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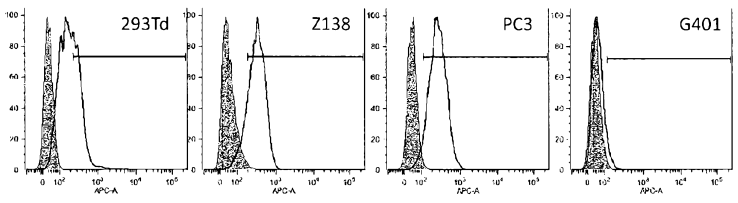

FIG. 10A

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

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NOVEL MODULATORS AND METHODS OF USE

CROSS REFERENCED APPLICATIONS

This application claims priority to U.S. Provisional Application Ser. No. 61/421,157 filed December 8, 2010 and Patent Cooperation Treaty (PCT); No. PCT/US2011/050451, filed September 2, 2011, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This application generally relates to novel compositions and methods of their use in preventing, treating or ameliorating hyperproliferative disorders and any expansion, recurrence, relapse or metastasis thereof. In a broad aspect, the present invention relates to the use of ephrin-A ligand (EFNA) modulators, including anti-EFNA antibodies and fusion constructs, for the treatment or prophylaxis of neoplastic disorders. Particularly preferred embodiments of the present invention provide for the use of such EFNA modulators directed to EFNA1 and EFNA3 for the immunotherapeutic treatment of malignancies comprising a reduction in tumor initiating cell frequency.

BACKGROUND OF THE INVENTION

Stem and progenitor cell differentiation and cell proliferation are normal ongoing processes that act in concert to support tissue growth during organogenesis and cell replacement and repair of most tissues during the lifetime of all living organisms. Differentiation and proliferation decisions are often controlled by numerous factors and signals that are balanced to maintain cell fate decisions and tissue architecture. Normal tissue architecture is largely maintained by 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 hyperproliferative disorders such as cancer.

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. For example, in some patients certain cancers exhibit gene mutations that render them non-responsive despite the general effectiveness of selected therapies. 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 which 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.

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 generating the 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 an inherent 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 differentiation to tumor progenitor cells and, subsequently, to terminally differentiated tumor cells.

In recent years it has become more evident these CSC (also known as tumor perpetuating cells or TPC) might be more resistant to traditional chemotherapeutic agents or radiation and thus persist after standard of care clinical therapies to later fuel the growth of refractory tumors, secondary tumors and promote metastases. Moreover, 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.

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. In numerous cancers 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. While progress has been made, inherent technical difficulties associated with handling primary and/or xenograft tumor tissue, along with a lack of experimental platforms to characterize CSC identity and differentiation potential, pose major challenges. As such, there remains a substantial need to selectively target cancer stem cells and develop diagnostic, prophylactic or therapeutic compounds or methods that may be used in the treatment, prevention and/or management of hyperproliferative disorders.

SUMMARY OF THE INVENTION

These and other objectives are provided for by the present invention which, in a broad sense, is directed to methods, compounds, compositions and articles of manufacture that may be used in the treatment of EFNA associated disorders (e.g., hyperproliferative disorders or neoplastic disorders). To that end, the present invention provides novel EFNA (or ephrin-A ligand) modulators that effectively target tumor cells or cancer stem cells and may be used to treat patients suffering from a wide variety of malignancies. As will be discussed in more detail herein, there are presently six known ephrin-A ligands (i.e., EFNAs 1-6) and the disclosed modulators preferably comprise or associate with EFNA1 and/or EFNA3. Moreover, in certain embodiments the disclosed EFNA modulators may comprise any compound that recognizes, competes, agonizes, antagonizes, interacts, binds or associates with an EFNA1 or EFNA3 polypeptide their receptors or genes and modulates, adjusts, alters, changes or modifies the impact of the EFNA protein on one or more physiological pathways. Thus, in a broad sense the present invention is directed to isolated EFNA modulators selected from the group consisting of EFNA1 modulators and EFNA3 modulators (or, as generally used herein, EFNA modulators unless otherwise dictated by context). In preferred embodiments the invention is more particularly directed to isolated EFNA1 modulators or isolated EFNA3 modulators comprising antibodies (i.e., antibodies that comprise or associate with at least EFNA1 or EFNA3). Moreover, as discussed extensively below, such modulators may be used to provide pharmaceutical compositions.

In selected embodiments of the invention, EFNA modulators may comprise an EFNA1 or EFNA3 ligand itself or fragments thereof, either in an isolated form or fused or associated with other moieties (e.g., Fc-EFNA, PEG-EFNA or EFNA associated with a targeting moiety). In other selected embodiments EFNA modulators may comprise EFNA antagonists which, for the purposes of the instant application, shall be held to mean any construct or compound that recognizes, competes, interacts, binds or associates with EFNA1 and/or EFNA3 and neutralizes, eliminates, reduces, sensitizes, reprograms, inhibits or controls the growth of neoplastic cells including tumor initiating cells. In preferred embodiments the EFNA modulators of the instant invention comprise anti-EFNA1 or anti-EFNA3 antibodies, or fragments or derivatives thereof, that have unexpectedly been found to silence, neutralize, reduce, decrease, deplete, moderate, diminish, reprogram, eliminate, or otherwise inhibit the ability of tumor initiating cells to propagate, maintain, expand, proliferate or otherwise facilitate the survival, recurrence,

regeneration and/or metastasis of neoplastic cells. In particularly preferred embodiments the antibodies or immunoreactive fragments may be associated with or conjugated to one or more anti-cancer agents.

In selected embodiments compatible EFNA1 modulators may comprise an antibody having a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 9, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 25, SEQ ID NO: 29, SEQ ID NO: 33, SEQ ID NO: 37, SEQ ID NO: 41 and SEQ ID NO: 45 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 19, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 31, SEQ ID NO: 35, SEQ ID NO: 39 and SEQ ID NO: 43.

In other embodiments compatible EFNA3 modulators may comprise an antibody having a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 49, SEQ ID NO: 53, SEQ ID NO: 57, SEQ ID NO: 61, SEQ ID NO: 65, SEQ ID NO: 69, SEQ ID NO: 73, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 97 and SEQ ID NO: 101 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 47, SEQ ID NO: 51, SEQ ID NO: 55, SEQ ID NO: 59, SEQ ID NO: 63, SEQ ID NO: 67, SEQ ID NO: 71, SEQ ID NO: 75, SEQ ID NO: 79, SEQ ID NO: 83, SEQ ID NO: 87, SEQ ID NO: 91, SEQ ID NO: 95 and SEQ ID NO: 99.

Of course, in view of the instant disclosure those skilled in the art could readily identify CDRs associated with each of the aforementioned heavy and light chain variable regions and use those CDRs to engineer or fabricate chimeric, humanized or CDR grafted antibodies without undue experimentation. As such, in selected embodiments the present invention is directed to anti-EFNA1 or anti-EFNA3 antibodies comprising one or more CDRs from a variable region sequence set forth in FIG. 6 or FIG. 7. In preferred embodiments such antibodies will comprise monoclonal antibodies and, in even more preferred embodiments will comprise chimeric, CDR grafted or humanized antibodies. As

discussed in more detail below still other embodiments will comprise such antibodies conjugated or associated with one or more cytotoxic agents.

In certain other embodiments the invention will comprise an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators that reduces the frequency of tumor initiating cells upon administration to a subject. Preferably the reduction in frequency will be determined using *in vitro* or *in vivo* limiting dilution analysis. In particularly preferred embodiments such analysis may be conducted using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice. Alternatively, the limiting dilution analysis may be conducted using *in vitro* limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions. In either case, the analysis, calculation or quantification of the reduction in frequency will preferably comprise the use of Poisson distribution statistics to provide an accurate accounting. It will be appreciated that, while such quantification methods are preferred, other, less labor intensive methodology such as flow cytometry or immunohistochemistry may also be used to provide the desired values and, accordingly, are expressly contemplated as being within the scope of the instant invention. In such cases the reduction in frequency may be determined using flow cytometric analysis or immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.

As such, in another preferred embodiment of the instant invention comprises a method of treating an EFNA associated disorder comprising administering a therapeutically effective amount of an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators to a subject in need thereof whereby the frequency of tumor initiating cells is reduced. Again, the reduction in the tumor initiating cell frequency will preferably be determined using *in vitro* or *in vivo* limiting dilution analysis.

In this regard it will be appreciated that the present invention is based, at least in part, upon the discovery that EFNA polypeptides (i.e., EFNA1 and EFNA3 as discussed below) are associated with tumor perpetuating cells (i.e., cancer stem cells) that are involved in the etiology of various neoplasia. More specifically, the instant application unexpectedly demonstrates that the administration of various exemplary EFNA modulators can mediate, reduce, inhibit or eliminate tumorigenic signaling by tumor initiating cells (i.e., reduce the frequency of tumor initiating cells). This reduced signaling, whether by reduction, elimination, reprogramming or silencing of the tumor

initiating cells or by modifying tumor cell morphology (e.g., induced differentiation, niche disruption), in turn allows for the more effective treatment of EFNA associated disorders by inhibiting tumorigenesis, tumor maintenance, expansion and/or metastasis and recurrence.

In other embodiments the disclosed modulators of EFNA1 or EFNA3 may promote, support or otherwise enhance EFNA mediated signaling that may limit or restrain tumor growth. In other embodiments the disclosed modulators may interfere, suppress or otherwise retard EFNA mediated signaling that may fuel tumor growth. Further, as will be discussed in more detail below, EFNA1 and EFNA3 polypeptides may be involved in generating adhesive and repulsive forces between cells through integrin and cytoskeleton rearrangements or cytostructural modifications. Intervention in such intercellular interactions, using the novel EFNA modulators described herein, may thereby ameliorate a disorder by more than one mechanism (i.e., tumor initiating cell reduction and disruption of cellular adhesion) to provide additive or synergistic effects. Still other preferred embodiments may take advantage of the cellular internalization of ephrin-A ligands to deliver a modulator mediated anti-cancer agent. In this regard it will be appreciated that the present invention is not limited by any particular mechanism of action but rather encompasses the broad use of the disclosed modulators to treat EFNA associated disorders (including various neoplasia).

Thus, another preferred embodiment of the invention comprises a method of treating an EFNA associated disorder in a subject in need thereof comprising the step of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators to said subject. In particularly preferred embodiments the EFNA modulator will be associated (e.g., conjugated) with an anti-cancer agent. In yet other embodiments the EFNA modulator will internalize following association or binding with the ephrin-A ligand on or near the surface of the cell. Moreover the beneficial aspects of the instant invention, including any cellular adhesion disruption and collateral benefits, may be achieved whether the subject tumor tissue exhibits elevated levels of EFNA or reduced or depressed levels of EFNA as compared with normal adjacent tissue.

As alluded to above and discussed in more detail below there are currently six known ephrin-A ligands (i.e., EFNAs 1-6). In accordance with the instant invention it will be appreciated that the disclosed modulators may be generated, fabricated and/or selected to react with a single ephrin-A ligand (e.g., EFNA1), a subset of ephrin-A ligands (e.g.,

EFNA1 and EFNA3) or all six ephrin-A ligands. More particularly, as described herein and set forth in the Examples below, preferred modulators such as antibodies may be generated and selected so that they react or bind with domains or epitopes that are expressed on a single ephrin-A ligand or with epitopes that are conserved (at least to some extent) and presented across multiple or all EFNA polypeptides (e.g., EFNAs 1 and 3 or EFNAs 1, 3 and 6). This is significant with respect to the instant invention in that, as shown in the Examples below, certain ephrin-A ligands including EFNA1 and EFNA3 have been found to be preferably expressed on TIC and, in combination, may serve as particularly effective therapeutic targets that provide for the selective reduction in tumorigenic cell frequency and/or depletion of cancer stem cell populations.

Of course it will be appreciated that the disclosed EFNA modulators may be generated, fabricated and/or selected to preferentially react or associate with a single ephrin-A ligand (e.g., EFNA1) and exhibit minimal or no association with any other ephrin-A ligand. Accordingly, selected embodiments of the invention are directed to EFNA modulators that immunospecifically associate with a selected ephrin-A ligand such as EFNA1 or EFNA3 and exhibit little or no association with any other ephrin-A ligand. In this regard preferred embodiments disclosed herein will comprise methods of treating an EFNA associated disorder in a subject in need thereof comprising the step of administering an EFNA modulator wherein the EFNA modulator immunospecifically associates with EFNA1 or EFNA3 and is substantially non-reactive with any other ephrin-A ligand. Further, methods of generating, fabricating and selecting such modulators are within the scope of the instant invention.

Other facets of the instant invention exploit the ability of the disclosed modulators to potentially disrupt cell interactions while simultaneously silencing tumor initiating cells. Such multi-active EFNA modulators (e.g., EFNA antagonists) may prove to be particularly effective when used in combination with standard of care anti-cancer agents or debulking agents. In addition, two or more EFNA antagonists (e.g. antibodies that specifically bind to two discrete epitopes on an ephrin-A ligand or that associate with discrete ligands) may be used in combination in accordance with the present teachings. Moreover, as discussed in some detail below, the EFNA modulators of the present invention may be used in a conjugated or unconjugated state and, optionally, as a sensitizing agent in combination with a variety chemical or biological anti-cancer agents.

Thus, another preferred embodiment of the instant invention comprises a method of sensitizing a tumor in a subject for treatment with an anti-cancer agent comprising the step

of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators to said subject. In a particularly preferred aspect of the invention the EFNA modulator will specifically result in a reduction of tumor initiating cell frequency is as determined using *in vitro* or *in vivo* limiting dilution analysis thereby sensitizing the tumor for concomitant or subsequent debulking.

Similarly, as the compounds of the instant invention may exert therapeutic benefits through various physiological mechanisms, the present invention is also directed to selected effectors or modulators that are specifically fabricated to exploit certain cellular processes. For example, in certain embodiments the preferred modulator may be engineered to associate with EFNA on or near the surface of the tumor initiating cell and stimulate the subject's immune response. In other embodiments the modulator may comprise an antibody directed to an epitope that neutralizes EFNA1 or EFNA3 activity and interactions with ephrin receptors which may impact adhesive and repulsive forces between cells through integrin and cytoskeleton rearrangements or cytostructural modifications. In yet other embodiments the disclosed modulators may act by depleting or eliminating the EFNA associated cells. As such, it is important to appreciate that the present invention is not limited to any particular mode of action but rather encompasses any method or EFNA modulator that achieves the desired outcome.

Within such a framework preferred embodiments of the disclosed embodiments are directed to a method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one neutralizing EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

In yet another aspect the present invention will comprise a method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one internalizing EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators. Preferred embodiments will comprise the administration of internalizing antibody modulators wherein, in other selected embodiments, the internalizing antibody modulators are conjugated or associated with a cytotoxic agent.

Other embodiments are directed to a method of treating a subject suffering from an EFNA associated disorder comprising the step of administering a therapeutically effective amount of at least one depleting EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators. A related method is directed to depleting

EFNA associated cells in a subject in need thereof comprising the step of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

In yet another embodiment the present invention provides methods of maintenance therapy wherein the disclosed effectors or modulators are administered over a period of time following an initial procedure (e.g., chemotherapeutic, radiation or surgery) designed to remove at least a portion of the tumor mass. Such therapeutic regimens may be administered over a period of weeks, a period of months or even a period of years wherein the EFNA modulators may act prophylactically to inhibit metastasis and/or tumor recurrence. In yet other embodiments the disclosed modulators may be administrated in concert with known debulking regimens to prevent or retard metastasis.

Beyond the therapeutic uses discussed above it will also be appreciated that the modulators of the instant invention may be used to diagnose EFNA related disorders and, in particular, hyperproliferative disorders. In some embodiments the modulator may be administered to the subject and detected or monitored *in vivo*. Those of skill in the art will appreciate that such modulators may be labeled or associated with markers or reporters as disclosed below and detected using any one of a number of standard techniques (e.g., MRI or CAT scan).

Thus, in some embodiments the invention will comprise a method of diagnosing, detecting or monitoring an EFNA associated disorder *in vivo* in a subject in need thereof comprising the step of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

In other instances the modulators may be used in an *in vitro* diagnostic setting using art-recognized procedures. As such, a preferred embodiment comprises a method of diagnosing a hyperproliferative disorder in a subject in need thereof comprising the steps of:

a. obtaining a tissue sample from said subject;
b. contacting the tissue sample with at least one EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators; and
c. detecting or quantifying the EFNA modulator associated with the sample.

Such methods may be easily discerned in conjunction with the instant application and may be readily performed using generally available commercial technology such as automatic plate readers, dedicated reporter systems, etc. In selected embodiments the

EFNA modulator will be associated with tumor perpetuating cells present in the sample. In other preferred embodiments the detecting or quantifying step will comprise a reduction of tumor initiating cell frequency and detection thereof. Moreover, limiting dilution analysis may be conducted as previously alluded to above and will preferably employ the use of Poisson distribution statistics to provide an accurate accounting as to the reduction of frequency.

In a similar vein the present invention also provides kits that are useful in the diagnosis and monitoring of EFNA associated disorders such as cancer. To this end the present invention preferably provides an article of manufacture useful for diagnosing or treating EFNA associated disorders comprising a receptacle comprising an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators and instructional materials for using said EFNA modulator to treat or diagnose the EFNA associated disorder.

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

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

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

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A - 1E depict, respectively, the nucleic acid sequence encoding human EFNA1 (SEQ ID NO: 1), the corresponding amino acid sequence of human EFNA1 isoform a (SEQ ID NO: 2), an alignment of human EFNA1 a, and b isoform sequences showing amino acid differences (SEQ ID NOS: 2-3), the amino acid sequence encoding human EFNA3 (SEQ ID NO: 4) and a corresponding nucleic acid sequence of human EFNA3 (SEQ ID NO: 5);

FIGS. 2A - 2E are graphical representations depicting, respectively, the gene expression levels of selected human ephrin-A ligands and ephrin-A receptors in untreated (FIG. 2A) and in irinotecan treated (FIG. 2B) colorectal tumors and EFNA1 in treated and untreated colorectal (FIG. 2C), pancreatic (FIG. 2D) and non-small cell lung cancer (FIG. 2E) tumors as measured using whole transcriptome sequencing of highly enriched tumor progenitor cell (TProg) and tumor perpetuating cell (TPC) and non-tumorigenic cell (NTG) populations obtained from a subset of whole tumor specimens;

FIGS. 3A - 3C are graphical representations depicting the gene expression levels of human ephrin-A3 ligand in treated and untreated colorectal tumor samples (FIG. 3A), pancreatic tumor samples (FIG. 3B) and non-small cell lung cancer samples (FIG. 3C) as measured using whole transcriptome sequencing of highly enriched tumor progenitor cell (TProg) and tumor perpetuating cell (TPC) and non-tumorigenic cell (NTG) populations or tumorigenic (TG) and non-tumorigenic cell (NTG) populations;

FIGS. 4A and 4B are graphical representations showing the relative gene expression levels of human EFNA1 (FIG. 4A) and EFNA3 (FIG.4B) as measured using RT-PCR in colorectal and pancreatic tumor specimens comprising tumor perpetuating cell (TPC) and non-tumorigenic cell (NTG) populations;

FIGS. 5A and 5B illustrate the relative gene expression of EFNA1 (FIG. 5A) and EFNA3 (FIG. 5B) represent gene expression levels of human EFNA genes as measured by RT-PCR in whole tumor specimens (grey dot) or matched NAT (white dots) from patients with one of a number of solid tumor types;

FIGS. 6A – 6J depict the murine heavy and light chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 6 - 45) of several exemplary EFNA1 modulators isolated and cloned as described herein;

FIGS. 7A – 7N depict the murine heavy and light chain variable region nucleic acid and amino acid sequences (SEQ ID NOS: 46 - 101) of several exemplary EFNA3 modulators isolated and cloned as described herein;

FIG. 8 sets forth biochemical and immunological properties of exemplary EFNA1 modulators as represented in a tabular format;

FIG. 9 sets forth biochemical and immunological properties of exemplary EFNA3 modulators as represented in a tabular format;

FIGS. 10A - 10C illustrate, respectively, cell surface binding properties of an exemplary EFNA1 modulator (open histogram) compared to isotype control antibody (shaded histogram) with regard to four selected traditional tumor cell lines (FIG. 10A) and exemplary EFNA1 (FIG. 10B) and EFNA3 (FIG. 10C) modulator binding to engineered cells expressing the respective ephrin-A ligand;

FIGS. 11A – 11C are graphical representations illustrating the ability of ephrin-A ligands to interact selectively with numerous EPHA receptors wherein HEK293T cells only bind EPHA-ECD-Fc receptor constructs via endogenously expressed ephrin-A ligands to a limited degree (FIG. 11A) while HEK293T cells overexpressing EFNA1 (FIG. 11B) and HEK293T cells overexpressing EFNA3 (FIG. 11C) bind tested EPHA receptor constructs to various degrees;

FIGS. 12A and 12B illustrate the ability the disclosed modulators to inhibit the cell surface binding of human EPHA receptors wherein FIG. 12A demonstrates the ability of 12 exemplary EFNA1 modulators to reduce EPHA receptor binding to EFNA1 expressing cells and FIG. 12B demonstrates the ability of 16 exemplary EFNA3 modulators to reduce EPHA receptor binding to EFNA3 expressing cells;

FIGS. 13A and 13B illustrate that exemplary EFNA1 modulators may effectively be used as targeting moieties to direct cytotoxic payloads to cells expressing significant levels of ephrin-A1 ligand (FIG. 13B) wherein the downward sloping curve is indicative of cell killing through internalized payload, while cells expressing low levels of ephrin-A1 ligand (FIG. 13A) are not eliminated;

FIGS. 14A and 14B illustrate that exemplary EFNA3 modulators may effectively be used as targeting moieties to direct cytotoxic payloads to cells expressing significant levels of ephrin-A3 ligand (FIG. 14B) wherein the downward sloping curve is indicative of cell killing through internalized payload, while cells expressing low levels of ephrin-A3 ligand (FIG. 13A) are not eliminated; and

FIGS. 15A and 15B provide evidence that exemplary EFNA1 modulators may effectively be used as targeting moieties to direct cytotoxic payloads to patient derived NTX lung (FIG. 15A) and ovarian (FIG. 15B) cancer stem cell populations expressing ephrin-A ligand.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

While the present invention may be embodied in many different forms, disclosed herein are specific illustrative embodiments thereof that exemplify the principles of the invention. It should be emphasized that the present invention is not limited to the specific embodiments illustrated. Moreover, any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

As previously alluded to, it has surprisingly been found that the expression of ephrin-A ligands (or EFNA) such as ephrin-A1 and ephrin-A3 are associated with neoplastic growth and hyperproliferative disorders and that such ligands provide useful tumor markers which may be exploited in the treatment of related diseases. More specifically, it has been discovered that EFNA modulators such as those disclosed herein may advantageously be used in the diagnosis, theragnosis, treatment or prevention of neoplastic disorders in subjects in need thereof. Accordingly, while preferred embodiments of the invention will be discussed extensively below, particularly in the context of cancer stem cells and their interactions with the disclosed modulators, those skilled in the art will appreciate that the scope of the instant invention is not limited by such exemplary embodiments. Rather, the present invention and the appended claims are broadly and expressly directed to EFNA modulators selected from the group consisting of EFNA1 modulators and EFNA3 modulators and their use in the diagnosis, theragnosis, treatment or prevention of a variety of EFNA associated or mediated disorders, including neoplastic or hyperproliferative disorders, regardless of any particular mechanism of action or specifically targeted tumor component.

It will further be appreciated that, in contrast to many prior art disclosures, the present invention is largely directed to ephrin ligand modulators (i.e. EFN) rather than ephrin receptor (i.e. EPH) modulators. That is, while ephrin receptors have been widely implicated in several types of disorders and generally targeted for therapeutic intervention, ephrin ligands have heretofore attracted much less attention. In part this may be as a result of the promiscuous behavior attributed to the ligands and the misplaced belief that such varied interactions made them untenable therapeutic targets as pathway redundancy would likely compensate for any ligand antagonism. However, as demonstrated herein the disclosed ephrin-A ligand modulators can effectively be used to target and eliminate or

otherwise incapacitate tumorigenic cells. Moreover, in selected embodiments the present invention may comprise modulators that associate or react with more than one ephrin-A ligand thereby providing an unexpected additive or synergistic effect that may allow for quiescence of more than one ephrin ligand mediated pathway.

Besides the general association discussed immediately above, the inventors have further discovered a heretofore unknown phenotypical association between selected "tumor initiating cells" (TIC) and ephrin-A ligands such as EFNA1 and EFNA3. In this regard, it has been found that selected TICs express elevated levels of ephrin-A ligands when compared to normal tissue and non-tumorigenic cells (NTG), which together comprise much of a solid tumor. Thus, the ephrin-A ligands comprise tumor associated markers (or antigens) and have been found to provide effective agents for the detection and suppression of TIC and associated neoplasia due to elevated levels of the proteins on cell surfaces or in the tumor microenvironment. More specifically, it has further been discovered that EFNA modulators, including immunoreactive antagonists and antibodies that associate or react with the proteins, effectively reduce the frequency of tumor initiating cells and are therefore useful in eliminating, incapacitating, reducing, promoting the differentiation of, or otherwise precluding or limiting the ability of these tumor-initiating cells to lie dormant and/or continue to fuel tumor growth, metastasis or recurrence in a patient. As discussed in more detail below, the TIC tumor cell subpopulation is composed of both tumor perpetuating cells (TPC) and highly proliferative tumor progenitor cells (TProg).

In view of these discoveries, those skilled in the art will appreciate that the present invention further provides EFNA modulators selected from the group consisting of EFNA1 modulators and EFNA3 modulators and their use in reducing the frequency of tumor initiating cells. As will be discussed extensively below, EFNA modulators of the invention broadly comprise any compound that recognizes, reacts, competes, antagonizes, interacts, binds, agonizes, or associates with EFNA1 or EFNA3 or their genes. By these interactions, the EFNA modulators thereby reduce or moderate the frequency of tumor initiating cells. Exemplary modulators disclosed herein comprise nucleotides, oligonucleotides, polynucleotides, peptides or polypeptides. In certain preferred embodiments the selected modulators will comprise antibodies to EFNA1 or EFNA3 or immunoreactive fragments or derivatives thereof. Such antibodies may be antagonistic or agonistic in nature and may optionally be conjugated or associated with a cytotoxic agent. In other embodiments, modulators within the instant invention will comprise an EFNA

construct comprising an ephrin-A ligand selected from the group consisting of EFNA1 modulators and EFNA3 or a reactive fragment thereof. It will be appreciated that such constructs may comprise fusion proteins and can include reactive domains from other polypeptides such as immunoglobulins or biological response modifiers. In still other aspects, the EFNA modulator will comprise a nucleic acid assembly that exerts the desired effects at a genomic level. Still other modulators compatible with the instant teachings will be discussed in detail below.

Whichever form of modulator is ultimately selected it will preferably be in an isolated and purified state prior to introduction into a subject. In this regard the term "isolated EFNA modulator" or "isolated EFNA1 modulator" or "isolated EFNA3 modulator" shall be construed in a broad sense and in accordance with standard pharmaceutical practice to mean any preparation or composition comprising the modulator in a state substantially free of unwanted contaminants (biological or otherwise). As will be discussed in some detail below these preparations may be purified and formulated as desired using various art recognized techniques. Of course, it will be appreciated that such "isolated" preparations may be intentionally formulated or combined with inert or active ingredients as desired to improve the commercial, manufacturing or therapeutic aspects of the finished product and provide pharmaceutical compositions.

II. EFNA Physiology

Ephrin receptor tyrosine kinases (EPH), type-I transmembrane proteins, comprise the largest family of receptor tyrosine kinases within animal genomes and interact with ephrin ligands (EFN), which are also cell surface associated. Receptors in the EPH subfamily typically have a single kinase domain and an extracellular region containing a Cys-rich domain and 2 fibronectin type III repeats. Convention holds that ephrin receptors are divided into two groups based on the similarity of their extracellular domain sequences and their affinities for binding ephrin-A and ephrin-B ligands. Previous research has shown that EPH mediated signaling events control multiple aspects of embryonic development, particularly in the nervous system and are important mediators of cell-cell communication regulating cell attachment, shape, and mobility. Moreover, many members of the ephrin receptor family, as opposed to ephrin ligands, have been identified as important markers and/or regulators of the development and progression of cancer. To date nine ephrin-A receptors and six ephrin-B receptors are known

For the purposes of the instant application the terms "ephrin receptor," "ephrin-A receptor," "ephrin-B receptor," "EPHA," or "EPHB" (or EphA or EphB) may be used interchangeably and held to mean the specified family, subfamily or individual receptor (i.e., EPHA1, EPHA2, EPHA3, EPHA4, EPHA5, EPHA6, EPHA7, EPHA8, EPHA9, EPHB1, EPHB2, EPHB3, EPHB4, EPHB5, EPHB6) as dictated by context.

Based upon sequence analyses, ephrin ligands can be divided into two groups: six ephrin-A ligands (or EFNA), typically anchored to the cell surface via glycosylphosphatidylinositol linkages (although some non-GPI-anchored proteins are produced through alternative splicing of ephrin mRNAs; e.g. EFNA1) and three ephrin-B ligands (or EFNB) containing a transmembrane domain and a short cytoplasmic region with conserved tyrosine residues and a PDZ-binding motif. EFNA ligands interact preferentially with any of the nine different EPHA receptors, whereas EFNB ligands interact preferentially with any of six different EPHB receptors, although some specific EFNA-EPHB and EFNB-EPHA cross-interactions have been reported.

For the purposes of the instant application the terms "ephrin ligand," "ephrin-A ligand," "ephrin-B ligand," "EFNA," or "EFNB" may be used interchangeably and held to mean the specified family, subfamily or individual receptor (i.e., EFNA1, EFNA2, EFNA3, EFNA4, EFNA5, EFNA6, EFNB1, EFNB2, EFNB3) as dictated by context. For example, the terms "ephrin-A1," ephrin-A1 ligand" or "EFNA1" shall all be held to designate the same family of protein isoforms (e.g., as set forth in FIG. 1B) while the terms "ephrin-A ligand" and "ENFA" shall be held to mean the ephrin subfamily (i.e. A as opposed to B) comprising all six A type ligands and any isoforms thereof.

A more detailed summary of ephrin receptor and ligand nomenclature may be found in Table 1 immediately below.

TABLE 1

Receptors		Ligands	
new name	*previous names*	*new name*	*previous names*
EphA1	Eph, Esk	ephrin-A1	B61; LERK-1, EFL-1
EphA2	Eck, Myk2, Sek2	ephrin-A2	ELF-1; Cek7-L, LERK-6
EphA3	Cek4, Mek4, Hek, Tyro4; Hek4	ephrin-A3	Ehk1-L, EFL-2, LERK-3
EphA4	Sek, Sek1, Cek8, Hek8, Tyro1	ephrin-A4	LERK-4; EFL-4
EphA5	Ehk1, Bsk, Cek7, Hek7; Rek7	ephrin-A5	AL-1, RAGS; LERK-7, EFL-5
EphA6	Ehk2; Hek12	ephrin-A6	
EphA7	Mdk1, Hek11, Ehk3, Ebk, Cek11		
EphA8	Eek; Hek3		
EphA9			
EphB1	Elk, Cek6, Net; Hek6	ephrin-B1	LERK-2, Elk-L, EFL-3, Cek5-L; STRA1
EphB2	Cek5, Nuk, Erk, Qek5, Tyro5, Sek3; Hek5, Drt	ephrin-B2	Htk-L, ELF-2; LERK-5, NLERK-1
EphB3	Cek10, Hek2, Mdk5, Tyro6, Sek4	ephrin-B3	NLERK-2, Elk-L3, EFL-6, ELF-3; LERK-8
EphB4	Htk, Myk1, Tyro11; Mdk2		
EphB5	Cek9; Hek9		
EphB6	Mep		

Eph Nomenclature Committee, Cell. 1997; 90 (3):403-4, which is incorporated herein in its entirety by reference.

As with all cell surface receptor-ligand interactions, engagement of the ephrin receptor by an ephrin ligand ultimately results in the activation of intracellular signaling cascades. Although receptor-ligand interactions may take place between molecules on the surface of the same cell (*cis* interactions), it is generally thought that *cis* interactions do not lead to the triggering of signaling cascades, or that *cis* interactions may actually antagonize signaling cascades initiated by *trans* interactions (e.g., between receptors and ligands on separate cells). One unique aspect of EPH-EFN *trans* interactions is the capacity for the triggering of two signaling cascades upon receptor-ligand engagement-- a forward signaling cascade in the cell expressing the ephrin receptor, and a reverse signaling cascade in the cell expressing the ephrin ligand. The activation of two separate signaling cascades may reflect cell sorting and cell positioning processes that EPH and EFN have evolved to co-ordinate in animal embryonic development.

EPH-EFN signaling frequently activates cell-signaling pathways that regulate cytoskeletal dynamics and lead to modulation of the adhesive and repulsive interactions between different types of cells. As a generalization, EPH and EFN proteins are found at much higher levels during embryogenesis versus those observed in adult tissues, although continued low-level expression in the adult may reflect roles for these molecules in the normal function of tissues such as the adult gut, which has a well defined architecture arising from the migration of differentiating cells from their source at the tissue stem cell in the crypt to their final location at the surface of the villi facing the intestinal lumen. Since ephrin receptors were first identified in hepatocellular carcinomas, and EPH and EFN expression is typically limited in adults, reactivation of the expression of ephrin ligands and/or ephrin receptors in human cancers may be linked to the dedifferentiation of the cancer cells and/or the ability of these cancer cells to invade surrounding normal tissue and to migrate from the site of the primary tumor to distant locations. Other studies have suggested that EPH-EFN interactions also have a role in neoangiogenesis.

Consistent with findings that EPH-EFN interactions in non-lymphoid tissues regulate cellular interactions by generating adhesive or repulsive forces between cells through integrin and cytoskeleton rearrangements, EPH and EFN molecules found on lymphoid cells have been shown to mediate cell adhesion to extracellular matrix components, chemotaxis and cell migration. For example, EFNA1 engagement on primary CD4 and CD8 T cells has been found to stimulate cell migration and enhance chemotaxis. Such properties implicate selected members of the EFNA ligand family as potential markers for various disorders and, in view of the instant disclosure and Examples below, as tumor markers. In this regard it has been found that EFNA1 and EFNA3 are of particular interest as potential therapeutic and diagnostic targets with respect to hyperproliferative disorders.

More specifically, as will be discussed in more detail below EFNA1 and EFNA3 have been found to display elevated expression in cancer stem cell populations, while concomitant upregulation of several EPHA receptors in the bulk tumor raises the possibility that these ligand receptor interactions may be triggering cell signaling cascades linked to tumor proliferation, neoangiogenesis and/or tumor metastasis. While not wishing to be bound by any particular theory, it is believed that EFNA1 and EFNA3 effectors, antagonists, and particularly EFNA1 or EFNA3 targeting moieties of the present invention act, at least in part, by either interfering with oncogenic survival outside the context of standard of care therapeutic regimens (e.g. irinotecan), thereby reducing or

eliminating tumor initiating cell frequency or signaling or delivering an entity able to kill EFNA1 or EFNA3 expressing cells. For example, elimination of TPC by antagonizing EFNA1 or EFNA3 may include simply promoting cell proliferation in the face of chemotherapeutic regimens that eliminate proliferating cells, or promote differentiation of TPC such that their self-renewal (i.e. unlimited proliferation and maintenance of multipotency) capacity is lost. Alternatively, recruitment of cytotoxic T-cells to EFNA1 or EFNA3 expressing cells, or delivery of a potent toxin conjugated to an anti-EFNA1 or anti-EFNA3 antibody that was able to internalize, may selectively kill TPC.

As used herein the term EFNA1 (also known as B61, ligand of eph-related kinase 1, LERK1; or eph-related receptor tyrosine kinase ligand 1) to naturally occurring human EFNA1 unless contextually dictated otherwise. Representative EFNA1 protein orthologs include, but are not limited to, human (i.e. hEFNA1, NP_004419 or NP_872626), mouse (NP_034237 or NP_001155897) chimpanzee (XP_001141980 and XP_003308473) and rat (NP_446051). The transcribed human EFNA1 gene comprises at minimum 7038 bp from chromosome 1 at q21-22; the transcript may then undergo alternative splicing into a minimum of two reported forms: (1) a 1590 bp variant (NM_004428; EFNA1 transcript variant 1) which encodes a 205 amino acid proprotein (NP_004419; EFNA1 isoform a); and (2) a 1524 bp variant (NM_182685; EFNA1 transcript variant 2) which in encodes a 183 amino acid proprotein (NP_872626; EFNA isoform b). An exemplary EFNA1 nucleic acid sequence (variant 1) is provided in FIG. 1A (SEQ ID NO: 1), an exemplary amino acid sequence is provided in FIG. 1B (SEQ ID NO: 2) and aligned isoforms a and b are provided in FIG. 1C (SEQ ID NOS: 2 and 3).

As used herein the term EFNA3 (also known as ligand of eph-related kinase 3, LERK3; or eph-related receptor tyrosine kinase ligand 3) to naturally occurring human EFNA3 unless contextually dictated otherwise. Representative EFNA3 protein orthologs include, but are not limited to, human (i.e. hEFNA3, NP_004943), mouse (NP_034238), chimpanzee (XP_003308464 and XP_003308465) and rat (XP_574979). The transcribed human EFNA3 gene comprises at minimum 8667 bp from chromosome 1 at q21-22; the transcript is subsequently spliced into the mature mRNA (NM_004952) encoding a 238 amino acid proprotein (NP_004943). An exemplary EFNA3 nucleic acid sequence is provided in FIG. 1D (SEQ ID NO: 5) while an exemplary amino acid sequence is provided in FIG. 1C (SEQ ID NO: 4).

It will be appreciated that both the of the human EFNA1 and EFNA3 proteins include a predicted signal or leader sequence, comprising amino acids 1-18 of EFNA1

(NP_004419) and amino acids 1 – 22 of EFNA3 (NP_004943) according to computer prediction algorithms, Peterson et al., 2011 PMID: 21959131 which is incorporated herein by reference. This signal peptide targets the polypeptide to the cell surface/secretory pathway. Additionally, the EFNA1 and EFNA3 proteins are post-translationally processed, like other EFNA family members, into globular proteins linked to the cell surface via glycosylphosphatidylinositol (GPI) anchors.

III. Tumor Perpetuating Cells

In contrast to teachings of the prior art, the present invention provides EFNA modulators that are particularly useful for targeting tumor initiating cells, and especially tumor perpetuating cells, thereby facilitating the treatment, management or prevention of neoplastic disorders. More specifically, as previously indicated it has surprisingly been found that specific tumor cell subpopulations express EFNA and likely modify localized coordination of morphogen signaling important to cancer stem cell self-renewal and cell survival. Thus, in preferred embodiments modulators of EFNA may be used to reduce tumor initiating cell frequency in accordance with the present teachings and thereby facilitate the treatment or management of hyperproliferative diseases.

As used herein, the term tumor initiating cell (TIC) encompasses both tumor perpetuating cells (TPC; i.e., cancer stem cells or CSC) and highly proliferative tumor progenitor cells (termed TProg), which together generally comprise a unique subpopulation (i.e. 0.1-40%) of a bulk tumor or mass. For the purposes of the instant disclosure the terms tumor perpetuating cells and cancer stem cells are equivalent and may be used interchangeably herein. Conversely, TPC differ from TProg in that they can completely recapitulate the composition of tumor cells existing within a tumor and have unlimited self-renewal capacity as demonstrated by serial transplantation (two or more passages through mice) of low numbers of isolated cells. As will be discussed in more detail below fluorescence-activated cell sorting (FACS) using appropriate cell surface markers is a reliable method to isolate highly enriched cell subpopulations (> 99.5% purity) due, at least in part, to its ability to discriminate between single cells and clumps of cells (i.e. doublets, etc.). Using such techniques it has been shown that when low cell numbers of highly purified TProg cells are transplanted into immunocompromised mice they can fuel tumor growth in a primary transplant. However, unlike purified TPC subpopulations the TProg generated tumors do not completely reflect the parental tumor in phenotypic cell heterogeneity and are demonstrably inefficient at reinitiating serial

tumorigenesis in subsequent transplants. In contrast, TPC subpopulations completely reconstitute the cellular heterogeneity of parental tumors and can efficiently initiate tumors when serially isolated and transplanted. Thus, those skilled in the art will recognize that a definitive difference between TPC and TProg, though both may be tumor generating in primary transplants, is the unique ability of TPC to perpetually fuel heterogeneous tumor growth upon serial transplantation at low cell numbers. Other common approaches to characterize TPC involve morphology and examination of cell surface markers, transcriptional profile, and drug response although marker expression may change with culture conditions and with cell line passage *in vitro*.

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

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

chemosensitivity of such EFNA positive tumor initiating cells regardless of the particular embodiment or mix represented in a tumor.

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

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

Among the methods that can be used to assess such a reduction in the frequency of tumor initiating cells is limiting dilution analysis either *in vitro* or *in vivo*, preferably

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

With respect to limiting dilution analysis, *in vitro* enumeration of tumor initiating cell frequency may be accomplished by depositing either fractionated or unfractionated human tumor cells (e.g. from treated and untreated tumors, respectively) into *in vitro* growth conditions that foster colony formation. In this manner, colony forming cells might be enumerated by simple counting and characterization of colonies, or by analysis consisting of, for example, the deposition of human tumor cells into plates in serial dilutions and scoring each well as either positive or negative for colony formation at least 10 days after plating. *In vivo* limiting dilution experiments or analyses, which are generally more accurate in their ability to determine tumor initiating cell frequency encompass the transplantation of human tumor cells, from either untreated control or treated conditions, for example, into immunocompromised mice in serial dilutions and subsequently scoring each mouse as either positive or negative for tumor formation at least 60 days after transplant. The derivation of cell frequency values by limiting dilution analysis *in vitro* or *in vivo* is preferably done by applying Poisson distribution statistics to the known frequency of positive and negative events, thereby providing a frequency for events fulfilling the definition of a positive event; in this case, colony or tumor formation, respectively.

As to other methods compatible with the instant invention that may be used to calculate tumor initiating cell frequency, the most common comprise quantifiable flow cytometric techniques and immunohistochemical staining procedures. Though not as precise as the limiting dilution analysis techniques described immediately above, these procedures are much less labor intensive and provide reasonable values in a relatively short time frame. Thus, it will be appreciated that a skilled artisan may use flow cytometric cell surface marker profile determination employing one or more antibodies or

reagents that bind art recognized cell surface proteins known to enrich for tumor initiating cells (e.g., potentially compatible markers as 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* (e.g., in a tissue section) by immunohistochemistry using one or more antibodies or reagents that are able to bind cell surface proteins thought to demarcate these cells.

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

IV. EFNA Modulators

In any event, the present invention is directed to the use of EFNA modulators selected from the group consisting of EFNA1 modulators and EFNA3 modulators, including EFNA antagonists, for the diagnosis, treatment and/or prophylaxis of any one of a number of EFNA associated malignancies. The disclosed modulators may be used alone or in conjunction with a wide variety of anti-cancer compounds such as chemotherapeutic or immunotherapeutic agents (e.g., therapeutic antibodies) or biological response modifiers. In other selected embodiments, two or more discrete EFNA modulators may be used in combination to provide enhanced anti-neoplastic effects or may be used to fabricate multispecific constructs.

In certain embodiments, the EFNA modulators of the present invention will comprise nucleotides, oligonucleotides, polynucleotides, peptides or polypeptides. Even more preferably the modulators will comprise soluble EFNA (sEFNA) or a form, variant, derivative or fragment thereof including, for example, EFNA fusion constructs (e.g., EFNA-Fc, EFNA-targeting moiety, etc.) or EFNA-conjugates (e.g., EFNA-PEG, EFNA-

cytotoxic agent, EFNA-brm, etc.). It will also be appreciated that, in other embodiments, the EFNA modulators comprise antibodies (e.g., anti-EFNA1 or anti-EFNA3 mAbs) or immunoreactive fragments or derivatives thereof. In particularly preferred embodiments the modulators of the instant invention will comprise neutralizing antibodies or derivatives or fragments thereof. In other embodiments the EFNA modulators may comprise internalizing antibodies or fragments thereof. In still other embodiments the EFNA modulators may comprise depleting antibodies or fragments thereof. Moreover, as with the aforementioned fusion constructs, these antibody modulators may be conjugated, linked or otherwise associated with selected cytotoxic agents, polymers, biological response modifiers (BRMs) or the like to provide directed immunotherapies with various (and optionally multiple) mechanisms of action. As alluded to above such antibodies may be pan-EFNA antibodies and associate with two or more ephrin-A ligands or immunospecific antibodies that selectively react with one of the six ephrin-A ligands. In yet other embodiments the modulators may operate on the genetic level and may comprise compounds as antisense constructs, siRNA, micro RNA and the like.

Based on the teachings herein, those skilled in the art will appreciate that particularly preferred embodiments of the invention may comprise soluble Fc-constructs (e.g., sEFNA1 or sEFNA3) or antibody modulators that associate with either, or both, of EFNA1 or EFNA3.

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

In the same sense disclosed embodiments of the instant invention may comprise one or more EFNA antagonists that associate with EFNA1 or EFNA3. To that end it will be appreciated that EFNA antagonists of the instant invention may comprise any ligand,

polypeptide, peptide, fusion protein, antibody or immunologically active fragment or derivative thereof that recognizes, reacts, binds, combines, competes, associates or otherwise interacts with the EFNA1 or EFNA3 protein or fragment thereof and eliminates, silences, reduces, inhibits, hinders, restrains or controls the growth of tumor initiating cells or other neoplastic cells including bulk tumor or NTG cells. In selected embodiments the EFNA modulators comprise EFNA antagonists.

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

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

Moreover, as demonstrated in the examples herein, some modulators of human EFNA may, in certain cases, cross-react with EFNA from a species other than human (e.g., murine). In other cases exemplary modulators may be specific for one or more isoforms of human EFNA and will not exhibit cross-reactivity with EFNA orthologs from other species. Of course, in conjunction with the teachings herein such embodiments may comprise pan-EFNA antibodies that associate with two or more ephrin-A ligands from a single species or antibodies that exclusively associate with a single ephrin-A ligand.

In any event, and as will be discussed in more detail below, those skilled in the art will appreciate that the disclosed modulators may be used in a conjugated or unconjugated form. That is, the modulator may be associated with or conjugated to (e.g. covalently or non-covalently) pharmaceutically active compounds, biological response modifiers, anti-

cancer agents, cytotoxic or cytostatic agents, diagnostic moieties or biocompatible modifiers. In this respect it will be understood that such conjugates may comprise peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, organic molecules and radioisotopes. Moreover, as indicated herein the selected conjugate may be covalently or non-covalently linked to the EFNA modulator in various molar ratios depending, at least in part, on the method used to effect the conjugation.

V. Antibodies

a. Overview

As previously alluded to particularly preferred embodiments of the instant invention comprise EFNA modulators in the form of antibodies that preferentially associate with EFNA1 or EFNA3. 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, CDR-grafted 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., immunospecific or immunopreferential EFNA1 or EFNA3 association or binding). In a broader sense, the antibodies of the present invention include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antigen binding site, where these fragments may or may not be fused to another immunoglobulin domain including, but not limited to, an Fc region or fragment thereof. Further, as outlined in more detail herein, the terms antibody and antibodies specifically include Fc variants as described below, including full length antibodies and variant Fc-Fusions comprising Fc regions, or fragments thereof, optionally comprising at least one amino acid residue modification and fused to an immunologically active fragment of an immunoglobulin.

As discussed in more detail below, the generic terms antibody or immunoglobulin comprises five distinct classes of antibody that can be distinguished biochemically and, depending on the amino acid sequence of the constant domain of their heavy chains, can readily be assigned to the appropriate class. For historical reasons, the major classes of

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

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

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

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

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

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

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

The framework regions comprising the remainder of the heavy and light variable domains show less inter-molecular variability in amino acid sequence. Rather, the framework regions largely adopt a β-sheet conformation and the CDRs form loops which connect, and in some cases form part of, the β-sheet structure. Thus, these framework regions act to form a scaffold that provides for positioning the six CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen-binding site formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen (i.e. EFNA1 or EFNA3). This complementary surface promotes the non-covalent binding of the antibody to the immunoreactive antigen epitope. It will be appreciated that the position and composition of CDRs can be readily identified by one of ordinary skill in the art using the definitions provided herein.

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

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

More specifically, in the context of the instant invention it will be appreciated that any of the disclosed heavy and light chain CDRs derived from the sequences set forth in FIG. 6 or FIG. 7 may be rearranged in this manner to provide optimized anti-EFNA (e.g. anti-hEFNA1 or anti-hEFNA3) antibodies in accordance with the instant teachings. That is, one or more of the CDRs derived from the sequences set forth in FIG. 6 (SEQ ID NOS: 6 – 45) comprising anti-EFNA1 antibodies or those set forth in FIG. 7 (SEQ ID NOS: 46 – 101) comprising anti-EFNA3 antibodies may be incorporated in an EFNA modulator and, in particularly preferred embodiments, in a CDR grafted or humanized antibody that immunospecifically associates with one or more ephrin-A ligands.

In any event, the complementarity determining regions residue numbers may be defined as those of Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.), specifically, residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain and 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Note that CDRs vary considerably from antibody to antibody (and by definition will not exhibit homology with the Kabat consensus sequences). Maximal alignment of framework residues frequently requires the insertion of spacer residues in the numbering system, to be used for the Fv region. In addition, the identity of certain individual residues at any given Kabat site number may vary from antibody chain to antibody chain due to interspecies or allelic divergence. *See also* Chothia et al., J. Mol. Biol. 196:901-917 (1987); Chothia et al., Nature 342, pp. 877-883 (1989) and by MacCallum et al., J. Mol. Biol. 262:732-745 (1996) where the definitions include overlapping or subsets of amino acid residues when compared against each other. Each of the aforementioned references is incorporated herein by reference in its entirety and the amino acid residues which encompass CDRs as defined by each of the above cited references are set forth for comparison.

CDR Definitions

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

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

As discussed one skilled in the art could readily define, identify derive and/or enumerate the CDRs as defined by Kabat et al., Chothia et al. or MacCallum et al. for each respective heavy and light chain sequence set forth in FIG. 6 or FIG. 7. Accordingly, each of the subject CDRs and antibodies comprising CDRs defined by all such nomenclature are expressly included within the scope of the instant invention. More broadly the term variable region CDR amino acid residue includes amino acids in a CDR as identified using any sequence or structure based method as set forth above.

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

For the specific example of a heavy chain variable region and for the CDRs as defined by Kabat et al., framework region 1 corresponds to the domain of the variable region encompassing amino acids 1-30; framework region 2 corresponds to the domain of the variable region encompassing amino acids 36-49; framework region 3 corresponds to the domain of the variable region encompassing amino acids 66-94, and framework region 4 corresponds to the domain of the variable region from amino acids 103 to the end of the variable region. The framework regions for the light chain are similarly separated by each of the light claim variable region CDRs. Similarly, using the definition of CDRs by

Chothia et al. or McCallum et al. the framework region boundaries are separated by the respective CDR termini as described above.

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

b. Antibody generation

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

After immunization of an animal with an EFNA immunogen (e.g., soluble EFNA1 or EFNA3) which may comprise selected isoforms and/or peptides, or live cells or cell

preparations expressing the desired protein, antibodies and/or antibody-producing cells can be obtained from the animal using art recognized techniques. In some embodiments, polyclonal anti-EFNA antibody-containing serum is obtained by bleeding or sacrificing the animal. The serum may be used for research purposes in the form obtained from the animal or, in the alternative, the anti-EFNA antibodies may be partially or fully purified to provide immunoglobulin fractions or homogeneous antibody preparations.

c. Monoclonal antibodies

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

In preferred embodiments, antibody-producing cell lines are prepared from cells isolated from the immunized animal. After immunization, the animal is sacrificed and lymph node and/or splenic B cells are immortalized by means well known in the art as shown in the appended Examples). 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 an ephrin-A ligand (including selected isoforms), or an immunoreactive portion thereof. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunoassay (ELISA) or a radioimmunoassay.

More generally, discrete monoclonal antibodies consistent with the present invention can be prepared using a wide variety of techniques known in the art including hybridoma,

recombinant techniques, phage display technologies, yeast libraries, transgenic animals (e.g. a XenoMouse® or HuMAb Mouse®) or some combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques such as broadly described above and taught in more detail in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) each of which is incorporated herein. Using the disclosed protocols, antibodies are preferably raised in mammals by multiple subcutaneous or intraperitoneal injections of the relevant antigen and an adjuvant. As previously discussed, this immunization generally elicits an immune response that comprises production of antigen-reactive antibodies (that may be fully human if the immunized animal is transgenic) from activated splenocytes or lymphocytes. While the resulting antibodies may be harvested from the serum of the animal to provide polyclonal preparations, it is generally more desirable to isolate individual lymphocytes from the spleen, lymph nodes or peripheral blood to provide homogenous preparations of monoclonal antibodies. Most typically, the lymphocytes are obtained from the spleen and immortalized to provide hybridomas.

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

d. Chimeric antibodies

In another embodiment, the antibody of the invention may comprise chimeric antibodies derived from covalently joined protein segments from at least two different species or types of antibodies. It will be appreciated that, as used herein, the term chimeric antibodies is directed to constructs in which a portion of the heavy and/or light

chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; Morrison et al., Proc. Natl. Acad. Sci. USA, 81:6851-6855 (1984)). In one exemplary embodiment, a chimeric antibody in accordance with the teachings herein may comprise murine V_H and V_L amino acid sequences and constant regions derived from human sources. In other compatible embodiments a chimeric antibody of the present invention may comprise a CDR grafted or humanized antibody as described herein.

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

e. Humanized antibodies

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

Generally humanization of an antibody comprises an analysis of the sequence homology and canonical structures of both the donor and recipient antibodies. In selected

embodiments, the recipient antibody may comprise consensus sequences. To create consensus human frameworks, frameworks from several human heavy chain or light chain amino acid sequences may be aligned to identify a consensus amino acid sequence. Moreover, in many instances, one or more framework residues in the variable domain of the human immunoglobulin are replaced by corresponding non-human residues from the donor antibody. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. Such substitutions help maintain the appropriate three-dimensional configuration of the grafted CDR(s) and often improve infinity over similar constructs with no framework substitutions. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance using well-known techniques.

CDR grafting and humanized antibodies are described, for example, in U.S.P.Ns. 6,180,370, 5,693,762, 5,693,761, 5,585,089, and 5,530,101. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDRs correspond to those of a non-human immunoglobulin, and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, e.g., Jones et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, Ann. Allergy, Asthma & Immunol. 1: 105-115 (1998); Harris, Biochem. Soc. Transactions 23:1035-1038 (1995); Hurle and Gross, Curr. Op. Biotech. 5:428-433 (1994); and U.S.P.Ns. 6,982,321 and 7,087,409. Still another method is termed humaneering and is described, for example, in U.S. 2005/0008625. For the purposes of the present application the term humanized antibodies will be held to expressly include CDR grafted antibodies (i.e. human antibodies comprising one or more grafted non-human CDRs) with no or minimal framework substitutions.

Additionally, a non-human anti-EFNA antibody may also be modified by specific deletion of human T cell epitopes or deimmunization by the methods disclosed in WO 98/52976 and WO 00/34317. Briefly, the heavy and light chain variable regions of an

antibody can be analyzed for peptides that bind to MHC Class II; these peptides represent potential T-cell epitopes (as defined in WO 98/52976 and WO 00/34317). For detection of potential T-cell epitopes, a computer modeling approach termed peptide threading can be applied, and in addition a database of human MHC class II binding peptides can be searched for motifs present in the V_H and V_L sequences, as described in WO 98/52976 and WO 00/34317. These motifs bind to any of the 18 major MHC class II DR allotypes, and thus constitute potential T cell epitopes. Potential T-cell epitopes detected can be eliminated by substituting small numbers of amino acid residues in the variable regions, or by single amino acid substitutions. As far as possible, conservative substitutions are made. Often, but not exclusively, an amino acid common to a position in human germline antibody sequences may be used. After the deimmunizing changes are identified, nucleic acids encoding V_H and V_L can be constructed by mutagenesis or other synthetic methods (e.g., de novo synthesis, cassette replacement, and so forth). A mutagenized variable sequence can, optionally, be fused to a human constant region.

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

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

Human germline sequences, for example, are disclosed in Tomlinson, I. A. et al. (1992) J. Mol. Biol. 227:776-798; Cook, G. P. et al. (1995) Immunol. Today 16: 237-242; Chothia, D. et al. (1992) J. Mol. Bio. 227:799-817; and Tomlinson et al. (1995) EMBO J

14:4628-4638. The V BASE directory provides a comprehensive directory of human immunoglobulin variable region sequences (See Retter et al., (2005) Nuc Acid Res 33: 671-674). These sequences can be used as a source of human sequence, e.g., for framework regions and CDRs. As set forth herein consensus human framework regions can also be used, e.g., as described in U.S.P.N. 6,300,064.

f. Human antibodies

In addition to the aforementioned antibodies, those skilled in the art will appreciate that the antibodies of the present invention may comprise fully human antibodies. For the purposes of the instant application the term human antibody comprises an antibody which possesses an amino acid sequence that corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

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

Recombinant human anti-EFNA antibodies of the invention may be isolated by screening a recombinant combinatorial antibody library prepared as above. In a preferred embodiment, the library is a scFv phage display library, generated using human V_L and V_H cDNAs prepared from mRNA isolated from B cells. Methods for preparing and screening such libraries are well known in the art and kits for generating phage display libraries are commercially available (e.g., the Pharmacia Recombinant Phage Antibody System, catalog no. 27-9400-01; and the Stratagene SurfZAP™ phage display kit, catalog no. 240612). There also are other methods and reagents that can be used in generating and screening antibody display libraries (see, e.g., U.S.P.N. 5,223,409; PCT Publication Nos. WO 92/18619, WO 91/17271, WO 92/20791, WO 92/15679, WO 93/01288, WO 92/01047, WO 92/09690; Fuchs et al., Bio/Technology 9:1370-1372 (1991); Hay et al., Hum. Antibod. Hybridomas 3:81-85 (1992); Huse et al., Science 246:1275-1281 (1989); McCafferty et al., Nature 348:552-554 (1990); Griffiths et al., EMBO J. 12:725-734 (1993); Hawkins et al., J. Mol. Biol. 226:889-896 (1992); Clackson et al., Nature 352:624-628 (1991); Gram et al., Proc. Natl. Acad. Sci. USA 89:3576-3580 (1992); Garrad et al., Bio/Technology 9:1373-1377 (1991); Hoogenboom et al., Nuc. Acid Res. 19:4133-4137 (1991); and Barbas et al., Proc. Natl. Acad. Sci. USA 88:7978-7982 (1991).

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

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

Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S.P.Ns. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and U.S.P.N 6,075,181 and 6,150,584 regarding Xenomouse® technology along with the following scientific publications: Marks et al., Bio/Technology 10: 779-783 (1992); Lonberg et al., Nature 368: 856-859 (1994); Morrison, Nature 368:812-13 (1994); Fishwild et al., Nature Biotechnology 14: 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B-lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual suffering from a neoplastic disorder or may have been immunized *in vitro*). See, e.g., Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985); Boerner et al., J. Immunol, 147 (l):86-95 (1991); and U.S.P.N. 5,750,373.

VI. Antibody Characteristics

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

a. Neutralizing antibodies

In particularly preferred embodiments the modulators of the instant invention will comprise neutralizing antibodies or derivative or fragment thereof. The term neutralizing antibody or neutralizing antagonist refers to an antibody or antagonist that binds to or interacts with an ephrin-A ligand and prevents binding or association of the ligand to its binding partner (e.g., EphA receptor) thereby interrupting the biological response that otherwise would result from the interaction of the molecules. In assessing the binding and specificity of an antibody or immunologically functional fragment or derivative thereof, an antibody or fragment will substantially inhibit binding of the ligand to its binding partner or substrate when an excess of antibody reduces the quantity of binding partner bound to the target molecule by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or more as measured, for example, in an *in vitro* competitive binding assay (see e.g., Examples 10 and 11 herein). In the case of antibodies to EFNA1 for example, a neutralizing antibody or antagonist will preferably diminish the ability of EFNA1 to bind to a selected EphA receptor by at least about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99% or more. It will be appreciated that this diminished activity may be measured directly using art recognized techniques or may be measured by the impact such reduction will have on EphA receptor activity.

b. Internalizing antibodies

While evidence indicates that selected ephrin-A ligands or their isoforms may be present in a soluble form, at least some EFNA (e.g., EFNA1 and EFNA3) likely remains associated with the cell surface thereby allowing for internalization of the disclosed modulators. Accordingly, the anti-EFNA antibodies of the instant invention may be internalized, at least to some extent, by cells that express an ephrin-A ligand. For

example, an anti-EFNA1 antibody that binds to EFNA1 on the surface of a tumor-initiating cell may be internalized by the tumor-initiating cell. In particularly preferred embodiments such anti-EFNA antibodies may be associated with or conjugated to anti-cancer agents such as cytotoxic moieties that kill the cell upon internalization.

As used herein, an anti-EFNA antibody that internalizes is one that is taken up by the cell upon binding to an EFNA associated with a mammalian cell. The internalizing antibody includes antibody fragments, human or humanized antibody and antibody conjugates. Internalization may occur *in vitro* or *in vivo*. For therapeutic applications, internalization may occur *in vivo*. The number of antibody molecules internalized may be sufficient or adequate to kill an EFNA-expressing cell, especially an EFNA-expressing tumor initiating cell. Depending on the potency of the antibody or antibody conjugate, in some instances, the uptake of a single antibody molecule into the cell is sufficient to kill the target cell to which the antibody binds. For example, certain toxins are highly potent in killing such that internalization of one molecule of the toxin conjugated to the antibody is sufficient to kill the tumor cell. Whether an anti-EFNA antibody internalizes upon binding EFNA on a mammalian cell can be determined by various assays including those described in the Examples below (e.g., Examples 12-14). Methods of detecting whether an antibody internalizes into a cell are also described in U.S.P.N. 7,619,068 which is incorporated herein by reference in its entirety.

c. Depleting antibodies

In other preferred embodiments the modulators of the instant invention will comprise depleting antibodies or derivatives or fragments thereof. The term depleting antibody refers to an antibody or fragment that binds to or associates with an EFNA on or near the cell surface and induces, promotes or causes the death, incapacitation or elimination of the cell (e.g., by complement-dependent cytotoxicity or antibody-dependent cellular cytotoxicity). In some embodiments discussed more fully below the selected depleting antibodies will be associated or conjugated to a cytotoxic agent. Preferably a depleting antibody will be able to remove, incapacitate, eliminate or kill at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, or 99% of tumor perpetuating cells in a defined cell population. In some embodiments the cell population may comprise enriched, sectioned, purified or isolated tumor perpetuating cells. In other embodiments the cell population may comprise whole tumor samples or heterogeneous tumor extracts that comprise tumor perpetuating cells. Those skilled in the art will appreciate that standard biochemical techniques as described in the Examples below (e.g., Example 14)

may be used to monitor and quantify the depletion of tumorigenic cells or tumor perpetuating cells in accordance with the teachings herein.

d. Epitope binding

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

Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope, e.g., by immunizing with a peptide comprising the epitope using techniques described in the present invention. Alternatively, during the discovery process, the generation and characterization of antibodies may elucidate information about desirable epitopes. From this information, it is then possible to competitively screen antibodies for binding to the same epitope. An approach to achieve this is to conduct competition studies to find antibodies that competitively bind with one another, i.e. the antibodies compete for binding to the antigen. A high throughput process for binning antibodies based upon their cross-competition is described in WO 03/48731.

As used herein, the term binning refers to a method to group antibodies based on their antigen binding characteristics. The assignment of bins is somewhat arbitrary,

depending on how different the observed binding patterns of the antibodies tested. Thus, while the technique is a useful tool for categorizing antibodies of the instant invention, the bins do not always directly correlate with epitopes and such initial determinations should be further confirmed by other art recognized methodology.

With this caveat one can determine whether a selected primary antibody (or fragment thereof) binds to the same epitope or cross competes for binding with a second antibody by using methods known in the art and set forth in the Examples herein. In one embodiment, one allows the primary antibody of the invention to bind to EFNA under saturating conditions and then measures the ability of the secondary antibody to bind to EFNA. If the test antibody is able to bind to EFNA at the same time as the primary anti-EFNA 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 EFNA at the same time, then the secondary antibody binds to the same epitope, an overlapping epitope, or an epitope that is in close proximity to the epitope bound by the primary antibody. As known in the art and detailed in the Examples below, the desired data can be obtained using solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay, a Biacore™ system (i.e., surface plasmon resonance – GE Healthcare), a ForteBio® Analyzer (i.e., bio-layer interferometry - ForteBio, Inc.) or flow cytometric methodology. The term surface plasmon resonance, as used herein, refers to an optical phenomenon that allows for the analysis of real-time specific 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 as demonstrated in the Examples below.

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

antibody for steric hindrance to occur. Additional details regarding methods for determining competitive binding are provided in the Examples herein. Usually, when a competing antibody is present in excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 40%, 45%, 50%, 55%, 60%, 65%, 70% or 75%. In some instance, binding is inhibited by at least 80%, 85%, 90%, 95%, or 97% or more.

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

e. Binding affinity

In this respect, the present invention further encompasses the use of antibodies that have a high binding affinity for a selected EFNA or, in the case of pan-antibodies, more than one type of ephrin-A ligand. 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 EFNA with a K_d of between about 10^{-8}M and 10^{-10}M, and in yet another embodiment it will bind with a $K_d \leq 2x10^{-10}$M. Still other selected embodiments of the present invention comprise antibodies that have a disassociation constant or K_d (k_{off}/k_{on}) of less than 10^{-2}M, less than $5x10^{-2}$M, less than 10^{-3}M, less than $5x10^{-3}$M, less than 10^{-4}M, less than $5x10^{-4}$M, less than 10^{-5}M, less than $5x10^{-5}$M, less than 10^{-6}M, less than $5x10^{-6}$M, less than 10^{-7}M, less than $5x10^{-7}$M, less than 10^{-8}M, less than $5x10^{-8}$M, less than 10^{-9}M, less than $5x10^{-9}$M, less than 10^{-10}M, less than $5x10^{-10}$M, less than 10^{-11}M, less than $5x10^{-11}$M, less than 10^{-12}M, less than $5x10^{-12}$M, less than 10^{-13}M, less than $5x10^{-13}$M, less than 10^{-14}M, less than $5x10^{-14}$M, less than 10^{-15}M or less than $5x10^{-15}$M.

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

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

$5x10^{-6}s^{-1}$ less than $10^{-7}s^{-1}$, less than $5x10^{-7}s^{-1}$, less than $10^{-8}s^{-1}$, less than $5x10^{-8}s^{-1}$, less than $10^{-9}s^{-1}$, less than $5x10^{-9}s^{-1}$ or less than $10^{-10}s^{-1}$.

In other selected embodiments of the present invention anti-EFNA 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 $5x10^{15}M^{-1}$.

f. Isoelectric points

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

The pI of a protein may be determined by a variety of methods including but not limited to, isoelectric focusing and various computer algorithms (see for example Bjellqvist et al., 1993, Electrophoresis 14:1023). In one embodiment, the pI of the anti-EFNA antibodies of the invention is between is higher than about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In another embodiment, the pI of the anti-EFNA antibodies of the invention is between is higher than 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0. In yet another embodiment, substitutions resulting in alterations in the pI of antibodies of the invention will not significantly diminish their binding affinity for EFNA. As discussed in more detail below, it is specifically contemplated that the substitution(s) of the Fc region that result in altered binding to FcγR may also result in a change in the pI. In a preferred embodiment, substitution(s) of the Fc region are specifically chosen to effect both the

desired alteration in FcγR binding and any desired change in pI. As used herein, the pI value is defined as the pI of the predominant charge form.

g. Thermal stability

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

VII. EFNA Modulator Fragments and Derivatives

Whether the agents of the present invention comprise intact fusion constructs, antibodies, fragments or derivatives, the selected modulators will react, bind, combine, complex, connect, attach, join, interact or otherwise associate with EFNA and thereby provide the desired anti-neoplastic effects. Those of skill in the art will appreciate that modulators comprising anti-EFNA antibodies interact or associate with EFNA through one or more binding sites expressed on the antibody. More specifically, as used herein the term binding site comprises a region of a polypeptide that is responsible for selectively binding to a target molecule of interest (e.g., enzyme, antigen, ligand, receptor, substrate or inhibitor). Binding domains comprise at least one binding site (e.g. an intact IgG antibody will have two binding domains and two binding sites). Exemplary binding domains include an antibody variable domain, a receptor-binding domain of a ligand, a

ligand-binding domain of a receptor or an enzymatic domain. For the purpose of the instant invention the typical active region of EFNA (e.g., as part of an Fc-EFNA fusion construct) may comprise a binding site for a substrate (e.g., an Eph receptor).

a. Fragments

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

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

More specifically, papain digestion of antibodies produces two identical antigen-binding fragments, called Fab fragments, each with a single antigen-binding site, and a residual Fc fragment, whose name reflects its ability to crystallize readily. Pepsin

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

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

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

b. Derivatives

In another embodiment, it will further be appreciated that the modulators of the invention may be monovalent or multivalent (e.g., bivalent, trivalent, etc.). As used herein the term valency refers to the number of potential target (i.e., EFNA) binding sites associated with an antibody. Each target binding site specifically binds one target molecule or specific position or locus on a target molecule. When an antibody of the instant invention comprises more than one target binding site (multivalent), each target

binding site may specifically bind the same or different molecules (e.g., may bind to different ligands or different antigens, or different epitopes or positions on the same antigen). For the purposes of the instant invention, the subject antibodies will preferably have at least one binding site specific for human EFNA. In one embodiment the antibodies of the instant invention will be monovalent in that each binding site of the molecule will specifically bind to a single EFNA position or epitope. In other embodiments, the antibodies will be multivalent in that they comprise more than one binding site and the different binding sites specifically associate with more than a single position or epitope. In such cases the multiple epitopes may be present on the selected EFNA polypeptide or spice variant or a single epitope may be present on EFNA while a second, different epitope may be present on another molecule or surface. See, for example, U.S.P.N. 2009/0130105.

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

In yet other embodiments, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, C_H2, and/or C_H3 regions. In one example, the first heavy-chain constant region (C_H1) containing the site necessary for light chain binding is present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide

fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when, the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm (e.g., EFNA1), and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., 1986, Methods in Enzymology, 121:210. According to another approach described in WO96/27011, a pair of antibody molecules can be engineered to maximize the percentage of heterodimers that are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

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

VIII. EFNA Modulators - Constant Region Modifications

a. Fc region and Fc receptors

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

The term Fc region herein is used to define a C-terminal region of an immunoglobulin heavy chain, including native sequence Fc regions and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody. Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. A functional Fc region possesses an effector function of a native sequence Fc region. Exemplary effector functions include C1q binding; CDC; Fc receptor binding; ADCC; phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g., an antibody variable domain) and can be assessed using various assays as disclosed, for example, in definitions herein.

Fc receptor or FcR describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI,

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

b. Fc functions

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

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

EFNA modulator variants with altered FcR binding affinity or ADCC activity is one which has either enhanced or diminished FcR binding activity and/or ADCC activity

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

As to FcRn, the antibodies of the instant invention also comprise or encompass Fc variants with modifications to the constant region that provide half-lives (e.g., serum half-lives) in a mammal, preferably a human, of greater than 5 days, greater than 10 days, greater than 15 days, preferably greater than 20 days, greater than 25 days, greater than 30 days, greater than 35 days, greater than 40 days, greater than 45 days, greater than 2 months, greater than 3 months, greater than 4 months, or greater than 5 months. The increased half-lives of the antibodies (or Fc containing molecules) of the present invention in a mammal, preferably a human, results in a higher serum titer of said antibodies or antibody fragments in the mammal, and thus, reduces the frequency of the administration of said antibodies or antibody fragments and/or reduces the concentration of said antibodies or antibody fragments to be administered. Antibodies having increased *in vivo* half-lives can be generated by techniques known to those of skill in the art. For example, antibodies with increased *in vivo* half-lives can be generated by modifying (e.g., substituting, deleting or adding) amino acid residues identified as involved in the interaction between the Fc domain and the FcRn receptor (see, e.g., International Publication Nos. WO 97/34631; WO 04/029207; U.S.P.N. 6,737,056 and U.S.P.N. 2003/0190311. Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 describes antibody variants with improved or diminished binding to FcRns. See also, e.g., Shields et al. J. Biol. Chem. 9(2):6591-6604 (2001).

c. Glycosylation modifications

In still other embodiments, glycosylation patterns or compositions of the antibodies of the invention are modified. More particularly, preferred embodiments of the present

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

Additionally or alternatively, an Fc variant can be made that has an altered glycosylation composition, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNAc structures. These and similar altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Engineered glycoforms may be generated by any method known to one skilled in the art, for example by using engineered or variant expression strains, by co-expression with one or more enzymes (for example N-acetylglucosaminyltransferase III (GnTI11)), by expressing a molecule comprising an Fc region in various organisms or cell lines from various organisms or by modifying carbohydrate(s) after the molecule comprising Fc region has been expressed. See, for example, Shields, R. L. et al. (2002) J. Biol. Chem. 277:26733-26740; Umana et al. (1999) Nat. Biotech. 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342, Umana et al, 1999, Nat. Biotechnol 17:176-180; Davies et al., 20017 Biotechnol Bioeng 74:288-294; Shields et al, 2002, J Biol Chem 277:26733-26740; Shinkawa et al., 2003, J Biol Chem 278:3466-3473) U.S.P.N. 6,602,684; U.S.S.Ns. 10/277,370; 10/113,929; PCT WO 00/61739A1; PCT WO 01/292246A1; PCT WO 02/311140A1; PCT WO 02/30954A1; Potillegent™ technology (Biowa, Inc.); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG); WO 00061739; EA01229125; U.S.P.N. 2003/0115614; Okazaki et al., 2004, JMB, 336: 1239-49.

IX. Modulator Expression

a. Overview

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

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

Whether the nucleic acid encoding the desired portion of the EFNA modulator is obtained or derived from phage display technology, yeast libraries, hybridoma based technology, synthetically or from commercial sources, it is to be understood that the present invention explicitly encompasses nucleic acid molecules and sequences encoding EFNA modulators including fusion proteins and anti-EFNA antibodies or antigen-binding fragments or derivatives thereof. The invention further encompasses nucleic acids or nucleic acid molecules (e.g., polynucleotides) that hybridize under high stringency, or alternatively, under intermediate or lower stringency hybridization conditions (e.g., as defined below), to polynucleotides complementary to nucleic acids having a polynucleotide sequence that encodes a modulator of the invention or a fragment or variant thereof. The term nucleic acid molecule or isolated nucleic acid molecule, as used

herein, is intended to include at least DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA. Moreover, the present invention comprises any vehicle or construct, incorporating such modulator encoding polynucleotide including, without limitation, vectors, plasmids, host cells, cosmids or viral constructs.

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

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

b. Hybridization and Identity

As indicated, the invention further provides nucleic acids that hybridize to other nucleic acids under particular hybridization conditions. Methods for hybridizing nucleic acids are well known in the art. See, e.g., Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For the purposes of the instant application, a moderately stringent hybridization condition uses a prewashing solution containing 5x sodium chloride/sodium citrate (SSC), 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization buffer of about 50% formamide, 6xSSC, and a hybridization temperature of 55°C (or other similar hybridization solutions, such as one containing about 50% formamide, with a

hybridization temperature of 42°C), and washing conditions of 60°C, in 0.5xSSC, 0.1% SDS. A stringent hybridization condition hybridizes in 6xSSC at 45°C, followed by one or more washes in 0.1xSSC, 0.2% SDS at 68°C. Furthermore, one of skill in the art can manipulate the hybridization and/or washing conditions to increase or decrease the stringency of hybridization such that nucleic acids comprising nucleotide sequences that are at least 65, 70, 75, 80, 85, 90, 95, 98 or 99% identical to each other typically remain hybridized to each other. More generally, for the purposes of the instant disclosure the term substantially identical with regard to a nucleic acid sequence may be construed as a sequence of nucleotides exhibiting at least about 85%, or 90%, or 95%, or 97% sequence identity to the reference nucleic acid sequence.

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

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

c. Expression

A nucleic acid, such as a nucleic acid expressing RNA and/or protein or peptide, and an expression control sequence are functionally linked to one another, if they are covalently linked to one another in such a way that expression or transcription of said nucleic acid is under the control or under the influence of said expression control sequence. If the nucleic acid is to be translated into a functional protein, then, with an expression control sequence functionally linked to a coding sequence, induction of said expression control sequence results in transcription of said nucleic acid, without causing a

frame shift in the coding sequence or said coding sequence not being capable of being translated into the desired protein or peptide.

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

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

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

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

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

In practicing the present invention it will be appreciated that many conventional techniques in molecular biology, microbiology, and recombinant DNA technology are optionally used. Such conventional techniques relate to vectors, host cells and recombinant methods as defined herein. These techniques are well known and are explained in, for example, Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif.; Sambrook et al., Molecular Cloning-A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 and Current Protocols in Molecular Biology, F. M. Ausubel et al., eds., supra Other useful references, e.g. for cell isolation and culture (e.g., for subsequent nucleic acid or protein isolation) include Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley-Liss, New York and the references cited therein; Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, N.Y.; Gamborg and Phillips (Eds.) (1995) Plant Cell, Tissue and Organ Culture; Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Atlas and Parks (Eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, Fla. Methods of making nucleic acids (e.g., by in vitro amplification, purification from cells, or chemical synthesis), methods for manipulating nucleic acids (e.g., site-directed mutagenesis, by restriction enzyme digestion, ligation, etc.), and various vectors, cell lines and the like useful in manipulating and making nucleic acids are described in the above references. In addition, essentially any polynucleotide (including, e.g., labeled or biotinylated polynucleotides) can be custom or standard ordered from any of a variety of commercial sources.

Thus, in one aspect, the present invention provides recombinant host cells allowing recombinant expression of antibodies of the invention or portions thereof. Antibodies

produced by expression in such recombinant host cells are referred to herein as recombinant antibodies. The present invention also provides progeny cells of such host cells, and antibodies produced by the same.

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

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

As indicated above, expression of an antibody of the invention (or fragment or variants thereof) preferably comprises expression vector(s) containing a polynucleotide that encodes the desired anti-EFNA antibody. Methods that are well known to those skilled in the art can be used to construct expression vectors comprising antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Embodiments of the invention, thus, provide replicable vectors comprising a nucleotide sequence encoding an anti-EFNA antibody of the invention (e.g., a whole antibody, a heavy or light chain of an antibody, a heavy or light chain variable domain of an antibody, or a portion thereof, or a heavy or light chain CDR, a single chain Fv, or fragments or variants thereof), operably linked to a promoter. In preferred embodiments such vectors may include a nucleotide sequence encoding the heavy chain of an antibody molecule (or fragment thereof), a nucleotide sequence encoding the light chain of an antibody (or fragment thereof) or both the heavy and light chain.

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

X. Modulator Production and Purification

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

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

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

heavy chain. The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

a. Host-expression systems

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

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of a modulator, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the E. coli expression vector pUR278 (Ruther et al., EMBO 1. 2:1791 (1983)), in which the coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109 (1985); Van Heeke & Schuster, J. Biol. Chem. 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed

cells by adsorption and binding to matrix glutathione agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

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

In mammalian host cells, a number of viral-based expression systems may be used to introduce the desired nucleotide sequence. In cases where an adenovirus is used as an expression vector, the coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the molecule in infected hosts (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 8 1:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et al., Methods in Enzymol. 153:51-544 (1987)). Thus, compatible mammalian cell lines available as hosts for expression are well known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC). These include, inter alia, Chinese hamster ovary (CHO) cells, NS0 cells, SP2 cells, HEK-293T cells, 293 Freestyle cells (Life Technologies), NIH-3T3 cells, HeLa cells, baby hamster kidney (BHK) cells, African green monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), A549 cells, and a number of other cell lines.

For long-term, high-yield production of recombinant proteins stable expression is preferred. Accordingly, cell lines that stably express the selected modulator may be engineered using standard art recognized techniques. Rather than using expression vectors

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

A number of selection systems are well known in the art and may be used including, but not limited to, the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223 (1977)), hypoxanthineguanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., Cell 22:8 17 (1980)) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA 77:357 (1980); O'Hare et al., Proc. Natl. Acad. Sci. USA 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 (Clinical Pharmacy 12:488-505; Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62: 191-217 (1993); TIB TECH 11(5):155-2 15 (May, 1993)); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin et al., J. Mol. Biol. 150:1 (1981). It will be appreciated that one particularly preferred method of establishing a stable, high yield cell line comprises the glutamine synthetase gene expression system (the GS system) which provides an efficient approach for

enhancing expression under certain conditions. The GS system is discussed in whole or part in connection with EP patents 0 216 846, 0 256 055, 0 323 997 and 0 338 841 each of which is incorporated herein by reference.

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

b. Chemical synthesis

Besides the aforementioned host cell systems, it will be appreciated that the modulators of the invention may be chemically synthesized using techniques known in the art (e.g., see Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman & Co., N.Y., and Hunkapiller, M., et al., 1984, Nature 310:105-111). For example, a peptide corresponding to a polypeptide fragment of the invention can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into a polypeptide sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoro-amino acids, designer

amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

c. Transgenic systems

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

In accordance with the teachings herein non-human transgenic animals or plants may be produced by introducing one or more nucleic acid molecules encoding an EFNA modulator of the invention into the animal or plant by standard transgenic techniques. See Hogan and U.S. Pat. No. 6,417,429. The transgenic cells used for making the transgenic animal can be embryonic stem cells or somatic cells or a fertilized egg. The transgenic non-human organisms can be chimeric, nonchimeric heterozygotes, and nonchimeric homozygotes. See, e.g., Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual 2nd ed., Cold Spring Harbor Press (1999); Jackson et al., Mouse Genetics and Transgenics: A Practical Approach, Oxford University Press (2000); and Pinkert, Transgenic Animal Technology: A Laboratory Handbook, Academic Press (1999). In some embodiments, the transgenic non-human animals have a targeted disruption and replacement by a targeting construct that encodes, for example, a heavy chain and/or a light chain of interest. In one embodiment, the transgenic animals comprise and express nucleic acid molecules encoding heavy and light chains that specifically bind to EFNA. While anti-EFNA antibodies may be made in any transgenic animal, in particularly preferred embodiments the non-human animals are mice, rats, sheep, pigs, goats, cattle or horses. In further embodiments the non-human transgenic animal expresses the desired pharmaceutical product in blood, milk, urine, saliva, tears, mucus and other bodily fluids from which it is readily obtainable using art recognized purification techniques.

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

d. Purification

Once a modulator of the invention has been produced by recombinant expression or any one of the other techniques disclosed herein, it may be purified by any method known in the art for purification of immunoglobulins, or more generally by any other standard technique for the purification of proteins. In this respect the modulator may be isolated. As used herein, an isolated EFNA modulator is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. Isolated modulators include a modulator *in situ* within recombinant cells because at least one component of the polypeptide's natural environment will not be present.

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

The modulator (e.g., fc-EFNA or anti-EFNA antibody) composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the selected construct. Protein A can be used to purify antibodies that are based on human IgG1, IgG2 or IgG4 heavy chains (Lindmark, et al., J Immunol Meth 62:1 (1983)). Protein G is recommended for all mouse isotypes and for human IgG3 (Guss, et al., EMBO J 5:1567 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker; Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, reverse phase HPLC, chromatography on silica, chromatography on heparin, sepharose chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE and ammonium sulfate precipitation are also available depending on the antibody to be recovered. In

particularly preferred embodiments the modulators of the instant invention will be purified, at least in part, using Protein A or Protein G affinity chromatography.

XI. Conjugated EFNA Modulators

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

In preferred embodiments it will be apparent that the modulators of the invention may be conjugated or associated with proteins, polypeptides or peptides that impart selected characteristics (e.g., biotoxins, biomarkers, purification tags, etc.). More generally, in selected embodiments the present invention encompasses the use of modulators or fragments thereof recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide wherein the polypeptide comprises at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids. The construct does not necessarily need to be directly linked, but may occur through linker sequences. For example, antibodies may be used to target heterologous polypeptides to particular cell types expressing EFNA, either *in vitro* or *in vivo*, by fusing or conjugating the modulators of the present invention to antibodies specific for particular cell surface receptors. Moreover, modulators fused or conjugated to heterologous polypeptides may also be used in *in vitro* immunoassays and may be compatible with purification methodology known in the art. See e.g., International publication No. WO 93/21232; European Patent No. EP 439,095; Naramura et al., 1994, Immunol. Lett. 39:91-99; U.S. Pat. No. 5,474,981; Gillies et al., 1992, PNAS 89:1428-1432; and Fell et al., 1991, J. Immunol. 146:2446-2452.

a. Biocompatible modifiers

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

b. Diagnostic or detection agents

In other preferred embodiments, modulators of the present invention, or fragments or derivatives thereof, are conjugated to a diagnostic or detectable agent, marker or reporter which may be a biological molecule (e.g., a peptide or nucleotide), a small molecule, flourophore, or radioisotope. Labeled modulators 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 (i.e. theragnostics). Such markers or reporters may also be useful in purifying the selected modulator, separating or isolating TIC or in preclinical procedures or toxicology studies.

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

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

c. Therapeutic Moieties

As previously alluded to the modulators or fragments or derivatives thereof may also be conjugated, linked or fused to or otherwise associated with a therapeutic moiety such as anti-cancer agents, a cytotoxin or cytotoxic agent, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha or beta-emitters. As used herein a cytotoxin or cytotoxic agent includes any agent or therapeutic moiety that is detrimental to cells and may inhibit cell growth or survival. Examples include paclitaxel, cytochalasin B,

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

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

The selected modulators can also be conjugated to therapeutic moieties such as radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (see above for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N",N"-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res. 4:2483; Peterson et al., 1999, Bioconjug. Chem. 10:553; and Zimmerman et al., 1999, Nucl. Med. Biol. 26:943.

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

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

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

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

XII. Diagnostics and Screening

a. Diagnostics

As indicated, the present invention provides *in vitro* or *in vivo* methods for detecting, diagnosing or monitoring hyperproliferative disorders and methods of screening cells from a patient to identify tumorigenic cells including TPCs. Such methods include identifying an individual having cancer for treatment or monitoring progression of a cancer comprising contacting the patient or a sample obtained from a patient with a selected EFNA modulator as described herein and detecting presence or absence, or level of association of the modulator to bound or free ephrin-A ligand in the sample. When the modulator comprises an antibody or immunologically active fragment thereof the

association with particular EFNA in the sample likely denotes that the sample may contain tumor perpetuating cells (e.g., a cancer stem cells) indicating that the individual having cancer may be effectively treated with an EFNA modulator as described herein. The methods may further comprise a step of comparing the level of binding to a control. Conversely, when the selected modulator is Fc-EFNA the binding properties of the selected ephrin-A ligand may be exploited and monitored (directly or indirectly, *in vivo* or *in vitro*) when in contact with the sample to provide the desired information. Other diagnostic or theragnostic methods compatible with the teachings herein are well known in the art and can be practiced using commercial materials such as dedicated reporting systems.

In a particularly preferred embodiment the modulators of the instant invention may be used to detect and quantify EFNA levels in a patient sample (e.g., plasma or blood) which may, in turn, be used to detect, diagnose or monitor EFNA associated disorders including hyperproliferative disorders.

Exemplary compatible assay methods include radioimmunoassays, enzyme immunoassays, competitive-binding assays, fluorescent immunoassay, immunoblot assays, Western Blot analysis, flow cytometry assays, and ELISA assays. More generally detection of EFNA in a biological sample or the measurement of EFNA enzymatic activity (or inhibition thereof) may be accomplished using any art-known assay. Compatible *in vivo* theragnostics or diagnostics may comprise art recognized imaging or monitoring techniques such as magnetic resonance imaging (MRI), computerized tomography (e.g. CAT scan), positron tomography (e.g., PET scan) radiography, ultrasound, etc. Those skilled in the art will readily be able to recognize and implement appropriate detection, monitoring or imaging techniques (often comprising commercially available sources) based on the etiology, pathological manifestation or clinical progression of the disorder.

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 embodiments, further *ex vivo* analysis is performed.

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 certain examples, the tumorigenic cells in a subject or a sample from a subject may be assessed or characterized using the disclosed modulators 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.

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

b. Screening

The EFNA modulators and cells, cultures, populations and compositions comprising the same, including progeny thereof, can also be used to screen for or identify compounds or agents (e.g., drugs) that affect a function or activity of tumor initiating cells or progeny thereof by interacting with an ephrin-A ligand (e.g., the polypeptide or genetic components thereof). The invention therefore further provides systems and methods for evaluation or identification of a compound or agent that can affect a function or activity

tumor initiating cells or progeny thereof by associating with EFNA or its substrates. Such compounds and agents can be drug candidates that are screened for the treatment of a hyperproliferative disorder, for example. In one embodiment, a system or method includes tumor initiating cells exhibiting EFNA and a compound or agent (e.g., drug), wherein the cells and compound or agent (e.g., drug) are in contact with each other.

The invention further provides methods of screening and identifying EFNA modulators or agents and compounds for altering an activity or function of tumor initiating cells or progeny cells. In one embodiment, a method includes contacting tumor initiating cells or progeny thereof with a test agent or compound; and determining if the test agent or compound modulates an activity or function of the ephrin-A ligand associated tumor initiating cells.

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

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

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

modulation of an activity or function of tumor initiating cells or progeny cells as disclosed herein or would be known to the skilled artisan.

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

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

XIII. Pharmaceutical Preparations and Therapeutic Uses

a. Formulations and routes of administration

Depending on the form of the modulator along with any optional conjugate, the mode of intended delivery, the disease being treated or monitored and numerous other variables, compositions of the instant invention may be formulated as desired using art recognized techniques. That is, in various embodiments of the instant invention compositions comprising EFNA modulators are formulated with a wide variety of pharmaceutically acceptable carriers (see, e.g., Gennaro, *Remington: The Science and Practice of Pharmacy with Facts and Comparisons: Drugfacts Plus*, 20th ed. (2003); Ansel et al., *Pharmaceutical Dosage Forms and Drug Delivery Systems,* 7th ed., Lippencott Williams and Wilkins (2004); Kibbe et al., *Handbook of Pharmaceutical Excipients,* 3rd ed., Pharmaceutical Press (2000)). Various pharmaceutically acceptable carriers, which include vehicles, adjuvants, and diluents, are readily available from

numerous commercial sources. Moreover, an assortment of pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are also available. Certain non-limiting exemplary carriers include saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

More particularly it will be appreciated that, in some embodiments, the therapeutic compositions of the invention may be administered neat or with a minimum of additional components. Conversely the EFNA modulators of the present invention may optionally be formulated to contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries that are well known in the art and are relatively inert substances that facilitate administration of the modulator or which aid processing of the active compounds into preparations that are pharmaceutically optimized for delivery to the site of action. For example, an excipient can give form or consistency or act as a diluent to improve the pharmacokinetics of the modulator. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, buffers, and skin penetration enhancers.

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

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

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

b. Dosages

Similarly, the particular dosage regimen, i.e., dose, timing and repetition, will depend on the particular individual and that individual's medical history. Empirical considerations such as pharmacokinetics (e.g., half-life, clearance rate, etc.) will contribute to the determination of the dosage. Frequency of administration may be determined and adjusted over the course of therapy, and is based on reducing the number of hyperproliferative or neoplastic cells, including tumor initiating cells, maintaining the reduction of such neoplastic cells, reducing the proliferation of neoplastic cells, or delaying the development of metastasis. Alternatively, sustained continuous release formulations of a subject therapeutic composition may be appropriate. As alluded to above various formulations and devices for achieving sustained release are known in the art.

From a therapeutic standpoint the pharmaceutical compositions are administered in an amount effective for treatment or prophylaxis of the specific indication. The therapeutically effective amount is typically dependent on the weight of the subject being treated, his or her physical or health condition, the extensiveness of the condition to be treated, or the age of the subject being treated. In general, the EFNA modulators of the invention may be administered in an amount in the range of about 10 μg/kg body weight to about 100 mg/kg body weight per dose. In certain embodiments, the EFNA modulators of the invention may be administered in an amount in the range of about 50 μg/kg body weight to about 5 mg/kg body weight per dose. In certain other embodiments, the EFNA modulators of the invention may be administered in an amount in the range of about 100 μg/kg body weight to about 10 mg/kg body weight per dose. Optionally, the EFNA modulators of the invention may be administered in an amount in the range of about 100

μg/kg body weight to about 20 mg/kg body weight per dose. Further optionally, the EFNA modulators of the invention may be administered in an amount in the range of about 0.5 mg/kg body weight to about 20 mg/kg body weight per dose. In certain embodiments the compounds of present invention are provided a dose of at least about 100 μg/kg body weight, at least about 250 μg/kg body weight, at least about 750 μg/kg body weight, at least about 3 mg/kg body weight, at least about 5 mg/kg body weight, at least about 10 mg/kg body weight is administered.

Other dosing regimens may be predicated on Body Surface Area (BSA) calculations as disclosed in U.S.P.N. 7,744,877 which is incorporated herein by reference in its entirety. As is well known in the art the BSA is calculated using the patient's height and weight and provides a measure of a subject's size as represented by the surface area of his or her body. In selected embodiments of the invention using the BSA the modulators may be administered in dosages from 10 mg/m^2 to 800 mg/m^2. In other preferred embodiments the modulators will be administered in dosages from 50 mg/m^2 to 500 mg/m^2 and even more preferably at dosages of 100 mg/m^2, 150 mg/m^2, 200 mg/m^2, 250 mg/m^2, 300 mg/m^2, 350 mg/m^2, 400 mg/m^2or 450 mg/m^2. Of course it will be appreciated that, regardless of how the dosages are calculated, multiple dosages may be administered over a selected time period to provide an absolute dosage that is substantially higher than the individual administrations.

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

Dosages and regimens may also be determined empirically for the disclosed therapeutic compositions in individuals who have been given one or more administration(s). For example, individuals may be given incremental dosages of a therapeutic composition produced as described herein. To assess efficacy of the selected composition, a marker of the specific disease, disorder or condition can be followed as

described previously. In embodiments where the individual has cancer, these include direct measurements of tumor size via palpation or visual observation, indirect measurement of tumor size by x-ray or other imaging techniques; an improvement as assessed by direct tumor biopsy and microscopic examination of the tumor sample; the measurement of an indirect tumor marker (e.g., PSA for prostate cancer) or an antigen identified according to the methods described herein, a decrease in pain or paralysis; improved speech, vision, breathing or other disability associated with the tumor; increased appetite; or an increase in quality of life as measured by accepted tests or prolongation of survival. It will be apparent to one of skill in the art that the dosage will vary depending on the individual, the type of neoplastic condition, the stage of neoplastic condition, whether the neoplastic condition has begun to metastasize to other location in the individual, and the past and concurrent treatments being used.

c. Combination therapies

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

According to the methods of the present invention, there is no requirement for the combined results to be additive of the effects observed when each treatment (e.g., anti-EFNA antibody and anti-cancer agent) is conducted separately. Although at least additive effects are generally desirable, any increased anti-tumor effect above one of the single therapies is beneficial. Furthermore, the invention does not require the combined

treatment to exhibit synergistic effects. However, those skilled in the art will appreciate that with certain selected combinations that comprise preferred embodiments, synergism may be observed.

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

Alternatively, the modulator may precede, or follow, the anti-cancer agent treatment by, e.g., intervals ranging from minutes to weeks. In certain embodiments wherein the anti-cancer agent and the antibody are applied separately to the subject, the time period between the time of each delivery is such that the anti-cancer agent and modulator are able to exert a combined effect on the tumor. In a particular embodiment, it is contemplated that both the anti-cancer agent and the EFNA modulator are administered within about 5 minutes to about two weeks of each other.

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

administered for as long as the tumor is present provided that the combination therapy causes the tumor or cancer to stop growing or to decrease in weight or volume.

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

The present invention contemplates at least one cycle, preferably more than one cycle during which the combination therapy is administered. An appropriate period of time for one cycle will be appreciated by the skilled artisan, as will the total number of cycles, and the interval between cycles. The invention contemplates the continued assessment of optimal treatment schedules for each modulator and anti-cancer agent. Moreover, the invention also provides for more than one administration of either the anti-EFNA antibody or the anti-cancer agent. The modulator and anti-cancer agent may be administered interchangeably, on alternate days or weeks; or a sequence of antibody treatment may be given, followed by one or more treatments of anti-cancer agent therapy. In any event, as will be understood by those of ordinary skill in the art, the appropriate doses of chemotherapeutic agents will be generally around those already employed in clinical therapies wherein the chemotherapeutics are administered alone or in combination with other chemotherapeutics.

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

Moreover such treatments could be continued for a period of weeks, months, years or even indefinitely depending on the patient response and clinical and diagnostic parameters.

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

d. Anti-cancer agents

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

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

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

Examples of anti-cancer agents that may be used in combination with (or conjugated to) the modulators of the present invention include, but are not limited to, alkylating agents, alkyl sulfonates, aziridines, ethylenimines and methylamelamines, acetogenins, a camptothecin, bryostatin, callystatin, CC-1065, cryptophycins, dolastatin, duocarmycin, eleutherobin, pancratistatin, a sarcodictyin, spongistatin, nitrogen mustards, antibiotics, enediyne antibiotics, dynemicin, bisphosphonates, an esperamicin, chromoprotein enediyne antiobiotic chromophores, aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN® doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites, folic acid analogues, purine analogs, androgens, anti-adrenals, folic acid replenisher such as frolinic acid, aceglatone, aldophosphamide glycoside, aminolevulinic acid, eniluracil, amsacrine, bestrabucil, bisantrene, edatraxate, defofamine, demecolcine, diaziquone, elfornithine, elliptinium acetate, an epothilone, etoglucid, gallium nitrate, hydroxyurea, lentinan, lonidainine, maytansinoids, mitoguazone, mitoxantrone, mopidanmol, nitraerine, pentostatin, phenamet, pirarubicin, losoxantrone, podophyllinic acid, 2- ethylhydrazide, procarbazine, PSK® polysaccharide complex (JHS Natural Products, Eugene, OR), razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, chloranbucil; GEMZAR® gemcitabine; 6-

thioguanine; mercaptopurine; methotrexate; platinum analogs, vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE® vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11), topoisomerase inhibitor RFS 2000; difluorometlhylornithine (DMFO); retinoids; capecitabine; combretastatin; leucovorin (LV); oxaliplatin; inhibitors of PKC-alpha, Raf, H-Ras, EGFR and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens and selective estrogen receptor modulators (SERMs), aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, and anti-androgens; as well as troxacitabine (a 1,3- dioxolane nucleoside cytosine analog); antisense oligonucleotides,; ribozymes such as a VEGF expression inhibitor and a HER2 expression inhibitor; vaccines, PROLEUKIN® rIL-2; LURTOTECAN® topoisomerase 1 inhibitor; ABARELIX® rmRH; Vinorelbine and Esperamicins and pharmaceutically acceptable salts, acids or derivatives of any of the above. Other embodiments comprise the use of immunotherapeutic agents, such as antibodies, approved for cancer therapy including, but not limited to, rituximab, trastuzumab, gemtuzumab ozogamcin, alemtuzumab, ibritumomab tiuxetan, tositumomab, bevacizumab, cetuximab, patitumumab, ofatumumab, ipilimumab and brentuximab vedotin. Those skilled in the art will be able to readily identify additional anti-cancer agents that are compatible with the teachings herein.

e. Radiotherapy

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

f. Neoplastic conditions

Whether administered alone or in combination with an anti-cancer agent or radiotherapy, the EFNA modulators of the instant invention are particularly useful for

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

More specifically, neoplastic conditions subject to treatment in accordance with the instant invention may be selected from the group including, but not limited to, adrenal gland tumors, AIDS-associated cancers, alveolar soft part sarcoma, astrocytic tumors, bladder cancer (squamous cell carcinoma and transitional cell carcinoma), bone cancer (adamantinoma, aneurismal bone cysts, osteochondroma, osteosarcoma), brain and spinal cord cancers, metastatic brain tumors, breast cancer, carotid body tumors, cervical cancer, chondrosarcoma, chordoma, chromophobe renal cell carcinoma, clear cell carcinoma, colon cancer, colorectal cancer, cutaneous benign fibrous histiocytomas, desmoplastic small round cell tumors, ependymomas, Ewing's tumors, extraskeletal myxoid chondrosarcoma, fibrogenesis imperfecta ossium, fibrous dysplasia of the bone, gallbladder and bile duct cancers, gestational trophoblastic disease, germ cell tumors, head and neck cancers, islet cell tumors, Kaposi's Sarcoma, kidney cancer (nephroblastoma, papillary renal cell carcinoma), leukemias, lipoma/benign lipomatous tumors, liposarcoma/malignant lipomatous tumors, liver cancer (hepatoblastoma, hepatocellular carcinoma), lymphomas, lung cancers (small cell carcinoma, adenocarcinoma, squamous cell carcinoma, large cell carcinoma etc.), medulloblastoma, melanoma, meningiomas, multiple endocrine neoplasia, multiple myeloma, myelodysplastic syndrome, neuroblastoma, neuroendocrine tumors, ovarian cancer, pancreatic cancers, papillary thyroid carcinomas, parathyroid tumors, pediatric cancers, peripheral nerve sheath tumors, phaeochromocytoma, pituitary tumors, prostate cancer, posterious unveal melanoma, rare hematologic disorders, renal metastatic cancer, rhabdoid tumor, rhabdomysarcoma, sarcomas, skin cancer, soft-tissue sarcomas, squamous cell cancer, stomach cancer,

synovial sarcoma, testicular cancer, thymic carcinoma, thymoma, thyroid metastatic cancer, and uterine cancers (carcinoma of the cervix, endometrial carcinoma, and leiomyoma). In certain preferred embodiments, the cancerous cells are selected from the group of solid tumors including but not limited to breast cancer, non-small cell lung cancer (NSCLC), small cell lung cancer, pancreatic cancer, colon cancer, prostate cancer, sarcomas, renal metastatic cancer, thyroid metastatic cancer, and clear cell carcinoma.

With regard to hematologic malignancies it will be further be appreciated that the compounds and methods of the present invention may be particularly effective in treating a variety of B-cell lymphomas, including low grade/NHL follicular cell lymphoma (FCC), mantle cell lymphoma (MCL), diffuse large cell lymphoma (DLCL), small lymphocytic (SL) NHL, intermediate grade/follicular NHL, intermediate grade diffuse NHL, high grade immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL, Waldenstrom's Macroglobulinemia, lymphoplasmacytoid lymphoma (LPL), mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large cell lymphoma (DLCL), Burkitt's lymphoma (BL), AIDS-related lymphomas, monocytic B cell lymphoma, angioimmunoblastic lymphoadenopathy, small lymphocytic, follicular, diffuse large cell, diffuse small cleaved cell, large cell immunoblastic lymphoblastoma, small, non-cleaved, Burkitt's and non-Burkitt's, follicular, predominantly large cell; follicular, predominantly small cleaved cell; and follicular, mixed small cleaved and large cell lymphomas. See, Gaidono et al., "Lymphomas", IN CANCER: PRINCIPLES & PRACTICE OF ONCOLOGY, Vol. 2: 2131-2145 (DeVita et al., eds., 5.sup.th ed. 1997). It should be clear to those of skill in the art that these lymphomas will often have different names due to changing systems of classification, and that patients having lymphomas classified under different names may also benefit from the combined therapeutic regimens of the present invention.

In yet other preferred embodiments the EFNA modulators may be used to effectively treat certain myeloid and hematologic malignancies including leukemias such as chronic lymphocytic leukemia (CLL or B-CLL). CLL is predominantly a disease of the elderly that starts to increase in incidence after fifty years of age and reaches a peak by late sixties. It generally involves the proliferation of neoplastic peripheral blood lymphocytes. Clinical finding of CLL involves lymphocytosis, lymphadenopatliy, splenomegaly, anemia and thrombocytopenia. A characteristic feature of CLL is monoclonal B cell proliferation and accumulation of B-lymphocytes arrested at an intermediate state of differentiation

where such B cells express surface IgM (sIgM) or both sIgM and sIgD, and a single light chain at densities lower than that on the normal B cells.

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

Still other preferred embodiments of the instant invention comprise the use of EFNA modulators to treat subjects suffering from solid tumors. In such subjects many of these solid tumors comprise tissue exhibiting various genetic mutations that may render them particularly susceptible to treatment with the disclosed effectors. For example, KRAS, APC and CTNNB1and CDH1 mutations are relatively common in patients with colorectal cancer. Moreover, patients suffering from tumors with these mutations are usually the most refractory to current therapies; especially those patients with KRAS mutations. KRAS activating mutations, which typically result in single amino acid substitutions, are also implicated in other difficult to treat malignancies, including lung adenocarcinoma, mucinous adenoma, and ductal carcinoma of the pancreas.

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

Loss of CDH1 (E-cadherin) expression is yet another common occurrence in colorectal cancer, often observed in more advanced stages of the disease. E-cadherin is the central member of adherin junctions that connect and organize cells in epithelial layers. Normally E-cadherin physically sequesters beta-catenin (CTNNB1) at the plasma membrane; loss of E-cadherin expression in colorectal cancer results in localization of beta-catenin to the nucleus and transcriptional activation of the beta -catenin/ WNT pathway. Aberrant beta-catenin/ WNT signaling is central to oncogenesis and nuclear beta-catenin has been implicated in cancer stemness (Schmalhofer et al., 2009 PMID 19153669). E-cadherin is required for the expression and function of EphA2 a known binding partner for EFNA ligands in epithelia cells (Dodge Zantek et al., 1999 PMID 10511313; Orsulic S and Kemler R, 2000 PMID 10769210). Using modulators that bind to EFNA ligands and agonize with or antagonize Eph receptor binding may modify, interrupt or reverse the pro-oncogenic processes. Alternatively, EFNA modulators may preferentially bind to tumor cells with aberrant EphA/EFNA interactions based on the binding preferences of the EFNA modulators. Hence patients with cancers carrying the above mentioned genetic traits may benefits from treatment with aforementioned EFNA modulators.

XIV. Articles of Manufacture

Pharmaceutical packs and kits comprising one or more containers, comprising one or more doses of an EFNA modulator are also provided. In certain embodiments, a unit dosage is provided wherein the unit dosage contains a predetermined amount of a composition comprising, for example, an anti-EFNA antibody, with or without one or more additional agents. For other embodiments, such a unit dosage is supplied in single-use prefilled syringe for injection. In still other embodiments, the composition contained in the unit dosage may comprise saline, sucrose, or the like; a buffer, such as phosphate, or the like; and/or be formulated within a stable and effective pH range. Alternatively, in certain embodiments, the composition may be provided as a lyophilized powder that may be reconstituted upon addition of an appropriate liquid, for example, sterile water. In certain preferred embodiments, the composition comprises one or more substances that inhibit protein aggregation, including, but not limited to, sucrose and arginine. Any label on, or associated with, the container(s) indicates that the enclosed composition is used for diagnosing or treating the disease condition of choice.

The present invention also provides kits for producing single-dose or multi-dose administration units of an EFNA modulator and, optionally, one or more anti-cancer agents. The kit comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition that is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). Such kits will generally contain in a suitable container a pharmaceutically acceptable formulation of the EFNA modulator and, optionally, one or more anti-cancer agents in the same or different containers. The kits may also contain other pharmaceutically acceptable formulations, either for diagnosis or combined therapy. For example, in addition to the EFNA modulator of the invention such kits may contain any one or more of a range of anti-cancer agents such as chemotherapeutic or radiotherapeutic drugs; anti-angiogenic agents; anti-metastatic agents; targeted anti-cancer agents; cytotoxic agents; and/or other anti-cancer agents. Such kits may also provide appropriate reagents to conjugate the EFNA modulator with an anti-cancer agent or diagnostic agent (e.g., see U.S.P.N. 7,422,739 which is incorporated herein by reference in its entirety).

More specifically the kits may have a single container that contains the EFNA modulator, with or without additional components, or they may have distinct containers for each desired agent. Where combined therapeutics are 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 EFNA modulator and any optional anti-cancer agent of the kit may be maintained separately within distinct containers prior to administration to a patient. The kits may also comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent such as bacteriostatic water for injection (BWFI), phosphate-buffered saline (PBS), Ringer's solution and dextrose solution.

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

As indicated briefly above the kits may also contain a means by which to administer the antibody and any optional components to an animal or patient, e.g., one or more needles or syringes, or even an eye dropper, pipette, or other such like apparatus, from which the formulation may be injected 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. Any label or package insert indicates that the EFNA modulator composition is used for treating cancer, for example colorectal cancer.

XV. Research Reagents

Other preferred embodiments of the invention also exploit the properties of the disclosed modulators 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).

XVI. Miscellaneous

Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. More specifically, as used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a protein" includes a plurality of proteins; reference to "a cell" includes mixtures of cells, and the like. In addition, ranges provided in the specification and appended claims include both end points and all points between the end points. Therefore, a range of 2.0 to 3.0 includes 2.0, 3.0, and all points between 2.0 and 3.0.

Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic

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

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

EXAMPLES

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

Example 1

Enrichment of Tumor Initiating Cell Populations

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

As the NTX tumor cell lines were established the constituent tumor cell phenotypes were analyzed using flow cytometry to identify discrete markers that might be used to characterize, isolate, purify or enrich tumor initiating cells (TIC) and separate or analyze TPC and TProg cells within such populations. In this regard the inventors employed a proprietary proteomic based platform (i.e. PhenoPrint™ Array) that provided for the rapid characterization of cells based on protein expression and the concomitant identification of potentially useful markers. The PhenoPrint Array is a proprietary proteomic platform comprising hundreds of discrete binding molecules, many obtained from commercial sources, arrayed in 96 well plates wherein each well contains a distinct antibody in the phycoerythrin fluorescent channel and multiple additional antibodies in different

fluorochromes arrayed in every well across the plate. This allows for the determination of expression levels of the antigen of interest in a subpopulation of selected tumor cells through rapid inclusion of relevant cells or elimination of non-relevant cells via non-phycoerythrin channels. When the PhenoPrint Array was used in combination with tissue dissociation, transplantation and stem cell techniques well known in the art (Al-Hajj et al., 2004, Dalerba et al., 2007 and Dylla et al., 2008, all supra, each of which is incorporated herein by reference in its entirety), it was possible to effectively identify relevant markers and subsequently isolate and transplant specific human tumor cell subpopulations with great efficiency.

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

The term enriching is used synonymously with isolating cells and means that the yield (fraction) of cells of one type is increased over the fraction of other types of cells as compared to the starting or initial cell population. Preferably, enriching refers to increasing the percentage by about 10%, by about 20%, by about 30%, by about 40%, by about 50% or greater than 50% of one type of cell in a population of cells as compared to the starting population of cells.

As used herein a marker, in the context of a cell or tissue, means any characteristic in the form of a chemical or biological entity that is identifiably associated with, or

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

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

Those skilled in the art will recognize that numerous markers (or their absence) have been associated with various populations of cancer stem cells and used to isolate or characterize tumor cell subpopulations. In this respect exemplary cancer stem cell markers comprise OCT4, Nanog, STAT3, EPCAM, CD24, CD34, NB84, TrkA, GD2, CD133, CD20, CD56, CD29, B7H3, CD46, transferrin receptor, JAM3, carboxypeptidase M, ADAM9, oncostatin M, Lgr5, Lgr6, CD324, CD325, nestin, Sox1, Bmi-1, eed, easyh1, easyh2, mf2, yy1, smarcA3, smarckA5, smarcD3, smarcE1, mllt3, FZD1, FZD2, FZD3,

FZD4, FZD6, FZD7, FZD8, FZD9, FZD10, WNT2, WNT2B, WNT3, WNT5A, WNT10B, WNT16, AXIN1, BCL9, MYC, (TCF4) SLC7A8, IL1RAP, TEM8, TMPRSS4, MUC16, GPRC5B, SLC6A14, SLC4A11, PPAP2C, CAV1, CAV2, PTPN3, EPHA1, EPHA2, SLC1A1, CX3CL1, ADORA2A, MPZL1, FLJ10052, C4.4A, EDG3, RARRES1, TMEPAI, PTS, CEACAM6, NID2, STEAP, ABCA3, CRIM1, IL1R1, OPN3, DAF, MUC1, MCP, CPD, NMA, ADAM9, GJA1, SLC19A2, ABCA1, PCDH7, ADCY9, SLC39A1, NPC1, ENPP1, N33, GPNMB, LY6E, CELSR1, LRP3, C20orf52, TMEPAI, FLVCR, PCDHA10, GPR54, TGFBR3, SEMA4B, PCDHB2, ABCG2, CD166, AFP, BMP-4, β-catenin, CD2, CD3, CD9, CD14, CD31, CD38, CD44, CD45, CD74, CD90, CXCR4, decorin, EGFR, CD105, CD64, CD16, CD16a, CD16b, GLI1, GLI2, CD49b, and CD49f. See, for example, Schulenburg et al., 2010, PMID: 20185329, U.S.P.N. 7,632,678 and U.S.P.Ns. 2007/0292414, 2008/0175870, 2010/0275280, 2010/0162416 and 2011/0020221 each of which is incorporated herein by reference. It will be appreciated that a number of these markers were included in the PhenoPrint Array described above.

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

Cells with negative expression (i.e."-") are herein defined as those cells expressing less than, or equal to, the 95^{th} 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%.

Using techniques such as those described above to quickly identify and rank colorectal tumor antigens based on expression intensity and heterogeneity across several NTX tumors from colorectal cancer patients, candidate TPC antigens were further assessed by comparison of tumor versus normal adjacent tissue and then selected based, at least in part, on the up- or down-regulation of the particular antigen in malignant cells. Moreover, systematic analysis of a variety of cell surface markers for their ability to enrich for the ability to transplant fully heterogeneous tumors into mice (i.e. tumorigenic ability), and subsequent combination of these markers substantially improved the resolution of the method and improved the ability to tailor fluorescence activated cell sorting (FACS) techniques to identify and characterize distinct, highly enriched tumor cell subpopulations that exclusively contained all tumor generating ability upon transplantation (i.e. tumor initiating cells). To reiterate, the term tumor initiating cell (TIC) or tumorigenic (TG) cell encompasses both Tumor Perpetuating Cells (TPC; i.e. cancer stem cells) and highly proliferative Tumor Progenitor cells (TProg), which together generally comprise a unique subpopulation (i.e. 0.1-25%) of a bulk tumor or mass; the characteristics of which are defined above. The majority of tumor cells characterized in this fashion are devoid of this tumor forming ability, and can thus be characterized as non-tumorigenic (NTG).

Surprisingly, it was observed that most distinct markers identified using the proprietary PhenoPrint Array did not demonstrate an ability to enrich tumor initiating cell populations in colorectal tumors using standard FACS protocols, but that distinct marker combinations could be used to identify two subpopulations of tumor initiating cells: TPC and TProg. Those skilled in the art will recognize that the defining difference between TPC and TProg, though both are tumor initiating in primary transplants, is the ability of TPC to perpetually fuel tumor growth upon serial transplantation at low cell numbers. Furthermore, the marker/proteins used in combination to enrich for both TPC and TProg were unknown to be associated with cells containing such activity in any tissue or neoplasm prior to discovery by current inventors though others have defined cell surface markers or enzymatic activity that can similarly be used to enrich for tumorigenic cells (Dylla et al 2008, supra). As set forth below, specific tumor cell subpopulations isolated using cell surface marker combinations alluded to above were then analyzed using whole transcriptome next generation sequencing to identify and characterize differentially expressed genes.

Example 2

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

Several established colorectal NTX cell lines (SCRX-CR4, CR11, CR33, PA3, PA6 & PA14) generated and passaged as described in Example 1 were used to initiate tumors in immune compromised mice. For mice bearing SCRX-CR4, PA3 or PA6 tumors, once the mean tumor burden reached ~ 300 mm^3 the mice were randomized and treated with 15 mg/kg irinotecan, 25 mg/kg Gemcitabine, or vehicle control (PBS) twice weekly for a period of at least twenty days prior to euthanization. Tumors arising from all six NTX lines, including those from mice undergoing chemotherapeutic treatment were removed and TPC, TProg and NTG cells, respectively, were isolated from freshly resected colorectal NTX tumors and, similarly, TG and NTG cells were isolated from pancreatic NTX tumors, generally using the technique set out in Example 1. 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 Technologies) again using the vendor's protocols and recommended instrument settings. The resulting total RNA preparation was suitable for genetic sequencing and analysis.

Total RNA samples obtained from the respective cell populations isolated as described above from vehicle or chemotherapeutic agent-treated mice were prepared for whole transcriptome sequencing using an Applied Biosystems SOLiD 3.0 (Sequencing by Oligo Ligation/Detection) next generation sequencing platform (Life Technologies), starting with 5 ng of total RNA per sample. The data generated by the SOLiD platform mapped to 34,609 genes from the human genome and was able to detect ephrin-A ligands, including EFNA1 and EFNA3, and provided verifiable measurements of ENFA levels in most samples.

Generally the SOLiD3 next generation sequencing platform enables parallel sequencing of clonally-amplified RNA/DNA fragments linked to beads. Sequencing by ligation with dye-labeled oligonucleotides is then used to generate 50 base reads of each fragment that exists in the sample with a total of greater than 50 million reads generating a much more accurate representation of the mRNA transcript level expression of proteins in the genome. The SOLiD3 platform is able to capture not only expression, but SNPs, known and unknown alternative splicing events, and potentially new exon discoveries based solely on the read coverage (reads mapped uniquely to genomic locations). Thus, use of this next generation platform allowed the determination of differences in transcript level expression as well as differences or preferences for specific splice variants of those expressed mRNA transcripts. Moreover, analysis with the SOLiD3 platform using a modified whole transcriptome protocol from Applied Biosystems only required approximately 5 ng of starting material pre-amplification. This is significant as extraction of total RNA from sorted cell populations where the TPC subset of cells is, for example, vastly smaller in number than the NTG or bulk tumors and thus results in very small quantities of usable starting material.

Duplicate runs of sequencing data from the SOLiD3 platform were normalized and transformed and fold ratios calculated as is standard industry practice. As seen in FIG. 2, levels of EFNA1, EFNA3 and EFNA4 from a tumor were measured as well as levels of Eph receptors EPHA1, EPHA2 and EPHA10. An analysis of the data showed that EFNA1 is the most highly expressed ephrin-A ligand in SCRx-CR4 NTX tumors, with slightly elevated expression in the TPC population (FIG. 2A). In mice being treated with 15 mg/kg irinotecan twice weekly, EFNA1 expression was maintained in TPC whereas NTG

cells saw reduced levels (FIG. 2B). It will further be appreciated that EFNA1 was also elevated in TPC versus TProg and NTG cells, respectively, in NTX tumors derived from additional patients (SCRx-CR7 and SCRx-CR11; FIG. 2C). This expression was also independent of whether tumors had been exposed to standard of care chemotherapeutic regimens such as FOLFIRI (i.e. 5-FU, oxaliplatin and irinotecan). Furthermore, when pancreatic (SCRx-PA3, PA4, PA6 & PA14; FIG. 2D) and non-small cell lung tumor samples (FIG. 2E) were analyzed by SOLiD3 whole-transcriptome sequencing, EFNA1 gene expression was similarly elevated in TPC versus NTG cells, and in non-small cell lung tumor subpopulations versus normal lung (FIG. 2E), in most patients, as defined using $CD46^{+}CD324^{+}$ cell populations as illustrated previously. Furthermore, EFNA1 gene expression was elevated in pancreatic tumor cell subpopulations exposed to the standard of care chemotherapeutic agent, gemcitibine (FIG. 2D).

Close examination of whole transcriptome gene expression data revealed that EPHA2 receptor (with which both EFNA1 and EFNA3 ligands interact) expression inversely reflects that of both EFNA1 and EFNA3 during the progression of differentiation from TPC to NTG cells in colorectal tumors (FIGS. 2A and 2B). This inverse expression pattern of the EFNA1/EFNA3 ligands and EPHA2 receptor suggests that crosstalk between these ligand/receptor pairs might play a role in cell fate decisions during colorectal cancer stem cell differentiation and that neutralizing these interactions might negatively impact tumor growth. Specifically, by blocking EphA2 interactions with EFNA1 and/or EFNA3 using neutralizing modulators as disclosed herein, TPC might be sensitized to chemotherapeutic agents, for example, or forced to differentiate. Moreover, by targeting TPC using EFNA1 and/or EFNA3-internalizing antibodies, TPC might be killed directly by the naked modulator or through the use of a toxin or antibody drug conjugate.

Analysis of whole transcriptome data, as discussed above, also showed elevated EFNA3 expression in some colorectal, pancreatic and non-small cell lung tumors, with elevated expression in the TIC subpopulations of these tumors. Specifically, EFNA3 expression was elevated in TIC subpopulations (TPC and TProg) versus NTG cells isolated from several human colorectal tumors (SCRx-CR11 and –CR33; FIG. 3A). This expression was independent of whether tumors had been exposed to standard of care chemotherapeutic regimens such as FOLFIRI (i.e. 5-FU, oxaliplatin and irinotecan). When pancreatic (SCRx-PA3, PA4 and PA6; FIG. 3B) and non-small cell lung tumor samples (FIG. 3C) were analyzed by SOLiD3 whole-transcriptome sequencing, EFNA3 gene

expression was similarly elevated in TPC versus NTG cells, and in non-small cell lung tumor subpopulations versus normal lung (FIG. 3C), in most patients, as defined using $CD46^{+}/CD324^{+}$ cell populations as described herein. Furthermore, EFNA3 gene expression was maintained or increased in human pancreatic TIC subpopulations isolated from xenograft tumor bearing mice that had been exposed to the standard of care chemotherapeutic agent, gemcitibine (FIG. 3B).

The observations detailed above show that EFNA1 and/or EFNA3 expression is generally elevated in TPC populations and suggests that these membrane-tethered ligands may play an important role in tumorigenesis and tumor maintenance, thus constituting excellent targets for novel therapeutic approaches.

Example 3

Real-Time PCR Analysis of Ephrin-A Ligands in Enriched Tumor Initiating Cell Populations

To validate the differential ephrin-A ligand expression observed by whole transcriptome sequencing in TPC populations versus TProg and NTG cells in colorectal cancer, and TG versus NTG cells in pancreatic cancer, TaqMan® quantitative real-time PCR was used to measure gene expression levels in respective cell populations isolated from various NTX lines as set forth above. It will be appreciated that such real-time PCR analysis allows for a more direct and rapid measurement of gene expression levels for discrete targets using primers and probe sets specific to a particular gene of interest. TaqMan® real-time quantitative PCR was performed on an Applied Biosystems 7900HT Machine (Life Technologies), which was used to measure EFNA1 and EFNA3 gene expression in multiple patient-derived NTX line cell populations and corresponding controls. Moreover, the analysis was conducted as specified in the instructions supplied with the TaqMan System and using commercially available EFNA1 and EFNA3 primer/probe sets (Life Technologies).

As seen in FIGS. 4A and 4B, quantitative real-time PCR interrogation of gene expression in NTG and TPC populations isolated from distinct colorectal NTX tumor lines (SCRx-CR2, CR4, & CR14) showed that EFNA1 and EFNA3 gene expression is elevated more than 1.5-fold in the TPC subpopulations versus NTG cells. Likewise, pancreatic NTX line (SCRx-PA14) showed that EFNA3 gene expression was elevated 1.3-fold in the TPC subpopulation versus the NTG cells. The observation of elevated EFNA1 and

EFNA3 expression in NTX TPC cell preparations as compared with NTG cell controls from both colorectal and pancreatic patient-derived NTX tumors using the more widely accepted methodology of real-time quantitative PCR confirms the more sensitive SOLiD3 whole transcriptome sequencing data of the previous Example, and supports the observed association between EFNA1 and EFNA3 expressing cells underlying tumorigenesis, resistance to therapy and recurrence.

Example 4

Expression of Ephrin-A Ligands in Unfractionated Colorectal Tumor Specimens

In light of the fact that ephrin-A ligand gene expression was found to be elevated in TPC populations from colorectal tumors when compared with TProg and NTG cells from the same tumors, experiments were conducted to determine whether elevated ephrin-A ligand (i.e., EFNA1 and EFNA3) expression was also detectable in unfractionated colorectal tumor samples versus normal adjacent tissue (NAT). To further assess EFNA1 and EFNA3 gene expression in additional colorectal cancer patient tumor samples and tumor specimens from patients diagnosed with 1 of 17 other different solid tumor types, Taqman qRT-PCR was performed using TissueScan™ qPCR (Origene Technologies) 384-well arrays, to determine how the expression of EFNA1 and EFNA3 in tumors compares with levels in normal tissue samples. More particularly, using the procedures detailed in Example 3 and the same EFNA1 and EFNA3 specific primer/probe sets, TaqMan real-time quantitative PCR was performed in the wells of the Origene plates.

FIGS. 5A and 5B show the relative gene expression levels, respectively, of human EFNA1 (FIG. 5A) and EFNA3 (FIG. 5B) in whole tumor specimens (grey dots) or matched normal adjacent tissue (NAT; white dots) from patients with one of eighteen different solid tumor types. Data is normalized against mean gene expression in NAT for each tumor type analyzed. Specimens not amplified were assigned a Ct value of 45, which represents the last cycle of amplification in the experimental protocol. Each dot represents an individual tissue specimen, with the mean value represented as a black line.

Using the Origene Array, it was observed that the majority of patients diagnosed with prostate, ovarian, cervical, colon, endometrial and bladder cancer, EFNA1 is overexpressed. EFNA3 is overexpressed in endometrial, uterine, prostate, lung, bladder, colon, breast, cervical, kidney and stomach cancer. This data suggests that EFNA1 and EFNA3 might play a role in tumorigenesis and/or progression in these tumors.

Example 6

Generation of anti-EFNA Antibodies using EFNA Immunogens

EFNA modulators in the form of murine antibodies were produced in accordance with the teachings herein through inoculation with soluble immunogens hEFNA1-ECD-His (NP_004419.2 – EFNA3 precursor), and hEFNA3-ECD-Fc (NP_004943.1). Immunogens were all prepared using commercially available starting materials (e.g., recombinant human Ephrin-A1/ EFNA1 Sino Biological Inc # 10882-H08H) and/or techniques well known to those skilled in the art.

More particularly murine antibodies were generated by immunizing 9 female mice (3 each: Balb/c, CD-1, FVB) with various preparations of EFNA1 or EFNA3 antigen. Immunogens included the aforementioned Fc or His constructs comprising at least part of the extracellular domain of human EFNA1 and human EFNA3. Mice were immunized via footpad route for all injections. 10 μg of EFNA1 or EFNA3 immunogen emulsified with an equal volume of TITERMAX™ or alum adjuvant were used for immunization. After immunization mice were euthanized, and draining lymph nodes (popliteal and inguinal, if enlarged) were dissected out and used as a source for antibody producing cells. Lymphocytes were released by mechanical disruption of the lymph nodes using a tissue grinder.

Electrofusion was then performed followed by growth of the hybridoma library in bulk and single cell deposition of the hybridomas with a subsequent screen of the clones. To that end a single cell suspension of harvested B cells were fused with non-secreting P3x63Ag8.653 myeloma cells at a ratio of 1:1. Electrofusion was performed using the Hybrimune System, model 47-0300, (BTX® Harvard Apparatus). Fused cells were resuspended in hybridoma selection medium supplemented with Azaserine (Sigma #A9666) (DMEM (Cellgro cat#15-017-CM) medium containing, 15% Fetal Clone I serum (Hyclone), 10% BM Condimed (Roche Applied Sciences), 1 mM sodium pyruvate, 4 mM L-glutamine, 100 IU Penicillin-Streptomycin, 50 μM 2-mercaptoethanol, and 100 μM hypoxanthine) and then plated in four T225 flasks at 90ml selection medium per flask. The flasks are then placed in a humidified 37°C incubator containing 5% CO_2 and 95% air for 6-7 days.

At 6-7 days of growth the library is plated at 1 cell per well in 48 Falcon 96 well U-bottom plates using the Aria I cell sorter. Briefly culture medium containing 15% Fetal

Clone I serum (Hyclone), 10% BM-Condimed (Roche Applied Sciences), 1 mM sodium pyruvate, 4 mM L-glutamine, 100 IU Penecillin-Streptamycin, 50 μM 2-mercaptoethanol, and 100 μM hypoxanthine is plated at 200ul per well in 48 Falcon 96 well U-bottom plates. Viable hybridomas are placed at 1 cell per well using the Aria I cell sorter and cultured for 10-11 days and the supernatants are assayed for antibodies reactive by FACS or ELISA for EFNA1 or EFNA3.

For the ELISA screening microtiter plates were coated with purified recombinant EFNA1 or EFNA3 His fusion proteins from transfected 293 cells at 100 ng/well in carbonate buffer. Plates incubated at 4°C overnight than blocked with 200μl/well of 3% BSA in PBS/Tween (0.05%). Supernatant from hybridoma plates were added to each well and incubated for 1-2 hours at ambient temperature. The plates were washed with PBS/Tween and than incubated with Goat anti mouse IgG, Fc Fragment Specific conjugated with horseradish peroxidase (HRP) Jackson ImmunoResearch) for one hour at room temperature. After washing, the plates were developed with TMB substrate (Thermo Scientific 34028) and analyzed by spectrophotometer at OD 450.

Selected EFNA1 and EFNA3 secreting hybridomas from positive wells were rescreened and subcloned by limited dilution or single cell FACS sorting.

Sub cloning was performed on selected antigen-positive wells using limited dilution plating. Plates were visually inspected for the presence of single colony growth and supernatants from single colony wells then screened by antigen-specific ELISAs described above and FACS confirmation as described below. The resulting clonal populations were expanded and cryopreserved in freezing medium (90% FBS, 10% DMSO) and stored in liquid nitrogen. Both the fusions from mice immunized with EFNA1 and mice immunized with EFNA3 yielded numerous murine monoclonal antibodies reactive for the respective antigen as determined using the ELISA protocol described above.

As indicated selected growth positive hybridoma wells secreting mouse immunoglobulins were also screened for human EFNA1 or EFNA3 specificity using a FACS assay as follows. Briefly $1x10^5$ per well Jurkat cells expressing human EFNA1 or EFNA3 were incubated for 30 minutes with 25-100ul hybridoma supernatant. Cells were washed PBS/2%FCS twice and then incubated with 50ul per sample DyeLight 649 labeled goat-anti-mouse IgG, Fc fragment specific secondary diluted 1:200 in PBS/2%FCS. After a 15 minute incubation cells were washed 2 times with PBS/2%FCS and re-suspended in PBS/2%FCS with DAPI and analyzed by FACS Canto II (BD Biosciences) under standard conditions and using the HTS attachment. The resulting specific clonal hybridomas were

expanded and cryopreserved in CS-10 freezing medium (Biolife Solutions) and stored in liquid nitrogen. The FACS analysis confirmed that purified antibody from most or all of these hybridomas bind EFNA1 or EFNA3 in a concentration-dependent manner.

Example 7

Sequencing of Ephrin-A Ligand Modulators

Based on the foregoing, a number of exemplary distinct monoclonal antibodies that bind immobilized human EFNA1 or EFNA3 with apparently high affinity were selected. As shown in FIGS. 6 and 7 sequence analysis of the DNA encoding mAbs from Example 6 confirmed that many had unique VDJ rearrangements and displayed novel complementarity determining regions.

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

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

The variable DNA sequences of the hybridoma amplified with consensus primer sets specific for murine immunoglobulin heavy chains and kappa light chains were obtained using a mix of variable domain primers. One step RT-PCR kit was used to amplify the V_H and V_K gene segments from each RNA sample. The Qiagen One-Step RT-PCR Kit provides a blend of Sensiscript and Omniscript Reverse Transcriptases, HotStarTaq DNA Polymerase, Qiagen OneStep RT-PCR Buffer, a dNTP mix, and Q-Solution, a novel additive that enables efficient amplification of "difficult" (e.g., GC-rich) templates.

Reaction mixtures were prepared that included 3 μL of RNA, 0.5 of 100 μM of

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

To prepare the PCR products for direct DNA sequencing, they were purified using the QIAquick™ PCR Purification Kit according to the manufacturer's protocol. The DNA was eluted from the spin column using 50 μL of sterile water and then sequenced directly from both strands. PCR fragments were sequenced directly and DNA sequences were analyzed using VBASE2 (Retter et al., Nucleic Acid Res. 33; 671-674, 2005 - data not shown).

As briefly alluded to above, the nucleic acid and corresponding amino acid sequences of murine heavy and light chain variable regions comprising exemplary modulators of the instant invention are set forth in FIGS. 6 and 7. More specifically FIGS. 6A – 6J provide the variable region sequences (SEQ ID NOS: 6 – 45) of exemplary antibodies that react with EFNA1 while FIGS. 7A – 7N provide the variable region sequences (SEQ ID NOS: 46 – 101) of exemplary antibodies that react with EFNA3. Note that for the purposes of the instant disclosure antibody modulators that primarily react with EFNA1 are designated SC9.xx while antibody modulators that primarily react with EFNA3 are designated SC11.xx where xx refers to the particular clone number.

Example 8

Characteristics of EFNA Modulators

Various methods were used to analyze the immunochemical characteristics of selected EFNA1 and EFNA3 modulators generated as set forth above. Specifically, a number of these antibodies were characterized as to affinity, kinetics, binning, and cross reactivity with regard to cynomolgus and mouse homologs (e.g., by ForteBio). The reactivity of the modulators was also measured by Western blot using reduced and non-reduced samples to provide some indication as to whether the epitopes were linear or not. In addition, the antibodies were tested for their ability to neutralize (e.g., block receptor ligand interaction as per Example 11), and were benchmarked for their relative EC_{50} of

killing by *in vitro* cytotoxicity assay (e.g., as per Examples 12 - 14). The results of this characterization are set forth in tabular form in FIG. 8 for modulators that primarily react with EFNA1 and in FIG. 9 for modulators that primarily react with EFNA3. Affinities and kinetic constants k_{on} and k_{off} of the selected modulators were measured using bio-layer interferometry analysis on a ForteBio RED (ForteBio, Inc.) with a standard antigen concentration series. In general, the selected modulators exhibited relatively high affinities in the nanomolar range.

As to antibody binning, ForteBio was used per manufacturer's instructions to identify antibodies, which bound to the same or different bins. Briefly, an antibody (Ab1) was captured onto an anti-mouse capture chip, a high concentration of nonbinding antibody was then used to block the chip and a baseline was collected. Monomeric, recombinant ephrin-A1-His or ephrinA3-His was then captured by the specific antibody (Ab1) and the tip was dipped into a well with either the same antibody (Ab1) as a control or into a well with a different antibody (Ab2). If additional binding was observed with a new antibody, then Ab1 and Ab2 were determined to be in a different bin. If no further binding occurred, similar to the control Ab1, then Ab2 was determined to be in the same bin. This process can be expanded to screen large libraries of unique antibodies using a full row of antibodies representing unique bins in a 96-well plate. This experiment showed the screened antibodies bound to at least three different bins or epitopes on the ephrin-A1 and ephrin-A3 proteins, respectively. This number of bins is consistent with an antigen less than 30kDa.

In order to determine whether the epitope recognized by the ephrin-A1 and ephrin-A3 modulator comprises contiguous amino acids or is formed by noncontiguous amino acids juxtaposed by secondary structure of the antigen, reduced and alkylated ELISA were run. More particularly, using 0.5M DTT to reduce recombinantly expressed protein and 0.25M iodoacetamide techniques well known in the art, ephrin-A1 and ephrin-A3 antigen was then used to coat an ELISA plate under alkali conditions. The respective modulators were then exposed to the plate, washed and developed with an anti-mouse IgG antibody conjugated to a developing agent. As detailed in FIG. 8 and FIG. 9, many ephrin-A1 and ephrin-A3 modulators substantially reacted with both denatured and reduced protein. The antibodies that were identified by binding to reduced and alkylated antigen on ELISA were used in western blot to test for target expression in cancer and normal tissues following verification on naïve and overexpressing cell lines.

Finally, cross-reactivity with regard to cynomolgus ephrin-A1 homologs were

evaluated in ForteBio using a concentration series with recombinantly expressed, monomeric ephrin-A1 antigens. This analysis was not completed for ephrin-A3 because the mature protein found in cynomolgus monkey is identical in sequence and so reactivity between human and cynomolgus protein would be identical. As listed in FIG. 8 the selected modulators were reactive with any number of the homologs. In particular, SC9.105 was cross-reactive with mouse EFNA1, while all antibodies cross-reacted with the highly similar cynomolgus EFNA1. ND in the tables indicates that the data was not determined.

Example 9

Ephrin-A Ligand Modulators Demonstrate Cell Surface Binding

Supernatants from hybridomas producing antibodies raised against EFNA1 or EFNA3 as set forth above were screened for cell surface binding as measured in a flow cytometric assay. To demonstrate the binding properties of selected modulators three tumor cell lines known to express moderate levels of EFNA1 (HEK293Td, Z138, PC3) and a control (G401) were analyzed by FACS using the EFNA1 antibody SC9.121. More specifically, fifty thousand cells of each type were incubated with 10 μg/ml purified modulator for 60 minutes at 4°C. The cells were washed once with PBS containing 2% FBS, 2mM EDTA and 0.05% sodium azide (wash buffer) and then stained for 60 minutes at 4°C in the dark with a Fc-region specific F(ab)2 fragment of Goat-anti-mouse IgG polyclonal antibody conjugated to DyLight649 (Jackson Immuno Research). Cells were washed twice with wash buffer, and counterstained with 2 μg/ml DAPI. Samples were collected on a FACS Canto II (BD Biosciences) under standard conditions and using the HTS attachment. FIG. 10A shows histograms of single live cells stained with modulator SC9.121 (open histograms) or isotype control antibody (shaded histograms) demonstrating moderate EFNA1 expression by HEK293Td, Z138 and PC3 cell lines.

Using a similar protocol and the same apparatus assays were run to demonstrate that exemplary modulators which associate with EFNA1 or EFNA3 bind to cells expressing the respective ligand. In this case HEK293T cells were engineered by means of retroviral transduction to express markedly higher levels of EFNA1 or EFNA3 than is expressed endogenously by the wild type parent. Binding of the selected EFNA1 and EFNA3 modulators is shown, respectively, in FIG. 10B and FIG. 10C wherein IgG2a is used as a negative control and the measurements are depicted as mean fluorescence intensity (MFI)

of the chosen fluorescent channel. The graphs illustrate that the modulators of the invention readily associate with ligands expressed on the surface of cells.

Example 10

Ephrin-A Ligands Interact Selectively with Multiple EphA Receptors

As discussed herein ephrin-A ligands are characterized as promiscuous as they are known to bind to various EphA receptors. To explore which EphA receptors have the potential to interact with EFNA1 and EFNA3, a flow cytometric binding assay similar to the one described in Example 9 was developed. More particularly soluble EphA receptors expressed as human IgG1 Fc fusion constructs (10 μg/ml; obtained commercially or generated in-house) were added to fifty thousand HEK293T cells per well (FIG. 11A) or HEK293T cells overexpressing EFNA1 (FIG. 11B) or EFNA3 (FIG. 11C) by means of retroviral transduction for 1 hour in staining buffer at 4°C. After washing, a secondary anti-human IgG polyclonal antibody conjugated to DyeLight 649 (Jackson Immuno Research) was added for one hour. After washing twice, samples were resuspended in staining buffer containing 2 μg/ml DAPI and analyzed on a FACS Canto II (BD Biosciences) under standard conditions using the HTS attachment. FIG. 11A demonstrates that EphA1, EphA3, EphA4, EphA6, EphA7and EphA10, but not EphA2, apparently bind to the low levels of endogenously expressed ephrin-A ligands on parental HEK293T cells. Engineering the cells to express elevated levels of either EFNA1 (FIG. 11B) or EFNA3 (FIG. 11C) resulted in the substantial binding of all tested EphA receptors in a dose dependent manner albeit to varying degrees. These multiple interactions again point to the advantages and potential multifaceted points of action inherent in modulators of the instant invention.

Example 11

EFNA Modulators Block Binding of EFNA to EphA Receptors

As seen in Example 10 most EphA receptors associate to some degree with EFNA1 and EFNA3 ligands expressed on cell surfaces. This binding can be inhibited using the ephrin-A modulators of the instant invention and, in particular, through the use of monoclonal antibodies that associate with EFNA1 or EFNA3. To illustrate this aspect of the invention fifty thousand HEK293T cells overexpressing EFNA1 or EFNA3 were

deposited per well and incubated with 20 μg/ml of the exemplary modulator in wash buffer for 1 hr at 4°C. Mouse IgG isotypes and no antibody (data not shown) serve as negative controls. Various EphA-Fc constructs (EphA1, EphA2, EphA4 and EphA7 in the case of EFNA1 and EphA2, EphA4, EphA7 and EphA10 in the case of EFNA3) were then added to the cells at 10 μg/ml in wash buffer for an additional 1 hr at 4°C. The cells were then washed twice with wash buffer, counterstained with 2 μg/ml DAPI, and analyzed on a FACS Canto II (BD Biosciences) under standard conditions using the HTS attachment. Results are presented as mean fluorescence intensity (MFI) of the chosen fluorescent channel.

The results graphically represented in FIG. 12A depict blocking of EphA receptor binding to EFNA1 ligand by EFNA1 modulators, and those in FIG. 12B depict blocking of EphA receptor binding to EFNA3 ligand. A review of FIG. 12A shows that modulators SC9.20, SC9.92, SC9.98, SC9.120, SC9.140 and SC9.141 substantially inhibit the binding of all or some of the tested EphA1, EphA2, EphA4 and EphA7 receptors to EFNA1 whereas modulators SC9.52, SC9.66, SC9.96, SC9.116, SC9.121 and SC9.122 exhibit relatively less inhibition. Some of these modulators selectively enhance binding of EphA4 and EphA7-Fc. A review of FIG. 12B shows that modulators SC11.18, SC11.27, SC11.30, SC11.32 and SC11.34 substantially inhibit the binding of all or some of the tested EphA2, EphA4, EphA7 and EphA10 receptors to EFNA3 whereas modulators SC11.9, SC11.19, SC11.37, SC11.47, SC11.51, SC11.53, SC11.54, SC11.55, SC11.57, SC11.67 and SC11.112 exhibit relatively less inhibition. Again some of these modulators selectively enhance binding of EphA7 and EphA10-Fc. These data, when combined with the results of the other Examples herein, suggest that these modulators' ability to agonize or antagonize the binding of various receptors may be significant in providing the observed therapeutic effects of the instant invention.

Example 12

EFNA1 Modulators as Targeting Moieties

Targeting of a cytotoxic drug stably linked to an antibody represents an approach that might have great therapeutic benefit for patients with solid tumors. To determine whether the EFNA1-specific antibodies described above were able to mediate the delivery of a cytotoxic agent to live cells, an *in vitro* cell killing assay was performed wherein an Anti-Mouse IgG Fab fragment conjugated to the ribosome-inactivating protein Saporin

(referred to as FAB-ZAP™) was added together with purified EFNA1 antibodies to target cells, and the ability of these Saporin complexes to internalize and kill cells was measured 72 hours later by measuring cell viability.

Specifically, 500 cells per well of the following cell types were plated into 96 well tissue culture plates in their respective culture media one day before the addition of antibodies and toxin conjugate: parental HEK293T cells (FIG. 13A), and HEK293T overexpressing EFNA1 cells (FIG. 13B). Purified mouse monoclonal antibodies at various concentrations and a fixed concentration of 10 nM Anti-Mouse IgG Fab fragment covalently linked to Saporin (Advanced Targeting Systems, #IT-48) were added to the cultures and allowed to incubate for 72 hours. Viable cell numbers were enumerated using CellTiter-Glo® (Promega Corp.) per the manufacturer's protocol. Raw luminescence counts using cultures containing cells with the Saporin Fab fragment (but no modulator) were set as 100% reference values and all other counts calculated accordingly (referred to as "Normalized RLU").

Using this assay it was demonstrated that, except for SC9.105, all tested EFNA1 antibodies (but not isotype control antibodies) are able to effectively mediate the killing of the engineered target cells (FIG. 13B). This assay demonstrates that internalization occurs upon binding of the EFNA1 antibody to the cell surface without the need for additional cross-linking and that cells expressing certain levels of EFNA are killed by EFNA modulator mediated cytotoxicity. In this case parental HEK293T cells expressing a low number of EFNA1 on their cell surface were not killed while engineered HEK293T cells expressing the ligand strongly (e.g., as per Example 9) were terminated in a modulator dose dependent manner. These data clearly demonstrate the effectiveness of the disclosed modulators when acting as vectors for the selective internalization of cytotoxic payloads in cells expressing EFNA1.

Example 13

EFNA3 Modulators as Targeting Moieties

Using the protocol essentially as set forth in Example 12 the ability of EFNA3 modulators to mediate the killing of cells in accordance with the present invention was demonstrated. In this regard parental HEK293T cells (FIG. 14A) and engineered HEK293T cells expressing EFNA3 (FIG. 14B) were deposited at approximately 500 cells per well in appropriate culture media. Again, purified modulators comprising murine

monoclonal antibodies at various concentrations and a fixed concentration of 10 nM Anti-Mouse IgG Fab fragment covalently linked to Saporin were added to the wells and incubated in culture for 72 hours. Again viable cell numbers were enumerated using CellTiter-Glo per the manufacturer's protocol and reference RLU was established using wells comprising a non-reactive control antibody.

This assay again demonstrated that the disclosed modulators, in this case EFNA3 modulators, could effectively be used to mediate cytotoxic induced killing of cells expressing certain levels of ephrin-A ligand (FIG. 14B). Such findings support the observation that, in accordance with the instant invention, EFNA modulators may be used to effectively deliver toxic payloads into the cell through association with cell surface ephrin-A ligand.

Example 14

EFNA1 Modulators as Targeting Moieties for Cancer Stem Cells

Based on the unexpected results detailed above an assay was devised to confirm the findings obtained with engineered cells and demonstrate that the disclosed modulators can effectively mediate delivery of cytotoxic agents to tumor initiating cells expressing ephrin-A ligand. More particularly the instant Example demonstrates that the disclosed modulators may be used to promote toxin internalization and cell killing of murine lineage-depleted NTX cells (i.e. human tumor initiating cells propagated as low-passage xenografts in immunocompromised mice).

In this respect NTX tumors, representing lung and ovarian tumor specimens, were dissociated into a single cell suspension and plated, at 2,500 cells per well, on BD Primaria™ plates (BD Biosciences) in growth factor supplemented serum free media. LU50 (FIG. 15A) was initially isolated from a 76 year old male non-small cell lung cancer patient and was passaged in mice three times prior to *in vitro* culture as discussed in Example 1 above. OV26 (FIG. 15B) was derived from a 69 year old female ovarian cancer patient and was similarly passaged twice in mice prior to *in vitro* culture. After 3-5 days of culture at 37°C/5%CO_2/5%O_2, cells were contacted with a control (a non-reactive IgG1a or a murine EFNA1 modulator (SC9.7, SC9.66, or SC9.96 at 100 pM or 10 pM) and Fab-ZAP (at 4 nM) as per the previous Examples. Modulator-mediated saporin cytotoxicity was then assessed by quantifying the remaining number of cells using CellTiter-Glo 5-7 days later.

As seen in FIGS. 15A and 15B exposure to EFNA1 modulators, particularly at the 100 pM concentration, resulted in reduced LU50 and OV26 cell numbers indicating that the cytotoxic payload had been internalized via EFNA1 binding and eliminated cancer stem cells. In contrast, the IgG2a isotype control antibody did not substantially impact the number of live cells after treatment. These data clearly demonstrate that the disclosed modulators effectively bind to ephrin-A ligands expressed on the surface of cancer stem cell populations and can facilitate the delivery of a cytotoxic payload (e.g., via internalization) resulting in tumorigenic cell death (i.e., a reduction in tumor cell frequency).

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

CLAIMS

1. An isolated EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.
2. The isolated EFNA modulator of claim 1, wherein the EFNA modulator comprises an EFNA antagonist.
3. The isolated EFNA modulator of claim 1, wherein the EFNA modulator comprises an antibody or immunoreactive fragment thereof.
4. The isolated EFNA modulator of claim 3 wherein the antibody or immunoreactive fragment thereof comprises a monoclonal antibody.
5. The isolated EFNA modulator of claim 4 wherein the monoclonal antibody is selected from the group consisting of chimeric antibodies, CDR-grafted antibodies, humanized antibodies and human antibodies.
6. The isolated EFNA modulator of claim 4 wherein said monoclonal antibody comprises a neutralizing antibody.
7. The isolated EFNA modulator of claim 4 wherein said monoclonal antibody comprises an internalizing antibody.
8. The isolated EFNA modulator of claim 4 wherein said monoclonal antibody comprises a depleting antibody.
9. The isolated EFNA modulator of claim 4 wherein said monoclonal antibody comprises an antibody that associates with EFNA1.
10. The isolated EFNA modulator of claim 9 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 9, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 25, SEQ ID NO: 29, SEQ ID NO: 33, SEQ ID NO: 37, SEQ ID NO: 41 and SEQ ID NO: 45 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 19, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 31, SEQ ID NO: 35, SEQ ID NO: 39 and SEQ ID NO: 43.
11. The isolated EFNA modulator of claim 4 wherein said monoclonal antibody comprises an antibody that associates with EFNA3.

12. The isolated EFNA modulator of claim 11 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 49, SEQ ID NO: 53, SEQ ID NO: 57, SEQ ID NO: 61, SEQ ID NO: 65, SEQ ID NO: 69, SEQ ID NO: 73, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 97 and SEQ ID NO: 101 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 47, SEQ ID NO: 51, SEQ ID NO: 55, SEQ ID NO: 59, SEQ ID NO: 63, SEQ ID NO: 67, SEQ ID NO: 71, SEQ ID NO: 75, SEQ ID NO: 79, SEQ ID NO: 83, SEQ ID NO: 87, SEQ ID NO: 91, SEQ ID NO: 95 and SEQ ID NO: 99.
13. The isolated EFNA modulator of claim 9, 10, 11 or 12 further comprising a cytotoxic agent.
14. A nucleic acid encoding an amino acid heavy chain variable region or an amino acid light chain variable region having at least 60% identity to an amino acid sequence of claim 10 or 12.
15. A vector comprising the nucleic acid of claim 14.
16. A host cell comprising the vector of claim 15.
17. The isolated EFNA modulator of claim 1 wherein said modulator reduces the frequency of tumor initiating cells upon administration to a subject in need thereof.
18. The isolated EFNA modulator of claim 17 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells.
19. The isolated EFNA modulator of claim 17 wherein the reduction in frequency is determined using immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.
20. The isolated EFNA modulator of claim 17 wherein said tumor initiating cells comprise tumor perpetuating cells.
21. The isolated EFNA modulator of claim 1 further comprising a cytotoxic agent.
22. A pharmaceutical composition comprising the isolated EFNA modulator of claim 1.

23. An isolated EFNA modulator comprising an amino acid sequence as set forth in SEQ ID NO: 2 or SEQ ID NO: 4 or fragments thereof.
24. The isolated EFNA modulator of claim 23 wherein the EFNA modulator further comprises at least a portion of an immunoglobulin constant region.
25. An isolated EFNA1 modulator.
26. The isolated EFNA1 modulator of claim 25 further comprising a cytotoxic agent.
27. A pharmaceutical composition comprising the isolated EFNA1 modulator of claim 25.
28. An isolated EFNA3 modulator.
29. The isolated EFNA3 modulator of claim 28 further comprising a cytotoxic agent.
30. A pharmaceutical composition comprising the isolated EFNA3 modulator of claim 28.
31. A method of treating an EFNA associated disorder comprising administering a therapeutically effective amount of an EFNA modulator to a subject in need thereof wherein said EFNA modulator is selected from the group consisting of EFNA1 modulators and EFNA3 modulators.
32. The method of claim 31 wherein said EFNA modulator comprises an EFNA antagonist.
33. The method of claim 31 wherein said EFNA modulator comprises an antibody or immunoreactive fragment thereof.
34. The method of claim 33 wherein the antibody or immunoreactive fragment thereof comprises a monoclonal antibody.
35. The method of claim 34 wherein the monoclonal antibody is selected from the group consisting of chimeric antibodies, CDR-grafted antibodies, humanized antibodies and human antibodies.
36. The method of claim 34 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 9, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 21, SEQ ID NO: 25, SEQ ID NO: 29, SEQ ID NO: 33, SEQ ID NO: 37, SEQ ID NO: 41 and SEQ ID NO: 45 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID

NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 19, SEQ ID NO: 13, SEQ ID NO: 17, SEQ ID NO: 31, SEQ ID NO: 35, SEQ ID NO: 39 and SEQ ID NO: 43.

37. The method of claim 34 wherein said monoclonal antibody comprises a light chain variable region and a heavy chain variable region wherein said light chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 49, SEQ ID NO: 53, SEQ ID NO: 57, SEQ ID NO: 61, SEQ ID NO: 65, SEQ ID NO: 69, SEQ ID NO: 73, SEQ ID NO: 77, SEQ ID NO: 81, SEQ ID NO: 85, SEQ ID NO: 89, SEQ ID NO: 93, SEQ ID NO: 97 and SEQ ID NO: 101 and wherein said heavy chain variable region comprises an amino acid sequence having at least 60% identity to an amino acid sequence selected from the group consisting of amino acid sequences as set forth in SEQ ID NO: 47, SEQ ID NO: 51, SEQ ID NO: 55, SEQ ID NO: 59, SEQ ID NO: 63, SEQ ID NO: 67, SEQ ID NO: 71, SEQ ID NO: 75, SEQ ID NO: 79, SEQ ID NO: 83, SEQ ID NO: 87, SEQ ID NO: 91, SEQ ID NO: 95 and SEQ ID NO: 99.

38. The method of claim 34 wherein said monoclonal antibody associates with EFNA1.

39. The method of claim 34 wherein said monoclonal antibody associates with EFNA3.

40. The method of claim 34 wherein said monoclonal antibody comprises a neutralizing antibody.

41. The method of claim 34 wherein said monoclonal antibody comprises an internalizing antibody.

42. The method of claim 41 wherein said internalizing antibody comprises a cytotoxic agent.

43. The method of claim 34 wherein said monoclonal antibody comprises a depleting antibody.

44. The method of claim 31 wherein said EFNA associated disorder comprises a hyperproliferative disorder.

45. The method of claim 44 wherein said hyperproliferative disorder comprises a neoplastic disorder.

46. The method of claim 45 wherein said neoplastic disorder comprises a solid tumor.

47. The method of claim 46 wherein neoplastic disorder comprises adrenal cancer,

bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer or breast cancer.

48. The method of claim 45 wherein said neoplastic disorder comprises a hematologic malignancy.

49. The method of claim 48 wherein said hematologic malignancy comprises leukemia or lymphoma.

50. The method of claim 45 wherein the subject suffering said neoplastic disorder exhibits tumors comprising tumor initiating cells.

51. The method of claim 50 further comprising the step of reducing the frequency of tumor initiating cells in said subject.

52. The method of claim 51 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells or immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.

53. The method of claim 51 wherein the reduction in frequency is determined using *in vitro* or *in vivo* limiting dilution analysis.

54. The method of claim 53 wherein the reduction in frequency is determined using *in vivo* limiting dilution analysis comprising transplant of live human tumor cells into immunocompromised mice.

55. The method of claim 54 wherein the reduction of frequency determined using *in vivo* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.

56. The method of claim 53 wherein the reduction of frequency is determined using *in vitro* limiting dilution analysis comprising limiting dilution deposition of live human tumor cells into *in vitro* colony supporting conditions.

57. The method of claim 56 wherein the reduction of frequency determined using *in vitro* limiting dilution analysis comprises quantification of tumor initiating cell frequency using Poisson distribution statistics.

58. The method of claim 45 further comprising the step of administering an anti-cancer agent.

59. The method of claim 45 wherein said EFNA modulator further comprises a cytotoxic agent.

60. A method of reducing the frequency of tumor initiating cells in a subject in need

thereof comprising the step of administering an EFNA modulator to said subject wherein said EFNA modulator is selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

61. The method of claim 60 wherein the tumor initiating cells comprise tumor perpetuating cells.

62. The method of claim 61 wherein said tumor perpetuating cells are $CD44^+$ or $CD133^+$ cells.

63. The method of claim 60 wherein said EFNA modulator comprises an antibody.

64. The method of claim 63 wherein said antibody comprises a monoclonal antibody.

65. The method of claim 64 wherein said EFNA modulator comprises an anti-EFNA1 antibody.

66. The method of claim 64 wherein said EFNA modulator comprises an anti-EFNA3 antibody.

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

68. The method of claim 60 wherein the subject is suffering from a hematologic malignancy.

69. The method of claim 60 wherein the frequency of tumor initiating cells is reduced by at least 10%.

70. The method of claim 60 wherein the reduction in frequency is determined using flow cytometric analysis of tumor cell surface markers known to enrich for tumor initiating cells or immunohistochemical detection of tumor cell surface markers known to enrich for tumor initiating cells.

71. The method of claim 60 wherein the reduction in frequency is determined using *in vitro* or *in vivo* limiting dilution analysis.

72. A method of treating a subject suffering from a hematologic malignancy comprising the step of administering an EFNA modulator to said subject wherein said EFNA modulator is selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

73. The method of claim 72 wherein said EFNA modulator is an EFNA1 modulator.

74. The method of claim 72 wherein said EFNA modulator is an EFNA3 modulator.

75. A method of sensitizing a tumor in a subject for treatment with an anti-cancer

agent comprising the step of administering an EFNA modulator to said subject wherein said EFNA modulator is selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

76. The method of claim 75 wherein said EFNA modulator comprises an antibody.

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

78. The method of claim 75 wherein said anti-cancer agent comprises a chemotherapeutic agent.

79. The method of claim 75 wherein said anti-cancer agent comprises an immunotherapeutic agent.

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

    a. obtaining a tissue sample from said subject;
    b. contacting the tissue sample with at least one EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators; and
    c. detecting or quantifying the EFNA modulator associated with the sample.

81. The method of claim 80 wherein the EFNA modulator comprises a monoclonal antibody.

82. The method of claim 81 wherein the antibody is operably associated with a reporter.

83. An article of manufacture useful for diagnosing or treating an EFNA associated disorder comprising a receptacle comprising an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators and instructional materials for using said EFNA modulator to treat or diagnose the EFNA associated disorder.

84. The article of manufacture of claim 83 wherein said EFNA modulator is a monoclonal antibody.

85. The article of manufacture of claim 83 wherein the receptacle comprises a readable plate.

86. A method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one internalizing EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

87. The method of claim 86 wherein said EFNA modulator comprises an antibody.

88. The method of claim 87 wherein said antibody comprises a monoclonal antibody.
89. The method of claim 88 wherein the monoclonal antibody further comprises a cytotoxic agent.
90. The method of claim 88 wherein the monoclonal antibody associates with EFNA1.
91. The method of claim 88 wherein the monoclonal antibody associates with EFNA3.
92. A method of treating a subject suffering from neoplastic disorder comprising the step of administering a therapeutically effective amount of at least one neutralizing EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.
93. The method of claim 92 wherein said EFNA modulator comprises an antibody.
94. The method of claim 93 wherein said antibody comprises a monoclonal antibody.
95. The method of claim 94 wherein said monoclonal antibody comprises an anti-EFNA1 antibody or an anti-EFNA3 antibody.
96. The method of claim 92 wherein the neoplastic disorder is selected from the group consisting of adrenal cancer, bladder cancer, cervical cancer, endometrial cancer, kidney cancer, liver cancer, lung cancer, ovarian cancer, colorectal cancer, pancreatic cancer, prostate cancer and breast cancer.
97. A method of identifying, isolating, sectioning or enriching a population of tumor initiating cells comprising the step of contacting said tumor initiating cells with an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators..
98. The method of claim 97 wherein said EFNA modulator comprises an antibody.
99. A method inhibiting or preventing metastasis in a subject in need thereof comprising the step of administering a pharmaceutically effective amount of an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.
100. The method of claim 99 wherein the subject undergoes a debulking procedure before or after administration of the EFNA modulator.
101. The method of claim 99 wherein said debulking procedure comprises the administration of at least one anti-cancer agent.
102. A method of performing maintenance therapy on a subject in need thereof comprising the step of administering a pharmaceutically effective amount of an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

103. The method of claim 102 wherein said subject was treated for a neoplastic disorder prior to the administration of the EFNA modulator.

104. A method of depleting tumor cells in a subject suffering from a hyperproliferative disorder comprising the step of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

105. The method of claim 104 wherein said tumor cells comprise tumor initiating cells.

106. A method of diagnosing, detecting or monitoring an EFNA associated disorder *in vivo* in a subject in need thereof comprising the step of administering an EFNA modulator selected from the group consisting of EFNA1 modulators and EFNA3 modulators.

Homo sapiens ephrin-A1 (EFNA1), transcript variant 1, mRNA

(SEQ ID NO: 1)

```
   1 gccagatctg tgagcccagc gctgactgcg ccgcggagaa agccagtggg aacccagacc
  61 cataggagac ccgcgtcccc gctcggcctg gccaggcccc gcgctatgga gttcctctgg
 121 gcccctctct tgggtctgtg ctgcagtctg gccgctgctg atcgccacac cgtcttctgg
 181 aacagttcaa atcccaagtt ccggaatgag gactacacca tacatgtgca gctgaatgac
 241 tacgtggaca tcatctgtcc gcactatgaa gatcactctg tggcagacgc tgccatggag
 301 cagtacatac tgtacctggt ggagcatgag gagtaccagc tgtgccagcc ccagtccaag
 361 gaccaagtcc gctggcagtg caaccggccc agtgccaagc atggcccgga gaagctgtct
 421 gagaagttcc agcgcttcac acctttcacc ctgggcaagg agttcaaaga aggacacagc
 481 tactactaca tctccaaacc catccaccag catgaagacc gctgcttgag gttgaaggtg
 541 actgtcagtg gcaaaatcac tcacagtcct caggcccatg acaatccaca ggagaagaga
 601 cttgcagcag atgacccaga ggtgcgggtt ctacatagca tcggtcacag tgctgcccca
 661 cgcctcttcc cacttgcctg gactgtgctg ctccttccac ttctgctgct gcaaaccccg
 721 tgaaggtgta tgccacacct ggccttaaag agggacaggc tgaagagagg gacaggcact
 781 ccaaacctgt cttggggcca ctttcagagc ccccagccct gggaaccact cccaccacag
 841 gcataagcta tcacctagca gcctcaaaac gggtcagtat taaggttttc aaccggaagg
 901 aggccaacca gcccgacagt gccatcccca ccttcacctc ggagggatgg agaaagaagt
 961 ggagacagtc ctttcccacc attcctgcct ttaagccaaa gaaacaagct gtgcaggcat
1021 ggtcccttaa ggcacagtgg gagctgagct ggaaggggcc acgtggatgg gcaaagcttg
1081 tcaaagatgc cccctccagg agagagccag gatgcccaga tgaactgact gaaggaaaag
1141 caagaaacag tttcttgctt ggaagccagg tacaggagag gcagcatgct tgggctgacc
1201 cagcatctcc cagcaagacc tcatctgtgg agctgccaca gagaagtttg tagccaggta
1261 ctgcattctc tcccatcctg gggcagcact ccccagagct gtgccagcag gggggctgtg
1321 ccaacctgtt cttagagtgt agctgtaagg gcagtgccca tgtgtacatt ctgcctagag
1381 tgtagcctaa agggcagggc ccacgtgtat agtatctgta tataagttgc tgtgtgtctg
1441 tcctgatttc tacaactgga gtttttttat acaatgttct ttgtctcaaa ataaagcaat
1501 gtgttttttc ggacatgctt ttctgccact ccatattaaa acatatgacc attgagtccc
1561 tgctaaaaaa aaaaaaaaaa aaaaaaaaaa
```

FIG. 1A

Homo sapiens ephrin-A1 (EFNA1), isoform a

(SEQ ID NO: 2)

MEFLWAPLLGLCCSLAAADRHTVFWNSSNPKFRNEDYTIHVQLNDYVDIICPHYEDHSVAVQLNDYVDIICPHYE
DHSVAQPQSKDQVRWQCNRPSAKHGPEKLSEKFQRFTPFTLGKEFKEGHSYYYISKPIHQHEDRCLRLKVTVSGK
ITHPQAHDNPQEKRLAADDPEVRVLHSIGHSAAPRLFPLAWTVLLLPLLHSAAPRLFPLAWTVLLLPLL

FIG. 1B

Homo sapiens ephrin-A1 (EFNA1), aligned isoform a and isoform b

```
hEFNA1 iso a NP_004419 (1): meflwapllg lccslaaadr htvfwnssnp kfrnedytih
hEFNA1 iso b NP_872626 (2): meflwapllg lccslaaadr htvfwnssnp kfrnedytih

hEFNA1 iso a NP_004419 (1): vqlndyvdii cphyedhsva vqlndyvdii cphyedhsva
hEFNA1 iso b NP_872626 (2): vqlndyvdii cphyedhsva daameqyily lveheeyqlc

hEFNA1 iso a NP_004419 (1): qpqskdqvrw qcnrpsakhg peklsekfqr ftpftlgkef
hEFNA1 iso b NP_872626 (2): qpqskdqvrw qcnrpsakhg peklsekfqr ftpftlgkef

hEFNA1 iso a NP_004419 (1): keghsyyyis kpihqhedrc lrlkvtvsgk ithspqahdn
hEFNA1 iso b NP_872626 (2): keghsyyyis                       hspqahdn

hEFNA1 iso a NP_004419 (1): pqekrlaadd pevrvlhsig hsaaprlfpl awtvlllpll
hEFNA1 iso b NP_872626 (2): pqekrlaadd pevrvlhsig hsaaprlfpl awtvlllpll

hEFNA1 iso a NP_004419 (1): hsaaprlfpl awtvlllpll     (SEQ ID NO: 2)
hEFNA1 iso b NP_872626 (2): llqtp                     (SEQ ID NO: 3)
```

FIG. 1C

gi: 156071497 / NM_004952.4

Homo sapiens ephrin-A3 (EFNA3)

(SEQ ID NO: 4)

MAAAPLLLLLLLVPVPLLPLLAQGPGGALGNRHAVYWNSSNQHLRREGYTVQVNVNDYLDIYCPHYNSSGVGPGA
GPGPGGGAEQYVLYMVSRNGYRTCNASQGFKRWECNRPHAPHSPIKFSEKFQRYSAFSLGYEFHAGHEYYYISTP
THNLHWKCLRMKVFVCCASTSHSGEKPVPTLPQFTMGPNVKINVLEDFEGENPQVPKLEKSISGTSPKREHLPLA
VGIAFFLMTFLAS

FIG. 1D

Homo sapiens ephrin-A3 (EFNA3), transcript variant 1, mRNA

(SEQ ID NO: 5)

```
...1 ggagctggga agcggagaag ccgggagcgc ggggctcagt cgggggggcgg cggcggcggc
..61 ggctccgggg atggcggcgg ctccgctgct gctgctgctg ctgctcgtgc ccgtgccgct
 121 gctgccgctg ctggcccaag ggcccggagg ggcgctggga aaccggcatg cggtgtactg
 181 gaacagctcc aaccagcacc tgcggcgaga gggctacacc gtgcaggtga acgtgaacga
 241 ctatctggat atttactgcc cgcactacaa cagctcgggg gtgggccccg gggcgggacc
 301 ggggcccgga ggcggggcag agcagtacgt gctgtacatg gtgagccgca acggctaccg
 361 cacctgcaac gccagccagg gcttcaagcg ctgggagtgc aaccggccgc acgccccgca
 421 cagccccatc aagttctcgg agaagttcca gcgctacagc gccttctctc tgggctacga
 481 gttccacgcc ggccacgagt actactacat ctccacgccc actcacaacc tgcactggaa
 541 gtgtctgagg atgaaggtgt tcgtctgctg cgcctccaca tcgcactccg gggagaagcc
 601 ggtccccact ctcccccagt tcaccatggg ccccaatgtg aagatcaacg tgctggaaga
 661 ctttgaggga gagaaccctc aggtgcccaa gcttgagaag agcatcagcg ggaccagccc
 721 caaacgggaa cacctgcccc tggccgtggg catcgccttc ttcctcatga cgttcttggc
 781 ctcctagctc tgccccctcc cctgggggggg gagagatggg gcggggcttg gaaggagcag
 841 ggagcctttg gcctctccaa gggaagccta gtgggcctag acccctcctc ccatggctag
 901 aagtggggcc tgcaccatac atctgtgtcc gccccctcta ccccttcccc ccacgtaggg
 961 cactgtagtg gaccaagcac ggggacagcc atgggtcccg ggcggccttg tggctctggt
1021 aatgtttggt accaaacttg ggggccaaaa agggcagtgc tcaggactcc ctggcccctg
1081 gtacctttcc ctgactcctg gtgccctctc cctttgtccc cccagagaga catatgcccc
1141 cagagagagc aaatcgaagc gtgggaggca cccccattgc tctcctccag gggcagaaca
1201 tggggagggg actagatggg caaggggcag cactgcctgc tgcttccttc ccctgtttac
1261 agcaataagc acgtcctcct cccccactcc cacttccagg attgtggttt ggattgaaac
1321 caagtttaca agtagacacc cctgggggggg cgggcagtgg acaaggatgg caaggggtgg
1381 gcattggggt gccaggcagg catgtacaga ctctatatct ctatatataa tgtacagaca
1441 gacagagtcc cttccctctt taaccccctg acctttcttg acttcccctt cagcttcaga
1501 ccccttcccc accaggctag gccccccaca cctgggggac cccctggccc ctcttttgtc
1561 ttctgtgaag acaggaccta tgcaacgcac agacactttt ggagaccgta aaacaacaac
1621 gccccctccc ttccagccct gagccgggaa ccatctccca ggaccttgcc ctgctcaccc
1681 tatgtggtcc cacctatcct cctgggcctt tttcaagtgc tttggctgtg actttcatac
1741 tctgctctta gtctaaaaaa aataaactgg agataaaaat aa
```

FIG. 1E

EFNA1 Gene Expression is Elevated in Colorectal Tumor Initiating Cells

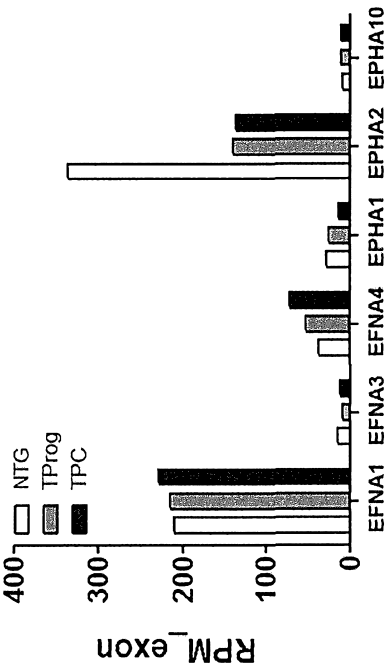

FIG. 2A

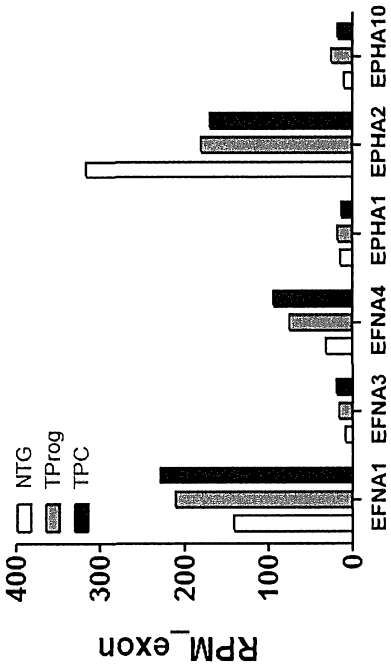

FIG. 2B

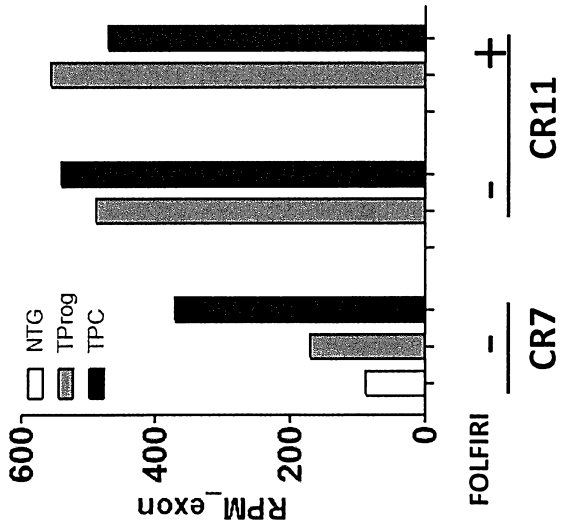

FIG. 2C

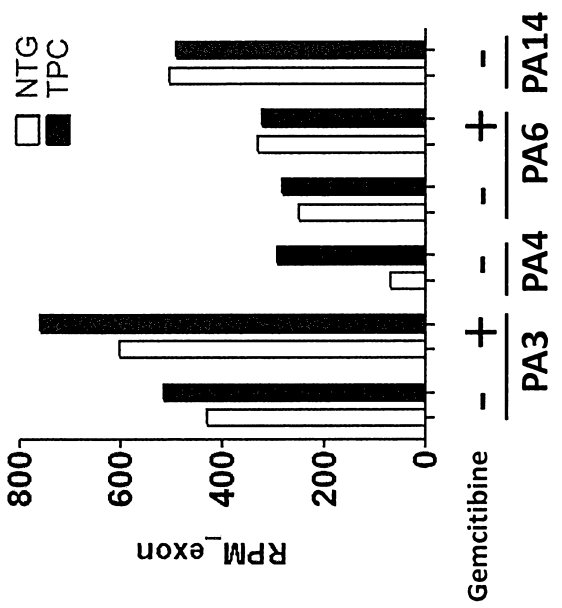

FIG. 2D

EFNA1 Gene Expression is Elevated in Colorectal, Pancreatic and NSCLC Tumor Initiating Cells

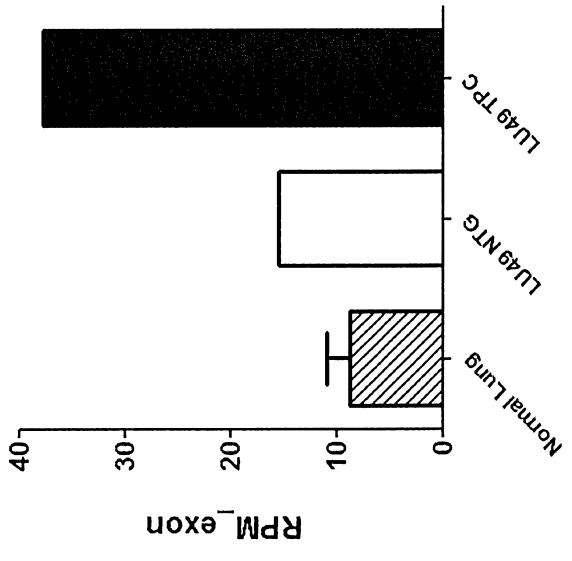

FIG. 2E

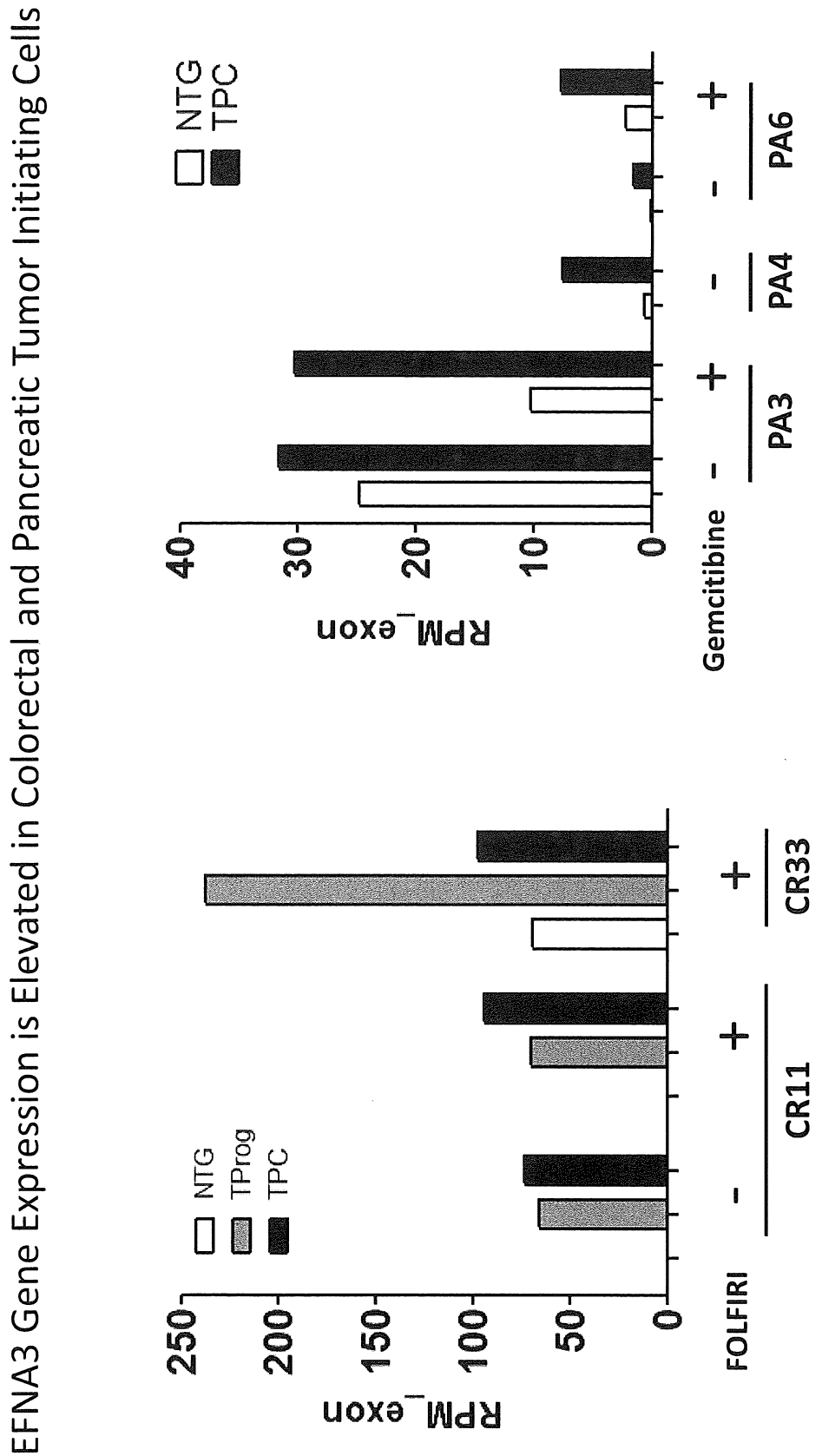
EFNA3 Gene Expression is Elevated in Colorectal and Pancreatic Tumor Initiating Cells
NTG
TProg
TPC
RPM_exon
250
200
150
100
50
0
FOLFIRI
-
+
+
CR11
CR33
NTG
TPC
RPM_exon
40
30
20
10
0
Gemcitibine
-
+
-
-
+
PA3
PA4
PA6

FIG. 3A

FIG. 3B

EFNA3 Gene Expression is Elevated in NSCLC Tumor Initiating Cells

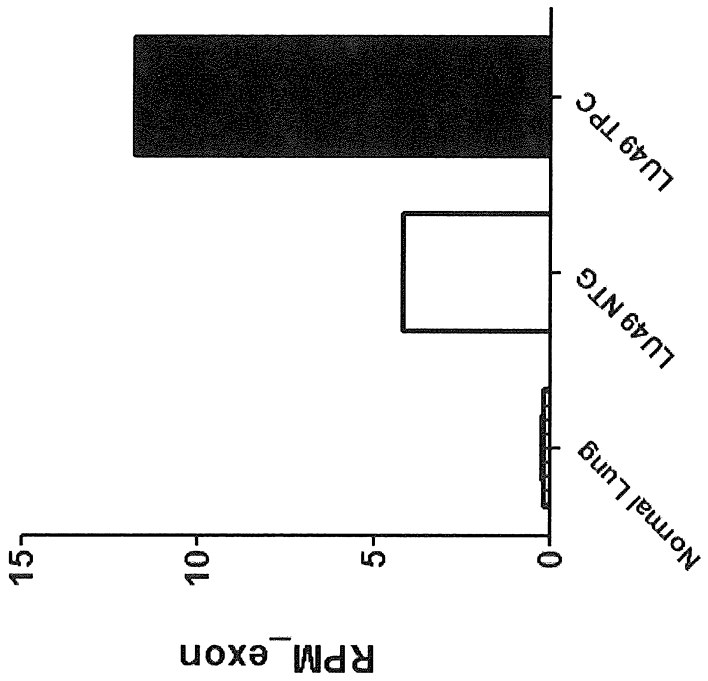

FIG. 3C

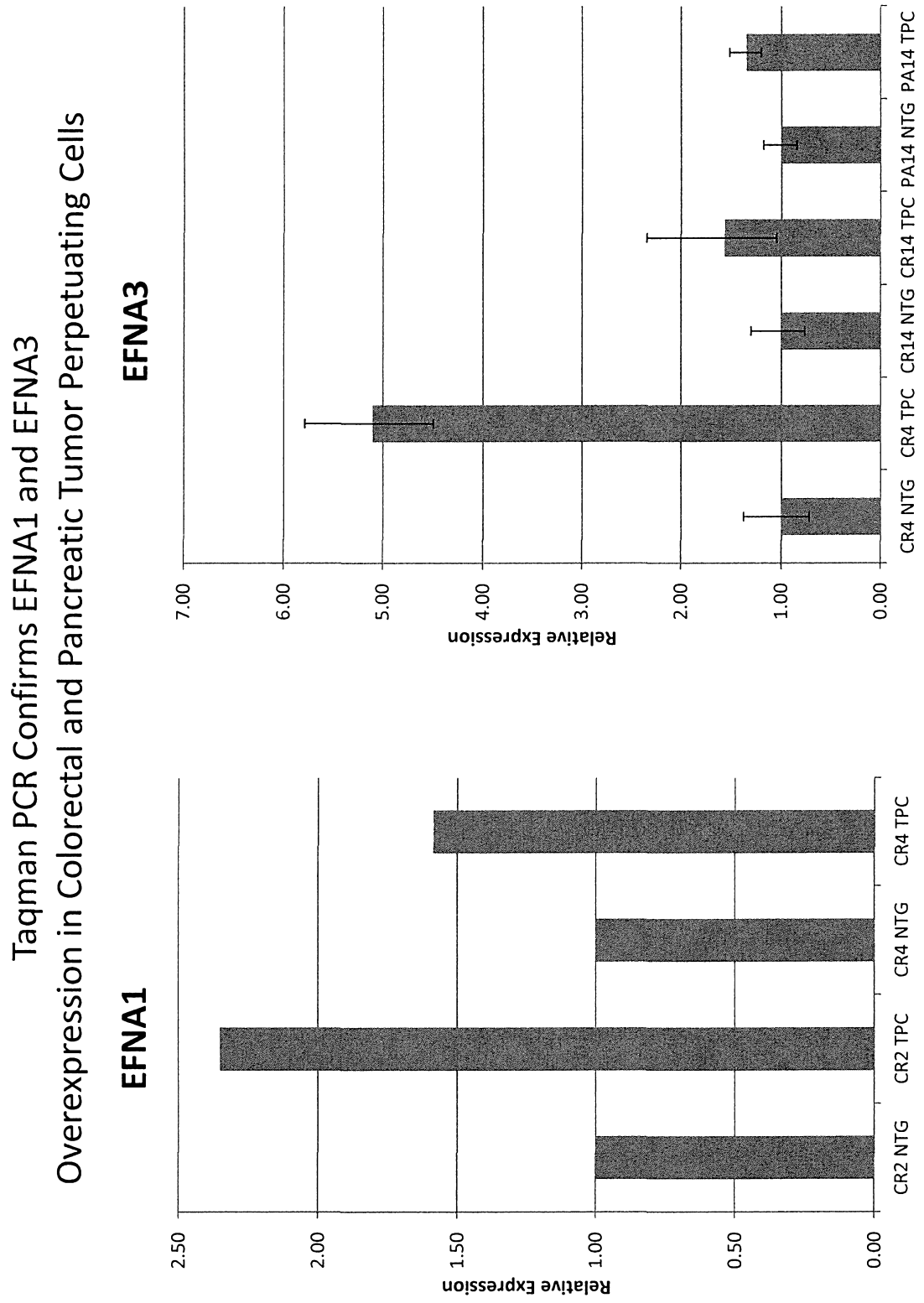
Taqman PCR Confirms EFNA1 and EFNA3
Overexpression in Colorectal and Pancreatic Tumor Perpetuating Cells
EFNA1
Relative Expression
2.50
2.00
1.50
1.00
0.50
0.00
CR2 NTG
CR2 TPC
CR4 NTG
CR4 TPC
EFNA3
Relative Expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
CR4 NTG
CR4 TPC
CR14 NTG
CR14 TPC
PA14 NTG
PA14 TPC

FIG. 4A

FIG. 4B

Taqman PCR Demonstrates EFNA1 and EFNA3 Overexpression in Various Cancers

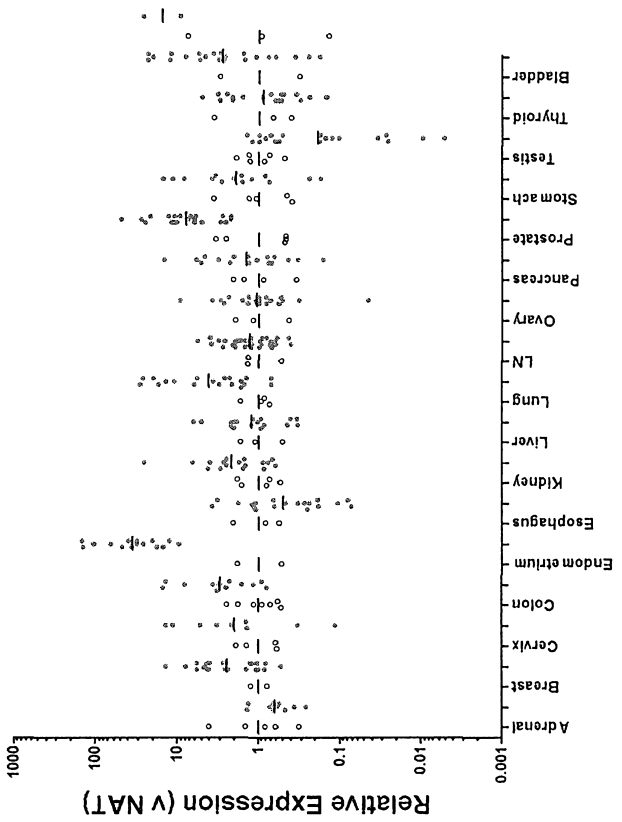

FIG. 5B

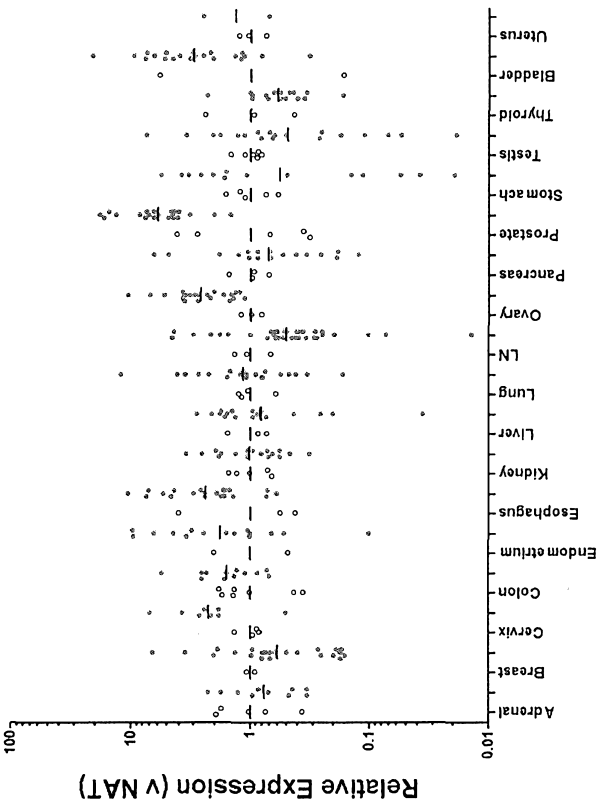

FIG. 5A

SC9.16 Heavy chain - nucleotide sequence (SEQ ID NO: 6)

GAGGTCCAGCTGCAACAGTTTGGAGCTGAGCTGGTGAAGCCTGGGGCTTCAGTAAAGATATCCTGCAAGGCTTCT
GGCTACGCATTCACTGACTACAACATAGACTGGGTGAAACAGAGCCATGGAAGGAGCCTTGAGTGGATTGGAGA
TATTAATCCTAATTATGAAAGTACTCGCTACAACCGGAAGTTCATGGGAAAGGCCACATTGACTGTAGACAAGTCC
TCCAACACAGCCTACATGGATCTCCGCAGCCTGACATCTGAGGACACTGCAGTCTATTACTGTACAAGAGATGGTT
CCTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC9.16 Heavy chain - protein sequence (SEQ ID NO: 7)

EVQLQQFGAELVKPGASVKISCKASGYAFTDYNIDWVKQSHGRSLEWIGDINPNYESTRYNR
KFMGKATLTVDKSSNTAYMDLRSLTSEDTAVYYCTRDGSYAMDYWGQGTSVTVSS

SC9.16 Light chain - nucleotide sequence (SEQ ID NO: 8)

GACATTGTGATGACACAGTCTCCATCCTCCCTGAGTGTGTCAGCAGGAGAGAAGGTCACTCTGAGCTGCAAGTCC
AGTCAGAGTCTGTTAAACAGTGGACATCAAAAGAACTACTTGGCCTGGTACCAGCAGAAACCAGGGCAGCCTCCT
AAACTGTTGATCTACGGGGCATCCACTAGGGAATCTGGGGTCCCTGATCGCTTCACAGGCAGTGGATCTGGAACC
GATTTCACTCTTACCATCAGCAGTGTGCAGGCTGAAGACCTGGCAGTTTATTACTGTCAGAATGATCATAGGTATC
CTCTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAAC

SC9.16 Light chain - protein sequence (SEQ ID NO: 9)

DIVMTQSPSSLSVSAGEKVTLSCKSSQSLLNSGHQKNYLAWYQQKPGQPPKLLIYGAST
RESGVPDRFTGSGSGTDFTLTISSVQAEDLAVYYCQNDHRYPLTFGAGTKLELK

FIG. 6A

SC9.18 Heavy chain - nucleotide sequence (SEQ ID NO: 10)

GAGGTCCAGCTGCAGCAGTCTGGACCTGAACTAGTGAAGACTGGGGCTTCAGTGAAGATATCCTGCAAGGCTTCT
GGTTACTCATTCAGTGGTTACTACATGCACTGGGTCAAGCAGAGCCGAGGAAAGAGCCTTGAGTGGATTGGATAT
ATTCGTTCTTACAATGGTGCTACTAGCTACAACCAGAAGTTCAAGGGCAAGGCCACCTTTACTGTAGACACATCCT
CCAGCACAGCCTACATGCAGTTCAACAGCCTGACATCTGAAGACTCTGCGGTCTATTTCTGTGCAAGAGAGGGGA
ATTACTACGGTAGTAGCCTTGACTTCTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC9.18 Heavy chain - protein sequence (SEQ ID NO: 11)

EVQLQQSGPELVKTGASVKISCKASGYSFSGYYMHWVKQSRGKSLEWIGYIRSYNGATSY
NQKFKGKATFTVDTSSSTAYMQFNSLTSEDSAVYFCAREGNYYGSSLDFWGQGTTLTVSS

FIG. 6B

SC9.18 Light chain - nucleotide sequence (SEQ ID NO: 12)

GATGTCCAGATAACCCAGTCTCCATCTTATCTTGCTGCATCTCCTGGAGAAATCATTACTATTAATTGCAGGGCAAG
TAAGAGCATTAGCAAATTTTTAGCCTGGTATCAAGCGAAACCTGGGAAAACTAATAAGCTTCTTATCCACTCTGGA
TCCACTTTGCAATCTGGAATTCCATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACTCTCACCATCAGTAG
CCTGGAGCCTGAAGATTTTGCAATGTATTACTGTCAACAGCATAATGAATACCCGTGGACGTTCGGTGGAGGCAC
CAAGTTGGAGATCAAAC

SC9.18 Light chain - protein sequence (SEQ ID NO: 13)

DVQITQSPSYLAASPGEIITINCRASKSISKFLAWYQAKPGKTNKLLIHSGSTLQS
GIPSRFSGSGSGTDFTLTISSLEPEDFAMYYCQQHNEYPWTFGGGTKLEIK

SC9.20 Heavy chain - nucleotide sequence (SEQ ID NO: 14)

TCTGATGTACAGCTTCAGGAGTCAGGACCTGGCCTCGTGAAACCTTCTCAGTCTCTGTCTCTCACCTGCTCTGTCACT
GGCTACTCCATCACCAGTGGTTATTACTGGAACTGGATCCGGCAGTTTCCAGGAAACAGACTGGAATGGATGGCC
TACATAAGTTACGACGGTAGCAATGACTACAACCCATCTCTCAAAAATCGTATCTCCATCACTCGTGACACCTCTAA
GAATCAGTTTTTCCTGAAGTTGAATTCTGTGACTACTGAGGACACAGCTACATATTACTGTGCAAGAGGTTACCCG
ATCCTCTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

SC9.20 Heavy chain - protein sequence (SEQ ID NO: 15)

SDVQLQESGPGLVKPSQSLSLTCSVTGYSITSGYYWNWIRQFPGNRLEWMAYISYDG
SNDYNPSLKNRISITRDTSKNQFFLKLNSVTTEDTATYYCARGYPILFAYWGQGTLVTVSA

FIG. 6C

SC9.20 Light chain - nucleotide sequence (SEQ ID NO: 16)

GACATCAAGATGACCCAGTCTCCATCTTCCATGAATGCATCTCTAGGAGAGAGAGTCTCTATCACTTGCAAGACGA
GTCAGGACATTAATAGCTATTTAAGCTGGCTCCAGCAGAAACCAGGGAAATCTCCAAAGACCCTGATCTATCGTG
CAAACAGATTGGTAGATGGGGTCCCTTCAAGGTTCAGTGGCAGTGGATCTGGGCAAGATTATTCTCTCACCATCA
GCAGCCTGGAGTATGAAGATATGGGAATTTATTATTGTCTACAGTATGATGAGTTTCCGCTCACGTTCGGGATTGG
GACCAAGCTGGAGCTGAAAC

SC9.20 Light chain - protein sequence (SEQ ID NO: 17)

DIKMTQSPSSMNASLGERVSITCKTSQDINSYLSWLQQKPGKSPKTLIYRANR
LVDGVPSRFSGSGSGQDYSLTISSLEYEDMGIYYCLQYDEFPLTFGIGTKLELK

SC9.52 Heavy chain - nucleotide sequence (SEQ ID NO: 18)

GAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCT
GGATTCACTTTCAGTAGCTATGGCATGTCTTGGGTTCGCCAGACTCCGGAGAAGAGGCTGGAGTGGGTCGCAGCC
ATTAATATTAATGGTGGTATCACCTACTATCCAGACACTGTGAAGGGCCGATTCACCATCTCCAGAGACAATGCCA
AGAACACCCTGTCCCTGCAAATGAGCAGTCTGAGGTCTGAGGACACAGCCTTCTATTACTGTGCAAGAGACATCTC
GGGCTATGCTATGGACTACTGGGGTCAAGGAACCTCGGTCACCGTCTCCTCA

SC9.52 Heavy chain - protein sequence (SEQ ID NO: 19)

EVQLVESGGGLVKPGGSLKLSCAASGFTFSSYGMSWVRQTPEKRLEWVAAININGGITYY
PDTVKGRFTISRDNAKNTLSLQMSSLRSEDTAFYYCARDISGYAMDYWGQGTSVTVSS

FIG. 6D

SC9.52 Light chain - nucleotide sequence (SEQ ID NO: 20)

GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAA
GTCAGGACATTAGCAATTATTTAAACTGGTATCAGCAGAAACCAGATGGAACTGTTAAACTCCTGATCTCCTACAG
ATCAAGATTACCCTCAGGAGTCCCATCAAGGTTCAGTGGCAGTGGGTCTGGAACATATTATTCTCTCACCATTAGC
AACCTGGCGCAAGAAGATTTTGCCACTTACTTTTGCCAACAGGGTCATACGCTTCCGTGGACGTTCGGTGGAGGCA
CCAAGCTGAAAATCAAAC

SC9.52 Light chain - protein sequence (SEQ ID NO: 21)

DIQMTQTTSSLSASLGDRVTISCRASQDISNYLNWYQQKPDGTVKLLISYRSR
LPSGVPSRFSGSGSGTYYSLTISNLAQEDFATYFCQQGHTLPWTFGGGTKLKIK

SC9.59 Heavy chain - nucleotide sequence (SEQ ID NO: 22)

GAGGTCCAGCTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCT
GGATACACATTCACTAGTTATGTTATGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGATTT
ATTAATCCTCACAATGAGGGTACTAAGTACAATGAGAAGTTCAAAGGCAAGGCCACACTGACTTCAGACAAATCC
TCCACCACAGCCTTCATGGAGCTCAGCAGCCTGACCTCTGAGGACTCTGCGGTCTATTACTGTGCAAGAACGTGGG
TCCCTTACGACGGCCTTGCTGACTGGGGCCAAGGGACTCTGATCACTGTCTCTGAA

SC9.59 Heavy chain - protein sequence (SEQ ID NO: 23)

FIG. 6E

EVQLQQSGPELVKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGFINPHNEG
TKYNEKFKGKATLTSDKSSTTAFMELSSLTSEDSAVYYCARTWVPYDGLADWGQGTLITVSE

SC9.59 Light chain - nucleotide sequence (SEQ ID NO: 24)

GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTTAGTCCAGGGGAAAGAGTCAGTTTCTCCTGCAGGGCCA
GTCAGAGCATTGGCACAAGCATACACTGGTATCAGCAAAGAACAACTGGTTCTCCAAGGCTTCTCATAAAGGATG
CTTCTGAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGAACAGGGACAGATTTTACTCTTACTATCAAC
AGTGTGGAGTCTGAAGATATTGCAGATTATTATTGTCAACAAAGTAATAGCTGGCCATACACGTTCGGCGGGGGG
ACCAAGCTGGAAATAAAACG

SC9.59 Light chain - protein sequence (SEQ ID NO: 25)

DILLTQSPAILSVSPGERVSFSCRASQSIGTSIHWYQQRTTGSPRLLIKDASESI
SGIPSRFSGSGTGTDFTLTINSVESEDIADYYCQQSNSWPYTFGGGTKLEIKR

SC9.64 Heavy chain - nucleotide sequence (SEQ ID NO: 26)

GAGGTCCAGCTGCAGCAGTCTGGACCTGAGCTGGTAAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCT
GGATACACGTTCACTAGTTATGTTATGCACTGGGTGAAGCAGAAGCCTGGGCAGGGCCTTGAGTGGATTGGATTT
ATTAATCCTCACAATGAGGGTACTAAGTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACTTCAGACAAATCC
TCCACCACAGCCTACATGGAGCTCAGCAGCCTGACCTCTGAGGACTCTGCGGTCTATTACTGTGCAAGAACGAGG
GTCCCTTACGACGGCCTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

SC9.64 Heavy chain - protein sequence (SEQ ID NO: 27)

FIG. 6F

EVQLQQSGPELVKPGASVKMSCKASGYTFTSYVMHWVKQKPGQGLEWIGFINPHNEGTK
YNEKFKGKATLTSDKSSTTAYMELSSLTSEDSAVYYCARTRVPYDGLAYWGQGTLVTVSA

SC9.64 Light chain - nucleotide sequence (SEQ ID NO: 28)

GACATCTTCCTGACTCAGTCTCCAGCCATCCTGTCTGTTAGTCCAGGAGAAAGAGTCAGTTTCTCCTGCAGGGCCA
GTCAGAGCATTGGCACAAGCTTACACTGGTATCAGCAAAGAACAAATGGTTCTCCAAGACTTCTCATAAAGGATG
CTTCTGAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTCACCATCAAC
AGTGTGGAGTCTGAAGATATTGCAGATTATTACTGTCAACAAAGTAATAGGTGGCCATACACATTCGGAGGGGG
GACCAAGCTGGAAATAAAACG

SC9.64 Light chain - protein sequence (SEQ ID NO: 29)

DIFLTQSPAILSVSPGERVSFSCRASQSIGTSLHWYQQRTNGSPRLLIKDASESIS
GIPSRFSGSGSGTDFTLTINSVESEDIADYYCQQSNRWPYTFGGGTKLEIKR

SC9.66 Heavy chain - nucleotide sequence (SEQ ID NO: 30)

CAGGTGCAGCTGGAGCAGTCAGGACCTGGCCTAGTGCAGCCCTCACAGAGCCTGTCCATAACCTGCACAGTCTCT
GGTTTCTCATTAATTAGCGATGGTGTACACTGGGTTCGCCAGTCTCCAGGAAAGGGTCTGGAGTGGCTGGGCGTG
ATATGGAAAAGTGGAAGCACAGACTACAATGGAGCTTTCATGTCCAGACTGAGCATCACCAAGGACAACTCCAA
GAGCCAAGTTTTCTTTGAAATGAACAGTCTGCAATCTGATGACACTGCCATGTACTACTGTGCCATTCATTCCTACG
GCTACGTGATTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

SC9.66 Heavy chain - protein sequence (SEQ ID NO: 31)

QVQLEQSGPGLVQPSQSLSITCTVSGFSLISDGVHWVRQSPGKGLEWLGVIWKSGSTDY
NGAFMSRLSITKDNSKSQVFFEMNSLQSDDTAMYYCAIHSYGYVIAYWGQGTLVTVSA

FIG. 6G

SC9.66 Light chain - nucleotide sequence (SEQ ID NO: 32)

GATATCCAGATGACACAGACTACATCCTCCCTGTCTGCCTCTCTGGGAGACAGAGTCACCATCAGTTGCAGGGCAA
GTCAAGACATTTACAAATATTTAAACTGGTATCAGCAGAAACCAGATGGAACTGTTAAACTCCTGATCTACTACAC
ATCAAGATTACACTCAGGAGTCCCATCAAGGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTACC
AACCTGGAGCAAGAAGACATTGCCACTTACTTTTGCCAACAGGGTGATACGCTTCCGTGGACGTTCGGTGGCGGC
ACCAAGC

SC9.66 Light chain - protein sequence (SEQ ID NO: 33)

DIQMTQTTSSLSASLGDRVTISCRASQDIYKYLNWYQQKPDGTVKLLIYYTSRLH
SGVPSRFSGSGSGTDYSLTITNLEQEDIATYFCQQGDTLPWTFGGGTK

SC9.76 Heavy chain - nucleotide sequence (SEQ ID NO: 34)

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCTGTGCAGCCTCT
GGATTCACTTTCAGTAACTTTGCCATGTCTTGGGTTCGCCAGACTCCGGAGAAGAGGCTGGAGTGGGTCGCAACC
ATTACTAGTGGTGGTACTTACACATACAATCCAGACAGTGTGAAGGGTCGATTCACCATCTCCAGAGACAATGCCA
AGAACATTTTGTACCTGCAAATGAGCAGTCTGAGGTCTGAGGACACGGCCATGTATTACTGTGCACGACAAAACT
ACTTTGACTACTGGGGCCAAGGCACCATTCTCACAGTCTCCTCA

SC9.76 Heavy chain - protein sequence (SEQ ID NO: 35)

EVQLVESGGGLVKPGGSLKLSCAASGFTFSNFAMSWVRQTPEKRLEWVATITSGGTYTY
NPDSVKGRFTISRDNAKNILYLQMSSLRSEDTAMYYCARQNYFDYWGQGTILTVSS

FIG. 6H

SC9.76 Light chain - nucleotide sequence (SEQ ID NO: 36)

GAAAATGTGCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAGAAGGTCACCATGAGCTGCAGGGCC
AGCTCAAGTGTAAATTACATGTACTGGTACCAGCAGAAGTCAGATGCCTCCCCCAAACTATGGATTTATTTCACAT
CCAACCTGGCTCCTGGAGTCCCAGGTCGCTTCAGTGGCAGTGGGTCTGGGAACTCTTATTCTCTCACAATCAGCAG
CATGGAGGGTGAAGATGCTGCCACTTATTTCTGCCAGCAGTTTACTAGTCCCCCATCCATCACGTTCGGTGCTGGG
ACCAAGCTGGAGCAGAAAC

SC9.76 Light chain - protein sequence (SEQ ID NO: 37)

ENVLTQSPAIMSASLGEKVTMSCRASSSVNYMYWYQQKSDASPKLWIYFTSN
LAPGVPGRFSGSGSGNSYSLTISSMEGEDAATYFCQQFTSPPSITFGAGTKLELK

SC9.79 Heavy chain - nucleotide sequence (SEQ ID NO: 38)

CAGGTTCAGCTGCAGCAGTCTGGAGCTGAGCTGATGAAGCCTGGGGCCTCAGTGAAGATATCCTGCAAGGCTACT
GGCTACACATTCAGTACCTACTGGATAGAGTGGGTAAAACAGAGGCCTGGACATGGCCTTGAGTGGATTGGAGA
GATTTTACCTGGAAGTGGTAATATTAAGTACAATGAGAGATTCAAGGGCAAGGCCACATTCACTGCAGATACATC
CTCCAACACAGCCTACATGCAACTCAGCAGCCTGACATCTGAAGACTCTGCCGTCTATTACTGTGCAACGACTACG
GTAGTATCTACGAACTTTGACTACTGGGGCCAAGGCACCACTCTCACTGTCTCCTCA

SC9.79 Heavy chain - protein sequence (SEQ ID NO:39)

QVQLQQSGAELMKPGASVKISCKATGYTFSTYWIEWVKQRPGHGLEWIGEILPGSGNIK
YNERFKGKATFTADTSSNTAYMQLSSLTSEDSAVYYCATTTVVSTNFDYWGQGTTLTVSS

FIG. 6I

SC9.79 Light chain - nucleotide sequence (SEQ ID NO: 40)

GACATTGTGATGACTCAGTCTCCAGCCACCCTGTCTGTGACTCCAGGAGATAGAGTCTCTCTTTCCTGCAGGGCCA
GCCAGAGTATTAGCGACTACTTACACTGGTATCAACAAAAATCACATGAGTCTCCAAGGCTTCTCATCAAATATGC
TTCCCAATCCATCTCTGGGATCCCCTCCAGGTTCAGTGGCAGTGGATCAGGGTCAGATTTCACTCTCAGTATCAACA
GTATGGAACCTGAAGATGTTGGAGTGTATTACTGTCAAAATGGTCACAGCTTTCCTCGGACGTTCGGTGGAGGCA
CCAAGCTGGAAATCAAAC

SC9.79 Light chain - protein sequence (SEQ ID NO: 41)

DIVMTQSPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRLLIKYASQSIS
GIPSRFSGSGSGSDFTLSINSMEPEDVGVYYCQNGHSFPRTFGGGTKLEIK

SC9.93 Heavy chain - nucleotide sequence (SEQ ID NO: 42)

GAGGTGAAGCTTTTCGAGTCTGGAGGTGGCCTGGTGCAGCCTGGAGGATCCCTGAAACTCTCCTGTGCAGCCTCA
GGATTCGATTTTAGTAGATACTGGATGACTTGGGTCCGGCAGGCTCCAGGGACAGGGCTAGAATGGATTGGAGA
AATTAATCCAGATAGCAGTACGATAAACTATACGCCATCTCTGAGGGATAAATTCATCATCTCCAGAGACAACGCC
AAAAATGCGCTGAACCTGCAAATGAGCAAAGTGAGATCTGAGGACACAGCCCTTTATTACTGTCACTCCTATGCTA
TGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC9.93 Heavy chain - protein sequence (SEQ ID NO: 43)

EVKLFESGGGLVQPGGSLKLSCAASGFDFSRYWMTWVRQAPGTGLEWIGEINPDSS
TINYTPSLRDKFIISRDNAKNALNLQMSKVRSEDTALYYCHSYAMDYWGQGTSVTVSS

FIG. 6J

SC9.93 Light chain - nucleotide sequence (SEQ ID NO: 44)

GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCCATCTCTTGCAAGTCAAG
TCAGAGCCTCTTAGATAGTGATGGAAAGACATATTTGAATTGGTTGTTACAGAGGCCAGGCCAGTCTCCAAAGCG
CCTAATCTATCTGGTGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGGACAGATTTC
ACACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTATTGCTGGCAAGGTACACATTTTCCTCAG
ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC

SC9.93 Light chain - protein sequence (SEQ ID NO: 45)

DVVMTQTPLTLSVTIGQPASISCKSSQSLLDSDGKTYLNWLLQRPGQSPKRLIYLVSK
LDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCWQGTHFPQTFGGGTKLEIK

SC11.9 Heavy chain - nucleotide sequence (SEQ ID NO: 46)

GAGGTGCAGCTTGTTGAGTCTGGTGGAGGATTGGTGCAGCCTAAAGGGTCATTGAAACTCTCATGTGCAGCCTCT
GGATTCACCTTCGATACCTACGCCATGAACTGGGTCCGCCAGGCTCCAGGAAAGGGTTTGGAATGGGTTGCTCGC
ATAAGAAGTAAAAGTAATGATTATGCAACATATTATGTCGATTCAGTGAAAGACAGGTTCACCATTTCCAGAGAT
GATTCACAAAACATGCTCTATCTGCACATGAACAACTTGAAAACTGAGGACACAGCCATATATTACTGTATGATCT
CTTCGACTTTTTTTGACTGTTGGGGCCAAGGCACCTCTCTC

FIG. 7A

SC11.9 Heavy chain - protein sequence (SEQ ID NO: 47)

EVQLVESGGGLVQPKGSLKLSCAASGFTFDTYAMNWVRQAPGKGLEWVARIRSKSNDYATYY
VDSVKDRFTISRDDSQNMLYLHMNNLKTEDTAIYYCMISSTFFDCWGQGTSL

SC11.9 Light chain - nucleotide sequence (SEQ ID NO: 48)

GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCTATCTCTTGCAGGTCAAG
TCAGAGCCTCTTATATAGTAATGGAAATACCTATTTGAATTGGTTATTACAGAGGCCAGGCCAGTCTCCAAAGCGC
CTAGTCTATCTGGTGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCACTGGATCAGGAACAGATTTTA
CCCTGAAGATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTACTGCGTGCAAGGTACACATTTTCCATTCA
CGTTCGGCTCGGGGACAAAGTTGGAAATAAAAC

SC11.9 Light chain - protein sequence (SEQ ID NO: 49)

DVVMTQTPLTLSVTIGQPASISCRSSQSLLYSNGNTYLNWLLQRPGQSPKRLVY
LVSKLDSGVPDRFTGTGSGTDFTLKISRVEAEDLGVYYCVQGTHFPFTFGSGTKLEIK

SC11.16 Heavy chain - nucleotide sequence (SEQ ID NO: 50)

GAGGTCCAGTTGCAACAGTCTGGACCTGAGCTAATGAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGACTTCT
GGATACACATTCACTGATTACAACATACACTGGGTGAAGCAGAACCAAGGAAAGAGCCTAGAGTGGATCGGAGA
AATTAATCCTTACACTGGTGGTACTGGCTACAACCAGAAATTCACAGGCAAGGCCACATTGACTGTAGACAAGTCC
TCCAGCACAGCCTACATGGAGCTCCGCAGCCTAACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAGATGGTT
ACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

FIG. 7B

SC11.16 Heavy chain - protein sequence (SEQ ID NO: 51)

EVQLQQSGPELMKPGASVKMSCKTSGYTFTDYNIHWVKQNQGKSLEWIGEINPYTGGTG
YNQKFTGKATLTVDKSSSTAYMELRSLTSEDSAVYYCARDGYFDYWGQGTTLTVSS

SC11.16 Light chain - nucleotide sequence (SEQ ID NO: 52)

GACATCCAGATGACCCAGTCTCCATCCTCCTTATCTGCCTCTCTGGGAGAAAGAGTCAGTCTCACTTGTCGGGCAA
GTCAGGAAATTAGTGATTACTTAAGCTGGCTTCAGCAGAAACCAGATGGAACTATTAAACGCCTGATCTACGCCG
CATCCACTTTAGATTCTGGTGTCCCAAAAAGGTTCAGTGGCAGTAGGTCTGGGTCAGATTATTCTCTCACCATCAGC
AGCCTTGAGTCTGAAGATTTTGCAGACTATTACTGTCTACAATATGCTAGTTCTCCGCTCACGTTCGGTGCTGGGAC
CAAGCTG

SC11.16 Light chain - protein sequence (SEQ ID NO: 53)

DIQMTQSPSSLSASLGERVSLTCRASQEISDYLSWLQQKPDGTIKRLIYAAS
TLDSGVPKRFSGSRSGSDYSLTISSLESEDFADYYCLQYASSPLTFGAGTKL

SC11.25 Heavy chain - nucleotide sequence (SEQ ID NO: 54)

GAGGTCCAGCTGCAACAGTCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATGTCCTGCAAGGCTTCT
GGATACACATTCACTGACTACAGCATACACTGGGTGAAGCAGAGCCTTGGAAAGAGCCTTGAGTGGATTGGATAT
ATTACCCCTAACACTGGTGGCACTAACTACAACCAGAACTTCAAGGACAAGGCCATATTGACTGTAAACAAGTCCT
CCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCGGAGGATTCTGCAGTCTATTACTGTGCAAGAAACTGGG
GTTTTTTCCTCTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC11.25 Heavy chain - protein sequence (SEQ ID NO: 55)

EVQLQQSGPELVKPGASVKMSCKASGYTFTDYSIHWVKQSLGKSLEWIGYITPNTGG
TNYNQNFKDKAILTVNKSSSTAYMELRSLTSEDSAVYYCARNWGFFLFDYWGQGTTLTVSS

FIG. 7C

SC11.25 Light chain - nucleotide sequence (SEQ ID NO: 56)

AGTATTGTGATGACCCAGACTCCCAAATTCCTGCCTGTAACAGCAGAAGACAGGGTTACCATAACCTGCAAGGCC
ACTCAGAGTGTGAGTAATGAAGTAGCTTGGTACCAACAGAAGGCAGGGCAGTCTCCTAAACTGATGATATACTAT
GCATCCAATCGCTACACTGGAGTCCCTGATCGCTTCACTGGCAGTGGATCTGGCACGGATTTCACTTTCACCATCAG
CAGTGTGCAGGTTGAAGACCTGGCAGTTTATTTCTGTCAGCATCATTACAGTTCTCCCACGTTCGGTGCTGGGACC
AAGCTGGAGCTGAAAC

SC11.25 Light chain - protein sequence (SEQ ID NO: 57)

SIVMTQTPKFLPVTAEDRVTITCKATQSVSNEVAWYQQKAGQSPKLMIYYA
SNRYTGVPDRFTGSGSGTDFTFTISSVQVEDLAVYFCQHHYSSPTFGAGTKLELK

SC11.32 Heavy chain - nucleotide sequence (SEQ ID NO: 58)

CAGGTCCAACTGCAGCAGCCTGGGGCTGAAATTGTGAGGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCT
GGCTACACCTTTACCGCCTATTGGATGCACTGGGTGAAACAGAGGCCTGGACAAGGCCCTGAGTGGATCGGAGCA
ATTGATCCTTCTGATAGTTATACTTACTACAATCAAAAGTTCAAGGGCAAGGCCACATTGACTGTAGACACATCCT
CCAACTCAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTTCTGTGCAAGATGGGATTA
CTACGATGGTAACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCAAAACAACACCCCCATCCGTCTATCCC
NGGGCCCCTGA

SC11.32 Heavy chain - protein sequence (SEQ ID NO: 59)

QVQLQQPGAEIVRPGASVKLSCKASGYTFTAYWMHWVKQRPGQGPEWIGAIDPSDSY
TYYNQKFKGKATLTVDTSSNSAYMQLSSLTSEDSAVYFCARWDYYDGNWGQGTTLTVSS

FIG. 7D

SC11.32 Light chain - nucleotide sequence (SEQ ID NO: 60)

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATCTCTTGCAGATCTAG
TCAGAGCCTTGTACACAGTGATGGAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTC
CTGATCTACAGAGTTTCCAACCGCTTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCA
CACTCAAGATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCGTACAC
GTTCGGAGGGGGGACCAAGCTGGAAATAAAACG

SC11.32 Light chain - protein sequence (SEQ ID NO: 61)

DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSDGNTYLHWYLQKPGQSPKLLIYRVS
NRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPYTFGGGTKLEIK

SC11.34 Heavy chain - nucleotide sequence (SEQ ID NO: 62)

TCTGATGTGCACCTTCAGGAGACGGGACCTGGCCTGGTGAAACCTTCTCAGTCTCTGTCCGTCACCTGCACTGTCAC
TGGTTACTCAATCACCAGTGATTATGCCTGGAACTGGATCCGGCAGTTTCCAGGAAACAAACTGGAGTGGATGGG
CTACATAAGCTACAGTGGTGGCACTAGGTACAACCCATCTGTCAAAAGTCGAATCTCTATCACTCGAGACACATCC
AAGAACCAGTTCTTCCTGCAGTTGAATTCTGTGACTACTGAGGACACAGCCACATATTACTGTGCAAGAGGGCCCT
ATGCTGGTTACCCCGCCTGGTTTGATTACTGGGGCCCAGGGACTCTGGTCACTGTCTCTGCA

SC11.34 Heavy chain - protein sequence (SEQ ID NO: 63)

SDVHLQETGPGLVKPSQSLSVTCTVTGYSITSDYAWNWIRQFPGNKLEWMGYISYSGGTRY
NPSVKSRISITRDTSKNQFFLQLNSVTTEDTATYYCARGPYAGYPAWFDYWGPGTLVTVSA

FIG. 7E

SC11.34 Light chain - nucleotide sequence (SEQ ID NO: 64)

GAAAATGTGCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCTAGGGGAGAAGGTCACCATGAGCTGCAGGGCC
AACTCAAGTATAAATTACATGTACTGGTACCAGCAGAAGTCAGATGCCTCCCCCAAACTATGGATTTATTACACAT
CCAACCTGGCTCCTGGAGTCCCAGCTCGCTTCAGTGGCAGTGGGTCTGGGAACTCTTATTCTCTCACAATCAGCAG
CATGGAGGGTGAAGATGCTGCCACTTATTACTGCCAGCAGTTTACTAGTTCCCCGTGGACGTTCGGTGGAGGCAC
CAAGCTGGAAATCAAAC

SC11.34 Light chain - protein sequence (SEQ ID NO: 65)

ENVLTQSPAIMSASLGEKVTMSCRANSSINYMYWYQQKSDASPKLWIYYTS
NLAPGVPARFSGSGSGNSYSLTISSMEGEDAATYYCQQFTSSPWTFGGGTKLEIK

SC11.37 Heavy chain - nucleotide sequence (SEQ ID NO: 66)

GCGGTGCAGCTTGTTGAGTCTGGTGGAGGATTGGTGCAGCCTGAAGGGTCATTGAAACTCTCGTGTGCAGCCTCT
GGATTCACCTTCAATGCCTACGCCATGAACTGGGTCCGCCAGGCTCCAGGAAAGGGTTTGGAATGGGTTGCTCGC
ATAAGAAGTAAAAGTAATGATTATGCAACATATTATGCCGATTCAGTGAAAGACAGGTTCACCATCTCCAGAGAT
GATTCACAAAGCATGCTCTATCTGCAAATGAACAACTTGAAAACTGAGGACACAGGCATGTATTACTGTTTGACCT
TCTCTGTAGATTTGCACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

FIG. 7F

SC11.37 Heavy chain - protein sequence (SEQ ID NO: 67)

AVQLVESGGGLVQPEGSLKLSCAASGFTFNAYAMNWVRQAPGKGLEWVARIRSKSNDYAT
YYADSVKDRFTISRDDSQSMLYLQMNNLKTEDTGMYYCLTFSVDLHYWGQGTTLTVSS

SC11.37 Light chain - nucleotide sequence (SEQ ID NO: 68)

GATGTTGTGATGACCCAGACTCCACTCACTTTGTCGGTTACCATTGGACAACCAGCCTCTATCTCTTGCAAGTCAAG
TCAGAGCCTCTTATATAGTAATGGAAAGACCTATTTGAATTGGTTATTACAGAGGCCAGGCCAGTCTCCAAAGCGC
CTAATCTATCTGATGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGAACAGATTTTA
CACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAATTTATTACTGCTTGCAAGGTACACATTTTCCGTACA
CGTTCGGAGGGGGGACCGAGCTGGAAATTAAACG

SC11.37 Light chain - protein sequence (SEQ ID NO: 69)

DVVMTQTPLTLSVTIGQPASISCKSSQSLLYSNGKTYLNWLLQRPGQSPKRLIYL
MSKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGIYYCLQGTHFPYTFGGGTELEIKR

SC11.44 Heavy chain - nucleotide sequence (SEQ ID NO: 70)

TCCCGGGGGCCCCTGGAGTCTGATGGAGGATTGGTGCAGCCTGAAGGGTCATTGAAACTCTCATGTGCAGCCTCT
GGATTCACCTTCAATACCTACGCCATGAACTGGGTCCGCCAGGCTCCAGGAAAGGGTTTGGAATGGGTTGCTCGC
ATAAGAAGTAAAAGTAATAATTATGTAACATATTATGCCGATTCAGTGAAAGACAGGTTCACCGTCTCCAGAGAT
GATTCACAAAGCATGCTCTATCTGCAAATGAACAACTTGAAAACTGAGGACACAGGCATGTATTACTGTGTGACCT
TCTCTGTGGATTTGCACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC11.44 Heavy chain - protein sequence (SEQ ID NO: 71)

SRGPLESDGGLVQPEGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKSNNY
VTYYADSVKDRFTVSRDDSQSMLYLQMNNLKTEDTGMYYCVTFSVDLHYWGQGTTLTVSS

FIG. 7G

SC11.44 Light chain - nucleotide sequence (SEQ ID NO: 72)

GATGTTGTGATGACCCAGACTCCGCTCACTTTGTCGGTTACCATTGGACAACCAGCCTCTATCTCTTGCAAGTCAGG
TCAGAGCCTCTTATATGGTAATGGAAAAACCTATTTGAATTGGTTATTTCAGAGGCCAGGCCAGTCTCCAAAGCGC
CTAATCTATCTGGTGTCTAAACTGGACTCTGGAGTCCCTGACAGGTTCACTGGCAGTGGATCAGGAACAGATTTTA
CACTGAAAATCAGCAGAGTGGAGGCTGAGGATTTGGGAGTTTATTACTGCTTGCAAGGTACACATTTTCCGTACA
CGTTCGGAGGGGGGACCGAGCTGGAAATAAAACG

SC11.44 Light chain - protein sequence (SEQ ID NO: 73)

DVVMTQTPLTLSVTIGQPASISCKSGQSLLYGNGKTYLNWLFQRPGQSPKRLIYLV
SKLDSGVPDRFTGSGSGTDFTLKISRVEAEDLGVYYCLQGTHFPYTFGGGTELEIKR

SC11.51 Heavy chain - nucleotide sequence (SEQ ID NO: 74)

GAGGTCCAGCTACAACAATCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCT
GGATACACGTTCACTGACTACTACATGAACTGGGTGAAACAGACTCATGGAAAGAGCCTTGAGTGGATTGGAGA
TATTACTCCTAACAGTGGTGGTCCTACCTACAACCAGAATTTCAAGGGCAAGGCCACATTGACTGTTGACAGGTCC
TCCACCACAGCCTTCATGGAGCTCCGCAGCCTGACCTCTGATGACTCTGCTGTCTATTACTGTGTAAGATCGGCTTA
TTACTACGGTACTAACTACGACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA

SC11.51 Heavy chain - protein sequence (SEQ ID NO: 75)

EVQLQQSGPELVKPGASVKISCKASGYTFTDYYMNWVKQTHGKSLEWIGDITPNSGGPTYNQN
FKGKATLTVDRSSTTAFMELRSLTSDDSAVYYCVRSAYYYGTNYDFDYWGQGTTLTVSS

FIG. 7H

SC11.51 Light chain - nucleotide sequence (SEQ ID NO: 76)

CAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCA
GCTCAAGTGTTACTTACATGTACTGGTACCAACAGAAGCCAGGATCCTCACCCAAACCCTGGATTTATCGCACATC
CAACCTTGCTTCTGGAGTCCCTACTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCG
TGGAGGCCGAAGATACTGCCACTTATTACTGCCAGCAGTACAGTGATTACCCGCTCACGTTCGGTGCTGGGACCAA
GCTGGAGCTGAAAC

SC11.51 Light chain - protein sequence (SEQ ID NO: 77)

QIVLTQSPAIMSASPGEKVTMTCSASSSVTYMYWYQQKPGSSPKPWIYRTS
NLASGVPTRFSGSGSGTSYSLTISSVEAEDTATYYCQQYSDYPLTFGAGTKLELK

SC11.67 Heavy chain - nucleotide sequence (SEQ ID NO: 78)

GAGGTGCAGCTTGTTGAGTCTGGTGGAGGATTGGTGCAGCCTGAAGGGTCATTGAAACTCTCATGTGCAGCCTCT
GGATTCACCTTCAATACCTACGCCATGAACTGGGTCCGCCAGGCTCCAGGAAAGGGTTTGGAATGGGTTGCTCGC
ATCAGAAGTAAAAGTAATAATTATGCAACATATTATGCCGATTCAGTGAAAGACAGGTTCACCATCTCCAGAGAT
GATTCACAAAGTATGCTCTATCTGCAAATGAACAACTTGAAAACTGAGGACACAGGCATGTATTACTGTTTGACCT
TCTCTGTAGATTTGCACTACTGGGGCCAAGGCACCGCTCTCACAGTCTCCTCA

SC11.67 Heavy chain - protein sequence (SEQ ID NO: 79)

EVQLVESGGGLVQPEGSLKLSCAASGFTFNTYAMNWVRQAPGKGLEWVARIRSKSNN
YATYYADSVKDRFTISRDDSQSMLYLQMNNLKTEDTGMYYCLTFSVDLHYWGQGTALTVSS

FIG. 7I

SC11.67 Light chain - nucleotide sequence (SEQ ID NO: 80)

GACATCTTGCTGACTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGTTTCTCCTGCAGGGCCA
GTCAGAGCATTGGCACGGACATACACTGGTTTCAGCTAAAAACAAATGGTTCTCCAAGACTTCTCATAAATTATAC
TTCTGAGTCTATCTCTGTGATCCCTTCCAGGCTTAGTGGCAGTGGATCAGGGACAGATTTTACTCTTAGCATCAACA
GTGTGGAGTCTGAAGATTTTGCAGATTGTCACTGTCAACAAAATAATAACTGGCCGCTCACGTTCGGTGCTGGGA
CCAAGCTGGAGCTGAAAC

SC11.67 Light chain - protein sequence (SEQ ID NO: 81)

DILLTQSPAILSVSPGERVSFSCRASQSIGTDIHWFQLKTNGSPRLLINYTSE
SISVIPSRLSGSGSGTDFTLSINSVESEDFADCHCQQNNNWPLTFGAGTKLELK

SC11.82 Heavy chain - nucleotide sequence (SEQ ID NO: 82)

GAGGTCCAGCTGCAACAATCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCT
GGATACACGTTCACTGACTACTACATGAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGA
TATTAATCCTAACATTGGTGGTACTAACTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACAAGTC
CTCCAGTACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAAGAAGCTGG
ATCTACTATGGTTACGACCCTGATATGGACTACTGGGGTCAAGGAACATCAGTCACCGTCTCCTCA

SC11.82 Heavy chain - protein sequence (SEQ ID NO: 83)

EVQLQQSGPELVKPGASVKISCKASGYTFTDYYMNWVKQSHGKSLEWIGDINPNIGGTNY
NQKFKGKATLTVDKSSSTAYMELRSLTSEDSAVYYCARSWIYYGYDPDMDYWGQGTSVTVSS

FIG. 7J

SC11.82 Light chain - nucleotide sequence (SEQ ID NO: 84)

CAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCA
GCTCAAGTCTAAGTTACATGTACTGGTACCAGCAGAAGCCAGGATCCTCACCCAAACCCTGGATTTATCGCACATC
CAACCTGGCTTCTGGAGTCCCTACTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCG
TGGAGGCCGAAGATGCTGCCACTTATTACTGCCAGCAGTACAGTAATTACCCGCTCACGTTCGGTGCT

SC11.82 Light chain - protein sequence (SEQ ID NO: 85)

QIVLTQSPAIMSASPGEKVTMTCSASSSLSYMYWYQQKPGSSPKPWIY
RTSNLASGVPTRFSGSGSGTSYSLTISSVEAEDAATYYCQQYSNYPLTFGA

SC11.89 Heavy chain - nucleotide sequence (SEQ ID NO: 86)

GAGGTCCAGCTGCAACAATCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCT
GGATACACGTTCACTGACTATTATATGAACTGGGTGAGACAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGA
TGTTCATCCTAACATTGGTACTATTAACTACAACCAGAAGTTCAAGGACAAGGCCACATTGACTATAGACAAGTCC
TCCAGTACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAAGACTCTGCAGTCTATTTCTGTGCAAGAGAAGGG
GCCTATTATGGTCCCCCTTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

SC11.89 Heavy chain - protein sequence (SEQ ID NO: 87)

EVQLQQSGPELVKPGASVKISCKASGYTFTDYYMNWVRQSHGKSLEWIGDVHPNIGTINY
NQKFKDKATLTIDKSSSTAYMELRSLTSEDSAVYFCAREGAYYGPPFAYWGQGTLVTVSA

FIG. 7K

SC11.89 Light chain - nucleotide sequence (SEQ ID NO: 88)

ACTGGATGGTGGGAAGATGGATACAGTTGGTGCAGCATCAGCCCGTTTAATTTCCAACTTGGTCCCCCCTCCGAAC
GTGGAGGAATCTCCCAACTGCGCTGACAGTCATATTTTGCAGTATCCTCCTCCTCCACAGGATGGATGTTGAGGGT
GCAGTCTGTCCCAGACCCACTGCCACTGAACCTGGCAGGGACCCCAGATTCTAGGTTGGATGCATACTTGATGAG
GAGCTTGGGTGGCTGTCCTGGTATCTGTTGGTACCAGTATATATGACTATAGCTAGATGAATTGACACTTTGGCTG
GCCCTGCATGAGATGGTGGCCCTCTGCCCCAGAGATACAGCTAAGGAAGCAGGAGACTGTGTCAGCACAATGTCA
CCAGTGNAACCTGGAACCCANAGCAGCA

SC11.89 Light chain - protein sequence (SEQ ID NO: 89)

DIVLTQSPASLAVSLGQRATISCRASQSVNSSSYSHIYWYQQIPGQPPKLLIKYA
SNLESGVPARFSGSGSGTDCTLNIHPVEEEDTAKYDCQRSWEIPPRSEGGPSWKLN

SC11.90 Heavy chain - nucleotide sequence (SEQ ID NO: 90)

GAGGTCCAACTGCAACAATCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCT
GGATACACGTTCACTGACTACTACATGAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGA
TATTCATCCTAACAATGGTGGTGCTAACTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACCAGTC
CTCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGACTCTGCAGTCTATTTCTGTGCAAGAGAGGG
GGATTACGGTGGTAACTCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC11.90 Heavy chain - protein sequence (SEQ ID NO: 91)

EVQLQQSGPELVKPGASVKISCKASGYTFTDYYMNWVKQSHGKSLEWIGDIHPNNGGANY
NQKFKGKATLTVDQSSSTAYMELRSLTSEDSAVYFCAREGDYGGNSMDYWGQGTSVTVSS

FIG. 7L

SC11.90 Light chain - nucleotide sequence (SEQ ID NO: 92)

CAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCGCCATGACCTGCAGTGCCA
GCTCAAGTGTAACTTACATGTACTGGTACCAGCAGAAGCCAGGATCCTCACCCAAACCCTGGATTTATCGCACATC
CAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCG
TGGAGGCCGAAGATGCTGCCACTTATTACTGCCAGCAGTACGATAATTACCCGCTCACGTTCGGTGCTGGGACCAA
GCTGGAGCTGAAAC

SC11.90 Light chain - protein sequence (SEQ ID NO: 93)

QIVLTQSPAIMSASPGEKVAMTCSASSSVTYMYWYQQKPGSSPKPWIYRTS
NLASGVPARFSGSGSGTSYSLTISSVEAEDAATYYCQQYDNYPLTFGAGTKLELK

SC11.91 Heavy chain - nucleotide sequence (SEQ ID NO: 94)

GAGGTCCAACTGCAACAATCTGGACCTGAGCTGGTGAAGCCTGGGGCTTCAGTGAAGATATCCTGTAAGGCTTCT
GGATACACGTTCACTGACTACTATATGAACTGGGTGAAGCAGAGCCATGGAAAGAGCCTTGAGTGGATTGGAGA
TATTCATCCTAACTATGGTGGTTCTAACTACAACCAGAAGTTCAAGGGCAAGGCCACATTGACTGTAGACCGGTCC
TCCAGCACAGCCTACATGGAGCTCCGCAGCCTGACATCTGAGGACTCTGCAGTCTATTTCTGTGCAAGAGAGGGG
GATTACGGTGGTAGCTCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA

SC11.91 Heavy chain - protein sequence (SEQ ID NO: 95)

VQLQQSGPELVKPGASVKISCKASGYTFTDYYMNWVKQSHGKSLEWIGDIHPNYGGSNYN
QKFKGKATLTVDRSSSTAYMELRSLTSEDSAVYFCAREGDYGGSSMDYWGQGTSVTVSS

FIG. 7M

SC11.91 Light chain - nucleotide sequence (SEQ ID NO: 96)

CAAATTGTTCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCGCCATGACCTGCAGTGCCA
GCTCAAGTGTAACTTACATGTACTGGTACCAGCAGAAGCCAGGATCCTCACCCAAACCCTGGATTTATCGCACATC
CAACCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGCG
TGGAGGCCGAAGATGCTGCCACTTATTACTGCCAGCAGTATGATAATTATCCGCTCACGTTCGGTGCTGGGACCAA
GC

SC11.91 Light chain - protein sequence (SEQ ID NO: 97)

QIVLTQSPAIMSASPGEKVAMTCSASSSVTYMYWYQQKPGSSPKPWIYR
TSNLASGVPARFSGSGSGTSYSLTISSVEAEDAATYYCQQYDNYPLTFGAGTK

SC11.104 Heavy chain - nucleotide sequence (SEQ ID NO: 98)

CAGGTGCAGCTGAAGCAGTCAGGACCTGGCCTTGTGCAGCCCTCACAGAGCCTGTCCATCACCTGCACAGTCTCTG
GTTTCTCATTATCTAACTATGGTGTACACTGGGTTCGCCAGTCTCCAGGAAAGGGTCTGGAGTGGCTGGGAGTGAT
ATGGAGTGGTGGAAGCACAGACTATAATGCAGCTTTCATATCCAGACTGAACATCAACAAGGACAATTCCAAGAG
CCAAGTTTTCTTTAAAATGAACAGTCTGCAATCTGATGACACAGCCATATATTACTGTGCCGACTACTATGATTACG
ATGGGGCCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCA

FIG. 7N

SC11.104 Heavy chain - protein sequence (SEQ ID NO: 99)

QVQLKQSGPGLVQPSQSLSITCTVSGFSLSNYGVHWVRQSPGKGLEWLGVIWSGGSTDYNAA
FISRLNINKDNSKSQVFFKMNSLQSDDTAIYYCADYYDYDGAWFAYWGQGTLVTVSA

SC11.104 Light chain - nucleotide sequence (SEQ ID NO: 100)

GACATTGTGATGACCCAGTCTCAAAAATTCATGTCCACTTCAGTAGGAGACAGGGTCAGCGTCACCTGCAAGGCC
AGTCAGAACGTGGGTACTAATGTAGCCTGGTTTCAACAGAAACCAGGGCAATCTCCTAAAGCACTGATTCACTCG
GCATCCTACCGGTACAGTGGAGTCCCTGATCGCTTCACAGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCA
GCAATGTGCAGCCTGAAGACTTGGCAGAGTATTTCTGTCAGCAACATAACAGCTTTCCTCTCACGTTCGGTGCTGG
GACTAAGCTGGAGCTGAAAC

SC11.104 Light chain - protein sequence (SEQ ID NO: 101)

DIVMTQSQKFMSTSVGDRVSVTCKASQNVGTNVAWFQQKPGQSPKALIHSAS
YRYSGVPDRFTGSGSGTDFTLTISNVQPEDLAEYFCQQHNSFPLTFGAGTKLELK

Properties of Selected Ephrin-A1 Ligand Modulators

Clone	Bin	Affinity (nM)	Western Reactivity	Ms XR	Cyno XR	Neutralizing	Killing
SC9.7	A	0.5	NR/R	No	Yes	No	Yes
SC9.9	A	0.5	NR	ND	Yes	No	ND
SC9.10	A	2	NR	ND	Yes	No	ND
SC9.20	ND	0.5	NR/R	ND	Yes	Yes	Yes
SC9.66	B	2	NR/R	No	Yes	No	Yes
SC9.88	A	2	NR	ND	Yes	No	ND
SC9.92	ND	5	No	ND	Yes	Yes	Yes
SC9.96	ND	10	No	ND	Yes	No	Yes
SC9.98	ND	0.5	NR/R	ND	Yes	Yes	ND
SC9.105	C	2	ND	Yes	No	No	No
SC9.109	A	2	NR/R	ND	Yes	No	Yes
SC9.121	B	1	NR/R	No	Yes	No	Yes
SC9.122	A	2	NR/R	No	Yes	No	Yes
SC9.140	B	0.5	NR/R	ND	Yes	Yes	ND
SC9.141	B	4	No	ND	Yes	Yes	ND

FIG. 8

Properties of Selected Ephrin-A3 Ligand Modulators

Clone	Bin	Affinity (nM)	Western Reactivity	Ms XR	Neutralizing	Killing
SC11.9	A	0.5	NR/R	Yes	No	Yes
SC11.18	C	0.5	NR	No	Yes	Yes
SC11.19	A	5.0	NR/R	Yes	No	ND
SC11.27	B	2.0	NR	no	Yes	Yes
SC11.30	C	2.0	NR/R	no	Yes	ND
SC11.32	B	0.2	NR/R	ND	Yes	Yes
SC11.34	B	2.0	NR	no	Yes	Yes
SC11.37	A	ND	ND	ND	No	ND
SC11.47	A	1.0	NR/R	Yes	No	Yes
SC11.51	B	1.0	NR	Yes	No	Yes
SC11.53	A	ND	NR	ND	No	ND
SC11.54	A	5.0	ND	Yes	No	ND
SC11.55	A	ND	NR/R	ND	No	Yes
SC11.112	A	2.0	ND	Yes	No	Yes

FIG. 9

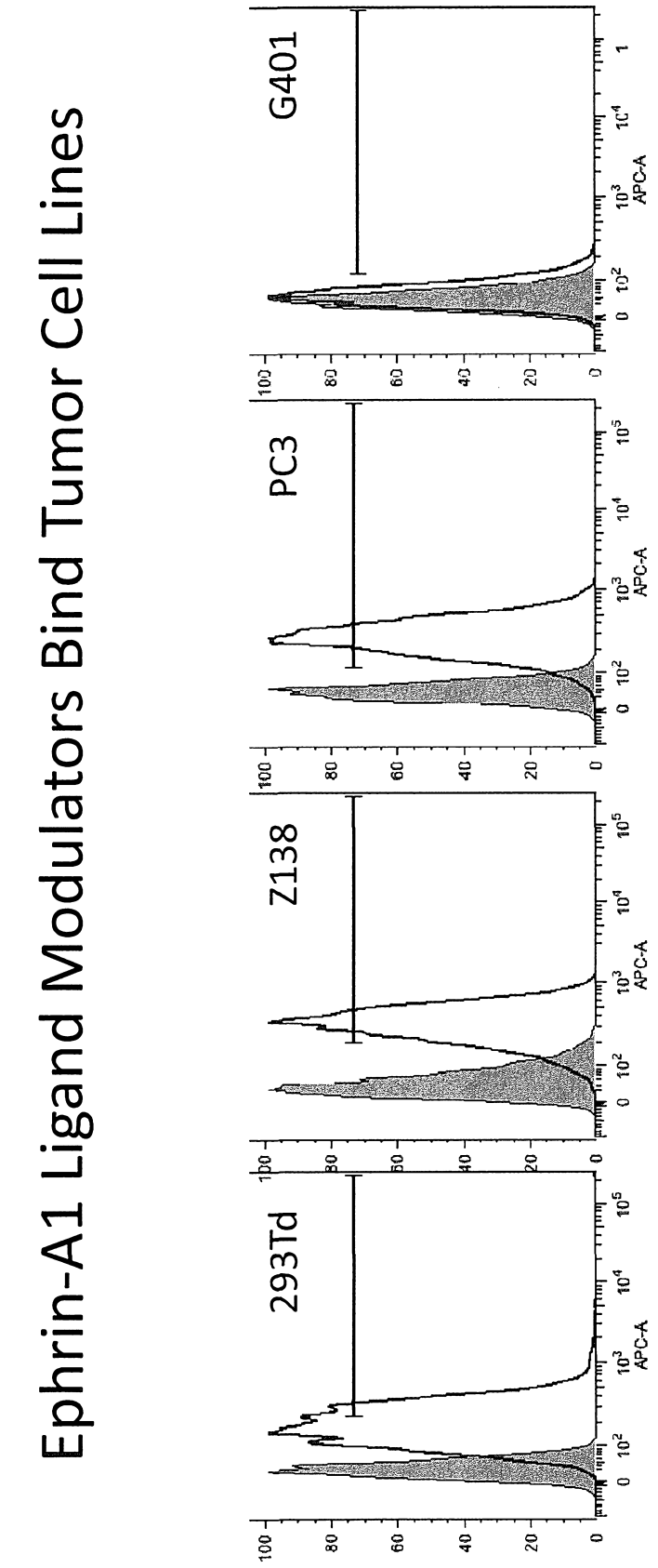
Ephrin-A1 Ligand Modulators Bind Tumor Cell Lines
293Td
Z138
PC3
G401
APC-A

FIG. 10A

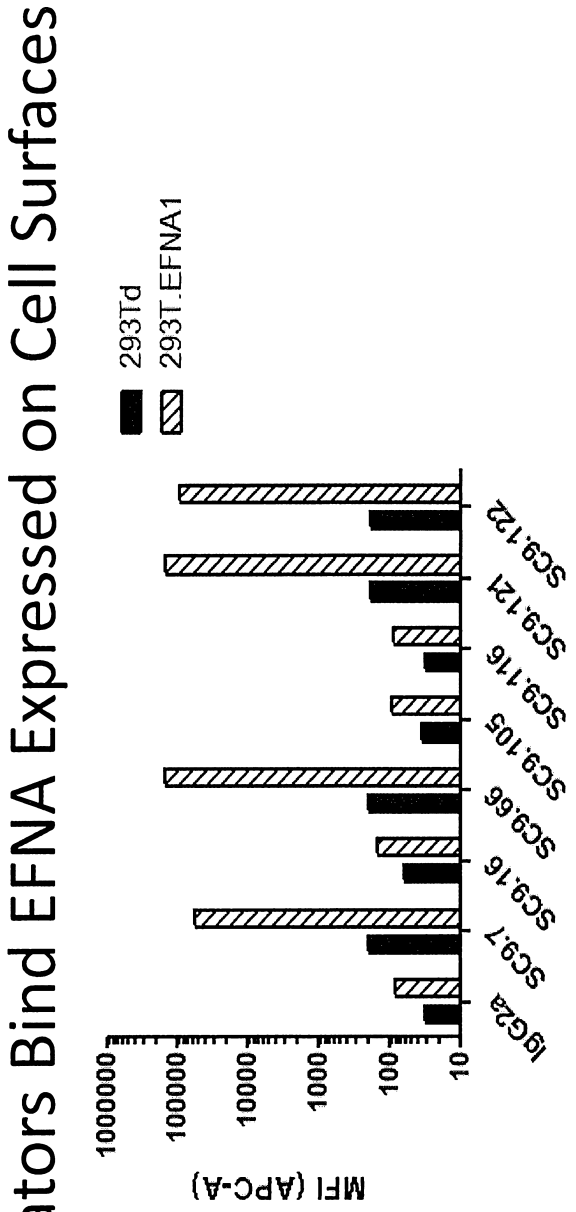

FIG. 10B

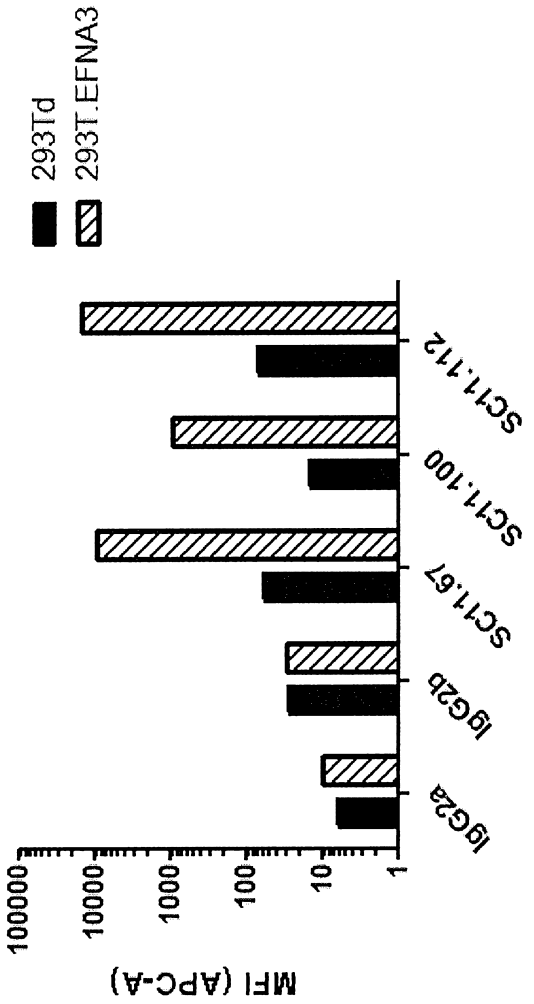

FIG. 10C

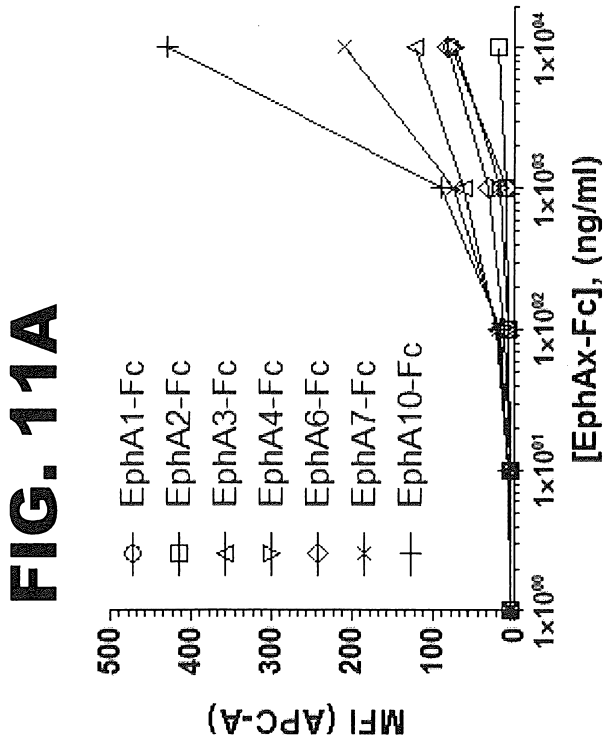

FIG. 11A

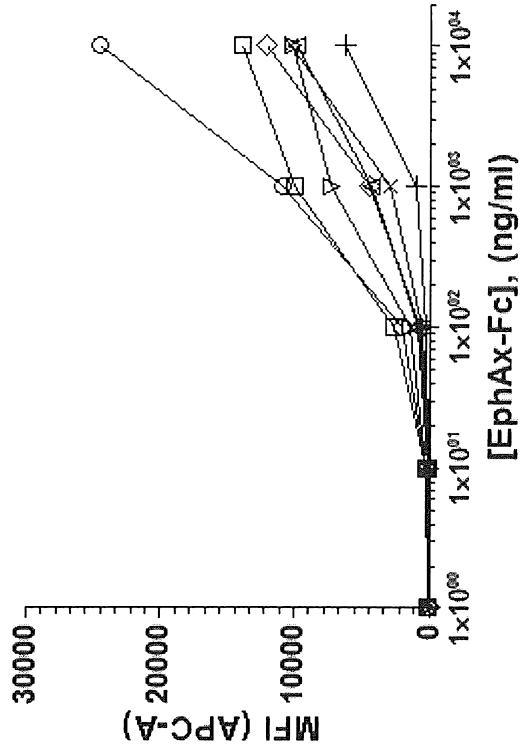

FIG. 11B

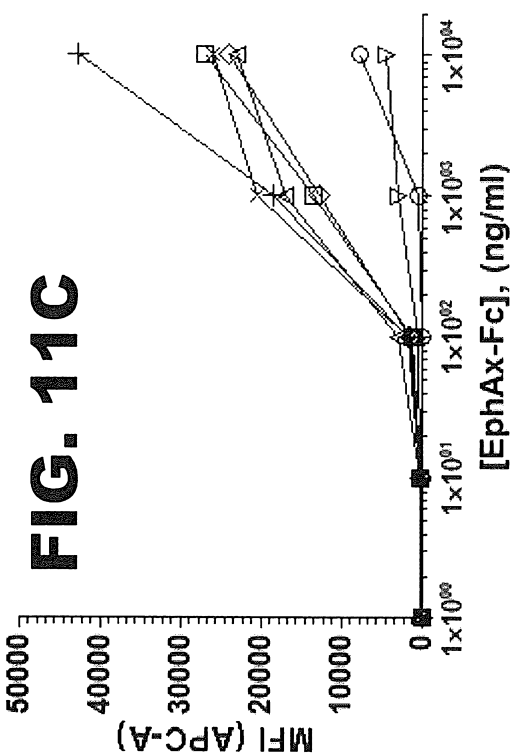

FIG. 11C

Ephrin-A Ligands Expressed on the Cell Surface Bind Ephrin-A Receptors

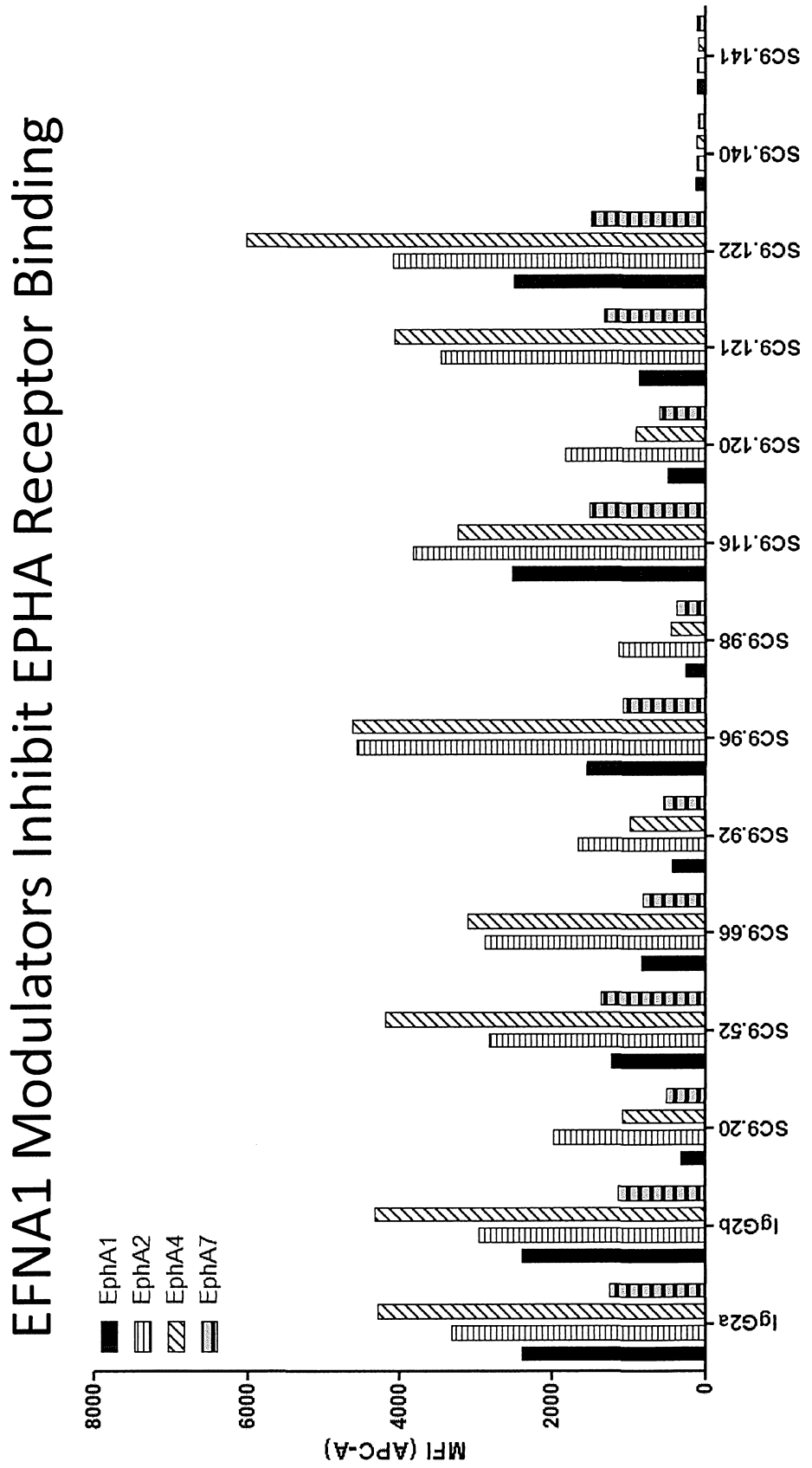
EFNA1 Modulators Inhibit EPHA Receptor Binding
EphA1
EphA2
EphA4
EphA7
MFI (APC-A)
8000
6000
4000
2000
0
IgG2a
IgG2b
SC9.20
SC9.52
SC9.66
SC9.92
SC9.96
SC9.98
SC9.116
SC9.120
SC9.121
SC9.122
SC9.140
SC9.141

FIG. 12A

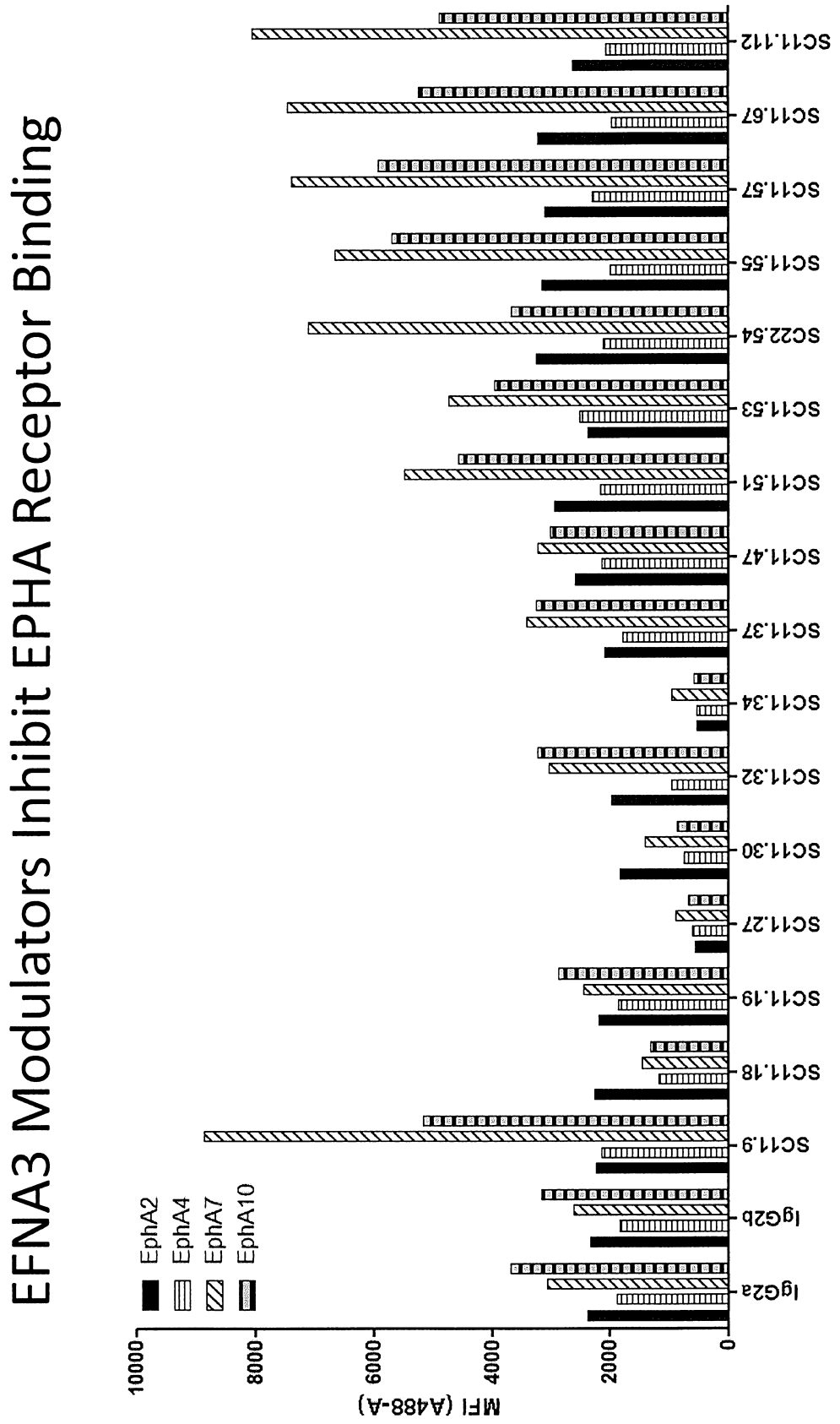
EFNA3 Modulators Inhibit EPHA Receptor Binding
EphA2
EphA4
EphA7
EphA10
MFI (A488-A)
10000
8000
6000
4000
2000
0
IgG2a
IgG2b
SC11.9
SC11.18
SC11.19
SC11.27
SC11.30
SC11.32
SC11.34
SC11.37
SC11.47
SC11.51
SC11.53
SC22.54
SC11.55
SC11.57
SC11.67
SC11.112

FIG. 12B

EFNA1 Modulators Target Cells With Cytotoxic Payloads

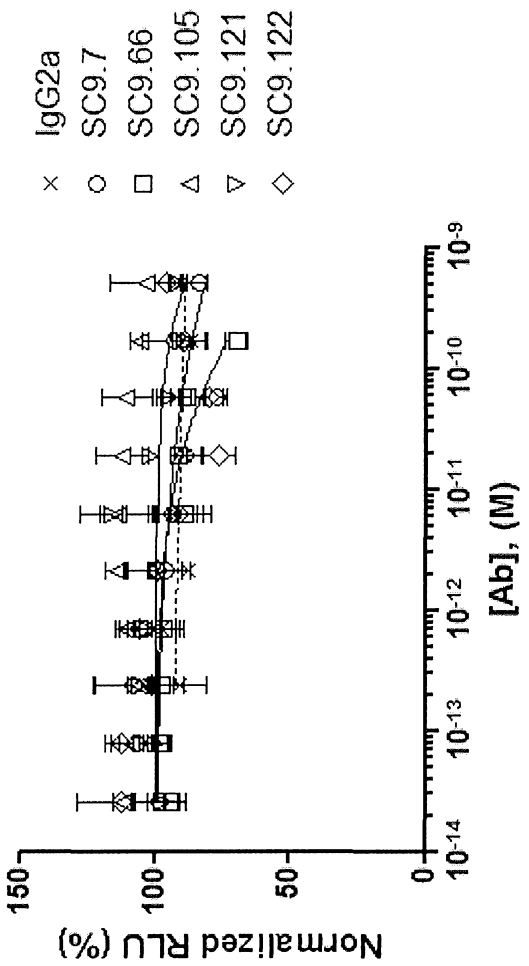

FIG. 13A

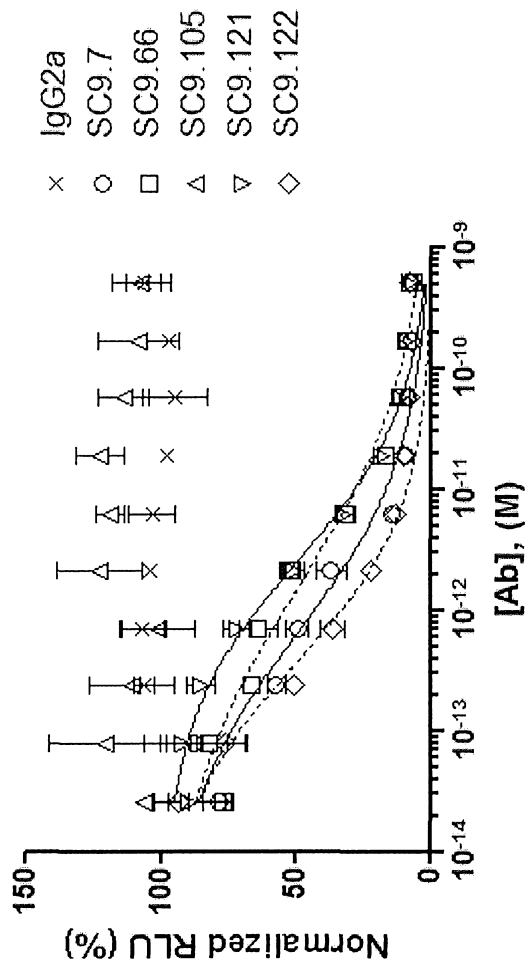

FIG. 13B

EFNA3 Modulators Target Cells With Cytotoxic Payloads

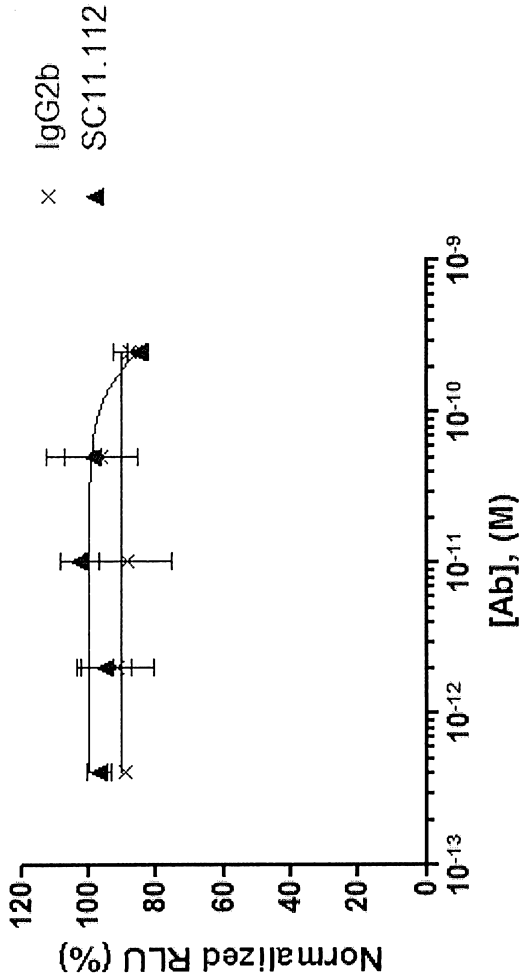

FIG. 14A

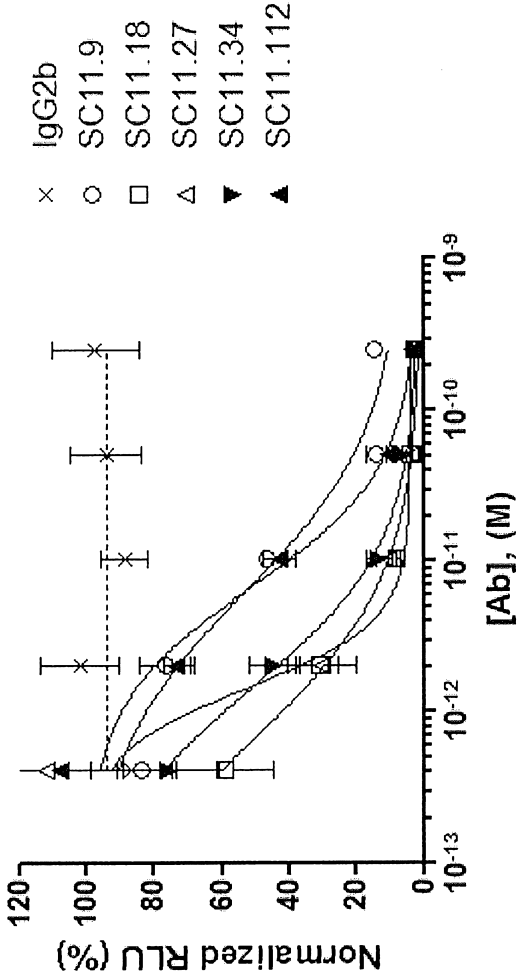

FIG. 14B

EFNA1 Modulators
Target Patient
Derived Tumor Cells

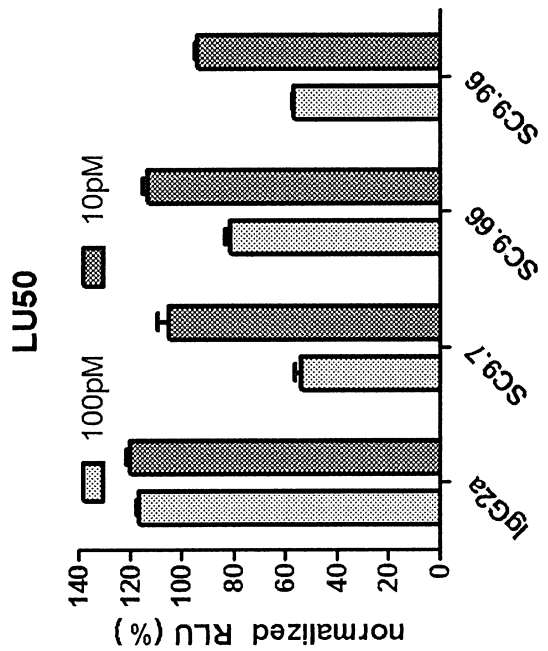

FIG. 15A

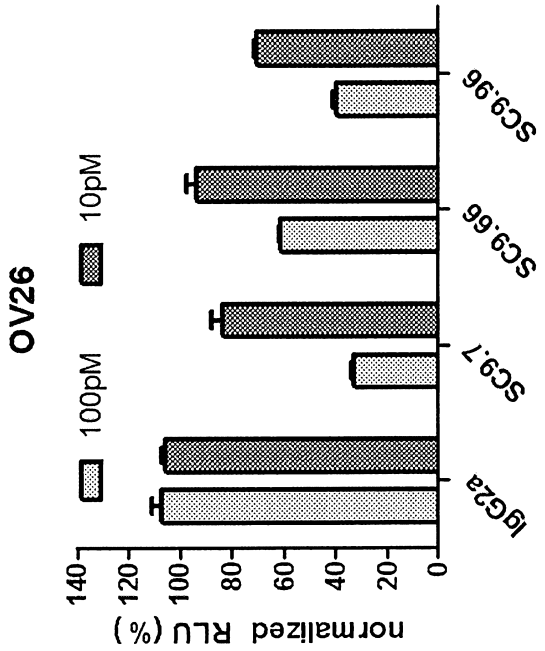

FIG. 15B