IDENTIFICATION AND ENRICHMENT OF CELL SUBPOPULATIONS

CROSS REFERENCED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. 119(e) of U.S. Provisional Application Nos. 61/380,181 filed September 3, 2010, and 61/510,413 filed July 21, 2011, each of which are incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[002] The present invention relates generally to the identification, characterization and, optionally the isolation or enrichment of cells or cell subpopulations, particularly tumor initiating cells derived from various neoplasia. In selected aspects the invention also relates to methods of isolating tumor initiating cells and the resulting preparations, as well as various methods for using tumor initiating cells and cell populations for drug research and development and in diagnostic, therapeutic and other clinical and non-clinical applications.

BACKGROUND OF THE INVENTION

[003] 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. Accordingly, cell proliferation and differentiation normally occurs only as necessary for the replacement of damaged or dying cells or for growth. Unfortunately, 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. When normal cellular proliferation and/or differentiation is disturbed or somehow disrupted it can lead to various diseases or disorders including cancer.

[004] Conventional treatments for cancer include chemotherapy, radiotherapy, surgery, immunotherapy (e.g., biological response modifiers, vaccines or targeted therapeutics) or combinations thereof. Sadly, 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. Limitations inherent in current standard of care therapeutics are particularly evident when

attempting to care for patients who have undergone previous treatments and have subsequently relapsed. In such cases the failed therapeutic regimens and resulting patient deterioration may contribute to refractory tumors often manifest themselves as a more aggressive disease that ultimately proves to be incurable. 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 relapse, tumor recurrence and metastases. Thus, it remains a challenge to develop more targeted and potent therapies.

[005] 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.

[006] 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 in its entirety by reference. 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 in its entirety by reference. Such observations

are confirmed by the consistent inability of traditional debulking agents to substantially increase patient survival when suffering from solid tumors, and through the development of an increasingly sophisticated understanding as to how tumors grow, recur and metastasize. Accordingly, recent strategies for treating neoplastic disorders have recognized the importance of eliminating, depleting, silencing or promoting the differentiation of tumor perpetuating cells so as to diminish the possibility of tumor recurrence, metastasis or patient relapse.

[007] Efforts to develop such strategies have incorporated recent work involving non-traditional xenograft (NTX) models, wherein primary human solid tumor specimens are implanted and passaged exclusively in immunocompromised mice. Such techniques confirm the existence of a subpopulation of cells with the unique ability to generate heterogeneous tumors and fuel their growth indefinitely. As previously hypothesized, work in NTX models has confirmed that identified CSC subpopulations of tumor cells appear more resistant to debulking regimens such as chemotherapy and radiation, potentially explaining the disparity between clinical response rates and overall survival. Further, employment of NTX models in CSC research has sparked a fundamental change in drug discovery and preclinical evaluation of drug candidates that may lead to CSC-targeted therapies having a major impact on tumor recurrence and metastasis thereby improving patient survival rates.

[008] Unfortunately, such techniques and related methodology are largely dependent on the ability to efficiently and reliably identify, characterize and/or partition, isolate or enrich tumor perpetuating cell subpopulations that may then be effectively analyzed. To ultimately carry out such work the identification and association of tumor cell markers that allow for dissection and organization of cellular hierarchy is critical. Identification of reliable markers associated with tumor perpetuating cells (i.e. CSC) therefore provides valuable information that is useful for a variety of applications in both clinical and basic research settings. The identification and subsequent isolation of tumor perpetuating cells from a particular tumor or metastatic lesion is useful, for example, in diagnosing the pathological basis for the disorder and/or developing a rational therapeutic treatment. In other instances, identification, characterization, isolation, partition or enrichment of TPC is necessary for conducting *in vitro* experiments on physiological and molecular mechanisms or for implantation into animals for *in vivo* studies of tumor generation, progression and/or treatment.

[009] The present invention addresses several of these issues and, by providing novel compositions, models and methods to facilitate the understanding, treatment, prevention and/or management of hyperproliferative disorders.

SUMMARY OF THE INVENTION

[0010] 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 to identify or characterize, and, optionally, to isolate, partition, separate or enrich certain tumorigenic cells or cell subpopulations associated with hyperproliferative or neoplastic disorders. In preferred embodiments the subject cell or cell subpopulation will comprise tumor initiating cells and/or tumor perpetuating cells and/or tumor progenitor cells.

[0011] More specifically, in accordance with the teachings herein the inventors have discovered a series of markers that may be used independently or collectively to accurately identify, sort enrich and/or and characterize cancer stem cells from a wide variety of tumors. Using selected biochemical techniques, the novel association of the disclosed markers with specific, self-renewing malignant cells in the tumor architecture provides for enrichment, isolation, or purification of phenotypically distinct cells or tumor cell subpopulations that are capable of perpetual self-renewal and tumor recapitulation. In contrast to the prior art, and as evidenced by the Examples below, the disclosed markers are capable of recognizing or identifying tumor initiating cells from a variety of tumors. The enrichment or isolation of these marker-defined, relatively homogeneous cell populations in turn allows for the extensive characterization of the constituent cancer stem cells, including the elucidation of potential therapeutic targets and screening of pharmaceutical compounds. In other embodiments the cells and compositions of the instant invention may be used in conjunction with animal models such as non-traditional xenograft models to facilitate research and drug development efforts. Moreover, the disclosed markers may further be used in clinical and non-clinical settings for the diagnosis, classification, monitoring and management of hyperproliferative disorders as well as providing associated kits or other articles of manufacture.

[0012] In particularly preferred embodiments the present invention provides for the identification, characterization, enrichment and/or isolation of, respectively, tumor initiating cells (TIC), tumor perpetuating cells (TPC) and tumor progenitor cells (TProg) through the novel use of selected marker or marker combinations as set forth herein. With respect to the cancer stem cell paradigm, each of the aforementioned cell subpopulations are tumorigenic to a greater or lesser extent and, as such, are responsible for tumor growth, maintenance, metastasis and recurrence. Significantly, the instant invention allows such cell subpopulations to be identified and optionally derived from a variety of different tumor types and stages of cancer. That is, through the identification of specific cancer stem cell markers, including tumor initiating cell associated markers (TICAM), tumor perpetuating cell associated markers (TPCAM) and tumor

progenitor cell associated markers (TProgAM), the present invention provides, among other aspects, for the uniform and reproducible recognition, characterization and sorting or isolation of highly pure tumorigenic cell subpopulations that, in turn, allow for the identification of cancer stem cell specific therapeutic targets and proteins of prospective diagnostic utility. In this regard these cells or enriched cell subpopulations, as defined by the disclosed markers alone or in combination, can be effectively employed in pharmaceutical research and development activities to identify novel therapeutic targets optimized for the suppression or depletion of tumor feeding cancer stem cells.

[0013] Accordingly, in a preferred embodiments the present invention provides an isolated tumorigenic cell population enriched for expression of at least one TICAM. In a related method the present invention will comprise a method for enriching a tumorigenic cell population comprising the steps of:

contacting a tumor cell population with a binding agent which preferably associates with at least one TICAM; and

sorting said cells associated with said at least one TICAM to provide an enriched tumorigenic cell population.

[0014] In particularly preferred embodiments the sorting step comprises fluorescence activated cell sorting, magnetic-assisted cell sorting, substrate-assisted cell sorting, laser mediated sectioning, fluorometry, flow cytometry or microscopy techniques. In other preferred embodiments the sorting step will comprise contacting the tumor cell population with a plurality of binding agents.

[0015] Also provided by the invention are enriched tumorigenic cell populations derived from a heterogeneous tumor mass as well as tumor initiating cells derived from these tumors. It will be appreciated that the disclosed methods of tumor initiating cell sorting along with *in vivo* cell passaging and *in vitro* culture techniques and the resultant populations of purified cells provide for a wide variety of uses, as further described below.

[0016] Another aspect of the invention comprises a personalized method of treatment of a subject with a tumor, which is made possible by the ready availability and manipulability of tumorigenic cells derived from the subject's own tumor using methods in accordance with the invention.

[0017] In this respect another embodiment of the invention comprises a method of treating or diagnosing a subject in need thereof comprising the steps of;

obtaining a tumor sample from the subject; and

contacting the tumor sample with at least one binding agent that preferably associates with a TICAM.

[0018] In preferred embodiments the tumor sample will comprise a solid tumor sample. In other preferred embodiments the method will further comprise the step of characterizing or assessing tumorigenic cells associated with said tumor sample.

[0019] Yet another aspect of the instant invention will comprise method of determining if a cell obtained from a tumor sample is tumorigenic comprising the step of contacting the tumor cell with at least one TICAM.

[0020] Yet another aspect of the present invention provides methods of treating a subject suffering from cancer wherein the method comprises assessing the TICAM positive cells in a sample obtained from the subject. Optionally the assessment may occur following treatment of the subject and, in other embodiments, the assessment will occur after the subject has been treated with one or more anti-cancer agents. In yet other embodiments the assessment will be undertaken prior to the patient being treated.

[0021] Another embodiment of the present invention relates to methods to determine if a subject is at risk of recurrence wherein the method comprising assessing the presence of TICAM positive cells through the introduction of a binding agent that preferably associates TICAM, wherein the detection of tumor initiating cells is indicative that the subject is at risk of metastasis or recurrent cancer. In such embodiments, the subject can be administered an anti-cancer agent or therapy as known by a person of ordinary skill in the art.

[0022] In a similar vein the present invention also provides kits that are useful in the diagnosis and monitoring of tumor initiating cell associated disorders such as cancer. To this end the present invention preferably provides an article of manufacture useful for diagnosing or treating such disorders comprising a receptacle or receptacles comprising one or more binding agents that preferably associate with a tumor initiating cell associated marker and instructional materials for using the same.

[0023] Still another embodiment of the instant invention comprises a method of conducting genotypic or phenotypic analysis comprising the steps of:

providing a tumorigenic cell or enriched tumorigenic cell population comprising one or more TICAM;

treating said cell or cell population to provide genetic or proteomic material; and

analyzing said genetic or proteomic material.

[0024] In preferred embodiments the analysis will comprise Next-Gen sequencing of at least a portion of the transcriptome. In other embodiments the invention will comprise mass

spectrometric analysis.

[0025] In yet another embodiment the present invention will comprise a method of screening potential pharmaceutical compounds comprising the steps of:

exposing a tumorigenic cell or tumorigenic cell population to a candidate compound; and

contacting the tumorigenic cell or tumorigenic cell population with at least one TICAM.

[0026] In selected embodiments the exposing step will be conducted *in vivo*. In other preferred embodiments the exposing step will be conducted *in vitro*. In yet other preferred embodiments the method will further comprise characterizing the tumorigenic cell or tumorigenic cell population.

[0027] In another embodiment, the instant invention provides a method of inducing cancer comprising the steps of:

providing a tumorigenic cell population enriched for one or more TICAM; and

introducing the tumorigenic cell population into a subject.

[0028] In yet another preferred embodiment the instant invention comprises a method of inducing cancer comprising the steps of:

isolating a tumorigenic cell exhibiting one or more TICAM; and

introducing isolated tumorigenic cell into a subject.

[0029] Preferably the cell is isolated by contacting said cell with one or more binding agents that preferably associate with one or more TICAM.

[0030] The instant invention further provides a method of generating tumor samples comprising the steps of :

providing a tumorigenic cell or enriched tumorigenic cell population comprising one or more TICAM;

introducing the tumorigenic cell or enriched tumorigenic cell population into a subject;

waiting for the tumorigenic cell or cell population to generate a heterogeneous tumor; and

harvesting said heterogeneous tumor.

[0031] In preferred embodiments the harvested tumor will be frozen and banked using standard techniques. In yet other preferred embodiments cells derived from the harvested tumors will be implanted in a subject. Still other preferred embodiments of the instant invention will comprise a tumor bank comprising such harvested tumors.

[0032] In still another particularly preferred embodiment the present invention provides an animal model for the analysis of pharmaceutical compounds comprising a subject implanted with a tumorigenic cell or cell population purified or enriched for one or more TICAM. In particularly preferred embodiments the subject will comprise an immunocompromised mouse. A

related preferred embodiment comprises a method of producing an animal model for the analysis of pharmaceutical compounds comprising the step of introducing a tumorigenic cell or cell population purified or enriched for one or more TICAM.

[0033] Besides the aforementioned aspects selected embodiments of the invention provide a method of analyzing cancer progression and/or pathogenesis *in vivo*, comprising the steps of:

introducing one or more tumor initiating cells comprising one or more TICAM into a subject; and

analyzing cancer progression and/or pathogenesis in the subject.

[0034] In preferred embodiments the animal will be treated with one or more anti-cancer agents following the introduction of the one or more tumor initiating cells.

[0035] Preferred TICAMs compatible with instant invention will comprise CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9. In particularly preferred embodiments cells and cell populations enriched or isolated as set forth herein shall comprise a marker phenotype comprising one or more TICAM. As discussed extensively below and illustrated by the appended Examples, each of the aforementioned makers may be used in accordance with the teachings herein to identify, characterize and, optionally separate or enrich tumorigenic cell populations.

[0036] With regard to all the aforementioned embodiments it will be appreciated that a particularly preferred TICAM comprises a TICAM selected from the group consisting of CD46, CD324 and CD66c. In this respect it will be appreciated that selected particularly preferred tumorigenic cells or enriched cell populations will comprise a $CD46^{hi}$ phenotype. In other preferred embodiments the disclosed cells or cell populations will comprise a $CD324^{+}$ phenotype. In yet other especially preferred embodiments the cells or cell compositions of the instant invention will comprise a $CD46^{hi}$ $CD324^{+}$ phenotype. In still other preferred embodiments the disclosed cells will have a CD 46^{hi} $CD324^{+}$ $CD66c^{-}$ phenotype while in yet other embodiments the cells will exhibit a $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$. Of course it will be appreciated that cell populations comprising the aforementioned phenotypes may be derived using binding agents reactive with each of the specified markers.

[0037] 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 by reference to the figures and in the teachings set forth herein.

BRIEF DESCRIPTION OF THE FIGURES

[0038] FIGS. 1A and 1B depict flow cytometry-based protein expression data for individual tumor cells displayed as histogram plots, wherein the background staining of isotype control antibodies is shown in gray, filled histograms and CD46 expression is displayed by the bold, black line;

[0039] FIG. 2 shows graphical representations of flow cytometry-based protein expression data for individual tumor cells displayed as histogram plots, wherein the background staining of isotype control antibodies is shown in the gray, filled histograms and CD324 expression is displayed by the bold, black line;

[0040] FIGS. 3A and 3B depict flow cytometry-based expression data for individual tumor cells displayed as histogram plots, wherein the background staining of isotype control antibodies is shown in the gray, filled histograms and CD24 or CD34 expression are displayed by the bold, black line;

[0041] FIGS. 4A and 4B are scatter plots and a graphical representation demonstrating the tumorigenicity of enriched $CD46^{hi}$ $CD324^{+}$ cell populations from a representative colorectal tumor;

[0042] FIGS. 5A and 5B are scatter plots and a graphical representation demonstrating the tumorigenicity of enriched $CD46^{hi}$ $CD324^{+}$ cell populations from a representative pancreatic tumor;

[0043] FIGS. 6A and 6B are scatter plots and a graphical representation demonstrating the tumorigenicity of enriched $CD46^{hi}$ $CD324^{+}$ cell populations from a representative lung tumor;

[0044] FIGS. 7A and 7B are scatter plots and a graphical representation demonstrating the tumorigenicity of enriched ESA^{+} ($CD46^{hi}$) $CD324^{+}$ cell populations from a representative triple negative breast tumor;

[0045] FIG. 8 is a tabular overview of representative colorectal, non-small cell lung, pancreatic and triple-negative breast tumor cell transplant experiments utilizing isolated tumor cell subpopulations expressing various combinations of CD46 and CD324;

[0046] FIGS. 9A and 9B provide a tabular overview of markers observed to be co-expressed

on $CD46^{hi}$ $CD324^{+}$ cells in the respective solid tumor types denoted;

[0047] FIG. 10 shows graphical representations of flow cytometry-based protein expression data for individual colorectal tumor cells displayed as scatter plots (above) or histogram plots (below), wherein in the histogram plots the background staining of isotype control antibodies is shown in the gray, filled histograms and the cell surface expression of the denoted protein antigen is displayed by the bold, black line;

[0048] FIG. 11 shows graphical representations of flow cytometry-based protein expression data for individual pancreatic tumor cells displayed as contour plots (above) or histogram plots (below), wherein in the histogram plots the background staining of isotype control antibodies is shown in the gray, filled histograms and the cell surface expression of the denoted protein antigen is displayed by the bold, black line;

[0049] FIG. 12 shows graphical representations of flow cytometry-based protein expression data for individual non-small cell lung tumor cells displayed as scatter plots (above) or histogram plots (below), wherein in the histogram plots the background staining of isotype control antibodies is shown in the gray, filled histograms and the cell surface expression of the denoted protein antigen is displayed by the bold, black line;

[0050] FIG. 13 A-C are graphical representations depicting the ability of colorectal tumor-derived $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ enriched cell populations to reconstitute heterogeneous tumors reflecting the parental tumor from which they were obtained;

[0051] FIGS. 14A and 14B are histogram plots showing $CD66c^{-}$ cell reconstitution by distinct colorectal tumor cell subpopulations in primary transplants and a graphical representation of tumorigenicity by the denoted tumor cell subpopulations in secondary transplants;

[0052] FIGS. 15 A-C provide scatter plots and a tabular summary showing the robust tumorigenicity demonstrated by $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ enriched cell populations through serial transplantation;

[0053] FIGS. 16 A-D graphically illustrate the ability of distinct tumor cell subpopulations expressing various levels of CD46, CD324 and CD66c to form colonies, differentiate and produce soluble CD66c *in vitro*;

[0054] FIGS. 17 A-D provides data demonstrating the impact of chemotherapeutic agents on colorectal tumor cell subpopulations by showing the increase in relative frequency of $CD46^{hi}$ $CD324^{+}$ $CD66^{-}$ cells in treated tumors;

[0055] FIGS. 18A and 18B provides data demonstrating the impact of chemotherapeutic agents on pancreatic tumor cell subpopulations by showing the increase in relative frequency of $CD46^{hi}$ cells in treated tumors;

[0056] FIGS. 19A and 19B are schematic representations reflecting the cellular hierarchy as delineated by the instant disclosure;

[0057] FIGS. 20A – 20D represent graphical representations of representative gene expression for the NOTUM (FIG. 20A), APCDD1 (FIG. 20B), exemplary pancreatic protein (FIG. 20C) and MUC20 (FIG. 20D) associated with NTG cells, TProg cells and TPC, respectively, within colorectal xenograft tumors.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0058] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that aspects of the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

[0059] Characterizing therapeutically significant aspects of tumor initiation and growth to define treatment strategies and provide effective therapeutic targets has proven extremely difficult. In the past the inability to develop successful therapeutic regimens for the treatment of various cancers could, at least in part, be attributed to a lack of understanding of the underlying forces driving tumor growth and recurrence and limitations in research techniques and tools. Yet with the development and validation of the cancer stem cell paradigm over the past several years, and an enhanced appreciation of the intricacies of tumor physiology brought about by increasingly effective research methodology, substantial clinical advances as exhibited by patient survival should be self-evident. Unfortunately, and with some notable exceptions, the expected improvements have not been recognized, particularly in the case of certain solid tumors.

[0060] In part, this relative lack of clinical success may be attributed to the misapplication of generally accurate and illuminating research techniques to ill-conceived or improperly defined cell populations that are commonly held to be tumorigenic. For example, while differential gene expression has been widely used to identify cancer-associated gene activity and to identify expressed proteins having potential therapeutic or diagnostic value, the technique has been of limited value in improving patient survival. This is because such analysis has often been conducted using genetic information obtained from a complex mixture of tumor cells or subpopulations sorted using non-specific or inappropriate cancer stem cell markers.

[0061] While these procedures may provide some insight as to general, homogeneously expressed tumor associated gene products, they do little to identify specific, relevant markers of

cancer stem cells that can be exploited to develop targeted therapeutics for the prevention of tumor recurrence and metastasis. That is, because of the poor quality of the starting material, such differential expression studies often implicate irrelevant or relatively insignificant genes and/or proteins (from a pathology standpoint) from phenotypically diverse differentiated cells that prove generally ineffective as cancer stem cell markers upon further study. Conversely, through the identification of specific cancer stem cell markers, including tumor initiating cell associated markers (TICAM), tumor perpetuating cell associated markers (TPCAM) and tumor progenitor cell associated markers (TProgAM), the present invention provides, among other aspects, for the uniform and reproducible recognition, characterization and sorting or isolation of highly pure tumorigenic cell subpopulations that, in turn, allow for the identification of cancer stem cell specific therapeutic targets and proteins of prospective diagnostic utility.

[0062] More generally, in accordance with the teachings herein the inventors have discovered a series of markers that may be used independently or collectively to accurately identify, sort enrich and/or and characterize cancer stem cells from a wide variety of tumors. Using selected biochemical techniques, the novel association of the disclosed markers with specific, self-renewing malignant cells in the tumor architecture provides for enrichment, isolation, or purification of phenotypically distinct cells or tumor cell subpopulations that are capable of perpetual self-renewal and tumor recapitulation. In contrast to the prior art, and as evidenced by the Examples below, the disclosed markers are capable of recognizing or identifying tumor initiating cells from a variety of tumors. The enrichment or isolation of these marker-defined, relatively homogeneous cell populations in turn allows for the extensive characterization of the constituent cancer stem cells, including the elucidation of potential therapeutic targets and screening of pharmaceutical compounds. In other embodiments the cells and compositions of the instant invention may be used in conjunction with animal models such as non-traditional xenograft models to facilitate research and drug development efforts. Moreover, the disclosed markers may further be used in clinical and non-clinical settings for the diagnosis, classification, monitoring and management of hyperproliferative disorders as well as providing associated kits or other articles of manufacture.

[0063] While certain features of the invention have been illustrated and described, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

II. Selected abbreviations

[0064] CSC, cancer stem cells; ETP, early tumor progenitor cells; FACS, fluorescence activated cell sorting; LTP, late tumor progenitor cells; MACS, magnetic-assisted cell sorting; TIC, tumor initiating cells; TICAM, tumor initiating cell associated marker; TPC, tumor perpetuating cells; TPCAM, tumor perpetuating cell associated marker; TProg, tumor progenitor cells; TProgAM, highly proliferative tumor progenitor cell associated marker; NTX, non-traditional xenograft;

III. Definitions

[0065] For convenience, certain terms employed in the entire application (including the specification, examples, and appended claims) are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0066] As used herein the term "tumor initiating cells" (TIC) shall be held to mean tumorigenic cells or populations thereof that are capable of at least significant proliferation capacity and an ability to initiate tumors in immunocompromised mice when transplanted. In this regard tumor initiating cell populations will comprise both tumor perpetuating cells and highly proliferative tumor progenitor cells.

[0067] For purposes of the instant disclosure, "tumor perpetuating cells" or "cancer stem cells" (TPC or CSC) may be used interchangeably and are defined as cells that can undergo self-renewal as well as abnormal proliferation and differentiation to form a tumor. Functional features of tumor perpetuating cells are that they are tumorigenic; they can give rise to additional tumorigenic cells by self-renewal; and they can fully recapitulate a heterogeneous tumor mass by giving rise to non-tumorigenic tumor cells.

[0068] With respect to the instant invention the terms "tumor progenitor cells" (TProg) refer to tumorigenic cells or populations thereof that are progeny of TPC and possess the capacity for at least some tumor recapitulation and limited self-renewal when cultured in vitro or passaged through a compatible animal host. Certain TProg cells or populations may be referred to as highly proliferative herein. As discussed in more detail below, TProg cell populations comprise both "early tumor progenitor cells" (ETP) and "late tumor progenitor cells" (LTP) that may be distinguished by phenotype (e.g., cell surface markers) and different capacities to recapitulate tumor cell architecture.

[0069] In the instant application a "tumor initiating cell associated marker" (TICAM) shall be held to comprise any marker that is associated with a tumor initiating cell or cell subpopulation and allows for the identification, characterization and optional isolation or

enrichment thereof.

[0070] As used herein a "tumor perpetuating cell associated marker" (TPCAM) shall be held to comprise any marker that is associated with a tumor perpetuating cell or cell subpopulation and allows for the identification, characterization and optional isolation or enrichment thereof. It will be appreciated that TICAM and TPCAM are not mutually exclusive and that an individual marker may comprise both simultaneously.

[0071] For the purposes of the instant application a "tumor progenitor cell associated marker" (TProgAM) comprises any marker that is associated with a tumor progenitor cell or cell subpopulation and allows for the identification, characterization and optional isolation or enrichment thereof. It will be appreciated that TICAM and TProgAM are not mutually exclusive and that an individual marker may comprise both simultaneously.

[0072] As used herein, particularly in reference to an isolated cell or isolated cell population, the term "tumorigenic" refers to a cell derived from a tumor that is capable of forming a tumor, when dissociated and transplanted into a suitable animal model such as an immunocompromised mouse.

[0073] In the instant application 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 immunocompromised mice, even when transplanted in excess cell numbers.

[0074] For the purposes of the instant application the terms "selecting," "sorting," "partitioning" "sectioning" or "isolating" selected cells, cell populations or cell subpopulations may be used interchangeably and mean, unless otherwise dictated by context, that a selected cell or defined subset of cells are removed from a tissue sample and separated from other cells and contaminants that are not within the parameters defining the cell or cell population. An isolated cancer stem cell will be generally free from contamination by other cell types and have the capability of self-renewal and tumor recapitulation allowing the generation of differentiated progeny. However, when the process or treatment results in a cell population it is understood that it is impractical to provide compositions of absolute purity. In such cases the cell population is "enriched" for the selected cells which then exist in the presence of various contaminants (including other cell types) that do not materially interfere with the function or properties of the selected cell subpopulation.

[0075] As used herein and applied to cells or cell populations, the term "enriched" may be construed broadly and held to mean any processed or treated cell population that contains a

higher percentage of a selected cell type than is found in an untreated, otherwise equivalent cell population or sample. In some preferred embodiments enriching a cell population 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. In other preferred embodiments enriched cell populations of the instant invention will comprise at least 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, or 99% of the selected cell type. In yet other embodiments, an enriched preparation of tumor perpetuating cells may be described as comprising about 1% or greater or about 0.5% to about 40% of the total cell population contained in a preparation. By way of comparison, a non-enriched preparation of exemplary tumor cells would include only about 0.2% to about 2.0% or less of tumor perpetuating cells that are capable of giving rise to a secondary colony forming sphere or perpetuating a heterogeneous tumor *in vivo*. In some embodiments, the enriched preparations comprise a 100-fold, 200-fold, 500-fold, 1,000-fold, or up to a 2,000-fold or 10,000-fold to 20,000-fold enriched preparation of cancer stem cells capable of giving rise to secondary colonies or perpetuating a heterogeneous tumor *in vivo*, starting with low cell numbers.

[0076] In other aspects of the invention the term "substantially pure," with respect to a particular cell population, refers to a population of cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to the cells making up a total cell population. Recast, the terms "substantially pure" or "essentially purified", with regard to a preparation of one or more partially and/or terminally differentiated cell types, refer to a population of cells that contain fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, 2%, 1%, or less than 1%, of cells that are not tumor initiating cells.

[0077] A "marker" or "cell marker" as used herein in the context of a cell or tissue, means any trait or 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. 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 (or is not) 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). Markers contemplated herein are specifically held to be positive or negative and may denote a cell or cell subpopulation by its presence (positive) or absence (negative).

[0078] Similarly 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, quantify, separate, isolate, purify or enrich a particular cell or cell population. In specific preferred embodiments, the marker phenotype is a cell surface phenotype which may be determined by detecting or identifying the expression of a combination of cell surface markers.

[0079] "Positive," "low" and "negative" expression levels as they apply to markers or marker phenotypes are defined as follows. Cells with negative expression (i.e."-") are herein defined as those cells expressing less than, or equal to, the 95th percentile of expression observed with an isotype control antibody in the channel of fluorescence in the presence of the complete antibody staining cocktail labeling for other proteins of interest in additional channels of fluorescence emission. Those skilled in the art will appreciate that this procedure for defining negative events is referred to as "fluorescence minus one", or "FMO", staining. Cells with expression greater than the 95th percentile of expression observed with an isotype control antibody using the FMO staining procedure described above are herein defined as "positive" (i.e."+"). As defined herein there are various populations of cells broadly defined as "positive." First, cells with low expression (i.e. "lo") are generally defined as those cells with observed expression above the 95th percentile determined using FMO staining with an isotype control antibody and within one standard deviation of the 95th percentile of expression observed with an isotype control antibody using the FMO staining procedure described above. Cells with "high" expression (i.e. "hi") may be defined as those cells with observed expression above the 95th percentile determined using FMO staining with an isotype control antibody and greater than one standard deviation above the 95th percentile of expression observed with an isotype control antibody using the FMO staining procedure described above. In other embodiments the 99th percentile may preferably be used as a demarcation point between negative and positive FMO staining and in particularly preferred embodiments the percentile may be greater than 99%.

[0080] The term "lineage" as used herein describes cells with a common ancestry. For example, cells that are derived from the same tumor initiating cell may be of the same lineage though the progeny have differentiated into various classes of cells.

[0081] For the purposes of the instant disclosure the terms "binding agent," "binding molecule" and "binding entity" are synonymous unless otherwise dictated by circumstance and may be used interchangeably. In the context of the instant invention the binding agent binds, interacts, recognizes, reacts, or otherwise preferably associates with a selected marker on a defined cell or cell subpopulation. Exemplary binding agents can include, but are not limited to, an antibody or fragment thereof, an antigen, an aptamer, a nucleic acid (e.g. DNA and RNA), a

protein (e.g. receptor, enzyme, enzyme inhibitor, enzyme substrate, ligand), a peptide, a lectin, a fatty acid or lipid and a polysaccharide. For example, in a selected embodiment a compatible binding agent may comprise the hemagglutinin protein of measles virus or CD46 binding fragment thereof. In other particularly preferred embodiments the binding agent or entity comprises an antibody or fragment thereof.

[0082] As set forth herein the term "antibody" 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., marker 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, 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.

[0083] As used herein the term "epitope" refers to that portion of the target marker or antigen capable of being recognized and specifically bound by a particular antibody. When the marker is a polypeptide such as a cell surface receptor, 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.

[0084] The term "derivative" as used herein refers to molecules, including proteins and nucleic acids which have been modified through standard molecular biology methodology or chemically, for example but not limited to by techniques such as ubiquitination, labeling (a flourophore), pegylation (derivatization with polyethylene glycol) or addition of other molecules.

[0085] The term "functional derivative" and "mimetic" are used interchangeably, and refer to compounds that possess a biological activity (either functional or structural) that is substantially similar to a biological activity of the entity or molecule for which it's a functional derivative. The term functional derivative is intended to include the fragments, variants, analogues or chemical derivatives of a molecule.

[0086] As used herein, "variant" with reference to a polynucleotide or polypeptide, refers to a polynucleotide or polypeptide that can vary in primary, secondary, or tertiary structure, as compared to a reference polynucleotide or polypeptide, respectively (e.g., as compared to a wild-type polynucleotide or polypeptide). A "variant" of an antibody for example, is meant to refer to a molecule substantially similar in structure and function, e.g., where the variant retains the ability to bind with a selected antigen or a fragment thereof yet has an altered biochemical effect.

[0087] A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules not found in the other, or if the sequence of amino acid residues is not identical.

[0088] A "fragment" of a molecule, is meant to refer to any contiguous polypeptide or nucelotide subset of the molecule. Fragments of, for example, a transmembrane protein may comprise constructs that only include the extracellular domains or some portion thereof. With respect to the instant invention marker fragments or derivatives may include any immunoreactive or immunologically active portion of a selected marker.

[0089] An "analog" of a molecule such as a marker is meant to refer to a molecule similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half life, etc. The moieties can alternatively decrease the toxicity of the

molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, Pa. (1990).

[0090] As used herein, "homologous", when used to describe a polynucleotide, indicates that two polynucleotides, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least 70% of the nucleotides, usually from about 75% to 99%, and more preferably at least about 98 to 99% of the nucleotides. The term "homolog" or "homologous" as used herein also refers to homology with respect to structure and/or function. With respect to sequence homology, sequences are homologs if they are at least 50%, at least 60 at least 70%, at least 80%, at least 90%, at least 95% identical, at least 97% identical, or at least 99% identical. The term "substantially homologous" refers to sequences that are at least 90%, at least 95% identical, at least 97% identical or at least 99% identical. Homologous sequences can be the same functional gene in different species.

[0091] As used herein, the term "substantial similarity" in the context of polypeptide sequences, indicates that the polypeptide comprises a sequence with at least 60% sequence identity to a reference sequence, or 70%, or 80%, or 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-100 amino acid residues (e.g., a heavy or light chain variable region of an antibody). In the context of amino acid sequences, "substantial similarity" further includes conservative substitutions of amino acids. Thus, a polypeptide is substantially similar to a second polypeptide, for example, where the two peptides differ by one or more conservative substitutions. The term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 65 percent sequence identity, preferably at least 80 or 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity or higher). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

[0092] Determination of homologs of the genes or polypeptides of the present invention can be easily ascertained by the skilled artisan. The terms "homology" or "identity" or "similarity" are used interchangeably herein and refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology and identity can each be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules

can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. A sequence which is "unrelated" or "non-homologous" shares less than 40% identity, though preferably less than 25% identity with a sequence of the present application.

[0093] As used herein, the terms "subject" or "patient" may be used interchangeably and refer to any living organism which can be administered any derived pharmaceutical compositions of the present invention and in which cancer or a proliferative disorder can occur. The term includes, but is not limited to, humans, non-human animals, for example non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses, domestic subjects such as dogs and cats, laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. The term "subject" also includes living organisms susceptible to conditions or disease states as generally disclosed, but not limited to, throughout this specification. Examples of subjects include humans, dogs, cats, cows, goats, and mice, including transgenic species The term "non-human animals" and "non-human mammals" are used interchangeably herein includes all vertebrates, e.g., mammals, such as non-human primates, (particularly higher primates), sheep, dog, rodent (e.g. mouse or rat), guinea pig, goat, pig, cat, rabbits, cows, and non-mammals such as chickens, amphibians, reptiles etc. In one embodiment, the subject is human. In another embodiment, the subject is an experimental animal or animal substitute as a disease model.

[0094] The term "effective amount" as used herein refers to the amount of an agent and/or a pharmaceutical composition required to retard, reduce or ameliorate at least one symptom of the disease or disorder. For example, an effective amount of a potential drug compound is the amount of required to reduce the rate of growth of tumor initiating cells implanted in a test animal. Thus, an effective amount is also the amount sufficient to prevent the development of a disease symptom, or to reduce a symptom or reduce the rate of symptom progression.

[0095] The terms "malignancy," "neoplasia" and "cancer" are used interchangeably herein and refer to diseases or disorders that are characterized by uncontrolled, hyperproliferative or abnormal growth or metastasis of cells. In other aspects the term is also intended to include any disease of an organ or tissue (e.g., colorectal, pancreatic, breast, etc.) in mammals characterized by poorly controlled or uncontrolled multiplication of normal or abnormal cells in that tissue and its effect on the body as a whole. Disorders within the scope of the definition comprise benign

neoplasms, dysplasias, hyperplasias as well as neoplasms showing metastatic growth or any other transformations like e.g. leukoplakias which often precede an episode or recurrence of cancer.

[0096] As used herein, the term "refractory" is most often determined by failure to reach clinical endpoint, e.g., response, extended duration of response, extended disease-free, survival, relapse-free survival, progression-free survival and overall survival. Another way to define being refractory to a therapy is that a patient has failed to achieve a response to a therapy such that the therapy is determined to not be therapeutically effective.

[0097] As used herein, the phrase "diagnostic agent" refers to any molecule, compound, and/or substance used for the purpose of diagnosing a disease or disorder. In preferred embodiments the diagnostic agent shall comprise a binding agent associated with a reporter. Other non-limiting examples of diagnostic agents include antibodies, antibody fragments, or other proteins, including those conjugated to a detectable agent. As used herein, the term "detectable agent" or "reporter" refer to any molecule, compound and/or substance that is detectable by any methodology available to one of skill in the art. Non-limiting examples of detectable agents or reporter molecules include dyes, fluorescent tags, gases, metals, or radioisotopes. As set forth herein, "diagnostic agent" "imaging agent" and "reporter" or "reporter molecule" are equivalent and may be used interchangeably unless otherwise dictated by context.

[0098] As used herein, the term "treating" includes preventing the progression and/or reducing or reversing at least one adverse effect or symptom of a condition, disease or disorder associated with inappropriate proliferation of cells such as in, for example, cancer.

[0099] As used herein, the terms "administering" and "introducing" are used interchangeably herein and refer to the placement of the pharmaceutical compositions as disclosed herein into a subject by a method or route which results in at least partial localization of the pharmaceutical compositions at a desired site. The compounds of the present invention can be administered by any appropriate route which results in an effective treatment in the subject.

[00100] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracerebrospinal, and intrasternal injection and infusion. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein mean the administration of the pharmaceutical compositions of the present invention comprising pyrazoloanthrones and optionally other agents or material other

than directly into the central nervous system, such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[00101] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00102] The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation, or be biologically inert.

IV. Tumorigenic cell and cell subpopulations

[00103] In particularly preferred embodiments the present invention provides for the identification, characterization, enrichment and/or isolation of, respectively, tumor initiating cells (TIC), tumor perpetuating cells (TPC) and tumor progenitor cells (TProg) through the novel use of selected marker or marker combinations as set forth herein. With respect to the cancer stem cell paradigm, each of the aforementioned cell subpopulations are tumorigenic to a greater or lesser extent and, as such, are responsible for tumor growth, maintenance, metastasis and recurrence. Significantly, the instant invention allows such cell subpopulations to be identified and optionally derived from a variety of different tumor types and stages of cancer. As such, accounting for and eliminating these cells are likely critical for the effective management and treatment of a variety of diverse neoplasia. In this regard these cells or enriched cell subpopulations, alone or in combination, can be effectively employed in pharmaceutical research and development activities to identify novel therapeutic targets optimized for the suppression or depletion of tumor feeding cancer stem cells.

[00104] As has been demonstrated for blood and hematopoietic diseases, a hierarchy of cells likely exists within many solid tumors wherein distinct tumor cell subpopulations possess different proliferation and differentiation potential. More specifically, as illustrated in FIG. 19, cell populations isolated using the markers disclosed herein support the hypothesis that in tumors where there exists significant differentiation capacity and the ability to engage these differentiation programs, tumor perpetuating cells (i.e. CSC) lie at the top of the cellular

hierarchy in cancer. These cells exhibit the classical characteristics defining stem cells, whereas tumor progenitor cells exhibit similar characteristics, minus the ability to self-renew as demonstrated by serial transplantation of defined and highly purified cells.

[00105] As previously alluded to, tumor initiating cell populations encompass both tumor perpetuating cells and highly proliferative tumor progenitor cells, 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 tumor cell subpopulations (> 99% purity) due, at least in part, to its ability to discriminate between single cells and clumps of cells (i.e. doublets, etc.).

[00106] 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/or 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, demonstration of differential proliferation/differentiation capacity in vitro using colony forming cell assays, transcriptional profiling, and characterization of drug resistance, although marker expression may change with culture conditions and with cell line passage *in vitro*.

[00107] 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 defined above a non-tumorigenic cell 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.

[00108] For purposes of the instant disclosure, TProg are also categorized as tumor initiating cells 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, as will be seen in the Examples below 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. In any event both types of tumor-initiating cells are likely represented in the typical tumor mass of a single patient and, as will be shown in the Examples below, are subject to identification, characterization, separation and/or enrichment or isolation as set forth herein.

[00109] More generally, 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). The ability to identify, characterize and separate or enrich these distinct cell subpopulations as disclosed herein is critical in understanding tumor etiology and subsequently identifying novel compounds or modulators that may form the basis for more effective therapeutic treatments.

[00110] As explained in more detail below, virtually all colorectal, pancreatic, non-small cell

lung cancer and triple-negative breast tumor cells are ESA^+ $CD24^+$ $CD34^-$ and thus these markers have little utility in identifying tumor cell subpopulations. Surprisingly, it has been discovered that the disclosed TICAM, TPCAM and TProgAM are particularly effective markers for identifying, characterizing and optionally isolating or enriching selected tumorigenic cell populations. For example, in one particularly preferred embodiment comprising colorectal cancer, it has been demonstrated that $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells are able to generate fully heterogeneous tumors consisting, in part, of $CD46^{hi}$ $CD324^+$ $CD66c^-$ and $CD66c^+$ cells. This is significant because, although $CD46^{hi}$ $CD324^+$ $CD66c^+$ cells are tumorigenic, they do not have the ability to generate $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells and are unable to efficiently fuel tumor growth through serial transplantation. These data, combined with the observations that the cells with the a) ability to consistently generate heterogeneous tumors upon serial transplantation; b) most colony forming cell potential; and c) potential to generate CD66c cells and protein *in vitro* are $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells supports the hypothesis that $CD46^{hi}$ $CD324^+$ $CD66c^+$ cells are daughters of $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells with restricted proliferation capacity and potential.

[00111] The general, reduction in *in vivo* tumorigenicity and *in vitro* colony forming potential along with a reduced ability to generate CD66c as cells lose expression of CD324 supports the hypothesis that $CD46^{hi}$ $CD324^-$ $CD66^+$ cells represent a late tumor progenitor (LTP) cell population that has some residual proliferative ability, but generally has no capacity for self-renewal. Finally, in support of the cellular hierarchy laid out in FIG. 19A, $CD46^-$ cells are also $CD324^-$ and are generally $CD66c^-$ as well. These NTG cells likely represent the terminally differentiated progeny, which in the colon include goblet cells (G) and enteroendocrine (eE) cells of the secretory lineage or enterocytes (E) of the absorptive lineage. Gene expression of the isolated cell subpopulations supports this hypothesis, as $CD46^-$ cells express many genes attributed to terminally differentiated secretory and/or absorptive cell types typical of colorectal tissue.

[00112] Unlike many conventional prior art therapeutic agents, novel compounds and compositions identified and developed in accordance with 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, cell surface protein, etc.) of the selected modulator. It will be appreciated that 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. With selected

modulators the reduction in the frequency of tumor initiating cells occurs as a result of a change in one or more physiological pathways. Pathway intervention or modulation, 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 neoplastic disorders by inhibiting tumorigenesis, tumor maintenance and/or metastasis and recurrence.

[00113] As will be discussed in more detail below and shown in the Examples, preferred methods used to assess such a reduction in the frequency of tumor initiating cells comprise limiting dilution analysis, either *in vitro* or *in vivo*, using either bulk tumor cells or tumor cell subpopulations characterized and isolated in accordance with the present invention. Preferably, the assessment employs Poisson distribution statistical analysis or otherwise assesses the frequency of measureable events such as the ability to generate tumors *in vivo* or not. While such limiting dilution analysis comprise preferred methods of calculating a 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. It is also possible to determine reduction of frequency through well-known flow cytometric or immunohistochemical means assessing the respective tumor cell subpopulations based upon their profile of marker expression, as laid out in the instant invention. 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.

V. Sources of Tumor Initiating Cells

[00114] Except where otherwise required, the invention can be practiced with tumor initiating cells of any vertebrate species. Included are cancer stem cells from humans; as well as non-human primates, domestic animals including those typically used in research, livestock, and other non-human mammals.

[00115] Those skilled in art will appreciate that the aforementioned cells and cell subpopulations may be obtained from a variety of tumors at different clinical or pathological stages. Moreover, as will be illustrated in the Examples below the starting heterogeneous cell populations may be obtained from a primary tumor sample or biopsy taken directly from the patient or from secondary tumors that have been passaged in vivo or cultured in vitro. In particularly preferred embodiments the tumor cell populations to be analyzed, characterized and/or enriched are derived from tumors arising from either bulk tumor material that contain

TIC, or enriched TIC populations that have been implanted in immunocompromised mice. In other embodiments the tumor sample will be obtained from a non-traditional xenograft (NTX) tumor bank. Whichever tumor source is selected, the tumors are preferably handled in accordance with good laboratory practices and may be dissociated into single cell suspensions using art recognized mechanical and enzymatic dissociation techniques prior to being characterized, separated and/or isolated.

[00116] Exemplary tumors which may be used as a source of cells, either directly or indirectly through in vitro or in vivo culturing or passaging, include, but are not limited to, sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, mesothelioma, Ewing's tumor, lymphangioendotheliosarcoma, synovioma, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, astrocytic tumors (e.g., diffuse, infiltrating gliomas, anaplastic astrocytoma, glioblastoma, gliosarcoma, pilocytic astrocytoma, pleomorphic xanthoastrocytoma), oligodendroglial tumors and mixed gliomas (e.g., oligodendroglioma, anaplastic oligodendroglioma, oligoastrocytoma, anaplastic oligoastrocytoma), ependymal tumors (e.g., ependymoma, anaplastic ependymoma, myxopapillary ependymoma, subependymoma), choroid plexus tumors, neuroepithelial tumors of uncertain origin (astroblastoma, chordoid glioma, gliomatosis cerebri), neuronal and mixed-neuronal-glial tumors (e.g., ganglioglioma and gangliocytoma, desmoplastic infantile astrocytoma and ganglioglioma, dysembryoplastic neuroepithelial tumor, central neurocytoma, cerebellar liponeurocytoma, paraganglioglioma), pineal parenchymal tumors, embryonal tumors (medulloepithelioma, ependymoblastoma, medulloblastoma, primitive neuroectodemmal tumor, atypical teratoid/rhabdoid tumor), peripheral neuroblastic tumors, tumors of cranial and peripheral nerves (e.g., schwannoma, neurinofibroma, perineurioma, malignant peripheral nerve sheath tumor), meningeal tumors (e.g., meningeomas, mesenchymal, non-meningothelial tumors, haemangiopericytomas, melanocytic lesions), germ cell tumors, tumors of the sellar region (e.g., craniopharyngioma, granular cell tumor of the neurohypophysis), hemangioblastoma, melanoma, and retinoblastoma.

[00117] In particularly preferred embodiments the tumor sample will be obtained from a

human colorectal, breast, non-small cell lung, small cell lung, pancreatic, melanoma, ovarian or head and neck tumor.

[00118] Further, as alluded to above the enriched cell populations of the instant invention may be derived from primary tumors obtained directly from subjects suffering from a neoplastic disorder. However, in other embodiments isolated cell populations are obtained from patient-derived tumors that have been cultured *in vitro* or passaged through non-human animals. With regard to the present invention, and as described in more detail and in the Examples below, a 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 tumors having well defined lineages greatly facilitate the identification and isolation of TPC as set forth herein as they allow for the reproducible and repeated characterization of cells purified from the cell lines.

[00119] More particularly, the existence of such models allows for consistent verification that the identified or isolated cells or cell subpopulations (as defined by marker phenotype) are actually TIC in that the TPC components 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. That is, the ability to use small populations of enriched or isolated cells to generate fully heterogeneous tumors in mice is strongly indicative of the fact that the isolated cells comprise TPC and validates associated markers and putative therapeutic targets. 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 conventional therapeutic agents such as irinotecan, which provides clinically relevant insights into underlying mechanisms driving tumor growth, resistance to current therapies and tumor recurrence.

VI. Defining tumorigenic cell populations

[00120] In preferred embodiments the present invention provides for the identification, characterization and, optionally, the enrichment or isolation of selected tumor initiating cells or cell subpopulations. This analysis and/or physical enrichment of the cancer stem cell populations is accomplished by differential cell analysis using one or more markers (i.e., TICAM, TPCAM and TProgAM) that are phenotypically heterogeneous with regard to different cell populations. As previously discussed it will be appreciated that any trait or discernable characteristic, or

combinations thereof, that allows for the differentiation of various cell populations constitutes a marker that may be used in accordance with the teachings herein. Thus, while in preferred embodiments such markers are cell surface proteins, other characteristics or traits may function to differentiate the cell subpopulations and are expressly contemplated as be within the scope of the invention. For example, in addition to cell surface phenotyping, it may be useful to quantitate or characterize cell subpopulations based on certain tumor perpetuating cell characteristics. These attributes can be determined, for instance, by measuring the ability of the cells to self-renew and proliferate in culture; or by determining the presence of particular activated pathway in these cells. For example, in preferred embodiments a nucleic acid construct may be introduced into a cell or population of cells, where the construct comprises sequences encoding a detectable marker, and wherein the marker is operably linked to a transcriptional response element regulated by or associated with the selected pathway (e.g., one that covers the expression of a cell surface protein TICAM). As the pathway is activated the detectable marker is expressed, and indicates that the cell or cell subpopulation comprises tumor perpetuating cells. In some embodiments of the invention, the detectable marker is a fluorescent protein, e.g. green fluorescent protein (GFP) and variants thereof. Viable cells expressing GFP can be sorted, in order to isolate or enrich for the cell subpopulations of interest. In this aspect of the invention, the disclosed methods may be used to enrich for tumor perpetuating cells.

[00121] As disclosed herein, certain aspects of the present invention are directed to identifying or characterizing and, optionally, enriching or isolating tumor initiating cells or cell subpopulations thereof (e.g., TPC and/or TProg). In this respect the inventors have surprisingly discovered selected markers and/or marker combinations that allow for the rapid and effective identification, characterization and optionally, the enrichment or isolation of tumor initiating cells or populations thereof. As discussed briefly above a "marker", as used herein 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).

[00122] It will be appreciated that markers, marker combinations or marker phenotypes may vary as to specific cells or cell subpopulations or with regard to cell lifecycles or hierarchy. In some embodiments markers will comprise stable or transitory characteristics, whether

phenotypical, morphological, functional or biochemical (e.g., enzymatic) particular to a specific cell type or population.

[00123] In particularly preferred embodiments compatible markers are proteins or polypeptides and, more preferably, possess one or more epitopes or active sites that allow for the association of binding molecules including, but not limited to, antibodies or immunoreactive fragments thereof, ligands, substrates or enzymes. However, for the purposes of the instant application a marker may consist of any molecule or metabolite associated with a cell including, but not limited to, proteins (peptides and polypeptides), lipids, polysaccharides, nucleic acids and steroids. In particularly preferred embodiments the marker of interest will be located on the surface of the cell at the appropriate time but in other embodiments the marker may be located or positioned intercellularly or intracellularly (e.g., on the nucleus). 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. As discussed herein it will be further appreciated that compatible markers may be detected by any method available to one of ordinary skill in the art. 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. Specific preferred markers will be discussed in more detail below.

[00124] The information thus derived from such markers is useful in prognosis and diagnosis, including susceptibility to acceleration of disease, status of a diseased state and response to changes in the environment, such as the passage of time, treatment with drugs or other modalities. The cells can also be classified as to their ability to respond to therapeutic agents and treatments, isolated for research purposes, screened for gene expression, and the like. Clinical samples can be further characterized by genetic analysis, proteomics, cell surface staining, or other means, in order to determine the presence of markers that are useful in classification. For example, genetic abnormalities can be causative of disease susceptibility or drug responsiveness, or can be linked to such phenotypes.

[00125] Whichever markers are ultimately chosen to identify or characterize the selected cell

subpopulations, the actual monitoring or analysis may be conducted using any one of a number of standard techniques well known to one of skill in the art. For example, cell surface marker expression can be assayed by immunoassays including, but not limited to, western blots, immunohistochemistry, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, immunofluorescence, protein A immunoassays, laser capture microdissection, massively multiparametric *mass* cytometry, flow cytometry, and FACS analysis. In certain embodiments the amount of cancer stem cells in a test sample from a subject may be determined by comparing the results to the amount of cancer stem cells in a reference sample (e.g., a sample from a subject who has no detectable cancer) or to a predetermined reference range, or to the patient him/herself at an earlier time point (e.g., prior to, or during therapy).

[00126] In specific embodiments, the cancer stem cell population is obtained from a tumor sample from a patient and is characterized by flow cytometry. As is well known in the art and demonstrated in the appended Examples, this method exploits the differential expression of certain surface markers on cancer stem cells relative to the bulk of the tumor. Labeled antibodies (e.g., fluorescent antibodies) can be used to react with, or binding agents recognizing other binding agents (i.e., secondary binding agents) that recognize, the markers on cells in the sample for the purpose of enriching or isolating cells by any number of methods including magnetic separation or FACS. In some embodiments, a combination of cell surface markers are utilized in order to further define or quantify the cancer stem cells in the sample in situ (e.g. by immunofluorescence or immunohistochemistry) or using methods analyzing single cells following tumor dissociation (e.g. flow cytometry or massively multiparametric *mass* cytometry). For example, both positive and negative cell sorting may be used to assess various TPC subpopulations or quantify the amount of cancer stem cells in the sample. The number or frequency of cancer stem cells in the sample might also be determined by transplanting un-enriched tumor cells in limiting dilution into immunocompromised mice, followed by the analysis of the frequency of tumorigenesis, independent of rate, by the different dilutions using Poisson distribution statistics. Moreover, as demonstrated in the Examples below specific tumor types can be characterized by assessing the frequency and nature of cells expressing tumor perpetuating cell and/or tumor progenitor cell markers. Preferably the tumors comprise tumor initiating cells identified using the markers disclosed herein, including those markers demonstrated to be co-expressed with respective TIC populations (i.e. TICAM), such as those

described herein and whose presence may be confirmed through isolation and transplant in immunocompromised mice.

[00127] In other selected embodiments the identification and characterization of tumor initiating cells in a sample, e.g., a tissue sample, such as a solid tumor biopsy, is determined using immunohistochemistry or immunofluorescence techniques. As known in the art, these methods exploit the differential expression of certain surface markers on tumor initiating cells relative to the bulk of the tumor. Antibodies (e.g., antibodies conjugated to a fluorophore) can be used to react with the cell subpopulation of interest. These antibodies may be directly labeled, or detected with secondary binding agents that detect the primary antibody. In preferred embodiments a combination of certain cell surface markers are used to further define and, if desired, quantify cancer stem cells in the un-dissociated sample (i.e. in situ). Further, such staining techniques allow for visualization of selected tumor cell subpopulations by assessing the expression of certain markers that distinguish the cancer stem or progenitor cells of interest. Such techniques facilitate the localization of cancer stem and/or progenitor cells within the context of the tumor, allowing spatial characteristics such as the distance from blood vessels to be established.

[00128] Using the aforementioned art-recognized techniques for cell and cell population recognition and characterization in conjunction with the teachings herein one can readily identify the disclosed tumorigenic cell subpopulations. More particularly, as discussed extensively herein the present invention is predicated upon, at least in part, the heretofore unknown association between certain tumor initiating cell associated markers (TICAM) and tumor initiating cells or cell subpopulations. These markers, whether by their absence or presence on the relevant cell populations, allow for the effective identification or characterization of tumor initiating cells and their respective TPC and TProg component subpopulations. Moreover, in preferred embodiments such as those set forth in the Examples below, these same markers allow for the rapid and efficient identification, separation, partitioning, sectioning, isolation or enrichment of defined tumor initiating cell populations or their respective TPC and/or TProg cell subpopulations. As set forth above and in accordance with the instant invention, TICAM shall be held to mean any marker or marker phenotype that allows for the identification, characterization and, optionally, isolation and enrichment of tumor initiating cells or cell populations. Similarly, marker or marker phenotypes that, by their presence or absence, may be used to identify or characterize and, optionally, isolate or enrich TPC or TProg or cell subpopulations comprise TPCAM or TProgAM as defined previously. As explicitly noted above it will be understood that three sets of markers are not mutually exclusive and that individual markers may be associated

with different cell subpopulations (e.g., a single marker may be a TICAM and a TPCAM). Preferably such markers are proteins and even more preferably are cell surface proteins.

[00129] For the purposes of the instant application particularly preferred TICAM comprise CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9. In particularly preferred embodiments the marker will comprise CD46. In other preferred embodiments the marker will comprise CD324. In yet other preferred embodiments the marker will comprise CD66c. In still other preferred embodiments a combination of CD46 marker and CD324 marker will be used to identify and, optionally, enrich or isolate tumorigenic cell subpopulations.

[00130] With regard to the listed TICAM FIGS. 9A and B provide tumor expression information in a tabular form while FIGS. 10-12 show expression data of the individual markers as derived from defined tumor initiating cell populations. In this respect it will be appreciated that, although the of tenets of the instant invention are most commonly exemplified or confirmed using the TICAM markers CD46 and CD324, the compatibility of other listed markers with the teachings herein is readily determined for specific tumors without undue experimentation. That is, despite the different etiology of the cancers and biology of the respective tissues in which these tumors arose, FIGS. 10-12 demonstrate that the compatibility and utility of a listed marker may readily discerned using standard techniques. In view of these teachings and associated data one skilled in the art would easily be able to determine the optimal TICAM or combinations of TICAM to use to analyze or separate tumorigenic cell subpopulations from a particular type of tumor. The unexpected finding that the disclosed markers are useful over a range of tumors is merely underscores the novelty of the invention.

[00131] Pursuant to the instant disclosure it will be appreciated that the aforementioned TICAM may be used, alone or in combination, to identify, characterize or, optionally, purify, separate, enrich or isolate defined tumorigenic cell populations using art-recognized techniques. In preferred embodiments the cell populations will comprise tumor perpetuating cells. In other preferred embodiments the cell populations identified or enriched using selected TICAM shall comprise tumor perpetuating cells and tumor progenitor cells. With respect to these latter embodiments, and as shown in the appended Examples, the TIC components may further be characterized and/or separated by contacting the TICAM enriched population to TPCAM and/or

TProgAM that provide for the isolation of the respective tumorigenic cell subpopulations.

[00132] For example, in particularly preferred embodiments it has been demonstrated that TIC may be identified and characterized through a combination of CD46 and CD324 and exhibit a $CD46^{hi}$ $CD324^{+}$ marker phenotype. Accordingly, in other particularly preferred embodiments the isolated or enriched tumor cell subpopulations are obtained by contacting the TIC with binding agents for CD324 and CD46 wherein the binding agents may contacted with the TIC in any order or simultaneously. Related preferred embodiments comprise isolated or enriched TIC populations comprising a $CD46^{hi}$ $CD324^{+}$ marker phenotype.

[00133] In another selected embodiment comprising TIC in colorectal cancer, it has been demonstrated that $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells are able to generate fully heterogeneous tumors consisting, in part, of $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ and $CD66c^{+}$ cells. This is significant because, although $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells are tumorigenic, they do not have the ability to generate $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells and are unable to efficiently fuel tumor growth through serial transplantation. Accordingly, other preferred embodiments of the instant invention comprise isolated or enriched TIC populations comprising a $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ marker phenotype.

[00134] As disclosed herein a particularly preferred marker associated with tumorigenic cells is CD46 that has been found to act advantageously as a TICAM. As shown throughout the Examples below, the CD46 marker may be used, alone or in combination with other markers, to identify, characterize, interrogate, recognize or distinguish tumor initiating cells, tumor perpetuating cells or discrete cell subpopulations thereof. Further, in other preferred embodiments the CD46 marker may be used, alone or in combination with other markers, to effectively section, partition, isolate, purify or enrich tumorigenic cells or subpopulations thereof. Preferably the identification, characterization or isolation of the cancer stem cells or cell subpopulation will be effected with a binding agent that immunospecifically reacts with full length CD46 or a splice variant or isoform thereof. In other preferred embodiments the CD46 binding agent will comprise an antibody or immunoreactive fragment thereof. In other preferred embodiments the CD46 binding agent will be associated with a detectable reporter entity.

[00135] CD46 is also known as membrane cofactor protein or MCP. It is a type I transmembrane protein that is widely expressed but has a number of isoforms as a result of alternate exon splicing and glycosylation. Recently Karosi et al., *Laryngoscope* 118: 1669-1676 (Sept. 2008), which is incorporated herein by reference in its entirety, reported detecting fourteen isoforms of the molecule. The mRNA is transcribed from a single gene located at chromosome 1q32 and undergoes extensive alternative splicing to produce multiple transcripts encoding the various protein isoforms. Of the 14 exons comprising the gene, it appears that exons 1-6 are

conserved in all CD46 protein isoforms, whereas exons 7 to 9 encode variably utilized serine-threonin-proline ("STP") rich regions, leading to the major hypervariability in the protein isoforms. Exons 11 and 12 encode the transmembrane region of CD46, while exons 13 and 14 encode the cytoplasmic tail of the protein. The longest mRNA transcript, variant A (NM_002389), contains sequences from all fourteen exons of the gene. Variable splicing of exons 7, 8, 9, and 13 is believed to yield the majority of CD46's fourteen isoforms, with the predominant observed protein isoforms of 66 and 56 kDa arising from alternative inclusion or exclusion of exon 8. Alternate inclusion/exclusion of exon 13 leads to changes in the encoded sequence of the cytoplasmic tail of the molecule, with the suggestion that these changes affect subcellular trafficking, stability, and the signaling properties of the protein.

[00136] As set forth in Karosi et al., CD46 mRNA isoform D comprises exons 1-6, 8-12 and 14 of the CD46 gene (equivalent to the sequence NM_153826, encoding the protein NP_722548), isoform F comprises exons 1-6, 9-12, and 14 (equivalent to the sequence NM_172353, encoding NP_758863), and isoform J comprises exons 1-6, 8, 10-12, and 14 (equivalent to the sequence NM_172356, encoding NP_758866). More specifically the CD46 molecule comprises four N-terminal short consensus repeat (SCR) modules ("Sushi" domains: 4 Cysteines in a 1-3, 2-4 linkage topology), where these SCR domains are encoded by the first six exons of the gene. The SCR2, 3, and 4 modules have the C3b/C4b binding and regulatory activity (discussed below), while the SCR1 module and sequences distal of SCR4 are not essential for complement regulatory function, The membrane-proximal extracellular sequence, encoded by the alternatively utilized exons 7-9 as well as exon 10, is heavily glycosylated, mainly via O-linked carbohydrates.

[00137] For the purposes of the instant disclosure the term "CD46" shall be held to mean any protein as set forth immediately above including any splice variant or immunoreactive fragment thereof as well as any nucleic acid sequence encoding such proteins, splice variants or fragments unless otherwise contextually dictated. Thus, as discussed above a "CD46 marker" would broadly include any detectible protein, peptide or nucleic acid sequence that constitutes or encodes for the CD46 antigen. In preferred embodiments the CD46 marker will comprise the full length glycoprotein (variant A) or reported splice variant or immunoreactive fragment thereof. Even more preferably the CD46 protein marker will be present on the cell surface of the selected tumorigenic cell population. In other preferred embodiments the CD46 marker will comprise a nucleic acid sequence (e.g., DNA or mRNA) encoding full length CD46, a splice variant or fragment thereof.

[00138] Another preferred marker that may optionally be used to characterize selected tumor

initiating cell subpopulations in accordance with the teachings herein is human CD324 (hCD324) which has an exemplary nucleic acid sequence corresponding to GenBank Accession No: NM_004360 and an exemplary preproprotein amino acid sequence corresponding to GenBank Accession No: NP_004351 each of which is incorporated herein by reference. CD324 (also known as E-cadherin, uvomorulin, cadherin-1 or CAM 120/80) is a transmembrane protein that mediates calcium-dependent cell adhesion. It is specifically expressed in epithelial cells, where it is involved in maintaining their phenotype. The cytoplasmic domain of E-cadherin binds to β-catenin, which is itself bound to the actin filament networks of the cytoskeleton. This E-cadherin / β-catenin binding plays an essential role in stabilizing cell/cell adhesions of the epithelial tissue. The loss of E-cadherin can therefore reduce cell adhesion and increase the invasive capacity of cancer cells. A reduction in expression of E-cadherin or of β-catenin is generally associated with greater aggressiveness and dedifferentiation of the tumor, in particular with respect to gastrointestinal cancers. It has also been shown that patients having colorectal cancer and underexpressing E-cadherin have a more unfavorable prognosis than patients having a normal expression level.

[00139] Still another preferred marker that may be used in accordance with the present invention is CD66c (also called or NCA-90 or CEACAM6). Human CD66c (hCD66c) nucleic acid sequence corresponds to GenBank Accession No: NM_002483 while the amino acid sequence of the protein precursor (344 aa) corresponds to GenBank Accession No: NP_002474 each of which is incorporated herein by reference. The CD66c glycoprotein belongs to the immunoglobulin family, characterized by variable domains in the N-terminal part of the protein, and constant domains containing disulfide bridges inducing the formation of loops (Hammarstrom and Baranov 2001). CD66c is normally expressed at the surface of the granulocytes, macrophages and polynuclear neutrophils, but also by the epithelial cells in the colon, the lungs and the spleen (Audette et al., 1987; Kolbinger et al., 1989; von Kleist et al., 1972; Jantscheff et al., 2003). CD66c is also expressed in proliferating cells of hyperplastic colonic polyps and adenomas, compared with normal mucosa, as well as by many human cancers (Scholzel et al., Am J Pathol 157:1051-52, 2000; Kuroki et al., Anticancer Res 19:5599-5606, 1999).

VII. Binding Agents

[00140] As discussed throughout the instant application the disclosed markers are preferably detected, recognized or interrogated by a binding agent as defined herein. Construed broadly for the purposes of the present invention a compatible binding agent may essentially comprise any

entity or molecule that preferably associates with a marker and is detectible. In this regard the binding agent may take on many forms including, but not limited to small molecules, peptides, proteins and nucleic acids including DNA and RNA. More particularly the binding agents of the present invention may comprise a receptor, enzyme, enzyme inhibitor, enzyme substrate, ligand, lectin, fatty acid, aptamer, lipid or polysaccharide. For example, in a selected embodiment a compatible binding agent may comprise the hemagglutinin protein of measles virus or CD46 binding fragment thereof. In other particularly preferred embodiments the binding agent or entity comprises an antibody or fragment thereof. In yet other preferred embodiments the binding agent may comprise a marker specific small molecule binding agents that could disrupt receptor-ligand interactions, receptor-activation or dimierization/multimerization, co-receptor binding, or other myriad intracellular processes. In view of the instant disclosure the selection of compatible binding agents is well within the purview of one skilled in the art without undue experimentation.

[00141] As alluded to particularly preferred embodiments of the instant invention comprise binding agents 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., marker 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 herein, 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.

[00142] Moreover, 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.

[00143] Within the context of the instant invention it will be appreciated that compatible antibodies specific for the disclosed TICAM are often commercially available (e.g., from Life Technologies or BioLegend, Inc.). In any event, antibodies or their derivatives and/or their fragments can be provided by methods that are well known to the person skilled in the art and include hybridoma technology in normal or transgenic mice or in rabbits, or phage display antibody technology. In one embodiment, genetic immunization is used. This technique comprises administration of a nucleic acid sequence, or a functional equivalent thereof, encoding at least one antigen of interest, to a non-human animal. The encoded antigen(s) is/are produced by the animal, which stimulates the animal's immune system against said antigen(s). In other embodiments antigens in the form of proteins or peptides are injected directly with and without adjuvants. However the immunogen is introduced an immune response against said antigen(s) is elicited in the animal. Subsequently, T-cells, B-cells and/or antibodies specific for an antigen of interest are preferably obtained from said animal. The T-cells, B-cells and/or antibodies are optionally further processed. In one preferred embodiment, an obtained B-cell of interest is used in hybridoma technology wherein said obtained B-cell is fused with a tumor cell in order to produce a hybrid antibody producing cell.

[00144] More particularly preferred embodiments of the invention employ monoclonal antibodies as binding agents. 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 CD46, or an immunoreactive portion thereof. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunoassay (ELISA) or a radioimmunoassay.

[00145] 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.

[00146] More generally, methods of producing polyclonal and monoclonal antibodies are known to those of ordinary skill in the art. See, e.g., Coligan, Current Protocols in Immunology Wiley/Greene, N.Y., 1991; and Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Press, NY, 1989; Stites et al., (eds.) Basic and Clinical Immunology (4th ed.) Lange Medical Publications, Los Altos, Calif., and references cited therein; Goding, Monoclonal Antibodies: Principles and Practice (2d ed.) Academic Press, New York, N.Y., 1986; and Kohler and Milstein, Nature 256: 495-497, 1975. Other suitable techniques for antibody preparation include selection of libraries of recombinant antibodies in phage or similar vectors. See, Huse et al., Science 246: 1275-1281, 1989; and Ward et al., Nature 341: 544-546, 1989. Immunoglobulins and certain variants thereof are known and many have been prepared in recombinant cell culture (e.g., see U.S. Pat. No. 4,745,055; U.S. Pat. No. 4,444,487; WO 88/03565; EP 256,654; EP 120,694; EP 125,023; Faoulkner et al., Nature 298:286, 1982; Morrison, J. Immunol. 123:793, 1979; Morrison et al., Ann Rev. Immunol 2:239, 1984). Detailed methods for preparation of chimeric (humanized) antibodies can be found in U.S. Pat. No. 5,482,856. Additional details on humanization and other antibody production and engineering techniques can be found in Borrebaeck (ed), Antibody Engineering, 2nd Edition Freeman and Company, NY, 1995; McCafferty et al., Antibody Engineering, A Practical Approach, IRL at Oxford Press, Oxford, England, 1996, and Paul Antibody Engineering Protocols Humana Press, Towata, N.J., 1995. Each of the forgoing is incorporated herein by reference in its entirety.

[00147] No matter how obtained or which of the aforementioned forms the antibody binding agent takes (e.g., humanized, human, etc.) preferred embodiments of the disclosed agents may

exhibit various characteristics. In this regard TICAM 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 such as affinity, epitope or domain specificity, etc. 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.

[00148] It will further be appreciated the disclosed antibodies will associate with, or bind to, discrete epitopes or determinants presented by the selected markers. 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 CD46, 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.

[00149] 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.

[00150] 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.

[00151] 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 selected embodiments, one allows the primary antibody of the invention to bind to the marker under saturating conditions and then measures the ability of the secondary antibody to bind to the same marker. If the test antibody is able to bind to the marker at the same time as the primary marker 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 the maker 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, 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. The term surface plasmon resonance, as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix. In a particularly preferred embodiment, the analysis is performed using a Biacore or ForteBio instrument.

[00152] 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.

[00153] 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.

[00154] In this respect, the present invention further encompasses the use of antibodies that have a high binding affinity for the selected marker. An antibody of the invention is said to specifically bind its target antigen when the dissociation constant K_d (k_{off}/k_{on}) 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 CD46 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.

[00155] In specific embodiments, an antibody of the invention that immunospecifically binds to CD46 has an association rate constant or k_{on} rate (CD46 (Ab) + antigen (Ag)$^{k}_{on}$←Ab-Ag) of at least $10^5M^{-1}s^{-1}$, at least $2x10^5M^{-1}s^{-1}$, at least $5x10^5M^{-1}s^{-1}$, at least $10^6M^{-1}s^{-1}$, at least $5x10^6M^{-1}s^{-1}$, at least $10^7M^{-1}s^{-1}$, at least $5x10^7M^{-1}s^{-1}$, or at least $10^8M^{-1}s^{-1}$.

[00156] In another embodiment, an antibody of the invention that immunospecifically binds to CD46 has a k_{off} rate (CD46 (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 $10^{-10}s^{-1}$.

[00157] In other selected embodiments of the present invention anti-CD46 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 $5x10^{15}M^{-1}$

[00158] Once an protein or immunoglobulin binding agent of the invention has been produced by recombinant expression, it may be purified by any method known in the art for purification of an immunoglobulin molecule, or more generally, for purification of a protein, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Further, antibody binding agents of the present invention may be fused to heterologous polypeptide sequences described herein or otherwise known in the art to facilitate purification. In particularly preferred embodiments the modulators of the instant invention will be purified, at least in part, using Protein A or Protein G affinity chromatography.

[00159] In other preferred embodiments, binding agents of the present invention, or fragments or derivatives thereof, are conjugated, coupled or otherwise associated with or to a diagnostic or detectable agent or reporter that may be a biological molecule (e.g., a peptide or nucleotide) or a small molecule or radioisotope. As set forth elsewhere herein such labeled binding agents 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 modulators. In particularly preferred embodiments the disclosed binding agents associated with a reporter or detectable agent may be useful in identifying or characterizing and, optionally, enriching, isolating, sectioning, partitioning or purifying selected tumorigenic cell populations. Such populations may be used for a myriad of purposes including, but not limited to target identification, drug research and development, toxicology studies, etc.

[00160] Such diagnosis, detection and/or separation or enrichment can be accomplished by coupling or associating the binding agent 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. In particularly preferred embodiments the labeled binding agents may be used in flow cytometric analysis or FACS.

[00161] As discussed in more detail below, the binding agents can be associated with marker sequences, such as a peptide or fluorophore to facilitate purification of cell populations or separation or diagnostic procedures such as immunohistochemistry or FACS. In particularly preferred embodiments such labels shall comprise the MAXPAR™ reagent system (University of Toronto, Stemspec). In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen), among others, many of which are commercially available. 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).

VIII. Analysis of tumorigenic cell subpopulations

[00162] As discussed immediately above the markers disclosed herein provide for the differentiation of TPC and TProg (e.g., in colorectal cancer) and TIC and NTG cells in a variety of other neoplasia. Although isolation of live cells is requisite to demonstrate functional reconstitution, and thus definitively demonstrate tumor perpetuating capability (i.e. CSC identity), knowledge of TIC, their composite TPC and TProg cell, and NTG cell identity as discerned using the disclosed markers facilitates the discovery of genes and/or proteins associated with these respective cell populations, thereby enabling discovery of diagnostic and/or therapeutic target proteins.

[00163] In using the markers disclosed herein as tools to identify TIC or TPC populations, cells or genetic and/or proteomic material isolated from these cells, can be identified and isolated

from NTG cells or contaminants through the use of art-recognized techniques such as magnetic separation, FACS (Example 2), laser capture microdissection or other techniques described above. Further, the isolated or enriched populations may be characterized and/or quantified using microarray technologies (Example 1), next-generation sequencing (Example 8), mass spectrometry, and technologies combining several aspects of these technologies, such as massively multiparametric mass cytometry. Such technologies, when applied to analyze the respective tumor cell subpopulations disclosed herein, for example, may lead to the identification of additional TIC markers, including proteins, RNA markers that may be identifiable by fluorescence in situ hybridization (FISH), biochemical markers that can be visualized with chromogenic or fluorogenic dyes, physicochemical properties such as differential adherence to material substrates (i.e. glass, plastic, etc.), or expression of transcriptional regulators that can be identified using fluorescent or luminescent reporter gene constructs inserted into the genome using any number of transfection or viral transduction methods.

[00164] Those skilled in the art will appreciate that the cell populations disclosed herein can be enriched or isolated using any single marker, any combination of the select disclosed markers, or surrogate markers that associate with the expression of those markers disclosed herein (i.e. TICAM). As set forth immediately above tumor cell subpopulations can be enriched or isolated by contacting them with binding agents and then using magnetic separation, FACS, or any other technology able to separate the disclosed tumor cell subpopulations based on inherent physicochemical properties (i.e. their charge or differential adhesion to particular substrates) or physicochemical properties conferred to distinct tumor cell subpopulations via binding agents that associate with TICAM. Though magnetic separation techniques are common and generally successful at enriching populations of cells expressing a particular marker, samples enriched in this manner are commonly contaminated by a variety of dead cells, non-target cells and cell clumps. Similarly, cells obtained by depleting a population of cells with all markers except that not expressed on a target cell population (e.g. CD66c) are rarely pure.

[00165] Accordingly, particularly preferred embodiments of the instant application employ fluorescence-activated cell sorting (FACS) for cell enrichment or isolation, as flow cytometry allows rapid detailed analysis of single cells, and discriminates size and complexity of individual cells, enabling live versus dead, large versus small, single cell versus cell clumps, and more or less granular cells to be discriminated from one other. In addition to size & complexity parameters, emitted fluorescence can be measured via an array of reflective mirrors and bandpass filters, such that multiple light emission wavelengths from distinct fluorescent proteins, intracellular chemical reactions, retained dyes, or fluorochrome-conjugated antibodies can be

interrogated in this manner on the order of many thousands of cells per second. Furthermore, using a flow cytometer with cell isolation capabilities (e.g. FACSAria; Becton Dickenson), single cells with distinct morphological, physicochemical and/or fluorescent characteristics can commonly be isolated to great purity (> 99%). Using such techniques in accordance with the teachings herein, it was possible to characterize the heterogeneity of populations of single cells obtained from primary and metastatic tumors obtained directly from cancer patients and from NTX tumors grown in mice. Moreover, it was possible to maintain the viability of these cells during the process, enabling transplantation of as few as 3 cells per mouse in demonstrating the robust tumorigenicity of cell populations isolated using the markers disclosed herein.

[00166] Because the markers disclosed in the instant invention enable precise identification and isolation of TPC in colorectal cancer, for example, and distinguish these cells from TProg cells, NTG cells and stromal cells, the resulting tumorigenic cell populations provide for detection of protein-encoding genes, micro-RNAs, long or short non-coding RNAs and/or proteins associated with CSC. That is, these genetic and/or proteomic determinants of self-renewal, differentiation, proliferation and/or survival that are preferentially expressed by the relatively homogeneous tumorigenic cell subpopulations can be identified as discussed herein evaluated for use as prospective diagnostic markers that may truly predict disease severity, response to a particular therapeutic regimen or disease outcome based on their privileged expression by TPC (i.e. CSC) in colorectal cancer, for example. Similarly, markers disclosed herein that facilitate the isolation of TIC and NTG cells from pancreatic, non-small cell lung and triple-negative breast cancer enable the discovery of better diagnostic markers associated with TIC.

[00167] The enriched or isolated solid tumor cell subpopulations provided for by the instant invention further allow for the precise elucidation cellular hierarchy and enhanced characterization of cell phenotypes. In this respect it will be understood that hematologic malignancies are among the best understood neoplastic malignancies precisely because the constituent cells are easy to obtain and the *in vivo* and *in vitro* assays to determine the fate and potential of these cells have been exhaustively developed. Similarly, oncology research has evolved to the point where, not only can the cells responsible for fueling tumor growth be identified, but they can be reliably isolated using the methods herein and their characteristics tested both *in vivo* and *in vitro*. Because tumor initiating cells (TIC) are preferably identified retrospectively by their ability to fuel tumor growth *in vivo*, techniques and instrumentation that facilitate isolation of competent TIC is essential for their precise identification. In this vein tumor perpetuating cells (i.e. the cancer stem cell subset of TIC), are best identified

retrospectively based upon their ability to fuel heterogeneous tumor growth through at least two rounds of serial transplantation using small numbers of well defined cells as demonstrated herein for colorectal cancer.

[00168] Although primary transplants are sufficient to give an initial read on tumorigenicity, thus facilitating the retrospective identification of TIC and NTG cells, a single round of transplantation is not sufficient to definitively distinguish between TPC and TProg cells, as both populations are tumorigenic. As indicated herein TProg cells, as identified and disclosed herein for colorectal cancer, differ from TPC (i.e. CSC) in their inability to completely reconstitute tumor heterogeneity, but because TPC levels are typically low, this loss in heterogeneity (i.e. loss of TPC in tumors generated by TProg cells) can be difficult to observe. A second round of transplantation using small numbers of cells is needed to demonstrate the reconstitution ability of TPC versus TProg cells, as the latter population of cells is devoid of self-renewal properties and its proliferation capacity should be exhausted prior to secondary transplantation. Done correctly, these serial transplantation experiments should use very small numbers of highly purified cells (< 200 cells isolated by FACS) and tumors from primary transplants should be allowed to grow to > 1,500 mm^3, therein providing an opportunity for TProg cells to exhaust their proliferative capacity in vivo prior to secondary transplants. Such analysis is greatly facilitated by the techniques disclosed herein.

[00169] With respect to such matters it will be appreciated that a panel of markers is preferably used to precisely identify any define population of stem and/or progenitor cells. More generally, cell populations defined by certain individual markers may not be pure but contain several different cell types, or cells with different potential, wherein additional markers are needed to parse apart the remaining heterogeneity. To determine whether a panel of markers is sufficient to precisely identify a particular cell population, the potential of that particular cell population is preferably demonstrated at the single cell level by single cell transplantation and/or lineage tracing in vivo. The above described serial transplantation methods enable identification of respective tumor cell subpopulations comprising TIC (TPC and TProg) and NTG cells.

[00170] When further modified to include the transplantation of at least 3 groups of mice with dilutions of identical cell populations isolated as described herein, one can also determine the actual frequency of TIC, or its constituent TPC or TProg cells, among the input population of cells. For example, this frequency is determined using Poisson distribution statistics, which enables the quantification of TIC among a known population of cells by empirically testing for the frequency of tumorigenicity (the functional demonstration of TIC capacity), independent of the rate at which tumors arise, using limiting dilutions of known input cell populations. The

number of defined outcomes (i.e. tumors) achieved at each dilution of input cells is used to calculate the number of TIC among the input population of cells. In this manner, the true frequency of a cell with defined potential (i.e. tumorigenicity) can be assessed. It is important to note that Poisson distribution statistics gains its power from the defined events (i.e. tumorigenicity) being limited in number over the course of dilutions assessed in the experiment. As demonstrated herein for the $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cell population isolated from the colorectal NTX-CR4 patient-derived xenograft line, TIC appear to be represented at a 1 in 5 frequency within this population of cells (FIG. 15), whereas cells expressing other markers are non-tumorigenic or very poorly tumorigenic (FIGS. 4 and 8). As evidence of the novelty of the instant invention demonstration of a 1 in 5 TIC frequency, as disclosed herein, represents a significant improvement over art recognized markers for other TIC populations, as cells with those markers demonstrate a 1:75 tumorigenic cell frequency in similar experiments (Dylla et al. 2008, PLoS ONE 3:e2428).

[00171] It will further be appreciated that, in preferred embodiments, it is possible to quantify specific tumor cell subpopulations based on their marker profile. CSC and NTG cells, for example, may be detected and quantified in tumors following treatment with a prospective therapeutic agent that may then be compared with pre-treatment controls. As discussed above, tumors may be dissociated and contacted with agents binding to the markers that define the respective tumor cell subpopulations, and the relative frequency of these cells can be assessed by flow cytometry. In accordance with the teachings herein this is demonstrated as set forth in the Examples below wherein CSC are enriched in colorectal and pancreatic tumors upon exposure to standard of care chemotherapeutic regimens involving irinotecan or gemcitabine, respectively (FIGS. 17 & 18).

[00172] With respect to limiting dilution analysis, *in vitro* enumeration of tumor initiating cell frequency may be accomplished by depositing characterized and/or fractionated 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. As alluded to above 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.

[00173] 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 (e.g., potentially compatible markers are set forth in Example 1 below) 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.

[00174] *In vitro* culture conditions have a long way to go before they mimic the physiological environment encountered in vivo; however, *in vitro* assays can serve as a decent surrogate for demonstration of functional reconstitution of a tumor in vivo by serial transplantation using the cell populations enriched or isolated using the markers disclosed herein. Specifically, cells of interest may be tested for functional properties such as proliferation and/or differentiation capacity in *in vitro* assays that might predict behavior *in vivo*. There exist examples where defined, serum-free media is able to maintain and expand tumorigenic cells in vitro (Dylla et al. 2008, PLoS ONE 3:e2428), demonstrating that tumorigenic cells can be maintained in vitro for at least brief periods of time. Such assays have typically been restricted to colony forming cell (CFC) or sphere forming assays, for example, which measure the ability of cell populations to initiate a colony (attached grouping of cells totally $\geq$ 50 cells) or a floating ball of cells (i.e. sphere) (Bao et al. 2006, Nature 444:756). The number of CFC or spheres formed can serve as a proxy for TIC frequency. Moreover, upon dissociation of these colonies and/or spheres, the ability of the composite cells to subsequently reinitiate colonies or spheres can serve as a surrogate readout for self-renewal. In this manner, the frequency of CFC/sphere forming cells and degree of self-renewal under any various culture conditions can be used to identify and characterize the potential of tumor cell subpopulations such as those isolated using the markers

herein disclosed.

[00175] As is also standard in the art, differentiation might be facilitated by the culture of tumor cell subpopulations on different attachment matrices (e.g. collagen or fibronectin), addition of certain growth factors and/or fetal calf serum, for example, thereby allowing the experimental interrogation of the differentiation potential of said tumor cell subpopulations. Furthermore, genes, proteins and/or factors that impact stem and/or progenitor cell fate decisions such as self-renewal versus differentiation might also be interrogated by co-culture of the defined TIC with such factors or following transfection or transduction with genes and/or proteins of interest. For example, traditional CFC-like assays may be informative wherein these assays can be linked to a differentiation capacity readout such as the generation of NTG cell-associated gene products (e.g. CEACAM6), as demonstrated herein (FIG. 16B and 16C). More clearly, cell differentiation potential might be measured *in vitro* based on the input of defined cell populations and monitoring differentiation processes that can be driven by culture conditions conducive to self-renewal or differentiation, followed by characterizing the resulting cells, proteins these cells are generating, and/or interrogating the resulting population's potential by *in vivo* transplantation. Other methods of characterization enabling the identification of cell populations expressing the markers disclosed herein include the use of high content imaging technologies. By utilizing parameters such as multispectral analysis facilitating the identification of specific markers and their localization in live or fixed cells, parameters such as overall colony numbers, cell number per colony and quantification of cellular morphology among cells within the colony (e.g. nuclear to cytoplasmic ratio) can be analyzed, for example. Many of these analyses can also be done manually. In *in vitro* assays where markers indicative of TIC, their composite TPC or TProg cells, and/or NTG cells, respectively, are monitored by high-content, multispectral imaging, the number or frequency or the respective tumor cell subpopulations might be determined in vitro, and responses of these cells to various stimuli that may impact self-renewal or differentiation of these cell populations can be assessed.

[00176] As evidenced by the instant disclosure, the ability to passage and analyze enriched or isolated tumor initiating cell subpopulations in animals is a valuable and significant aspect of the instant invention. In this regard compatible animal hosts may comprise model organisms such as nematode, fruit fly, zebrafish; preferably a laboratory mammal such as a mouse (nude mouse, SCID mouse, NOD/SCID mouse, Beige/SCID mouse, FOX/SCID mouse), rat, rabbit, or primate. Severely immunodeficient NOD-SCID mice are particularly suitable animal recipients of transplanted human cancer stem cells. Immunodeficient mice do not reject human tissues, and SCID and NOD-SCID mice have been characterized as hosts for in vivo studies of human

hematopoiesis and tissue engraftment. McCune et al., Science 241: 1632-9 (1988); Kamel-Reid & Dick, Science 242: 1706-9 (1988); Larochelle et al., Nat. Med. 2: 1329-37 (1996). In particularly preferred embodiments the NOD/SCID or Beige/SCID mice can be further immunosuppressed using VP-16, antibodies against asialo-GM1 protein, radiation therapy, chemotherapy, or other immunosuppressive biological agents. Additional murine models capable of propagating xenografted human tumors include Nude.Beige, NOD/SCID/IL2Rg-/- , RAG2-/-/IL2Rg-/-, RAG1-/-/IL2Rg-/-, NOD/beta-2-microglobulin-/-, and athymic nude mice. Each of these murine model systems have severe deficiencies in adaptive immune responses, with further selective deficiencies in innate immunity which permit engraftment of tissues which would otherwise be rejected by the host immune system.

[00177] Typically, single-cell suspensions (or suspensions with a few aggregates of cells, such as 20,000 cells; ideally less than 100; preferably less than 10 cells) of the isolated cancer stem cells are prepared in a mixture comprising a 1:1 ratio of cell suspension and the matrix carrier Matrigel (Invitrogen), and transplanted into appropriate anatomical sites in the mice. General techniques for formulation and injection of cells may be found in Remington's Pharmaceutical Sciences, 20th ed. (Mack Publishing Co., Easton, Pa.). Suitable routes may include parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, intracerebral, or intraocular injections, for example. For injection, the cells of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. As set forth herein once tumorigenicity is established, the animal model can be used for a wide array of biological and molecular assays to characterize the tumorigenic stem cells and the tumors that arise therefrom.

[00178] For example, in the case of treatment of advanced tumors, tumors are allowed to develop to the desired size, with animals having tumors exceeding the desired size range or those tumors that are insufficiently developed being eliminated. The selected animals are distributed at random to undergo the treatments and controls. Animals not bearing tumors may also be subjected to the same treatments as the tumor-bearing animals in order to be able to dissociate any toxicity from the test agent versus toxicity arising from tumor-associated material or responses to the said agent. Chemotherapy generally begins from 21 - 60 days after grafting, depending on the type of NTX tumor, and the animals are observed daily. The targeted cargo proteins can be administered to the animals, for example, by i.p. injection, intravenous injection,

direct injection into the tumor (or into the organ having the tumor), or bolus infusion. The amount of test compound that is injected can be readily be determined by those of skill in the art. Typically the different animal groups are weighed about 1 or 2 times a week until the end of the trial. Tumors are measured once they are palpable and are monitored continuously by direct measurement using electronic calipers or 3D scanning about 1 or 2 times a week until the tumor reaches a pre-determined size and/or weight, or until the animal dies or is euthanized if this occurs before the tumor reaches the pre-determined size/weight. The animals are then sacrificed and not only is tissue is saved for RNA, DNA, protein, IHC and other analyses, but tumor cell subpopulations are enriched or isolated using the methods described herein for similar analyses of the respective populations.

IX. Analysis of potential therapeutic compounds

[00179] The TICAM disclosed herein provide extremely effective methods for the identification, characterization, monitoring and separation or isolation of tumorigenic cell subpopulations. It will be appreciated that the disclosed observational techniques and highly defined cell subpopulations provide powerful tools that may be exploited to identify and validate therapeutic or diagnostic targets as will as pharmaceutical compounds for the treatment, prevention or diagnosis of selected disorders.

[00180] In one embodiment of the instant invention the isolated cells or cell populations may be subjected to genotypic or phenotypic analysis (e.g., Example 8 below). More particularly the isolated or enriched cells will be treated or prepared to provide genetic or proteomic material (i.e., information such as a transcriptome) using techniques known in the art. This prepared or treated material is then analyzed using modern techniques such as Next-Gen sequencing or mass spectrometry to provide selected information about the tumorigenic cell or cell subpopulation such as protein expression or morphology.

[00181] Further, the defined tumorigenic cell subpopulations disclosed herein may be used to evaluate test or candidate compounds *in vitro* and/or *in vivo* for their ability to reduce the amount of cancer cells and/or cancer cells, or inhibit their proliferation. The ability of a candidate compound to stabilize or reduce the amount of cancer cells, cancer cells and/or immune cells (e.g., lymphocytes) or inhibit their proliferation can be assessed by: detecting the expression of antigens on cancer cells, cancer cells, and immune cells; detecting the proliferation cancer cells, cancer cells and immune cells; detecting the cancer cells and cancer cells using functional assays. In particularly preferred embodiments the analysis will comprise the identification, characterization and/or isolation and enrichment of tumorigenic cell subpopulations. Techniques

known to those of skilled in the art can be used for measuring these activities. For example, cellular proliferation can be assayed by ^{3}H-thymidine incorporation assays and trypan blue cell counts. Antigen expression can be assayed, for example, by immunoassays including, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, immunohistochemistry radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, immunofluorescence, flow cytometry, and FACS analysis.

[00182] A potential pharmaceutical compound, pharmaceutical composition, or proposed regimen is preferably tested in vitro and then in vivo for the desired therapeutic or prophylactic activity prior to use in humans. For example, assays which can be used to determine whether administration of a specific compound is indicated include cell culture assays in which a patient tissue sample (e.g., whole tumor or enriched cell subpopulations) is passaged in immunocompromised mice and exposed to, or otherwise contacted with, a compound of the invention, and the effect of such compound upon the tissue sample is observed. The tissue sample can be obtained by biopsy from the patient. This test allows the identification of the therapeutically most effective therapy (e.g., prophylactic or therapeutic agent) for each individual patient. Generally, a therapy is preferably tested *in vitro* and then *in vivo* for the desired therapeutic or prophylactic activity prior to use in humans.

[00183] More specifically the markers and associated cells, cultures, populations and compositions comprising the same, including progeny thereof, can 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. 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 interfering with selected pathways or functions as displayed in the enriched populations. 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 enriched populations of tumor initiating cells and a compound or agent (e.g., drug), wherein the cells and compound or agent (e.g., drug) are in contact with each other.

[00184] In another preferred embodiment the method includes contacting enriched populations of tumor initiating cells or progeny thereof *in vivo* or *in vitro* with a test agent or compound; and determining if the test agent or compound modulates an activity or function of

the tumor initiating cells. 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.

[00185] In some embodiments it will be advantageous to screen for and select compounds that may impact more than one tumorigenic subpopulation. Thus, if overall survival is to be impacted by therapeutic regimens, the targets of these drugs will be best selected based on their expression on or in, primarily TPC, but also TProg. One might expect that selected targeting of TPC would result in the eventual regression of a tumor, but this may take some time to occur, as TProg are herein demonstrated in colorectal cancer to have significant proliferative capacity. Therapeutic targets not only expressed by TPC, but also by TProg may be better drug candidates as both the self-renewing component of the tumor and the highly proliferative TProg compartment might be eradicated in parallel, resulting in noticeable tumor regression and prolonged overall survival. These drug targets may be, for example, genes, proteins, micro-RNA, and/or long or short non-coding RNAs. Drug targets may also include ligands that are not expressed by the TPC or TProg, but which bind receptors on TPC or TProg and which are critical for the self-renewal of TPC or proliferation of TProg.

[00186] More generally, contacting, when used in reference to cells or a cell culture or method step or treatment, means a direct or indirect interaction between the enriched or selected tumorigenic cells or cell subpopulations another referenced entity. A particular example of a direct interaction is physical interaction. Moreover, such contact may take place *in vitro* or *in vivo* (i.e. a test compound administered to an NTX animal). A particular example of an indirect interaction is where a composition acts upon an intermediary molecule that in turn acts upon the referenced entity (e.g., cell or cell culture).

[00187] 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.

[00188] 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)).

[00189] In addition to complex biological agents candidate agents include organic molecules comprising functional groups necessary for structural interactions, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, frequently at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules, including peptides, polynucleotides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[00190] Included are pharmacologically active drugs, genetically active molecules, etc. Compounds of interest include chemotherapeutic agents, hormones or hormone antagonists, etc. Exemplary of pharmaceutical agents suitable for this invention are those described in, "The Pharmacological Basis of Therapeutics," Goodman and Gilman, McGraw-Hill, New York, N.Y., (1996), Ninth edition, under the sections: Water, Salts and Ions; Drugs Affecting Renal Function and Electrolyte Metabolism; Drugs Affecting Gastrointestinal Function; Chemotherapy of Microbial Diseases; Chemotherapy of Neoplastic Diseases; Drugs Acting on Blood-Forming organs; Hormones and Hormone Antagonists; Vitamins, Dermatology; and Toxicology, all incorporated herein by reference. Also included are toxins, and biological and chemical warfare agents, for example see Somani, S. M. (Ed.), "Chemical Warfare Agents," Academic Press, New York, 1992).

[00191] 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. In other preferred embodiments the method comprises screening a chemical compound library of interest for activity in a culture comprising an enriched preparation tumorigenic cells. Such a chemical library may include the Spectrum™ Collection library, the Lopac™ Collection library, the Prestwick Chemical Library® and the Maybridge® Collection library (each are libraries of compounds for screening).

[00192] Further, compounds, including candidate agents, are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds, including biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

[00193] In such embodiments parameters to be measured comprise quantifiable components of cells, particularly components that can be accurately measured, desirably in a high throughput system. A parameter can be any cell component or cell product including cell surface determinant, receptor, protein or conformational or posttranslational modification thereof, lipid, carbohydrate, organic or inorganic molecule, nucleic acid, e.g. mRNA, DNA, etc. or a portion derived from such a cell component or combinations thereof. While most parameters will provide a quantitative readout, in some instances a semi-quantitative or qualitative result will be acceptable. Readouts may include a single determined value, or may include mean, median value or the variance, etc. Characteristically a range of parameter readout values will be obtained for each parameter from a multiplicity of the same assays. Variability is expected and a range of values for each of the set of test parameters will be obtained using standard statistical methods with a common statistical method used to provide single values.

[00194] Agents are screened for biological activity by adding the agent to at least one and usually a plurality of cell samples, usually in conjunction with cells lacking the agent. The change in parameters in response to the agent is measured, and the result evaluated by comparison to reference cultures, e.g. in the presence and absence of the agent, obtained with other agents, etc.

[00195] The agents are conveniently added in solution, or readily soluble form, to the medium of cells in culture. The agents may be added in a flow-through system, as a stream, intermittent or continuous, or alternatively, adding a bolus of the compound, singly or incrementally, to an otherwise static solution. In a flow-through system, two fluids are used, where one is a physiologically neutral solution, and the other is the same solution with the test compound added. The first fluid is passed over the cells, followed by the second. In a single solution method, a bolus of the test compound is added to the volume of medium surrounding the cells. The overall concentrations of the components of the culture medium should not change significantly with the addition of the bolus, or between the two solutions in a flow through method.

[00196] Various methods can be utilized for quantifying the presence of the selected markers. For measuring the amount of a molecule that is present, a convenient method is to label a molecule with a detectable moiety, which may be fluorescent, luminescent, radioactive, enzymatically active, etc., particularly a molecule specific for binding to the parameter with high affinity. Fluorescent moieties are readily available for labeling virtually any biomolecule, structure, or cell type. Immunofluorescent moieties can be directed to bind not only to specific proteins but also specific conformations, cleavage products, or site modifications like phosphorylation. Individual peptides and proteins can be engineered to autofluoresce, e.g. by expressing them as green fluorescent protein chimeras inside cells. (for a review see Jones et al. (1999) Trends Biotechnol. 17(12):477-81). Thus, antibodies can be genetically modified to provide a fluorescent dye as part of their structure. Depending upon the label chosen, parameters may be measured using other than fluorescent labels, using such immunoassay techniques as radioimmunoassay (RIA) or enzyme linked immunosorbance assay (ELISA), homogeneous enzyme immunoassays, and related non-enzymatic techniques. The quantitation of nucleic acids, especially messenger RNAs, is also of interest as a parameter. These can be measured by hybridization techniques that depend on the sequence of nucleic acid nucleotides. Techniques include polymerase chain reaction methods as well as gene array techniques. See Current Protocols in Molecular Biology, Ausubel et al., eds, John Wiley & Sons, New York, N.Y., 2000; Freeman et al. (1999) Biotechniques 26(1):112-225; Kawamoto et al. (1999) Genome Res 9(12):1305-12; and Chen et al. (1998) Genomics 51(3):313-24, for examples.

X. Diagnostic uses

[00197] In another preferred aspect, the present invention provides a method of diagnosing cancer or detecting a cancerous or pre-cancerous cell comprising obtaining bulk tumor cells or

tumor cell subpopulations enriched or purified from a subject's tumor (e.g. TIC, TPC, TProg, NTG cells, tumor stroma, or whole tumor tissue) and assessing the frequency of these tumor cell subpopulations and/or the genetic and/or proteomic molecular profile from these respective cell populations. Tumorigenic cells can also be isolated based on the TICAM disclosed herein and various methods of isolating tumorigenic cells from a subject, for example, a human, are known in the relevant field.

[00198] In another embodiment, the invention provides a method of analyzing cancer progression and/or pathogenesis in-vivo. In another embodiment, analysis of cancer progression and/or pathogenesis in-vivo comprises determining the extent of tumor progression. In another embodiment, analysis comprises the identification of the tumor. In another embodiment, analysis of tumor progression is performed on the primary tumor. In another embodiment, analysis is performed over time depending on the type of cancer as known to one skilled in the art. In another embodiment, further analysis of secondary tumors originating from metastasizing cells of the primary tumor is analyzed *in-vivo*. In another embodiment, the size and shape of secondary tumors are analyzed. In some embodiment, further *ex-vivo* analysis is performed. In another embodiment, the patient's tumor is transplanted into immunocompromised mice and grown as a xenograft such that the above embodiments analyzing cancer progression, pathogenesis and/or experiments to predict response to prospective or actual therapies are performed.

[00199] Other preferred embodiments of the invention also exploit the properties of the disclosed TIC markers and TICAM, or TIC- or TICAM-associated genes, proteins, micro-RNAs, or short and long non-coding RNAs discovered using cells enriched or isolated using these TIC or TICAM, to quantify the relative number or frequency of TIC in a given specimen (e.g. tumor biopsy) based on the expression of said TIC- or TICAM-associated genes, proteins, micro-RNAs, or short and long non-coding RNAs versus a control specimen or a specimen obtained at a different point in time. In this manner, it may be possible to predict and/or actively assess response to therapy without directly assessing the actual number of TIC within the sample as described herein.

[00200] Any *in vitro* or *in vivo* assays known to one of ordinary skill in the art that can detect and/or quantify tumorigenic cells can be used to monitor the course of a disease in order to evaluate the impact of a selected treatment and/or regimen. These methods can be used to assess the impact in a research setting as well as in a clinical setting. The results of these assays then may be used to alter the dosing, drugs or timing of the treatment of a subject. The sample can be subjected to one or more pretreatment steps prior to the detection, isolation and/or measurement of the cancer stem cell population in the sample. In certain examples, a biological fluid is

pretreated by centrifugation, filtration, precipitation, dialysis, or chromatography, or by a combination of such pre-treatment steps. In other examples, a tissue sample is pretreated by freezing, chemical fixation, paraffin embedding, dehydration, permeabilization, or homogenization followed by centrifugation, filtration, precipitation, dialysis, or chromatography, or by a combination of such pretreatment steps. In certain examples, the sample is pretreated by removing cells other than tumorigenic cell subpopulations from the sample, or removing debris from the sample prior to the determination of the amount of cancer stem cells in the sample.

[00201] In another embodiment, the invention provides a method of analyzing cancer progression and/or pathogenesis *in-vivo* including determining cell metastasis. In yet another embodiment, analysis of cell metastasis comprises determination of progressive growth of cells at a site that is discontinuous from the primary tumor. In another embodiment, the site of cell metastasis analysis comprises the route of neoplastic spread. In some embodiment, cells can disperse via blood vasculature, lymphatics, within body cavities or combinations thereof. In another embodiment, cell metastasis analysis is performed in view of cell migration, dissemination, extravasation, proliferation or combinations thereof. In yet another embodiment, the metastatic potential of the tumorigenic cell subpopulations are analyzed following intravenous, orthotopic, or kidney capsule transplantation into immunocompromised mice.

[00202] In certain examples, the tumorigenic cells in a subject or a sample from a subject may be assessed or characterized prior to therapy or regimen to establish a baseline. In other examples the sample is derived from a subject that was treated. In some examples the sample is taken from the subject at least about 1, 2, 4, 6, 7, 8, 10, 12, 14, 15, 16, 18, 20, 30, 60, 90 days, 6 months, 9 months, 12 months, or >12 months after the subject begins or terminates treatment. In certain examples, the tumorigenic cells are assessed or characterized after a certain number of doses (e.g., after 2, 5, 10, 20, 30 or more doses of a therapy). In other examples, the tumorigenic cells are characterized or assessed after 1 week, 2 weeks, 1 month, 2 months, 1 year, 2 years, 3 years, 4 years or more after receiving one or more therapies.

XI. Articles of manufacture

[00203] The present invention also provides kits for identifying, characterizing and/or enriching, or isolating tumorigenic cells or cell subpopulations as described herein. Such kits may be use used in a clinical setting for patient diagnostic purposes or in research for characterization and/or enrichment of tumorigenic cell populations. Kits according to the invention will comprise one or more containers comprising TICAM binding agents and a label or package insert on or associated with the container. Suitable containers include, for example,

bottles, vials, syringes, 96 well plates, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds one or more compositions comprising binding agents that are effective for analyzing tumorigenic cells and optionally providing the enriched or isolated cells or cell subpopulations as described herein. Such kits will generally contain in a suitable container a formulation of one or more TICAM binding agents where in the case of multiple binding agents the binding agents may be in the same or different containers. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis or for labeling or modifying the enclosed binding agents.

[00204] More specifically the kits may have a single container that contains the one or more TICAM binding agent, with or without additional components, or they may have distinct containers for each component. Where combined reporters provided for conjugation, a single solution may be pre-mixed, either in a molar equivalent combination, or with one component in excess of the other. Alternatively, the binding agent and any optional labeling agent of the kit may be maintained separately within distinct containers prior to administration to a subject or *in vitro* use. 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.

[00205] 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.

[00206] As indicated briefly above the kits may also contain a means by which to administer the binding agent and any optional components to a subject, 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 or introduced 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. In particularly preferred embodiments any label or package insert indicates that the binding agent composition is used for in the diagnosis of cancer, for example colorectal cancer. In other preferred embodiments the enclosed instructions instructs or indicates how to use the components in an *in vivo* or *in vitro* research setting. one or more therapies.

XII. Research reagents

[00207] Other preferred embodiments of the invention also exploit the properties of the disclosed TICAM as an instrument useful for identifying, isolating, sectioning or enriching populations or subpopulations of tumor initiating cells through methods such as fluorescent activated cell sorting (FACS), magnetic activated cell sorting (MACS) or laser mediated sectioning. Those skilled in the art will appreciate that the modulators may be used in several compatible techniques for the characterization and manipulation of TIC 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).

XIII. Miscellaneous

[00208] 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.

[00209] 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.

[00210] 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

[00211] 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

Characterization of Tumor Initiating Cell Populations

[00212] 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 diagnostic biomarkers and 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 primary tumors, was propagated in immunocompromised mice through multiple passages of heterogeneous tumor cells originally obtained from cancer patients suffering from a variety of solid tumor malignancies. The availability of a large number of discrete early passage NTX tumor cell lines having well defined lineages greatly facilitate the identification and isolation of TPC as NTX tumor lines, the cells of which closely reflect the biology of a tumor taken directly from a patient, allow for the reproducible and repeated characterization of tumor cell subpopulations *in vivo*. More particularly, isolated or purified TPC 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, while retaining their ability to fuel tumor growth in serial transplants of low cell numbers (i.e. < 200 cells). Thus, the ability to use small populations of isolated cells to generate fully heterogeneous tumors in mice through at least two series of transplants is strongly indicative of the fact that the isolated cells comprise TPC. In such work the use of minimally passaged NTX cell lines that are never expanded *in vitro* (e.g. for the purpose of increasing cell numbers) greatly simplifies *in vivo* experimentation and provides verifiable results in a physiologically relevant setting. 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.

[00213] As the NTX tumor lines were established the constituent tumor cell phenotypes were analyzed using flow cytometry to identify discrete markers that might be used to identify, characterize, isolate, purify or enrich tumor initiating cells (TIC) and separate or analyze TPC and tumor progenitor 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 cellular protein expression and the concomitant identification of potentially useful markers.

[00214] 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 binding molecule in the phycoerythrin (PE) fluorescent channel. Other binding molecules in non-PE fluorescent channels are uniformly applied to all cells prior to their distribution into wells of the PhenoPrint Array for the purpose of identifying subpopulations within the tumor cell sample. The use of the PhenoPrint Array allows for the rapid identification of proteins or markers that prospectively distinguished TIC from non-tumorigenic (NTG) bulk tumor cells and tumor stroma (*e.g.*, fibroblasts and endothelial cells). More specifically, proteins exhibiting heterogeneous cell surface expression over whole tumor cell samples allow for the identification, isolation and transplantation of distinct, and highly purified, TIC subpopulations based on differential protein expression. It will be appreciated that characterizing and enriching or isolating cell subpopulations (*e.g.*, by flow cytometry or fluorescence activated cell sorting: FACS) based on marker phenotypes of the instant application allows for their transplant into immunocompromised mice, thereby facilitating the assessment of whether TIC were enriched in one tumor cell subpopulation versus another by functionally testing their tumorigenic capacity *in vivo*. 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 in accordance with the instant invention and subsequently isolate and transplant specific human tumor cell subpopulations with great efficiency.

[00215] In the instant case various NTX tumor lines comprising human tumors were established in severely immunocompromised mice using art recognized techniques. Upon reaching 800 - 2,000 mm^3, tumors were resected from mice and dissociated into single cell suspensions using art recognized mechanical and enzymatic dissociation techniques involving the use of collagenase, hyaluronidase and DNAseI (see for example U.S.P.N. 2007/0292414 which is incorporated herein). Using standard flow cytometric techniques, individual tumor cells were characterized on a BD FACSCanto™ II flow cytometer (BD Biosciences) for the expression of hundreds of cell surface proteins. In contrast to most cell surface proteins that were uniformly expressed or absent, selected proteins including CD46, CD324 and those set forth in FIGS. 9-12 were, to a greater or lesser extent, positively and/or heterogeneously expressed on the surface of numerous primary human colorectal ("CR"), pancreatic ("PA"), triple negative breast ("BR"), and non-small cell lung cancer ("LU") tumor cells. The heterogeneous expression of two of these markers, CD46 and CD324, are illustrated in FIG. 1 and FIG. 2 for several different tumor types. More particularly, FIG. 1 and FIG. 2 depict flow cytometry-based protein expression data for individual tumor cells displayed as histogram plots wherein fluorescence minus one (FMO) staining using isotype control antibodies is shown in the gray, filled histograms and target antigen expression (i.e. CD46 and CD324) as determined using commercially available antigen-specific, PE-conjugated antibodies, is displayed using bold, black lines.

[00216] As may be seen in FIGS. 1A and in accordance with the instant invention, CD46 expression was generally observed in subjects presenting with various types of solid tumors. Moreover, although the levels of expression varied it was generally above background staining. A review of the plots using tumor cells from freshly isolated tumors reveals that CD46 expression was especially heterogeneous in tumors derived from colorectal, pancreatic, non-small cell lung and triple negative breast cancer patients (FIG. 1B), with subpopulations generally demonstrating negative/lo or positive expression. Specifically, those cells positively expressing CD46 often had staining ranging from low levels to high levels as quantified using isotype control/FMO staining and standard flow cytometric methodology. Similar observations were made with CD324 as seen in FIG. 2, wherein tumor cell subpopulations expressing CD324 were often observed to comprise a minority population. Surprisingly, and in contrast to accounts

in the art, proteins generally thought to be associated only with a subpopulation of tumorigenic cells (e.g. CD24 and CD34) generally exhibited uniform expression as exemplified in FIGS. 3A (CD24) and 3B (CD34) when measured using the same techniques in similar solid tumor types. As such, these prior art markers may be incompatible with the teachings herein.

[00217] In any event, the combined use of NTX tumor models that accurately recapitulate tumor physiology with the PhenoPrint Array analysis of tumor cells as described above, demonstrate the possibility and utility in characterizing cell surface expression levels of many hundreds of tumor antigens, including CD46 and CD324. Unlike markers exhibiting homogeneous expression, the markers of the instant invention are generally heterogeneous across tumor cell subpopulations from numerous tumor types and thus provide a dramatic advantage when identifying, characterizing and/or isolating or enriching TIC or components thereof.

Example 2

Enrichment for Tumor Initiating Cell Populations by FACS and Transplantation

[00218] In tumors exhibiting heterogeneous expression of a particular protein or proteins of interest (e.g., CD46 and/or CD324), cells were enriched or isolated based on such markers and then transplanted into immunocompromised mice. More particularly, to determine whether high or low levels of surface CD46 and/or CD324 could be correlated with enhanced tumorigenicity, NTX tumor samples were disassociated using state of the art techniques as described above and isolated using a FACSAria Flow Cytometer (BD Biosciences) to provide distinct marker enriched subpopulations that were subsequently transplanted into immunocompromised mice. In this respect cells were injected subcutaneously into the mammary fat pad of recipient female immunocompromised NOD/SCID mice at doses typically ranging between 1,000 to 50 cells per mouse. When tumors arising from these transplants reached 800 - 2,000 mm^3, mice were euthanized and the tumors were removed and dissociated by enzymatic digestion to a single cell suspension for the purpose of phenotypic characterization to assess whether the constitution of cells was representative of the parental tumor from which the transplanted cells were originally isolated.

[00219] FIGS. 4-8 illustrate the results of representative experiments using NTX tumors derived from colorectal, pancreatic, non-small cell lung and triple negative breast cancer patients. Empty spaces in FIG. 8 denote that the indicated experimental condition was not tested. Significantly, tumorigenicity was consistently associated with the subpopulation of cells

expressing CD46 and CD324, and the tumors generated by cells with $CD46^{hi}$ $CD324^{+}$ phenotype were analogous in composition to their parental tumors. Moreover, because all human cells within these solid tumors were epithelial specific antigen (ESA; EpCAM) positive and all, or at least the vast majority, were $CD24^{+}$ and $CD34^{-}$ (FIG. 3), the tumor initiating cell (TIC) subpopulation from these tumors, and the vast majority of those tumors analyzed, can be identified using the phenotypic profile of ESA^{+} $CD46^{hi}$ $CD324^{+}$ $CD24^{+}$ $CD34^{-}$. Yet, because ESA, CD24 and CD34 vary little in their surface expression and are of limited utility in defining tumor cell subpopulations, the TIC subpopulations disclosed herein may be identified as $CD324^{+}$ and, optionally, as $CD46^{hi}$. As described above and repeated using NTX lines derived from many colorectal, pancreatic, triple negative breast and non-small cell lung cancer patients, $CD46^{hi}$ cells consistently generated heterogeneous tumors when transplanted into mice, thereby indicating that this isolated subpopulation of cells is significantly enriched for TICs. Although CD46 and CD324 may each be used alone to enrich and characterize TIC subpopulations in accordance with the teachings herein, various aspects of the instant invention will use these markers in combination and, in alternate embodiments, with additional markers to facilitate more precise stratification and isolation of tumor cell subpopulations (FIG.8). Conversely, tumor cells expressing either no, or low levels of, CD46 or CD324 were much less tumorigenic than their high or positive counterparts, respectively (FIGS. 4-8). Based on the generated data it was surprisingly found that subpopulations of tumor cells expressing the $CD46^{hi}$ $CD324^{+}$ phenotype generally contain the vast majority of tumorigenic capability.

Example 3

Representative Cell Surface Markers Expressed on Tumor Initiating Cells

[00220] Tumor initiating cells, like all cells, can express hundreds if not thousands of markers or antigens, preferably on their cell surface. As such, while CD46 and CD324 are preferred markers for TIC, other concomitant markers may be used to identify and/or isolate the same tumor cell subpopulation independent of using molecules able to detect CD46 and/or CD324. Following confirmation of TIC populations using the cell surface marker profiles described above, the PhenoPrint Array was repeated using reagents to identify proteins exhibiting comparable expression profiles to CD46 and/or CD324, indicating that they may be used as surrogates for the same in terms of the instant invention.

[00221] To identify proteins that are associated with TIC cell populations or substantially co-express with CD46 and/or CD324, non-PE-conjugated antibodies against these antigens were

included in all wells of the PhenoPrint Array and unique antibodies recognizing various distinct proteins of interest were arrayed and assessed using the fluorescent molecule PE. Proteins with higher expression within the human $CD46^{hi}$ $CD324^{+}$ (TIC) subfractions, for example, compared to the $CD46^{-/lo}$ (NTG) and/or $CD324^{-}$ subfractions, of xenografted colorectal, pancreatic, or non-small cell lung cancer are listed in FIGS 9A and 9B. Representative FACS plots demonstrating the co-expression of each marker with CD46 and CD324 in colorectal cancer are included in FIG. 10. Representative FACS plots demonstrating the co-expression of each marker with CD46 and CD324 in pancreatic cancer are included in FIG. 11. Representative FACS plots demonstrating the co-expression of each marker with CD46 and CD324 in non-small cell lung cancer are included in FIG. 12. Characterization or enrichment or isolation of cells with substantial expression of each listed marker also indicates the characterization or enrichment or isolation of $CD46^{hi}$ $CD324^{+}$ cells (i.e. TIC). In this regard, the markers of FIGS. 9 - 12 and other markers correlated with expression of either CD46 or CD324 may be used as surrogates and effectively used to identify TIC as set forth herein. Accordingly, the markers set forth in FIGS. 9 - 12 may be used for isolating TIC subpopulations and possess substantial utility as diagnostic markers and therapeutic targets.

Example 4

CD66c Can Distinguish Between Subpopulations of $CD46^{hi}$ $CD324^{+}$ Colorectal

[00222] While the majority of tumor cells are devoid of tumor forming ability and can thus be characterized as NTG, there is precedent in both normal cellular development and hematopoietic tumors for highly proliferative cells able to reconstitute a tissue and/or tumor upon transplantation, but which do not have self-renewal capacity (i.e. a finite lifespan) and thus are eventually exhausted: i.e. short-term reconstituting cells, transit amplifying cells or progenitor cells. In the context of cancer, cells with a progenitor cell phenotype might reacquire self-renewal properties normally restricted to stem cell populations and thereafter fulfill the definition of a TPC or CSC (Jamieson et al., N Engl J Med; 351, 2004, which is incorporated herein by reference in its entirety) in that these cells and their progeny will be long-lived; however, these mutagenic events that confer self-renewal properties to progenitor cells are rare. Nevertheless, because: a) the proliferative capacity of progenitor cells may be significant; b) immunocompromised mice must be euthanized once tumors reach ~ 1,500 – 2,000 mm^3; and c) the lifespan of immunocompromised mice is short relative to that of humans (~ 9 – 12 months for NOD/SCID mice), the ability of a TIC to generate tumors in mice is not a robust enough readout

to demonstrate that the TIC is a TPC. As discussed in the art, demonstration of self-renewal capacity by a stem cell must be shown in serial transplants whenever possible.

[00223] To determine whether subpopulations of $CD46^{hi}$ $CD324^{+}$ cells were more or less tumorigenic and/or had altered potential, cells with this phenotype were systematically screened for heterogeneity to identify additional markers enabling cell isolation and transplantation experiments. Specifically, cell surface markers of interest and potential utility were identified using the PhenoPrint Array as set forth in previous examples discussed above. During the course of these experiments, CD66c was identified as a cell surface marker that commonly displayed heterogeneity among the $CD46^{hi}$ $CD324^{+}$ cell population (FIG. 11A) and enabled the isolation of two distinct subpopulations of $CD46^{hi}$ $CD324^{+}$ cells possessing different functional reconstitution capabilities. The respective populations transplanted at 200 cells per mouse are denoted in FIG. 11A. Colorectal tumors arising from the transplantation of $CD66c^{-}$ cell subset of $CD46^{hi}$ $CD324^{+}$ cells were fully heterogeneous and reflected the parental tumors from which they were derived (FIGS. 11B & 11C [white bar vs. black bar] and FIG. 12A). Surprisingly, and in contrast to tumors derived from the $CD66c^{-}$ subset of $CD46^{hi}$ $CD324^{+}$ cells, transplants with small numbers of the $CD66c^{+}$ subset did not generate fully heterogeneous tumors in that there were significantly less $CD66c^{-}$ cells present in tumors generated from $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells (FIG. 11C [gray bar vs. black bar] and FIG. 12A), suggesting that these cells are tumor progenitor cells (TProg) with properties analogous to normal progenitor cells; i.e. significant proliferative capacity, but devoid of self-renewal capability and unable to dedifferentiate into $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells.

[00224] Serial transplantation of prospective TPC ($CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$) and TProg ($CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$) cells at low cell numbers confirmed the proposed identity of these tumor cell subpopulations, as $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells arising from tumors generated by $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells efficiently generated tumors upon serial transplantation of only 50 cells (p2 versus p1 tumor), whereas neither the $CD66c^{-}$ nor $CD66c^{+}$ cell subpopulations from tumors generated by $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells could efficiently reinitiate tumors upon serial transplantation (FIG. 12). To be clear, 50 $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ or 50 $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells isolated from tumors generated from 200 $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells were rarely tumorigenic. Surprisingly, these data are indicative of the fact that, in solid tumors from some colorectal cancer patients, $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells are TPC and $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells are TProg cells.

[00225] To determine the accuracy of the above described TPC phenotype in colorectal cancer, $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells were isolated by FACS (pre- vs. post-FACS; FIGS. 13A

vs. 13B, respectively) and transplanted in dilutions at 1,000, 200, 50, 20, 8 and 3 cells per mouse, respectively. Use of Poisson distribution statistics based on positive events being defined as successful tumorigenesis independent of rate resulted in the calculation that the true TIC frequency among $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ TPC was roughly 1 in 5.4 ± 2.5 cells (FIG. 13C). We have unexpectedly demonstrated in a representative colorectal NTX tumor that using the novel marker combination of $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ facilitates a measurable enrichment in TIC frequency, and use of CD66c further facilitates the parsing of TIC subpopulations in some patients into TPC versus TProg. This development has significant implications in that not all TIC are equal and that only the TPC subpopulation in any given patient is the long-lived cancer stem cell truly driving disease progression and recurrence.

[00226] The combination of cell surface proteins used to enrich for TPC and TProg cell populations defined above in colorectal tumors has not been known to be associated with cells containing such activity in any tissue or neoplasm previously. This work represents a substantial improvement in the resolution of the method of isolating TIC and subpopulations thereof, and further improves techniques to identify, isolate and characterize distinct, highly enriched solid tumor cell subpopulations that exclusively contain tumor generating ability upon transplantation, distinguishing between tumorigenic cell subpopulations without or with self-renewal capacity: i.e. TProg and TPC, respectively. We further herein describe a method by which to enumerate TIC by transplanting human tumor cells in progressively lower dilutions of cell numbers and assessing the subsequent frequency, independent of rate, at which these dilutions of cells are able to initiate tumorigenesis in immunocompromised mice. Accordingly, while most cell surface markers identified using the proprietary PhenoPrint Array did not demonstrate an ability to enrich TIC populations from tumors using standard FACS protocols, distinct markers and combinations thereof could be used to identify at least two subpopulations of TIC, including TPC and TProg, which were functionally demonstrated as fulfilling the definitions standard in the art of a stem cell and a progenitor, respectively.

Example 5

TPC and TProg Cell Populations Have Differing Potential

[00227] *In vitro* colony forming cell (CFC) assays have proven to have great utility in helping parse apart the differentiation potential of distinct cell populations in the hematopoietic cell hierarchy; however these assays are not amenable to epithelial tissues or solid tumor cells in that the assay conditions are not conducive to cell growth and/or differentiation. Using NTX tumor

models in which many phenotypically distinct tumor cell subpopulations exist as they do in patient tumors, FACS and *in vitro* assay conditions that support either self-renewal or differentiation of TIC aid in the determination of the potential of defined tumor cell subpopulations. These *in vitro* CFC assays, at the very least, provide an effective advantage in the search for new markers defining tumor cell subpopulations, determining the differentiation potential of these respective cell populations, and for the screening of anti-cancer agents.

[00228] To illustrate such aspects of the instant invention, single cells with the defined cell surface phenotypes of $CD46^{hi}$ $CD324^{+}$ (TIC) or $CD46^{-/lo}$ $CD324^{-}$ (NTG) were isolated from NTX CR tumors and deposited directly into 96-well plates in dilutions of 468, 162, 54, 18, 6 or 2 cells per well using a FACSAria flow cytometer (BD Biosciences). Cells were then cultured in defined serum-free conditions, as is standard in the art, for 15 days at 37° C/5% CO_2/5% O_2, and wells positive for colony formation were then scored as such (FIG. 16A). The frequency of colony-forming units for each population was finally determined utilizing L-Calc™ Software (Stemcell Technologies), utilizing the experimental setup information including: a) the number of individual wells initiated; b) the number of cells used to initiate each well; and c) the number of wells scored positive, independent of rate, from each dilution.

[00229] Colonies were observed in wells seeded with as few as 18 sorted $CD46^{hi}$ $CD324^{+}$ cells, while 162 cells were the fewest needed to observe a colony from sorted $CD46^{-/lo}$ $CD324^{-}$ cells. Colony forming cells were thus demonstrated to exist at frequencies of 7.3 and 1.1 per 1,000 cells for TIC and NTG cell populations, respectively (FIG. 16A). For the purposes of clarity, 1 in 137 $CD46^{hi}$ $CD324^{+}$ (TIC) cells formed colonies *in vitro*, while only 1 in 918 $CD46^{-/lo}$ $CD324^{-}$ (NTG) cells had this capacity. These results strongly indicate that colony-forming cells are significantly enriched in the $CD46^{hi}$ $CD324^{+}$ fraction of colorectal cancer cells and this activity correlates with tumorigenic capacity, as is also demonstrated above by *in vivo* transplantation.

[00230] To further assess the proliferative and differentiation potential of more distinct tumor cell subpopulations within colorectal tumors, cells expressing various combinations of CD46, CD324 and CD66c were sorted into plates containing standard serum-free media conditions that support stem cell self-renewal (Dylla et al., 2008, supra). The following cell populations were sorted from among single, live, human ESA^{+} cells: $CD46^{-/lo}$ (NTG cells; predominantly also $CD324^{-}$), $CD46^{hi}$ $CD324^{-}$, $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ and $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$. The ability of these respective tumor cell subpopulations to initiate colonies was then assessed 21 days after plating, as was the potential of these respective tumor cell subpopulations to generate soluble CD66c (i.e. CEACAM6) protein. CD66c is routinely shed from the cell surface, was readily

measureable in the supernatant of the above described *in vitro* cultures, and was used as a surrogate marker of differentiation.

[00231] Specifically, 2,000 cells from NTX tumor CR33, for example, were sorted into flat-bottom 96-well Primaria plates (BD Biosciences) in serum-free media conditions and incubated in the environmental conditions noted above. After 21 days, colonies (defined as > 50 cells per colony) of tightly packed, attached cells had formed and expanded in the wells. The number of colonies (white bars) in each well was counted manually and the concentration of CD66c protein in the supernatant (black bars) was measured by ELISA (FIG. 16B). The frequency of colony formation corresponded well with the frequency of tumorigenic cells observed *in vivo*. That is, $CD46^{-/lo}$ cells (i.e. NTG cells) generated only a single colony (1 in 2,000 cell frequency) and soluble CD66c protein was not detected in the media despite 21 days of culture (FIG. 16B). Just as $CD46^{hi}$ $CD324^{-}$ cells rarely demonstrate *in vivo* tumor initiation potential, we observed a small number of colonies *in vitro* and relatively low levels of CD66c were detected in the media. This is not surprising given that a subpopulation of $CD46^{hi}$ $CD324^{-}$ cells in many NTX tumors express CD66c and these cells generally appear to be short-lived. Furthermore, $CD46^{hi}$ $CD324^{+}$ cells, regardless of CD66c expression, were better able to form colonies *in vitro* and generated significantly more soluble CD66c than either the $CD46^{-/lo}$ or the $CD46^{hi}$ $CD324^{-}$ tumor cell subpopulations (FIG. 16B). In addition, the amount of CD66c generated in the supernatant appeared to correlate with the number of colonies, suggesting that both $CD66^{+}$ and $CD66^{-}$ cell populations that were initially seeded into the wells were capable of generating CD66c. This is consistent with the observation that $CD66c^{-}$ cells are capable of generating $CD66c^{+}$ cells as demonstrated above *in vivo*.

[00232] Colony formation can serve as a surrogate for in vivo transplantation and determination of whether TIC are present in one tumor cell subpopulation versus another and is generally able to assess the potential of candidate stem and progenitor cell populations. However, even the best *in vitro* cell culture conditions do not mimic the physiological environment encountered *in vivo*. Furthermore, these serum-free culture conditions generally support self-renewal and prevent differentiation, but do not actively facilitate differentiation.

[00233] To better assess the differentiation potential of single cells with the defined cell surface phenotypes described above, the media conditions were modified to contain 10% fetal calf serum (FCS), so as to actively facilitate differentiation as is common in the art. Fourteen days later, media was removed and the amount of CD66c in the supernatant was assessed by ELISA. Although elevated levels of CD66c were expected in the media from $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells, the fact that $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells were able to generate similar levels of

CD66c *in vitro* is consistent with the notion that $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells can differentiate into $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$ cells (FIG. 16C). Expansion in media conditions supportive of self-renewal (i.e. in defined, serum-free media) as described above does not appear to be supportive of robust CD66c production; however, addition of FCS to the media facilitated differentiation and greatly aided the ability of cells to produce CD66c. Perhaps not surprisingly given the demonstration above that $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells are often the only tumor cell subpopulation with self-renewal capacity (demonstrated by serial transplantation), the tumor cell subpopulation with the greatest CD66c production potential *in vitro* were also $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells. Although this result was unexpected given that the cells seeded into the well were negative for CD66c protein expression, and Taqman qRT PCR analysis of this cell population shows little to no presence of the CD66c transcript (data not shown), these results confirm *in vivo* observations demonstrating that $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells have the most proliferative and differentiation potential. This also implies that plating of $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ cells *in vitro* results in progeny that express CD66c, and this process is augmented by cell growth conditions that promote differentiation.

[00234] Induced differentiation of TIC *in vitro* by the addition of FCS is not only observed in with NTX colorectal cancer cells, as described above, but also with pancreatic cancer TIC. After fourteen days of pancreatic xenograft tumor cell culture without or with serum, the percentage of $CD46^{hi}$ cells (also predominantly $CD324^{+}$) was decreased on both NTX PA14 and PA20 tumor cells in conditions where FCS was present (FIG. 16D). This data further supports the use of CD46 and CD324 as markers to distinguish between less differentiated and more differentiated NTX-derived pancreatic cancer cells *in vitro* and supports the claim that these markers can serve distinguish between TIC and NTG cells in several epithelial tumor types.

[00235] This example demonstrates that TIC subpopulations (e.g. TPC & TProg) can be distinguished in vitro based upon their ability to initiate colonies, differentiate and produce proteins normally associated with differentiated cells. Moreover, culture conditions without or with FCS can also be developed that facilitate differentiation.

Example 6

Colorectal and Pancreatic Tumor Initiating Cells are Relatively Resistant to Standard of Care Chemotherapeutic Agents

[00236] A central tenet of the cancer stem cell paradigm is that CSC are generally resistant to standard of care therapeutic regimens such as chemotherapy and radiation. To assess which

colorectal and pancreatic tumor cell subpopulation was most resistant to standard of care regimens such as irinotecan or gemcitabine, respectively, mice were initiated with colorectal or pancreatic NTX tumors and then randomized once tumors reached approximately 180 – 350 mm^3. Mice then received twice weekly intraperitoneal dosing of 15 mg/kg irinotecan or 25 mg/kg gemcitabine, respectively, for a period of approximately 20 days before they were euthanized for tumor resection and cellular analysis.

[00237] To assess chemoresistance of tumor cell subpopulations, in vivo cohorts of mice bearing NTX colorectal tumors were randomized into two groups, such that the average tumor volume for each group was roughly equal. The groups were then treated twice weekly with either 15 mg/kg irinotecan or an equivalent volume of vehicle buffer. Tumor volumes and animal health were monitored and recorded twice weekly, a day prior to each injection. In the case of colorectal tumors, the vehicle-treated tumors continued to grow while the irinotecan-treated tumors showed either retarded growth or a decrease in volume (FIG. 17A). When tumors in irinotecan-treated mice began to decrease in volume, all mice in the experiment were euthanized and tumors were harvested, dissociated into single-cell suspensions and their individual cell phenotypes were analyzed by flow cytometry using art recognized techniques. The frequency of cells with a TPC phenotype ($CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$) among live human cells was determined to be more than 2.5-fold higher in tumors from irinotecan-treated mice, compared to saline-treated counterparts (FIG. 17B and 17C). This is an indication that cells with the TPC phenotype ($CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$) resist irinotecan-induced cytotoxicity while other tumor cell subpopulations succumb to irinotecan. In addition, among the $CD46^{hi}$ $CD324^{+}$ population of vehicle-treated tumors, most residual cells were $CD66c^{+}$; however, among the $CD46^{hi}$ $CD324^{+}$ population of irinotecan-treated mice, most residual cells were $CD66c^{-}$ (i.e. TPC or CSC) (FIG. 17D). This data indicates that cells with the TPC phenotype (i.e. $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$) are more resistant to irinotecan-mediated cytotoxicity than other tumor cell subpopulations, which include both the TProg subset of TIC (i.e. $CD46^{hi}$ $CD324^{+}$ $CD66c^{+}$) and NTG cells.

[00238] Analogous results were observed in a representative pancreatic NTX tumor, wherein residual pancreatic NTX tumors were removed during the course of gemcitabine treatment and the residual tumor cell subpopulations were analyzed based on their marker phenotype. As expected, pancreatic tumors from vehicle treated mice continued to grow unabated, while tumors from mice treated with gemcitabine decreased in volume in response to this chemotherapeutic agent (FIG. 18A). Harvested tumors were then dissociated and constituent single cells were analyzed by flow cytometry. As observed in colorectal cancer NTX tumors treated with

irinotecan, gemcitabine treatment resulted in enrichment for tumor cells expressing markers indicative of TIC (e.g. CD46). A representative example of CD46 expression in vehicle vs. gemcitabine treated tumors is shown in FIG. 18B. As with colorectal cancer, pancreatic TIC appear relatively resistant to the standard of care chemotherapeutic agent, gemcitabine.

[00239] We demonstrate here that not all tumor cell subpopulations are equally sensitive or resistant to standard of care chemotherapeutic agents such as irinotecan or gemcitabine. Moreover, we demonstrate in colorectal cancer that although TPC and TProg are both tumorigenic, TPC (i.e. CSC) are more resistant to chemotherapy. Our ability to precisely identify the most tumorigenic, chemoresistant subpopulation of tumors facilitates the identification of genes and proteins associated with tumorigenicity and chemoresistance and will therefore enable the identification of genes/proteins of great diagnostic and/or therapeutic utility.

Example 7

Proposed Hierarchy of Cells within Colorectal Tumors

[00240] As discussed above, TProg are categorized as a subpopulation of TIC due, in part, 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, there is precedent in the hematopoietic cell hierarchy, for example, for several distinct progenitor cell populations that maintain multilineage differentiation potential, but have differing capacity for proliferation prior to committing to defined cell fates.. Representative examples of progenitors in the hematopoietic system are short-term reconstituting hematopoietic stem cells (ST-HSC) and multipotent progenitor (MPP) cells. Although both cell populations can fully reconstitute the hematopoietic system, neither can do so indefinitely (*i.e.* they lack of self-renewal capacity inherent to true stem cells) and ST-HSC can do so for a longer period of time than MPP (which are progeny of ST-HSC). Although the hierarchy of cells in both solid tissues and tumors originating in these tissues is ill-defined, a hierarchy of cells likely exists within many organs and tumors wherein distinct cell subpopulations possess different proliferation and differentiation potential. We demonstrate here for the first time that not only can TProg exist as a subset of TIC within colorectal cancer, but TProg cells may be further subdivided into early tumor progenitor (ETP) and late tumor progenitor (LTP) cells. Each of these respective TProg cell populations may be distinguished by phenotype (e.g., cell surface markers) and their progressively lesser capacity for proliferation and differentiation potential, and thus differing in their overall capacity to recapitulate certain tumor architecture.

[00241] As illustrated in FIGS. 19A and 19B, the above described *in vivo* and *in vitro* data, together with tumor histomorphological observations, supports the hypothesis that in tumors where there exists significant differentiation capacity and the ability to enact these differentiation programs, the TPC may reflect the identity of the normal colon stem cell, for example, which we herein propose has the identity of ESA^+ $CD46^{hi}$ $CD324^+$ $CD66c^-$ $CD24^+$ $CD34^-$. We have herein demonstrated that virtually all colorectal tumor cells are ESA^+ $CD24^+$ $CD34^-$ and thus these markers serve little utility in identifying tumor cell subpopulations. We further demonstrated that $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells are able to generate fully heterogeneous tumors consisting, in part, of $CD46^{hi}$ $CD324^+$ $CD66c^-$ and $CD66c^+$ cells, whereas although $CD46^{hi}$ $CD324^+$ $CD66c^+$ cells are tumorigenic, they do not have the ability to generate $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells and are unable to efficiently fuel tumor growth through serial transplantation. This data, combined with the observations that the cells with the a) ability to consistently generate heterogeneous tumors upon serial transplantation; b) most colony forming cell potential; and c) potential to generate CD66c cells and protein *in vitro* are $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells supports the hypothesis that $CD46^{hi}$ $CD324^+$ $CD66c^+$ cells are daughters of $CD46^{hi}$ $CD324^+$ $CD66c^-$ cells with restricted proliferation capacity and potential. The further reduction in *in vivo* tumorigenicity, *in vitro* colony forming potential and reduced ability to generate CD66c as cells lose expression of CD324 supports the hypothesis that $CD46^{hi}$ $CD324^-$ $CD66^+$ cells represent a late tumor progenitor (LTP) cell population that has some residual proliferative ability, as has been demonstrated on occasion, but generally has no capacity for self-renewal. Finally, in support of the cellular hierarchy laid out in FIG. 19A, $CD46^-$ cells are also $CD324^-$ and are generally $CD66c^-$ as well. These NTG cells likely represent the terminally differentiated progeny, which in the colon include goblet cells (G) and enteroendocrine (eE) cells of the secretory lineage or enterocytes (E) of the absorptive lineage. Gene expression of the isolated cell subpopulations supports this hypothesis, as $CD46^-$ cells express many genes attributed to terminally differentiated secretory and/or absorptive cell types (e.g. MUC2 and MUC20; FIG. 20A).

[00242] The examples above surprisingly document the existence of multiple populations of tumor initiating cells that are characterized by their differing abilities to generate tumors when implanted in mice and are associated with unique marker phenotypes. Moreover, the discrete subpopulations of TIC differ in their ability to self-renew and fully reconstitute a tumor. Tumor perpetuating cells (TPC), as they're defined here, fulfill the prevailing definition of a cancer stem cell (CSC) in their demonstrated capacity for self-renewal, as tested by serial transplantation with small numbers of well defined cells and analysis of resulting tumor heterogeneity. Conversely, tumor progenitor (TProg) cells, although tumorigenic in primary transplants, differ from true

TPC or CSC in that they appear deficient of self-renewal capacity and their differentiation potential may, on occasion, be restricted. The above examples also demonstrate the existence of discrete TProg cell populations with progressively less proliferation and differentiation potential: ETP and LTP. Knowledge of these cell identities in the context of cancer, and potentially normal tissue biology, facilitate their use to identify proteins and molecules of diagnostic and therapeutic utility. As diagrammed in FIGS. 19A & 19B, and documented in the instant disclosure, heterogeneous tumors comprising tumorigenic and NTG cell populations provide TPC, whose progeny progress from a $CD46^{hi}$ $CD324^{+}$ $CD66c^{-}$ phenotype to gain expression of CD66c (taking the identity of ETP) and eventually lose expression of CD324 (LTP phenotype). Finally, terminal differentiation is apparently accompanied by the concomitant loss of CD66c and CD46.

Example 8

Identification of Prospective Diagnostic and Therapeutic Targets From Enriched Tumor Initiating Cell Populations

[00243] The established colorectal NTX tumor line SCRx-CR4, for example, was passaged as described in Example 1 and used to initiate tumors in immunocompromised mice. Once the mean tumor burden reached ~ 300 mm^3 the mice were randomized and treated with 15 mg/kg irinotecan or vehicle control (PBS) twice weekly for a period of at least twenty days before they were sacrificed. Tumors were then removed and TPC, TProg and NTG cells, respectively, were isolated generally using the technique set out in Example 2. More particularly, cell populations were isolated by FACS 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, total RNA was extracted using the Qiagen RNeasy isolation kit (Qiagen, Inc.) following 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.

[00244] Total RNA samples obtained from the respective 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 $\geq$ 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 transcripts of interest along with verifiable measurements of

transcript expression levels in all samples.

[00245] 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 per sample 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.

[00246] Duplicate runs of sequencing data from the SOLiD3 platform were normalized and transformed and fold ratios calculated, as is standard industry practice. In this manner, transcript gene expression levels (expressed as reads per million mapped to exons; RPM_Exon) were measured in NTG cells (white), TProg (gray) and TPC (black bars), which were isolated from SCRx-CR4 tumors. Analysis of whole transcriptome data in this manner identified several proteins and potential therapeutic targets of interest, including APCDD1, Notum, REG1A and CD46 splice variants. An analysis of the data showed that these proteins of interest were up-regulated at the transcript level at least 2-fold greater than expression in NTG cells in vehicle and irinotecan-treated mice (FIG. 20A – 20 C). Representative examples of prospective diagnostic and therapeutic target transcripts identified in this manner include NOTUM (FIG. 20A), APCDD1 (FIG. 20B), an exemplary pancreatic protein (FIG. 20C) and MUC20. Expression of these transcripts in TPC implies that these proteins are also preferentially expressed (i.e. APCDD1) or secreted (i.e. exemplary pancreatic protein and NOTUM) by TPC cells at the protein level and that these proteins are critical for TPC maintenance and/or self renewal. It was concomitantly possible to identify transcripts preferentially expressed by NTG cell populations (e.g. MUC20; FIG 20D), which may be used as markers to identify terminally differentiated cells, and in so doing facilitate a negative selection where in tumors are depleted of their bulk tumor cell population such that the more tumorigenic components can been more easily

identified and/or targeted with therapeutic regimens. Furthermore, because NTG cells are generally more numerous than their TProg and TPC counterparts, proteins expressed by these cell populations will be easier to detect, thus serving as biomarkers of disease burden and/or status.

[00247] 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 tumorigenic cell population enriched for expression of at least one TICAM.
2. The enriched tumorigenic cell population of claim 1 wherein said at least one TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.
3. The enriched tumorigenic cell population of claim 1 wherein said at least one TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.
4. The enriched tumorigenic cell population of claim 1 wherein said cells have a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.
5. A composition comprising the enriched tumorigenic cell population of claim 1 and a pharmaceutically acceptable carrier.
6. An isolated tumorigenic cell comprising at least one TICAM.
7. The cell of claim 6 wherein said at least one TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.
8. The cell of claim 7 wherein said at least one TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.
9. The cell of claim 6 wherein said cell has a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.
10. A composition comprising the cell of claim 6 and a pharmaceutically acceptable carrier.
11. A method of detecting a tumorigenic cell, comprising the steps of:
    a. contacting a tumor cell population with a binding agent which preferably associates with at least one TICAM; and
    b. detecting said binding agent associated with said tumorigenic cell.
12. The method of claim 11, wherein said binding agent comprises a nucleic acid.

13. The method of claim 11 wherein said binding agent comprises a protein.
14. The method of claim 13 wherein said protein comprises a ligand.
15. The method of claim 13 wherein said protein comprises an antibody.
16. The method of claim 15 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.
17. The method of claim 16 wherein said antibody comprises a monoclonal antibody.
18. The method of claim 17 wherein said monoclonal antibody comprises a reporter molecule.
19. The method of claim 18 wherein said detecting step comprises fluorescence activated cell sorting, magnetic-assisted cell sorting, substrate-assisted cell sorting, laser mediated sectioning, fluorometry, flow cytometry or microscopy techniques.
20. The method of claim 11, wherein said tumor cell population is derived from a solid tumor.
21. The method of claim 20 wherein said solid tumor is obtained from a subject 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.
22. The method of claim 11 wherein said detected cell is a metastatic cell.
23. The method of claim 11 wherein said contacting step takes place *in vivo*.
24. The method of claim 23 wherein said contacting step takes place in an immunocompromised mouse.
25. A method for enriching a tumorigenic cell population comprising the steps of:
    a. contacting a tumor cell population with a binding agent which preferably associates with at least one TICAM; and
    b. sorting said cells associated with said TICAM to provide an enriched tumorigenic cell population.
26. The method of claim 25, wherein said binding agent comprises a nucleic acid.
27. The method of claim 25 wherein said binding agent comprises a protein.

28. The method of claim 27 wherein said protein comprises a ligand.
29. The method of claim 27 wherein said protein comprises an antibody.
30. The method of claim 29 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.
31. The method of claim 30 wherein said TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.
32. The method of claim 30 wherein said antibody comprises a monoclonal antibody.
33. The method of claim 32 wherein said monoclonal antibody comprises a reporter molecule.
34. The method of claim 33 wherein said sorting step comprises fluorescence activated cell sorting, magnetic-assisted cell sorting, substrate-assisted cell sorting, laser mediated sectioning, fluorometry, flow cytometry or microscopy techniques.
35. The method of claim 25 wherein said tumor cell population is derived from a solid tumor.
36. The method of claim 35 wherein said solid tumor is obtained from a subject 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.
37. The method of claim 25 wherein said enriched tumorigenic cell population have a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.
38. A composition comprising the enriched tumorigenic cell population of claim 37 and a pharmaceutically acceptable carrier.
39. A method of sorting tumorigenic cells comprising the steps of:
    a. contacting a tumor cell population with a binding agent which preferably associates with at least one TICAM; and
    b. sorting cells associated with said at least one TICAM.
40. The method of claim 39, wherein said binding agent comprises a nucleic acid.
41. The method of claim 39 wherein said binding agent comprises a protein.
42. The method of claim 41 wherein said protein comprises a ligand.

43. The method of claim 41 wherein said protein comprises an antibody.

44. The method of claim 43 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.

45. The method of claim 43wherein said antibody comprises a monoclonal antibody.

46. The method of claim 45 wherein said monoclonal antibody comprises a reporter molecule.

47. The method of claim 39 wherein said sorting step comprises fluorescence activated cell sorting, magnetic-assisted cell sorting, substrate-assisted cell sorting, laser mediated sectioning, fluorometry, flow cytometry or microscopy techniques.

48. A method of determining if a cell obtained from a tumor sample is tumorigenic comprising the step of contacting the tumor cell with at least one TICAM.

49. The method of claim 48 wherein said TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.

50. The method of claim 48 wherein said contacting step comprises more than one TICAM.

51. The method of claim 48 wherein said tumor cell comprises a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.

52. A method of treating or diagnosing a disorder subject in need thereof comprising the steps of;

    a. obtaining a tumor sample from the subject; and
    b. contacting the tumor sample with at least one binding agent that preferably associates with a TICAM.

53. The method of claim 52, wherein said binding agent comprises a nucleic acid.

54. The method of claim 52 wherein said binding agent comprises a protein.
55. The method of claim 54 wherein said protein comprises a ligand.
56. The method of claim 54 wherein said protein comprises an antibody.
57. The method of claim 56 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.
58. The method of claim 52 wherein said TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.
59. The method of claim 56 wherein said antibody comprises a monoclonal antibody.
60. The method of claim 59 wherein said monoclonal antibody comprises a reporter molecule.
61. The method of claim 25 wherein said disorder comprises a solid tumor.
62. The method of claim 35 wherein said disorder comprises 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.
63. A method of determining the tumorigenicity of a cell obtained from a tumor sample comprising the step of contacting the tumor cell with at least one TICAM binding agent.
64. The method of claim 63, wherein said binding agent comprises a nucleic acid.
65. The method of claim 63 wherein said binding agent comprises a protein.
66. The method of claim 65 wherein said protein comprises a ligand.
67. The method of claim 65 wherein said protein comprises an antibody.
68. The method of claim 67 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra,

Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.

69. The method of claim 63 wherein said TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.

70. The method of claim 67 wherein said antibody comprises a monoclonal antibody.

71. The method of claim 70 wherein said monoclonal antibody comprises a reporter molecule.

72. The method of claim 63 wherein said tumor cell is a solid tumor cell.

73. The method of claim 72 wherein said solid tumor is obtained from a subject 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.

74. The method of claim 72 wherein said cell has a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.

75. A method of determining whether a subject suffering from cancer is at risk of recurrence comprising the steps of;

    a. obtaining a tumor sample from the subject; and

    b. contacting the tumor sample with at least one binding agent that preferably associates with a TICAM.

76. The method of claim 75, wherein said binding agent comprises a nucleic acid.

77. The method of claim 75 wherein said binding agent comprises a protein.

78. The method of claim 77 wherein said protein comprises a ligand.

79. The method of claim 77 wherein said protein comprises an antibody.

80. The method of claim 79 wherein said antibody preferably associates with a TICAM is selected from the group consisting of CD9, CD13, CD15, CD24, CD26, CD29, CD46, CD49a, CD49b, CD49c, CD49f, CD51, CD54, CD55, CD58, CD63, CD66a, CD66c, CD66e, CD71, CD73, CD81, CD82, CD91, CD98, CD99, CD104, CD105, CD108, CD111, CD130, CD136, CD151, CD155, CD157, CD164, CD166, CD172a, CD186, CD221, CD230, CD262, CD273, CD275, CD295, CD298, CD317, CD318, CD324, CD340, CCR10, c-Met, Eph-B2, Eph-B4, FLOR1, Glut-2, Glypican 5, HER3, IL-20Ra, Integrin a9b1, Integrin b5, LDL-R, Mer, Semaphorin 4B, TROP-2 and Vb9.

81. The method of claim 80 wherein said TICAM is selected from the group consisting of CD46, CD324, CD66c and combinations thereof.

82. The method of claim 79 wherein said antibody comprises a monoclonal antibody.

83. The method of claim 82 wherein said monoclonal antibody comprises a reporter

molecule.

84. The method of claim 75 wherein said contacting step takes place *in vivo*.

85. The method of claim 84 wherein said contacting step takes place in an immunocompromised mouse.

86. The method of claim 75 wherein said contacting step takes place *in vitro*.

87. The method of claim 75 wherein said tumor sample is a solid tumor sample.

88. The method of claim 87 wherein said tumor sample is obtained from a subject 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.

89. The method of claim 75 wherein said tumor sample has a marker phenotype comprising $CD46^{hi}$ $CD324^{+}$.

90. The method of claim 75 wherein the patient has undergone a debulking procedure.

91. A kit useful for the diagnosis or monitoring of hyperproliferative disorders comprising a receptacle comprising one or more TICAM binding agents and instructional materials for using the one or more TICAM binding agents to diagnose or monitor the hyperproliferative disorder.

92. The kit of claim 91 wherein said one or more TICAM binding agents comprises a monoclonal antibody.

93. The kit of claim 92 wherein said monoclonal antibody is associated with a reporter molecule.

94. The kit of claim 91 wherein the receptacle comprises a readable plate.

95. A method of conducting genotypic or phenotypic analysis comprising the steps of:
    a. providing a tumorigenic cell or enriched tumorigenic cell population comprising one or more TICAM;
    b. treating said cell or cell population to provide genetic or proteomic material; and
    c. analyzing said genetic or proteomic material.

96. The method of claim 95 wherein material is genetic material.

97. The method of claim 96 wherein said genetic material comprises transcriptome material.

98. A method of screening potential pharmaceutical compounds comprising the steps of:
    a. exposing a tumorigenic cell or tumorigenic cell population to a candidate compound; and
    b. contacting the tumorigenic cell or tumorigenic cell population with at least one TICAM binding agent.

99. The method of claim 98 wherein said candidate compounds are small molecules.

100. The method of claim 98 further comprising the step of sorting said tumorigenic cell or tumorigenic cell population.

101. The method of claim 100 wherein said sorting step comprises fluorescence activated cell sorting, magnetic-assisted cell sorting, substrate-assisted cell sorting, laser mediated sectioning, fluorometry, flow cytometry or microscopy techniques.

102. A method of inducing cancer comprising the steps of:
    a. providing a tumorigenic cell population enriched for one or more TICAM; and
    b. introducing the tumorigenic cell population into a subject.

103. A method of inducing cancer comprising the steps of:
    a. isolating a tumorigenic cell exhibiting one or more TICAM; and
    b. introducing isolated tumorigenic cell into a subject.

104. A method a method of generating tumor samples comprising the steps of :
    a. providing a tumorigenic cell or enriched tumorigenic cell population comprising one or more TICAM;
    b. introducing the tumorigenic cell or enriched tumorigenic cell population into a subject;
    c. waiting for the tumorigenic cell or cell population to generate a heterogeneous tumor; and
    d. harvesting said heterogeneous tumor.

105. The method of claim 104 wherein said subject is administered a candidate compound during said waiting step.

106. The method of claim 105 wherein said heterogeneous is sorted.

107. An animal model for the analysis of pharmaceutical compounds comprising a subject implanted with a tumorigenic cell or cell population purified or enriched for one or more TICAM.

108. The method of claim 107 wherein said subject comprises an immunocompromised mouse.

109. A method of producing an animal model for the analysis of pharmaceutical compounds comprising the step of introducing a tumorigenic cell or cell population purified or enriched for one or more TICAM.

110. The method of claim 109 wherein said subject comprises an immunocompromised mouse.

111. A method of analyzing cancer progression and/or pathogenesis *in vivo*, comprising the steps of:

a. introducing one or more tumorigenic cells comprising one or more TICAM into a subject; and

b. analyzing cancer progression and/or pathogenesis in the subject.