invention broadly comprise any compound that associates, binds, complexes or otherwise reacts or competes with APCDD1 and, optionally, provides for a reduction in tumor initiating cell frequency. Exemplary effectors disclosed herein comprise nucleotides, oligonucleotides, polynucleotides, peptides or polypeptides. In certain preferred embodiments the selected effectors will comprise antibodies to APCDD1 or immunoreactive fragments or derivatives thereof. Such antibodies may be antagonistic or agonistic in nature and may be used in a conjugated or unconjugated form. In other preferred embodiments effectors compatible with the instant invention will comprise APCDD1 constructs comprising APCDD1 itself or a reactive fragment thereof. It will be appreciated that such APCDD1 constructs may comprise fusion proteins and can include reactive domains from other polypeptides such as immunoglobulins, stapled peptides or biological response modifiers. In yet other embodiments the effectors may operate on the genetic level and may comprise compounds as antisense constructs, siRNA, miRNA and the like. The foregoing APCDD1 effectors may attenuate the growth, propagation or survival of tumor perpetuating cells and/or associated neoplasia through competitive mechanisms, agonizing or antagonizing selected pathways or eliminating or depleting specific cells (including non-TPC support cells) depending, for example, on the form of APCDD1 effector or dosing and method of delivery. Still other effectors compatible with the instant teachings will be discussed in detail below.

In a related note, the following discussion pertains to APCDD1 effectors, APCDD1 antagonists and anti-APCDD1 antibodies. While a more detailed definition of each term is provided below, it will be appreciated that the terms are largely interchangeable for the purposes of this disclosure and should not be construed narrowly unless dictated by the context. For example, if a point is made relating to APCDD1 antagonists it is also applicable to those antibodies of the instant invention that happen to be antagonistic. Similarly, the term APCDD1 effectors expressly include disclosed APCDD1 antagonists and anti-APCDD1 antibodies and references to the latter are also applicable to effectors to the extent not precluded by context.

II. APCDD1 Physiology

As used herein the term APCDD1 (adenomatosis polyposis coli down-regulated 1) refers to naturally occurring human APCDD1 unless contextually dictated otherwise. Representative APCDD1 protein orthologs include, but are not limited to, human (i.e. hAPCDD1, NP_694545.1)), mouse (NP_573500.2), chimpanzee (XP_512021.2) and rat (XP_001071384.1). The human ortholog of the gene comprises a 1542 base pair open reading frame, which provides for a 514 amino acid (aa) polypeptide construct having a molecular weight of approximately 59 kDa. An exemplary nucleic acid sequence encoding human APCDD1 protein is shown in SEQ ID NO: 1 while the corresponding amino acid sequence is shown in SEQ ID NO: 2. It will be appreciated that the human APCDD1 protein includes a predicted signal or leader sequence comprising amino acids 1-14 of SEQ ID NO: 2 which is clipped off to provide the mature form of the protein (i.e. 500 aa). As previously indicated, unless otherwise indicated by direct reference or contextual necessity the term APCDD1 shall be directed to human APCDD1 and immunoreactive equivalents. That is, the exemplary human homolog of APCDD1 (GenBank Accession No: NM_153000; GeneID 147495) that is more fully described in Takshashi et al., Cancer Res. 60(20), 56512 (2002) which is incorporated herein by reference. It will further be appreciated that the term may also refer to a fragment of a native or variant form of APCDD1 that contains an epitope to which an antibody or immunoreactive fragment can specifically bind.

Originally identified as a downstream target of the beta-catenin/Tcf4 signaling pathway, APCDD1 is a membrane-bound glycoprotein that appears to interact with Wnt3A and LRP5: which are a ligand and co-receptor, respectively, needed to efficiently stimulate beta-catenin signaling downstream of the Frizzled receptor family. That is, APCDD1 is a single pass transmembrane protein that interacts with LRP and Wnt proteins at the cell surface, apparently acting to compete with Frizzled for LRP co-receptor interactions and/or inhibit Wnt interactions with Frizzled. By sequestering Wnt3A, APCDD1 appears to effectively block Wnt/Frizzled interactions and inhibit downstream signaling via the beta-catenin/Tcf4 signaling pathway. In so doing, APCDD1 appears to act as a negative feedback regulator of Wnt/Frizzled/beta-catenin signaling.

It will be appreciated that Wnt/Fzd signaling plays a large role in cell fate determination decisions within many tissues during organogenesis and development, and perturbation of these pathways often results in cancer. Moreover, multiple mouse genetic models wherein stem cells of the lower gastrointestinal tract have been identified and/or manipulated show that signaling via the Wnt/beta-catenin pathway impact tissue-resident

stem cell differentiation decisions leading to the generation of Paneth cells, which themselves have been suggested to support stem cell self-renewal and expansion at the base of tissue structures known as crypts; which is where the stem cells are known to reside. Disregulation of Wnt signaling by APCDD1 and/or impaired feedback regulation of this pathway by increased expression of APCDD1 in the TPC population may contribute to tumorigenesis, continued tumor growth and tumor recurrence. Inhibiting this contribution with APCDD1 antagonists may have therapeutic benefit by preventing feedback inhibition resulting from its association with Wnt/Frizzled/LRP complexes at the cell surface of tumor cells.

While not wishing to be bound by any particular theory, it is believed that APCDD1 effectors, antagonists, and particularly APCDD1 targeting moieties of the present invention act, at least in part, by either interfering with oncogenic survival outside the context of standard of care therapeutic regimens (e.g. irinotecan), thereby reducing or eliminating tumor initiating cell frequency or signaling or through the depletion of APCDD1 expressing cells. For example, elimination of TPC by antagonizing APCDD1 may include simply promoting cell proliferation in the face of chemotherapeutic regimens that eliminate proliferating cells, or promote differentiation of TPC such that their self-renewal (i.e. unlimited proliferation and maintenance of multipotency) capacity is lost. Alternatively, recruitment of cytotoxic T-cells to APCDD1 expressing cells, or delivery of a potent toxin conjugated to an anti-APCDD1 antibody that was able to internalize, may selectively kill TPC

III. Tumor Initiating Cells

While the role of APCDD1 has been somewhat elucidated and linked to various oncogenic pathways, the polypeptide has not, to the knowledge of the inventors, been previously associated with tumor initiating cells (e.g., cancer stem cells). Thus, in contrast to teachings of the prior art the present invention provides APCDD1 effectors that are particularly useful for targeting and neutralizing or reducing the frequency of tumor initiating cells (including TPCs) thereby facilitating the treatment of neoplastic disorders. More particularly it has surprisingly been found that tumor-initiating cells often express altered levels of the tumor associated antigen APCDD1 which, in preferred embodiments, may be exploited to provide a reduction in tumor initiating cell frequency in accordance with the present teachings and thereby facilitate the treatment or management of hyperproliferative disorders.

As used herein, the term tumor initiating cell (TIC) encompasses both tumor perpetuating cells (TPC; i.e., cancer stem cells or CSC) and highly proliferative tumor progenitor cells (termed TProg), which together generally comprise a unique subpopulation (i.e. 0.1-40%) of a bulk tumor or mass. For the purposes of the instant disclosure the terms tumor perpetuating cells and cancer stem cells are equivalent and may be used interchangeably herein. Conversely, TPC differ from TProg in that they can completely recapitulate the composition of tumor cells existing within a tumor and have unlimited self-renewal capacity as demonstrated by serial transplantation (two or more passages through mice) of low numbers of isolated cells. As will be discussed in more detail below fluorescence-activated cell sorting (FACS) using appropriate cell surface markers is a reliable method to isolate highly enriched cell subpopulations (> 99.5% purity) due, at least in part, to its ability to discriminate between single cells and clumps of cells (i.e. doublets, etc.). Using such techniques it has been shown that when low cell numbers of highly purified TProg cells are transplanted into immunocompromised mice they can fuel tumor growth in a primary transplant. However, unlike purified TPC subpopulations the TProg generated tumors do not completely reflect the parental tumor in phenotypic cell heterogeneity and are demonstrably inefficient at reinitiating serial tumorigenesis in subsequent transplants. In contrast, TPC (or CSC) subpopulations completely reconstitute the cellular heterogeneity of parental tumors and can efficiently initiate tumors when serially isolated and transplanted. Thus, those skilled in the art will recognize that a definitive difference between TPC and TProg, though both may be tumor generating in primary transplants, is the unique ability of TPC to perpetually fuel heterogeneous tumor growth upon serial transplantation at low cell numbers. Other common approaches to characterize TPC involve morphology and examination of cell surface markers, transcriptional profile, and drug response although marker expression may change with culture conditions and with cell line passage *in vitro*.

Accordingly, for the purposes of the instant invention tumor perpetuating cells, like normal stem cells that support cellular hierarchies in normal tissue, are preferably defined by their ability to self-renew indefinitely while maintaining the capacity for multilineage differentiation. Tumor perpetuating cells are thus capable of generating both tumorigenic progeny (i.e., tumor initiating cells: TPC and TProg) and non-tumorigenic (NTG) progeny. As used herein a non-tumorigenic cell (NTG) refers to a tumor cell that arises from tumor initiating cells, but does not itself have the capacity to self-renew or generate the heterogeneous lineages of tumor cells that comprise a tumor. Experimentally, NTG cells

are incapable of reproducibly forming tumors in mice, even when transplanted in excess cell numbers.

As indicated, TProg are also categorized as tumor initiating cells (or TIC) due to their limited ability to generate tumors in mice. TProg are progeny of TPC and are typically capable of a finite number of non-self-renewing cell divisions. Moreover, TProg cells may further be divided into early tumor progenitor cells (ETP) and late tumor progenitor cells (LTP), each of which may be distinguished by phenotype (e.g., cell surface markers) and different capacities to recapitulate tumor cell architecture. In spite of such technical differences, both ETP and LTP differ functionally from TPC in that they are generally less capable of serially reconstituting tumors when transplanted at low cell numbers and typically do not reflect the heterogeneity of the parental tumor. Notwithstanding the foregoing distinctions, it has also been shown that various TProg populations can, on rare occasion, gain self-renewal capabilities normally attributed to stem cells and themselves become TPC (or CSC). In any event both types of tumor-initiating cells are likely represented in the typical tumor mass of a single patient and are subject to treatment with the effectors as disclosed herein. That is, the disclosed compositions are generally effective in reducing the frequency or altering the chemosensitivity of such APCDD1 positive tumor initiating cells regardless of the particular embodiment or mix represented in a tumor.

In the context of the instant invention, TPC are more tumorigenic, relatively more quiescent and often more chemoresistant than the TProg (both ETP and LTP), NTG cells and the tumor-infiltrating non-TPC derived cells (e.g. fibroblasts/stroma, endothelial & hematopoietic cells) that comprise the bulk of a tumor. Given that conventional therapies and regimens have, in large part, been designed to both debulk tumors and attack rapidly proliferating cells, TPC are likely to be more resistant to conventional therapies and regimens than the faster proliferating TProg and other bulk tumor cell populations. Further, TPC often express other characteristics that make them relatively chemoresistant to conventional therapies, such as increased expression of multi-drug resistance transporters, enhanced DNA repair mechanisms and anti-apoptotic proteins. These properties, each of which contribute to drug tolerance by TPC, constitute a key reason for the failure of standard oncology treatment regimens to ensure long-term benefit for most patients with advanced stage neoplasia--i.e. the failure to adequately target and eradicate those cells that fuel continued tumor growth and recurrence (i.e. TPC or CSC).

Unlike many of the aforementioned prior art treatments, the novel compositions of the present invention preferably reduce the frequency of tumor initiating cells upon administration to a subject regardless of the form or specific target (e.g., genetic material, APCDD1 or APCDD1 ligand) of the selected effector. As noted above, the reduction in tumor initiating cell frequency may occur as a result of a) elimination, depletion, sensitization, silencing or inhibition of tumor initiating cells; b) controlling the growth, expansion or recurrence of tumor initiating cells; c) interrupting the initiation, propagation, maintenance, or proliferation of tumor initiating cells; or d) by otherwise hindering the survival, regeneration and/or metastasis of the tumorigenic cells. In some embodiments, the reduction in the frequency of tumor initiating cells occurs as a result of a change in one or more physiological pathways. The change in the pathway, whether by reduction or elimination of the tumor initiating cells or by modifying their potential (e.g., induced differentiation, niche disruption) or otherwise interfering with their ability to exert affects on the tumor environment or other cells, in turn allows for the more effective treatment of APCDD1-associated disorders by inhibiting tumorigenesis, tumor maintenance and/or metastasis and recurrence.

Among the methods that can be used to assess such a reduction in the frequency of tumor initiating cells is limiting dilution analysis either *in vitro* or *in vivo*, preferably followed by enumeration using Poisson distribution statistics or assessing the frequency of predefined definitive events such as the ability to generate tumors *in vivo* or not. While such limiting dilution analysis are the preferred methods of calculating reduction of tumor initiating cell frequency, other, less demanding methods, may also be used to effectively determine the desired values, albeit slightly less accurately, and are entirely compatible with the teachings herein. Thus, as will be appreciated by those skilled in the art, it is also possible to determine reduction of frequency values through well-known flow cytometric or immunohistochemical means. As to all the aforementioned methods see, for example, Dylla et al. 2008, PMCID: PMC2413402 & Hoey et al. 2009, PMID: 19664991; each of which is incorporated herein by reference in its entirety.

With respect to limiting dilution analysis, *in vitro* enumeration of tumor initiating cell frequency may be accomplished by depositing either fractionated or unfractionated human tumor cells (e.g. from treated and untreated tumors, respectively) into *in vitro* growth conditions that foster colony formation. In this manner, colony forming cells might be enumerated by simple counting and characterization of colonies, or by analysis consisting of, for example, the deposition of human tumor cells into plates in serial

dilutions and scoring each well as either positive or negative for colony formation at least 10 days after plating. *In vivo* limiting dilution experiments or analyses, which are generally more accurate in their ability to determine tumor initiating cell frequency, encompass the transplantation of human tumor cells, from either untreated control or treated conditions, for example, into immunocompromised mice in serial dilutions and subsequently scoring each mouse as either positive or negative for tumor formation at least 60 days after transplant. The derivation of cell frequency values by limiting dilution analysis *in vitro* or *in vivo* is preferably done by applying Poisson distribution statistics to the known frequency of positive and negative events, thereby providing a frequency for events fulfilling the definition of a positive event; in this case, colony or tumor formation, respectively.

As to other methods compatible with the instant invention that may be used to calculate tumor initiating cell frequency, the most common comprise quantifiable flow cytometric techniques and immunohistochemical staining procedures. Though not as precise as the limiting dilution analysis techniques described immediately above, these procedures are much less labor intensive and provide reasonable values in a relatively short time frame. Thus, it will be appreciated that a skilled artisan may use flow cytometric cell surface marker profile determination employing one or more antibodies or reagents that bind art recognized cell surface proteins known to enrich for tumor initiating cells and thereby measure TIC levels from various samples. In still another compatible method one skilled in the art might enumerate TIC frequency *in situ* (i.e. tissue section) by immunohistochemistry using one or more antibodies or reagents that are able to bind cell surface proteins thought to demarcate these cells.

Using any of the above-referenced methods it is then possible to quantify the reduction in frequency of TIC provided by the disclosed APCDD1 effectors in accordance with the teachings herein. In some instances, the compounds of the instant invention may reduce the frequency of TIC (by a variety of mechanisms noted above, including elimination, induced differentiation, niche disruption, silencing, etc.) by 10%, 15%, 20%, 25%, 30% or even by 35%. In other embodiments, the reduction in frequency of TICs may be on the order of 40%, 45%, 50%, 55%, 60% or 65%. In certain embodiments, the disclosed compounds my reduce the frequency of TICs by 70%, 75%, 80%, 85%, 90% or even 95%. Of course it will be appreciated that any reduction of the frequency of the TICs likely results in a corresponding reduction in the tumorigenicity, persistence, recurrence and aggressiveness of the neoplasia.

IV. APCDD1 Effectors

In any event, the present invention is directed to the use of APCDD1 effectors, including APCDD1 antagonists, for the treatment and/or prophylaxis of any one of a number of APCDD1 associated malignancies. The disclosed effectors may be used alone or in conjunction with a wide variety of anti-cancer compounds such as chemotherapeutic or immunotherapeutic agents or biological response modifiers. In other selected embodiments, two or more discrete APCDD1 effectors may be used in combination to provide enhanced anti-neoplastic effects or may be used to fabricate multispecific constructs.

In certain embodiments, the APCDD1 effectors of the present invention will comprise nucleotides, oligonucleotides, polynucleotides, peptides or polypeptides. Even more preferably the effectors will comprise soluble APCDD1 (sAPCDD1) or a form, variant, derivative or fragment thereof including, for example, APCDD1 fusion constructs (e.g., APCDD1-Fc, APCDD1-targeting moiety, etc.) or APCDD1-conjugates (e.g., APCDD1-PEG, APCDD1-cytotoxic agent, APCDD1-brm, etc.). It will also be appreciated that, in other embodiments, the APCDD1 effectors comprise antibodies (e.g., anti-APCDD1 mAbs) or immunoreactive fragments or derivatives thereof. In particularly preferred embodiments the effectors of the instant invention will comprise neutralizing antibodies or derivatives or fragments thereof. In other embodiments the APCDD1 effectors may comprise internalizing antibodies. Moreover, as with the aforementioned fusion constructs, these antibody effectors may be conjugated, linked or otherwise associated with selected cytotoxic agents, polymers, biological response modifiers (BRMs) or the like to provide directed immunotherapies with various (and optionally multiple) mechanisms of action. In yet other embodiments the effectors may operate on the genetic level and may comprise compounds as antisense constructs, siRNA, micro RNA and the like.

Based on the teachings herein those skilled in the art will appreciate that particularly preferred embodiments of the invention may comprise sAPCDD1. As indicated above beta-catenin induced signaling plays a large role in the proliferation and cell fate determination decisions within many tissues during organogenesis and development, and perturbation of this pathway often results in cell dysfunction and/or cancer. Although many perturbations of this pathway result from mutations of intracellular proteins (e.g. APC or CTNNB1) likely not sensitive to protein agonist or antagonists of the

Frizzled/beta-catenin pathway, quite a few diseases and/or neoplasia results from overstimulation due to excess Frizzled ligands and/or increased sensitivity to these ligands. The extracellular domain of APCDD1 has been shown to neutralize extracellular/soluble Wnt3A, and may be used as a soluble antagonist of many Frizzled ligands; of which Wnt3A is one of many family members. In tumors where excess Frizzled/beta-catenin signaling as a result of excess local Frizzled ligand concentrations is playing a role in disease, these ligand-induced signals might be dampened by introducing sAPCDD1 to neutralize, for example, Wnt3A. As discussed elsewhere in more detail, in an especially preferred embodiment the sAPCDD1 will comprise a fusion protein having all or part of the APCDD1 extracellular domain operably linked to all or part of the fc region of an immunoglobulin.

It will further be appreciated that the disclosed APCDD1 effectors may deplete or eliminate or inhibit growth, propagation or survival of tumor cells, particularly TPC, and/or associated neoplasia through a variety of mechanisms, including agonizing or antagonizing selected pathways or eliminating specific cells depending, for example, on the form of APCDD1 effector, any associated payload or dosing and method of delivery. Accordingly, while preferred embodiments disclosed herein are directed to the depletion, inhibition or silencing of specific tumor cell subpopulations such as tumor perpetuating cells it must be emphasized that such embodiments are merely illustrative and not limiting in any sense. Rather, as set forth in the appended claims, the present invention is broadly directed to APCDD1 effectors and their use in the treatment, management or prophylaxis of various APCDD1 mediated hyperproliferative disorders irrespective of any particular mechanism or target tumor cell population.

In the same sense disclosed embodiments of the instant invention comprise one or more APCDD1 antagonists. To that end it will be appreciated that APCDD1 antagonists of the instant invention may comprise any ligand, polypeptide, peptide, fusion protein, antibody or immunologically active fragment or derivative thereof that recognizes, reacts, binds, combines, competes, associates or otherwise interacts with the APCDD1 protein or fragment thereof and eliminates, silences, reduces, inhibits, hinders, restrains or controls the growth of tumor initiating cells or other neoplastic cells including bulk tumor or NTG cells. In selected embodiments the APCDD1 effector comprises an APCDD1 antagonist. In other embodiments the APCDD1 effector comprises a neutralizing antibody or fragment thereof.

As used herein an antagonist refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with the activities of a particular or specified protein, including the binding of receptors to ligands or the interactions of enzymes with substrates. More generally antagonists of the invention may comprise antibodies and antigen-binding fragments or derivatives thereof, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, antisense constructs, siRNA, miRNA, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. Antagonists may also include small molecule inhibitors, fusion proteins, receptor molecules and derivatives which bind specifically to the protein thereby sequestering its binding to its substrate target, antagonist variants of the protein, antisense molecules directed to the protein, RNA aptamers, and ribozymes against the protein.

As used herein and applied to two or more molecules or compounds, the term recognizes or specifically recognizes shall be held to mean the reaction, binding, specific binding, combination, association, interaction, connection, linkage, uniting, coalescence, merger or joining, covalently or non-covalently, of the molecules whereby one molecule exerts an effect on the other molecule.

Moreover, as demonstrated in the examples herein, some effectors of human APCDD1 may, in certain cases, cross-react with APCDD1 from a species other than human (e.g., murine). In other cases exemplary effectors may be specific for one or more isoforms of human APCDD1 and will not exhibit cross reactivity with APCDD1 orthologs from other species.

In any event, those skilled in the art will appreciate that the disclosed effectors may be used in a conjugated or unconjugated form. That is, the effector may be associated with or conjugated to (e.g. covalently or non-covalently) pharmaceutically active compounds, biological response modifiers, cytotoxic or cytostatic agents, diagnostic moieties or biocompatible modifiers. In this respect it will be understood that such conjugates may comprise peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, organic molecules and radioisotopes. Moreover, as indicated above the selected conjugate may be covalently or non-covalently linked to the APCDD1 effector in various molar ratios depending, at least in part, on the method used to effect the conjugation

It will further be appreciated that the disclosed effectors may be used alone or in conjunction with a wide variety of anti-cancer compounds such as chemotherapeutic or

immunotherapeutic agents. In other selected embodiments, two or more discrete APCDD1 effectors may be used in combination to provide enhanced anti-neoplastic effects or may be used to fabricate multispecific constructs. As discussed above, particularly preferred embodiments of the instant invention comprise one or more APCDD1 antagonists. To that end it will be appreciated that APCDD1 antagonists of the instant invention may comprise any ligand, polypeptide, peptide, antibody or immunologically active fragment or derivative thereof that recognizes, reacts, binds, combines, associates or otherwise interacts with the APCDD1 protein or fragment thereof and eliminates, silences, reduces, inhibits, hinders, restrains or controls the growth of tumor initiating cells or other neoplastic cells including bulk tumor or NTG cells.

V. APCDD1 Antibody Effectors

a. Overview

As previously alluded to particularly preferred embodiments of the instant invention comprise APCDD1 effectors in the form of antibodies. The term antibody herein is used in the broadest sense and specifically covers synthetic antibodies, monoclonal antibodies, oligoclonal or polyclonal antibodies, multiclonal antibodies, recombinantly produced antibodies, intrabodies, multispecific antibodies, bispecific antibodies, monovalent antibodies, multivalent antibodies, human antibodies, humanized antibodies, chimeric antibodies, primatized antibodies, Fab fragments, F(ab') fragments, single-chain FvFcs (scFvFc), single-chain Fvs (scFv), anti-idiotypic (anti-Id) antibodies and any other immunologically active antibody fragments so long as they exhibit the desired biological activity (i.e., APCDD1 association or binding). In a broader sense, the antibodies of the present invention include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antigen binding site, where these fragments may or may not be fused to another immunoglobulin domain including, but not limited to, an Fc region or fragment thereof. Further, as outlined in more detail herein, the terms antibody and antibodies specifically include Fc variants as described below, including full length antibodies and variant Fc-Fusions comprising Fc regions, or fragments thereof, optionally comprising at least one amino acid residue modification and fused to an immunologically active fragment of an immunoglobulin.

As will be discussed in more detail below, the generic term antibodies or immunoglobulin comprises five distinct classes of antibody that can be distinguished biochemically and, depending on the amino acid sequence of the constant domain of their

heavy chains, can readily be assigned to the appropriate class. For historical reasons, the major classes of intact antibodies are termed IgA, IgD, IgE, IgG, and IgM. In humans, the IgG and IgA classes may be further divided into recognized subclasses (isotypes), i.e., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2 depending on structure and certain biochemical properties. It will be appreciated that the IgG isotypes in humans are named in order of their abundance in serum with IgG1 being the most abundant.

While all five classes of antibodies (i.e. IgA, IgD, IgE, IgG, and IgM) and all isotypes (i.e., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), as well as variations thereof, are within the scope of the present invention, preferred embodiments comprising the IgG class of immunoglobulin will be discussed in some detail solely for the purposes of illustration. It will be understood that such disclosure is, however, merely demonstrative of exemplary compositions and methods of practicing the present invention and not in any way limiting of the scope of the invention or the claims appended hereto.

In this respect, human IgG immunoglobulins comprise two identical light polypeptide chains of molecular weight approximately 23,000 Daltons, and two identical heavy chains of molecular weight 53,000-70,000 depending on the isotype. Heavy-chain constant domains that correspond to the different classes of antibodies are denoted by the corresponding lower case Greek letter α, δ, ε, γ, and μ, respectively. The light chains of the antibodies from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains. Those skilled in the art will appreciate that the subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The four chains are joined by disulfide bonds in a Y configuration wherein the light chains bracket the heavy chains starting at the mouth of the Y and continuing through the variable region to the dual ends of the Y. Each light chain is linked to a heavy chain by one covalent disulfide bond while two disulfide linkages in the hinge region join the heavy chains. The respective heavy and light chains also have regularly spaced intrachain disulfide bridges the number of which may vary based on the isotype of IgG.

Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. In this regard, it will be appreciated that the variable domains of both the light (V_L) and heavy (V_H) chain portions determine antigen

recognition and specificity. Conversely, the constant domains of the light chain (C_L) and the heavy chain (C_H1, C_H2 or C_H3) confer and regulate important biological properties such as secretion, transplacental mobility, circulation half-life, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. Thus, the amino or N-terminus of the antibody comprises the variable region and the carboxy or C-terminus comprises the constant region. Thus, the C_H3 and C_L domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

The term variable refers to the fact that certain portions of the variable domains differ extensively in sequence among immunoglobulins and these hot spots largely define the binding and specificity characteristics of a particular antibody. These hypervariable sites manifest themselves in three segments, known as complementarity determining regions (CDRs), in both the light-chain and the heavy-chain variable domains respectively. The more highly conserved portions of variable domains flanking the CDRs are termed framework regions (FRs). More specifically, in naturally occurring monomeric IgG antibodies, the six CDRs present on each arm of the antibody are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding site as the antibody assumes its three dimensional configuration in an aqueous environment.

The framework regions comprising the remainder of the heavy and light variable domains show less inter-molecular variability in amino acid sequence. Rather, the framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β-sheet structure. Thus, these framework regions act to form a scaffold that provides for positioning the six CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding site formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen (i.e. APCDD1). This complementary surface promotes the non-covalent binding of the antibody to the immunoreactive antigen epitope. It will be appreciated that the position of CDRs can be readily identified by one of ordinary skill in the art.

As discussed in more detail below all or part of the heavy and light chain variable regions may be recombined or engineered using standard recombinant and expression techniques to provide effective antibodies. That is, the heavy or light chain variable region from a first antibody (or any portion thereof) may be mixed and matched with any selected portion of the heavy or light chain variable region from a second antibody. For

example, in one embodiment, the entire light chain variable region comprising the three light chain CDRs of a first antibody may be paired with the entire heavy chain variable region comprising the three heavy chain CDRs of a second antibody to provide an operative antibody. Moreover, in other embodiments, individual heavy and light chain CDRs derived from various antibodies may be mixed and matched to provide the desired antibody having optimized characteristics. Thus, an exemplary antibody may comprise three light chain CDRs from a first antibody, two heavy chain CDRs derived from a second antibody and a third heavy chain CDR from a third antibody.

More specifically, in the context of the instant invention it will be appreciated that any of the disclosed heavy and light chain CDRs disclosed in FIG. 7 may be rearranged in this manner to provide optimized anti-APCDD1 (e.g. anti-APCDD1) antibodies in accordance with the instant teachings.

In any event, it is to be understood that the complementarity determining regions residue numbers referred to herein are those of Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.), specifically, residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain and 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Note that CDRs vary considerably from antibody to antibody (and by definition will not exhibit homology with the Kabat consensus sequences). Maximal alignment of framework residues frequently requires the insertion of spacer residues in the numbering system, to be used for the Fv region. With this in mind, the skilled artisan will understand that the CDRs referred to herein are those of Kabat et al. supra. In addition, the identity of certain individual residues at any given Kabat site number may vary from antibody chain to antibody chain due to interspecies or allelic divergence. *See also* Chothia et al., J. Mol. Biol. 196:901-917 (1987) and by MacCallum et al., J. Mol. Biol. 262:732-745 (1996) where the definitions include overlapping or subsets of amino acid residues when compared against each other. The amino acid residues which encompass the CDRs as defined by each of the above cited references are set forth for comparison.

CDR Definitions

	Kabat[1]	Chothia[2]	MacCallum[3]
V_H CDR1	31-35	26-32	30-35
V_H CDR2	50-65	53-55	47-58
V_H CDR3	95-102	96-101	93-101
V_L CDR1	24-34	26-32	30-36
V_L CDR2	50-56	50-52	46-55
V_L CDR3	89-97	91-96	89-96

[1]Residue numbering follows the nomenclature of Kabat et al., supra
[2]Residue numbering follows the nomenclature of Chothia et al., supra
[3]Residue numbering follows the nomenclature of MacCallum et al., supra

The term variable region CDR amino acid residue includes amino acids in a CDR as identified using sequence or structure based methods, in light of the above.

As used herein the term variable region framework (FR) amino acid residues refers to those amino acids in the framework region of an Ig chain. The term framework region or FR region as used herein, includes the amino acid residues that are part of the variable region, but are not part of the CDRs (e.g., using the Kabat definition of CDRs). Therefore, a variable region framework is a non-contiguous sequence between about 100-120 amino acids in length but includes only those amino acids outside of the CDRs.

For the specific example of a heavy chain variable region and for the CDRs as defined by Kabat et al., framework region 1 corresponds to the domain of the variable region encompassing amino acids 1-30; framework region 2 corresponds to the domain of the variable region encompassing amino acids 36-49; framework region 3 corresponds to the domain of the variable region encompassing amino acids 66-94, and framework region 4 corresponds to the domain of the variable region from amino acids 103 to the end of the variable region. The framework regions for the light chain are similarly separated by each of the light claim variable region CDRs. Similarly, using the definition of CDRs by Chothia et al. or McCallum et al. the framework region boundaries are separated by the respective CDR termini as described above. In preferred embodiments set forth herein the CDRs are as defined by Kabat.

With the aforementioned structural considerations in mind, those skilled in the art will appreciate that the antibodies of the present invention may comprise any one of a number of functional embodiments. In this respect, compatible antibodies may comprise

any immunoreactive antibody (as the term is defined herein) that provides the desired physiological response in a subject. While any of the disclosed antibodies may be used in conjunction with the present teachings, certain embodiments of the invention will comprise chimeric, humanized or human monoclonal antibodies or immunoreactive fragments thereof. Yet other embodiments may, for example, comprise homogeneous or heterogeneous multimeric constructs, Fc variants and conjugated or glycosylationally altered antibodies. Moreover, it will be understood that such configurations are not mutually exclusive and that compatible individual antibodies may comprise one or more of the functional aspects disclosed herein. For example, a compatible antibody may comprise a single chain diabody with humanized variable regions or a fully human full length IgG3 antibody with Fc modifications that alter the glycosylation pattern to modulate serum half-life. Other exemplary embodiments are readily apparent to those skilled in the art and may easily be discernable as being within the scope of the invention.

b. Antibody generation

As is well known various host animals, including rabbits, mice, rats, etc. may be inoculated and used to provide antibodies in accordance with the teachings herein. Art known adjuvants that may be used to increase the immunological response, depending on the inoculated species include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants may protect the antigen from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Preferably, if a polypeptide is being administered, the immunization schedule will involve two or more administrations of the polypeptide, spread out over several weeks.

After immunization of an animal with an APCDD1 immunogen, antibodies and/or antibody-producing cells can be obtained from the animal. In some embodiments, polyclonal anti-APCDD1 antibody-containing serum is obtained by bleeding or sacrificing the animal. The serum may be used for research purposes in the form obtained from the animal or, in the alternative, the anti-APCDD1 antibodies may be partially or fully purified to provide immunoglobulin fractions or homogeneous antibody preparations.

c. Monoclonal antibodies

While polyclonal antibodies may be used in conjunction with certain aspects of the present invention, preferred embodiments comprise the use of APCDD1 reactive monoclonal antibodies. As used herein, the term monoclonal antibody or mAb refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier monoclonal indicates the character of the antibody as not being a mixture of discrete antibodies and may be used in conjunction with any type of antibody. In certain embodiments, such a monoclonal antibody includes an antibody comprising a polypeptide sequence that binds or associates with APCDD1, wherein the APCDD1-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences.

In preferred embodiments, antibody-producing cell lines are prepared from cells isolated from the immunized animal. After immunization, the animal is sacrificed and lymph node and/or splenic B cells are immortalized by means well known in the art. Methods of immortalizing cells include, but are not limited to, transfecting them with oncogenes, infecting them with an oncogenic virus and cultivating them under conditions that select for immortalized cells, subjecting them to carcinogenic or mutating compounds, fusing them with an immortalized cell, e.g., a myeloma cell, and inactivating a tumor suppressor gene. If fusion with myeloma cells is used, the myeloma cells preferably do not secrete immunoglobulin polypeptides (a non-secretory cell line). Immortalized cells are screened using APCDD1, or an immunoreactive portion thereof. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunoassay (ELISA) or a radioimmunoassay.

More generally, discrete monoclonal antibodies consistent with the present invention can be prepared using a wide variety of techniques known in the art including hybridoma, recombinant techniques, phage display technologies, yeast libraries, transgenic animals (e.g. a XenoMouse® or HuMAb Mouse®) or some combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques such as broadly described above and taught in more detail in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) each of which is incorporated herein. Using the disclosed protocols, antibodies are preferably

raised in mammals by multiple subcutaneous or intraperitoneal injections of the relevant antigen and an adjuvant. As previously discussed, this immunization generally elicits an immune response that comprises production of antigen-reactive antibodies (that may be fully human if the immunized animal is transgenic) from activated splenocytes or lymphocytes. While the resulting antibodies may be harvested from the serum of the animal to provide polyclonal preparations, it is generally more desirable to isolate individual lymphocytes from the spleen, lymph nodes or peripheral blood to provide homogenous preparations of monoclonal antibodies. Most typically, the lymphocytes are obtained from the spleen and immortalized to provide hybridomas.

For example, as described above, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected APCDD1 binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations, which typically include discrete antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins that may be cross-reactive.

d. Chimeric antibodies

In another embodiment, the antibody of the invention may comprise chimeric antibodies derived from covalently joined protein segments from at least two different species or types of antibodies. It will be appreciated that, as used herein, the term chimeric antibodies is directed to constructs in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). In one exemplary embodiment, a chimeric antibody in accordance with the

teachings herein may comprise murine V_H and V_L amino acid sequences and constant regions derived from human sources. In other compatible embodiments a chimeric antibody of the present invention may comprise a CDR grafted or humanized antibody as described below.

Generally, a goal of making a chimeric antibody is to create a chimera in which the number of amino acids from the intended subject species is maximized. One example is the CDR-grafted antibody, in which the antibody comprises one or more complementarity determining regions (CDRs) from a particular species or belonging to a particular antibody class or subclass, while the remainder of the antibody chain(s) is/are identical with or homologous to a corresponding sequence in antibodies derived from another species or belonging to another antibody class or subclass. For use in humans, the variable region or selected CDRs from a rodent antibody often are grafted into a human antibody, replacing the naturally occurring variable regions or CDRs of the human antibody. These constructs generally have the advantages of providing full strength effector functions (e.g., CDC, ADCC, etc.) while reducing unwanted immune responses to the antibody by the subject.

e. Humanized antibodies

Similar to the CDR grafted antibody is a humanized antibody. Generally, a humanized antibody is produced from a monoclonal antibody raised initially in a non-human animal. As used herein humanized forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a CDR of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity.

In selected embodiments, the acceptor antibody may comprise consensus sequences. To create consensus human frameworks, frameworks from several human heavy chain or light chain amino acid sequences may be aligned to identify a consensus amino acid sequence. Moreover, in many instances, one or more framework residues in the variable domain of the human immunoglobulin are replaced by corresponding non-human residues from the donor antibody. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. Such substitutions help maintain the appropriate three-dimensional configuration of the grafted CDR(s) and often

improve infinity over similar constructs with no framework substitutions. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance using well-known techniques.

CDR grafting and humanized antibodies are described, for example, in U.S.P.Ns. 6,180,370, 5,693,762, 5,693,761, 5,585,089, and 5,530,101. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin, and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, e.g., 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). See also, e.g., Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1: 105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994); and U.S.P.Ns. 6,982,321 and 7,087,409. Still another method is termed humaneering and is described, for example, in U.S. 2005/0008625. For the purposes of the present application the term humanized antibodies will be held to expressly include CDR grafted antibodies (i.e. human antibodies comprising one or more grafted non-human CDRs) with no or minimal framework substitutions.

Additionally, a non-human anti-APCDD1 antibody may also be modified by specific deletion of human T cell epitopes or deimmunization by the methods disclosed in WO 98/52976 and WO 00/34317. Briefly, the heavy and light chain variable regions of an antibody can be analyzed for peptides that bind to MHC Class II; these peptides represent potential T-cell epitopes (as defined in WO 98/52976 and WO 00/34317). For detection of potential T-cell epitopes, a computer modeling approach termed peptide threading can be applied, and in addition a database of human MHC class II binding peptides can be searched for motifs present in the V_H and V_L sequences, as described in WO 98/52976 and WO 00/34317. These motifs bind to any of the 18 major MHC class II DR allotypes, and thus constitute potential T cell epitopes. Potential T-cell epitopes detected can be eliminated by substituting small numbers of amino acid residues in the variable regions, or by single amino acid substitutions. As far as possible, conservative substitutions are made. Often, but not exclusively, an amino acid common to a position in human germline

antibody sequences may be used. After the deimmunizing changes are identified, nucleic acids encoding V_H and V_L can be constructed by mutagenesis or other synthetic methods (e.g., de novo synthesis, cassette replacement, and so forth). A mutagenized variable sequence can, optionally, be fused to a human constant region.

In selected embodiments, at least 60%, 65%, 70%, 75%, or 80% of the humanized antibody variable region residues will correspond to those of the parental framework region (FR) and CDR sequences. In other embodiments at least 85% or 90% of the humanized antibody residues will correspond to those of the parental framework region (FR) and CDR sequences. In a further preferred embodiment, greater than 95% of the humanized antibody residues will correspond to those of the parental framework region (FR) and CDR sequences.

Humanized antibodies may be fabricated using common molecular biology and biomolecular engineering techniques as described herein. These methods include isolating, manipulating, and expressing nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from a hybridoma, eukaryotic cell or phage producing an antibody or immunoreactive fragment against a predetermined target, as described above, from germline immunoglobulin genes, or from synthetic constructs. The recombinant DNA encoding the humanized antibody can then be cloned into an appropriate expression vector.

Human germline sequences, for example, are disclosed in Tomlinson, I. A. et al. (1992) J. Mol. Biol. 227:776-798; Cook, G. P. et al. (1995) Immunol. Today 16: 237-242; Chothia, D. et al. (1992) J. Mol. Bio. 227:799-817; and Tomlinson et al. (1995) EMBO J 14:4628-4638. The V BASE directory provides a comprehensive directory of human immunoglobulin variable region sequences (See Retter et al., (2005) Nuc Acid Res 33: 671-674). These sequences can be used as a source of human sequence, e.g., for framework regions and CDRs. As set forth herein consensus human framework regions can also be used, e.g., as described in U.S.P.N. 6,300,064.

f. Human antibodies

In addition to the aforementioned antibodies, those skilled in the art will appreciate that the antibodies of the present invention may comprise fully human antibodies. For the purposes of the instant application the term human antibody comprises an antibody which possesses an amino acid sequence that corresponds to that of an antibody produced by a

human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

Human antibodies can be produced using various techniques known in the art. As alluded to above, phage display techniques may be used to provide immunoactive binding regions in accordance with the present teachings. Thus, certain embodiments of the invention provide methods for producing anti-APCDD1 antibodies or antigen-binding portions thereof comprising the steps of synthesizing a library of (preferably human) antibodies on phage, screening the library with APCDD1 or an antibody-binding portion thereof, isolating phage that bind APCDD1, and obtaining the immunoreactive fragments from the phage. By way of example, one method for preparing the library of antibodies for use in phage display techniques comprises the steps of immunizing a non-human animal comprising human or non-human immunoglobulin loci with APCDD1 or an antigenic portion thereof to create an immune response, extracting antibody-producing cells from the immunized animal; isolating RNA encoding heavy and light chains of antibodies of the invention from the extracted cells, reverse transcribing the RNA to produce cDNA, amplifying the cDNA using primers, and inserting the cDNA into a phage display vector such that antibodies are expressed on the phage. More particularly, DNA encoding the V_H and V_L domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (e.g., p CANTAB 6 or pComb 3 HSS). The vector may then be electroporated in E. coli and then the E. coli is infected with helper phage. Phage used in these methods are typically filamentous phage including fd and M13 and the V_H and V_L domains are usually recombinantly fused to either the phage gene III or gene VIII.

Recombinant human anti-APCDD1 antibodies of the invention may be isolated by screening a recombinant combinatorial antibody library prepared as above. In a preferred embodiment, the library is a scFv phage display library, generated using human V_L and V_H cDNAs prepared from mRNA isolated from B cells. Methods for preparing and screening such libraries are well known in the art and kits for generating phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, catalog no. 27-9400-01; and the Stratagene SurfZAP™ phage display kit, catalog no. 240612). There also are other methods and reagents that can be used in generating and screening antibody display libraries (see, e.g., U.S.P.N. 5,223,409; PCT Publication Nos. WO 92/18619, WO 91/17271, WO 92/20791, WO 92/15679, WO 93/01288, WO 92/01047, WO 92/09690; Fuchs et al., Bio/Technology 9:1370-1372 (1991); Hay et al.,

Hum. Antibod. Hybridomas 3:81-85 (1992); Huse et al., Science 246:1275-1281 (1989); McCafferty et al., Nature 348:552-554 (1990); Griffiths et al., EMBO J. 12:725-734 (1993); Hawkins et al., J. Mol. Biol. 226:889-896 (1992); Clackson et al., Nature 352:624-628 (1991); Gram et al., Proc. Natl. Acad. Sci. USA 89:3576-3580 (1992); Garrad et al., Bio/Technology 9:1373-1377 (1991); Hoogenboom et al., Nuc. Acid Res. 19:4133-4137 (1991); and Barbas et al., Proc. Natl. Acad. Sci. USA 88:7978-7982 (1991).

The antibodies produced by naive libraries (either natural or synthetic) can be of moderate affinity (K_d^{-1} of about 10^6 to 10^7 M^{-1}), but affinity maturation can also be mimicked in vitro by constructing and reselecting from secondary libraries as described in the art. For example, mutation can be introduced at random in vitro by using error-prone polymerase (reported in Leung et al., Technique, 1: 11-15 (1989)) in the method of Hawkins et al., J. Mol. Biol., 226: 889-896, (1992) or in the method of Gram et al., Proc. Natl. Acad. Sci. USA, 89: 3576-3580 (1992). Additionally, affinity maturation can be performed by randomly mutating one or more CDRs, e.g. using PCR with primers carrying random sequence spanning the CDR of interest, in selected individual Fv clones and screening for higher affinity clones. WO 9607754 described a method for inducing mutagenesis in a complementarity determining region of an immunoglobulin light chain to create a library of light chain genes. Another effective approach is to recombine the V_H or V_L domains selected by phage display with repertoires of naturally occurring V domain variants obtained from unimmunized donors and screen for higher affinity in several rounds of chain reshuffling as described in Marks et al., Biotechnol., 10: 779-783 (1992). This technique allows the production of antibodies and antibody fragments with affinities of about 10^{-9} M or less.

It will further be appreciated that similar procedures may be employed using libraries comprising eukaryotic cells (e.g., yeast) that express binding pairs on their surface. As with phage display technology, the eukaryotic libraries are screened against the antigen of interest (i.e., APCDD1) and cells expressing candidate-binding pairs are isolated and cloned. Steps may be taken to optimize library content and for affinity maturation of the reactive binding pairs. See, for example, U.S.P.N. 7,700,302 and U.S.S.N. 12/404,059. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. Nature Biotechnology 14:309-314 (1996): Sheets et al. Proc. Natl. Acad. Sci. 95:6157-6162 (1998)); Hoogenboom and Winter, J. Mol. Biol, 227:381 (1991); Marks et al., J. Mol. Biol, 222:581 (1991)). In other embodiments human binding pairs may be isolated from

combinatorial antibody libraries generated in eukaryotic cells such as yeast. See e.g., U.S.P.N. 7,700,302. Such techniques advantageously allow for the screening of large numbers of candidate effectors and provide for relatively easy manipulation of candidate sequences (e.g., by affinity maturation or recombinant shuffling).

Human antibodies can also be made by introducing 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.P.Ns. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and U.S.P.N 6,075,181 and 6,150,584 regarding Xenomouse® technology along with 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); Lonberg and Huszar, Intern. Rev. Immunol. 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B-lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual suffering from a neoplastic disorder or may have been immunized *in vitro*). See, e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol, 147 (l):86-95 (1991); and U.S.P.N. 5,750,373.

VI. Antibody Characteristics

No matter how obtained or which of the aforementioned forms the antibody effector takes (e.g., humanized, human, etc.) the preferred embodiments of the disclosed effectors may exhibit various characteristics. In this regard anti-APCDD1 antibody-producing cells (e.g., hybridomas or yeast colonies) may be selected, cloned and further screened for desirable characteristics including, for example, robust growth, high antibody production and, as discussed in more detail below, desirable antibody characteristics. Hybridomas can be expanded *in vivo* in syngeneic animals, in animals that lack an immune system, e.g., nude mice, or in cell culture *in vitro*. Methods of selecting, cloning and expanding hybridomas and/or colonies, each of which produces a discrete antibody species, are well known to those of ordinary skill in the art.

a. Neutralizing antibodies

In particularly preferred embodiments the antagonists of the instant invention will comprise neutralizing antibodies or derivative or fragment thereof. The term neutralizing antibody or neutralizing antagonist refers to an antibody or antagonist that recognizes, binds to or interacts with a ligand or enzyme, prevents binding of the ligand or enzyme to its binding partner or substrate and interrupts the biological response that otherwise would result from the interaction of the two molecules. In assessing the binding and specificity of an antibody or immunologically functional fragment or derivative thereof, an antibody or fragment will substantially inhibit binding of a ligand or enzyme to its binding partner or substrate when an excess of antibody reduces the quantity of binding partner bound to the target molecule by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or more (as measured in an *in vitro* competitive binding assay). In the case of antibodies to APCDD1, a neutralizing antibody or antagonist will diminish the ability of APCDD1 to facilitate Wnt/ Frizzled interactions by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or more. It will be appreciated that this diminished WnT/Frizzled interaction may be measured directly using art recognized techniques or may be measured indirectly by the impact such reduction will have on the pathway.

b. Internalizing antibodies

While evidence indicates that APCDD1 may be secreted by the cell, at least some APCDD1 remains likely remains associated with the cell surface thereby allowing for internalization of the disclosed effectors. Accordingly, anti-APCDD1 antibodies may be internalized, at least to some extent, by cells that express APCDD1. For example, an anti-APCDD1 antibody that binds to APCDD1 on the surface of a tumor-initiating cell may be internalized by the tumor-initiating cell. In particularly preferred embodiments such anti-APCDD1 antibodies may be associated with or conjugated to cytotoxic moieties that kill the cell upon internalization.

As used herein, an anti-APCDD1 antibody that internalizes is one that is taken up by the cell upon binding to APCDD1 associated with a mammalian cell. The internalizing antibody includes antibody fragments, human or humanized antibody and antibody conjugates. Internalization may occur *in vitro* or *in vivo*. For therapeutic applications, internalization may occur *in vivo*. The number of antibody molecules internalized may be sufficient or adequate to kill an APCDD1-expressing cell, especially an APCDD1-expressing tumor initiating cell. Depending on the potency of the antibody or antibody

conjugate, in some instances, the uptake of a single antibody molecule into the cell is sufficient to kill the target cell to which the antibody binds. For example, certain toxins are highly potent in killing such that internalization of one molecule of the toxin conjugated to the antibody is sufficient to kill the tumor cell. Whether an anti-APCDD1 antibody internalizes upon binding APCDD1 on a mammalian cell can be determined by various assays including those described in the experimental examples below. For example, methods of detecting whether an antibody internalizes into a cell are described in U.S.P.N. 7,619,068.

c. Epitope binding

It will further be appreciated the disclosed anti-APCDD1 antibodies will associate with, or bind to, discrete epitopes or determinants presented by the selected target(s). As used herein the term epitope refers to that portion of the target antigen capable of being recognized and specifically bound by a particular antibody. When the antigen is a polypeptide such as APCDD1, epitopes can be formed both from contiguous amino acids and noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained upon protein denaturing, whereas epitopes formed by tertiary folding are typically lost upon protein denaturing. An epitope typically includes at least 3, and more usually, at least 5 or 8-10 amino acids in a unique spatial conformation. More specifically, the skilled artisan will appreciate the term epitope includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor or otherwise interacting with a molecule. Epitopic determinants generally consist of chemically active surface groupings of molecules such as amino acids or carbohydrate or sugar side chains and generally have specific three dimensional structural characteristics, as well as specific charge characteristics. Additionally an epitope may be linear or conformational. In a linear epitope, all of the points of interaction between the protein and the interacting molecule (such as an antibody) occur linearly along the primary amino acid sequence of the protein. In a conformational epitope, the points of interaction occur across amino acid residues on the protein that are linearly separated from one another.

Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope, e.g., using the techniques described in the present invention. Alternatively, during the discovery process, the generation and characterization of antibodies may elucidate information about desirable epitopes. From this information, it is then possible to competitively screen antibodies for binding to the same epitope. An

approach to achieve this is to conduct competition studies to find antibodies that competitively bind with one another, i.e. the antibodies compete for binding to the antigen. A high throughput process for binning antibodies based upon their cross-competition is described in WO 03/48731.

As used herein, the term binning refers to a method to group antibodies based on their antigen binding characteristics. The assignment of bins is somewhat arbitrary, depending on how different the observed binding patterns of the antibodies tested. Thus, while the technique is a useful tool for categorizing antibodies of the instant invention, the bins do not always directly correlate with epitopes and such initial determinations should be further confirmed by other art recognized methodology.

With this caveat one can determine whether a selected primary antibody (or fragment thereof) binds to the same epitope or cross competes for binding with a second antibody by using methods known in the art and set forth in the Examples herein. In one embodiment, one allows the primary antibody of the invention to bind to APCDD1 under saturating conditions and then measures the ability of the secondary antibody to bind to APCDD1. If the test antibody is able to bind to APCDD1 at the same time as the primary anti-APCDD1 antibody, then the secondary antibody binds to a different epitope than the primary antibody. However, if the secondary antibody is not able to bind to APCDD1 at the same time, then the secondary antibody binds to the same epitope, an overlapping epitope, or an epitope that is in close proximity to the epitope bound by the primary antibody. As known in the art and detailed in the Examples below, the desired data can be obtained using solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay, a Biacore™ system (i.e., surface plasmon resonance – GE Healthcare), a ForteBio® Analyzer (i.e., bio-layer interferometry - ForteBio, Inc.) or flow cytometric methodology. In a particularly preferred embodiment, the analysis is performed using a Biacore or ForteBio instrument as demonstrated in the Examples below.

The term compete when used in the context of antibodies that compete for the same epitope means competition between antibodies is determined by an assay in which the antibody or immunologically functional fragment under test prevents or inhibits specific binding of a reference antibody to a common antigen. Typically, such an assay involves the use of purified antigen bound to a solid surface or cells bearing either of these, an unlabeled test immunoglobulin and a labeled reference immunoglobulin. Competitive inhibition is measured by determining the amount of label bound to the solid surface or

cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Antibodies identified by competition assay (competing antibodies) include antibodies binding to the same epitope as the reference antibody and antibodies binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antibody for steric hindrance to occur. Additional details regarding methods for determining competitive binding are provided in the examples herein. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75%. In some instance, binding is inhibited by at least 80%, 85%, 90%, 95%, or 97% or more.

Besides epitope specificity the disclosed antibodies may be characterized using a number of different physical characteristics including, for example, binding affinities, melting temperature (Tm), and isoelectric points.

d. Binding affinity

In this respect, the present invention further encompasses the use of antibodies that have a high binding affinity for APCDD1. An antibody of the invention is said to specifically bind its target antigen when the dissociation constant (K_d) is $\leq 10^{-8}$ M. The antibody specifically binds antigen with high affinity when the K_d is $\leq 5x10^{-9}$M, and with very high affinity when the K_d is $\leq 5x10^{-10}$ M. In one embodiment of the invention, the antibody has a K_d of $\leq 10^{-9}$ M and an off-rate of about $1x10^{-4}$/sec. In one embodiment of the invention, the off-rate is $<1x10^{-5}$/sec. In other embodiments of the invention, the antibodies will bind to APCDD1 with a K_d of between about 10^{-8} M and 10^{-10} M, and in yet another embodiment it will bind with a $K_d \leq 2x10^{-10}$ M. Still other selected embodiments of the present invention comprise antibodies that have a disassociation constant or K_d (k_{off}/k_{on}) of less than 10^{-2}M, less than $5x10^{-2}$M, less than 10^{-3}M, less than $5x10^{-3}$M, less than 10^{-4}M, less than $5x10^{-4}$M, less than 10^{-5}M, less than $5x10^{-5}$M, less than 10^{-6}M, less than $5x10^{-6}$M, less than 10^{-7}M, less than $5x10^{-7}$M, less than 10^{-8}M, less than $5x10^{-8}$M, less than 10^{-9}M, less than $5x10^{-9}$M, less than 10^{-10}M, less than $5x10^{-10}$M, less than 10^{-11}M, less than $5x10^{-11}$M, less than 10^{-12}M, less than $5x10^{-12}$M, less than 10^{-13}M, less than $5x10^{-13}$M, less than 10^{-14}M, less than $5x10^{-14}$M, less than 10^{-15}M or less than $5x10^{-15}$M.

In specific embodiments, an antibody of the invention that immunospecifically binds to APCDD1 has an association rate constant or k_{on} rate (APCDD1 (Ab) + antigen $(Ag)^{k}{}_{on} \leftarrow$Ab-Ag) of at least $10^5 M^{-1}s^{-1}$, at least $2x10^5 M^{-1}s^{-1}$, at least $5x10^5 M^{-1}s^{-1}$, at least $10^6 M^{-1}s^{-1}$, at least $5x10^6 M^{-1}s^{-1}$, at least $10^7 M^{-1}s^{-1}$, at least $5x10^7 M^{-1}s^{-1}$, or at least $10^8 M^{-1}s^{-1}$.

In another embodiment, an antibody of the invention that immunospecifically binds to APCDD1 has a k_{off} rate (APCDD1 (Ab) + antigen (Ag)$^{k}{}_{off}$←Ab-Ag) of less than $10^{-1}s^{-1}$, less than $5x10^{-1}s^{-1}$, less than $10^{-2}s^{-1}$, less than $5x10^{-2}s^{-1}$, less than $10^{-3}s^{-1}$, less than $5x10^{-3}s^{-1}$, less than $10^{-4}s^{-1}$, less than $5x10^{-4}s^{-1}$, less than $10^{-5}s^{-1}$, less than $5x10^{-5}s^{-1}$, less than $10^{-6}s^{-1}$, less than $5x10^{-6}s^{-1}$ less than $10^{-7}s^{-1}$, less than $5x10^{-7}s^{-1}$, less than $10^{-8}s^{-1}$, less than $5x10^{-8}s^{-1}$, less than $10^{-9}s^{-1}$, less than $5x10^{-9}s^{-1}$ or less than $10^{-10}s^{-1}$.

In other selected embodiments of the present invention anti-APCDD1 antibodies will have an affinity constant or K_a (k_{on}/k_{off}) of at least 10^2M^{-1}, at least $5x10^2M^{-1}$, at least 10^3M^{-1}, at least $5x10^3M^{-1}$, at least 10^4M^{-1}, at least $5x10^4M^{-1}$, at least 10^5M^{-1}, at least $5x10^5M^{-1}$, at least 10^6M^{-1}, at least $5x10^6M^{-1}$, at least 10^7M^{-1}, at least $5x10^7M^{-1}$, at least 10^8M^{-1}, at least $5x10^8M^{-1}$, at least 10^9M^{-1}, at least $5x10^9M^{-1}$, at least $10^{10}M^{-1}$, at least $5x10^{10}M^{-1}$, at least $10^{11}M^{-1}$, at least $5x10^{11}M^{-1}$, at least $10^{12}M^{-1}$, at least $5x10^{12}M^{-1}$, at least $10^{13}M^{-1}$, at least $5x10^{13}M^{-1}$, at least $10^{14}M^{-1}$, at least $5x10^{14}M^{-1}$, at least $10^{15}M^{-1}$ or at least $5x10^{15}M^{-1}$.

e. Isoelectric points

In addition to the aforementioned binding properties, anti-APCDD1 antibodies and fragments thereof, like all polypeptides, have an Isoelectric Point (pI), which is generally defined as the pH at which a polypeptide carries no net charge. It is known in the art that protein solubility is typically lowest when the pH of the solution is equal to the isoelectric point (pI) of the protein. Therefore it is possible to optimize solubility by altering the number and location of ionizable residues in the antibody to adjust the pI. For example the pI of a polypeptide can be manipulated by making the appropriate amino acid substitutions (e.g., by substituting a charged amino acid such as a lysine, for an uncharged residue such as alanine). Without wishing to be bound by any particular theory, amino acid substitutions of an antibody that result in changes of the pI of said antibody may improve solubility and/or the stability of the antibody. One skilled in the art would understand which amino acid substitutions would be most appropriate for a particular antibody to achieve a desired pI.

The pI of a protein may be determined by a variety of methods including but not limited to, isoelectric focusing and various computer algorithms (see for example Bjellqvist et al., 1993, Electrophoresis 14:1023). In one embodiment, the pI of the anti-APCDD1 antibodies of the invention is between is higher than about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In another embodiment, the pI of the anti-APCDD1 antibodies of the invention is between is higher than 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0. In yet

another embodiment, substitutions resulting in alterations in the pI of antibodies of the invention will not significantly diminish their binding affinity for APCDD1. As discussed in more detail below, it is specifically contemplated that the substitution(s) of the Fc region that result in altered binding to FcγR may also result in a change in the pI. In a preferred embodiment, substitution(s) of the Fc region are specifically chosen to effect both the desired alteration in FcγR binding and any desired change in pI. As used herein, the pI value is defined as the pI of the predominant charge form.

f. Thermal stability

It will further be appreciated that the Tm of the Fab domain of an antibody can be a good indicator of the thermal stability of an antibody and may further provide an indication of the shelf-life. Tm is merely the temperature of 50% unfolding for a given domain or sequence. A lower Tm indicates more aggregation/less stability, whereas a higher Tm indicates less aggregation/more stability. Thus, antibodies or fragments or derivatives having higher Tm are preferable. Moreover, using art recognized techniques it is possible to alter the composition of the anti-APCDD1 antibodies or domains thereof to increase or optimize molecular stability. See, for example, U.S.P.N. 7,960,142. Thus, in one embodiment, the Fab domain of a selected antibody has a Tm value higher than at least 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 100°C, 105°C, 110°C, 115°C or 120°C. In another embodiment, the Fab domain of an antibody has a Tm value higher than at least about 50°C, about 55°C, about 60°C, about 65°C, about 70°C, about 75°C, about 80°C, about 85°C, about 90°C, about 95°C, about 100°C, about 105°C, about 110°C, about 115°C or about 120°C. Thermal melting temperatures (Tm) of a protein domain (e.g., a Fab domain) can be measured using any standard method known in the art, for example, by differential scanning calorimetry (see, e.g., Vermeer et al., 2000, Biophys. J. 78:394-404; Vermeer et al., 2000, Biophys. J. 79: 2150-2154 both incorporated herein by reference).

VII. APCDD1 Effector Fragments and Derivatives

Whether the agents of the present invention comprise intact fusion constructs, antibodies, fragments or derivatives, the selected effectors will react, bind, combine, complex, connect, attach, join, interact or otherwise associate with APCDD1 and thereby provide the desired anti-neoplastic effects. Those of skill in the art will appreciate that effectors comprising anti-APCDD1 antibodies interact or associate with APCDD1 through one or more binding sites expressed on the antibody. More specifically, as used herein the

term binding site comprises a region of a polypeptide that is responsible for selectively binding to a target molecule of interest (e.g., enzyme, antigen, ligand, receptor, substrate or inhibitor). Binding domains comprise at least one binding site (e.g. an intact IgG antibody will have two binding domains and two binding sites). Exemplary binding domains include an antibody variable domain, a receptor-binding domain of a ligand, a ligand-binding domain of a receptor or an enzymatic domain. For the purpose of the instant invention the active region of APCDD1 (e.g., as part of an Fc-APCDD1 fusion construct) may comprise a binding site for a substrate.

a. Fragments

Regardless of which form of the effector (e.g. chimeric, humanized, etc.) is selected to practice the invention, it will be appreciated that immunoreactive fragments of the same may be used in accordance with the teachings herein. In the broadest sense, the term antibody fragment comprises at least a portion of an intact antibody (e.g. a naturally occurring immunoglobulin). More particularly the term fragment refers to a part or portion of an antibody or antibody chain (or APCDD1 molecule in the case of Fc fusions) comprising fewer amino acid residues than an intact or complete antibody or antibody chain. The term antigen-binding fragment refers to a polypeptide fragment of an immunoglobulin or antibody that binds antigen or competes with intact antibody (i.e., with the intact antibody from which they were derived) for antigen binding (i.e., specific binding). As used herein, the term fragment of an antibody molecule includes antigen-binding fragments of antibodies, for example, an antibody light chain (V_L), an antibody heavy chain (V_H), a single chain antibody (scFv), a F(ab')2 fragment, a Fab fragment, an Fd fragment, an Fv fragment, single domain antibody fragments, diabodies, linear antibodies, single-chain antibody molecules and multispecific antibodies formed from antibody fragments. Similarly, an enzymatically active fragment of APCDD1 comprises a portion of the APCDD1 molecule that retains its ability to interact with APCDD1 substrates and modify them (e.g., clip them) in a manner similar to that of an intact APCDD1 (though maybe with somewhat less efficiency).

Those skilled in the art will appreciate fragments can be obtained via chemical or enzymatic treatment of an intact or complete effector (e.g., antibody or antibody chain) or by recombinant means. In this regard, while various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, explicitly includes antibodies or

fragments or derivatives thereof either produced by the modification of whole antibodies or synthesized de novo using recombinant DNA methodologies.

More specifically, papain digestion of antibodies produces two identical antigen-binding fragments, called Fab fragments, each with a single antigen-binding site, and a residual Fc fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-binding sites and is still capable of cross-linking antigen. The Fab fragment also contains the constant domain of the light chain and the first constant domain (C_H1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy-chain C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. See, e.g., Fundamental Immunology, W. E. Paul, ed., Raven Press, N.Y. (1999), for a more detailed description of other antibody fragments.

It will further be appreciated that an Fv fragment is an antibody fragment that contains a complete antigen recognition and binding site. This region is made up of a dimer of one heavy and one light chain variable domain in tight association, which can be covalent in nature, for example in scFv. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs or a subset thereof confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although usually at a lower affinity than the entire binding site.

In other embodiments an antibody fragment, for example, is one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise on antigen binding arm linked to an Fc sequence capable of conferring *in vivo* stability to the fragment.

b. Derivatives

In another embodiment, it will further be appreciated that the effectors of the invention may be monovalent or multivalent (e.g., bivalent, trivalent, etc.). As used herein the term valency refers to the number of potential target (i.e., APCDD1) binding sites associated with an antibody. Each target binding site specifically binds one target molecule or specific position or locus on a target molecule. When an antibody of the instant invention comprises more than one target binding site (multivalent), each target binding site may specifically bind the same or different molecules (e.g., may bind to different ligands or different antigens, or different epitopes or positions on the same antigen). For the purposes of the instant invention, the subject antibodies will preferably have at least one binding site specific for human APCDD1. In one embodiment the antibodies of the instant invention will be monovalent in that each binding site of the molecule will specifically bind to a single APCDD1 position or epitope. In other embodiments, the antibodies will be multivalent in that they comprise more than one binding site and the different binding sites specifically associate with more than a single position or epitope. In such cases the multiple epitopes may be present on the selected APCDD1 polypeptide or a single epitope may be present on APCDD1 while a second, different epitope may be present on another molecule or surface. See, for example, U.S.P.N. 2009/0130105.

As alluded to above, multivalent antibodies may immunospecifically bind to different epitopes of the desired target molecule or may immunospecifically bind to both the target molecule as well as a heterologous epitope, such as a heterologous polypeptide or solid support material. While preferred embodiments of the anti-APCDD1 antibodies only bind two antigens (i.e. bispecific antibodies), antibodies with additional specificities such as trispecific antibodies are also encompassed by the instant invention. Examples of bispecific antibodies include, without limitation, those with one arm directed against APCDD1 and the other arm directed against any other antigen (e.g., an effector cell marker). Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., 1983, Nature, 305:537-539). Other more sophisticated compatible multispecific constructs and methods of their fabrication are set forth in U.S.P.N. 2009/0155255.

In yet other embodiments, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, C_H2, and/or C_H3 regions. In one example, the first heavy-chain constant region (C_H1) containing the site necessary for light chain binding is present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when, the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm (e.g., APCDD1), and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., 1986, Methods in Enzymology, 121:210. According to another approach described in WO96/27011, a pair of antibody molecules can be engineered to maximize the percentage of heterodimers that are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies also include cross-linked or heteroconjugate antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S.P.N. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S.P.N. 4,676,980, along with a number of cross-linking techniques.

VIII. APCDD1 Effectors - Constant Region Modifications

a. Fc region and Fc receptors

In addition to the various modifications, substitutions, additions or deletions to the variable or binding region of the disclosed effectors (e.g., Fc-APCDD1 or anti-APCDD1 antibodies) set forth above, those skilled in the art will appreciate that selected embodiments of the present invention may also comprise substitutions or modifications of the constant region (i.e. the Fc region). More particularly, it is contemplated that the APCDD1 effectors of the invention may contain inter alia one or more additional amino acid residue substitutions, mutations and/or modifications which result in a compound with preferred characteristics including, but not limited to: altered pharmacokinetics, increased serum half life, increase binding affinity, reduced immunogenicity, increased production, altered Fc ligand binding, enhanced or reduced ADCC or CDC activity, altered glycosylation and/or disulfide bonds and modified binding specificity. In this regard it will be appreciated that these Fc variants may advantageously be used to enhance the effective anti-neoplastic properties of the disclosed effectors.

The term Fc region herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and

antibody populations having a mixture of antibodies with and without the K447 residue. A functional Fc region possesses an effector function of a native sequence Fc region. Exemplary effector functions include C1q binding; CDC; Fc receptor binding; ADCC; phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays as disclosed, for example, in definitions herein.

Fc receptor or FcR describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FCRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγII receptors include FcγRIIA (an activating receptor) and FcγRIIB (an inhibiting receptor), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fcγ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daeron, Annu. Rev. Immunol. 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, Annu. Rev. Immunol 9:457-92 (1991); Capel et al., Immunomethods 4:25-34 (1994); and de Haas et al., J. Lab. Clin. Med. 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term FcR herein. The term Fc receptor or FcR also includes the neonatal receptor, FcRn, which, in certain instances, is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., Immunol. Today 18(12):592-598 (1997); Ghetie et al., Nature Biotechnology, 15(7):637-640 (1997); Hinton et al., J. Biol. Chem. 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

b. Fc functions

As used herein complement dependent cytotoxicity and CDC refer to the lysing of a target cell in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule, an antibody for example, complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., 1996, J. Immunol. Methods, 202:163, may be performed.

Further, antibody-dependent cell-mediated cytotoxicity or ADCC refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Specific high-affinity IgG antibodies directed to the target arm cytotoxic cells and are absolutely required for such killing. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement.

APCDD1 effector variants with altered FcR binding affinity or ADCC activity is one which has either enhanced or diminished FcR binding activity and/or ADCC activity compared to a parent or unmodified antibody or to an effector comprising a native sequence Fc region. The effector variant, which displays increased binding to an FcR, binds at least one FcR with better affinity than the parent or unmodified antibody or to an effector comprising a native sequence Fc region. A variant, which displays decreased, binding to an FcR, binds at least one FcR with worse affinity than the parent or unmodified antibody or to an effector comprising a native sequence Fc region. Such variants which display decreased binding to an FcR may possess little or no appreciable binding to an FcR, e.g., 0-20% binding to the FcR compared to a native sequence IgG Fc region, e.g. as determined techniques well known in the art.

As to FcRn, the antibodies of the instant invention also comprise or encompass Fc variants with modifications to the constant region that provide half-lives (e.g., serum half-lives) in a mammal, preferably a human, of greater than 5 days, greater than 10 days, greater than 15 days, preferably greater than 20 days, greater than 25 days, greater than 30 days, greater than 35 days, greater than 40 days, greater than 45 days, greater than 2 months, greater than 3 months, greater than 4 months, or greater than 5 months. The increased half-lives of the antibodies (or Fc containing molecules) of the present invention in a mammal, preferably a human, results in a higher serum titer of said antibodies or antibody fragments in the mammal, and thus, reduces the frequency of the administration of said antibodies or antibody fragments and/or reduces the concentration of said antibodies or antibody fragments to be administered. Antibodies having increased *in vivo* half-lives can be generated by techniques known to those of skill in the art. For example, antibodies with increased *in vivo* half-lives can be generated by modifying (e.g., substituting, deleting or adding) amino acid residues identified as involved in the interaction between the Fc domain and the FcRn receptor (see, e.g., International

Publication Nos. WO 97/34631; WO 04/029207; U.S.P.N. 6,737,056 and U.S.P.N. 2003/0190311. Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 describes antibody variants with improved or diminished binding to FcRns. See also, e.g., Shields et al. J. Biol. Chem. 9(2):6591-6604 (2001).

c. Glycosylation modifications

In still other embodiments, glycosylation patterns or compositions of the antibodies of the invention are modified. More particularly, preferred embodiments of the present invention may comprise one or more engineered glycoforms, i.e., an altered glycosylation pattern or altered carbohydrate composition that is covalently attached to a molecule comprising an Fc region. Engineered glycoforms may be useful for a variety of purposes, including but not limited to enhancing or reducing effector function, increasing the affinity of the antibody for a target antigen or facilitating production of the antibody. In cases where reduced effector function is desired, it will be appreciated that the molecule may be engineered to express in an aglycosylated form. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. That is, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby èliminate glycosylation at that site (see e.g. U.S.P.Ns. 5,714,350 and 6,350,861. Conversely, enhanced effector functions or improved binding may be imparted to the Fc containing molecule by engineering in one or more additional glycosylation sites.

Additionally or alternatively, an Fc variant can be made that has an altered glycosylation composition, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNAc structures. These and similar altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Engineered glycoforms may be generated by any method known to one skilled in the art, for example by using engineered or variant expression strains, by co-expression with one or more enzymes (for example N-acetylglucosaminyltransferase III (GnTI11)), by expressing a molecule comprising an Fc region in various organisms or cell lines from various organisms or by modifying carbohydrate(s) after the molecule comprising Fc region has been expressed. See, for example, Shields, R. L. et al. (2002) J. Biol. Chem. 277:26733-26740; Umana et al. (1999) Nat. Biotech. 17:176-1, as well as,

European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342, Umana et al, 1999, Nat. Biotechnol 17:176-180; Davies et al., 20017 Biotechnol Bioeng 74:288-294; Shields et al, 2002, J Biol Chem 277:26733-26740; Shinkawa et al., 2003, J Biol Chem 278:3466-3473) U.S.P.N. 6,602,684; U.S.S.Ns. 10/277,370; 10/113,929; PCT WO 00/61739A1; PCT WO 01/292246A1; PCT WO 02/311140A1; PCT WO 02/30954A1; Potillegent™ technology (Biowa, Inc.); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG); WO 00061739; EA01229125; U.S.P.N. 2003/0115614; Okazaki et al., 2004, JMB, 336: 1239-49.

IX. Effector Expression

a. Overview

DNA encoding the desired APCDD1 effectors may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding antibody heavy and light chains). Isolated and subcloned hybridoma cells (or phage or yeast derived colonies) may serve as a preferred source of such DNA if the effector is an antibody. If desired, the nucleic acid can further be manipulated as described herein to create agents including fusion proteins, or chimeric, humanized or fully human antibodies. More particularly, the isolated DNA (which may be modified) can be used to clone constant and variable region sequences for the manufacture antibodies as described in U.S.P.N. 7,709,611.

This exemplary method entails extraction of RNA from the selected cells, conversion to cDNA, and amplification by PCR using antibody specific primers. Suitable primers are well known in the art and, as exemplified herein, are readily available from numerous commercial sources. It will be appreciated that, to express a recombinant human or non-human antibody isolated by screening of a combinatorial library, the DNA encoding the antibody is cloned into a recombinant expression vector and introduced into host cells including mammalian cells, insect cells, plant cells, yeast, and bacteria. In yet other embodiments, the effectors are introduced into and expressed by simian COS cells, NS0 cells, Chinese Hamster Ovary (CHO) cells or myeloma cells that do not otherwise produce the desired construct. As will be discussed in more detail below, transformed cells expressing the desired effector may be grown up in relatively large quantities to provide clinical and commercial supplies of the fusion construct or immunoglobulin.

Whether the nucleic acid encoding the desired portion of the APCDD1 effector is obtained or derived from phage display technology, yeast libraries, hybridoma based

technology, synthetically or from commercial sources, it is to be understood that the present invention explicitly encompasses nucleic acid molecules and sequences encoding APCDD1 effectors including fusion proteins and anti-APCDD1 antibodies or antigen-binding fragments or derivatives thereof. The invention further encompasses nucleic acids or nucleic acid molecules (e.g., polynucleotides) that hybridize under high stringency, or alternatively, under intermediate or lower stringency hybridization conditions (e.g., as defined below), to polynucleotides complementary to nucleic acids having a polynucleotide sequence that encodes an effector of the invention or a fragment or variant thereof. The term nucleic acid molecule or isolated nucleic acid molecule, as used herein, is intended to include at least DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA. Moreover, the present invention comprises any vehicle or construct, incorporating such effector encoding polynucleotide including, without limitation, vectors, plasmids, host cells, cosmids or viral constructs.

The term isolated nucleic acid means a that the nucleic acid was (i) amplified in vitro, for example by polymerase chain reaction (PCR), (ii) recombinantly produced by cloning, (iii) purified, for example by cleavage and gel-electrophoretic fractionation, or (iv) synthesized, for example by chemical synthesis. An isolated nucleic acid is a nucleic acid that is available for manipulation by recombinant DNA techniques.

More specifically, nucleic acids that encode an effector, including one or both chains of an antibody of the invention, or a fragment, derivative, mutein, or variant thereof, polynucleotides sufficient for use as hybridization probes, PCR primers or sequencing primers for identifying, analyzing, mutating or amplifying a polynucleotide encoding a polypeptide, anti-sense nucleic acids for inhibiting expression of a polynucleotide, and complementary sequences of the foregoing are also provided. The nucleic acids can be any length. They can be, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 750, 1,000, 1,500, 3,000, 5,000 or more nucleotides in length, and/or can comprise one or more additional sequences, for example, regulatory sequences, and/or be part of a larger nucleic acid, for example, a vector. These nucleic acids can be single-stranded or double-stranded and can comprise RNA and/or DNA nucleotides, and artificial variants thereof (e.g., peptide nucleic acids). Nucleic acids encoding effectors of the invention, including antibodies or immunoreactive fragments or derivatives thereof, have preferably been isolated as described above.

b. Hybridization and Identity

As indicated, the invention further provides nucleic acids that hybridize to other nucleic acids under particular hybridization conditions. Methods for hybridizing nucleic acids are well known in the art. See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For the purposes of the instant application, a moderately stringent hybridization condition uses a prewashing solution containing 5x sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization buffer of about 50% formamide, 6xSSC, and a hybridization temperature of 55°C (or other similar hybridization solutions, such as one containing about 50% formamide, with a hybridization temperature of 42°C), and washing conditions of 60°C, in 0.5xSSC, 0.1% SDS. A stringent hybridization condition hybridizes in 6xSSC at 45°C, followed by one or more washes in 0.1xSSC, 0.2% SDS at 68°C. Furthermore, one of skill in the art can manipulate the hybridization and/or washing conditions to increase or decrease the stringency of hybridization such that nucleic acids comprising nucleotide sequences that are at least 65, 70, 75, 80, 85, 90, 95, 98 or 99% identical to each other typically remain hybridized to each other. More generally, for the purposes of the instant disclosure the term substantially identical with regard to a nucleic acid sequence may be construed as a sequence of nucleotides exhibiting at least about 85%, or 90%, or 95%, or 97% sequence identity to the reference nucleic acid sequence.

The basic parameters affecting the choice of hybridization conditions and guidance for devising suitable conditions are set forth by, for example, Sambrook, Fritsch, and Maniatis (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., chapters 9 and 11; and Current Protocols in Molecular Biology, 1995, Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4), and can be readily determined by those having ordinary skill in the art based on, for example, the length and/or base composition of the nucleic acid.

It will further be appreciated that nucleic acids may, according to the invention, be present alone or in combination with other nucleic acids, which may be homologous or heterologous. In preferred embodiments, a nucleic acid is functionally linked to expression control sequences that may be homologous or heterologous with respect to said nucleic acid. In this context the term homologous means that a nucleic acid is also functionally linked to the expression control sequence naturally and the term heterologous means that a nucleic acid is not functionally linked to the expression control sequence naturally.

c. Expression

A nucleic acid, such as a nucleic acid expressing RNA and/or protein or peptide, and an expression control sequence are functionally linked to one another, if they are covalently linked to one another in such a way that expression or transcription of said nucleic acid is under the control or under the influence of said expression control sequence. If the nucleic acid is to be translated into a functional protein, then, with an expression control sequence functionally linked to a coding sequence, induction of said expression control sequence results in transcription of said nucleic acid, without causing a frame shift in the coding sequence or said coding sequence not being capable of being translated into the desired protein or peptide.

The term expression control sequence comprises according to the invention promoters, ribosome binding sites, enhancers and other control elements that regulate transcription of a gene or translation of mRNA. In particular embodiments of the invention, the expression control sequences can be regulated. The exact structure of expression control sequences may vary as a function of the species or cell type, but generally comprises 5'-untranscribed and 5'- and 3'-untranslated sequences which are involved in initiation of transcription and translation, respectively, such as TATA box, capping sequence, CAAT sequence, and the like. More specifically, 5'-untranscribed expression control sequences comprise a promoter region that includes a promoter sequence for transcriptional control of the functionally linked nucleic acid. Expression control sequences may also comprise enhancer sequences or upstream activator sequences.

According to the invention the term promoter or promoter region relates to a nucleic acid sequence which is located upstream (5') to the nucleic acid sequence being expressed and controls expression of the sequence by providing a recognition and binding site for RNA-polymerase. The promoter region may include further recognition and binding sites for further factors that are involved in the regulation of transcription of a gene. A promoter may control the transcription of a prokaryotic or eukaryotic gene. Furthermore, a promoter may be inducible and may initiate transcription in response to an inducing agent or may be constitutive if transcription is not controlled by an inducing agent. A gene that is under the control of an inducible promoter is not expressed or only expressed to a small extent if an inducing agent is absent. In the presence of the inducing agent the gene is switched on or the level of transcription is increased. This is mediated, in general, by binding of a specific transcription factor.

Promoters which are preferred according to the invention include promoters for SP6, T3 and T7 polymerase, human U6 RNA promoter, CMV promoter, and artificial hybrid promoters thereof (e.g. CMV) where a part or parts are fused to a part or parts of promoters of genes of other cellular proteins such as e.g. human GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and including or not including (an) additional intron(s).

According to the invention, the term expression is used in its most general meaning and comprises the production of RNA or of RNA and protein/peptide. It also comprises partial expression of nucleic acids. Furthermore, expression may be carried out transiently or stably.

In a preferred embodiment, a nucleic acid molecule is according to the invention present in a vector, where appropriate with a promoter, which controls expression of the nucleic acid. The term vector is used here in its most general meaning and comprises any intermediary vehicle for a nucleic acid which enables said nucleic acid, for example, to be introduced into prokaryotic and/or eukaryotic cells and, where appropriate, to be integrated into a genome. Vectors of this kind are preferably replicated and/or expressed in the cells. Vectors may comprise plasmids, phagemids, bacteriophages or viral genomes. The term plasmid as used herein generally relates to a construct of extrachromosomal genetic material, usually a circular DNA duplex, which can replicate independently of chromosomal DNA.

In practicing the present invention it will be appreciated that many conventional techniques in molecular biology, microbiology, and recombinant DNA technology are optionally used. Such conventional techniques relate to vectors, host cells and recombinant methods as defined herein. These techniques are well known and are explained in, for example, Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif.; Sambrook et al., Molecular Cloning-A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 and Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2006). Other useful references, e.g. for cell isolation and culture (e.g., for subsequent nucleic acid or protein isolation) include Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley-Liss, New York and the references cited therein; Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, N.Y.; Gamborg and Phillips (Eds.) (1995) Plant Cell, Tissue and Organ Culture;

Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Atlas and Parks (Eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, Fla. Methods of making nucleic acids (e.g., by in vitro amplification, purification from cells, or chemical synthesis), methods for manipulating nucleic acids (e.g., site-directed mutagenesis, by restriction enzyme digestion, ligation, etc.), and various vectors, cell lines and the like useful in manipulating and making nucleic acids are described in the above references. In addition, essentially any polynucleotide (including, e.g., labeled or biotinylated polynucleotides) can be custom or standard ordered from any of a variety of commercial sources.

Thus, in one aspect, the present invention provides recombinant host cells allowing recombinant expression of antibodies of the invention or portions thereof. Antibodies produced by expression in such recombinant host cells are referred to herein as recombinant antibodies. The present invention also provides progeny cells of such host cells, and antibodies produced by the same.

The term recombinant host cell (or simply host cell), as used herein, means a cell into which a recombinant expression vector has been introduced. It should be understood that recombinant host cell and host cell mean not only the particular subject cell but also the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term host cell as used herein. Such cells may comprise a vector according to the invention as described above.

In another aspect, the present invention provides a method for making an antibody or portion thereof as described herein. According to one embodiment, said method comprises culturing a cell transfected or transformed with a vector as described above, and retrieving the antibody or portion thereof.

As indicated above, expression of an antibody of the invention (or fragment or variants thereof) preferably comprises expression vector(s) containing a polynucleotide that encodes the desired anti-APCDD1 antibody. Methods that are well known to those skilled in the art can be used to construct expression vectors comprising antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Embodiments of the invention, thus, provide replicable vectors comprising a nucleotide sequence encoding an anti-APCDD1 antibody of the

invention (e.g., a whole antibody, a heavy or light chain of an antibody, a heavy or light chain variable domain of an antibody, or a portion thereof, or a heavy or light chain CDR, a single chain Fv, or fragments or variants thereof), operably linked to a promoter. In preferred embodiments such vectors may include a nucleotide sequence encoding the heavy chain of an antibody molecule (or fragment thereof), a nucleotide sequence encoding the light chain of an antibody (or fragment thereof) or both the heavy and light chain.

Once the nucleotides of the present invention have been isolated and modified according to the teachings herein, they may be used to produce selected effectors including anti-APCDD1 antibodies or fragments thereof.

X. Effector Production and Purification

Using art recognized molecular biology techniques and current protein expression methodology, substantial quantities of the desired effectors may be produced. More specifically, nucleic acid molecules encoding effectors, such as antibodies obtained and engineered as described above, may be integrated into well known and commercially available protein production systems comprising various types of host cells to provide preclinical, clinical or commercial quantities of the desired pharmaceutical product. It will be appreciated that in preferred embodiments the nucleic acid molecules encoding the effectors are engineered into vectors or expression vectors that provide for efficient integration into the selected host cell and subsequent high expression levels of the desired APCDD1 effector.

Preferably nucleic acid molecules encoding APCDD1 effectors and vectors comprising these nucleic acid molecules can be used for transfection of a suitable mammalian, plant, bacterial or yeast host cell though it will be appreciated that prokaryotic systems may be used for effector production. Transfection can be by any known method for introducing polynucleotides into a host cell. Methods for the introduction of heterologous polynucleotides into mammalian cells are well known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. In addition, nucleic acid molecules may be introduced into mammalian cells by viral vectors. Methods of transforming mammalian cells are well known in the art. See, e.g., U.S.P.Ns 4,399,216, 4,912,040, 4,740,461, and 4,959,455. Further, methods of transforming plant

cells are well known in the art, including, e.g., Agrobacterium-mediated transformation, biolistic transformation, direct injection, electroporation and viral transformation. Methods of transforming bacterial and yeast cells are also well known in the art.

Moreover, the host cell may be co-transfected with two expression vectors of the invention, for example, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers that enable substantially equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain is preferably placed before the heavy chain to avoid an excess of toxic free heavy chain. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

a. Host-expression systems

A variety of host-expression vector systems, many commercially available, are compatible with the teachings herein and may be used to express the effectors of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be expressed and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a molecule of the invention *in situ*. Such systems include, but are not limited to, microorganisms such as bacteria (e.g., E. coli, B. subtilis, streptomyces) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing effector coding sequences; yeast (e.g., Saccharomyces, Pichia) transfected with recombinant yeast expression vectors containing effector coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing effector coding sequences; plant cell systems (e.g., Nicotiana, Arabidopsis, duckweed, corn, wheat, potato, etc.) infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transfected with recombinant plasmid expression vectors (e.g., Ti plasmid) containing effector coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the molecule being expressed. For example,

when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an effector, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278 (Ruther et al., EMBO 1. 2:1791 (1983)), in which the coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109 (1985); Van Heeke & Schuster, J. Biol. Chem. 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) may be used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The coding sequences may be cloned individually into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be used to introduce the desired nucleotide sequence. In cases where an adenovirus is used as an expression vector, the coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the molecule in infected hosts (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 8 1:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g.,

Bittner et al., Methods in Enzymol. 153:51-544 (1987)). Thus, compatible mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NS0 cells, SP2 cells, HEK-293T cells, 293 Freestyle cells (Life Technologies, San Diego), NIH-3T3 cells, HeLa cells, baby hamster kidney (BHK) cells, African green monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, and a number of other cell lines.

For long-term, high-yield production of recombinant proteins stable expression is preferred. Accordingly, cell lines that stably express the selected effector may be engineered using standard art recognized techniques. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compositions that interact directly or indirectly with the molecule.

A number of selection systems are well known in the art and may be used including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223 (1977)), hypoxanthineguanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., Cell 22:8 17 (1980)) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA 77:357 (1980); O'Hare et al., Proc. Natl. Acad. Sci. USA 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 (Clinical Pharmacy 12:488-505; Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62: 191-217 (1993); TIB TECH 11(5):155-2

15 (May, 1993)); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin et al., J. Mol. Biol. 150:1 (1981). It will be appreciated that one particularly preferred method of establishing a stable, high yield cell line comprises the glutamine synthetase gene expression system (the GS system) which provides an efficient approach for enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with EP patents 0 216 846, 0 256 055, 0 323 997 and 0 338 841 each of which is incorporated herein by reference.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function and/or purification of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. As known in the art appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the expressed polypeptide. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product are particularly effective for use in the instant invention. Accordingly, particularly preferred mammalian host cells include, but are not limited to, CHO, VERY, BHK, HeLa, COS, NS0, MDCK, 293, 3T3, W138, as well as breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT2O and T47D, and normal mammary gland cell line such as, for example, CRL7O3O and HsS78Bst. Depending on the effector and the selected production system, those of skill in the art may easily select and optimize appropriate host cells for efficient expression of the effector.

b. Chemical synthesis

Besides the aforementioned host cell systems, it will be appreciated that the effectors of the invention may be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., and Hunkapiller, M., et al., 1984, Nature 310:105-111). For example, a peptide

corresponding to a polypeptide fragment of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into an polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-amino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

c. Transgenic systems

The APCDD1 effectors of the invention also can be produced transgenically through the generation of a mammal or plant that is transgenic for the immunoglobulin heavy and light chain sequences (or fragments or derivatives or variants thereof) of interest and production of the desired compounds in a recoverable form. In connection with the transgenic production in mammals, anti-APCDD1 antibodies, for example, can be produced in, and recovered from, the milk of goats, cows, or other mammals. See, e.g., U.S.P.Ns. 5,827,690, 5,756,687, 5,750,172, and 5,741,957. In some embodiments, non-human transgenic animals that comprise human immunoglobulin loci are immunized with APCDD1 or an immunogenic portion thereof, as described above. Methods for making antibodies in plants are described, e.g., in U.S.P.Ns. 6,046,037 and 5,959,177.

In accordance with the teachings herein non-human transgenic animals or plants may be produced by introducing one or more nucleic acid molecules encoding an APCDD1 effector of the invention into the animal or plant by standard transgenic techniques. See Hogan and U.S.P.N. 6,417,429. The transgenic cells used for making the transgenic animal can be embryonic stem cells or somatic cells or a fertilized egg. The transgenic non-human organisms can be chimeric, nonchimeric heterozygotes, and nonchimeric homozygotes. See, e.g., Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1999); Jackson et al., Mouse Genetics and Transgenics: A Practical Approach, Oxford University Press (2000); and Pinkert, Transgenic Animal Technology: A Laboratory Handbook, Academic Press (1999). In some embodiments, the transgenic non-human animals have a targeted disruption and replacement by a targeting construct that encodes, for example, a heavy

chain and/or a light chain of interest. In one embodiment, the transgenic animals comprise and express nucleic acid molecules encoding heavy and light chains that specifically bind to APCDD1. While anti-APCDD1 antibodies may be made in any transgenic animal, in particularly preferred embodiments the non-human animals are mice, rats, sheep, pigs, goats, cattle or horses. In further embodiments the non-human transgenic animal expresses the desired pharmaceutical product in blood, milk, urine, saliva, tears, mucus and other bodily fluids from which it is readily obtainable using art recognized purification techniques.

It is likely that effectors, including antibodies, expressed by different cell lines or in transgenic animals will have different glycosylation patterns from each other. However, all effectors encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein are part of the instant invention, regardless of the glycosylation state of the molecule, and more generally, regardless of the presence or absence of post-translational modification(s). In addition the invention encompasses effectors that are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc. Various post-translational modifications are also encompassed by the invention include, for example, e.g., N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression. Moreover, as set forth in the text and Examples below the polypeptides may also be modified with a detectable label, such as an enzymatic, fluorescent, radioisotopic or affinity label to allow for detection and isolation of the effector.

d. Purification

Once an effector of the invention has been produced by recombinant expression or any one of the other techniques disclosed herein, it may be purified by any method known in the art for purification of immunoglobulins, or more generally by any other standard technique for the purification of proteins. In this respect the effector may be isolated. As

used herein, an isolated APCDD1 effector is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. Isolated effectors include an effector *in situ* within recombinant cells because at least one component of the polypeptide's natural environment will not be present.

When using recombinant techniques, the APCDD1 effector (e.g. an anti-APCDD1 antibody or derivative or fragment thereof) can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the desired molecule is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, may be removed, for example, by centrifugation or ultrafiltration. For example, Carter, et al., Bio/Technology 10:163 (1992) describe a procedure for isolating antibodies that are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 minutes. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The effector (e.g., fc-APCDD1 or anti-APCDD1 antibody) composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the selected construct. Protein A can be used to purify antibodies that are based on human IgG1, IgG2 or IgG4 heavy chains (Lindmark, et al., J Immunol Meth 62:1 (1983)). Protein G is recommended for all mouse isotypes and for human IgG3 (Guss, et al., EMBO J 5:1567 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3

domain, the Bakerbond ABX^{TM} resin (J. T. Baker; Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, reverse phase HPLC, chromatography on silica, chromatography on heparin, sepharose chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE and ammonium sulfate precipitation are also available depending on the antibody to be recovered. In particularly preferred embodiments the effectors of the instant invention will be purified, at least in part, using Protein A or Protein G affinity chromatography.

XI. Conjugated APCDD1 Effectors

Once the effectors of the invention have been purified according to the teachings herein they may be linked with, fused to, conjugated to (e.g. covalently or non-covalently) or otherwise associated with pharmaceutically active or diagnostic moieties or biocompatible modifiers. As used herein the term conjugate will be used broadly and held to mean any molecule associated with the disclosed effectors regardless of the method of association. In this respect it will be understood that such conjugates may comprise peptides, polypeptides, proteins, polymers, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, organic molecules and radioisotopes. Moreover, as indicated above the selected conjugate may be covalently or non-covalently linked to the effector and exhibit various molar ratios depending, at least in part, on the method used to effect the conjugation.

In preferred embodiments it will be apparent that the effectors of the invention may be conjugated or associated with proteins, polypeptides or peptides that impart selected characteristics (e.g. biotoxins, biomarkers, purification tags, etc.). More generally, in selected embodiments the present invention encompasses the use of effectors or fragments thereof recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide wherein the polypeptide comprises at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids. The construct does not necessarily need to be directly linked, but may occur through linker sequences. For example, antibodies may be used to target heterologous polypeptides to particular cell types expressing APCDD1, either *in vitro* or *in vivo*, by fusing or conjugating the effectors of the present invention to antibodies specific for particular cell surface receptors. Moreover, effectors fused or conjugated to heterologous polypeptides may also be used in *in vitro*

immunoassays and may be compatible with purification methodology known in the art. See e.g., International publication No. WO 93/21232; European Patent No. EP 439,095; Naramura et al., 1994, Immunol. Lett. 39:91-99; U.S. Pat. No. 5,474,981; Gillies et al., 1992, PNAS 89:1428-1432; and Fell et al., 1991, J. Immunol. 146:2446-2452.

a. Biocompatible modifiers

In a preferred embodiment, the effectors of the invention may be conjugated or otherwise associated with biocompatible modifiers that may be used to adjust, alter, improve or moderate effector characteristics as desired. For example, antibodies or fusion constructs with increased *in vivo* half-lives can be generated by attaching relatively high molecular weight polymer molecules such as commercially available polyethylene glycol (PEG) or similar biocompatible polymers. Those skilled in the art will appreciate that PEG may be obtained in many different molecular weight and molecular configurations that can be selected to impart specific properties to the antibody (e.g. the half-life may be tailored). PEG can be attached to effectors or antibody fragments or derivatives with or without a multifunctional linker either through site-specific conjugation of the PEG to the N- or C-terminus of said antibodies or antibody fragments or via epsilon-amino groups present on lysine residues. Linear or branched polymer derivatization that results in minimal loss of biological activity may be used. The degree of conjugation can be closely monitored by SDS-PAGE and mass spectrometry to ensure optimal conjugation of PEG molecules to antibody molecules. Unreacted PEG can be separated from antibody-PEG conjugates by, e.g., size exclusion or ion-exchange chromatography. In a similar manner, the disclosed effectors can be conjugated to albumin in order to make the antibody or antibody fragment more stable in vivo or have a longer half life in vivo. The techniques are well known in the art, see e.g., International Publication Nos. WO 93/15199, WO 93/15200, and WO 01/77137; and European Patent No. 0 413, 622. Other biocompatible conjugates are evident to those of ordinary skill and may readily be identified in accordance with the teachings herein.

b. Diagnostic or detection agents

In other preferred embodiments, effectors of the present invention, or fragments or derivatives thereof, are conjugated to a diagnostic or detectable agent which may be a biological molecule (e.g., a peptide or nucleotide) or a small molecule or radioisotope. Such effectors can be useful for monitoring the development or progression of a hyperproliferative disorder or as part of a clinical testing procedure to determine the efficacy of a particular therapy including the disclosed effectors. Such markers may also

be useful in purifying the selected effector, separating or isolating TIC or in preclinical procedures or toxicology studies.

Such diagnosis and detection can be accomplished by coupling the effector to detectable substances including, but not limited to, various enzymes comprising for example horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidinlbiotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not limited to iodine (^{131}I, ^{125}I, ^{123}I, ^{121}I,), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In,), and technetium (^{99}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc, ^{186}Re, ^{188}Re, ^{142}Pr, ^{105}Rh, ^{97}Ru, ^{68}Ge, ^{57}Co, ^{65}Zn, ^{85}Sr, ^{32}P, ^{153}Gd, ^{169}Yb, ^{51}Cr, ^{54}Mn, ^{75}Se, ^{113}Sn, and ^{117}Tin; positron emitting metals using various positron emission tomographies, noradioactive paramagnetic metal ions, and molecules that are radiolabeled or conjugated to specific radioisotopes. In such embodiments appropriate detection methodology is well known in the art and readily available from numerous commercial sources.

As indicated above, in other embodiments the effectors or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification or diagnostic procedures such as immunohistochemistry or FACs. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz et al., 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, Cell 37:767) and the "flag" tag (U.S.P.N. 4,703,004).

c. Therapeutic moieties

As previously alluded to the effectors or fragments or derivatives thereof may also be conjugated, linked or fused to or otherwise associated with a therapeutic moiety such as a cytotoxin or cytotoxic agent, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha or beta-emitters. As used herein a cytotoxin or cytotoxic

agent includes any agent or therapeutic moiety that is detrimental to cells and may inhibit cell growth or survival. Examples include paclitaxel, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin, maytansinoids (e.g. DM-1, DM-4, Immunogen, Inc.), dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, epirubicin, and cyclophosphamide and analogs or homologs thereof. Additional cytoxins comprise auristatins, including monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) (Seattle Genetics, Inc.), amanitins such as alpha-amanitin, beta-amanitin, gamma-amanitin or epsilon-amanitin (Heidelberg Pharma AG), DNA minor groove binding agents such as duocarmycin derivatives (Syntarga, B.V.) and modified pyrrolobenzodiazepine dimers (PBDs, Spirogen, Ltd). Furthermore, in one embodiment the APCDD1 effectors of the instant invention may be associated with anti-CD3 binding molecules to recruit cytotoxic T-cells and have them target the tumor initiating cells (BiTE technology; see e.g., Fuhrmann, S. et. al. *Annual Meeting of AACR* Abstract No. 5625 (2010) which is incorporated herein by reference).

Additional compatible therapeutic moieties comprise cytotoxic agents including, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). A more extensive list of therapeutic moieties can be found in PCT publication WO 03/075957 and U.S.P.N. 2009/0155255 each of which is incorporated herein by reference.

The selected effectors can also be conjugated to therapeutic moieties such as radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (see above for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N",N"-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res. 4:2483; Peterson et al., 1999, Bioconjug. Chem. 10:553; and Zimmerman et al., 1999, Nucl. Med. Biol.

26:943. Exemplary radioisotopes that may be compatible with this aspect of the invention include, but are not limited to, iodine (^{131}I, ^{125}I, ^{123}I, ^{121}I,), carbon (^{14}C), copper (^{62}Cu, ^{64}Cu, ^{67}Cu), sulfur (^{35}S), tritium (^{3}H), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In,), bismuth (^{212}Bi, ^{213}Bi), technetium (^{99}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc, ^{186}Re, ^{188}Re, ^{142} Pr, ^{105}Rh, ^{97}Ru, ^{68}Ge, ^{57}Co, ^{65}Zn, ^{85}Sr, ^{32}P, ^{153}Gd, ^{169}Yb, ^{51}Cr, ^{54}Mn, ^{75}Se, ^{113}Sn, ^{117}Tin, ^{225}Ac, ^{76}Br, and ^{211}At. Other radionuclides are also available as diagnostic and therapeutic agents, especially those in the energy range of 60 to 4,000 keV. Depending on the condition to be treated and the desired therapeutic profile, those skilled in the art may readily select the appropriate radioisotope for use with the disclosed effectors.

APCDD1 effectors of the present invention may also be conjugated to a therapeutic moiety or drug that modifies a given biological response. That is, therapeutic agents or moieties compatible with the instant invention are not to be construed as limited to classical chemical therapeutic agents. For example, in particularly preferred embodiments the drug moiety may be a protein or polypeptide or fragment thereof possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, Onconase (or another cytotoxic RNase), pseudomonas exotoxin, cholera toxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF- α, TNF-β, AIM I (see, International Publication No. WO 97/33899), AIM II (see, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., 1994, J. Immunol., 6:1567), and VEGI (see, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, a biological response modifier such as, for example, a lymphokine (e.g., interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), and granulocyte colony stimulating factor ("G-CSF")), or a growth factor (e.g., growth hormone ("GH")). As set forth above, methods for fusing or conjugating effectors to polypeptide moieties are known in the art. In addition to the previously disclosed subject references see, e.g., U.S.P.Ns. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851, and 5,112,946; EP 307,434; EP 367,166; PCT Publications WO 96/04388 and WO 91/06570; Ashkenazi et al., 1991, PNAS USA 88:10535; Zheng et al., 1995, J Immunol 154:5590; and Vil et al., 1992, PNAS USA 89:11337 each of which is incorporated herein by reference. The association of an

effector with a moiety does not necessarily need to be direct, but may occur through linker sequences. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res 4:2483; Peterson et al., 1999, Bioconjug Chem 10:553; Zimmerman et al., 1999, Nucl Med Biol 26:943; Garnett, 2002, Adv Drug Deliv Rev 53:171 each of which is incorporated herein.

More generally, techniques for conjugating therapeutic moieties or cytotoxic agents to effectors are well known. Moieties can be conjugated to effectors by any art-recognized method, including, but not limited to aldehyde/Schiff linkage, sulphydryl linkage, acid-labile linkage, cis-aconityl linkage, hydrazone linkage, enzymatically degradable linkage (see generally Garnett, 2002, Adv Drug Deliv Rev 53:171). Also see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., 1982, Immunol. Rev. 62:119. In preferred embodiments an APCDD1 effector that is conjugated to a therapeutic moiety or cytotoxic agent may be internalized by a cell upon binding to an APCDD1 molecule associated with the cell surface thereby delivering the therapeutic payload.

XII. Diagnostics and Screening

As indicated, the present invention provides methods for detecting or diagnosing hyperproliferative disorders and methods of screening cells from a patient to identify a tumor initiating cell. Such methods include identifying an individual having cancer for treatment or monitoring progression of a cancer comprising contacting a sample obtained from a patient with an APCDD1 effector as described herein and detecting presence or absence, or level of association of the effector to bound or free APCDD1 in the sample. When the effector comprises an antibody or immunologically active fragment thereof the association with APCDD1 in the sample indicates that the sample may contain tumor perpetuating cells (e.g., a cancer stem cells) indicating that the individual having cancer

may be effectively treated with an APCDD1 effector as described herein. The methods may further comprise a step of comparing the level of binding to a control. Conversely, when the selected effector is Fc-APCDD1 the enzymatic properties of the molecule as described herein may be monitored (directly or indirectly) when in contact with the sample to provide the desired information. Other diagnostic methods compatible with the teachings herein are well known in the art and can be practiced using commercial materials such as dedicated reporting systems.

Exemplary compatible assay methods include radioimmunoassays, enzyme immunoassays, competitive-binding assays, fluorescent immunoassay, immunoblot assays, Western Blot analysis, flow cytometry assays, and ELISA assays. More generally detection of APCDD1 in a biological sample or the measurement of APCDD1 enzymatic activity (or inhibition thereof) may be accomplished using any art-known assay.

In another aspect, and as discussed in more detail below, the present invention provides kits for detecting, monitoring or diagnosing a hyperproliferative disorder, identifying individual having such a disorder for possible treatment or monitoring progression (or regression) of the disorder in a patient, wherein the kit comprises an effector as described herein, and reagents for detecting the impact of the effector on a sample.

The APCDD1 effectors and cells, cultures, populations and compositions comprising the same, including progeny thereof, can also be used to screen for or identify compounds or agents (e.g., drugs) that affect a function or activity of tumor initiating cells or progeny thereof by interacting with APCDD1 (e.g., the polypeptide or genetic components thereof). The invention therefore further provides systems and methods for evaluation or identification of a compound or agent that can affect a function or activity tumor initiating cells or progeny thereof by associating with APCDD1 or its substrates. Such compounds and agents can be drug candidates that are screened for the treatment of a hyperproliferative disorder, for example. In one embodiment, a system or method includes tumor initiating cells exhibiting APCDD1 and a compound or agent (e.g., drug), wherein the cells and compound or agent (e.g., drug) are in contact with each other.

The invention further provides methods of screening and identifying APCDD1 effectors or agents and compounds for altering an activity or function of tumor initiating cells or progeny cells. In one embodiment, a method includes contacting tumor initiating cells or progeny thereof with a test agent or compound; and determining if the test agent or compound modulates an activity or function of the $APCDD1^{+}$ tumor initiating cells.

A test agent or compound modulating an APCDD1 related activity or function of such tumor initiating cells or progeny thereof within the population identifies the test agent or compound as an active agent. Exemplary activity or function that can be modulated include changes in cell morphology, expression of a marker, differentiation or de-differentiation, maturation, proliferation, viability, apoptosis or cell death neuronal progenitor cells or progeny thereof.

Contacting, when used in reference to cells or a cell culture or method step or treatment, means a direct or indirect interaction between the composition (e.g., APCDD1$^+$ cell or cell culture) and another referenced entity. A particular example of a direct interaction is physical interaction. A particular example of an indirect interaction is where a composition acts upon an intermediary molecule which in turn acts upon the referenced entity (e.g., cell or cell culture).

In this aspect of the invention modulates indicates influencing an activity or function of tumor initiating cells or progeny cells in a manner compatible with detecting the effects on cell activity or function that has been determined to be relevant to a particular aspect (e.g., metastasis or proliferation) of the tumor initiating cells or progeny cells of the invention. Exemplary activities and functions include, but are not limited to, measuring morphology, developmental markers, differentiation, proliferation, viability, cell respiration, mitochondrial activity, membrane integrity, or expression of markers associated with certain conditions. Accordingly, a compound or agent (e.g., a drug candidate) can be evaluated for its effect on tumor initiating cells or progeny cells, by contacting such cells or progeny cells with the compound or agent and measuring any modulation of an activity or function of tumor initiating cells or progeny cells as disclosed herein or would be known to the skilled artisan.

Methods of screening and identifying agents and compounds include those suitable for high throughput screening, which include arrays of cells (e.g., microarrays) positioned or placed, optionally at pre-determined locations or addresses. High-throughput robotic or manual handling methods can probe chemical interactions and determine levels of expression of many genes in a short period of time. Techniques have been developed that utilize molecular signals (e.g., fluorophores) and automated analyses that process information at a very rapid rate (see, e.g., Pinhasov et al., Comb. Chem. High Throughput Screen. 7:133 (2004)). For example, microarray technology has been extensively utilized to probe the interactions of thousands of genes at once, while providing information for specific genes (see, e.g., Mocellin and Rossi, Adv. Exp. Med. Biol. 593:19 (2007)).

Such screening methods (e.g., high-throughput) can identify active agents and compounds rapidly and efficiently. For example, cells can be positioned or placed (pre-seeded) on a culture dish, tube, flask, roller bottle or plate (e.g., a single multi-well plate or dish such as an 8, 16, 32, 64, 96, 384 and 1536 multi-well plate or dish), optionally at defined locations, for identification of potentially therapeutic molecules. Libraries that can be screened include, for example, small molecule libraries, phage display libraries, fully human antibody yeast display libraries (Adimab, LLC), siRNA libraries, and adenoviral transfection vectors.

XIII. Pharmaceutical Preparations and Therapeutic Uses

a. Formulations and routes of administration

Depending on the form of the effector along with any optional conjugate, the mode of intended delivery, the disease being treated or monitored and numerous other variables, compositions of the instant invention may be formulated as desired using art recognized techniques. That is, in various embodiments of the instant invention compositions comprising APCDD1 effectors are formulated with a wide variety of pharmaceutically acceptable carriers (see, e.g., Gennaro, *Remington: The Science and Practice of Pharmacy with Facts and Comparisons: Drugfacts Plus*, 20th ed. (2003); Ansel et al., *Pharmaceutical Dosage Forms and Drug Delivery Systems,* 7th ed., Lippencott Williams and Wilkins (2004); Kibbe et al., *Handbook of Pharmaceutical Excipients,* 3rd ed., Pharmaceutical Press (2000)). Various pharmaceutically acceptable carriers, which include vehicles, adjuvants, and diluents, are readily available from numerous commercial sources. Moreover, an assortment of pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are also available. Certain non-limiting exemplary carriers include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

More particularly it will be appreciated that, in some embodiments, the therapeutic compositions of the invention may be administered neat or with a minimum of additional components. Conversely the APCDD1 effectors of the present invention may optionally be formulated to contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that are well known in the art and are relatively inert substances that facilitate administration of the effector or which aid processing of the active compounds into preparations that are pharmaceutically optimized for delivery to the site of action. For example, an excipient can give form or consistency or act as a diluent to

improve the pharmacokinetics of the effector. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers.

Disclosed effectors for systemic administration may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulation may be used simultaneously to achieve systemic administration of the active ingredient. Excipients as well as formulations for parenteral and nonparenteral drug delivery are set forth in Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing (2000). Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate for oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension and include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

Suitable formulations for enteral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

In general the compounds and compositions of the invention, comprising APCDD1 effectors may be administered *in vivo,* to a subject in need thereof, by various routes, including, but not limited to, oral, intravenous, intra-arterial, subcutaneous, parenteral, intranasal, intramuscular, intracardiac, intraventricular, intratracheal, buccal, rectal, intraperitoneal, intradermal, topical, transdermal, and intrathecal, or otherwise by implantation or inhalation. The subject compositions may be formulated into preparations in solid, semi-solid, liquid, or gaseous forms; including, but not limited to, tablets, capsules, powders, granules, ointments, solutions, suppositories, enemas, injections, inhalants, and aerosols. The appropriate formulation and route of administration may be selected according to the intended application and therapeutic regimen.

b. Dosages

Similarly, the particular dosage regimen, i.e., dose, timing and repetition, will depend on the particular individual and that individual's medical history. Empirical considerations, such as the half-life, generally will contribute to the determination of the

dosage. Frequency of administration may be determined and adjusted over the course of therapy, and is based on reducing the number of hyperproliferative or neoplastic cells, including tumor initiating cells, maintaining the reduction of such neoplastic cells, reducing the proliferation of neoplastic cells, or delaying the development of metastasis. Alternatively, sustained continuous release formulations of a subject therapeutic composition may be appropriate. As alluded to above various formulations and devices for achieving sustained release are known in the art.

From a therapeutic standpoint the pharmaceutical compositions are administered in an amount effective for treatment or prophylaxis of the specific indication. The therapeutically effective amount is typically dependent on the weight of the subject being treated, his or her physical or health condition, the extensiveness of the condition to be treated, or the age of the subject being treated. In general, the APCDD1 effectors of the invention may be administered in an amount in the range of about 10 µg/kg body weight to about 100 mg/kg body weight per dose. In certain embodiments, the APCDD1 effectors of the invention may be administered in an amount in the range of about 50 µg/kg body weight to about 5 mg/kg body weight per dose. In certain other embodiments, the APCDD1 effectors of the invention may be administered in an amount in the range of about 100 µg/kg body weight to about 10 mg/kg body weight per dose. Optionally, the APCDD1 effectors of the invention may be administered in an amount in the range of about 100 µg/kg body weight to about 20 mg/kg body weight per dose. Further optionally, the APCDD1 effectors of the invention may be administered in an amount in the range of about 0.5 mg/kg body weight to about 20 mg/kg body weight per dose. In certain embodiments the compounds of present invention are provided a dose of at least about 100 µg/kg body weight, at least about 250 µg/kg body weight, at least about 750 µg/kg body weight, at least about 3 mg/kg body weight, at least about 5 mg/kg body weight, at least about 10 mg/kg body weight is administered.

Other dosing regimens may be predicated on Body Surface Area (BSA) calculations as disclosed in U.S.P.N. 7,744,877 which is incorporated herein by reference in its entirety. As is well known in the art the BSA is calculated using the patient's height and weight and provides a measure of a subject's size as represented by the surface area of his or her body. In selected embodiments of the invention using the BSA the effectors may be administered in dosages from 10 mg/m^2 to 800 mg/m^2. In other preferred embodiments the effectors will be administered in dosages from 50 mg/m^2 to 500 mg/m^2 and even more preferably at dosages of 100 mg/m^2, 150 mg/m^2, 200 mg/m^2, 250 mg/m^2, 300 mg/m^2, 350

mg/m^2, 400 mg/m^{2}or 450 mg/m^2. Of course it will be appreciated that, regardless of how the dosages are calculated, multiple dosages may be administered over a selected time period to provide an absolute dosage that is substantially higher than the individual administrations.

In any event, the APCDD1 effectors are preferably administered as needed to subjects in need thereof. Determination of the frequency of administration may be made by persons skilled in the art, such as an attending physician based on considerations of the condition being treated, age of the subject being treated, severity of the condition being treated, general state of health of the subject being treated and the like. Generally, an effective dose of the APCDD1 effector is administered to a subject one or more times. More particularly, an effective dose of the effector is administered to the subject once a month, more than once a month, or less than once a month. In certain embodiments, the effective dose of the APCDD1 effector may be administered multiple times, including for periods of at least a month, at least six months, or at least a year.

Dosages and regimens may also be determined empirically for the disclosed therapeutic compositions in individuals who have been given one or more administration(s). For example, individuals may be given incremental dosages of a therapeutic composition produced as described herein. To assess efficacy of the selected composition, a marker of the specific disease, disorder or condition can be followed. In embodiments where the individual has cancer, these include direct measurements of tumor size via palpation or visual observation, indirect measurement of tumor size by x-ray or other imaging techniques; an improvement as assessed by direct tumor biopsy and microscopic examination of the tumor sample; the measurement of an indirect tumor marker (e.g., PSA for prostate cancer) or an antigen identified according to the methods described herein, a decrease in pain or paralysis; improved speech, vision, breathing or other disability associated with the tumor; increased appetite; or an increase in quality of life as measured by accepted tests or prolongation of survival. It will be apparent to one of skill in the art that the dosage will vary depending on the individual, the type of neoplastic condition, the stage of neoplastic condition, whether the neoplastic condition has begun to metastasize to other location in the individual, and the past and concurrent treatments being used.

c. Combination therapies

Combination therapies contemplated by the invention may be particularly useful in decreasing or inhibiting unwanted neoplastic cell proliferation (e.g. endothelial cells),

decreasing the occurrence of cancer, decreasing or preventing the recurrence of cancer, or decreasing or preventing the spread or metastasis of cancer. In such cases the compounds of the instant invention may function as sensitizing or chemosensitizing agent by removing the TPCs propping up and perpetuating the tumor mass (e.g. NTG cells) and allow for more effective use of current standard of care debulking or anti-cancer agents. That is, a combination therapy comprising an APCDD1 effector and one or more anti-cancer agents may be used to diminish established cancer e.g., decrease the number of cancer cells present and/or decrease tumor burden, or ameliorate at least one manifestation or side effect of cancer. As such, combination therapy refers to the administration of an APCDD1 effector and one or more anti-cancer agent that include, but are not limited to, cytotoxic agents, cytostatic agents, chemotherapeutic agents, targeted anti-cancer agents, biological response modifiers, immunotherapeutic agents, cancer vaccines, anti-angiogenic agents, cytokines, hormone therapies, radiation therapy and anti-metastatic agents.

According to the methods of the present invention, there is no requirement for the combined results to be additive of the effects observed when each treatment (e.g., anti-APCDD1 antibody and anti-cancer agent) is conducted separately. Although at least additive effects are generally desirable, any increased anti-tumor effect above one of the single therapies is beneficial. Furthermore, the invention does not require the combined treatment to exhibit synergistic effects. However, those skilled in the art will appreciate that with certain selected combinations that comprise preferred embodiments, synergism may be observed.

To practice combination therapy according to the invention, an APCDD1 effector (e.g., anti-APCDD1 antibody) in combination with one or more anti-cancer agent may be administered to a subject in need thereof in a manner effective to result in anti-cancer activity within the subject. The APCDD1 effector and anti-cancer agent are provided in amounts effective and for periods of time effective to result in their combined presence and their combined actions in the tumor environment as desired. To achieve this goal, the APCDD1 effector and anti-cancer agent may be administered to the subject simultaneously, either in a single composition, or as two or more distinct compositions using the same or different administration routes.

Alternatively, the effector may precede, or follow, the anti-cancer agent treatment by, e.g., intervals ranging from minutes to weeks. In certain embodiments wherein the anti-cancer agent and the antibody are applied separately to the subject, the time period

between each delivery is such that the anti-cancer agent and effector are able to exert a combined effect on the tumor. In a particular embodiment, it is contemplated that both the anti-cancer agent and the APCDD1 effector are administered within about 5 minutes to about two weeks of each other.

In yet other embodiments, several days (2, 3, 4, 5, 6 or 7), several weeks (1, 2, 3, 4, 5, 6, 7 or 8) or several months (1, 2, 3, 4, 5, 6, 7 or 8) may lapse between administration of the effector and the anti-cancer agent. The APCDD1 effector and one or more anti-cancer agent (combination therapy) may be administered once, twice or at least the period of time until the condition is treated, palliated or cured. Preferably, the combination therapy is administered multiple times. The combination therapy may be administered from three times daily to once every six months. The administering may be on a schedule such as three times daily, twice daily, once daily, once every two days, once every three days, once weekly, once every two weeks, once every month, once every two months, once every three months, once every six months or may be administered continuously via a minipump. As previously indicated the combination therapy may be administered via an oral, mucosal, buccal, intranasal, inhalable, intravenous, subcutaneous, intramuscular, parenteral, intratumor or topical route. The combination therapy may be administered at a site distant from the site of the tumor. The combination therapy generally will be administered for as long as the tumor is present provided that the combination therapy causes the tumor or cancer to stop growing or to decrease in weight or volume.

In one embodiment an APCDD1 effector is administered in combination with one or more anti-cancer agents for a short treatment cycle to a cancer patient to treat cancer. The duration of treatment with the antibody may vary according to the particular anti-cancer agent used. The invention also contemplates discontinuous administration or daily doses divided into several partial administrations. An appropriate treatment time for a particular anti-cancer agent will be appreciated by the skilled artisan, and the invention contemplates the continued assessment of optimal treatment schedules for each anti-cancer agent.

The present invention contemplates at least one cycle, preferably more than one cycle during which the combination therapy is administered. An appropriate period of time for one cycle will be appreciated by the skilled artisan, as will the total number of cycles, and the interval between cycles. The invention contemplates the continued assessment of optimal treatment schedules for each effector and anti-cancer agent. Moreover, the invention also provides for more than one administration of either the anti-APCDD1 antibody or the anti-cancer agent. The effector and anti-cancer agent may be

administered interchangeably, on alternate days or weeks; or a sequence of antibody treatment may be given, followed by one or more treatments of anti-cancer agent therapy. In any event, as will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics.

In another preferred embodiment the APCDD1 effectors of the instant invention may be used in maintenance therapy to reduce or eliminate the chance of tumor recurrence following the initial presentation of the disease. Preferably the disorder will have been treated and the initial tumor mass eliminated, reduced or otherwise ameliorated so the patient is asymptomatic or in remission. As such time the subject may be administered pharmaceutically effective amounts of the disclosed effectors one or more times even though there is little or no indication of disease using standard diagnostic procedures. In some embodiments the effectors will be administered on a regular schedule over a period of time. For example the APCDD1 effectors could be administered weekly, every two weeks, monthly, every six weeks, every two months, every three months every six months or annually. Given the teachings herein one skilled in the art could readily determine favorable dosages and dosing regimens to reduce the potential of disease recurrence. Moreover such treatments could be continued for a period of weeks, months years or even indefinitely depending on the patient response and clinical and diagnostic parameters.

In yet another preferred embodiment the effectors of the present invention may be used to prophylactically to prevent or reduce the possibility of tumor metastasis following a debulking procedure. As used in the instant disclosure a debulking procedure is defined broadly and shall mean any procedure, technique or method that eliminates, reduces, treats or ameliorates a tumor or tumor proliferation. Exemplary debulking procedures include, but are not limited to, surgery, radiation treatments (i.e., beam radiation), chemotherapy or ablation. At appropriate times readily determined by one skilled in the art in view of the instant disclosure the APCDD1 effectors may be administered as suggested by clinical and diagnostic procedures to reduce tumor metastasis. The effectors may be administered one or more times at pharmaceutically effective dosages as determined using standard techniques. Preferably the dosing regimen will be accompanied by appropriate diagnostic or monitoring techniques that allow it to be modified as necessary.

d. Anti-cancer agents

As used herein the term anti-cancer agent means any agent that can be used to treat a cell proliferative or hyperproliferative disorder such as cancer, including cytotoxic agents, cytostatic agents, anti-angiogenic agents, debulking agents, chemotherapeutic agents, radiotherapy and radiotherapeutic agents, targeted anti-cancer agents, biological response modifiers, antibodies, and immunotherapeutic agents. It will be appreciated that, in selected embodiments as discussed above, anti-cancer agents may comprise conjugates and may be associated with effectors prior to administration.

The term cytotoxic agent means a substance that decreases or inhibits the function of cells and/or causes destruction of cells, i.e., the substance is toxic to the cells. Typically, the substance is a naturally occurring molecule derived from a living organism. Examples of cytotoxic agents include, but are not limited to, small molecule toxins or enzymatically active toxins of bacteria (e.g., Diptheria toxin, Pseudomonas endotoxin and exotoxin, Staphylococcal enterotoxin A), fungal (e.g.,α-sarcin, restrictocin), plants (e.g., abrin, ricin, modeccin, viscumin, pokeweed anti-viral protein, saporin, gelonin, momoridin, trichosanthin, barley toxin, Aleurites fordii proteins, dianthin proteins, Phytolacca mericana proteins (PAPI, PAPII, and PAP-S), Momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitegellin, restrictocin, phenomycin, neomycin, and the tricothecenes) or animals, e.g., cytotoxic RNases, such as extracellular pancreatic RNases; DNase I, including fragments and/or variants thereof.

A chemotherapeutic agent means a chemical compound that non-specifically decreases or inhibits the growth, proliferation, and/or survival of cancer cells (e.g., cytotoxic or cytostatic agents). Such chemical agents are often directed to intracellular processes necessary for cell growth or division, and are thus particularly effective against cancerous cells, which generally grow and divide rapidly. For example, vincristine depolymerizes microtubules, and thus inhibits cells from entering mitosis. In general, chemotherapeutic agents can include any chemical agent that inhibits, or is designed to inhibit, a cancerous cell or a cell likely to become cancerous or generate tumorigenic progeny (e.g., TIC). Such agents are often administered, and are often most effective, in combination, e.g., in the formulation CHOP.

Examples of anti-cancer agents that may be used in combination with the effectors of the present invention include, but are not limited to, alkylating agents, alkyl sulfonates, aziridines, ethylenimines and methylamelamines, acetogenins, a camptothecin, bryostatin, callystatin, CC-1065, cryptophycins, dolastatin, duocarmycin, eleutherobin, pancratistatin,

a sarcodictyin, spongistatin, nitrogen mustards, antibiotics, enediyne antibiotics, dynemicin, bisphosphonates, an esperamicin, chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites, folic acid analogues, purine analogs, androgens, anti-adrenals, folic acid replenisher such as frolinic acid, aceglatone, aldophosphamide glycoside, aminolevulinic acid, eniluracil, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elfornithine, elliptinium acetate, an epothilone, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidainine, maytansinoids, mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet, pirarubicin, losoxantrone, podophyllinic acid, 2- ethylhydrazide, procarbazine, PSK® polysaccharide complex (JHS Natural Products, Eugene, OR), razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs, vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11), topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids; capecitabine; combretastatin; leucovorin (LV); oxaliplatin; inhibitors of PKC-alpha, Raf, H-Ras, EGFR and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor effectors (SERMs), aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, and anti-androgens; as well as troxacitabine (a 1,3- dioxolane nucleoside cytosine analog); antisense oligonucleotides,; ribozymes such as a VEGF expression inhibitor and a HER2 expression inhibitor; vaccines, PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor;

ABARELIX® rmRH; Vinorelbine and Esperamicins and pharmaceutically acceptable salts, acids or derivatives of any of the above. Those skilled in the art will be able to readily identify additional anti-cancer agents that are compatible with the teachings herein.

e. Radiotherapy

The present invention also provides for the combination of APCDD1 effectors with radiotherapy (i.e., any mechanism for inducing DNA damage locally within tumor cells such as gamma.-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions and the like). Combination therapy using the directed delivery of radioisotopes to tumor cells is also contemplated, and may be used in connection with a targeted anti-cancer agent or other targeting means. Typically, radiation therapy is administered in pulses over a period of time from about 1 to about 2 weeks. The radiation therapy may be administered to subjects having head and neck cancer for about 6 to 7 weeks. Optionally, the radiation therapy may be administered as a single dose or as multiple, sequential doses.

f. Neoplastic conditions

Whether administered alone or in combination with an anti-cancer agent or radiotherapy, the APCDD1 effectors of the instant invention are particularly useful for generally treating neoplastic conditions in patients or subjects which may include benign or malignant tumors (e.g., renal, liver, kidney, bladder, breast, gastric, ovarian, colorectal, prostate, pancreatic, lung, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders; and inflammatory, angiogenic, immunologic disorders and disorders caused by pathogens. Particularly preferred targets for treatment with therapeutic compositions and methods of the present invention are neoplastic conditions comprising solid tumors. In other preferred embodiments the effectors of the present invention may be used for the diagnosis, prevention or treatment of hematologic malignancies. Preferably the subject or patient to be treated will be human although, as used herein, the terms are expressly held to comprise any mammalian species.

More specifically, neoplastic conditions subject to treatment in accordance with the instant invention may be selected from the group including, but not limited to, adrenal gland tumors, AIDS-associated cancers, alveolar soft part sarcoma, astrocytic tumors, bladder cancer (squamous cell carcinoma and transitional cell carcinoma), bone cancer (adamantinoma, aneurismal bone cysts, osteochondroma, osteosarcoma), brain and spinal cord cancers, metastatic brain tumors, breast cancer, carotid body tumors, cervical cancer,

chondrosarcoma, chordoma, chromophobe renal cell carcinoma, clear cell carcinoma, colon cancer, colorectal cancer, cutaneous benign fibrous histiocytomas, desmoplastic small round cell tumors, ependymomas, Ewing's tumors, extraskeletal myxoid chondrosarcoma, fibrogenesis imperfecta ossium, fibrous dysplasia of the bone, gallbladder and bile duct cancers, gestational trophoblastic disease, germ cell tumors, head and neck cancers, islet cell tumors, Kaposi's Sarcoma, kidney cancer (nephroblastoma, papillary renal cell carcinoma), leukemias, lipoma/benign lipomatous tumors, liposarcoma/malignant lipomatous tumors, liver cancer (hepatoblastoma, hepatocellular carcinoma), lymphomas, lung cancers (small cell carcinoma, adenocarcinoma, squamous cell carcinoma, large cell carcinoma etc.), medulloblastoma, melanoma, meningiomas, multiple endocrine neoplasia, multiple myeloma, myelodysplastic syndrome, neuroblastoma, neuroendocrine tumors, ovarian cancer, pancreatic cancers, papillary thyroid carcinomas, parathyroid tumors, pediatric cancers, peripheral nerve sheath tumors, phaeochromocytoma, pituitary tumors, prostate cancer, posterious unveal melanoma, rare hematologic disorders, renal metastatic cancer, rhabdoid tumor, rhabdomysarcoma, sarcomas, skin cancer, soft-tissue sarcomas, squamous cell cancer, stomach cancer, synovial sarcoma, testicular cancer, thymic carcinoma, thymoma, thyroid metastatic cancer, and uterine cancers (carcinoma of the cervix, endometrial carcinoma, and leiomyoma). In certain preferred embodiments, the cancerous cells are selected from the group of solid tumors including but not limited to breast cancer, non-small cell lung cancer (NSCLC), small cell lung cancer, pancreatic cancer, colon cancer, prostate cancer, sarcomas, renal metastatic cancer, thyroid metastatic cancer, and clear cell carcinoma.

With regard to hematologic malignancies it will be further be appreciated that the compounds and methods of the present invention may be particularly effective in treating a variety of B-cell lymphomas, including low grade/NHL follicular cell lymphoma (FCC), mantle cell lymphoma (MCL), diffuse large cell lymphoma (DLCL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, Waldenstrom's Macroglobulinemia, lymphoplasmacytoid lymphoma (LPL), mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large cell lymphoma (DLCL), Burkitt's lymphoma (BL), AIDS-related lymphomas, monocytic B cell lymphoma, angioimmunoblastic lymphoadenopathy, small lymphocytic, follicular, diffuse large cell, diffuse small cleaved cell, large cell immunoblastic lymphoblastoma, small, non-cleaved, Burkitt's and non-Burkitt's, follicular, predominantly large cell;

follicular, predominantly small cleaved cell; and follicular, mixed small cleaved and large cell lymphomas. See, Gaidono et al., "Lymphomas", IN CANCER: PRINCIPLES & PRACTICE OF ONCOLOGY, Vol. 2: 2131-2145 (DeVita et al., eds., 5.sup.th ed. 1997). It should be clear to those of skill in the art that these lymphomas will often have different names due to changing systems of classification, and that patients having lymphomas classified under different names may also benefit from the combined therapeutic regimens of the present invention.

In yet other preferred embodiments the APCDD1 effectors may be used to effectively treat certain myeloid and hematologic malignancies including leukemias such as chronic lymphocytic leukemia (CLL or B-CLL). CLL is predominantly a disease of the elderly that starts to increase in incidence after fifty years of age and reaches a peak by late sixties. It generally involves the proliferation of neoplastic peripheral blood lymphocytes. Clinical finding of CLL involves lymphocytosis, lymphadenopatliy, splenomegaly, anemia and thrombocytopenia. A characteristic feature of CLL is monoclonal B cell proliferation and accumulation of B-lymphocytes arrested at an intermediate state of differentiation where such B cells express surface IgM (sIgM) or both sIgM and sIgD, and a single light chain at densities lower than that on the normal B cells. However, as discussed above and shown in the Examples appended hereto, selected APCDD1 expression (e.g., APCDD1) is upregulated on B-CLL cells thereby providing an attractive target for the disclosed effectors.

The present invention also provides for a preventative or prophylactic treatment of subjects who present with benign or precancerous tumors. It is not believed that any particular type of tumor or neoplastic disorder should be excluded from treatment using the present invention. However, the type of tumor cells may be relevant to the use of the invention in combination with secondary therapeutic agents, particularly chemotherapeutic agents and targeted anti-cancer agents.

As discussed herein, preferred embodiments of the instant invention comprise the use of APCDD1 effectors to treat subjects suffering from solid tumors. In such subjects many of these solid tumors comprise tissue exhibiting various genetic mutations that may render them particularly susceptible to treatment with the disclosed effectors. For example, KRAS, APC or mutations that augment nuclear localization of B-Catenin are relatively common in patients with colorectal cancer. Moreover, patients suffering from tumors with these mutations are usually the most refractory to current therapies; especially those patients with KRAS mutations. KRAS activating mutations, which typically result

in single amino acid substitutions, are also implicated in other difficult to treat malignancies, including lung adenocarcinoma, mucinous adenoma, and ductal carcinoma of the pancreas.

Currently, the most reliable prediction of whether colorectal cancer patients will respond to EGFR- or VEGF-inhibiting drugs, for example, is to test for certain KRAS "activating" mutations. KRAS is mutated in 35-45% of colorectal cancers, and patients whose tumors express mutated KRAS do not respond well to these drugs. For example, KRAS mutations are predictive of a lack of response to panitumumab and cetuximab therapy in colorectal cancer (Lievre et al. *Cancer Res* 66:3992–5; Karapetis et al. *NEJM* 359:1757-1765). Approximately 85% of patients with colorectal cancer have mutations in the APC gene (Markowitz & Bertagnolli. *NEJM* 361:2449-60), and more than 800 APC mutations have been characterized in patients with familial adenomatous polyposis and colorectal cancer. A majority of these mutations result in a truncated APC protein with reduced functional ability to mediate the destruction of beta-catenin. Mutations in the beta-catenin gene, CTNNB1, can also result in increased stabilization of the protein, resulting in nuclear import and subsequent activation of several oncogenic transcriptional programs, which is also the mechanism of oncogenesis resulting from failure of mutated APC to appropriately mediate beta-catenin destruction, which is required to keep normal cell proliferation and differentiation programs in check. As indicated by the Examples herein, tumors comprising such mutations may prove to be particularly susceptible to treatment with the APCDD1 effectors of the instant invention.

Besides such aggressive malignancies the present invention also provides for a preventative or prophylactic treatment of subjects who present with benign or precancerous tumors. It is not believed that any particular type of tumor or neoplastic disorder should be excluded from treatment using the present invention. However, the type of tumor cells may be relevant to the use of the invention in combination with secondary therapeutic agents, particularly chemotherapeutic agents and targeted anti-cancer agents.

XIV. Articles of Manufacture

Pharmaceutical packs and kits comprising one or more containers, comprising one or more doses of APCDD1 effectors are also provided. In certain embodiments, a unit dosage is provided wherein the unit dosage contains a predetermined amount of a composition comprising, for example, an anti-APCDD1 antibody, with or without one or

more additional agents. For other embodiments, such a unit dosage is supplied in single-use prefilled syringe for injection. In still other embodiments, the composition contained in the unit dosage may comprise saline, sucrose, or the like; a buffer, such as phosphate, or the like; and/or be formulated within a stable and effective pH range. Alternatively, in certain embodiments, the composition may be provided as a lyophilized powder that may be reconstituted upon addition of an appropriate liquid, for example, sterile water. In preferred embodiments, the composition comprises one or more substances that inhibit protein aggregation, including, but not limited to, sucrose and arginine. Any label on, or associated with, the container(s) indicates that the enclosed composition is used for diagnosing or treating the disease condition of choice.

The present invention also provides kits for producing single-dose or multi-dose administration units of APCDD1 effectors and, optionally, one or more anti-cancer agents. The kit comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition that is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits will generally contain in a suitable container a pharmaceutically acceptable formulation of the APCDD1 effector and, optionally, one or more anti-cancer agents in the same or different containers. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis or combined therapy. For example, in addition to the APCDD1 effectors of the inventions such kits may contain any one or more of a range of chemotherapeutic or radiotherapeutic drugs; anti-angiogenic agents; anti-metastatic agents; targeted anti-cancer agents; cytotoxic agents; and/or other anti-cancer agents.

More specifically the kits may have a single container that contains the APCDD1 effector, with or without additional components, or they may have distinct containers for each desired agent. Where combined therapeutics are provided a single solution may be pre-mixed, either in a molar equivalent combination, or with one component in excess of the other. Alternatively, each of the APCDD1 effector and any optional other anti-neoplastic agent of the kit may be maintained separately within distinct containers prior to administration to a patient. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent such as

bacteriostatic water for injection (BWFI), phosphate-buffered saline (PBS), Ringer's solution and dextrose solution.

When the components of the kit are provided in one or more liquid solutions, the liquid solution is preferably an aqueous solution, with a sterile aqueous solution being particularly preferred. However, the components of the kit may be provided as dried powder(s). When reagents or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. It is envisioned that the solvent may also be provided in another container.

As indicated briefly above the kits may also contain a means by which to administer the APCDD1 effector and any optional components to an animal or patient, e.g., one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected into the animal or applied to a diseased area of the body. The kits of the present invention will also typically include a means for containing the vials, or such like, and other component in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials and other apparatus are placed and retained. Any label or package insert indicates that the APCDD1 effector composition is used for treating cancer, for example colorectal cancer.

XV. Research Reagents

Other preferred embodiments of the invention also exploit the properties of the disclosed effectors as an instrument useful for indentifying, isolating, sectioning or enriching populations or subpopulations of tumor initiating cells through methods such as fluorescent activated cell sorting (FACS) or laser mediated sectioning. Those skilled in the art will appreciate that the effectors may be used in several compatible techniques for the characterization and manipulation of TICs including cancer stem cells (e.g., see U.S.S.Ns. 12/686,359, 12/669,136 and 12/757,649 each of which is incorporated herein by reference in its entirety).

XVI. Miscellaneous

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. More specifically, as

used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protein" includes a plurality of proteins; reference to "a cell" includes mixtures of cells, and the like. In addition, ranges provided in the specification and appended claims include both end points and all points between the end points. Therefore, a range of 2.0 to 3.0 includes 2.0, 3.0, and all points between 2.0 and 3.0.

Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. See, e.g., Sambrook J. & Russell D. Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (2000); Ausubel et al., Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Wiley, John & Sons, Inc. (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998); and Coligan et al., Short Protocols in Protein Science, Wiley, John & Sons, Inc. (2003). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art.

All references or documents disclosed or cited within this specification are, without limitation, incorporated herein by reference in their entirety. Moreover, any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

EXAMPLES

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the instant invention. The examples are not intended to represent that the experiments below are all or the only experiments performed. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average

molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1

Enrichment of Tumor Initiating Cell Populations

To characterize the cellular heterogeneity of solid tumors as they exist in cancer patients, elucidate the identity of tumor perpetuating cells (TPC; i.e. cancer stem cells: CSC) using particular phenotypic markers and identify clinically relevant therapeutic targets, a large non-traditional xenograft (NTX) tumor bank was developed and maintained using art recognized techniques. The NTX tumor bank, comprising a large number of discrete tumor cell lines, was propagated in immunocompromised mice through multiple passages of heterogeneous tumor cells originally obtained from numerous cancer patients afflicted by a variety of solid tumor malignancies. The continued availability of a large number of discrete early passage NTX tumor cell lines having well defined lineages greatly facilitate the identification and isolation of TPCs as they allow for the reproducible and repeated characterization of cells purified from the cell lines. More particularly, isolated or purified TPCs are most accurately defined retrospectively according to their ability to generate phenotypically and morphologically heterogeneous tumors in mice that recapitulate the patient tumor sample from which the cells originated. Thus, the ability to use small populations of isolated cells to generate fully heterogeneous tumors in mice is strongly indicative of the fact that the isolated cells comprise TPCs. In such work the use of minimally passaged NTX cell lines greatly simplifies *in vivo* experimentation and provides readily verifiable results. Moreover, early passage NTX tumors also respond to therapeutic agents such as irinotecan (i.e. Camptosar®), which provides clinically relevant insights into underlying mechanisms driving tumor growth, resistance to current therapies and tumor recurrence.

As the NTX tumor cell lines were established the constituent tumor cell phenotypes were analyzed using flow cytometry to identify discrete markers that might be used to characterize, isolate, purify or enrich tumor initiating cells (TICs) and separate or analyze TPCs and TProg cells within such populations. In this regard the inventors employed a proprietary proteomic based platform (i.e. PhenoPrint™ Array) that provided for the rapid characterization of cells based on protein expression and the concomitant identification of

potentially useful markers. The PhenoPrint Array is a proprietary proteomic platform comprising hundreds of discrete binding molecules, many obtained from commercial sources, arrayed in 96 well plates wherein each well contains a distinct antibody in the phycoerythrin fluorescent channel and multiple additional antibodies in different fluorochromes arrayed in every well across the plate. This allows for the determination of expression levels of the antigen of interest in a subpopulation of selected tumor cells through rapid inclusion of relevant cells or elimination of non-relevant cells via non-phycoerythrin channels. When the PhenoPrint Array was used in combination with tissue dissociation, transplantation and stem cell techniques well known in the art (Al-Hajj et al., 2004, Dalerba et al., 2007 and Dylla et al., 2008, all supra., each of which is incorporated herein by reference in its entirety), it was possible to effectively identify relevant markers and subsequently isolate and transplant specific human tumor cell subpopulations with great efficiency.

Accordingly, upon establishing various NTX tumor cell lines as is commonly done for human tumors in severely immune compromised mice, the tumors were resected from mice upon reaching 800 - 2,000 mm^3 and the cells were dissociated into single cell suspensions using art-recognized enzymatic digestion techniques (See for example U.S.P.N. 2007/0292414 which is incorporated herein). Data obtained from these suspensions using the PhenoPrint Array provided both absolute (per cell) and relative (vs. other cells in the population) surface protein expression on a cell-by-cell basis, leading to more complex characterization and stratification of cell populations. More specifically, use of the PhenoPrint Array allowed for the rapid identification of proteins or markers that prospectively distinguished TIC or TPC from NTG bulk tumor cells and tumor stroma and, when isolated from NTX tumor models, provided for the relatively rapid characterization of tumor cell subpopulations expressing differing levels of specific cell surface proteins. In particular, proteins with heterogeneous expression across the tumor cell population allow for the isolation and transplantation of distinct, and highly purified, tumor cell subpopulations expressing either high and low levels of a particular protein or marker into immune-compromised mice, thereby facilitating the assessment of whether TPCs were enriched in one subpopulation or another.

The term enriching is used synonymously with isolating cells and means that the yield (fraction) of cells of one type is increased over the fraction of other types of cells as compared to the starting or initial cell population. Preferably, enriching refers to increasing the percentage by about 10%, by about 20%, by about 30%, by about 40%, by

about 50% or greater than 50% of one type of cell in a population of cells as compared to the starting population of cells.

As used herein a marker, in the context of a cell or tissue, means any characteristic in the form of a chemical or biological entity that is identifiably associated with, or specifically found in or on a particular cell, cell population or tissue including those identified in or on a tissue or cell population affected by a disease or disorder. As manifested, markers may be morphological, functional or biochemical in nature. In preferred embodiments the marker is a cell surface antigen that is differentially or preferentially expressed by specific cell types (e.g., TPCs) or by cells under certain conditions (e.g., during specific points of the cell life cycle or cells in a particular niche). Preferably, such markers are proteins, and more preferably, possess an epitope for antibodies, aptamers or other binding molecules as known in the art. However, a marker may consist of any molecule found on the surface or within a cell including, but not limited to, proteins (peptides and polypeptides), lipids, polysaccharides, nucleic acids and steroids. Examples of morphological marker characteristics or traits include, but are not limited to, shape, size, and nuclear to cytoplasmic ratio. Examples of functional marker characteristics or traits include, but are not limited to, the ability to adhere to particular substrates, ability to incorporate or exclude particular dyes, for example but not limited to exclusions of lipophilic dyes, ability to migrate under particular conditions and the ability to differentiate along particular lineages. Markers can also be a protein expressed from a reporter gene, for example a reporter gene expressed by the cell as a result of introduction of the nucleic acid sequence encoding the reporter gene into the cell and its transcription resulting in the production of the reporter protein that can be used as a marker. Such reporter genes that can be used as markers are, for example but not limited to fluorescent proteins enzymes, chromomeric proteins, resistance genes and the like.

In a related sense the term marker phenotype in the context of a tissue, cell or cell population (e.g., a stable TPC phenotype) means any marker or combination of markers that may be used to characterize, identify, separate, isolate or enrich a particular cell or cell population. In specific embodiments, the marker phenotype is a cell surface phenotype which may be determined by detecting or indentifying the expression of a combination of cell surface markers.

Those skilled in the art will recognize that numerous markers (or their absence) have been associated with various populations of cancer stem cells and used to isolate or characterize tumor cell subpopulations. In this respect exemplary cancer stem cell

markers comprise OCT4, Nanog, STAT3, EPCAM, CD24, CD34, NB84, TrkA, GD2, CD133, CD20, CD56, CD29, B7H3, CD46, transferrin receptor, JAM3, carboxypeptidase M, ADAM9, oncostatin M, Lgr5, Lgr6, CD324, CD325, nestin, Sox1, Bmi-1, eed, easyh1, easyh2, mf2, yy1, smarcA3, smarckA5, smarcD3, smarcE1, mllt3, FZD1, FZD2, FZD3, FZD4, FZD6, FZD7, FZD8, FZD9, FZD10, WNT2, WNT2B, WNT3, WNT5A, WNT10B, WNT16, AXIN1, BCL9, MYC, (TCF4) SLC7A8, IL1RAP, TEM8, TMPRSS4, MUC16, GPRC5B, SLC6A14, SLC4A11, PPAP2C, CAV1, CAV2, PTPN3, EPHA1, EPHA2, SLC1A1, CX3CL1, ADORA2A, MPZL1, FLJ10052, C4.4A, EDG3, RARRES1, TMEPAI, PTS, CEACAM6, NID2, STEAP, ABCA3, CRIM1, IL1R1, OPN3, DAF, MUC1, MCP, CPD, NMA, ADAM9, GJA1, SLC19A2, ABCA1, PCDH7, ADCY9, SLC39A1, NPC1, ENPP1, N33, GPNMB, LY6E, CELSR1, LRP3, C20orf52, TMEPAI, FLVCR, PCDHA10, GPR54, TGFBR3, SEMA4B, PCDHB2, ABCG2, CD166, AFP, BMP-4, β-catenin, CD2, CD3, CD9, CD14, CD31, CD38, CD44, CD45, CD74, CD90, CXCR4, decorin, EGFR, CD105, CD64, CD16, CD16a, CD16b, GLI1, GLI2, CD49b, and CD49f. See, for example, Schulenburg et al., 2010, PMID: 20185329, U.S.P.N. 7,632,678 and U.S.P.Ns. 2007/0292414, 2008/0175870, 2010/0275280, 2010/0162416 and 2011/0020221 each of which is incorporated herein by reference. It will be appreciated that a number of these markers were included in the PhenoPrint Array described above.

Similarly, non-limiting examples of cell surface phenotypes associated with cancer stem cells of certain tumor types include $CD44^{+}CD24^{low}$, $ALDH^{+}$, $CD133^{+}$, $CD123^{+}$, $CD34^{+}CD38^{-}$, $CD44^{+}CD24^{-}$, $CD46^{+}CD324^{+}CD66c^{-}$, $CD133^{+}CD34^{+}CD10^{-}CD19^{-}$, $CD138^{-}CD34^{-}CD19^{+}$, $CD133^{+}RC2^{+}$, $CD44^{+}\alpha_2\beta_1^{hi}CD133^{+}$, $CD44^{+}CD24^{+}ESA^{+}$, $CD271^{+}$, $ABCB5^{+}$ as well as other cancer stem cell surface phenotypes that are known in the art. See, for example, Schulenburg et al., 2010, supra., Visvader et al., 2008, PMID: 18784658 and U.S.P.N. 2008/0138313, each of which is incorporated herein in its entirety by reference. Those skilled in the art will appreciate that marker phenotypes such as those exemplified immediately above may be used in conjunction with standard flow cytometric analysis and cell sorting techniques to characterize, isolate, purify or enrich TIC and/or TPC cells or cell populations for further analysis. Of interest with regard to the instant invention CD46, CD324 and, optionally, CD66c are either highly or heterogeneously expressed on the surface of many human colorectal ("CR"), breast ("BR"), non-small cell lung (NSCLC), small cell lung (SCLC), pancreatic ("PA"), melanoma ("Mel"), ovarian ("OV"), and head and neck cancer ("HN") tumor cells, regardless of whether the tumor

specimens being analyzed were primary patient tumor specimens or patient-derived NTX tumors.

Example 2

Isolation and Analysis of RNA Samples From Enriched Tumor Initiating Cell Populations

Several established colorectal NTX cell lines (SCRx-CR2, CR4, CR11 and CR14) generated and passaged as described in Example 1 were used to initiate tumors in immune compromised mice. For mice bearing SCRx-CR4 and CR14 tumors, once the mean tumor burden reached ~ 300 mm^3 the mice were randomized and treated with either 15 mg/kg irinotecan or vehicle control (PBS) twice weekly for a period of twenty days, at which point in time the mice were euthanized. Tumors arising from all four NTX lines, including those from mice undergoing chemotherapeutic treatment as described above, were removed and TPC, TProg and NTG cells, respectively, were isolated from freshly resected NTX tumors generally using the technique set out in Example 1. More particularly, cell populations were isolated by FACS using CD46, CD324 and CD66c markers and immediately pelleted and lysed in Qiagen RLTplus RNA lysis buffer (Qiagen, Inc.). The lysates were then stored at -80°C until used. Upon thawing the RNA cell lysate, total RNA was extracted using the Qiagen RNEasy isolation kit (Qiagen, Inc.) following the vendor's instructions and quantified on the Nanodrop (Thermo Scientific) and a Bioanalyzer 2100 (Agilent) again using the vendor's protocols and recommended instrument settings. The resulting total RNA preparation was suitable for genetic sequencing and analysis.

The RNA samples obtained from the TPC, TProg and NTG cell populations isolated as described above from vehicle or irinotecan-treated mice were prepared for whole transcriptome sequencing using an Applied Biosystems SOLiD 3.0 (Sequencing by Oligo Ligation/Detection) next generation sequencing platform (Life Technologies), starting with 5 ng of total RNA per sample. The data generated by the SOLiD platform mapped to 34,609 genes from the human genome, was able to detect APCDD1, and provided verifiable measurements of APCDD1 levels in all samples.

Generally the SOLiD3 next generation sequencing platform enables parallel sequencing of clonally-amplified RNA/DNA fragments linked to beads. Sequencing by ligation with dye-labeled oligonucleotides is then used to generate 50 base reads of each fragment that exists in the sample with a total of greater than 50 million reads generating a

much more accurate representation of the mRNA transcript level expression of proteins in the genome. The SOLiD3 platform is able to capture not only expression, but SNPs, known and unknown alternative splicing events, and potentially new exon discoveries based solely on the read coverage (reads mapped uniquely to genomic locations). Thus, use of this next generation platform allowed the determination of differences in transcript level expression as well as differences or preferences for specific splice variants of those expressed mRNA transcripts. Moreover, analysis with the SOLiD3 platform using a modified whole transcriptome protocol from Applied Biosystems only required approximately 5 ng of starting material pre-amplification. This is significant as extraction of total RNA from sorted cell populations where the TPC subset of cells is, for example, vastly smaller in number than the NTG or bulk tumors and thus results in very small quantities of usable starting material.

Duplicate runs of sequencing data from the SOLiD3 platform were normalized and transformed and fold ratios calculated as is standard industry practice. As seen in FIG. 2, an analysis of the data showed that APCDD1 was up-regulated at the transcript level by 3- to 7-fold in the TPC (i.e., CSC in FIG. 2) over the NTG population, and 60% to 200% in TPC over the TProg population, irrespective of whether cells were obtained from mice being treated with 15 mg/kg irinotecan or vehicle. Significantly, elevated expression of APCDD1 was only observed in NTX lines derived from patients that had KRAS and APC gene mutations (G12V & E991^{STOP} for SCRx-CR4 and G13D & Q1191^{STOP} for SCRx-CR11 NTX lines, respectively), suggesting a correlation between one or both of these mutations and elevated APCDD1 expression in the TPC subpopulation of colorectal tumors. The observed overexpression of APCDD1 in the TPC subpopulation of NTX tumor samples with KRAS and/or APC mutations using the extremely sensitive SOLiD3 analytical platform suggests that APCDD1 may play an important role in colorectal tumorigenesis and maintenance.

Example 3

Real-Time PCR Analysis of APCDD1 in Enriched Tumor Initiating Cell Populations

To confirm enhanced expression of APCDD1 in TPC populations versus TProg and NTG cells, TaqMan® quantitative real-time PCR was used to measure gene expression levels in respective cell populations isolated from various NTX lines as set forth above. It

will be appreciated that such real-time PCR analysis allows for a more direct and rapid measurement of gene expression levels for discrete targets using primers and probe sets specific to a particular gene of interest. TaqMan real-time quantitative PCR was performed on an Applied Biosystems 7900HT Machine (ABI: a Life Technologies Company), which was used to measure APCDD1 gene expression in multiple patient-derived NTX line cell populations and corresponding controls. Moreover, the analysis was conducted as specified in the instructions supplied with the TaqMan System and using commercially available APCDD1 primer/probe sets (Life Technologies).

As seen in FIG. 3 quantitative real-time PCR interrogating gene expression in NTG, TProg and TPC populations isolated from 3 distinct colorectal NTX tumor lines (SCRx-CR2, CR4 & CR14) shows that APCDD1 gene expression is elevated more than 4-fold in TPC cells from patient tumors containing KRAS and/or APC mutations (i.e. SCRx-CR4). APCDD1 was also elevated more than 2-fold in TPC populations in mice undergoing treatment with irinotecan in the same KRAS and APC mutated tumors. The observation of elevated APCDD1 expression in NTX TPC cell preparations as compared with TProg and NTG cell controls from patient tumors containing KRAS and/or APC mutations using the more widely accepted methodology of real-time quantitative PCR confirms the more sensitive SOLiD3 whole transcriptome sequencing data of Example 2. This further implicates APCDD1 expression as a driving factor in the most aggressive and refractory colorectal neoplasias characterized by the above mutations. Moreover, increased APCDD1 expression in tumors treated with an anti-cancer agent shows that APCDD1 effectors or antagonists may prove valuable as an adjunct therapy.

Example 4

Expression of APCDD1 in Unfractionated Colorectal Tumor Samples

In light of the fact that APCDD1 gene expression was found to be elevated in TPC populations from colorectal tumors characterized as having KRAS and APCD mutations when compared with TProg and NTG cells from the same tumors, experiments were conducted to determine whether APCDD1 expression levels were also elevated in unfractionated colorectal tumor samples versus normal adjacent tissue (NAT) and other normal tissue samples. Custom TumorScan qPCR (Origene Technologies) 384-well arrays containing 110 colorectal patient tumor specimens, normal adjacent tissue, and 48

normal tissues were designed and custom fabricated according to a provided protocol. Using the procedures detailed in Example 3 and the same APCDD1 specific primer/probe sets, TaqMan real-time quantitative PCR was then performed in the wells of the custom plates.

FIGS. 4A and 4B show the results of the expression data in a graphical format normalized against the mean expression in normal colon and rectum tissue. More specifically, FIG. 4A summarizes data generated using 168 tissue specimens, obtained from 110 colorectal cancer patients, (35 tissue specimens of which are normal (NL) adjacent tissue from colorectal cancer patients) and 48 normal tissues. In the plot, data from each tissue specimen/patient is represented by a dot, with the geometric mean value of each population demarcated on the X-axis represented as a line. Similarly, FIG. 4B contains data from 24 matched colorectal patient specimens obtained from tumor (T) or normal adjacent tissue (N) at various stages of the disease (I-IV). Here the plotted data is presented on a sample by sample basis with linkage between the respective tumor and normal adjacent tissue from individual patients. Both FIGS. 4A and 4B indicate that, in all four stages presented, the expressed level of the APCDD1 gene is elevated in a subset of colorectal tumors and in matched tumor specimens versus normal adjacent tissue. Based on data presented in Examples 2 and 3, the subset of patients with elevated APCDD1 expression is most likely to be those patients with KRAS and/or APC mutations.

APCDD1 gene expression was low to moderate in most normal tissues, with only normal uterus tissue containing gene expression levels at or above the mean expression levels observed in colorectal cancer patient tumors (average relative expression of 9.12-fold higher than normal colorectal tissue). Elevated expression of APCDD1 in a subset of unfractionated colorectal tumor samples and low to moderate expression levels in normal control tissue is again suggestive as to the role of the APCDD1 gene product in the development and support of malignancies in a subset of cancer patients.

Example 5

Differential Expression of APCDD1 in Exemplary Tumor Samples

To further assess APCDD1 gene expression in additional colorectal cancer patient tumor samples and tumor specimens from patients diagnosed with 1 of 17 other different solid tumor types, Taqman qRT-PCR was performed using TissueScan qPCR (Origene

Technologies) 384-well arrays, which were custom assembled according to a provided protocol as in Example 4. The results of the measurements are presented in FIG. 5A and 5B and show that gene expression of APCDD1 is significantly elevated or repressed in a number of tumor samples.

In this regard, FIGS. 5A and 5B show the relative and absolute gene expression levels, respectively, of human APCDD1 in whole tumor specimens (grey dots) or matched normal adjacent tissue (NAT; white dots) from patients with one of eighteen different solid tumor types. In FIG. 5A, data is normalized against mean NAT gene expression for each tumor type analyzed. In FIG. 5B, the absolute expression of APCDD1 was assessed in various tissues/tumors, with the data being plotted as the number of cycles (Ct) needed to reach exponential amplification by quantitative real-time PCR. Specimens not amplified were assigned a Ct value of 45, which represents the last cycle of amplification in the experimental protocol. Each dot represents an individual tissue specimen, with the mean value represented as a black line.

In addition to a subset of patients diagnosed with colorectal cancer, subsets of patients diagnosed with endometrial, kidney, liver and prostate cancer also had significantly more APCDD1 gene expression in their tumors versus NAT, suggesting that APCDD1 might also play a pathological role by impacting TPC self-renewal and proliferation in these tumors. In contrast, expression of APCDD1 appeared significantly repressed in tumors from patients with adrenal, breast, cervix, lymphoid, ovarian, pancreatic and bladder cancer. What was also clear from the these studies is that APCDD1 gene expression was generally low to moderate in most NAT samples; with the highest expression being observed in the adrenal gland, cervix and bladder. Again, these data suggest that differential APCDD1 expression (high or low) is indicative, and potentially dispositive, as to tumorigenesis or perpetuation in patients presenting selected hyperproliferative disorders.

Example 6

Construction and Expression of Tagged APCDD1 Extra Cellular Domain

Constructs comprising APCDD1 extra cellular domain (ECD) were fabricated and expressed as set forth below for use in generating tumor initiating cell effectors. As a starting point, human APCDD1 cDNA encoding the entire open reading frame was

obtained from a commercial source (Open Biosystems; Accession No. BC053324). Based on this material, APCDD1 extra cellular domain (ECD) proteins were produced for use as assay reagents and for *in vivo* studies. More particularly, a carboxy terminal His tagged protein comprising the extracellular domains of the APCCD1 protein was made using human APCDD1 cDNA. The sequence encoding the majority of the ECD of the mature APCDD1 protein (amino acids 27 - 486) was amplified using PCR primers which also appended a sequence encoding 6 His residues to the 3' end of the cDNA. The PCR product was subcloned between the Asc I and Xho I sites of pcDNA-CAG-Zeo, a vector derived from pcDNA3.1 (Life Technologies) in which the CMV promoter was exchanged for a sequence containing CAG promoter. The resulting vector, pSCRxv005-APCDD1ecd, comprises an IgK signal peptide sequence, fused in frame to the sequences encoding the ECD of the mature APCDD1 protein. A translation of the protein encoded by this vector is shown in FIG. 1C along with the corresponding nucleic acid sequence set forth as SEQ ID NO: 3.

Recombinant soluble human APCDD1-ECD His tagged protein was produced in HEK293T cells that were transfected with pSCRxv005-APCDD1ecd using lipofectamine 2000 (Life Technologies). Three days after transfection, the recombinant His-tagged protein was purified from the supernatant using Ni-NTA HisTrap columns (GE Amersham) and an AKTA prime instrument. Recombinant protein was eluted from the column using a linear gradient of imidazole (final concentration 500 mM) and the fractions containing the APCDD1-ECD pooled, concentrated, and further purified on a Superdex200 size exclusion column using an AKTA FPLC to collect protein. Material eluted from the column was concentrated (to approximately 1 mg/mL) and the buffer exchanged to PBS.

Example 7

Construction and Expression of a Fc-APCDD1 Fusion Effector

Additional, relatively more soluble, APCDD1fusion proteins were produced for use as immunogens, assay reagents and for *in vivo* studies. As in Example 6, APCDD1 cDNA encoding the entire open reading frame (ORF) set forth as SEQ ID NO: 1 in FIG. 1A was obtained from a commercial source. The cDNA clone ORF sequence was confirmed by DNA sequencing to be without mutation relative to the reference sequence (GenBank

NM_153000). Based on this sequence relatively soluble, Fc constructs were made using human APCDD1 cDNA extracellular domain.

The sequences of the full length cDNA encoding the ECD of the mature APCDD1 protein (amino acids 17- 487) were amplified using PCR and subcloned in frame between the EcoRI and BamH I sites of SCRxv003, a vector derived from the pFUSE-mIgG_2b vector (Invivogen) but in which the sequences encoding the mouse IgG2b Fc domain had been replaced by a DNA sequence encoding the human IgG2 Fc domain. The resulting vector, pSCRxv003-APCDD1, comprises an IL-2 signal peptide sequence, fused in frame to the sequences encoding the ECD of the mature APCDD1 protein, fused in frame with sequences encoding the Fc domains derived from a human IgG2 gene. The protein encoded by this vector (i.e. hAPCDD1-Fc) is shown as a translation of the nucleic acid in SEQ ID NO: 4 as set forth in FIG 1D.

Recombinant human APCDD1-Fc protein was produced in HEK293T cells that were transfected with pSCRxv003-APCDD1 plasmid using lipofectamine 2000 (Life Technologies). Two days after transfection, the recombinant protein was purified from the supernatant using a Protein A columns and manufacturer's instructions (GE Amersham). Material eluted from the column was concentrated (to approximately 1 mg/mL) and the buffer exchanged to PBS.

Example 8

Generation of anti-APCDD1 Effectors using APCDD1 Constructs

APCDD1 effectors in the form of murine antibodies were produced in accordance with the teachings herein through inoculation with hAPCDD1-Fc from Example 7. In this regard three strains of mice were used to generate high affinity, murine, monoclonal antibodies that can be used therapeutically to inhibit the action of APCDD1 for the treatment of hyperproliferative disorders. Specifically, Balb/c, CD-1 and FVB mouse strains were immunized with human recombinant APCDD1 and used to produce hybridomas as follows:

Murine antibodies were generated by immunizing 9 female mice (3 each: Balb/c, CD-1, FVB). Immunization protocols used purified hAPCDD1-Fc recombinant fusion protein expressed in 293 cells as antigen. Mice were immunized with ten doses of the immunogen (10 μg per mouse for each immunization) emulsified with an equal volume of

TITERMAX or alum adjuvant via footpad injections.

Draining lymph nodes (popliteal and inguinal, if enlarged) were dissected out and used as a source for antibody producing cells. Single cell suspension of B cells were fused with non-secreting P3x63Ag8.653 myeloma cells (ATCC #CRL-1580) at a ratio of 1:1 by Electro-fusion. Electro cell fusion was performed using a fusion generator; model ECM2001, (Genetronic, Inc.). Cells were resuspended in hybridoma selection medium supplemented with HAT (Sigma #A9666) (DMEM (Cellgro cat#15-017-CM) medium containing 15% Fetal Clone I serum (Hyclone), 1 mM sodium pyruvate, 4 mM L-glutamine, 10 µg/mL gentamicin, 50 µM 2-mercaptoethanol, 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine) Cells were plated at $2X10^4$/well in flat bottom microtiter plates, then placed in a humidified 37°C incubator containing 5% CO_2 and 95% air for 7-10 days.

Individual wells were than screened by ELISA and FACS for anti-hAPCDD1 monoclonal antibodies. Supernatants from fifteen 96 well plates were screened. ELISA microtiter plates were coated with purified recombinant APCDD1-IgGFc fusion proteins (from Example 7) from transfected 293 cells at 100 ng/well in carbonate buffer. Plates incubated at 4°C overnight then blocked with 200 µl/well of 3% BSA in PBS/Tween (0.05%). Supernatant from hybridoma plates were added to each well and incubated for 1-2 hours at ambient temperature. The plates were washed with PBS/Tween and than incubated with goat anti-mouse IgG polyclonal antibody conjugated with horseradish proxidase (HRP) for one hour at room temperature. After washing, the plates were developed with TMB substrate (Thermo Scientific 34028) and analyzed by spectrophotometer at OD 450. Alternatively, ELISA plates were coated with goat anti-human IgG Fc, to capture APCDD1-hFc to ELISA plate. The plates were washed and blocked with 3% BSA-PBS for one hour at RT, and used to screen undiluted hybridoma supernatants. The plates are washed and probed with HRP labeled goat anti-mouse IgG diluted 1:10,000 in 3% BSA-PBS for one hour at RT. The plates are then incubated with substrate solution as described above.

Anti-hAPCDD1 secreted hybridoma from positive wells (165 hits OD405@20min >0.75) were re-plated, rescreened and subcloned by limited dilution or single cell FACS sorting. The resulting clonal populations were expanded and cryopreserved in freezing medium (90% FBS, 10% DMSO) and stored in liquid nitrogen.

ELISA analysis confirms that purified antibody from most or all of these hybridomas bind hAPCDD1 in a concentration-dependent manner. It should be noted that

binding APCDD1 directly to the ELISA plate can cause protein denaturation lead to apparent binding affinities may not be accurately reflective of binding to undenatured protein.

Example 9

Sequencing of APCDD1 Effectors

Based on the assays in the previous Example a number of exemplary monoclonal antibodies that bind immobilized human APCDD1 were selected and sequenced. The full sequence of the heavy and light chain variable regions for thirteen of these antibodies (SC7.18, SC7.107, SC7.143, SC7.12, SC7.13, SC7.43, SC7.46, SC7.48, SC7.58, SC7.75, SC7.85, SC7.142 and SC7.106) are shown in FIGS. 9A – 9M. Similarly, the genetic arrangement and complementarity determining regions of the anti-APCDD1 antibodies from Example 8 (including the three mentioned directly above) are shown in a tabular fashion in FIGS. 10A and 10B. Note that the complementarity determining regions set forth in FIG. 10B are defined as per Chothia et al., supra.

For initiation of sequencing TRIZOL reagent was purchased from Invitrogen (Life Technologies). One step RT PCR kit and QIAquick PCR Purification Kit were purchased from Qiagen, Inc. with RNasin were from Promega. Custom oligonucleotides were purchased from Integrated DNA Technologies.

Hybridoma cells were lysed in TRIZOL reagent for RNA preparation. Between 10^4 and 10^5 cells were resuspended in 1 ml TRIZOL. Tubes were shaken vigorously after addition of 200 µl of chloroform. Samples were centrifuged at 4°C for 10 minutes. The aqueous phase was transferred to a fresh microfuge tube and an equal volume of isopropanol was added. Tubes were shaken vigorously and allowed to incubate at room temperature for 10 minutes. Samples were then centrifuged at 4°C for 10 minutes. The pellets were washed once with 1 ml of 70% ethanol and dried briefly at room temperature. The RNA pellets were resuspended with 40 µl of DEPC-treated water. The quality of the RNA preparations was determined by fractionating 3 µl in a 1% agarose gel. The RNA was stored in a –80°C freezer until used.

The variable DNA sequences of the hybridoma amplified with consensus primer sets specific for murine immunoglobulin heavy chains and kappa light chains were obtained using a mix of variable domain primers. One step RT-PCR kit was used to amplify the

VH and VK gene segments from each RNA sample. The Qiagen One-Step RT-PCR Kit provides a blend of Sensiscript and Omniscript Reverse Transcriptases, HotStarTaq DNA Polymerase, Qiagen OneStep RT-PCR Buffer, a dNTP mix, and Q-Solution, a novel additive that enables efficient amplification of "difficult" (e.g., GC-rich) templates.

Reaction mixtures were prepared that included 3 μl of RNA, 0.5 of 100 μM of either heavy chain or kappa light chain primers 5 μl of 5× RT-PCR buffer, 1 μl dNTPs, 1 μl of enzyme mix containing reverse transcriptase and DNA polymerase, and 0.4 μl of ribonuclease inhibitor RNasin (1 unit). The reaction mixture contains all of the reagents required for both reverse transcription and PCR. The thermal cycler program was RT step 50°C for 30 minutes 95°C for 15 minutes followed by 30 cycles of (95°C for 30 seconds, 48°C for 30 seconds, 72°C for 1.0 minutes). There was then a final incubation at 72°C for 10 minutes.

To prepare the PCR products for direct DNA sequencing, they were purified using the QIAquick™ PCR Purification Kit according to the manufacturer's protocol. The DNA was eluted from the spin column using 50 μl of sterile water and then sequenced directly from both strands. PCR fragments were sequenced directly and DNA sequences were analyzed using VBASE2 (Retter et al., Nucleic Acid Res. 33; 671-674, 2005).

As discussed above the amino acid and nucleic acid sequences for thirteen exemplary antibody heavy and light chain variable regions are set forth in FIGS. 9A - 9M respectively (SEQ ID NOs: 5-16, 97-136) while the genetic arrangements and derived CDRs (Chothia et al. nomenclature) of these anti-APCDD1 antibodies are set forth in a tabular form in FIGS. 10A and 10B (SEQ ID NOs: 17-88).

Example 10

Characterization of APCDD1 Effector Specificity by ELISA and Flow Cytometry

For flow cytometric assays, ten million SW480 cells, known to naturally express high levels of APCDD1, were mixed with ten million hamster cells (CHO-S) that did not express human APCDD1, to a final concentration of 3.3 million cells per mL. 15 μL of this cell mixture was added to 35 μL of APCDD1 antibody-containing supernatant derived from different clones obtained as set forth in Example 8 in each well of two 96-well plates. The samples were mixed by gentle vortexing and the plates incubated for 30 minute at

4°C. The cells were then washed once with PBS and then stained for 30 minute at 4°C in the dark with DyLight649 anti-mouse IgG (BioLegend Inc.). After a 30 minute incubation at 4°C in the dark, the cells were washed with PBS and counterstained with DAPI (to eliminate dead cells from the analysis). A positive control sample was prepared with 1:50 dilution of a commercially available polyclonal AlloPhyco-Cyanin (APC)-conjugated APCDD1 antibody (Novus Biologicals LLC), and the negative control was labeled with only DyLight649 anti-mouse IgG. Samples that tested positive using ELISA were then analyzed by flow cytometry using a FACSCanto II (BD Biosciences) as per the manufacturer's instructions. Using this protocol, 115 of the 165 prospective APCDD1 antibody expressing hybridoma clones tested were found to contain antibodies with some cell surface binding as demonstrated by flow cytometry (data not shown). FACs plots for two representative clones (SC7.26 & SC7.43) secreting discrete antibodies reactive with APCDD1 are shown in FIG. 6, and demonstrate that effectors of the instant invention may be used to effectively characterize cell surface APCDD1 expression and to isolate APCDD1 positive cells.

Example 11

APCDD1 Effectors Inhibit Proliferation of SW480 cells

Supernatants from hybridomas producing antibodies raised against APCDD1 ECD-hFc (as set forth in Example 8) were screened for their ability to inhibit the proliferation of SW480 cells, based upon the reported ability of antisense oligonucleotides directed against APCDD1 to inhibit proliferation of SW480 cells (Takahashi *et al.*, Cancer Res. 62:5651-56(2002)). Three thousand SW480 cells were plated in 100 µL of growth medium (IMDM + 10% heat inactivated FBS) per well of a 96 well plate. Sixteen hours after plating, the plating medium was replaced with 25 µL of DMEM+10% heat inactivated FBS and 25 µL of selected antibody supernatant in each well. Seventy-two hours after the addition of the antibody containing supernatants, 50 µL of Cell-Titer Glo® reagent (Promega, Inc.) was added to each well to determine total cell counts, and after a 5 minute incubation, the relative light units per well quantitated using a VICTOR^3 Wallac 1420 (Perkin Elmer). All antibody supernatants were assayed in triplicate and plotted in FIG. 7 against 80% of negative controls. As may be seen in FIG. 7, 105 clones from a total of 165 clones screened (i.e. clones SC7.1 to SC7.165) showed some inhibition of

proliferation in this assay while 38 clones showed greater than 25% inhibition of growth. This is strongly indicative of the ability of effectors of the present invention to inhibit the proliferation of APCDD1 positive cells including TPC cells.

Example 12

APCDD1 Effectors Are Internalized By SW480 cells

Supernatant from hybridomas producing antibodies raised against APCDD1 ECD-hFc were screened for their ability to internalize in SW480 cells, which express APCDD1 on the cell surface. SW480 cells at a starting concentration of 10e6/ml (single cells suspension) were blocked with Human TruStain (BioLegend; San Diego, CA) for 10 minutes at room temperature, diluted to $5x10^4$ cells per condition, and duplicate samples were then stained for 30 minutes on ice with antibody containing supernatant for a final volume of 50μl. Cells were then washed with FACS staining medium (FSM; 2% fetal bovine serum/Hank's buffered saline solution/25mM HEPES [pH7.4]) to remove unbound antibody. This was followed by a second stain with donkey anti-mouse Alexa647 (Life Technologies) for 30 minutes on ice. Samples were washed again to remove unbound antibody and then re-suspended in internalization medium (2% fetal bovine serum/ Iscove's Modified Dulbecco's Medium). To allow internalization, samples were incubated in 5% CO2 @ 37°C (or 4°C for the Control) for 1 hour. Internalization was stopped by transferring samples to ice and adding excess ice cold FSM. To remove any antibody that did not internalize and remained on the cell surface, samples were treated with low pH phosphate buffered saline (PBS [pH2.0]) for 10 minutes on ice. Following this "acid strip" procedure, samples were washed extensively with FSM, resuspended in 150μl of FSM containing 2μg/ml of DAPI, and analyzed by flow cytometry. Any signal detected beyond background results from antibody internalization: a process, which protects the fluorescent conjugate from removal from the cell surface during the acid strip process. All incubations were performed in FSM unless stated otherwise.

Screening of 165 APCDD1 antibody-containing hybridoma supernatant clones using the acid strip protocol described above, many supernatants showed a positive shift in fluorescence vs. the IgG1 and IgG2b negative control antibodies (FIG. 8). The exemplary SC7.26 and SC7.43 clones, for instance, demonstrated excellent internalization in as far as supernatants from these clones was able to internalize and protect the Alexa647 secondary

antibody from acid stripping (FIG 8A). Compared to the IgG controls, approximately half of the APCDD1 antibody-containing supernatants induced internalization to varying degrees (FIG 8B), with the top 20 demonstrating a Delta Mean Fluorescent Intensity (MFI at 37°C vs. 4°C) above 200 (FIG 8C). This data indicates that a subset of antibodies generated against human APCDD1 ECD bind the antigen as it is presented on cells and is able to internalize.

Example 13

Determination of Binding Characteristics of APCDD1 Effectors

Various methods were used to analyze the biding characteristics of selected APCDD1 effectors generated as set forth above. Specifically, a number of APCDD1 antibodies were characterized as to affinity, kinetics, binning, and cross reactivity with regard to mouse and cynomolgus homologs by ForteBIO . The chemical nature of the epitope, whether linear or dependent on target secondary structure was determined through western blotting (reduced vs. non-reduced). The results of this characterization are set forth in tabular form in FIG. 11.

With regard to the data, affinity was measured in three ways to ensure accuracy. First, binding signal was measured for a fixed amount of antibody probed against serial dilutions of antigen in an ELISA to determine relative effector activity (data not shown). Second, the affinities of the selected effectors were then measured using bio-layer interferometry analysis on a ForteBIO RED (ForteBIO, Inc.) with a standard antigen concentration series. Finally, the affinity of selected effectors was measured by surface plasmon resonance (Biacore System, GE Healthcare). Based on a standard antigen concentration series and using a 1:1 Langmuir binding model, the K_d of the antibody binding to antigen and the kinetics constants k_{on} and k_{off} were determined. FIG. 11 identifies the method used to generate the number included in the table which was chosen for accuracy. In general the selected effectors exhibited relatively high affinities in the nanomolar range.

As to antibody binning, ForteBIO was used per manufacturer's instructions to identify antibodies, which bound to the same or different bins. Briefly, an antibody (Ab1) was captures onto an anti-mouse capture chip, a high concentration of nonbinding antibody was then used to block the chip and a baseline was collected. Monomeric,

recombinant APCDD1-His was then capture by the specific antibody (Ab1) and the tip was dipped into a well with either the same antibody (Ab1) as a control or into a well with a different antibody (Ab2). If additional binding response was observed with a new antibody, then Ab1 and Ab2 were determined to be in a different bin. If no further binding occurred similar to the control Ab1, then Ab2 was determined to be in the same bin. This process can be expanded to screen large libraries of unique antibodies using a full row of antibodies representing unique bins in a 96-well plate. This experiment showed the screened antibodies bound to at least seven different bins or epitopes on the APCDD1 protein.

In order to determine whether the epitope recognized by the APCDD1 effector comprises contiguous amino acids or is formed by noncontiguous amino acids juxtaposed by secondary structure of the antigen, Western blots were run under reducing and non-reducing conditions. More particularly, using standard electrophoresis techniques well known in the art, APCDD1 antigen in both states was exposed to the selected effector. As detailed in FIG. 11 some APCDD1 effectors reacted with both non-reduced and reduced antigen (NR/R) whereas others substantially reacted only with antigen where disulphide bonds were intact (NR).

Finally, cross-reactivity with regard to cynomolgus and murine APCDD1 homologs were evaluated in a standard ELISA format for cynomolgus and in ForteBIO for mouse using in-house produced, recombinantly expressed APCDD1 antigens. As listed in FIG. 11 the selected effectors were reactive with one or both homologs. ND in the table indicates that the data was not determined.

Example 14

Humanization of APCDD1 Effectors

Murine antibodies SC7.13.1 and SC7.48.1 were humanized using a computer-aided CDR-grafting method (Abysis Database, UCL Business Plc.) and standard molecular engineering techniques to provide hSC7.13 and hSC7.48 APCDD1 effectors. The human framework regions of the variable regions were selected based on their highest sequence homology to the mouse framework sequence and its canonical structure. For the purposes of the analysis the assignment of amino acids to each of the CDR domains is in accordance with the Kabat numbering. Several humanized antibody variants were made in

order to generate the optimal humanized antibody.

Molecular engineering procedures were conducted using art-recognized techniques. To that end total mRNA was extracted from the selected hybridomas according to the manufacturer's protocol (Trizol® Plus RNA Purification System, Life Technologies). A primer mix comprising thirty-two mouse specific 5' leader sequence primers, designed to target the complete mouse repertoire, was used in combination with 3' mouse Cγ primer mix to amplify and sequence the variable region of the antibody heavy chains. Similarly thirty-two 5' Vk leader sequence primer mix designed to amplify each of the Vk mouse families combined with a single reverse primer specific to the mouse kappa constant region were used to amplify and sequence the kappa light chain. The V_H and V_L transcripts were amplified from 100 ng total RNA using reverse transcriptase polymerase chain reaction (RT-PCR).

A total of eight RT-PCR reactions were run for each hybridoma: four for the V kappa light chain and four for the V gamma heavy chain. The Qiagen One Step RT-PCR kit was used for amplification, (Qiagen Inc.). The extracted PCR products were directly sequenced using specific V region primers. Nucleotide sequences were analyzed using IMGT to identify germline V, D and J gene members with the highest sequence homology. The derived sequences were compared to known germline DNA sequences of the Ig V- and J-regions using the V-BASE2 and by alignment of V_H and V_L genes to the mouse germ line database.

Sequence analysis: from the nucleotide sequence information, data regarding V, D and J gene segment of the heavy and light chain of SC7.13 and SC7.48 were obtained. Based on the sequence data new primer sets specific to the leader sequence of the Ig V_H and V_K chains of the antibodies were designed for cloning of the recombinant mouse monoclonal antibodies. Subsequently the V-(D)-J sequences were aligned with mouse Ig germ line sequences. Heavy chain genes of SC7.13 were identified as IGHV5-17, P1inv and JH4 whereas the heavy chain genes of SC7.48 were identified as IGHV1-14, DQ52a.1 and JH2. Light chain genes were, respectively, from IGKV3-5 and JK2 and IGKV6-32 and JK1 germline gene families.

The obtained heavy and light chain sequences from SC7.13 were aligned to the functional human variable region sequences and reviewed for homology and canonical structure. Based on this analysis the V_H3-48 germline and the JH6 J segment were selected with no framework amino acid substitutions for use in the SC7.13 humanized construct. The variable region of the hSC7.13 heavy chain shows 94% homology to the

human VH3-48 germline sequence (Z score value of 1.076) and 90% sequence homology to the mouse variable region. For the light chain of SC7.13 a similar process was followed and resulted in the selection of human germ line V_K L6 and J segment JK2. Several amino acid substitutions were made in the framework. The resulting variable region of the hSC7.13 kappa light chain shows 84% homology to the human V_K L6 germ line sequence (positive Z score value of 0.197) and 87% sequence homology to the mouse variable region. The amino acid sequences (along with a compatible nucleic acid sequence) of the humanized heavy chain (SEQ ID NO: 90) and the humanized light chain (SEQ ID NO: 92) are shown in FIG. 12A wherein the CDRs (as defined by Chothia et al.) are underlined.

A similar procedure was followed to derive hSC7.48. This analysis provided a humanized heavy chain comprising the V_H1-46 gene segment and JH5 J segment with several amino acid substitutions. Of particular note the residue at position 58 is preferably Glu or Ser, the residue at position 60 is preferably Asp or Ala, the residue at position 61 is preferably Glu or Gln and the residue at position 64 is preferably Lys or Gln. The variable region of hSC7.48 heavy chain shows 90% homology to the human V_H1-46 germ line sequence (Z score value of -0.418) and 82% sequence homology to the mouse variable region. For the light chain variable region the analysis indicated that V_K L11 and J segment JK1 with no framework substitutions would be effective. The variable region of the humanized I48 kappa light chain shows 87% homology to the human V_K L11germ line sequence (positive Z score value of 0.942) and 80% sequence homology to the mouse variable region. The amino acid sequences (along with a compatible nucleic acid sequence) of the humanized heavy chain (SEQ ID NO: 94) and the humanized light chain (SEQ ID NO: 96) are shown in FIG. 12B wherein the CDRs (as defined by Chothia et al.) are underlined.

For antibody production directional cloning of the murine and humanized variable gene PCR products into human immunoglobulin expression vectors was undertaken. All primers used in Ig gene-specific PCRs included restriction sites (AgeI and XhoI for IgH, XmaI and DraIII for IgK, which allowed direct cloning into expression vectors containing the human IgG1, and IGK constant regions, respectively. In brief, PCR products were purified with Qiaquick PCR purification kit (Qiagen, Inc.) followed by digestion with AgeI and XhoI (IgH), XmaI and DraIII (IgK), respectively. Digested PCR products were purified prior to ligation into expression vectors. Ligation reactions were performed in a total volume of 10 µL with 200U T4-DNA Ligase (New England Biolabs), 7.5 µL of digested and purified gene-specific PCR product and 25ng linearized vector DNA.

Competent E. coli DH10B bacteria (Life Technologies) were transformed via heat shock at 42°C with 3 µL ligation product and plated onto ampicillin plates (100 µg/mL). The AgeI-EcoRI fragment of the V_H region was than inserted into the same sites of pEE6.4HuIgG1 expression vector while the synthetic XmaI-DraIII V_K insert was cloned into the XmaI-DraIII sites of -the respective pEE12.4Hu-Kappa expression vector.

Cells producing the humanized antibodies were generated by transfection of HEK 293 cells with the appropriate plasmids using 293fectin. In this respect plasmid DNA was purified with QIAprep Spin columns (Qiagen, Inc.). Human embryonic kidney (HEK) 293T (ATCC No: CRL-11268) cells were cultured in 150mm plates (Falcon, Becton Dickinson) under standard conditions in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FCS, 100 µg/mL streptomycin, 100 U/mL penicillin G (all from Life Technologies).

For transient transfections cells were grown to 80% confluency. Equal amounts of IgH and corresponding IgL chain vector DNA (12.5 µg of each vector DNA) was added to 1.5 mL Opti-MEM mixed with 50 µL HEK 293 transfection reagent in 1.5 mL opti-MEM. The mix was incubated for 30 min at room temperature and distributed evenly to the culture plate. Supernatants were harvested three days after transfection, replaced by 20 mL of fresh DMEM supplemented with 10% FBS and harvested again at day 6 after transfection. Culture supernatants were cleared from cell debris by centrifugation at 800×g for 10 min and stored at 4°C. Recombinant antibodies were purified with Protein G beads (GE Healthcare).

Example 15

Characterization of Monoclonal Antibody APCDD1 Effectors

Using techniques set forth in Example 13 the humanized constructs hSC7.13 and hSC7.48 were analyzed to determine their binding characteristics. Moreover, hSC7.13 binding was directly compared with the parent murine antibody SC7.13 to identify any subtle changes in rate constants brought about by the humanization process.

More specifically, the affinity of murine SC7.13 was measured by a Biacore using surface plasmon resonance (SPR) to provide the results set forth in FIG. 13A. Based on a concentration series of 6.25, 3.125 and 1.5625 nM (generating the curves from top to bottom in the FIGS. 13A and 13B) and using a 1:1 Langmuir binding model, the K_d of the

antibody binding to antigen was estimated to be 1.6 nM. Similar experiments then run with the humanized construct showed equivalent results (FIG. 13B) indicating that the humanization process had not adversely impacted the affinity. In this regard the measurements indicated that the humanized construct had a K_d of 1.7×10^{-9} vs. a K_d of 1.6×10^{-9} for the parent murine antibody.

Along with techniques set out in Example 13, these measurements showed that both the humanized APCDD1 effectors from Example 14 possess desirable qualities. As set out in FIG. 13C SC7.48 strongly cross-reacts with both cynomolgus and murine APCDD1 homologs thereby facilitating toxicology studies. While the measured affinity of this effector is not as strong as that of its murine parent or the humanized SC7.13, it is still well within an acceptable therapeutic window. Similarly, SC7.13 shows excellent cross-reactivity with cynomolgus antigen allowing for relevant toxicology studies.

Example 16

Colorectal Tumor Initiating Cells Express APCDD1

As discussed above exemplary APCDD1 effectors, such as the monoclonal antibodies characterized in previous Examples, may be used with art-recognized flow cytometric techniques to interrogate and enrich tumor cell subpopulations as disclosed herein. In particular embodiments flow cytometry may be used to quantify cell surface expression of APCDD1 on cells or cell populations where the amount of detected fluorophore may be correlated to the number of bound effectors.

To determine the level of APCDD1 protein on the surface of respective colorectal tumor cell subpopulations, non-traditional xenograft colorectal tumors were dissociated to provide single cell suspensions and contacted with biotinylated anti-APCDD1 antibodies along with labeled antibodies able to distinguish between tumorigenic or non-tumorigenic colorectal tumor cells (i.e. CD46 and CD324). The relative amount of APCDD1 on the cell surface of these respective tumor cell subpopulations was then assessed on a FACSCanto II (BD Biosciences) flow cytometer using streptavidin-conjugated phycoerythrin (PE) to detect APCDD1 and fluorophore conjugated antibodies specific for CD46 and CD324 that emit light in emission bands distinct from PE.

Specifically, murine APCDD1 effectors SC7.13 (gray bars) and SC7.58 (white bars)

were used to detect APCDD1 protein expression on the surface of cells freshly isolated from human tumors that had been propagated in immunocompromised mice. As is shown in FIG. 14 compared to an isotype control antibody that does not bind APCDD1 (Control; black bars), CD46-negative ($CD46^-$) cells express significantly less APCDD1 protein on their cell surface than either $CD46^{hi}$ $CD324^-$ or $CD46^{hi}$ $CD324^+$ cells, as measured for respective cell populations using the mean fluorescence intensity (MFI) of PE.

As set forth in Example 1 $CD46^{hi}$ $CD324^+$ cell subpopulations appear to be highly enriched for tumor perpetuating cells, whereas $CD46^{hi}$ $CD324^-$ and $CD46^-$ cell subpopulations are not. Accordingly, expression of APCDD1 is strongly correlated with TPC and tumorigenic potential. The fact APCDD1 cell surface expression is associated with tumorigenicity is indicative of the propensity of the disclosed effectors to effectively function as diagnostic or therapeutic agents that are able to target TIC.

Example 17

APCDD1 Effectors Can Internalize and Mediate Anti-Cancer Agent Induced Cytotoxicity

To determine whether the APCDD1 effectors described herein are able to internalize and mediate the delivery of a cytotoxic agent to live cells, *in vitro* cell killing assays were performed. In this regard the ribosome-inactivating protein saporin was used as an exemplary anti-cancer agent and associated with APCDD1 effectors using a saporin conjugated anti-mouse antibody fragment (Fab-ZAP; Advanced Targeting Systems). The ability of these saporin associated APCDD1 effectors to be internalized and kill cells was then assessed by measuring cell viability following incubation of cells for 72 hours using art-recognized techniques (FIGS. 15A-C).

More particularly, human 293T cells (293) or 293T cells stably overexpressing human APCDD1 (293-A1) were plated in wells of a 96-well plate. APCDD1 effectors in the form of murine anti-APCDD1 antibodies, as described above, were purified and concentrated from supernatants, serially diluted and then added to the cell-plated wells. Concurrently, Fab-ZAP was added to the wells at a uniform concentration of 10 nM and the mixture was allowed to incubate at 37°C/5%CO_2 for 72 hours before viable cell numbers were quantified using CellTiter-Glo® (Promega Inc.) per the manufacturer's protocol. Negative controls in the graphs include parental 293 cells that express APCDD1

at significantly lower levels (FIG. 15A) and use of non-specific isotype control antibodies (i.e. IgG2a or MOPC in FIGS. 15B and 15C), which were not able to specifically mediate cell killing.

As evidenced by cell death, several representative internalizing APCDD1 antibodies were shown to mediate saporin toxin internalization and cell mortality, including antibodies from clones SC7.13, SC7.48 and SC7.58 (FIG. 15A), SC7.13.2 and SC7.161 (FIG. 15B), and SC.13.2, SC7.49 and SC7.142.1 (FIG. 15C). In FIG. 15A the data is presented as normalized percentage of live cells, while in FIGS. 15B and 15C the data is shown using non-normalized relative luminescence units. In any event, the curves of all three graphs illustrate the ability of the disclosed effectors to effectively mediate $APCDD1^{+}$ (i.e., 293-A1) cell killing in a dose dependent manner.

Example 18

APCDD1 Effectors Can Mediate Anti-Cancer Agent Induced Cytotoxicity in a Breast Tumor Cells

To corroborate the results of the previous Example and determine whether APCDD1effectors can mediate toxin internalization and cell killing of primary human tumor cells, mouse lineage-depleted NTX BR22 cells (i.e. human breast tumor cells propagated as low-passage xenografts in immunocompromised mice) were plated and subsequently exposed to anti-APCDD1 antibodies and Fab-ZAP.

Specifically, NTX BR22 tumors were dissociated into a single cell suspension and plated on BD Primaria™ plates (BD Biosciences) in growth factor supplemented serum free media as is known in the art. After 5 days of culture at 37°C/5%CO_2/5%O_2, cells were contacted with a control (IgG2a; black bars) or a murine APCDD1 effector at two different concentrations (SC7.13.2 at 0.2 nM or 1.0 nM; white bars), and Fab-ZAP (at 40nM) as described in Example 17. Effector-mediated saporin cytotoxicity was then assessed by quantifying the remaining number of cells using CellTiter Glo 12-14 days later. As seen in FIG. 16 exposure to the SC7.13.2 antibody resulted reduced cell numbers, whereas the IgG2a isotype control antibody did not impact the number of live cells after treatment. Not only does this data demonstrate that exemplary antibodies described herein are specific to APCDD1, are able to bind APCDD1 antigen on the cell

surface and facilitate the delivery of a cytotoxic payload resulting in cell death, but the above data also demonstrated that an anti-APCDD1 antibody can mediate killing of a NTX breast tumor cells.

Example 19

Humanized APCDD1 Effectors Can Mediate Anti-Cancer Agent Induced Cytotoxicity in APCDD1$^+$ Cells

To confirm the results seen in the previous Examples and demonstrate that humanized APCDD1 effectors can mediate the delivery of an anti-cancer agent to live cells *in vitro* cell killing assays were performed. In this Example the toxin saporin was bound to biotinylated humanized or murine anti-APCDD1 antibodies via streptavidin (SA). The ability of these saporin anti-APCDD1 antibody complexes to be internalized and kill cells in an APCDD1-dependent fashion was then assessed by measuring cell viability following incubation of cells for 72 to provide the data shown in FIGS. 17A and 17B.

As per Example 17 human 293T cells (293) or 293T cells stably overexpressing human APCDD1 (293-A1) were plated in wells of a 96-well plate. APCDD1 effectors in the form of murine and humanized anti-APCDD1 antibodies, as described above, were purified and concentrated from supernatants and biotinylated as per the manufacturer's instructions. An aliquot of each biotinylated antibody was mixed 1:1 with SA-ZAP (Advanced Targeting Systems), vortexed for 5 seconds and then incubated at room temperature for 30 minutes. The antibody-SA saporin preparations were then serially diluted and added to the 293T cells that were previously plated in 96-wells. The cell/antibody-saporin mixture was then incubated at 37°C/5%CO_2 for 72 hours and viable cell numbers were then quantified using CellTiter-Glo per the manufacturer's protocol. Negative controls in the graphs comprise the parental 293 cells that express APCDD1 at significantly lower levels. Results are presented as a normalized percentage of live cells remaining upon termination of the assay.

As evidenced by the resulting curves, the humanized and parental murine effectors were substantially equivalent in their ability to mediate cytotoxic agent induced cell death. More specifically, both FIG. 17A and FIG. 17B show that hSC7.13 and hSC7.48 provide for the internalization of saporin and resulting cell death of APCDD1$^+$ cells in a dose

dependent manner that is substantially similar to that demonstrated by the murine parental antibodies SC7.13 and SC7.48. Moreover, in each case neither the parental antibody nor the humanized variant was able to induce mortality in the APCDD1^{-} cells (i.e., the 293 cells). Such results indicate that the effectors of the instant invention may be humanized or otherwise derivatized using art recognized techniques without losing biological activity or therapeutic efficacy. Moreover, Examples 16-19 graphically illustrate the potential of the effectors of the instant invention to be used as antibody-drug conjugates that allow for the highly accurate targeting of cytotoxic payloads to tumor initiating cells.

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments that have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the invention.

CLAIMS

1. An isolated APCDD1 effector that reduces the tumorigenicity of neoplastic cells upon administration to a subject.
2. The isolated APCDD1 effector of claim 1, wherein the APCDD1 effector comprises an APCDD1 antagonist.
3. The isolated APCDD1 effector of claim 1, wherein the APCDD1 effector comprises an antibody or immunoreactive fragment thereof.
4. The isolated APCDD1 effector of claim 3 wherein the antibody or immunoreactive fragment thereof comprises a monoclonal antibody.
5. The isolated APCDD1 effector of claim 4 wherein the monoclonal antibody is selected from the group consisting of chimeric antibodies, humanized antibodies and human antibodies.
6. The isolated APCDD1 effector of claim 4 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NOS: 8, 12, 16, 100, 104, 108, 112, 116, 120, 124, 128, 132, and 136 and wherein said heavy chain variable region comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NOS: 6, 10, 14, 98, 102, 106, 110, 114, 118, 122, 126, 130 and 134.
7. A humanized antibody derived from a monoclonal antibody of claim 6.
8. The isolated APCDD1 effector of claim 4 wherein said monoclonal antibody comprises a light chain variable region having three complementarity determining regions and a heavy chain variable region having three complementarity determining regions wherein the heavy and light chain complementarity determining regions comprise complementarity determining regions set forth in FIG. 10B.
9. A nucleic acid molecule encoding an amino acid heavy chain variable region or an amino acid light chain variable region of claim 8.
10. A vector comprising the nucleic acid molecule of claim 9.
11. A host cell comprising the vector of claim 10.
12. The isolated APCDD1 effector of claim 1 comprising an amino acid sequence as set forth in SEQ ID NO: 2 or a fragment thereof.

13. The isolated APCDD1 effector of claim 12 wherein the APCDD1 effector further comprises at least a portion of an immunoglobulin constant region.
14. The isolated APCDD1 effector of claim 1 wherein the effector reduces the frequency of tumor initiating cells upon administration to a subject.
15. The isolated APCDD1 effector of claim 14 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells.
16. The isolated APCDD1 effector of claim 14 wherein the reduction in frequency is determined using immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.
17. The isolated APCDD1 effector of claim 14 wherein the reduction in frequency is determined using *in vitro* or *in vivo* limiting dilution analysis.
18. The isolated APCDD1 effector of claim 17 wherein the reduction in frequency is determined using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice.
19. The isolated APCDD1 effector of claim 18 wherein the reduction of frequency determined using *in vivo* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.
20. The isolated APCDD1 effector of claim 17 wherein the reduction of frequency is determined using *in vitro* limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions.
21. The isolated APCDD1 effector of claim 20 wherein the reduction of frequency determined using *in vitro* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.
22. The isolated APCDD1 effector of claim 1 wherein said neoplastic cells comprise tumor perpetuating cells.
23. A method of treating an APCDD1 associated disorder comprising administering a therapeutically effective amount of an APCDD1 effector to a subject in need thereof.
24. The method of claim 23 wherein said APCDD1 effector comprises an APCDD1 antagonist.
25. The method of claim 23 wherein said APCDD1 effector comprises an antibody or immunoreactive fragment thereof.
26. The method of claim 25 wherein the antibody or immunoreactive fragment thereof

comprises a monoclonal antibody.

27. The method of claim 24 wherein the monoclonal antibody is selected from the group consisting of chimeric antibodies, humanized antibodies and human antibodies.

28. The method of claim 24 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NOS: 8, 12, 16, 100, 104, 108, 112, 116, 120, 124, 128, 132, and 136 and wherein said heavy chain variable region comprises an amino acid sequence having at least 80% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NOS: 6, 10, 14, 98, 102, 106, 110, 114, 118, 122, 126, 130 and 134.

29. The method of claim 28 wherein the monoclonal antibody comprises a humanized antibody derived from a monoclonal antibody of claim 28.

30. The method of claim 26 wherein said monoclonal antibody comprises a light chain variable region having three complementarity determining regions and a heavy chain variable region having three complementarity determining regions wherein the heavy and light chain complementarity determining regions comprise complementarity determining regions set forth in FIG. 10B.

31. The method of claim 23 wherein said APCDD1 disorder comprises a neoplastic disorder.

32. The method of claim 31 wherein neoplastic disorder comprises adrenal cancer, bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer or breast cancer.

33. The method of claim 31 wherein said neoplastic disorder comprises a solid tumor.

34. The method of claim 33 wherein tissue from said solid tumor exhibits APCDD1 levels that are elevated when compared with normal adjacent tissue.

35. The method of claim 33 wherein tissue from said solid tumor exhibits APCDD1 levels that are depressed when compared with normal adjacent tissue.

36. The method of claim 33 wherein tissue from said solid tumor comprises at least one mutation selected from the group consisting of KRAS mutations, APC mutations, CTNNB1 mutations and combinations thereof.

37. The method of claim 32 wherein said solid tumor comprises a colorectal tumor.

38. The method of claim 21 wherein said APCDD1 associated disorder comprises a cell expressing APCDD1 on its surface and the APCDD1 effector is internalized by the cell upon association with the APCDD1.

39. The method of claim 34 wherein said APCDD1 effector is conjugated to a cytotoxic agent.

40. The method of claim 23 wherein said APCDD1 effector is conjugated to a cytotoxic agent.

41. The method of claim 31 further comprising the step of administering an anti-cancer agent.

42. The method of claim 23 wherein said APCDD1 effector comprises an amino acid sequence as set forth in SEQ ID NO: 2 or a fragment thereof.

43. The method of claim 42 wherein said APCDD1 effector further comprises at least a portion of an Fc region.

44. The method of claim 23 further comprising the step of administering a second APCDD1 effector wherein said second APCDD1 effector is distinct from said APCDD1 effector.

45. The method of claim 23 wherein the APCDD1 effector reduces the frequency of tumor initiating cells upon administration to a subject.

46. The method of claim 45 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells.

47. The method of claim 45 wherein the reduction in frequency is determined using immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.

48. The method of claim 45 wherein the reduction in frequency is determined using *in vitro* or *in vivo* limiting dilution analysis.

49. The method of claim 48 wherein the reduction in frequency is determined using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice.

50. The method of claim 49 wherein the reduction of frequency determined using *in vivo* limiting dilution analysis comprising quantification of tumor initiating cell frequency using Poisson distribution statistics.

51. The method of claim 48 wherein the reduction of frequency is determined using *in*

vitro limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions.

52. The method of claim 51 wherein the reduction of frequency determined using *in vitro* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.

53. A method of reducing the frequency of tumor initiating cells in a subject in need thereof comprising the step of administering an APCDD1 effector to said subject.

54. The method of claim 53 wherein the tumor initiating cells comprise tumor perpetuating cells.

55. The method of claim 54wherein said tumor perpetuating cells comprise $CD44^+$ or $CD133^+$ cells.

56. The method of claim 53 wherein said APCDD1 effector comprises an APCDD1 antagonist.

57. The method of claim 53 wherein said APCDD1 effector comprises an antibody.

58. The method of claim 57 wherein said antibody comprises a monoclonal antibody.

59. The method of claim 58 wherein said monoclonal antibody is an internalizing antibody.

60. The method of claim 53 wherein the subject is suffering from a neoplastic disorder selected from the group consisting of adrenal cancer, bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and breast cancer.

61. The method of claim 53 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells.

62. The method of claim 53 wherein the reduction in frequency is determined using immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.

63. The method of claim 53 wherein the reduction of tumor initiating cell frequency is as determined using *in vitro* or *in vivo* limiting dilution analysis.

64. The method of claim 63 wherein the reduction in frequency is determined using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice.

65. The method of claim 64wherein the reduction of frequency determined using *in vivo* limiting dilution analysis comprises quantification of tumor initiating cell

frequency using Poisson distribution statistics.

66. The method of claim 63 wherein the reduction of frequency is determined using *in vitro* limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions.

67. The method of claim 66 wherein the reduction of frequency determined using *in vitro* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.

68. The method of claim 53 wherein the frequency of tumor initiating cells is reduced by at least 10%.

69. A method of sensitizing a tumor in a subject to treatment with an anti-cancer agent comprising the step of administering an APCDD1 effector to said subject.

70. The method of claim 69wherein said APCDD1 effector is an antibody.

71. The method of claim 69 wherein said tumor is a solid tumor.

72. The method of claim 69 wherein said anti-cancer agent comprises a chemotherapeutic agent.

73. The method of claim 69 wherein said anti-cancer agent comprises a biological agent.

74. A method of diagnosing a hyperproliferative disorder in a subject in need thereof comprising the steps of:

    a. obtaining a tissue sample from said subject comprising tumorigenic cells;
    b. contacting the tissue sample with at least one APCDD1 effector; and
    c. detecting or quantifying an amelioration of the tumorigenic cells.

75. The method of claim 74 wherein said tumorigenic cells comprise tumor initiating cells.

76. The method of claim 75 wherein the amelioration of the tumorigenic cells comprises reducing the frequency of the tumor initiating cells.

77. The method of claim 74 wherein the APCDD1 effector comprises a monoclonal antibody.

78. The method of claim 77 wherein the antibody is operably associated with a reporter.

79. An article of manufacture useful for diagnosing or treating APCDD1 associated disorders comprising a receptacle comprising an APCDD1 effector and instructional materials for using said APCDD1 effector to treat or diagnose the APCDD1 associated disorder through a reduction in frequency of tumor initiating

cells as determined using *in vitro* or *in vivo* limiting dilution analysis .

80. The article of manufacture of claim 79 wherein said APCDD1 effector is a monoclonal antibody.

81. The article of manufacture of claim 79 wherein the receptacle comprises a readable plate.

82. A method of treating a subject suffering from neoplastic disorder comprising a solid tumor exhibiting a KRAS mutation, an APC mutation or a CTNNB1 mutation wherein said method comprises the step of administering a therapeutically effective amount of at least one APCDD1 effector.

83. The method of claim 82 wherein said APCDD1 effector comprises an APCDD1 antagonist.

84. The method of claim 82 wherein said APCDD1 effector comprises an antibody.

85. The method of claim 84 wherein said antibody comprises a monoclonal antibody.

86. The method of claim 82 wherein the neoplastic disorder is selected from the group consisting of adrenal cancer, bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and breast cancer.

87. A method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one internalizing APCDD1 effector.

88. The method of claim 87 wherein said APCDD1 effector comprises an APCDD1 antagonist.

89. The method of claim 87 wherein said APCDD1 effector comprises an antibody.

90. The method of claim 89 wherein said antibody comprises a monoclonal antibody.

91. The method of claim 87 wherein the neoplastic disorder is selected from the group consisting of adrenal cancer, bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and breast cancer.

92. A method of identifying, isolating, sectioning or enriching a population of tumor initiating cells comprising the step of contacting said tumor initiating cells with an APCDD1 effector.

93. The method of claim 92 wherein said APCDD1 effector comprises an antibody.

94. The method of claim 93 wherein said antibody comprises a monoclonal antibody.

95. An APCDD1 effector comprising a humanized antibody wherein said antibody

comprises a heavy chain variable region substantially similar to the amino acid sequence as set forth in SEQ ID NO: 90 and a light chain variable region substantially similar to the amino acid sequence set forth in SEQ ID NO: 92.

96. An APCDD1 effector comprising a humanized antibody wherein said antibody comprises a heavy chain variable region substantially similar to the amino acid sequence as set forth in SEQ ID NO: 94 and a light chain variable region substantially similar to the amino acid sequence set forth in SEQ ID NO: 96.

97. A composition comprising hSC7.13 antibody and a pharmaceutically acceptable carrier.

98. A composition comprising hSC7.48 antibody and a pharmaceutically acceptable carrier.

99. A method inhibiting or preventing metastasis in a subject in need thereof comprising the step of administering a pharmaceutically effective amount of an APCDD1 effector.

100. The method of claim 99 wherein the subject undergoes a debulking procedure before or after the administration of the APCDD1 effector.

101. A method of performing maintenance therapy on a subject in need thereof comprising the step of administering a pharmaceutically effective amount of an APCDD1 effector.

>gi|189409110|ref|NM_153000.4| Homo sapiens adenomatosis polyposis coli down-regulated 1 (APCDD1), mRNA

(SEQ ID NO: 1)

GAAATATGAAGAGACGCTGCAGCTGCGGTGGCGGTGGCGGCCACTGCAGCTCAGAGCGGCGCACGCGGCG
GCCGGGGCGGGACGCGGGGCCGGGCGCGGAGAAGTCGGGGCGGGCGGCAGAGAGGCCGGGACGCGGACCG
GGCCGGGGCGCCCACAGCCGCCCGACGGCGCCCAGAGAGCGCGCGCCCCGCAGCCCCGCGCCTAGCCCGC
CGGGCATGGGGCGCGCGGCAGCCGCCTGAAGCCCCGGCCTGGCCCGGCCGCACCCGGCCGGAGGCGGAGG
GCAGAGCGCGCGCCCAGTTGCCCGGGCACCAAATCGGAGCGCGGCGTGCGGGAGGCCCCAGAGCAGGACT
GGAAATGTCCTGGCCGCGCCGCCTCCTGCTCAGATACCTGTTCCCGGCCCTCCTGCTTCACGGGCTGGGA
GAGGGTTCTGCCCTCCTTCATCCAGACAGCAGGTCTCATCCTAGGTCCTTAGAGAAAAGTGCCTGGAGGG
CTTTTAAGGAGTCACAGTGCCATCACATGCTCAAACATCTCCACAATGGTGCAAGGATCACAGTGCAGAT
GCCACCTACAATCGAGGGCCACTGGGTCTCCACAGGCTGTGAAGTAAGGTCAGGCCCAGAGTTCATCACA
AGGTCCTACAGATTCTACCACAATAACACCTTCAAGGCCTACCAATTTTATTATGGCAGCAACCGGTGCA
CAAATCCCACTTATACTCTCATCATCCGGGGCAAGATCCGCCTCCGCCAGGCCTCCTGGATCATCCGAGG
GGGCACGGAAGCCGACTACCAGCTGCACAACGTCCAGGTGATCTGCCACACAGAGGCGGTGGCCGAGAAG
CTCGGCCAGCAGGTGAACCGCACATGCCCGGGCTTCCTCGCAGACGGGGGTCCCTGGGTGCAGGACGTGG
CCTATGACCTCTGGCGAGAGGAGAACGGCTGTGAGTGCACCAAGGCCGTGAACTTTGCCATGCATGAACT
TCAGCTCATCCGGGTGGAGAAGCAGTACCTTCACCACAACCTCGACCACCTGGTCGAGGAGCTCTTCCTT
GGTGACATTCACACTGATGCCACCCAGAGGATGTTCTACCGGCCCTCCAGTTACCAGCCCCCTCTGCAGA
ATGCCAAGAACCACGACCATGCCTGCATCGCCTGTCGGATCATCTATCGGTCAGACGAGCACCACCCTCC
CATCCTGCCCCCAAAGGCAGACCTGACCATCGGCCTGCACGGGGAGTGGGTGAGCCAGCGCTGTGAGGTG
CGCCCCGAAGTCCTCTTCCTCACCCGCCACTTCATCTTCCATGACAACAACAACACCTGGGAGGGCCACT
ACTACCACTACTCAGACCCGGTGTGCAAGCACCCCACCTTCTCCATCTACGCCCGGGGCCGCTACAGCCG
CGGCGTCCTCTCGTCCAGGGTCATGGGAGGCACCGAGTTCGTGTTCAAAGTGAATCACATGAAGGTCACC
CCCATGGATGCGGCCACAGCCTCACTGCTCAACGTCTTCAACGGGAATGAGTGCGGGGCCGAGGGCTCCT
GGCAGGTGGGCATCCAGCAGGATGTGACCCACACCAATGGCTGCGTGGCCCTGGGCATCAAACTACCTCA
CACGGAGTACGAGATCTTCAAAATGGAACAGGATGCCCGGGGGCGCTATCTGCTGTTCAACGGTCAGAGG
CCCAGCGACGGGTCCAGCCCAGACAGGCCAGAGAAGAGAGCCACGTCCTACCAGATGCCCTTGGTCCAGT
GTGCCTCCTCTTCGCCGAGGGCAGAGGACCTCGCAGAAGACAGTGGAAGCAGCCTGTATGGCCGGGCCCC
TGGGAGGCACACCTGGTCCCTGCTGCTGGCTGCACTTGCCTGCCTTGTCCCTCTGCTGCATTGGAACATC
CGCAGATAGAAGTTTTAGAAAGTTCTATTTTTCCAAACCAGGATTCCTTACTATTGACAGATTTGCTTTA
CCAAAAGAAAAGACATTTATTCTTTTGATGCACTTGAATGCCAGAGAACTGTCCTTCTTTTTCTCCTCTC
CCTCCCTCCCAGCCCCTGAGTCATGAACAGCAAGGAGTGTTTGAAGTTTCTGCTTTGAACTCCGTCCAGC
CTGATCCCTGGCCTGAGCAACTTCACAACAGTAATTGCACTTTAAGACAGCCTAGAGTTCTGGACGAGCG
TGTTTGGTAGCAGGGATGAAAGCTAGGGCCTCTTATTTTTTTCTCTTAATTATTATTATATTTCTGAGTT
AAACTTAGAAGAAACAACTATCAAGCTACAACTTTTCCTGCCATTTTCCTGTGGTTGCAGCCTGTCTTCC
TTTGAAATTGTTTTACTCTCTGAGTTTTATATGCTGGAATCCAATGCAGAGTTGGTTTGGGACTGTGATC
AAGACACCTTTTATTAATAAAGAAGAGACACAGGTGTAGATATGTATATACAAAAAGATGTACGGTCTGG
CCAAACCACCTTCCCAGCCTTTATGCAAAAAAAGGGGAGAATCAAAGCTTTCATTTCAGAAATGTTGCGT
GGAAAAGTATCTGTAATTAAAGTTTCGAAGTAATTTAACCTAAAAAAAAAAAAAAAAA

FIG. 1A

[NP_694545.1] [GI:23308597] - protein APCDD1 precursor [Homo sapiens].

(SEQ ID NO: 2)

MSWPRRLLLRYLFPALLLHGLGEGSALLHPDSRSHPRSLEKSAWRAFKESQCHHMLKHLHNGAR
ITVQMPPTIEGHWVSTGCEVRSGPEFITRSYRFYHNNTFKAYQFYYGSNRCTNPTYTLIIRGKI
RLRQASWIIRGGTEADYQLHNVQVICHTEAVAEKLGQQVNRTCPGFLADGGPWVQDVAYDLWRE
ENGCECTKAVNFAMHELQLIRVEKQYLHHNLDHLVEELFLGDIHTDATQRMFYRPSSYQPPLQN
AKNHDHACIACRIIYRSDEHHPPILPPKADLTIGLHGEWVSQRCEVRPEVLFLTRHFIFHDNNN
TWEGHYYHYSDPVCKHPTFSIYARGRYSRGVLSSRVMGGTEFVFKVNHMKVTPMDAATASLLNV
FNGNECGAEGSWQVGIQQDVTHTNGCVALGIKLPHTEYEIFKMEQDARGRYLLFNGQRPSDGSS
PDRPEKRATSYQMPLVQCASSSPRAEDLAEDSGSSLYGRAPGRHTWSLLLAALACLVPLLHWNI
RR

FIG. 1B

>APCDD1-ECD ORF (SEQ ID NO: 3)

atggagacagacacactcctgctatgggtactgctgctctgggttccaggttccactggt
M E T D T L L L W V L L L W V P G S T G
gacgcggcccagccggccaggcgcgccctccttcatccagacagcaggtctcatcctagg
D A A Q P A R R A L L H P D S R S H P R
tccttagagaaaagtgcctggagggcttttaaggagtcacagtgccatcacatgctcaaa
S L E K S A W R A F K E S Q C H H M L K
catctccacaatggtgcaaggatcacagtgcagatgccacctacaatcgagggccactgg
H L H N G A R I T V Q M P P T I E G H W
gtctccacaggctgtgaagtaaggtcaggcccagagttcatcacaaggtcctacagattc
V S T G C E V R S G P E F I T R S Y R F
taccacaataacaccttcaaggcctaccaatttattatggcagcaaccggtgcacaaat
Y H N N T F K A Y Q F Y Y G S N R C T N
cccacttatactctcatcatccggggcaagatccgcctccgccaggcctcctggatcatc
P T Y T L I I R G K I R L R Q A S W I I
cgagggggcacggaagccgactaccagctgcacaacgtccaggtgatctgccacacagag
R G G T E A D Y Q L H N V Q V I C H T E
gcggtggccgagaagctcggccagcaggtgaaccgcacatgcccgggcttcctcgcagac
A V A E K L G Q Q V N R T C P G F L A D
ggggggtccctgggtgcaggacgtggcctatgacctctggcgagaggagaacggctgtgag
G G P W V Q D V A Y D L W R E E N G C E
tgcaccaaggccgtgaactttgccatgcatgaacttcagctcatccgggtggagaagcag
C T K A V N F A M H E L Q L I R V E K Q
taccttcaccacaacctcgaccacctggtcgaggagctcttccttggtgacattcacact
Y L H H N L D H L V E E L F L G D I H T
gatgccacccagaggatgttctaccggccctccagttaccagccccctctgcagaatgcc
D A T Q R M F Y R P S S Y Q P P L Q N A
aagaaccacgaccatgcctgcatcgcctgtcggatcatctatcggtcagacgagcaccac
K N H D H A C I A C R I I Y R S D E H H
cctcccatcctgcccccaaaggcagacctgaccatcggcctgcacggggagtgggtgagc
P P I L P P K A D L T I G L H G E W V S
cagcgctgtgaggtgcgccccgaagtcctcttcctcacccgccacttcatcttccatgac
Q R C E V R P E V L F L T R H F I F H D
aacaacaacacctgggagggccactactaccactactcagacccggtgtgcaagcacccc
N N N T W E G H Y Y H Y S D P V C K H P
accttctccatctacgcccgggggccgctacagccgcggcgtcctctcgtccagggtcatg
T F S I Y A R G R Y S R G V L S S R V M
ggaggcaccgagttcgtgttcaaagtgaatcacatgaaggtcacccccatggatgcggcc
G G T E F V F K V N H M K V T P M D A A
acagcctcactgctcaacgtcttcaacgggaatgagtgcggggccgagggctcctggcag
T A S L L N V F N G N E C G A E G S W Q
gtgggcatccagcaggatgtgacccacaccaatggctgcgtggccctgggcatcaaacta
V G I Q Q D V T H T N G C V A L G I K L
cctcacacggagtacgagatcttcaaaatggaacaggatgcccggggcgctatctgctg
P H T E Y E I F K M E Q D A R G R Y L L
ttcaacggtcagaggcccagcgacgggtccagcccagacaggccagagaagagagccacg
F N G Q R P S D G S S P D R P E K R A T
tcctaccagatgcccttggtccagtgtgcctcctcttcgccgagggcagaggacctcgca
S Y Q M P L V Q C A S S S P R A E D L A
gaagacagtggaagcagcctgtatggcacgcgtcatcatcaccaccatcattga
E D S G S S L Y G T R H H H H H H -

FIG. 1C

>APCDD1-hFc ORF with translation (SEQ ID NO: 4)

```
atgtacaggatgcaactcctgtcttgcattgcactaagtcttgcacttgtcacgaattcg
 M  Y  R  M  Q  L  L  S  C  I  A  L  S  L  A  L  V  T  N  S
ctccttcatccagacagcaggtctcatcctaggtccttagagaaaagtgcctggagggct
 L  L  H  P  D  S  R  S  H  P  R  S  L  E  K  S  A  W  R  A
tttaaggagtcacagtgccatcacatgctcaaacatctccacaatggtgcaaggatcaca
 F  K  E  S  Q  C  H  H  M  L  K  H  L  H  N  G  A  R  I  T
gtgcagatgccacctacaatcgagggccactgggtctccacaggctgtgaagtaaggtca
 V  Q  M  P  P  T  I  E  G  H  W  V  S  T  G  C  E  V  R  S
ggcccagagttcatcacaaggtcctacagattctaccacaataacaccttcaaggcctac
 G  P  E  F  I  T  R  S  Y  R  F  Y  H  N  N  T  F  K  A  Y
caattttattatggcagcaaccggtgcacaaatcccacttatactctcatcatccggggc
 Q  F  Y  Y  G  S  N  R  C  T  N  P  T  Y  T  L  I  I  R  G
aagatccgcctccgccaggcctcctggatcatccgagggggcacggaagccgactaccag
 K  I  R  L  R  Q  A  S  W  I  I  R  G  G  T  E  A  D  Y  Q
ctgcacaacgtccaggtgatctgccacacagaggcggtggccgagaagctcggccagcag
 L  H  N  V  Q  V  I  C  H  T  E  A  V  A  E  K  L  G  Q  Q
gtgaaccgcacatgcccgggcttcctcgcagacggggggtccctgggtgcaggacgtggcc
 V  N  R  T  C  P  G  F  L  A  D  G  G  P  W  V  Q  D  V  A
tatgacctctggcgagaggagaacggctgtgagtgcaccaaggccgtgaactttgccatg
 Y  D  L  W  R  E  E  N  G  C  E  C  T  K  A  V  N  F  A  M
catgaacttcagctcatccgggtggagaagcagtaccttcaccacaacctcgaccacctg
 H  E  L  Q  L  I  R  V  E  K  Q  Y  L  H  H  N  L  D  H  L
gtcgaggagctcttccttggtgacattcacactgatgccacccagaggatgttctaccgg
 V  E  E  L  F  L  G  D  I  H  T  D  A  T  Q  R  M  F  Y  R
ccctccagttaccagcccctctgcagaatgccaagaaccacgaccatgcctgcatcgcc
 P  S  S  Y  Q  P  P  L  Q  N  A  K  N  H  D  H  A  C  I  A
tgtcggatcatctatcggtcagacgagcaccaccctcccatcctgcccccaaaggcagac
 C  R  I  I  Y  R  S  D  E  H  H  P  P  I  L  P  P  K  A  D
ctgaccatcggcctgcacggggagtgggtgagccagcgctgtgaggtgcgccccgaagtc
 L  T  I  G  L  H  G  E  W  V  S  Q  R  C  E  V  R  P  E  V
ctcttcctcacccgccacttcatcttccatgacaacaacaacacctgggagggccactac
 L  F  L  T  R  H  F  I  F  H  D  N  N  N  T  W  E  G  H  Y
taccactactcagacccggtgtgcaagcaccccaccttctccatctacgcccggggccgc
 Y  H  Y  S  D  P  V  C  K  H  P  T  F  S  I  Y  A  R  G  R
tacagccgcggcgtcctctcgtccagggtcatgggaggcaccgagttcgtgttcaaagtg
 Y  S  R  G  V  L  S  S  R  V  M  G  G  T  E  F  V  F  K  V
aatcacatgaaggtcacccccatggatgcggccacagcctcactgctcaacgtcttcaac
 N  H  M  K  V  T  P  M  D  A  A  T  A  S  L  L  N  V  F  N
gggaatgagtgcggggccgagggctcctggcaggtgggcatccagcaggatgtgacccac
 G  N  E  C  G  A  E  G  S  W  Q  V  G  I  Q  Q  D  V  T  H
accaatggctgcgtggccctgggcatcaaactacctcacacggagtacgagatcttcaaa
 T  N  G  C  V  A  L  G  I  K  L  P  H  T  E  Y  E  I  F  K
atggaacaggatgcccggggggcgctatctgctgttcaacggtcagaggcccagcgacggg
 M  E  Q  D  A  R  G  R  Y  L  L  F  N  G  Q  R  P  S  D  G
tccagcccagacaggccagagaagagagccacgtcctaccagatgcccttggtccagtgt
 S  S  P  D  R  P  E  K  R  A  T  S  Y  Q  M  P  L  V  Q  C
gcctcctcttcgccgagggcagaggacctcgcagaagacagtggaagcagcctgtatggc
 A  S  S  S  P  R  A  E  D  L  A  E  D  S  G  S  S  L  Y  G
cggggatctgtcgagtgcccaccgtgcccagcaccacctgtggcaggaccgtcagtcttc
 R  G  S  V  E  C  P  P  C  P  A  P  P  V  A  G  P  S  V  F
ctcttccccccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacgtgc
 L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  T  C
gtggtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgtggacggc
 V  V  V  D  V  S  H  E  D  P  E  V  Q  F  N  W  Y  V  D  G
gtggaggtgcataatgccaagacaaagccacgggaggagcagttcaacagcacgttccgt
 V  E  V  H  N  A  K  T  K  P  R  E  E  Q  F  N  S  T  F  R
gtggtcagcgtcctcaccgttgtgcaccaggactggctgaacggcaaggagtacaagtgc
 V  V  S  V  L  T  V  V  H  Q  D  W  L  N  G  K  E  Y  K  C
aaggtctccaacaaaggcctcccagcccccatcgagaaaaccatctccaaaaccaaaggg
 K  V  S  N  K  G  L  P  A  P  I  E  K  T  I  S  K  T  K  G
cagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaac
 Q  P  R  E  P  Q  V  Y  T  L  P  P  S  R  E  E  M  T  K  N
caggtcagcctgacctgcctggtcaaaggcttctaccccagcgacatcgccgtggagtgg
 Q  V  S  L  T  C  L  V  K  G  F  Y  P  S  D  I  A  V  E  W
gagagcaatgggcagccggagaacaactacaagaccacgcctcccatgctggactccgac
 E  S  N  G  Q  P  E  N  N  Y  K  T  T  P  P  M  L  D  S  D
ggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaac
 G  S  F  F  L  Y  S  K  L  T  V  D  K  S  R  W  Q  Q  G  N
gtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctc
 V  F  S  C  S  V  M  H  E  A  L  H  N  H  Y  T  Q  K  S  L
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 S  L  S  P  G  K  -
```

FIG. 1D

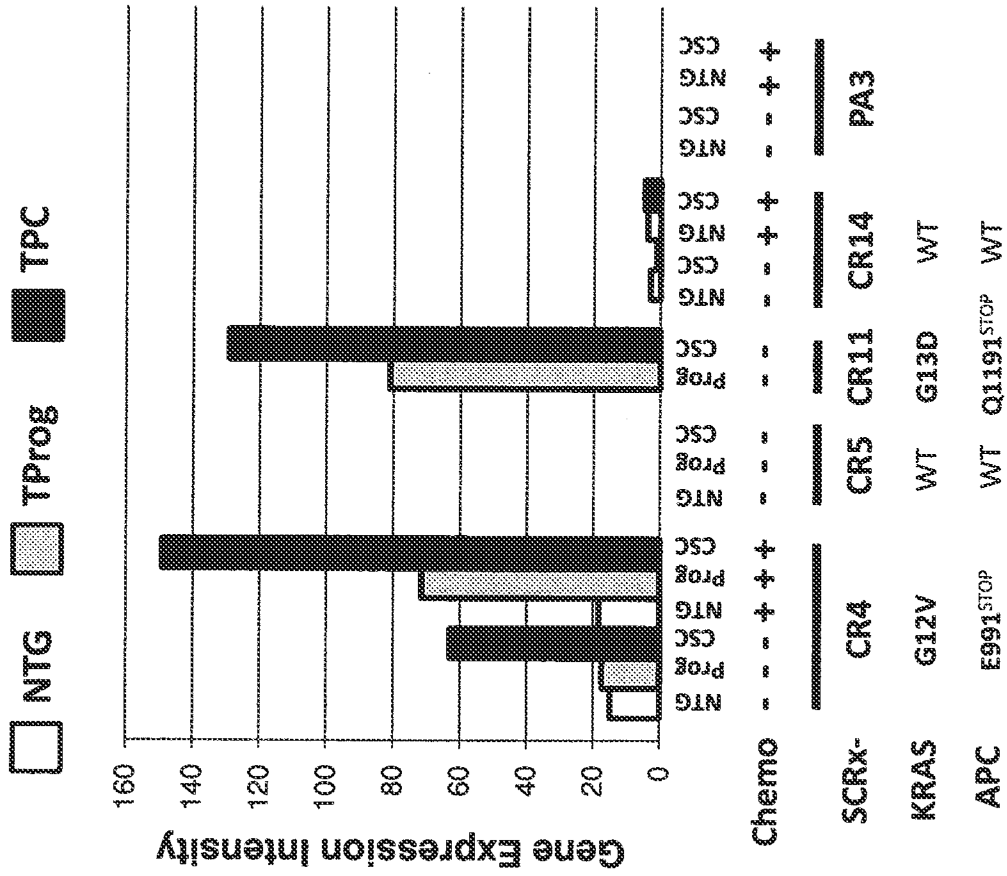


FIG. 2

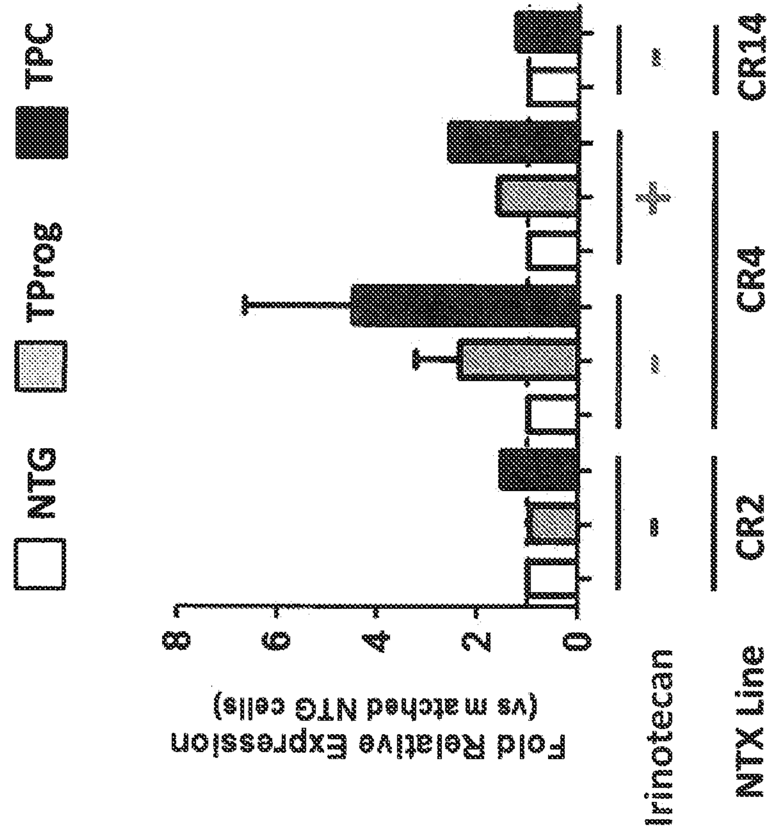


FIG. 3

Taqman PCR Confirms APCDD1
Overexpression in Whole Colorectal Tumor Specimens

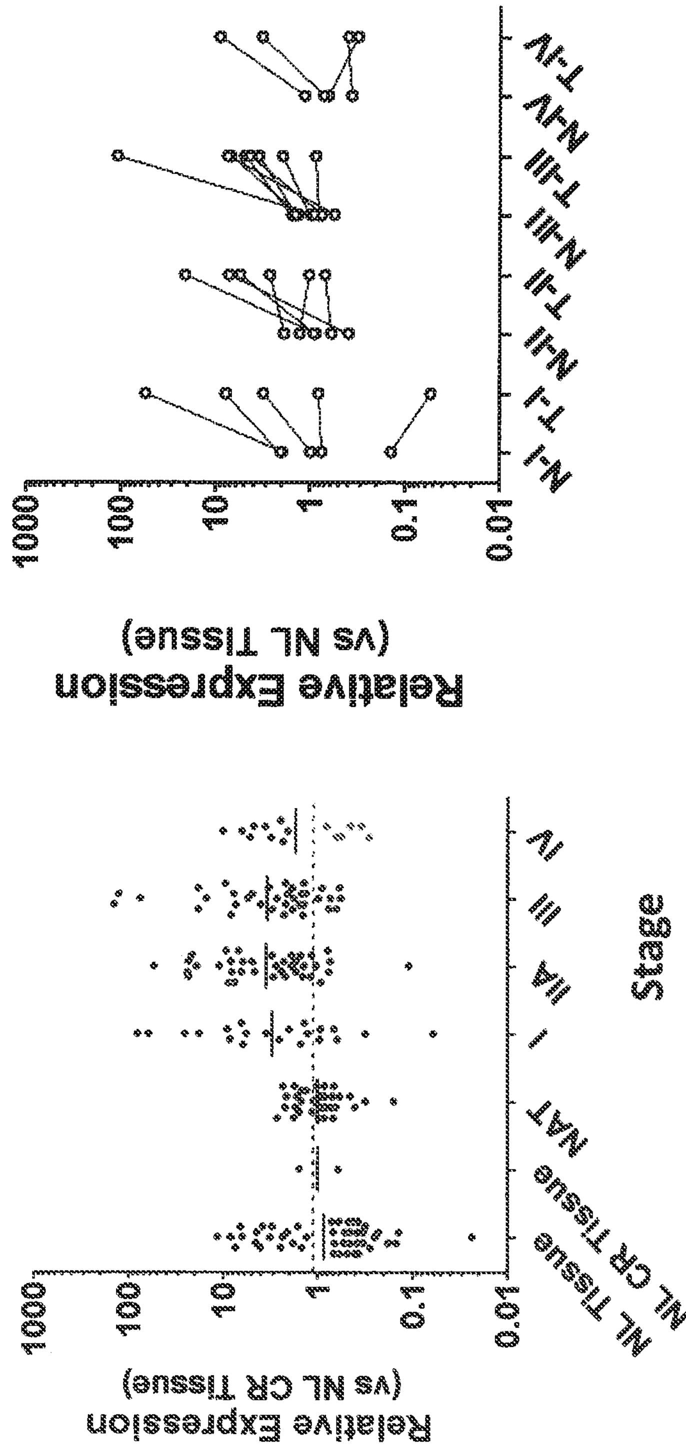


FIG. 4A

FIG. 4B

Taqman PCR Reveals Different APCDD1 Levels in Various Tumor Samples

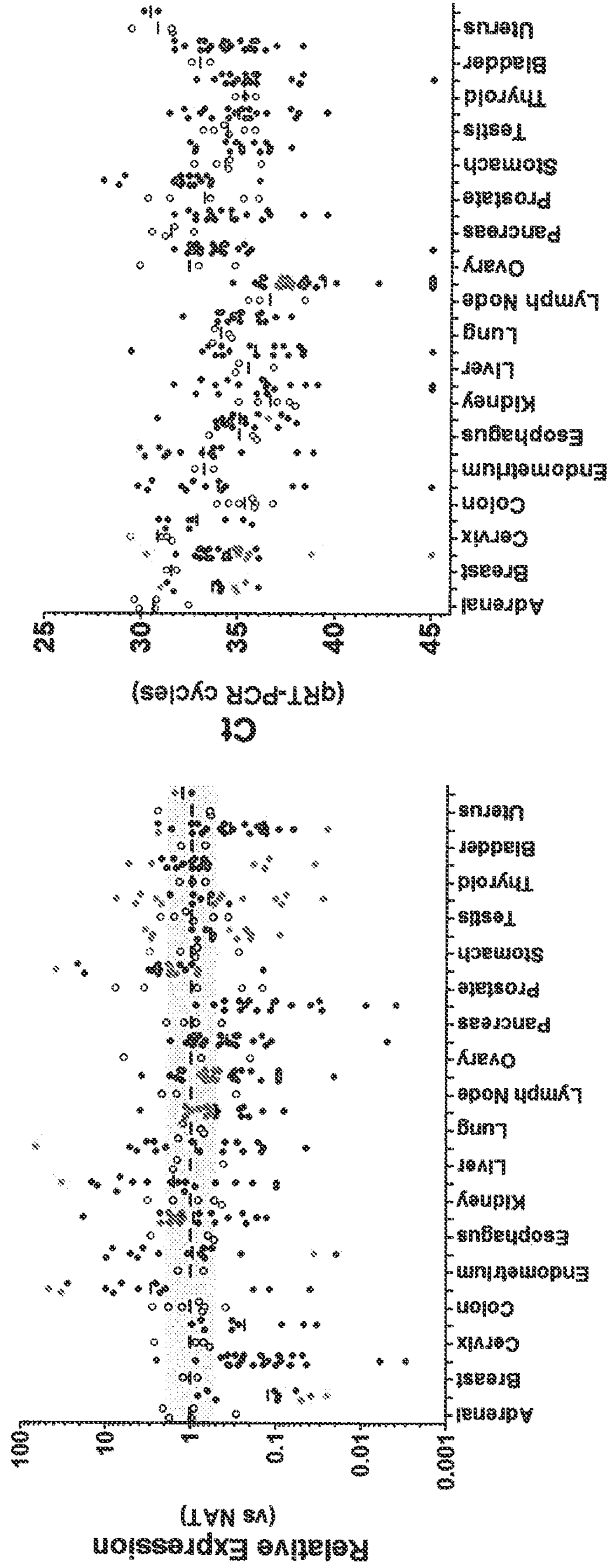


FIG. 5A

FIG. 5B

Examples from Flow Cytometry Screen for Human APCDD1 Cross-Reactive Antibodies on SW480 Cells

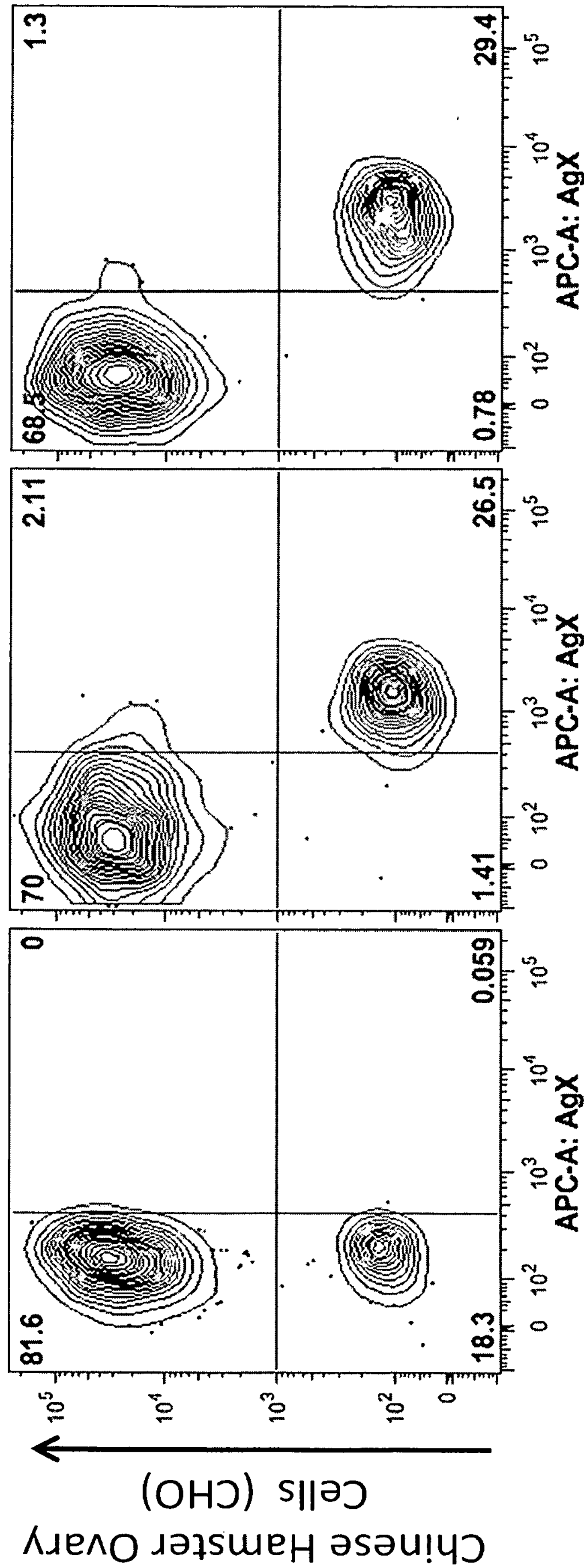


FIG. 6

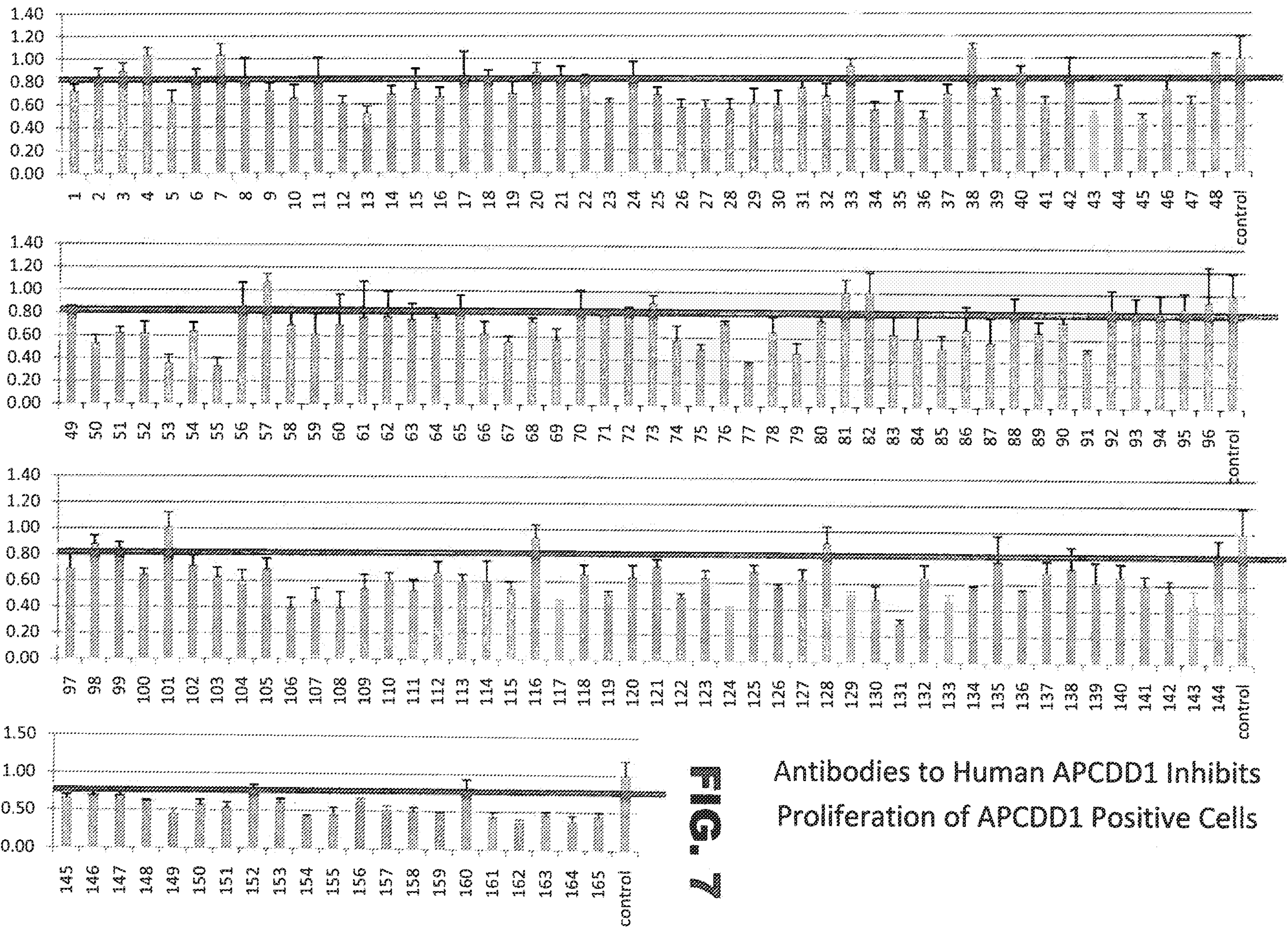


FIG. 7

Characterization of Internalizing Antibodies to APCDD1

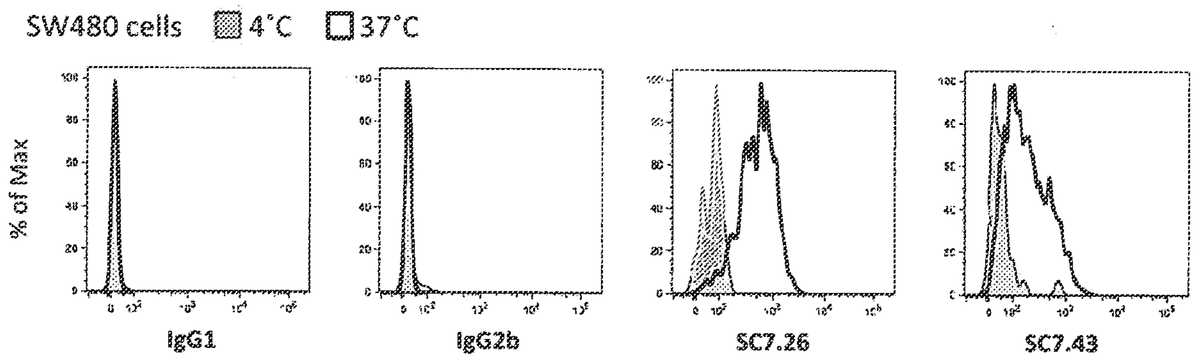


FIG. 8A

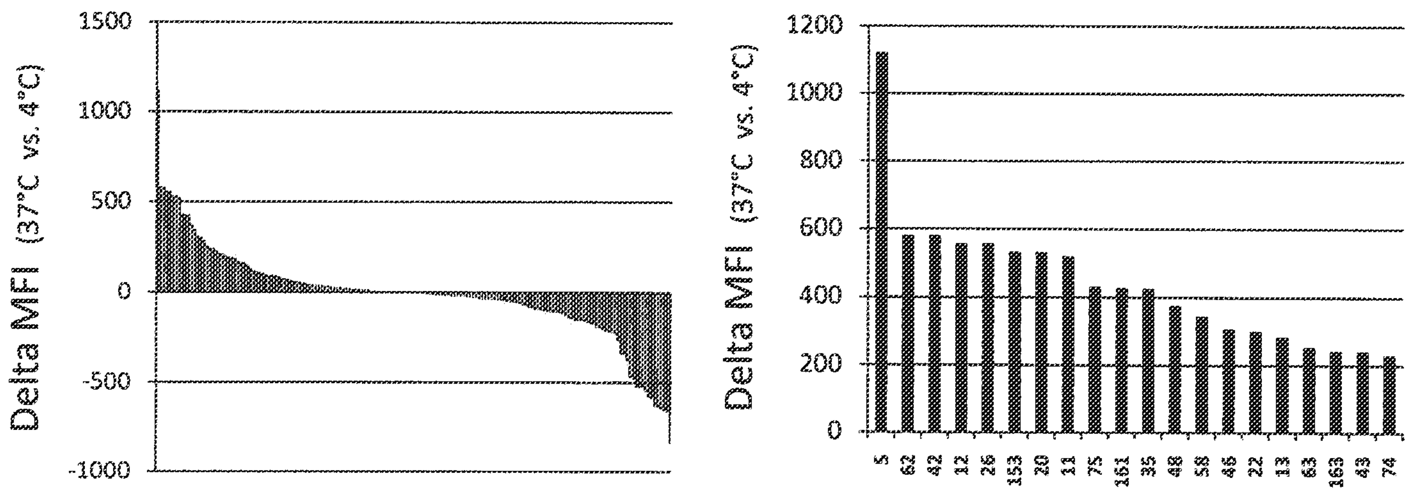


FIG. 8B

FIG. 8C

SC7.18 Heavy chain - nucleotide sequence (SEQ ID NO:5)

1 GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTC
61 TCCTGTGCAGCCTCTGGATTCACTTTCAGTGACTATTACATGTATTGGGTTCGCCAGACT
121 CCGGAAAAGAGGCTGGAGTGGGTCGCAACCATTAGTGATGGTGGTAGTTACACCTACTAT
181 CCAGACAGTGTGAAGGGGCGATTCACCATCTCCAGAGACAATGCCAAGAACAACCTGTAC
241 CTGCAAATGAGCAGTCTGAAGTCTGAGGACGCAGCCATGTATTACTGTGCAAGAGGGTAT
301 GGTTACTACTGGTACTTCGATGTCTGGGGCGCAGGGATCACGGTCACCGTCTCCTCA

SC7.18 Heavy chain - protein sequence (SEQ ID NO:6)

1 EVQLVESGGGLVKPGGSLKLSCAASGFTFSDYYMYWVRQTPEKRLEWVAT
51 ISDGGSYTYYPDSVKGRFTISRDNAKNNLYLQMSSLKSEDAAMYYCARGY
101 GYYWYFDVWGAGITVTVSS

FIG. 9A

SC7.18 Light chain - nucleotide sequence (SEQ ID NO:7)

1 GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACC
61 ATCAGTTGCAGGGCAAGTCAGGACATTAGCAATTGTTTAAACTGGTATCAGCAGAAACCA
121 GATGGAACTGTTAAACTCCTGATCTACTACACATCAAGATTACACTCAGGAGTCCCATCA
181 AGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCAGCATTAGCAACCTGGAGCAA
241 GAAGATATTGCCACTTACTTTTGCCAACAGGGTAATACGCTACCTTGGACGTTCGGTGGA
301 GGCACCAAACTGGAAATCATACGG

SC7.18 Light chain - protein sequence (SEQ ID NO:8)

1 DIQMTQTTSSLSASLGDRVTISCRASQDISNCLNWYQQKPDGTVKLLIYY
51 TSRLHSGVPSRFSGSGSGTDYSLSISNLEQEDIATYFCQQGNTLPWTFGG
101 GTKLEIIR

SC7.107 Heavy chain - nucleotide sequence (SEQ ID NO:9)

1 CAGGTCCAGCTGCAGCAGTCTGCAGCTGAACTGGCAAGACCTGGGGCCTCAGTGAAGATG
61 TCCTGCAAGGCTTCTGGCTACACCTTTACTAGCTACACGATGCACTGGGTAAAACAGAGG
121 CCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTTACACTGGATATTCTGAATAC
181 AATCTGAGGTTCAAGGACAAGACCACATTGACTGCAGACAAATCCTCCAGCACAGCCTAC
241 ATGCAACTGCGCAGCCTGACATCTGAGGACTCTGCGGCCTATTACTGTGCAAGAGAAAAC
301 TACAAGGATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC7.107 Heavy chain - protein sequence (SEQ ID NO:10)

1 QVQLQQSAAELARPGASVKMSCKASGYTFTSYTMHWVKQRPGQGLEWIGY
51 INPYTGYSEYNLRFKDKTTLTADKSSSTAYMQLRSLTSEDSAAYYCAREN
101 YKDAMDYWGQGTSVTVSS

FIG. 9B

SC7.107 Light chain - nucleotide sequence (SEQ ID NO:11)

1 GAAAATGTGCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAGAAGGTCACC
61 ATGAGCTGCAGGGCCAGCTCAAGTGTAAATTACATGTACTGGTACCAGCAGAAGTCAGAT
121 GCCTCCCCCAAACTTTGGATTTATTACACATCCAACCTGGCTCCTGGAGTCCCAGCTCGC
181 TTCAGTGGCAGTGGGTCTGGGGACTCTTATTCTCTCACAATCAGCAGCATGGAGGGTGAG
241 GATGCTGCCACTTATTACTGCCAGCAGTTAACTAGTTCCCCGTACACGTTCGGAGGGGGG
301 ACCAAGCTGGAAATAAG

SC7.107 Light chain - protein sequence (SEQ ID NO:12)

1 ENVLTQSPAIMSASLGEKVTMSCRASSSVNYMYWYQQKSDASPKLWIYYT
51 SNLAPGVPARFSGSGSGDSYSLTISSMEGEDAATYYCQQLTSSPYTFGGG
101 TKLEIR

SC7.143 Heavy chain - nucleotide sequence (SEQ ID NO:13)
1 GATGTGCAGCTGGTGGAGTCTGGGGGAGGATTAGTGCAGCCTGGAGGGTCCCGGAAACTC
61 TCCTGTGCAGCCTCTGGATTCACTTTCAGTAGGTTTGGAATGCACTGGGTTCGTCAGGCT
121 CCAGAGAAGGGGCTGGAGTGGGTCGCATACATTACTAGTGGCAGTAGCACCATCTACTAT
181 GCAGACACAGTGAAGGGCCGATTCACCATCTCCAGAGACAATCCCAAGAACACCCTGTTC
241 CTGCAAATGACCAGTCTAAGGTCTGAGGACACGGCCATGTATTACTGTGCAAAGTATGGA
301 AACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC7.143 Heavy chain - protein sequence (SEQ ID NO:14)
1 DVQLVESGGGLVQPGGSRKLSCAASGFTFSRFGMHWVRQAPEKGLEWVAY
51 ITSGSSTIYYADTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCAKYG
101 NYAMDYWGQGTSVTVSS

FIG. 9C

SC7.143 Light chain - nucleotide sequence (SEQ ID NO:15)
1 GACATTGTGCTGACCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACC
61 ATATCCTGCAGAGCCAGTGAAAGTGTCGATAGAGATGGCAACAGTTTGATGAATTGGTAC
121 CAGCAGAAACCAGGACAGCCACCCAAACTCCTCATCTATCGTGCATTCAACCTAGAATCT
181 GGGATCCCTGCCAGGTTCAGTGGCAGTGGGTCTAGGACAGACTTCACCCTCACCATTACT
241 CCTGTGGAGGCTGATGATGTTGCAACCTATTACTGTCAGCAAAGTAATGAGGCTCCGTAC
301 ACGTTCGGAGGGGGGACCAAGCTACAAATAAAA

SC7.143 Light chain - protein sequence (SEQ ID NO:16)
1 DIVLTQSPASLAVSLGQRATISCRASESVDRDGNSLMNWYQQKPGQPPKL
51 LIYRAFNLESGIPARFSGSGSRTDFTLTITPVEADDVATYYCQQSNEAPY
101 TFGGGTKLQIK

SC7.12 Heavy chain - nucleotide sequence (SEQ ID NO:97)
1 GACGTGAAGGTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTC
61 TCCTGTGCAGCCTCTGGATTCACTTTCAGTAGCTATACCATGTCTTGGGTTCGCCAGACT
121 CCGGAGAAGAGGCTGGAGTGGGTCGCAACCATTAGTAGTGGTGGTGGTTACACCTACTAT
181 CCAGACAGTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTAC
241 CTGCAAATGAGCGGTCTGAAGTCTGAGGACACAGCCATGTATTACTGTACAAGAGATAGG
301 CATGATGGCCCAGGGACCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

SC7.12 Heavy chain - protein sequence (SEQ ID NO:98)
1 DVKVVESGGGLVKPGGSLKLSCAASGFTFSSYTMSWVRQTPEKRLEWVAT
51 ISSGGGYTYYPDSVKGRFTISRDNAKNTLYLQMSGLKSEDTAMYYCTRDR
101 HDGPGTWFAYWGQGTLVTVSA

FIG. 9D

SC7.12 Light chain - nucleotide sequence (SEQ ID NO:99)
1 GATATTGTGCTAACTCAGTCTCCAGCCACCCTGTCGGTGACTCCAGGAGATAGCGTCAGT
61 CTTTCCTGCAGGGCCAGCCAAAGTGTTAGCAACAACCTACACTGGTATCAACAAAAATCA
121 CATGCGTCTCCAAGGCTTCTCATCAAGTATGCTTCCCAGTCCATCTCTGGGATCCCCTCC
181 AGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACTCTCAGTATCAACAGTGTGGAGACT
241 GAAGATTTTGGAATGTATTTCTGTCAACAGAGTTACAGCTGGCCTCGGACGTTCGGTGGA
301 GGCACCAAGCTGGAAATCAAACGG

SC7.12 Light chain - protein sequence (SEQ ID NO:100)
1 DIVLTQSPATLSVTPGDSVSLSCRASQSVSNNLHWYQQKSHASPRLLIKY
51 ASQSISGIPSRFSGSGSGTDFTLSINSVETEDFGMYFCQQSYSWPRTFGG
101 GTKLEIKR

SC7.13 Heavy chain - nucleotide sequence (SEQ ID NO:101)

1 GATGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCGGAAACTC
61 TCCTGTGCAGCCTCTGGATTCACTTTCAGTAGGTTTGGAATGCACTGGGTTCGTCAGGCT
121 CCAGAGAAGGGGCTGGAGTGGGTCGCATACATTAGTAGTGGCAGTAGTACCATCTACTAT
181 GCAGACACAGTGAAGGGCCGATTCACCATCTCCAGAGACAATCCCAAGAACACCCTGTTC
241 CTGCAAATGACCAGTCTACGGTCTGAGGACACGGCCATGTATTACTGTGCAAGAAAGGAC
301 TCTTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC7.13 Heavy chain - protein sequence (SEQ ID NO:102)

1 DVQLVESGGGLVQPGGSRKLSCAASGFTFSRFGMHWVRQAPEKGLEWVAY
51 ISSGSSTIYYADTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCARKD
101 SYAMDYWGQGTSVTVSS

FIG. 9E

SC7.13 Light chain - nucleotide sequence (SEQ ID NO:103)

1 GACATTGTGCTGACCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACC
61 ATATCCTGCAGAGCCAGTGAAAGTGTTGATCGTTATGGCAATAGTTTTATGCACTGGTAC
121 CAGCAGAAACCAGGACAGCCACCCAAACTCCTCATCTATCGTGCATCCAACCTAGAATCT
181 GGGATCCCTGCCAGGTTCAGTGGCAGTGGGTCTAGGACAGACTTCACCCTCACCATTAAT
241 CCTGTGGAGGCTGATGATGTTGCAACCTATTACTGTCAGCAAAGTAATGAGGTTCCGGAC
301 ACGTTCGGAGGGGGGACCAAGCTGGAAATAAAACGG

SC7.13 Light chain - protein sequence (SEQ ID NO:104)

1 DIVLTQSPASLAVSLGQRATISCRASESVDRYGNSFMHWYQQKPGQPPKL
51 LIYRASNLESGIPARFSGSGSRTDFTLTINPVEADDVATYYCQQSNEVPD
101 TFGGGTKLEIKR

SC7.43 Heavy chain - nucleotide sequence (SEQ ID NO:105)

1 ATGGCAGTGGTTACAGGGGTCAATTCAGAGGTTCAACTGCAGCAGTCTGGGGCTGAGCTT
61 GTGAGGCCAGGGGCCTTAGTCAAGTTGTCCTGCAAAGCTTCTGGCTTCAACTTTAAAGAC
121 TACTTTATGCACTGGGTGAGGCAGAGGCCTGAACAGGGCCTGGAGTGGATTGGACGGATT
181 GATCCTGAGAATGGTAATACTCTATATGACCCGAAGTTCCAGGACAAGGCCAGTATAACA
241 GCAGACACATCCTCCAACACAGCCTACCTGCAGCTCAGCAGCCTGACATCTGCGGACACT
301 GCCGTCTATTACTGTGCTAGAATATATGGTGACTACGGGGCTATGGACTACTGGGGTCAA
361 GGAACCTCAGTCACCGTCTCCTCAG

SC7.43 Heavy chain - protein sequence (SEQ ID NO:106)

1 MAVVTGVNSEVQLQQSGAELVRPGALVKLSCKASGFNFKDYFMHWVRQRP
51 EQGLEWIGRIDPENGNTLYDPKFQDKASITADTSSNTAYLQLSSLTSADT
101 AVYYCARIYGDYGAMDYWGQGTSVTVSSAKTTPPSVYPR

FIG. 9F

SC7.43 Light chain - nucleotide sequence (SEQ ID NO:107)

1 GACATTGTGATGACCCAGTCTCAAAAATTCATGTCCACATCAGTAGGAGACAGGGTCAGC
61 ATCACCTGCAAGGCCAGTCAGAATGTTCGTACTGTTGTAGCCTGGTATCAACAGAAACCA
121 GGGCAGTCTCCTAAAGCACTGATTTACTTGGCATCCAACCGGCACACTGGAGTCCCTGAT
181 CGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATTAGCAATGTGCAATCT
241 GAAGACCTGGCAGATTATTTCTGTCTGCAACATTGGAATTATCCGTACACGTTCGGAGGG
301 GGGACCAAGCTGGAAATAAAACGG

SC7.43 Light chain - protein sequence (SEQ ID NO:108)

1 DIVMTQSQKFMSTSVGDRVSITCKASQNVRTVVAWYQQKPGQSPKALIYL
51 ASNRHTGVPDRFTGSGSGTDFTLTISNVQSEDLADYFCLQHWNYPYTFGG
101 GTKLEIKR

SC7.46 Heavy chain - nucleotide sequence (SEQ ID NO:109)
1 CAGGTCCAGCTTCAGCAGTCTGGGGCTGAACTGGCAAAACCTGGGGCCTCAGTGAAGATG
61 TCCTGCAAGGCTTCTGGCTACACCTTTACTAGCTACTGGATGCACTGGATAAAACAGAGG
121 CCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTACCACTGGTTTAACTGACTAC
181 AATCAGAAGTTCACGGACAAGGCCACATTGACTGCAGACAAATCCTCCAGTACAGCCTAC
241 ATGCAACTGACCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAGAGGGC
301 TATAAGGATGCTCTGGACTACTGGGGTCAAGGAAACTCAGTCACCGTCTCCTCA

SC7.46 Heavy chain - protein sequence (SEQ ID NO:110)
1 QVQLQQSGAELAKPGASVKMSCKASGYTFTSYWMHWIKQRPGQGLEWIGY
51 INPTTGLTDYNQKFTDKATLTADKSSSTAYMQLTSLTSEDSAVYYCAREG
101 YKDALDYWGQGNSVTVSS

FIG. 9G

SC7.46 Light chain - nucleotide sequence (SEQ ID NO:111)
1 AGTATTGTGATGACCCAGACTCCCAAATTCCTGCTTGTATCAGCAGGAGACAGGGTTACC
61 ATAACCTGCAAGGCCAGTCAGAGTGTGAGCAATGCTGTAGCTTGGTACCAACAGAAACCA
121 GGGCAGTCTCCTACAATGGTGATATATCATGCATCCAATCGCTACACTGGAGTCCCTGAT
181 CGCTTCACTGGCAGTGGATATGGGACGGATTTCACTTTCACCATCAGCAGTGTGCAGGCT
241 GAAGACCTGGCAGTTTATTTCTGTCAGCAGGATTATAGTTCTCCGCTCACGTTCGGTGCT
301 GGGACCAAGCTGGAGCTGAAACGG

SC7.46 Light chain - protein sequence (SEQ ID NO:112)
1 SIVMTQTPKFLLVSAGDRVTITCKASQSVSNAVAWYQQKPGQSPTMVIYH
51 ASNRYTGVPDRFTGSGYGTDFTFTISSVQAEDLAVYFCQQDYSSPLTFGA
101 GTKLELKR

SC7.48 Heavy chain - nucleotide sequence (SEQ ID NO:113)
1 GAGGTCCAGCTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATG
61 TCCTGCAAGGCTTCAGGATACACATTCATTAGCGATGTTTTGCACTGGGTGAAGCAGAAG
121 CCTGGGCAGGGCCTTGAGTGGATTGGATATATTAATCCTTACAATGATGCTACTGAGTAC
181 GATGAGAAGTTCAAGGGCAAGGCCACACTGACTTCAGACAGATCCTCCAGCACAGCCTAC
241 ATGGAGCTCAGCAGCCTGACCTCTGAGGACTCTGCGGTCTATTACTGTGCAAGAAGAGGT
301 CTAATTGGGGCAGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC7.48 Heavy chain - protein sequence (SEQ ID NO:114)
1 EVQLQQSGPELVKPGASVKMSCKASGYTFISDVLHWVKQKPGQGLEWIGY
51 INPYNDATEYDEKFKGKATLTSDRSSSTAYMELSSLTSEDSAVYYCARRG
101 LIGADYWGQGTTLTVSS

FIG. 9H

SC7.48 Light chain - nucleotide sequence (SEQ ID NO:115)
1 AGTATTGTGATGACCCAGACTCCCAACTTCCTGCTTCTAGCAACAGGAGACAGGGTTAAC
61 ATAACCTGCAAGGCCAGTCAGAGTGTGAGTAATGATCTAGCTTGGTACCAACAGAAGCCA
121 GGGCAGTCTCCTAAACTGCTGATATACTATGCATCCAATCGCTACACTGGAGTCCCTGAT
181 CGCTTCTCTGGCAGTGGATATGGGACGGATTTCACTTTCACCATCAGCACTGTGCAGGCT
241 GAAGACCTGGCAGTTTACTTCTGTCTGCAGGATTATACCTCTCCGTGGACGTTCGGTGGA
301 GGCACCAAGCTGGAAATCAAGCGG

SC7.48 Light chain - protein sequence (SEQ ID NO:116)
1 SIVMTQTPNFLLLATGDRVNITCKASQSVSNDLAWYQQKPGQSPKLLIYY
51 ASNRYTGVPDRFSGSGYGTDFTFTISTVQAEDLAVYFCLQDYTSPWTFGG
101 GTKLEIKR

SC7.58 Heavy chain - nucleotide sequence (SEQ ID NO:117)

1 GAGGTGCGGCTTCAGGAGTCAGGACCTAGCCTCGTGAAACCTTCTCAGACTCTGTCCCTC
61 ACCTGTTCTGTCACTGGCGACTCCATCACCAGTGATTACTGGAACTGGATCCGGAAATTC
121 CCAGGGAATAAACTTGAGTACATGGGGTACATAAGCTACAGTGGTAATACTTACTACAAT
181 CCATCTCTCAAAAGTCGAATCTCCATCACTCGAGACACATCCAAGAACCAGTATTATTTG
241 CACTTGAATTCTGTGACTACTGAGGACGCAGCCACATATTACTGTGCAAGATCTTATCTC
301 TATGATGGTTACCTTGACTACTGGGGCCAGGGCACCAGTCTCACAGTCTCCTCA

SC7.58 Heavy chain - protein sequence (SEQ ID NO:118)

1 EVRLQESGPSLVKPSQTLSLTCSVTGDSITSDYWNWIRKFPGNKLEYMGY
51 ISYSGNTYYNPSLKSRISITRDTSKNQYYLHLNSVTTEDAATYYCARSYL
101 YDGYLDYWGQGTSLTVSS

FIG. 9I

SC7.58 Light chain - nucleotide sequence (SEQ ID NO:119)

1 GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACC
61 ATCAGTTGCAGAGCAAGTCAGGGCATTAGTAATTATTTAAACTGGTATCAGCAGAAACCA
121 GATGGAACTGTTAAACTCCTGATCTATTACACATCAAGTTTACACTCAGGAGTCCCATCA
181 AGGTTCAGTGGCAGTGGGTCTGGGACAGATTTTTCTCTCACCATCAGCAACCTGGAACCT
241 GAAGATTTTGCCACTTACTATTGTCAGCAGTATACTAAGCTTCCGTACACGTTCGGAGGG
301 GGGACCAAGCTGGAAATAAG

SC7.58 Light chain - protein sequence (SEQ ID NO:120)

1 DIQMTQTTSSLSASLGDRVTISCRASQGISNYLNWYQQKPDGTVKLLIYY
51 TSSLHSGVPSRFSGSGSGTDFSLTISNLEPEDFATYYCQQYTKLPYTFGG
101 GTKLEIR

SC7.75 Heavy chain - nucleotide sequence (SEQ ID NO:121)

1 ACTGGCTACACATTCAGTAGTTACTGGATAGAGTGGGTAAAGCAGAGGCCTGGACATGGC
61 CTTGAGTGGATTGGACAGATTTTACCTGGAAGTGGTACTACTTACTACAATGAGAAGTTC
121 AAGGGCAAGGCCACATTCACTGCAGATACATCCTCCAACACAGCCTACATGCAACTCAGC
181 AGCCTGACATCTGAGGACTCTGCCGTCTATTACTGTACAATCTATGGTAACTACTTCTAC
241 TATACTATGGACTATTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC7.75 Heavy chain - protein sequence (SEQ ID NO:122)

1 TGYTFSSYWIEWVKQRPGHGLEWIGQILPGSGTTYYNEKFKGKATFTADT
51 SSNTAYMQLSSLTSEDSAVYYCTIYGNYFYYTMDYWGQGTSVTVSS

FIG. 9J

SC7.75 Light chain - nucleotide sequence (SEQ ID NO:123)

1 ATTGGGCGACCAGCCTCCATCTCTTGCAAGTCAAGTCAGAGCCTCTTACATAGTGATGGA
61 AACACATATTTGAATTGGTTGTTACAGAGGCCAGGCCAGTCTCCAAAGCGCCTGATCTAT
121 CTGGTGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGGACA
181 GATTTCACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTATTGCTGT
241 CAAGGTACACATTTTCCTCGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGG

SC7.75 Light chain - protein sequence (SEQ ID NO:124)

1 IGRPASISCKSSQSLLHSDGNTYLNWLLQRPGQSPKRLIYLVSKLDSGVP
51 DRFTGSGSGTDFTLKISRVEAEDLGVYYCCQGTHFPRTFGGGTKLEIKR

SC7.85 Heavy chain - nucleotide sequence (SEQ ID NO:125)

1 CAGGTCCAACTGCAGCAGTCTGGGGCTGAGCTGGCAAGACCTGGGGCTTCAGTGAAGTTG
61 TCCTGCAAGGCTTCTGGCTCCACCTTTACTAGCTACTGGATGCAGTGGGTGAAACAGAGG
121 CCTGGACAGGGTCTGGAATGGATTGGGGCTATTTATCCTGGAGATGGTGATACTAGGTAC
181 GCTCAGGAGTTCAAGGGCAAGGCCACATTGACTGCAGATAAATCCTCCAGCACAGCCTAC
241 ATGCAGCTCAACACCTTGGCATCTGAGGACTCTGCGGTCTATTACTGTGCAGAGGGCCCT
301 TACTTCTTTGACTGCTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC7.85 Heavy chain - protein sequence (SEQ ID NO:126)

1 QVQLQQSGAELARPGASVKLSCKASGSTFTSYWMQWVKQRPGQGLEWIGA
51 IYPGDGDTRYAQEFKGKATLTADKSSSTAYMQLNTLASEDSAVYYCAEGP
101 YFFDCWGQGTTLTVSS

FIG. 9K

SC7.85 Light chain - nucleotide sequence (SEQ ID NO:127)

1 GACACTGTGATGACCCAGTCTCAAAAATTCATGTCCACAACAGTAGGAGACAGGGTCAGC
61 ATCACCTGCAAGGCCAGTCAGAATGTGGGTACTGCTGTAGCCTGGTATCAACAGAAACCA
121 GGACAATCTCCTAAATTATTGATTTACTTAGCATCCAATCGGTACACTGGAGTCCCTGAT
181 CGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATTAGCAATATGCAGTCT
241 GAAGACCTGGCAGATTATTTCTGTCAGCAATATAACAGCTATCCTCTGACGTTCGGTGGA
301 GGCACCAAGCTGGAGATCAAACGG

SC7.85 Light chain - protein sequence (SEQ ID NO:128)

1 DTVMTQSQKFMSTTVGDRVSITCKASQNVGTAVAWYQQKPGQSPKLLIYL
51 ASNRYTGVPDRFTGSGSGTDFTLTISNMQSEDLADYFCQQYNSYPLTFGG
101 GTKLEIKR

SC7.142 Heavy chain - nucleotide sequence (SEQ ID NO:129)

1 GAGGTTCAGCTGCAGCAGTCTGGGGCAGAGCTTGTGAAGCCAGGGGCCTCAGTCAAGTTG
61 TCCTGCACAGCTTCTGGCTTCAACATTAAAGACACCTATATGGATTGGGTGAAGCAGAGG
121 CCTGAACAGGGCCTGGAGTGGATTGGAAGGATTGATCCTGCGAATGGTAATACTAAATAT
181 GACCCGAAGTTCCAGGGCAAGGCCACTATAACAACAGACACATCCTCCAACACAGCCTAC
241 CTGCAGCTCAGCAGCCTGACATCTGAGGACACTGCCGTCTATTACTGTGCTAGACCCTTT
301 GGTAGCTTCGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC7.142 Heavy chain - protein sequence (SEQ ID NO:130)

1 EVQLQQSGAELVKPGASVKLSCTASGFNIKDTYMDWVKQRPEQGLEWIGR
51 IDPANGNTKYDPKFQGKATITTDTSSNTAYLQLSSLTSEDTAVYYCARPF
101 GSFAMDYWGQGTSVTVSS

FIG. 9L

SC7.142 Light chain - nucleotide sequence (SEQ ID NO:131)

1 GACATCCAGATGACACAATCTTCATCCTACTTGTCTTTATCTCTAGGAGGCAGAGTCACC
61 ATTACTTGCAAGGCAAGTGACCACATTAATAATTGGTTAGCCTGGTATCAGCAGAAACCA
121 GGAACTGCTCCTAGACTCTTAATATCTGGTGCAACCAGTTTGGAAACTGGGGTTCCTTCA
181 AGATTCAGTGGCAGTGGATCTGGAAAGGATTTCACTCTCAGCATTACCAGTCTTCAGGCT
241 GAAGATGTTGCTACTTATTACTGTCAACAGTATTGGAGTACTCCGTACACGTTCGGAGGG
301 GGGACCAAACTGGAAATAAAACGG

SC7.142 Light chain - protein sequence (SEQ ID NO:132)

1 DIQMTQSSSYLSLSLGGRVTITCKASDHINNWLAWYQQKPGTAPRLLISG
51 ATSLETGVPSRFSGSGSGKDFTLSITSLQAEDVATYYCQQYWSTPYTFGG
101 GTKLEIKR

SC7.106 Heavy chain - nucleotide sequence (SEQ ID NO:133)
1 GGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCTGGATTC
61 GCTTTCAGTAACTATGCCATGTCTTGGGTTCGCCAGACTCCGGAGAAGAGGCTGGAGTGG
121 GTCGCTACCATTTTCAGTGGTGTTATTTACACCTACTATCCAGACAGTGTGAAGGGGCGA
181 TTCACCATCTCCAGAGACAATGCCAAGAACACCCTGTACCTACAAATGAGCAGTCTGAGG
241 TCTGAGGACACGGCCATGTATTACTGTGCAAGATATGATGCCTTGTACTACTTTGACTAC
301 TGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC7.106 Heavy chain - protein sequence (SEQ ID NO:134)
1 GGGLVKPGGSLKLSCAASGFAFSNYAMSWVRQTPEKRLEWVATIFSGVIY
51 TYYPDSVKGRFTISRDNAKNTLYLQMSSLRSEDTAMYYCARYDALYYFDY
101 WGQGTTLTVSS

FIG. 9M

SC7.106 Light chain - nucleotide sequence (SEQ ID NO:135)
1 ACATTAGTAGGTGACAGGGTCAACATCACCTGCAAGGCCAGTCAGGATGTGAGTACTGCT
61 GTTGCCTGGTATCAACAAAAACCAGGGCTATCTCCTAAACTGCTGATTTACTGGGCATCC
121 ACCCGGCACACTGGAGTCCCTGATCGCTTCACAGGCAGTGGGTCTGGGACAGATTATATT
181 CTCACCATCAGCAGTGTGCAGGCTGAAGACCTGGCACTCTATTACTGTCAGCAACATTAT
241 AGCACTCCATTCACGTTCGGCTCGGGGACAAAGTTGGAGATAAAACGG

SC7.106 Light chain - protein sequence (SEQ ID NO:136)
1 TLVGDRVNITCKASQDVSTAVAWYQQKPGLSPKLLIYWASTRHTGVPDRF
51 TGSGSGTDYILTISSVQAEDLALYYCQQHYSTPFTFGSGTKLEIKR

FIG. 10A

Genetic Arrangements of Distinct Anti-APCDD1 Antibodies

	Clone	VH	DH	JH	VK	JK
1	SC7.12	VH69-1	IGHD2-14	JH3	IGKV5-43	JK1
2	SC7.13.2	IGHV5-17	P1inv	JH4	IGKV3-5	JK2
3	SC7.18.1	IGHV5-4	DSP2.4	JH1	IGKV10-96	JK1
4	SC7.43.1	IGHV14-1	DSP2.13	JH4	IGKV6-14	JK2
5	SC7.46.1	VHJ558	DST4.3	JH4	IGKV6-32	JK5
6	SC7.48.1	IGHV1-14	DQ52a.1	JH2	IGKV6-32	JK1
7	SC7.58.1	IGHV3-8	DSP2.9	JH2	IGKV10-94	JK2
8	SC7.75.3	J558.17	DSP2.7	JH4	IGKV1-135	Jk1
9	SC7.85.3	J558.19	P8inv	JH2	IGKV6-13	JK1
10	SC7.106	IGHV5-9-1	DSP2.9	JH2	IGKV6-25	JK4
11	SC7.107.1	VHJ558	DSP2.8	JH4	IGKV4-50	JK2
12	SC7.142.1	IGHV14-3	DFL16.1e	JH4	IGKV13-85	JK2
13	SC7.143.2	IGHV5-17	DSP2.8	JH4	IGKV3-5	JK2

FIG. 10B

Complementarity Determining Regions of Distinct Anti-APCDD1 Antibodies

	Clone	CDRH1 SEQ ID NOS 17-28	CDRH2 SEQ ID NOS 29-40	CDRH3 SEQ ID NOS 41-52	CDRL1 SEQ ID NOS 53-64	CDRL2 SEQ ID NOS 65-76	CDRL3 SEQ ID NOS 77-88
1	SC7.12	GFTFSSYT	ISSGGGYT	TRDRHDGPGTWFAY	QSVSNN	YASQSIS	QQSYSWPRT
2	SC7.13.2	GFTFSRFG	ISSGSSTI	ARKDSYAMDY	ESVDRYGNSF	RASNLES	QQSNEVPDT
3	SC7.18.1	GFTFSDYY	ISDGGSYT	ARGYGYYWYFDV	QDISNC	YTSRLHS	QQGNTLPWT
4	SC7.43.1	GFNFKDYF	IDPENGNT	ARIYGDYGAMDY	QNVRTV	LASNRHT	LQHWNYPYT
5	SC7.46.1	GYTFTSYW	INPTTGLT	AREGYKDALDY	QSVSNA	HASNRYT	QQDYSSPLT
6	SC7.48.1	GYTFISDV	INPYNDAT	ARRGLIGADY	QSVSND	YASNRYT	LQDYTSPWT
7	SC7.58.1	GDSITSDY	ISYSGNT	ARSYLYDGYLDY	QGISNY	YTSSLHS	QQYTKLPYT
8	SC7.75.3	GYTFSSYW	ILPGSGTT	TIYGNYFYYTMDY	QSLLHSDGNTY	LVSKLDS	CQGTHFPRT
9	SC7.85.3	GSTFTSYW	IYPGDGDT	AEGPYFFDC	QNVGTA	LASNRYT	QQYNSYPLT
10	SC7.106	GFAFSNYA	IFSGVIYT	ARYDALYYFDY	QDVSTA	WASTRHT	QQHYSTPFT
11	SC7.107.1	GYTFTSYT	INPYTGYS	ARENYKDAMDY	SSVNY	YTSNLAP	QQLTSSPYT
12	SC7.142.1	GFNIKDTY	IDPANGNT	ARPFGSFAMDY	DHINNW	GATSLET	QQYWSTPYT
13	SC7.143.2	GFTFSRFG	ITSGSSTI	AKYGNYAMDY	ESVDRDGNSL	RAFNLES	QQSNEAPYT

APCDD1 Effector Characteristics

Clone	Bin	Affinity (nM)	Western Reactivity	Ms XR	Cyno XR
8	E	2.0^{F}	NR	ND	ND
13.2	A	0.3^{B}	NR	No	Yes
43.3	B	$<0.2^{F}$	NR/R	Yes	Yes
45	F	2.0^{F}	ND	ND	ND
46.2	A	2.0^{F}	NR	No	ND
48	G	0.6^{B}	NR/R	Yes	Yes
58.2	C	$<0.2^{B}$	NR	No	Yes
75.4	A	4.0^{F}	NR	ND	ND
85	B	$<0.2^{B}$	NR/R	No	ND
107	A	0.3^{F}	NR	No	ND
124	B	1.0^{F}	NR	No	Yes
142.1	B	1.0^{B}	NR/R	Yes	ND
143.2	D	$<0.2^{B}$	NR/R	ND	ND
153.38	A	1.0^{F}	NR	No	ND

^{B} Biacore affinity; ^{F} ForteBIO in-house comparison

FIG. 11

hSC7.13 V_H Chain

```
1                                                 50
GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTC
-E--V--Q--L--V--E--S--G--G--G--L--V--Q--P--G--G--S
51                                               100
CCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGTCGCTTTGGCA
--L--R--L--S--C--A--A--S--G--F--T--F--S--R--F--G-
101                                              150
TGCACTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCATAC
M--H--W--V--R--Q--A--P--G--K--G--L--E--W--V--S--Y-
151                                              200
ATTAGTAGTGGTAGTAGTACCATATACTACGCAGACACTGTGAAGGGCCG
-I--S--S--G--S--S--T--I--Y--Y--A--D--T--V--K--G--R
201                                              250
ATTCACCATCTCCAGAGACAATGCCAAGAACTCACTGTATCTGCAAATGA
--F--T--I--S--R--D--N--A--K--N--S--L--Y--L--Q--M--
251                                              300
ACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGAAAGGAC
N--S--L--R--A--E--D--T--A--V--Y--Y--C--A--R--K--D-
301                                              350
TCTTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTC     (SEQ ID NO: 89)
-S--Y--A--M--D--Y--W--G--Q--G--T--S--V--T--V--S—S     (SEQ ID NO: 90)
```

hSC7.13 V_L Chain

```
1                                                 50
GAAATTGTGTTGACACAGAGCCCGGCGACCCTGAGCCTGAGTCCGGGCGA
-E--I--V--L--T--Q--S--P--A--T--L--S--L--S--P--G--E
51                                               100
ACGCGCGACCATTAGCTGCCGCGCGAGCGAAAGCGTGGATCGCTATGGCA
--R--A--T--I--S--C--R--A--S--E--S--V--D--R--Y--G--
101                                              150
ACAGCTTTATGCATTGGTATCAGCAGAAACCGGGCCAGGCGCCGCGCCTG
N--S--F--M--H--W--Y--Q--Q--K--P--G--Q--A--P--R--L-
151                                              200
CTGATTTATCGCGCGAGCAACCTGGAAAGCGGCATTCCGGCGCGCTTTAG
-L--I--Y--R--A--S--N--L--E--S--G--I--P--A--R--F--S
201                                              250
CGGCAGCGGCAGCCGCACCGATTTTACCCTGACCATTAGCAGCCTGGAAC
--G--S--G--S--R--T--D--F--T--L--T--I--S--S--L--E--
251                                              300
CGGAAGATTTTGCGGTGTATTATTGCCAGCAGAGCAACGAAGTGCCGGAT
P--E--D--F--A--V--Y--Y--C--Q--Q--S--N--E--V--P--D-
301
ACCTTTGGCGGCGGCACCAAACTGGAAATTAAACGG                   (SEQ ID NO: 91)
-T--F--G--G--G--T--K--L--E--I--K--R-                   (SEQ ID NO: 92)
```

FIG. 12A

hSC7.48 V_H Chain

```
1                                                 50
CAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGGCCTC
-Q--V--Q--L--V--Q--S--G--A--E--V--K--K--P--G--A--S
51                                               100
AGTGAAGGTTTCCTGCAAGGCATCTGGATACACCTTCATCAGCGATGTTC
--V--K--V--S--C--K--A--S--G--Y--T--F--I--S--D--V--
101                                              150
TGCACTGGGTGCGACAGGCCCCTGGACAAGGGCTTGAGTGGATTGGATAT
L--H--W--V--R--Q--A--P--G--Q--G--L--E--W--I--G--Y-
151                                              200
ATCAACCCTTATAATGATGCTACATCATACGCCCAGAAGTTCCAGGGCAG
-I--N--P--Y--N--D--A--T--S--Y--A--Q--K--F--Q--G--R
201                                              250
AGTCACCATGACCAGGGACACGTCCACGAGCACAGTCTACATGGAGCTGA
--V--T--M--T--R--D--T--S--T--S--T--V--Y--M--E--L--
251                                              300
GCAGCCTGAGATCTGAGGACACGGCCGTGTATTACTGTGCGAGAAGAGGT
S--S--L--R--S--E--D--T--A--V--Y--Y--C--A--R--R--G-
301                                              350
CTAATTGGGGCAGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTC      (SEQ ID NO: 93)
-L--I--G--A--D--Y--W--G--Q--G--T--T--L--T--V--S—S       (SEQ ID NO: 94)
```

hSC7.48 V_L Chain

```
1                                                 50
GCCATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGA
-A--I--Q--M--T--Q--S--P--S--S--L--S--A--S--V--G--D
51                                               100
CAGAGTCACCATCACTTGCAAGGCAAGTCAGAGCGTTAGCAATGATTTAG
--R--V--T--I--T--C--K--A--S--Q--S--V--S--N--D--L--
101                                              150
CCTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCTGATCTATTAT
A--W--Y--Q--Q--K--P--G--K--A--P--K--L--L--I--Y--Y-
151                                              200
GCATCCAATCGATATACTGGGGTCCCATCAAGGTTCAGCGGCAGTGGATC
-A--S--N--R--Y--T--G--V--P--S--R--F--S--G--S--G--S
201                                              250
TGGCACAGATTTCACTCTCACCATCAGCAGCCTGCAGCCTGAAGATTTTG
--G--T--D--F--T--L--T--I--S--S--L--Q--P--E--D--F--
251                                              300
CAACTTATTACTGTCTACAAGATTACACCTCTCCGTGGACGTTCGGTGGA
A--T--Y--Y--C--L--Q--D--Y--T--S--P--W--T--F--G--G-
301
GGCACCAAGCTGGAAATCAAGCGG                                (SEQ ID NO: 95)
-G--T--K--L--E--I--K--R-                                (SEQ ID NO: 96)
```

FIG. 12B

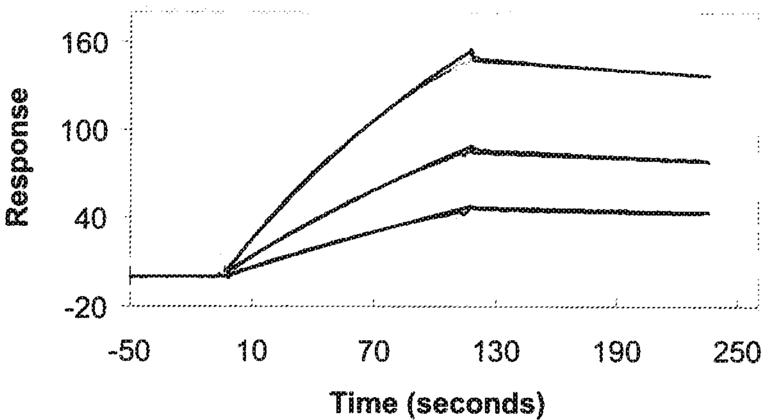


FIG. 13A

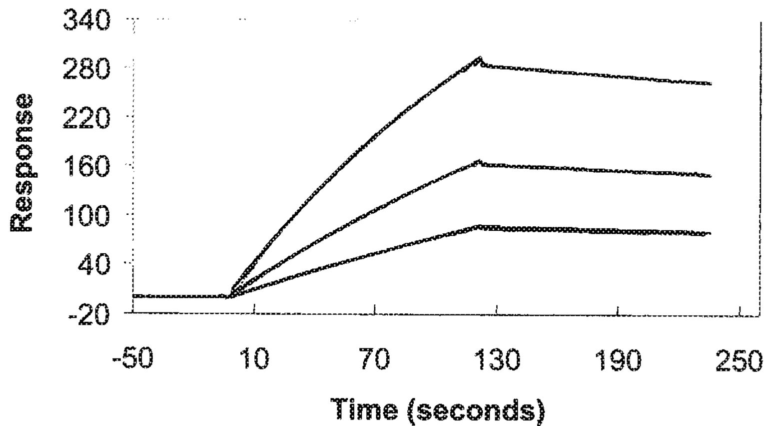


FIG. 13B

hClone	Bin	Ms MAb Isotype	Mouse Ag Affinity	Cyno Ag Affinity	Hu Ag Affinity (Murine mAb)	Hu Ag Affinity (Human mAb)
SC7.13	A	IgG_{2a}	>100 nM	0.3 nM	0.3 nM	0.3 nM
SC7.48	G	IgG_{2a}	0.7 nM	0.7 nM	0.6 nM	3.7nM

FIG. 13C

APCDD1 is Expressed on Colorectal Tumor Initiating Cells

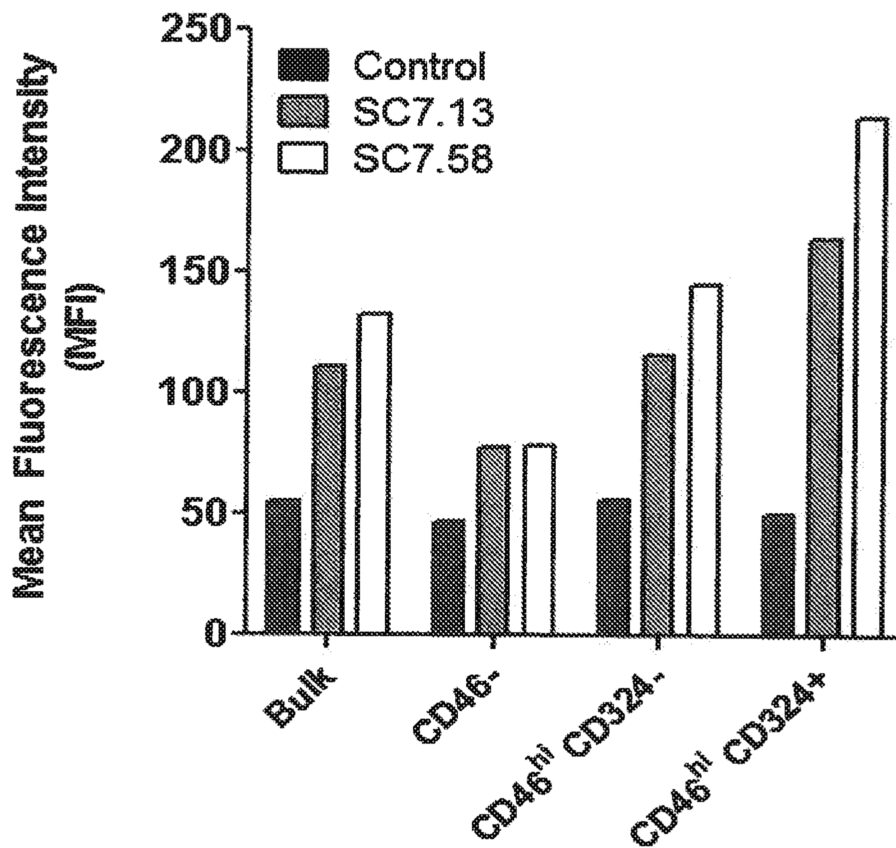


FIG. 14

APCDD1 Effectors Mediate Fab-Saporin Cytotoxicity in APCDD1+ Cells

FIG. 15A

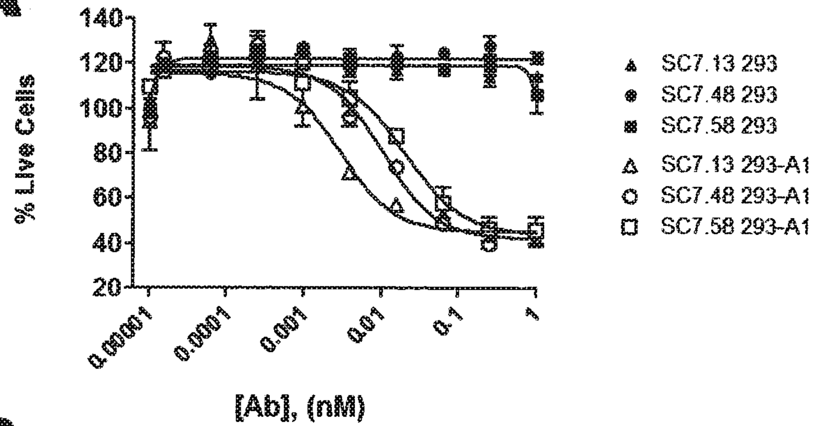


FIG. 15B

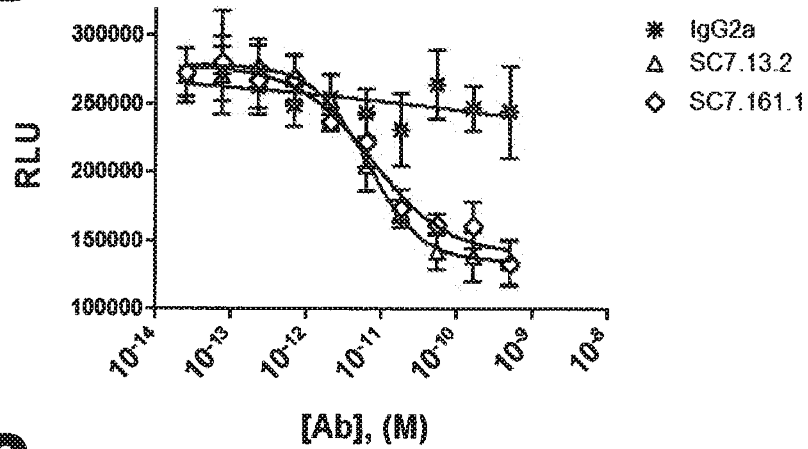
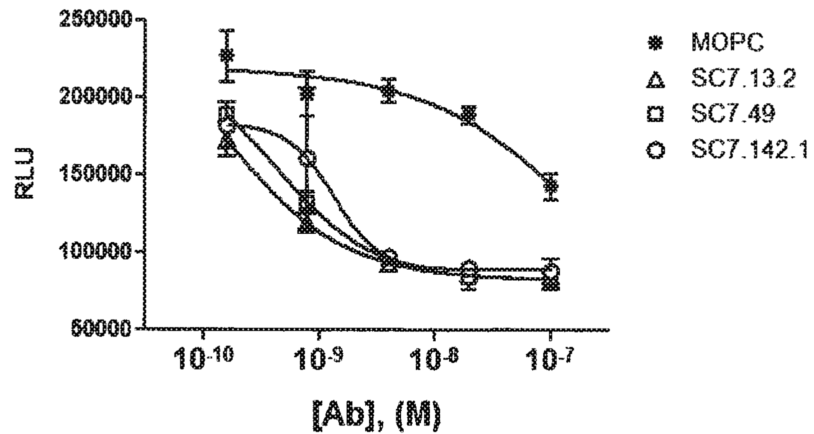


FIG. 15C



APCDD1 Effectors Mediate Fab-Saporin Cytotoxicity in Tumor Cells

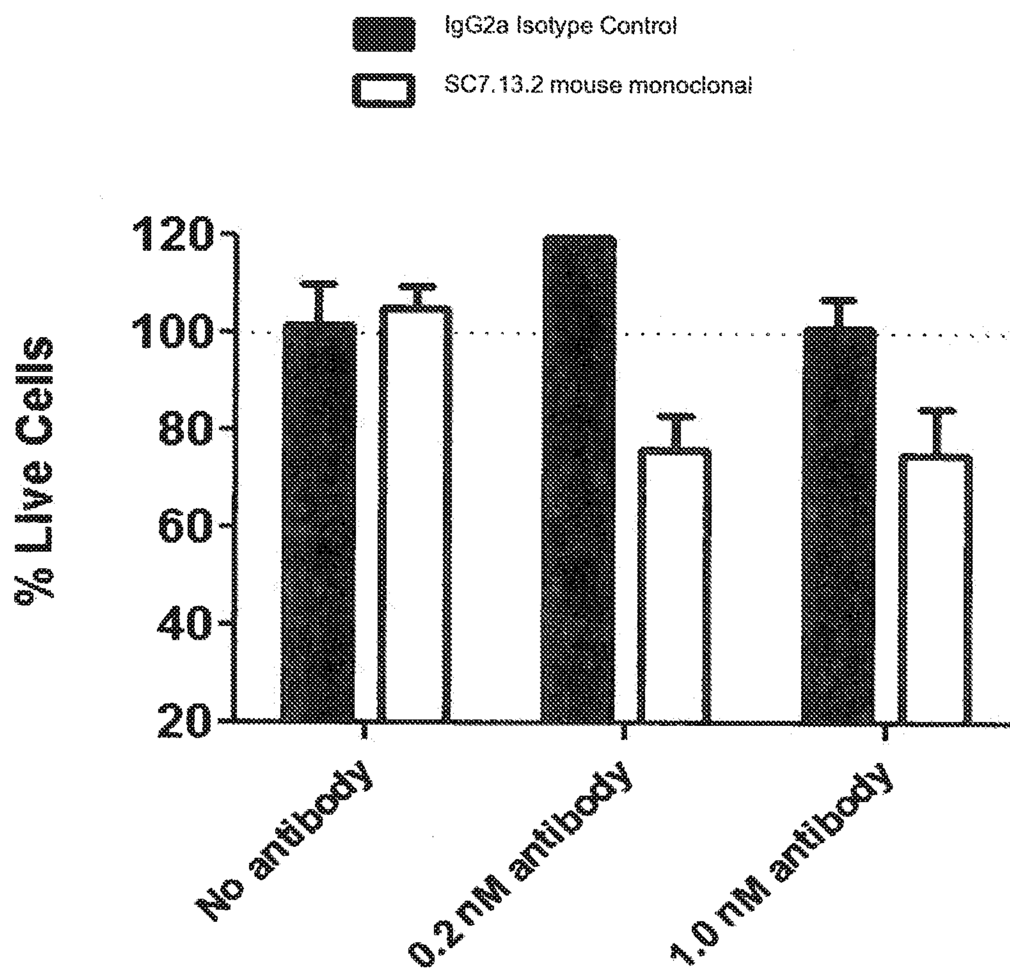


FIG. 16

Humanized APCDD1 Effectors Mediate Saporin Cytotoxicity in APCDD1+ Cells

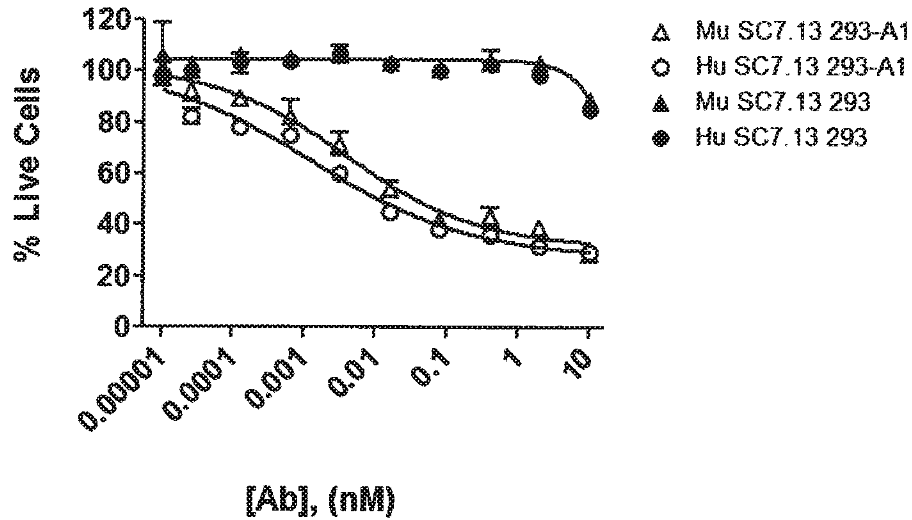


FIG. 17A

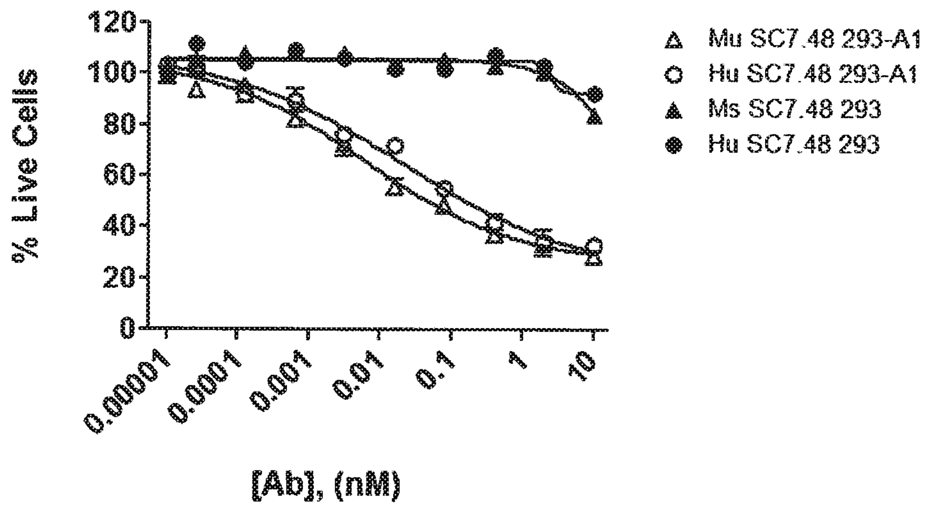


FIG. 17B