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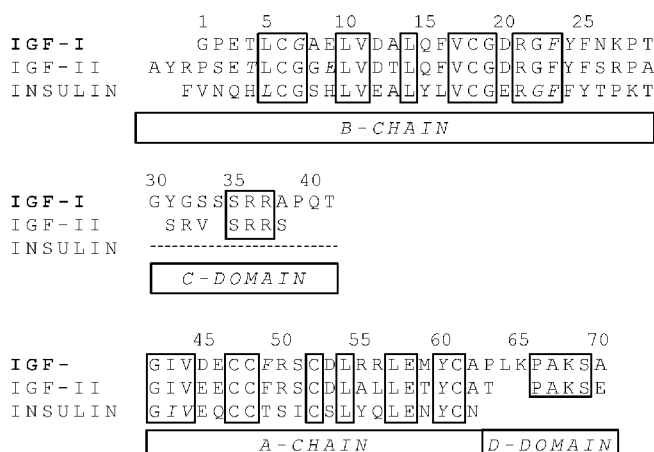
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FIG 1



(57) Abstract: The present invention resides in the discovery that the specific interaction between insulin-like growth factor 2 (IGF2) and integrin is involved in integrin-mediated cellular signaling, such as enhanced proliferation of cells expressing integrin, especially integrin $\alpha v \beta 3$. Thus, this invention provides for a novel method for inhibiting integrin signaling by using an inhibitor of IGF1-integrin binding. A method for identifying inhibitors of IGF1-integrin binding is also described. Further disclosed are polypeptides, nucleic acids, and corresponding compositions for inhibiting integrin signaling.

INSULIN-LIKE GROWTH FACTOR 2 (IGF2) SIGNALING AND MODULATION

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No.
5 62/104,608, filed on January 16, 2015, the contents of which are hereby incorporated by
reference in the entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under Grant No. CA13015 by
10 the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

[0003] Insulin-like growth factor-1 (IGF1) and insulin-like growth factor-2 (IGF2) are two
polypeptide hormones with a high level of structural similarity (75-kD). They also share a
substantial degree of structural similarity to human proinsulin.

15 [0004] Integrins are a family of cell adhesion receptors that mediate cell-extracellular
matrix interaction and cell-cell interaction. It has been proposed that signaling from inside
cells regulates the ligand-binding affinity of integrins (inside-out signaling). Each integrin is
a heterodimer containing α and β subunits. At present 18 α and 8 β subunits have been
identified, which combine to form 24 integrins.

20 [0005] It has been reported that integrin may play a role in cancer proliferation and
invasiveness. For instance, high levels of integrin $\alpha\beta3$ have been reported to correlate with
growth and/or progression of melanoma, neuroblastoma, breast cancer, colon cancer, ovarian
cancer, and cervical cancer.

[0006] IGF1 has been implicated in cancer progression. One of the major actions of IGF1
25 is to inhibit apoptosis. IGF1 confers resistance to chemotherapy and radiation therapy. IGF1
expression levels are increased in breast, lung, prostate, and many other cancers. It is
understood that IGF1 binds to both integrins and its receptor IGF1R during IGF1 signaling,
and integrin binding-defective mutants of IGF1 have been shown to be dominant-negative
inhibitors of IGF1R-mediated IGF1 signaling.

[0007] One known role of IGF2 is as a growth promoting hormone during gestation. In humans, the IGF2 gene is located on chromosome 11p15.5, and the GenBank Accession Numbers for human IGF2 mRNA sequence and amino acid sequence are NM_000612 and NP_000603, respectively.

5 [0008] The insulin receptor (IR) and the type 1 insulin-like growth factor receptor (IGF1R) are both members of the tyrosine kinase class of membrane receptors [2]. IR exists in two splice variant isoforms; the 'B' isoform (IR-B) recognizes only insulin, but the 'A' isoform (IR-A) recognizes both insulin and insulin-like growth factor-2 (IGF2) [3]. Heterodimers comprised of a half IR and a half IGF1R can form, and these are known as hybrid receptors
10 [3, 4]. The ligand binding to IR or IGF1R to the extracellular domain of the receptor leads to the activation of the tyrosine kinase in the cytoplasmic domain. This leads to phosphorylation of members of the IR substrate (IRS) family of proteins, and activation of PI3K, AKT and various downstream networks [5]. IR-A is a predominant IR isoform expressed in a variety of cancers, including cancers of the breast, colon, and lung. Abnormal autocrine or paracrine
15 expression of ligands, particularly IGF2, is common in many cancers [6], and the presence of IR-A/IGF2 loop may denote an 'addiction' to IR/IGF1R activation. In cells with a high IR-A:IGF1R ratio, autocrine production of IGF2 stimulates cell growth through IR-A stimulation. Blocking either IGF2 or the IR markedly inhibits growth, which demonstrates the relevance of the IR-A/IGF2 loop in cancer. In contrast, IR-B is predominantly expressed
20 in the liver, and also expressed in muscle, adipose tissue, and kidney, and only binds to insulin. Blocking IR-B has, thus, been carefully avoided as a therapy, since it will affect normal glucose metabolism in these tissues.

[0009] Since IR-A is a therapeutic target in cancer, efforts are made to produce mutants of IGF2 that bind to IR-A, but not IR-B, for the purpose of modulating cellular signaling in
25 cancer cells to achieve potential therapeutic benefits by way of suppressing tumorigenesis. Unfortunately, currently available kinase inhibitors to IR or IGF1R do not distinguish IGF1R, IR-A or IR-B, whereas anti-IGF1R antibodies do not block IR-A or IR-B. While dominant-negative IGF1 mutants have been made to act as inhibitors of IGF1R (IGF1-decoys), there is significant interest in generating IGF2 mutants, such as dominant-negative mutants similar to
30 the IGF1-decoys, that are defective in their ability to bind integrins but still capable of binding IGF1R and IR-A, to further study IGF2 signaling and explore the possibility of therapeutic use of this type of inhibitors, for instance, for treating conditions involving inappropriate cellular proliferation including various forms of cancer.

BRIEF SUMMARY OF THE INVENTION

[0010] This invention provides new methods and compositions useful for inhibiting IGF2 signaling in a cell, based on the discovery that the interaction between IGF2 and certain integrin molecules is involved in IGF2-mediated signaling. Thus, in one aspect, the present invention relates to a method for inhibiting IGF2 signaling in a cell, comprising the step of contacting the cell with an effective amount of an inhibitor of IGF2-integrin binding.

[0011] In some embodiments, the integrin is $\alpha v \beta 3$. In some embodiments, the integrin is $\alpha 5 \beta 1$ or $\alpha 6 \beta 4$. In some embodiments, the inhibitor is an IGF2 mutant comprising two substitutions of R37E and R38E in the amino acid sequence of a wild-type IGF2 protein (*e.g.*, SEQ ID NO:1). In some embodiments, the inhibitor is IGF2 mutant R24E/R37E/R38E, or IGF2 mutant R34E/R37E/R38E, or IGF2 mutant R24E/R34E/R37E/R38E. In some embodiments, the cell is within a patient's body. In some embodiments, the contacting step is performed by oral administration. In some embodiments, the contacting step is performed by intravenous, subcutaneous, intraperitoneal, or intratumor injection. For example, administration may be performed by way of using a device similar to that of an insulin pump, a medical device used for the administration of a therapeutic agent in a method known as continuous subcutaneous infusion therapy.

[0012] As it is recognized that excessive IGF2 signaling may lead to undesirable cellular responses such as abnormal cell proliferation and inflammatory responses, which in turn can cause or contribute to a variety of diseases and disorders, for example, hyperproliferative diseases including cancers such as melanoma, neuroblastoma, breast cancer, colon cancer, ovarian cancer, and cervical cancer; inflammatory diseases such as arthropathies, rheumatoid arthritis, and osteoarthritis; autoimmune diseases, such as rheumatoid spondylitis, autoimmune uveitis, multiple sclerosis, autoimmune diabetes, as well as rheumatoid arthritis; osteoporosis; angiogenesis (related to inflammation and cancer, as well as other diseases involving hypervascularization); and also various types of conditions involving the abnormal formation of fibrous tissues, *e.g.*, fibrosis.

[0013] In a second aspect, the present invention relates to a method for identifying an inhibitor of IGF2-integrin binding. This method comprises the following steps: (1) contacting an integrin and a polypeptide comprising an integrin-binding sequence of an IGF2, in the presence of a test compound, under conditions permissible for IGF2-integrin binding; and (2) detecting the level of polypeptide-integrin binding, wherein a decrease in the level of

binding when compared with the level of binding in the absence of the test compound indicates the compound as an inhibitor of IGF2-integrin binding.

[0014] In some embodiments, the integrin is $\alpha v \beta 3$. In other embodiments, the integrin is $\alpha 5 \beta 1$ or $\alpha 6 \beta 4$. In some embodiments, the polypeptide comprises the sequence of C-domain of a human IGF2 protein (*e.g.*, SEQ ID NO:2). In some embodiments, the polypeptide comprises the full length of a human IGF2 protein (*e.g.*, SEQ ID NO:1). In some embodiments, the polypeptide further comprises a heterologous amino acid sequence, such as a glutathione S-transferase (GST). In some embodiments, the polypeptide further comprises modification such as PEGylation (covalent attachment or amalgamation of polyethylene glycol (PEG) polymer chains) at one or more residues, *e.g.*, one or more of the Arg residues at positions 24, 34, 37, and 38, which may be directly PEGylated or substituted with another amino acid such as Lys, which permits PEGylation. PEGylation can take place on amino acids including lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine. Further, the N-terminal amino group and the C-terminal carboxylic acid can also be used, directly or upon functionalization, as a site for PEGylation. In some embodiments, the integrin is expressed on a cell surface.

[0015] In a third aspect, the present invention relates to an isolated polypeptide comprising an amino acid sequence that (1) has at least 95% sequence identity to the sequence of a naturally occurring wild-type IGF2 protein, such as a wild-type human IGF2 protein; (2) comprises substitutions of at least two Arg residues at positions 37 and 38 of a wild type human IGF2 protein; and (3) inhibits IGF2-integrin binding. The invention also relates to an isolated nucleic acid encoding this polypeptide, as well as a recombinant expression cassette comprising the nucleic acid or an isolated host cell comprising such a recombinant expression cassette.

[0016] In some embodiments, the integrin is $\alpha v \beta 3$. In other embodiments, the integrin is $\alpha 5 \beta 1$ or $\alpha 6 \beta 4$. In some embodiments, at least 3 of the Arg residues at positions 24, 34, 37, and 38 are substituted. In some embodiments, the Arg residues at positions 24, 34, 37, and 38 are substituted. In some embodiments, each of the Arg residues is substituted with a Glu residue.

[0017] In a fourth aspect, the present invention relates to a composition comprising (A) a physiologically acceptable excipient and (B) a polypeptide comprising an amino acid sequence that (1) has at least 95% sequence identity to the sequence of a naturally occurring

wild-type IGF2 protein, especially a wild-type human IGF2 protein; (2) comprises substitutions of at least two Arg residues at positions 37 and 38 of a wild-type human IGF2 protein; and (3) inhibits IGF2-integrin binding. The invention also relates to a composition comprising a nucleic acid encoding the polypeptide described above with a pharmaceutically acceptable excipient. These compositions are useful for treating various diseases and disorders that involve excessive IGF2 signaling resulting in undesirable cell proliferation and inflammatory responses, including but not limited to the conditions named above.

[0018] In some embodiments, the polypeptide is IGF2 mutant R24E/R37E/R38E, or IGF2 mutant R34E/R37E/R38E, or IGF2 mutant R24E/R34E/R37E/R38E. In some embodiments, the polypeptide is PEGylated as detailed in other sections.

[0019] In a fifth aspect, the present invention relates to a kit for inhibiting IGF2 signaling, comprising the composition of a polypeptide or nucleic acid as described above with a pharmaceutically acceptable excipient. Instruction manual or user information in other forms is generally included in the kit. The kit of this invention is for treating various diseases and disorders that involve excessive IGF2 signaling resulting in undesirable cell proliferation and inflammatory responses, including but not limited to the conditions named above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] **Fig.1 The sequence and chain organization of IGF1, IGF2, and insulin [1].** The IGF-specific C- and D-domains are colored grey and pink, respectively; the B and A chains of insulin and their equivalents in IGF1/2 are highlighted in yellow and blue, respectively. Residues important for the IGF-1R or IR binding are in red, with residues responsible for association with IGFBPs in green (mutations of the highlighted residues result in a minimum 90% drop in binding; residues for which substitution results in even higher impact on affinities toward receptors and IGFBPs are in italic. The amino acid sequences of IGF2, IGF1, and insulin are shown in Figure 1 (SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:4, respectively).

[0021] **Fig. 2 R36E/R37E of IGF1 suppresses cell survival and tumorigenesis, while WT IGF1 enhances them in mouse breast cancer Met-1 cells that express WT IGF1 or R36E/R37E [10].** Top: Cell survival. The Met-1 transfectants were cultured in polyHEMA-coated plates in DMEM for 48 h and cell survival was measured by MTS assays (n=6). Bottom: Tumorigenesis *in vivo*. Met-1 cells that stably secrete IGF1 (WT or mutant) were injected to mammary fat pads of FVB mice (two injections per mouse, 10⁵ cells per injection)

without further selection. Tumor growth was monitored by measuring the size of tumors using calipers. Statistical analysis was performed using t-test (n=10) in days 25-31, * P<0.05.

[0022] Fig. 3 IGF2 binds to integrin $\alpha\beta 3$ and generation of integrin-binding

5 defective mutants of IGF2. a) Binding of soluble integrin $\alpha\beta 3$ to immobilized IGF2 in ELISA-type binding assays. Wells of 96-well microtiter plates were coated with IGF2 at increasing concentrations. Immobilized IGF2 was incubated with soluble recombinant $\alpha\beta 3$ (5 $\mu\text{g/ml}$) in Tyrode-HEPES buffer containing Mg^{2+} . Bound $\alpha\beta 3$ was measured using anti-integrin $\beta 3$ mAb. b) CHO cells that express $\alpha\beta 3$ ($\beta 3$ -CHO cells) adhere to IGF2, those
 10 expressing $\alpha\beta 1$ ($\beta 1$ -CHO cells) do not. Wells of 96-well microtiter plates were coated with IGF2 at increasing concentrations. $\beta 3$ -CHO cells or $\beta 1$ -CHO cells (10^5 cells/well) were incubated with immobilized IGF2 in DMEM and bound cells were counted. c) and d) Mutations within the predicted integrin-binding interface of IGF2 suppress integrin binding of IGF2. Based on the alignment of IGF1 and IGF2 (Fig. 1), several Arg residues of IGF2
 15 were selected for mutagenesis. Combined mutations of several Arg residues effectively suppressed integrin binding of IGF2.

[0023] Fig. 4 The integrin-binding defective IGF2 mutants are functionally defective and dominant-negative.

a) Several integrin-binding defective IGF2 mutants are defective in enhancing cell survival. The survival of $\beta 3$ -CHO cells was measured by MTS assays in
 20 polyHEMA-coated wells as described [10]. b) Excess IGF2 mutants suppress cell survival increased by WT IGF2 (25 ng/ml).

[0024] Fig. 5 IGF1 signaling and pertinent biological processes.

[0025] Fig. 6 IGF1 decoy affects morphology and Oct-4 and Nanog expression in Met-1 mouse breast cancer cells.

It was reported that expression of IGF1 decoy suppresses
 25 tumorigenesis of Met-1 cells, while expression of WT IGF1 enhances it. a. **Cell shapes on tissue culture plates.** Met-1 cells that express R36E/R37E (IGF1 decoy) have epithelial-like cell shapes that are different from those of vector only WT IGF1 expressing cells. b. **Western blot analysis of cell lysates.** Met-1 cells that express IGF1 decoy show low Oct-4 and Nanog expression by western blotting of cell lysate. α -tubulin was used as loading controls.
 30 This indicates that IGF1 decoy potentially suppressed the dedifferentiation induced by endogenous IGF1 and/or IGF2.

[0026] **Fig. 7 Continuous infusion of IGF2 decoy using insulin pump technology.** An insulin pump is a small device about the size of a small cell phone that is worn externally. It delivers precise doses of insulin to closely match the body's needs. This technology can be used to infuse IGF1 or IGF2 decoy.

5 [0027] **Fig. 8 (A) Schematic illustration of the micro-patterning assay.** Grids of BSACy5 are printed on epoxy-coated glass coverslips (microcontact printing). The interspaces are filled with streptavidin and biotinylated monoclonal antibodies against the membrane protein bait. In cells grown on such micro-biochips, the bait will be arranged in the plasma membrane according to the antibody micro-pattern. Interactions with a second
 10 fluorescently labeled prey protein are probed by measuring the degree of co-patterning. **(B) Live cell images of HeLa cells transiently expressing GFP-IRS-3 and grown on anti-IR antibody functionalized coverslips.** IR-IRS-3 interaction is probed by strong co-patterning of GFPIRS-3 in IR-enriched micro-domains.

[0028] **Fig. 9: Representative total internal reflection fluorescence (TIRF) images of**
 15 **IGF-IR-mediated β 3-integrin co-recruitment.** HeLa cells were transiently transfected with IGF-IR-RFP and β 3-integrin-GFP and grown on anti-IGF-IR antibody coated micro-biochips. β 3-integrin-GFP co-recruitment into IGF-IR enriched regions indicates interaction between IGF-IR and β 3-integrin.

DEFINITIONS

20 [0029] The term "**inhibiting**" or "**inhibition**," as used herein, refers to any detectable negative effect on a target biological process, such as the binding between IGF2 and integrin α β 3, or on its downstream processes including IGF1 receptor (IGF1R) or insulin receptor type A (IR-A) phosphorylation, AKT and ERK1/2 activation,, as well as cell proliferation, tumorigenicity, and metastatic potential. Typically, an inhibition is reflected in a decrease of
 25 at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or higher in IGF2-integrin binding, or any one of the downstream parameters mentioned above, when compared to a control.

[0030] The term "**nucleic acid**" or "**polynucleotide**" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known
 30 analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses

conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-
5 base and/or deoxyinosine residues (Batzner *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* **8**:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

[0031] The term “**gene**” means the segment of DNA involved in producing a polypeptide chain. It may include regions preceding and following the coding region (leader and trailer)
10 as well as intervening sequences (introns) between individual coding segments (exons).

[0032] The term “**amino acid**” refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the
15 genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified
20 R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. “Amino acid mimetics” refers to chemical compounds having a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

[0033] There are various known methods in the art that permit the incorporation of an
25 unnatural amino acid derivative or analog into a polypeptide chain in a site-specific manner, *see, e.g.*, WO 02/086075.

[0034] Amino acids may be referred to herein by either the commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly
30 accepted single-letter codes.

[0035] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, “conservatively modified

variants” refers to those nucleic acids that encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are “silent variations,” which are one species of conservatively modified variations. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid that encodes a polypeptide is implicit in each described sequence.

[0036] As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a “conservatively modified variant” where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

[0037] The following eight groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Glycine (G);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V);
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W);
- 7) Serine (S), Threonine (T); and
- 8) Cysteine (C), Methionine (M)

(see, e.g., Creighton, *Proteins*, W. H. Freeman and Co., N. Y. (1984)).

[0038] Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

- 5 **[0039]** In the present application, amino acid residues are numbered according to their relative positions from the left most residue, which is numbered 1, in an unmodified wild-type polypeptide sequence.

[0040] As used in herein, the terms “**identical**” or percent “**identity**,” in the context of describing two or more polynucleotide or amino acid sequences, refer to two or more
10 sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (for example, a core amino acid sequence responsible for IGF-integrin binding has at least 80% identity, preferably 85%, 90%, 91%, 92%, 93, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity, to a reference sequence, *e.g.*, C-domain sequence of a wild-type IGF2 protein), when compared and aligned for maximum
15 correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Such sequences are then said to be “substantially identical.” With regard to polynucleotide sequences, this definition also refers to the complement of a test sequence. Preferably, the identity exists over a region that is at least about 50 amino acids or nucleotides in length, or
20 more preferably over a region that is 75-100 amino acids or nucleotides in length.

[0041] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program
25 parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. For sequence comparison of nucleic acids and proteins, the BLAST and BLAST 2.0 algorithms and the default parameters discussed below are used.

- 30 **[0042]** A “comparison window”, as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may

be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981),
 5 by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see,
 10 e.g., *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[0043] Examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, (1990) *J. Mol. Biol.* 215: 403-410 and Altschul *et al.* (1977) *Nucleic Acids Res.* 25: 3389-3402, respectively. Software for performing BLAST analyses is publicly
 15 available at the National Center for Biotechnology Information website, ncbi.nlm.nih.gov. The algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*).

20 These initial neighborhood word hits acts as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). For amino
 25 acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the
 30 sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word size (W) of 28, an expectation (E) of 10, M=1, N=-2, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as

defaults a word size (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

[0044] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787

(1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0045] An indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

[0046] “**Polypeptide**,” “**peptide**,” and “**protein**” are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0047] The term “**effective amount**,” as used herein, refers to an amount that produces therapeutic effects for which a substance is administered. The effects include the prevention, correction, or inhibition of progression of the symptoms of a disease/condition and related complications to any detectable extent. The exact amount will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (*see, e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science*

and Technology of Pharmaceutical Compounding (1999); and Pickar, *Dosage Calculations* (1999)).

[0048] An “**expression cassette**” is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular polynucleotide sequence in a host cell. An expression cassette may be part of a plasmid, viral genome, or nucleic acid fragment. Typically, an expression cassette includes a polynucleotide to be transcribed, operably linked to a promoter.

[0049] As used herein, a “**polypeptide comprising the IGF2-integrin binding region**” refers to a polypeptide containing a core amino acid sequence that generally corresponds to the amino acid sequence of the C-domain of a wild-type IGF2 protein. IGF2 amino acid sequence is shown in Figure 1 (SEQ ID NO:1), and its C-domain sequence is SRVSRRS (SEQ ID NO:2). IGF1 and insulin amino acid sequences are also shown in Figure 1 (SEQ ID NO:3 and SEQ ID NO:4, respectively). Full length amino acid sequence of pre-IGF2 protein is set forth in GenBank Accession No. NP_000603 or P01344 in Swissprot protein database. The mature IGF2 protein amino acid sequence corresponds to the 25-91 segment of the pre-IGF2 protein sequence. This core amino acid sequence may contain some variations such as amino acid deletion, addition, or substitution, but should maintain a substantial level sequence homology (*e.g.*, at least 80%, 85%, 90%, 95%, or higher sequence homology) to the C-domain sequence and is capable of binding integrin $\alpha v \beta 3$. In addition to this core sequence that is responsible for the polypeptide's ability to bind to integrin, one or more amino acid sequences of a homologous origin (*e.g.*, additional sequence from the same protein, IGF2) or a heterologous origin (*e.g.*, sequence from another unrelated protein) can be included in the polypeptide. Some examples of the “polypeptide comprising the IGF2-integrin binding site” include the C-domain sequence or the full length wild type IGF2. Optionally, an affinity or epitope tag (such as a GST tag) can be included in the polypeptide to facilitate purification, isolation, or immobilization of the polypeptide. The polypeptide may be further modified in order to enhance its characteristics for ease of use, improved stability and /or bioavailability via glycosylation, PEGylation, *etc.* or incorporation of one or more non-naturally occurring amino acids such as D-amino acids, so long as its capability of binding integrin $\alpha v \beta 3$ is retained.

[0050] An “**antibody**” refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda,

alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

5 **[0051]** An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H)
10 refer to these light and heavy chains respectively.

[0052] Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H - C_{H1} by a disulfide bond. The $F(ab)'_2$
15 may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see*, Paul (Ed.) *Fundamental Immunology*, Third Edition, Raven Press, NY (1993)). 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
20 synthesized *de novo* either chemically or by utilizing recombinant DNA methodology.

[0053] Further modification of antibodies by recombinant technologies is also well known in the art. For instance, chimeric antibodies combine the antigen binding regions (variable regions) of an antibody from one animal with the constant regions of an antibody from another animal. Generally, the antigen binding regions are derived from a non-human
25 animal, while the constant regions are drawn from human antibodies. The presence of the human constant regions reduces the likelihood that the antibody will be rejected as foreign by a human recipient. On the other hand, "humanized" antibodies combine an even smaller portion of the non-human antibody with human components. Generally, a humanized antibody comprises the hypervariable regions, or complementarity determining regions
30 (CDR), of a non-human antibody grafted onto the appropriate framework regions of a human antibody. Antigen binding sites may be wild type or modified by one or more amino acid substitutions, *e.g.*, modified to resemble human immunoglobulin more closely. Both

chimeric and humanized antibodies are made using recombinant techniques, which are well-known in the art (*see, e.g., Jones et al. (1986) Nature 321:522-525*).

[0054] Thus, the term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or antibodies synthesized *de novo* using recombinant DNA methodologies (*e.g., single chain Fv, a chimeric or humanized antibody*).

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0055] Efforts were made to develop dominant-negative IGF2 mutants using a strategy that was successfully used to develop dominant-negative IGF1 mutants that retain the ability to bind IGF1R but have diminished ability to bind integrins. IGF2 is structurally similar to IGF1, and amino acid residues of IGF1 that are critical for integrin binding to IGF1 are conserved in IGF2. The dominant-negative IGF2 mutants are studied for their ability to suppress IR-A together with IGF1R and block the IR-A/IGF2 loop in cancer. In their preliminary studies, the present inventors have confirmed that IGF2 binds to integrins. They have also generated several integrin-binding defective IGF2 mutants. It has been shown that these mutants are defective in signaling functions and suppress cell viability increased by wild-type (WT) IGF2, *i.e., a dominant-negative effect*. These dominant-negative IGF2 mutants or IGF2-decoys are useful as therapeutic agents in cancer treatment, due to their ability to effectively suppress IR-A (and IGF1R).

II. Production of IGF2-Related Polypeptides

A. General Recombinant Technology

[0056] Basic texts disclosing general methods and techniques in the field of recombinant genetics include Sambrook and Russell, *Molecular Cloning, A Laboratory Manual* (3rd ed. 2001); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and Ausubel *et al., eds., Current Protocols in Molecular Biology* (1994).

[0057] For nucleic acids, sizes are given in either kilobases (kb) or base pairs (bp). These are estimates derived from agarose or acrylamide gel electrophoresis, from sequenced nucleic acids, or from published DNA sequences. For proteins, sizes are given in kilodaltons (kDa) or amino acid residue numbers. Proteins sizes are estimated from gel electrophoresis, from sequenced proteins, from derived amino acid sequences, or from published protein sequences.

[0058] Oligonucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* **22**: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et al.*, *Nucleic Acids Res.* **12**: 6159-6168 (1984).

5 Purification of oligonucleotides is performed using any art-recognized strategy, *e.g.*, native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* **255**: 137-149 (1983).

[0059] The sequence of an IGF2 gene, a polynucleotide encoding a polypeptide comprising the integrin-binding domain of IGF2 (*i.e.*, the C domain), and synthetic oligonucleotides can
10 be verified after cloning or subcloning using, *e.g.*, the chain termination method for sequencing double-stranded templates of Wallace *et al.*, *Gene* **16**: 21-26 (1981).

B. Coding Sequence for a IGF2-Related Polypeptide

[0060] Polynucleotide sequences encoding a wild-type IGF2 protein, especially a wild-type human IGF2 protein, have been determined and may be obtained from a commercial supplier.
15 For example, the GenBank Accession Nos. for human IGF2 mRNA and protein sequences are NM_000612 and NP_000603, respectively.

[0061] The rapid progress in the studies of human genome has made possible a cloning approach where a human DNA sequence database can be searched for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one
20 encoding a previously identified human IGF. Any DNA sequence so identified can be subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human cDNA or genomic library using a synthetic probe may be necessary to obtain a larger
25 gene.

[0062] Alternatively, a nucleic acid sequence encoding a human IGF2 can be isolated from a human cDNA or genomic DNA library using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence encoding an IGF2. Most commonly used techniques for this
30 purpose are described in standard texts, *e.g.*, Sambrook and Russell, *supra*.

[0063] cDNA libraries suitable for obtaining a coding sequence for a human IGF may be commercially available or can be constructed. The general methods of isolating mRNA,

making cDNA by reverse transcription, ligating cDNA into a recombinant vector, transfecting into a recombinant host for propagation, screening, and cloning are well known (*see, e.g.,* Gubler and Hoffman, *Gene*, **25**: 263-269 (1983); Ausubel *et al., supra*). Upon obtaining an amplified segment of nucleotide sequence by PCR, the segment can be further used as a probe to isolate the full length polynucleotide sequence encoding the IGF2 from the cDNA library. A general description of appropriate procedures can be found in Sambrook and Russell, *supra*.

[0064] A similar procedure can be followed to obtain a full-length sequence encoding a human IGF2 from a human genomic library. Human genomic libraries are commercially available or can be constructed according to various art-recognized methods. In general, to construct a genomic library, the DNA is first extracted from a tissue where an IGF2 is likely found. The DNA is then either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb in length. The fragments are subsequently separated by gradient centrifugation from polynucleotide fragments of undesired sizes and are inserted in bacteriophage λ vectors. These vectors and phages are packaged *in vitro*. Recombinant phages are analyzed by plaque hybridization as described in Benton and Davis, *Science*, **196**: 180-182 (1977). Colony hybridization is carried out as described by Grunstein *et al., Proc. Natl. Acad. Sci. USA*, **72**: 3961-3965 (1975).

[0065] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (*see, e.g.,* White *et al., PCR Protocols: Current Methods and Applications*, 1993; Griffin and Griffin, *PCR Technology*, CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full-length nucleic acid encoding an IGF2 is obtained.

[0066] Upon acquiring a nucleic acid sequence encoding an IGF2, the coding sequence can be further modified by a number of well known techniques such as restriction endonuclease digestion, PCR, and PCR-related methods to generate coding sequences for IGF2-related polypeptides, including IGF2 mutants (especially the dominant-negative type) and polypeptides comprising an integrin-binding sequence derived from an IGF2. The polynucleotide sequence encoding a desired IGF2-related polypeptide can then be subcloned into a vector, for instance, an expression vector, so that a recombinant polypeptide can be produced from the resulting construct. Further modifications to the coding sequence, *e.g.,*

nucleotide substitutions, may be subsequently made to alter the characteristics of the polypeptide.

[0067] A variety of mutation-generating protocols are established and described in the art, and can be readily used to modify a polynucleotide sequence encoding an IGF-related polypeptide. *See, e.g., Zhang et al., Proc. Natl. Acad. Sci. USA*, **94**: 4504-4509 (1997); and Stemmer, *Nature*, **370**: 389-391 (1994). The procedures can be used separately or in combination to produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[0068] Mutational methods of generating diversity include, for example, site-directed mutagenesis (Botstein and Shortle, *Science*, **229**: 1193-1201 (1985)), mutagenesis using uracil-containing templates (Kunkel, *Proc. Natl. Acad. Sci. USA*, **82**: 488-492 (1985)), oligonucleotide-directed mutagenesis (Zoller and Smith, *Nucl. Acids Res.*, **10**: 6487-6500 (1982)), phosphorothioate-modified DNA mutagenesis (Taylor *et al.*, *Nucl. Acids Res.*, **13**: 8749-8764 and 8765-8787 (1985)), and mutagenesis using gapped duplex DNA (Kramer *et al.*, *Nucl. Acids Res.*, **12**: 9441-9456 (1984)).

[0069] Other possible methods for generating mutations include point mismatch repair (Kramer *et al.*, *Cell*, **38**: 879-887 (1984)), mutagenesis using repair-deficient host strains (Carter *et al.*, *Nucl. Acids Res.*, **13**: 4431-4443 (1985)), deletion mutagenesis (Eghtedarzadeh and Henikoff, *Nucl. Acids Res.*, **14**: 5115 (1986)), restriction-selection and restriction-purification (Wells *et al.*, *Phil. Trans. R. Soc. Lond. A*, **317**: 415-423 (1986)), mutagenesis by total gene synthesis (Nambiar *et al.*, *Science*, **223**: 1299-1301 (1984)), double-strand break repair (Mandecki, *Proc. Natl. Acad. Sci. USA*, **83**: 7177-7181 (1986)), mutagenesis by polynucleotide chain termination methods (U.S. Patent No. 5,965,408), and error-prone PCR (Leung *et al.*, *Biotechniques*, **1**: 11-15 (1989)).

C. Modification of Nucleic Acids for Preferred Codon Usage in a Host Organism

[0070] The polynucleotide sequence encoding an IGF2-related polypeptide can be further altered to coincide with the preferred codon usage of a particular host. For example, the preferred codon usage of one strain of bacterial cells can be used to derive a polynucleotide that encodes a recombinant polypeptide of the invention and includes the codons favored by this strain. The frequency of preferred codon usage exhibited by a host cell can be calculated by averaging frequency of preferred codon usage in a large number of genes expressed by the

host cell (*e.g.*, calculation service is available from web site of the Kazusa DNA Research Institute, Japan). This analysis is preferably limited to genes that are highly expressed by the host cell.

[0071] At the completion of modification, the coding sequences are verified by sequencing and are then subcloned into an appropriate expression vector for recombinant production of the IGFs-related polypeptides.

D. Chemical Synthesis of IGF2-Related Polypeptides

[0072] The amino acid sequence of integrin-bind site derived from human IGF2, such as the C-domain sequence of IGF2, is provided. A polypeptide comprising this IGF2-integrin binding sequence thus can also be chemically synthesized using conventional peptide synthesis or other protocols well known in the art.

[0073] Polypeptides may be synthesized by solid-phase peptide synthesis methods using procedures similar to those described by Merrifield *et al.*, *J. Am. Chem. Soc.*, **85**:2149-2156 (1963); Barany and Merrifield, *Solid-Phase Peptide Synthesis*, in *The Peptides: Analysis, Synthesis, Biology* Gross and Meienhofer (eds.), Academic Press, N.Y., vol. **2**, pp. 3-284 (1980); and Stewart *et al.*, *Solid Phase Peptide Synthesis* 2nd ed., Pierce Chem. Co., Rockford, Ill. (1984). During synthesis, N- α -protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to a solid support, *i.e.*, polystyrene beads. The peptides are synthesized by linking an amino group of an N- α -deprotected amino acid to an α -carboxy group of an N- α -protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N- α -protecting groups include Boc, which is acid labile, and Fmoc, which is base labile.

[0074] Materials suitable for use as the solid support are well known to those of skill in the art and include, but are not limited to, the following: halomethyl resins, such as chloromethyl resin or bromomethyl resin; hydroxymethyl resins; phenol resins, such as 4-(α -[2,4-dimethoxyphenyl]-Fmoc-aminomethyl)phenoxy resin; tert-alkyloxycarbonyl-hydrazidated resins, and the like. Such resins are commercially available and their methods of preparation are known by those of ordinary skill in the art.

[0075] Briefly, the C-terminal N- α -protected amino acid is first attached to the solid support. The N- α -protecting group is then removed. The deprotected α -amino group is

coupled to the activated α -carboxylate group of the next N- α -protected amino acid. The process is repeated until the desired peptide is synthesized. The resulting peptides are then cleaved from the insoluble polymer support and the amino acid side chains deprotected. Longer peptides can be derived by condensation of protected peptide fragments. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (See, Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press (1989), and Bodanszky, *Peptide Chemistry, A Practical Textbook*, 2nd Ed., Springer-Verlag (1993)).

III. Expression and Purification of IGF2-Related Polypeptides

[0076] Following verification of the coding sequence, an IGF2-related polypeptide of the present invention can be produced using routine techniques in the field of recombinant genetics, relying on the polynucleotide sequences encoding the polypeptide disclosed herein.

A. Expression Systems

[0077] To obtain high level expression of a nucleic acid encoding an IGF2-related polypeptide of the present invention, one typically subclones a polynucleotide encoding the polypeptide into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook and Russell, *supra*, and Ausubel *et al.*, *supra*. Bacterial expression systems for expressing the polypeptide are available in, *e.g.*, *E. coli*, *Bacillus sp.*, *Salmonella*, and *Caulobacter*. Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. In one embodiment, the eukaryotic expression vector is an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[0078] The promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is optionally positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0079] In addition to the promoter, the expression vector typically includes a transcription unit or expression cassette that contains all the additional elements required for the expression of the IGF2-related polypeptide in host cells. A typical expression cassette thus

contains a promoter operably linked to the nucleic acid sequence encoding the IGF2-related polypeptide and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. The nucleic acid sequence encoding the IGF2-related polypeptide is typically linked to a cleavable signal peptide sequence to promote secretion of the polypeptide by the transformed cell. Such signal peptides include, among others, the signal peptides from tissue plasminogen activator, insulin, and neuron growth factor, and juvenile hormone esterase of *Heliothis virescens*. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

[0080] In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

[0081] The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as GST and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, *e.g.*, c-myc.

[0082] Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, *e.g.*, SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

[0083] Some expression systems have markers that provide gene amplification such as thymidine kinase, hygromycin B phosphotransferase, and dihydrofolate reductase.

Alternatively, high yield expression systems not involving gene amplification are also suitable, such as a baculovirus vector in insect cells, with a polynucleotide sequence encoding

the NRG-related polypeptide under the direction of the polyhedrin promoter or other strong baculovirus promoters.

[0084] The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are optionally chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary. Similar to antibiotic resistance selection markers, metabolic selection markers based on known metabolic pathways may also be used as a means for selecting transformed host cells.

[0085] When periplasmic expression of a recombinant protein (*e.g.*, an IGF2-related polypeptide of the present invention) is desired, the expression vector further comprises a sequence encoding a secretion signal, such as the *E. coli* OppA (Periplasmic Oligopeptide Binding Protein) secretion signal or a modified version thereof, which is directly connected to 5' of the coding sequence of the protein to be expressed. This signal sequence directs the recombinant protein produced in cytoplasm through the cell membrane into the periplasmic space. The expression vector may further comprise a coding sequence for signal peptidase 1, which is capable of enzymatically cleaving the signal sequence when the recombinant protein is entering the periplasmic space. More detailed description for periplasmic production of a recombinant protein can be found in, *e.g.*, Gray *et al.*, *Gene* **39**: 247-254 (1985), U.S. Patent Nos. 6,160,089 and 6,436,674.

[0086] A person skilled in the art will recognize that various conservative substitutions can be made to any wild-type or mutant IGF2 or a polypeptide comprising an integrin-binding sequence of IGF2, to produce a modified polypeptide that, while still retaining the ability to bind integrin, does not trigger IGF2 downstream signaling. Moreover, modifications of a polynucleotide coding sequence may also be made to accommodate preferred codon usage in a particular expression host without altering the resulting amino acid sequence.

B. Transfection Methods

[0087] Standard transfection methods are used to produce bacterial, mammalian, yeast, insect, or plant cell lines that express large quantities of an IGF2-related polypeptide, which are then purified using standard techniques (*see, e.g.*, Colley *et al.*, *J. Biol. Chem.* **264**:

17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (*see, e.g.*, Morrison, *J. Bact.* **132**: 349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* **101**: 347-362 (Wu *et al.*, eds, 1983).

- 5 [0088] Any of the well known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, or other foreign genetic material into a host cell (*see, e.g.*, Sambrook and
10 Russell, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing the IGF2-related polypeptide.

C. Purification of Recombinantly Produced IGF2-Related Polypeptides

- [0089] Once the expression of a recombinant IGF2-related polypeptide in transfected host
15 cells is confirmed, *e.g.*, via an immunoassay such as Western blotting assay, the host cells are then cultured in an appropriate scale for the purpose of purifying the recombinant polypeptide.

1. **Purification of Recombinantly Produced Polypeptides from Bacteria**

- [0090] When the IGF2-related polypeptides of the present invention are produced
20 recombinantly by transformed bacteria in large amounts, typically after promoter induction, although expression can be constitutive, the polypeptides may form insoluble aggregates. There are several protocols that are suitable for purification of protein inclusion bodies. For example, purification of aggregate proteins (hereinafter referred to as inclusion bodies) typically involves the extraction, separation and/or purification of inclusion bodies by
25 disruption of bacterial cells, *e.g.*, by incubation in a buffer of about 100-150 µg/ml lysozyme and 0.1% Nonidet P40, a non-ionic detergent. The cell suspension can be ground using a Polytron grinder (Brinkman Instruments, Westbury, NY). Alternatively, the cells can be sonicated on ice. Additional methods of lysing bacteria are described in Ausubel *et al.* and Sambrook and Russell, both *supra*, and will be apparent to those of skill in the art.
- 30 [0091] The cell suspension is generally centrifuged and the pellet containing the inclusion bodies resuspended in buffer which does not dissolve but washes the inclusion bodies, *e.g.*, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 150 mM NaCl and 2% Triton-X 100, a non-ionic

detergent. It may be necessary to repeat the wash step to remove as much cellular debris as possible. The remaining pellet of inclusion bodies may be resuspended in an appropriate buffer (*e.g.*, 20 mM sodium phosphate, pH 6.8, 150 mM NaCl). Other appropriate buffers will be apparent to those of skill in the art.

5 **[0092]** Following the washing step, the inclusion bodies are solubilized by the addition of a solvent that is both a strong hydrogen acceptor and a strong hydrogen donor (or a combination of solvents each having one of these properties). The proteins that formed the inclusion bodies may then be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to, urea (from about 4 M to about 8 M),
10 formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents that are capable of solubilizing aggregate-forming proteins, such as SDS (sodium dodecyl sulfate) and 70% formic acid, may be inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine
15 hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of the immunologically and/or biologically active protein of interest. After solubilization, the protein can be separated from other bacterial proteins by standard separation techniques. For further description of purifying recombinant polypeptides from
20 bacterial inclusion body, *see, e.g., Patra et al., Protein Expression and Purification* **18**: 182-190 (2000).

[0093] Alternatively, it is possible to purify recombinant polypeptides, *e.g.*, an IGF2-related polypeptide, from bacterial periplasm. Where the recombinant protein is exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by
25 cold osmotic shock in addition to other methods known to those of skill in the art (*see e.g., Ausubel et al., supra*). To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell
30 suspension is centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

2. Standard Protein Separation Techniques for Purification

[0094] When a recombinant polypeptide of the present invention, *e.g.*, an IGF2 mutant or a polypeptide comprising an IGF2-integrin binding sequence, is expressed in host cells in a soluble form, its purification can follow the standard protein purification procedure described below. This standard purification procedure is also suitable for purifying IGF2-related polypeptides obtained from chemical synthesis.

i. Solubility Fractionation

[0095] Often as an initial step, and if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest, *e.g.*, an IGF2-related polypeptide of the present invention. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol is to add saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This will precipitate the most hydrophobic proteins. The precipitate is discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, through either dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

ii. Size Differential Filtration

[0096] Based on a calculated molecular weight, a protein of greater and lesser size can be isolated using ultrafiltration through membranes of different pore sizes (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of a protein of interest, *e.g.*, an IGF2-related polypeptide. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

iii. Column Chromatography

[0097] The proteins of interest (such as an IGF-related polypeptide of the present invention) can also be separated from other proteins on the basis of their size, net surface charge, hydrophobicity, or affinity for ligands. In addition, antibodies raised against a segment of IGF2 such as the integrin-binding site can be conjugated to column matrices and the IGF2-related polypeptide immunopurified. All of these methods are well known in the art.

[0098] It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (*e.g.*, Pharmacia Biotech).

IV. Identification of Inhibitors for IGF-Integrin Binding**A. IGF-Integrin Binding Assays**

[0099] An *in vitro* assay can be used to detect IGF2-integrin binding and to identify compounds that are capable of inhibiting IGF2-integrin binding. In general, such an assay can be performed in the presence of an IGF2, such as human IGF2, and an integrin, such as $\alpha v \beta 3$, that are known to bind each other, under conditions permitting such binding. For convenience, one of the binding partners may be immobilized onto a solid support and/or labeled with a detectable moiety. A third molecule, such as an antibody (which may include a detectable label) to one of the binding partners, can also be used to facilitate detection.

[0100] In some cases, the binding assays can be performed in a cell-free environment; whereas in other cases, the binding assays can be performed on cell surface, frequently using cells recombinantly or endogenously expressing an appropriate integrin molecule. More details and some examples of such binding assays can be found in the Examples section of this application.

[0101] To screen for compounds capable of inhibiting IGF2-integrin binding, the above-described assays are performed both in the presence and absence of a test compound, the level of IGF2-integrin binding is then compared. If IGF2-integrin binding is suppressed at the presence of the test compound at a level of at least 10%, more preferably at least 20%, 30%, 40%, or 50%, or even higher, the test compound is then deemed an inhibitor of IGF2-integrin binding and may be subject to further testing to confirm its ability to inhibit IGF2 signaling.

[0102] The binding assay is also useful for confirming that a polypeptide comprising an integrin-binding sequence derived from an IGF can indeed specifically bind integrin. For instance, a polypeptide comprising the C-domain of an IGF2 protein but not the full length IGF2 sequence may be recombinantly expressed, purified, and placed in a binding assay with integrin $\alpha\beta 3$, substituting a full length wild type IGF2 protein, which is used in a control assay to provide a comparison basis. If deemed to have sufficient integrin-binding ability, a polypeptide comprising an IGF2-integrin binding sequence can then be used, in place of a wild-type full length IGF2 protein, in a binding assay for identifying inhibitors of IGF2-integrin binding. Similarly, a polypeptide comprising a core sequence with a high level of homology (*e.g.*, 90%, 95% or higher) to C-domain sequence of a wild-type IGF2 protein can be tested and, if appropriate, can be used, in place of a wild-type full length IGF2 protein, in a binding assay for identifying inhibitors of IGF2-integrin binding.

[0103] Inhibitors of IGF2-integrin binding can have diverse chemical and structural features. For instance, an inhibitor can be a non-functional IGF2 mutant that retaining integrin-binding ability, an antibody to either IGF2 or intergrin that interferes with IGF2-integrin binding, or any small molecule or macromolecule that simply hinders the interaction between IGF2 and integrin. Essentially any chemical compound can be tested as a potential inhibitor of IGF2-integrin binding. Most preferred are generally compounds that can be dissolved in aqueous or organic (especially DMSO-based) solutions. Inhibitors can be identified by screening a combinatorial library containing a large number of potentially effective compounds. Such combinatorial chemical libraries can be screened in one or more assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

[0104] Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)) and carbohydrate libraries (*see, e.g.*, Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (PCT Publication No. WO 91/19735), encoded peptides (PCT Publication WO 93/20242), random bio-oligomers (PCT Publication No. WO

92/00091), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with β -D-glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (*see*, Ausubel, Berger and Sambrook, *all supra*), peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083), antibody libraries (*see, e.g.*, Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; and benzodiazepines, U.S. Patent 5,288,514).

15 B. IGF2 Signaling Assays

[0105] The inhibitors of IGF2-integrin binding are useful for their ability to inhibit IGF2 signaling, especially as anti-cancer therapeutics for cancer patients overexpressing one or more integrin molecules. Assays for confirming such inhibitory effect of an inhibitor can be performed *in vitro* or *in vivo*. An *in vitro* assay typically involves exposure of cultured cells to an inhibitor and monitoring of subsequent biological and biochemical changes in the cells. For example, following exposure to 0.1-20 μ g/ml an inhibitor for 0.5-48 hours, suitable cells (such as those expressing integrin α v β 3) are examined for their proliferation/survival status using methods such as direct cell number counting, BrdU or H³-thymidine incorporation, tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation assay, chicken embryo allantoic membrane (CAM) assay, TUNNEL assay, annexin V binding assay, *etc.* Further downstream changes due to IGF2 signaling, *e.g.*, phosphorylation of IGF1R, IR-A, AKT or ERK1/2 activation, can also be monitored to provide an indication of suppressed IGF2 signaling. In addition, tumorigenicity of cancer cells is useful parameters for monitoring and can be tested by methods such as colony formation assays or soft agar assays. Detailed description of some exemplary assays can be found in the Examples section of this disclosure. An inhibitory effect is detected when a decrease in IGFs signaling, as indicated by any one

aforementioned parameter, of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more is observed.

[0106] The effects of a IGF2-integrin binding inhibitor of the present invention can also be demonstrated in *in vivo* assays. For example, an inhibitor of IGF2-integrin can be injected into animals that have a compromised immune system (*e.g.*, nude mice, SCID mice, or NOD/SCID mice) and therefore permit xenograft tumors. Injection methods can be intravenous, intraperitoneal, or intratumoral in nature. Tumor development is subsequently monitored by various means, such as measuring tumor volume and scoring secondary lesions due to metastases, in comparison with a control group of animals with similar tumors but not given the inhibitors. The Examples section of this disclosure provides detailed description of some exemplary *in vivo* assays. An inhibitory effect is detected when a negative effect on tumor growth or metastasis is established in the test group. Preferably, the negative effect is at least a 10% decrease; more preferably, the decrease is at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%.

V. Pharmaceutical Compositions and Administration

[0107] The present invention also provides pharmaceutical compositions or physiological compositions comprising an effective amount of a compound that inhibits IGF-integrin binding, such as a dominant negative IGF2 mutant R24E/R37E/R38E, R34E/R37E/R38E, or R24E/R34E/R37E/R38E, or its encoding nucleic acid, inhibiting IGF2 signaling in both prophylactic and therapeutic applications. Such pharmaceutical or physiological compositions also include one or more pharmaceutically or physiologically acceptable excipients or carriers. Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, *see*, Langer, *Science* **249**: 1527-1533 (1990).

[0108] The pharmaceutical compositions of the present invention can be administered by various routes, *e.g.*, oral, subcutaneous, transdermal, intramuscular, intravenous, or intraperitoneal. The preferred routes of administering the pharmaceutical compositions are local delivery to an organ or tissue suffering from a condition exacerbated by IGF2 overexpression (*e.g.*, intratumor injection to a tumor) at daily doses of about 0.01 - 5000 mg, preferably 5-500 mg, of an IGF2-integrin binding inhibitor for a 70 kg adult human per day.

The appropriate dose may be administered in a single daily dose or as divided doses presented at appropriate intervals, for example as two, three, four, or more subdoses per day.

[0109] For preparing pharmaceutical compositions containing an IGF2-integrin inhibitor, inert and pharmaceutically acceptable carriers are used. The pharmaceutical carrier can be either solid or liquid. Solid form preparations include, for example, powders, tablets, dispersible granules, capsules, cachets, and suppositories. A solid carrier can be one or more substances that can also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, or tablet disintegrating agents; it can also be an encapsulating material.

[0110] In powders, the carrier is generally a finely divided solid that is in a mixture with the finely divided active component, *e.g.*, an IGF2 dominant negative mutant polypeptide. In tablets, the active ingredient (an inhibitor of IGF2-integrin binding) is mixed with the carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired.

[0111] For preparing pharmaceutical compositions in the form of suppositories, a low-melting wax such as a mixture of fatty acid glycerides and cocoa butter is first melted and the active ingredient is dispersed therein by, for example, stirring. The molten homogeneous mixture is then poured into convenient-sized molds and allowed to cool and solidify.

[0112] Powders and tablets preferably contain between about 5% to about 70% by weight of the active ingredient of an inhibitor of IGF2-integrin binding. Suitable carriers include, for example, magnesium carbonate, magnesium stearate, talc, lactose, sugar, pectin, dextrin, starch, tragacanth, methyl cellulose, sodium carboxymethyl cellulose, a low-melting wax, cocoa butter, and the like.

[0113] The pharmaceutical compositions can include the formulation of the active compound of an IGF2-integrin binding inhibitor with encapsulating material as a carrier providing a capsule in which the inhibitor (with or without other carriers) is surrounded by the carrier, such that the carrier is thus in association with the compound. In a similar manner, cachets can also be included. Tablets, powders, cachets, and capsules can be used as solid dosage forms suitable for oral administration.

[0114] Liquid pharmaceutical compositions include, for example, solutions suitable for oral or parenteral administration, suspensions, and emulsions suitable for oral administration. Sterile water solutions of the active component (*e.g.*, a dominant-negative IGF2 mutant polypeptide) or sterile solutions of the active component in solvents comprising water,

buffered water, saline, PBS, ethanol, or propylene glycol are examples of liquid compositions suitable for parenteral administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like.

[0115] Sterile solutions can be prepared by dissolving the active component (*e.g.*, an IGF2-integrin binding inhibitor) in the desired solvent system, and then passing the resulting solution through a membrane filter to sterilize it or, alternatively, by dissolving the sterile compound in a previously sterilized solvent under sterile conditions. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9, and most preferably from 7 to 8.

[0116] The pharmaceutical compositions containing IGF2-integrin binding inhibitors can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions are administered to a patient already suffering from a condition that may be exacerbated by the overexpression of IGF2 or integrin family members in an amount sufficient to prevent, cure, reverse, or at least partially slow or arrest the symptoms of the condition and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend on the severity of the disease or condition and the weight and general state of the patient, but generally range from about 0.1 mg to about 2,000 mg of the inhibitor per day for a 70 kg patient, with dosages of from about 5 mg to about 500 mg of the inhibitor per day for a 70 kg patient being more commonly used.

[0117] In prophylactic applications, pharmaceutical compositions containing IGF2-integrin binding inhibitors are administered to a patient susceptible to or otherwise at risk of developing a disease or condition in which overexpression of IGF2 or integrin is undesirable, in an amount sufficient to delay or prevent the onset of the symptoms. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts of the inhibitor again depend on the patient's state of health and weight, but generally range from about 0.1 mg to about 2,000 mg of the inhibitor for a 70 kg patient per day, more commonly from about 5 mg to about 500 mg for a 70 kg patient per day.

[0118] Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of an IGF2-integrin binding sufficient to effectively inhibit IGF2 signaling in the patient, either therapeutically or prophylatically.

5 VI. Therapeutic Applications Using Nucleic Acids

[0119] A variety of diseases can be treated by therapeutic approaches that involve introducing a nucleic acid encoding a polypeptide inhibitor of integrin-IGF2 binding into a cell such that the coding sequence is transcribed and the polypeptide inhibitor is produced in the cell. Diseases amenable to treatment by this approach include a broad spectrum of solid
10 tumors, the survival and growth of which rely on to some extent the continued signaling of IGF2 or integrin family members. For discussions on the application of gene therapy towards the treatment of genetic as well as acquired diseases, *see*, Miller *Nature* **357**:455-460 (1992); and Mulligan *Science* **260**:926-932 (1993).

A. Vectors for Gene Delivery

15 [0120] For delivery to a cell or organism, a polynucleotide encoding a polypeptide that inhibits IGF2-integrin binding (such as the dominant-negative mutant R24E/R37E/R38E, R34E/R37E/R38E, or R24E/R34E/R37E/R38E) can be incorporated into a vector. Examples of vectors used for such purposes include expression plasmids capable of directing the expression of the nucleic acids in the target cell. In other instances, the vector is a viral
20 vector system wherein the polynucleotide is incorporated into a viral genome that is capable of transfecting the target cell. In a preferred embodiment, the polynucleotide encoding a polypeptide inhibitor can be operably linked to expression and control sequences that can direct expression of the polypeptide in the desired target host cells. Thus, one can achieve expression of the polypeptide inhibitor under appropriate conditions in the target cell.

25 B. Gene Delivery Systems

[0121] Viral vector systems useful in the expression of a polypeptide inhibitor of IGF2-integrin binding include, for example, naturally occurring or recombinant viral vector systems. Depending upon the particular application, suitable viral vectors include replication competent, replication deficient, and conditionally replicating viral vectors. For example,
30 viral vectors can be derived from the genome of human or bovine adenoviruses, vaccinia virus, herpes virus, adeno-associated virus, minute virus of mice (MVM), HIV, sindbis virus, and retroviruses (including but not limited to Rous sarcoma virus), and MoMLV. Typically,

the genes of interest (*e.g.*, one encoding for a polypeptide inhibitor of the present invention) are inserted into such vectors to allow packaging of the gene construct, typically with accompanying viral DNA, followed by infection of a sensitive host cell and expression of the gene of interest.

5 [0122] As used herein, “gene delivery system” refers to any means for the delivery of a nucleic acid of the invention to a target cell. In some embodiments of the invention, nucleic acids are conjugated to a cell receptor ligand for facilitated uptake (*e.g.*, invagination of coated pits and internalization of the endosome) through an appropriate linking moiety, such as a DNA linking moiety (Wu *et al.*, *J. Biol. Chem.* **263**:14621-14624 (1988); WO
10 92/06180). For example, nucleic acids can be linked through a polylysine moiety to asialo-
oromucoid, which is a ligand for the asialoglycoprotein receptor of hepatocytes.

[0123] Similarly, viral envelopes used for packaging gene constructs that include the nucleic acids of the invention can be modified by the addition of receptor ligands or antibodies specific for a receptor to permit receptor-mediated endocytosis into specific cells
15 (*see, e.g.*, WO 93/20221, WO 93/14188, and WO 94/06923). In some embodiments of the invention, the DNA constructs of the invention are linked to viral proteins, such as adenovirus particles, to facilitate endocytosis (Curiel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**:8850-8854 (1991)). In other embodiments, molecular conjugates of the instant invention can include microtubule inhibitors (WO/9406922), synthetic peptides mimicking influenza
20 virus hemagglutinin (Plank *et al.*, *J. Biol. Chem.* **269**:12918-12924 (1994)), and nuclear localization signals such as SV40 T antigen (WO93/19768).

[0124] Retroviral vectors may also be useful for introducing the coding sequence of a polypeptide inhibitor of the invention into target cells or organisms. Retroviral vectors are produced by genetically manipulating retroviruses. The viral genome of retroviruses is RNA.
25 Upon infection, this genomic RNA is reverse transcribed into a DNA copy which is integrated into the chromosomal DNA of transduced cells with a high degree of stability and efficiency. The integrated DNA copy is referred to as a provirus and is inherited by daughter cells as is any other gene. The wild type retroviral genome and the proviral DNA have three genes: the *gag*, the *pol* and the *env* genes, which are flanked by two long terminal repeat
30 (LTR) sequences. The *gag* gene encodes the internal structural (nucleocapsid) proteins; the *pol* gene encodes the RNA directed DNA polymerase (reverse transcriptase); and the *env* gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs. Adjacent to the 5' LTR are sequences

necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsulation of viral RNA into particles (the Psi site) (*see*, Mulligan, In: *Experimental Manipulation of Gene Expression*, Inouye (ed), 155-173 (1983); Mann *et al.*, *Cell* **33**:153-159 (1983); Cone and Mulligan, *Proceedings of the National Academy of Sciences, U.S.A.*, **81**:6349-6353 (1984)).

[0125] The design of retroviral vectors is well known to those of ordinary skill in the art. In brief, if the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a *cis* acting defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all virion proteins. Retroviral genomes from which these sequences have been deleted, as well as cell lines containing the mutant genome stably integrated into the chromosome are well known in the art and are used to construct retroviral vectors. Preparation of retroviral vectors and their uses are described in many publications including, *e.g.*, European Patent Application EPA 0 178 220; U.S. Patent 4,405,712, Gilboa *Biotechniques* **4**:504-512 (1986); Mann *et al.*, *Cell* **33**:153-159 (1983); Cone and Mulligan *Proc. Natl. Acad. Sci. USA* **81**:6349-6353 (1984); Eglitis *et al.* *Biotechniques* **6**:608-614 (1988); Miller *et al.* *Biotechniques* **7**:981-990 (1989); Miller (1992) *supra*; Mulligan (1993), *supra*; and WO 92/07943.

[0126] The retroviral vector particles are prepared by recombinantly inserting the desired nucleotide sequence into a retrovirus vector and packaging the vector with retroviral capsid proteins by use of a packaging cell line. The resultant retroviral vector particle is incapable of replication in the host cell but is capable of integrating into the host cell genome as a proviral sequence containing the desired nucleotide sequence. As a result, the patient is capable of producing, for example, a polypeptide or polynucleotide of the invention and thus restore the cells to a normal phenotype.

[0127] Packaging cell lines that are used to prepare the retroviral vector particles are typically recombinant mammalian tissue culture cell lines that produce the necessary viral structural proteins required for packaging, but which are incapable of producing infectious virions. The defective retroviral vectors that are used, on the other hand, lack these structural genes but encode the remaining proteins necessary for packaging. To prepare a packaging cell line, one can construct an infectious clone of a desired retrovirus in which the packaging site has been deleted. Cells comprising this construct will express all structural viral proteins, but the introduced DNA will be incapable of being packaged. Alternatively, packaging cell

lines can be produced by transforming a cell line with one or more expression plasmids encoding the appropriate core and envelope proteins. In these cells, the *gag*, *pol*, and *env* genes can be derived from the same or different retroviruses.

[0128] A number of packaging cell lines suitable for the present invention are also available in the prior art. Examples of these cell lines include Crip, GPE86, PA317 and PG13 (*see Miller et al., J. Virol.* **65**:2220-2224 (1991)). Examples of other packaging cell lines are described in Cone and Mulligan *Proceedings of the National Academy of Sciences, USA*, **81**:6349-6353 (1984); Danos and Mulligan *Proceedings of the National Academy of Sciences, USA*, **85**:6460-6464 (1988); Eglitis *et al.* (1988), *supra*; and Miller (1990), *supra*.

[0129] Packaging cell lines capable of producing retroviral vector particles with chimeric envelope proteins may be used. Alternatively, amphotropic or xenotropic envelope proteins, such as those produced by PA317 and GPX packaging cell lines may be used to package the retroviral vectors.

C. Pharmaceutical formulations

[0130] When used for pharmaceutical purposes, the nucleic acid encoding an IGF-integrin binding inhibitor polypeptide is generally formulated in a suitable buffer, which can be any pharmaceutically acceptable buffer, such as phosphate buffered saline or sodium phosphate/sodium sulfate, Tris buffer, glycine buffer, sterile water, and other buffers known to the ordinarily skilled artisan such as those described by Good *et al. Biochemistry* **5**:467 (1966).

[0131] The compositions can additionally include a stabilizer, enhancer or other pharmaceutically acceptable carriers or vehicles. A pharmaceutically acceptable carrier can contain a physiologically acceptable compound that acts, for example, to stabilize the nucleic acids of the invention and any associated vector. A physiologically acceptable compound can include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives, which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. Examples of carriers, stabilizers or adjuvants can be found in Remington's *Pharmaceutical Sciences*, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985).

D. Administration of Formulations

[0132] The formulations containing a nucleic acid encoding a polypeptide inhibitor of the binding between IGF2 and integrin can be delivered to any tissue or organ using any delivery method known to the ordinarily skilled artisan. In some embodiments of the invention, the nucleic acids encoding the inhibitor polypeptides are formulated for intravenous, intraperitoneal, or intratumor injection.

[0133] The formulations containing the nucleic acid of the invention are typically administered to a cell. The cell can be provided as part of a tissue, such as an epithelial membrane, or as an isolated cell, such as in tissue culture. The cell can be provided *in vivo*, *ex vivo*, or *in vitro*.

[0134] The formulations can be introduced into the tissue of interest *in vivo* or *ex vivo* by a variety of methods. In some embodiments of the invention, the nucleic acids of the invention are introduced into cells by such methods as microinjection, calcium phosphate precipitation, liposome fusion, ultrasound, electroporation, or biolistics. In further embodiments, the nucleic acids are taken up directly by the tissue of interest.

[0135] In some embodiments of the invention, the nucleic acids of the invention are administered *ex vivo* to cells or tissues explanted from a patient, then returned to the patient. Examples of *ex vivo* administration of therapeutic gene constructs include Nolta *et al.*, *Proc Natl. Acad. Sci. USA* **93**(6):2414-9 (1996); Koc *et al.*, *Seminars in Oncology* **23**(1):46-65 (1996); Raper *et al.*, *Annals of Surgery* **223**(2):116-26 (1996); Dalesandro *et al.*, *J. Thorac. Cardi. Surg.*, **11**(2):416-22 (1996); and Makarov *et al.*, *Proc. Natl. Acad. Sci. USA* **93**(1):402-6 (1996).

[0136] Effective dosage of the formulations will vary depending on many different factors, including means of administration, target site, physiological state of the patient, and other medicines administered. Thus, treatment dosages will need to be titrated to optimize safety and efficacy. In determining the effective amount of the vector to be administered, the physician should evaluate the particular nucleic acid used, the disease state being diagnosed; the age, weight, and overall condition of the patient, circulating plasma levels, vector toxicities, progression of the disease, and the production of anti-vector antibodies. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector. To practice the present invention, doses ranging from about 10 ng - 1 g, 100 ng - 100 mg, 1 µg - 10 mg, or 30 - 300

µg DNA per patient are typical. Doses generally range between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg / kg of body weight or about 10^8 - 10^{10} or 10^{12} particles per injection. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 µg - 100 µg for a typical 70 kg patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of nucleic acid encoding a polypeptide that inhibits the binding between integrin and IGF2 (*e.g.*, human IGF2).

VII. KITS

[0137] The invention also provides kits for inhibiting IGF2 signaling according to the method of the present invention. The kits typically include a container that contains a pharmaceutical composition having an effective amount of an inhibitor of IGF2-integrin binding (such as a dominant-negative mutant R24E/R37E/R38E, R34E/R37E/R38E, or R24E/R34E/R37E/R38E or a polynucleotide sequence encoding the polypeptide) as well as informational material containing instructions on how to dispense the pharmaceutical composition, including description of the type of patients who may be treated (*e.g.*, cancer patients with IGF2 or integrin overexpression), the schedule (*e.g.*, dose and frequency) and route of administration, and the like.

EXAMPLES

[0138] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially the same or similar results.

Example 1

[0139] IGF1 and IGF2 have similar signaling functions: IGF1 and IGF2 are polypeptide hormones (75-kD) that have a high degree of structural similarity to human proinsulin (Fig. 1). They act through binding to IGF1R, a receptor tyrosine kinase, that is ubiquitously present on multiple cell types. IGF1 and IGF2 are involved in cell growth and, consequently, IGF1R inhibition is being pursued as a potential measure for treating and preventing cancer. Ligand binding induces phosphorylation of specific tyrosine residues of IGF1R. These phosphotyrosines then bind to adapter molecules such as Shc and insulin receptor substrate (IRS)-1. Phosphorylation of these proteins leads to activation of PI3K and MAPK signaling pathways [11].

[0140] Development of dominant-negative inhibitory IGF1 mutant: Integrins $\alpha v\beta 3$ and $\alpha 6\beta 4$ are overexpressed in a variety of human cancers and associated with poor patient prognosis [12], but the roles of these integrins in cancer has not been established. The present inventors have previously reported that IGF1 directly and specifically binds to $\alpha v\beta 3$ [7] and $\alpha 6\beta 4$ [8]. The integrin binding-defective mutant (R36E/R37E) of IGF1 is defective in enhancing cell viability and inducing IGF signaling, although R36E/R37E still binds to IGF1R. Interestingly, WT IGF1 induces a ternary complex formation ($\alpha v\beta 3$ -IGF1-IGF1R and $\alpha 6\beta 4$ -IGF1-IGF1R) while R36E/R37E does not. This indicates that the direct binding of these integrins to IGF1 and subsequent ternary complex formation are critical for IGF signaling [7, 8].

[0141] A model of IGF1 signaling has been proposed, in which IGF1 binds to IGF1R on the cell surface and integrins are recruited to the IGF1-IGF1R complex through direct binding to IGF1, making the IGF1R-IGF1-integrin ternary complex. If formation of the ternary complex is critical for IGF signaling, then the integrin binding-defective R36E/R37E mutant of IGF1 is antagonistic, since R36E/R37E can bind to IGF1R well and compete with WT IGF1 for binding to IGF1R. The present inventors demonstrated that excess R36E/R37E suppressed signaling induced by WT IGF1 *in vitro*. Notably, R36E/R37E suppressed anchorage-independent growth *in vitro* and tumorigenesis *in vivo* of cancer cells, while WT IGF1 markedly enhanced them (Fig. 2) [10]. R36E/R37E IGF1 has potential as a therapeutic agent in cancer ("IGF1-decoy"). It has been observed that excess IGF1-decoy suppresses the binding of WT IGF1 to the cell surface in cancer cells, suggesting that IGF1-decoy and WT IGF1 compete for binding to IGF1R on the cell surface [10].

[0142] Insulin receptor (IR) is over-expressed in cancer cells: The insulin receptor (IR) is structurally very similar to IGF1R and they are both members of the tyrosine kinase class of membrane receptors [2]. IRs are usually abnormally expressed in cancer cells, where they mediate both the metabolic and non-metabolic effects of insulin. When compared, mean IR content in cancerous breast tissue is more than 6-fold higher than normal breast tissue [13]. Approx. 80% of breast cancer samples had an IR content much higher than in normal breast tissue, and approx. 20% had IR values over 10-fold higher than in normal breast tissue [13]. Functional studies indicated a higher IR responsiveness to insulin in breast cancer than in normal breast cells [14].

[0143] IR-A and IR-B have distinct functions: The IR occurs in two isoforms (IR-A and IR-B). The most relevant functional difference between these two isoforms is the high

affinity of IR-A for IGF2 (a ligand to IR-A, but not to IR-B, Table 1). IR-A is predominantly expressed during prenatal life. It enhances the effects of IGF2 during embryogenesis and fetal development. It is also significantly expressed in adult tissues, especially in the brain. Conversely, IR-B is predominantly expressed in well-differentiated adult tissues, including the liver, where it enhances the metabolic effects of insulin. IR-A preferentially induces mitogenic and anti-apoptotic signals, whereas IR-B predominantly induces cell differentiation signals [15].

[0144] *IR-A is overexpressed in cancer:* IR splicing is altered in cancer cells, thus increasing IR-A:IR-B ratio, which profoundly affects the cell response to circulating insulin and IGF2. IR-A is the predominant IR isoform expressed in a variety of cancers, including carcinomas of the breast, colon, and lung [16]. In particular, IR-A is the predominant IR isoform in a panel of breast cancer cell lines (ranging from 64–100% of total IR) and in a series of breast cancer tissue specimens (ranging from 40–80%) [17]. In contrast, IR-A represents 30–50% of total IR content in normal breast cells and tissue specimens [17]. This indicates that IR-A plays a role in cancer.

[0145] *Autocrine IR-A/IGF2 loop plays a role in cancer:* IR-A is an IGF2 receptor [16]. This provides further insight into the role of IR overexpression in cancer. Notably, breast cancer cells produce IGF2 in an autocrine manner. In cells with a high IR-A:IGF1R ratio, autocrine production of IGF2 stimulates cell growth through IR-A stimulation. In these cells, blocking either IGF2 or the IR markedly inhibited growth, demonstrating the relevance of this autocrine loop (IR-A/IGF2 loop) in cancer [17]. IR-A binding to IGF2 is associated with stimulation of growth and cell invasion [16], whereas IR-B, which does not bind IGF2, is associated with differentiation and metabolic signals [13].

[0146] Heterodimers comprised of a half IR and a half IGF1R can form (Table 1), and these are known as hybrid receptors [3, 4]. As most cancers express both IR and the IGF1R, they display many of the hybrid receptor species, rather than a single receptor type. In each case, the kinase activity of the receptor leads to phosphorylation of members of the IR substrate (IRS) family of proteins, and this leads to activation of PI3K, AKT and various downstream networks [5]. Abnormal autocrine or paracrine expression of IGF2 [6] and the IR-A/IGF2 loop may denote ‘addiction’ to IR/IGF1R activation in many cancers.

Table 1. Ligand specificity of the IGF system in mammals [3]. Note that IGF2 binds to IR-A, and hybrid receptors that contains IR-A (but not IR-B) or IGF1R. Dominant-negative IGF2 is expected to suppress IR-A/IR-A, IGF1R/IGF1R, IR-B/IR-A, IR-B/IGF1R, and IR-A/IGF1R, but not IR-B/IR-B.

Receptor	Homotetramers			Heterotetramers (hybrids)		
	IR-B/IR-B	IR-A/IR-A	IGF-IR/IGF-IR	IR-B/IR-A	IR-B/IGF-IR	IR-A/IGF-IR
Name	IR-B	IR-A	IGF-IR	IR-AB	IR-B	IR-A
Ligand(s)	Insulin	Insulin	IGF-I	Insulin	IGF-I	IGF-I
		IGF-II	IGF-II	IGF-II	IGF-II	IGF-II
						Insulin

5 **[0147] Dominant-negative IGF2 (IGF2-decoy) have therapeutic use:** If one can block IR-A without affecting IR-B, it will be possible to efficiently suppress tumorigenesis. Unfortunately, currently available kinase inhibitors to IR or IGF1R do not distinguish IGF1R, IR-A or IR-B. Also, anti-IGF1R antibodies do not block IR-A or IR-B. The inventors have already developed IGF1-decoy that effectively suppresses IGF1-induced tumor cell survival and tumorigenesis in vivo [10]. IGF1-decoy, however, does not suppress IR-A or IR-B. To suppress IR-A, a valid therapeutic target in cancer, the property of IGF2 to bind to IR-A, but not IR-B (Table 1), is exploited. The inventors developed IGF2-decoys using a strategy that was successfully used for IGF1-decoy and other dominant-negative growth factors (see below). IGF2 is structurally similar to IGF1 and amino acid residues of IGF1 that are critical for integrin binding to IGF1 are conserved in IGF2. The IGF2-decoy suppresses IR-A together with IGF1R and blocks the IR-A/IGF2 loop in cancer. In their preliminary studies, the inventors found that IGF2 binds to integrin $\alpha\beta3$. Integrin-binding defective IGF2 mutants were generated using the strategy used for IGF1 (Fig. 3). The IGF2 mutants were found defective in signaling function, and they suppressed cell survival increased by WT IGF2 (dominant-negative effect) (Fig. 4).

15 **[0148] Dominant-negative technology--a novel platform for drug discovery:** Using docking simulation, the inventors identified several growth factors as new integrin ligands (FGF1 [18], IGF1 [7], and neuregulin-1(NRG1)) [19]. These growth factors directly bind to integrins (*e.g.*, $\alpha\beta3$ and/or $\alpha6\beta4$), and this interaction plays a role in growth factor signaling. integrin binding-defective growth factors were generated by introducing mutations in the predicted integrin-binding site. The integrin binding-defective FGF1 mutant (R50E) is defective in both inducing signals and in inducing ternary complex (integrin-FGF1-FGF1 receptor), while it still binds to FGF1 receptor [20]. Also, R50E is dominant-negative and suppresses signaling induced by WT growth factors, and suppresses tumorigenesis [20] and angiogenesis [21]. US Patent No. 8,168,591 (Compositions and methods related to anti-FGF

agents) has been issued in 2012 on the dominant-negative FGF1 mutants. The integrin binding-defective NRG1 mutant (3KE) is defective in ErbB3 signaling, while the mutant still binds to Erbβ3 [19]. WT NRG1 induces a ternary complex (integrin-NRG1-Erbβ3), while 3KE does not. 3KE is also a dominant-negative mutant (inventors' unpublished results).

5 Most recently, the inventors reported that the chemokine domain of fractalkine (CX3CL1), a transmembrane chemokine, binds to integrins αvβ3 and α4β1 [22]. CX3CL1 induces a ternary complex formation (integrin, CX3CL1, and its specific receptor CX3CR1). Also, the integrin binding-defective mutant of fractalkine is a dominant-negative antagonist of CX3CR1 [22]. These findings indicate that integrin-growth factor receptor crosstalk through
10 direct binding to growth factor may potentially be a common mechanism in many growth factors. This strategy is a novel platform for drug discovery.

[0149] *Mutants of human proteins can be used as therapeutic agents:* There is a precedent that a mutant of human protein was used for human diseases. A mutant of human growth hormone (hGH) has been used as an antagonist of GH receptor in the treatment of
15 acromegaly (Pegvisomant, trade name Somavert) [23]. The Gly-120 of h GH was mutated to Arg (G120R) and this mutant was further modified by poly(ethylene glycol) (PEG)-5000 to elongate half-life. Pegvisomant prevents functional dimerization of hGH receptor by sterically inhibiting conformational changes within the GHR dimers [23]. Pegvisomant is generally well tolerated with a safety profile similar to that reported in clinical trials and can
20 effectively reduce IGF1 in patients with acromegaly refractory to conventional therapy [24]. Thus, the IGF2 dominant-negative mutants can serve as therapeutic agents.

[0150] *Innovation:* IR-A is overexpressed in cancers and is a therapeutic target in cancer. Current therapeutics cannot target IR-A. Antibodies to IGF1R do not affect IR-A or IR-B, and antibodies to IR do not distinguish IR-A and IR-B. Kinase inhibitors do not distinguish
25 IR-A, IR-B, and IGF1R. IGF2 binds to IR-A and IGF1R, but not to IR-B. To target IR-A, dominant-negative IGF2 mutants have been developed. The so-called "IGF2-decoys" have advantages over antibodies and kinase inhibitors. In addition to being a specific antagonist to IR-A, an IGF2-decoy may have better penetrance to the tumor tissues than IgG because of its smaller size. IGF2-decoy are potential therapeutics. To identify dominant-negative IGF2,
30 the same strategy that has successfully worked for other growth factors (*e.g.*, FGF1, IGF1, and neuregulin-1) is employed. Several candidate IGF2-decoys so generated are particularly useful in studies to establish roles of integrins in IGF2 and IR-A signaling.

[0151] ***IGF2 uniquely distinguishes IR-A and IR-B.*** The dominant-negative mutants of IGF2 target IR-A, which is overexpressed in cancer, and plays a role in cancer progression.

[0152] ***Rationale:*** Blocking IGF2 functions reduces IR-A signaling together with IGF1R signaling and serves as a reasonable approach for suppressing cancer progression. Dominant-negative form of IGF2 is developed to accomplish this goal. The dominant-negative form of IGF1 targets IGF1R, but not IR-A or IR-B [7-10]. The dominant-negative form of IGF2 targets IR-A (not IR-B) and IGF1R and suppresses their activation. In their preliminary studies, the inventors have identified several candidate dominant-negative IGF2 mutants and are continuing to characterize these IGF2 mutants in *in vitro* and *in vivo* experiments.

[0153] ***Research design:*** In preliminary studies, a bacterial expression construct of IGF2 was generated by subcloning the cDNA encoding IGF2 into the NdeI/XhoI site of PET28a. The protein was expressed as an insoluble inclusion body and the insoluble IGF2 was refolded as described [7]. The ability of IGF2 to bind to integrins was tested in ELISA-type binding assays using recombinant soluble integrin, and in cell adhesion assays using CHO cells that express recombinant integrins as described [7]. The results showed that CHO cells that express $\alpha\beta 3$ ($\beta 3$ -CHO cells) strongly adhered to IGF2 in a dose dependent manner, but those expressing recombinant $\alpha\beta 1$ ($\beta 1$ -CHO cells) did not (Fig. 3). These findings indicate that $\alpha\beta 3$ interacts with IGF2. The recombinant IGF2 induced IGF1R phosphorylation in a dose-dependent manner (data not shown), which also indicates that the IGF2 preparation is functional. Various mutations were introduced into IGF2, and several IGF2 mutants were identified as defective in integrin binding (Fig. 3c and d). The ability of these IGF2 mutants to induce signals was tested in $\beta 3$ -CHO cells, and it was found that the integrin-binding defective mutants are defective in enhancing cell viability (Fig. 4a). It was observed that excess integrin-binding defective IGF2 mutants suppress cell viability increased by WT IGF2 (Fig. 4b). In conclusion, the integrin-binding defective mutants are not only defective in signaling functions but are dominant-negative.

[0154] 1) Characterization of integrin-binding defective IGF2 mutants: the mutants are further studies for their binding to IGF1R in ELISA-type assays using soluble IGF1R as previously described [7] (commercially available). The ability of WT IGF2 and the integrin binding-defective mutants of IGF2 to induce ternary complex formation are tested as described [7] and to confirm that WT IGF2 induces ternary complex, but the integrin binding-defective mutants do not.

[0155] 2) Testing the ability of IGF2 to activate IGF1R and IR: the IGF2 mutants are tested for their ability to induce tyrosine phosphorylation of IR and IGF1R. MCF-7 cells are stimulated with IGF2 (WT and mutants) and cell lysates are analyzed in Western blotting using antibodies specific to phospho-IR and phospho-IGF1R (commercially available). The integrin binding-defective IGF2 mutants are verified as defective in inducing IR and IGF1R phosphorylation, but the IGF2 mutants that are not defective in integrin binding retain intact signaling functions. This is consistent with previous findings that the integrin binding-defective IGF1 is defective in IGF1R activation [7].

[0156] 3) Studying the role of integrin-IGF2 binding in IGF2/IR-A or IGF1R signaling:

WT IGF2 is studied for its ability to induce ternary complex formation (integrin-IGF2-IGF1R or integrin-IGF2-IR-A). Cells (*e.g.*, β 3-CHO cells or MCF-7 cells) are incubated with WT IGF2, and integrin β 3 or IGF1R (or IR) are immunopurified from cell lysates. The purified materials are then analyzed by western blotting as described [7]. The integrin binding-defective IGF2 mutant is tested to reveal whether it induces ternary complex formation. WT IGF2 induces ternary complex formation, but the mutant does not. These experiments illustrate a new role of integrins in IR signaling, and the IGF2 mutants are important tools for these studies.

[0157] 4) Studying the ability of the IGF2 mutants to suppress signaling induced by WT IGF2: the IGF2 mutants are tested for their suppression of signaling induced by WT IGF2.

Non-transformed cells (*e.g.*, NIH3T3 in regular tissue culture plates) or transformed cells (*e.g.*, MCF-7 in plastic wells coated with hydrogel (polyHEMA) to reduce cell-matrix interaction) are cultured as described [10], and serum-starved. The cells are then stimulated with WT IGF2 and/or IGF2 mutants. Cell viability is measured using MTS assays. Cell proliferation is assayed using BrdU incorporation. When excess IGF2 mutants suppress cell viability and proliferation induced by WT IGF2, it is a dominant-negative effect by definition as described [8-10]. The IGF2 mutants are tested for their ability to suppress IR and IGF1R phosphorylation induced by WT IGF2 in non-transformed and transformed cells as described above. Cell lysates are analyzed using western blotting with antibodies specific to phosphorylated or non-phosphorylated IR and IGF1R as described [8-10].

[0158] 5) Testing the dominant-negative IGF2 mutants *in vivo*: The IGF2 mutants that show dominant-negative effects *in vitro* are chosen to be tested for their ability to suppress tumorigenesis *in vivo*. IGF2 (6His-tagged, WT and the mutants) in secretion vector (*e.g.*, pSecTag) are stably expressed in cancer cells (*e.g.*, MDA-MB231) and tested to verify that

IGF2 is secreted from the cells using anti-His tag antibodies. MDA-MB231 cells are chosen because they express high IR-A. The transfected cells are characterized *in vitro* in colony formation in soft agar, in survival in response to serum starvation and chemotherapeutics (by MTS assays), and in the ability to proliferate (by BrdU incorporation assays) as described for IGF1 [10]. WT IGF2 enhances cell survival and proliferation, whereas dominant-negative IGF2 suppresses them. WT IGF2 and dominant-negative IGF2 are injected into mice, and their effect on tumorigenesis is monitored as described [10]. Analysis of tumor tissues indicates that IGF2 mutants suppress tumorigenesis and angiogenesis by affecting IR-A signaling in tumor cells and microenvironment.

[0159] Statistical analysis and power analysis: Tumor growth curves are compared across groups using standard repeated measures mixed models [25]. These models allow for possibly unequal spacing of measurements or unequal lengths of follow-up, as, for example, if some mice develop unsustainable tumor burdens and are sacrificed early. These models are formulated to test specifically for IGF2-treated mice versus control, then test for the added impact of increasing doses, to identify an optimal dose level, on the rate of tumor growth. It has been found that power analysis in the mouse breast cancer Met-1 orthograft is such that a 20% difference between treatment and control groups can be detected with 8 mice in each group [26, 27]. Typically 10-12 mice are used per group, unless pilot data suggest a much better than 20% effect, in which case 8 mice are used per group.

[0160] It has been reported that direct binding of IGF1 to integrins together with IGF1R (and resulting ternary complex formation) is critical for IGF1 signaling [7]. IGF1 and IGF2 are similar in structure and signaling functions (except that IGF2 binds to IR-A but IGF1 does not). IGF2 requires direct integrin binding and the resulting complex formation (integrin-IGF2-IGF1R/IR-A) plays a critical role in IGF2 signaling. Also, Arg residues critical for integrin binding in IGF1 (Arg36 and Arg37) are conserved in IGF2. Several integrin-binding defective IGF2 mutants have been generated by mutating these residues and nearby Arg residues in IGF2. It has also been shown that these IGF2 mutants suppressed cell viability increased by WT IGF2. Thus, these mutants are dominant-negative. These mutants are further characterized in experiments described above to identify which is the most potent antagonist of IR-A and IGF1R. Since the IGF2 mutants suppress the IR-A/IGF2 loop, they can serve as an important therapeutic target in cancer. The IGF2-decoy can provide great therapeutic benefits to cancer patients.

[0161] It is possible that IGF binding proteins (IGFBPs) may affect half-life of IGF2-decoy. Only a small fraction (0.5-2%, depending on the literature) of total IGF2 is present in free form, and the rest are bound to IGFBP, with about 80% to IGFBP3. It is possible to suppress IGFBP binding to IGF2 by modifying the IGFBP-binding site in IGF2. Two amino acid residues of IGF2 (Fig. 1 in green) are critical for IGFBP binding [1]. Additional mutations are to be introduced into FGF2-decoy if IGFBP binding affects availability of IGF2-decoy *in vivo*. It has been reported that the IGF1 mutant that cannot bind to IGFBP has a much longer half-life than WT IGF1 (20 min for WT IGF1 and 20 h for the IGF1 mutant that cannot bind to IGFBP), and is substantially more potent than WT IGF1 [28]. Thus, similar mutations in IGF2 can improve *in vivo* stability of IGF2.

[0162] When antibodies of IGF1R are used, IGF1R signaling is inhibited on both normal and neoplastic IGF1R-positive cells, and also on IGF1R-positive cells in the hypothalamic-pituitary axis that are involved in the feedback inhibition of IGFI on growth hormone (GH) secretion. This results in substantial increases in GH, which stimulate the liver to increase IGF production and also cause insulin resistance in insulin-target tissues, which raise glucose levels, and thereby lead to increases in insulin production. The IGF1R tyrosine kinase inhibitors have similar effects, but they also block insulin receptors (reviewed in [29]). When IGF2-decoy is used *in vivo*, it suppresses IGF1R and IR-A, which reduces the effect of enhanced insulin production on normal and cancer cells. However, the effect of IGF2-decoy on normal cells is minimal since IR-B is not be affected by IGF2-decoy. This is confirmed by monitoring metabolic effects of IGF2-decoy *in vitro* and *in vivo* (e.g., levels of serum glucose and insulin).

Example 2 Development of dominant-negative IGF2 mutants (IGF2-decoy)

[0163] **Objective:** IGF2 uniquely distinguishes IR-A and IR-B. Dominant-negative mutants of IGF2 are designed to target IR-A, which is overexpressed in cancer and plays a role in cancer progression.

[0164] **Rationale:** Blocking IGF2 functions is expected to reduce IR-A signaling together with IR-B signaling without affecting IGF1, and may be a reasonable approach for suppressing cancer progression. This goal is accomplished by developing dominant-negative form of IGF2. The dominant-negative form of IGF1 targets IGF1R, but not IR-A or IR-B [9, 10, 30]. The dominant-negative form of IGF2 targets IR-A (not IR-B) and IGF1R and suppresses their activation. In the preliminary studies, several candidate dominant-negative

IGF2 mutants were identified. These IGF2 mutants are characterized *in vitro* and *in vivo* in detail.

Methods and Materials

[0165] **Cell lines:** non-transformed cells (NIH3T3) and several transformed cells (including human breast cancer MCF7, mouse breast cancer Met-1, and CHO cells) were previously used for studying IGF1-decoy, since they respond well to WT IGF1. These cell lines are used in this IGF2-decoy study. In addition, human breast cancer MDA-MB231 cells are used for studying IGF2, since they express IR-A in addition to IGF1R. HEK293 cells that express Tet-On transactivator (available from Clontech) are also used to test the inducible IGF2-decoy expression system.

a) Characterization of integrin-binding defective IGF2 mutants:

[0166] IGF1R binding. Mutants binding to IGF1R is detected in ELISA-type assays using soluble IGF1R as previously described [30] (commercially available). Briefly, WT IGF2 is will biotinylate and tested if IGF2 mutants can compete for binding to immobilized IGF1R in 96 wells in ELISA-type competitive binding assays. Most of the IGF2 mutants compete well with WT IGF2 for binding to IGF1R.

[0167] Tyrosine phosphorylation. It is tested whether IGF2 mutants induce tyrosine phosphorylation of IR and IGF1R. Cells (*e.g.*, MCF-7) with IGF2 (WT and mutants) are stimulated and cell lysates are analyzed in Western blotting using antibodies specific to phospho-IR and phospho-IGF1R (commercially available). The integrin binding-defective IGF2 mutants are defective in inducing IR and IGF1R phosphorylation, but the IGF2 mutants that are not defective in integrin binding have intact signaling functions.

b) Studying the role of integrin-IGF2 interaction in IGF2/IR-A or IGF1R signaling:

[0168] IGF2 mutants are tested to study their suppression of signaling induced by WT IGF2. Nontransformed cells (*e.g.*, NIH3T3 in regular tissue culture plates) or transformed cells (*e.g.*, MCF-7 in plastic wells coated with hydrogel (polyHEMA) to reduce cell-matrix interaction as described [10]) are cultured and serum-starved. Cells are then stimulated with WT IGF2 and/or IGF2 mutants. Cell viability are measured using MTS assays. Cell proliferation are assayed using BrdU incorporation. When excess IGF2 mutants suppress cell viability and proliferation induced by WT IGF2, it is a dominant-negative effect by definition as described [8, 9, 10]. IGF2 mutants are tested for their ability to suppress IR and IGF1R phosphorylation induced by WT IGF2 in nontransformed and transformed cells as described

above. Cell lysates are analyzed using western blotting with antibodies specific to phosphorylated or non-phosphorylated IR and IGF1R as described [8, 9, 10]. Non-transformed HEK 293 cells that inducibly express IGF2 (WT and mutants) were generated using the Tet-On system (data not shown). While originally intended to test the inducible expression of IGF2-decoy induced by doxycycline, the transfected HEK293 cells are further characterized, since HEK293 cells are expected to respond to IGF2-decoy. The effect of IGF2-decoy is detected on cellular morphology and signaling as in NIH3T3 cells.

Results

[0169] IGF1 and IGF2 are similar in structure and signaling functions (except that IGF2 binds to IR-A but IGF1 does not). Thus, IGF2 is expected to require direct integrin binding, and the resulting complex formation (integrin-IGF2-IGF1R/IR-A) plays a critical role in IGF2 signaling. Also, Arg residues critical for integrin binding in IGF1 (Arg36 and Arg37) are conserved in IGF2. Several integrin-binding defective IGF2 mutants were made by mutating these residues and nearby Arg residues in IGF2 during preliminary studies. It was showed that these IGF2 mutants suppressed cell viability increased by WT IGF2. Thus, these mutants are dominant-negative. One purpose of this study is to establish that IGF2 signaling through IGF1R and/or IR-A requires direct integrin binding, and that IGF2 mutants that are defective in integrin binding are dominant-negative as in IGF1. Another purpose is to identify the most effective IGF2-decoy, which is useful for various practical applications.

Discussions

[0170] One possible means of enhancing the effect of IGF2 mutants is introducing PEGylation in the predicted integrin-binding site. For this purpose one of the K residues in the integrin binding site remains unchanged while all other K residues are mutated to R. The remaining K residue is then cross-linked to PEG. This strategy suppresses integrin binding and therefore suppresses IGF signaling, resulting in a longer half-life of the protein.

Example 3 Study effects of IGF2 decoy on cancer stem cells and tumorigenesis in vivo Preliminary studies:

[0171] A dominant-negative IGF1 was previously generated, which affects phenotype of cancer. WT IGF1 enhances, and IGF1 decoy suppresses, tumorigenesis using Met-1 mouse breast cancer cells that were transfected with IGF1 decoy (Fig. 2). IGF1 decoy is expected to inhibit IGF1R signaling in CSCs and suppress CSC-associated phenotypes including self-renewal ability, enhanced epithelial-to-mesenchymal transition (EMT) potential, and

increased resistance towards therapeutic interventions (chemo- and radiation-therapies). It was studied if IGF1 decoy affects morphology and expression of stem cell marker in Met-1 cells. IGF1 decoy induced epithelial-like morphology (Fig. 6a), and reduced Oct-4 and Nanog expression in Met-1 cells that were transfected with IGF1 decoy (Fig. 6b). It is plausible that IGF1 decoy suppresses stemness and dedifferentiation of CSCs. It is also likely that Met-1 cells express endogenous IGF1 or 2, since transfection of WT IGF1 did not effectively affect stemness and morphology.

Rationale

[0172] In the preliminary studies, IGF2 mutants were constitutively expressed in secretion vector in triple negative breast cancer cells MDA-MB231. The transfected cells did not grow well (data not shown). This observation strongly supports the hypothesis that they are effective inhibitors of IGFR/IR-A signaling. This may present challenges for in vitro studies. Alternatively, it was possible to manipulate the expression of IGF2 WT and mutants using the Tet-On inducible system [31](Clontech Lab). HEK293 cells that inducibly express IGF2 (WT and mutants) have already been generated.

[0173] HEK293 transfectants are useful to establish the dominant-negative property of the IGF2 mutants in nontransformed cells. IGF2 expression is induced by using doxycycline in culture medium, and the ability of the IGF2 mutants to suppress IGF signaling and proliferation is tested. As transformed cells, MDA-MB231 human triple negative breast cancer cells are chosen because they express high IR-A. Met-1 mouse breast cancer cells are also chosen to use syngeneic mice that have intact immune system. The transfected cells are characterized in vitro in making colonies in soft agar, in surviving in response to serum starvation and chemotherapeutics (by MTS assays), and in the ability to proliferate (by BrdU incorporation assays) as described for IGF1 [10]. WT IGF2 enhances cell survival, proliferation, and tumorigenesis, while dominant-negative IGF2 mutants (IGF2 decoy) suppress them.

Research design

a) Effect of mutant IGF2 on cancer stem cell (CSC) phenotypes

[0174] 1) Effect of IGF2 decoy on expression of stemness markers: the effect of IGF1 decoy on the phenotype of MDA-MB231 cells is studied. Met-1 mouse breast cancer cells are generated from widely used polyomavirus middle T (PyV-mT) transgenic mouse model of breast cancer [32] that inducibly express IGF2 WT and mutants, since syngeneic mouse

strain that have intact immune system can be used in the in vivo tumorigenesis study (Fig. 2). First, cancer cells that inducibly secrete WT IGF2 or IGF2 decoy are fully characterized without further enrichment or cloning in stemness, tumorigenicity, morphology and gene expression. IGF2 expression in the transfected cells is induced, the cells are then fixed and stained for Oct-4 and Nanog and other stemness markers. Secretion of IGF2 (6His-tagged) is detected by western blotting of culture medium using anti-His antibody. A laser scanning cytometry is used to quantitatively analyze expression profile of the stemness markers in the cells. As controls, cells treated with WT IGF2 or cells that are not treated with IGF2 are used. WT IGF2 enhances, and IGF2 decoy suppresses, expression of stemness markers. Alternatively, levels of stem cell markers are monitored using qtPCR. These experiments establish that IGF2 decoy targets CSCs.

[0175] 2) Effect of IGF2 decoy on the CSC population. Side population (SP) methodology will be utilized as one of the tools for analyzing CSCs in this study. It has been shown that active ABCG2 efflux pumps are active in many types of stem cells and has been shown to pump the cell-permeant DNA-binding dye Hoechst 33342 out of stem cells (SP). SP cells are detected using flow cytometry. IGF2 decoy reduces, and WT IGF1 enhances, the percentage of SP cells.

[0176] 3) Effect of IGF2 decoy on chemoresistance in vitro. One of the major hallmarks of CSCs is the resistance against and rebound from chemotherapy-induced cell killing. The functional hallmark of CSCs are tested in parallel with measurement of the CSC markers. Cell viability is compared the between the parental cells (non-CSCs) and CSCs in the presence of IGF2 decoy or WT IGF2. It is also tested if IGF2 decoy competitively suppresses the cell survival increased by WT IGF2 in the presence of chemotherapeutic agents. WT IGF2 enhances chemoresistance and IGF2 decoy reduces it. The expression of stem cell markers are monitored the during the treatment.

[0177] 4) In vivo evaluation of IGF2 decoy on tumor-initiating ability. Cells with CD44⁺/CD24⁻ expression profile have been widely accepted as cancer stem-like cells in breast cancer. Two approaches are employed to evaluate the efficacy of IGF2 decoy on preventing and/or suppressing the tumor-initiating ability of murine Met-1 cells (in syngeneic mouse model) or human MDA-MB-231 (in immune-compromised xenograft model). CD44⁺/CD24⁻ cells are sorted and collected via Aria III FACS system. Sorted cells (alternatively, mammospheres generated under serum-deprived conditions) are orthotopically injected into the mammary fat pad of the mouse. Tumor inoculated mice receive either IGF2

decoy (experimental group), vehicle control and IGF2 mutant (experimental group) for in vivo monitoring of tumorigenesis. Since these cells have been modified to contain dual-reporter system (firefly luciferase 2 and enhanced GFP, L2G, a generous gift from Dr. Sanjiv Gambhir, the Molecular Imaging Program at Stanford (MIPS), non-invasive in vivo
5 monitoring of tumorigenesis (growth and metastasis) can be achieved longitudinally within the same animals. Tumor biopsies are harvested after the experimental period and used for further analyses.

b) Effect of IGF2 decoy on tumorigenesis

[0178] Various in vivo tumorigenesis experiments are performed to analyze the pathology
10 of tumors. Cancer cells that inducibly express IGF2 (WT or mutants) are injected into mice, and tumorigenesis is monitored as described [10]. Cancer cells are subcutaneously injected. IGF2 expression is induced by doxycycline in the medium in vitro, and in drinking water in vivo. Analysis of tumor tissues can show that the IGF2 mutants suppress tumorigenesis and angiogenesis by affecting IR-A signaling in tumor cells and microenvironment. To keep the
15 host immune system intact, the combination of Met-1 mouse breast cancer cells that inducibly secrete IGF2 decoy and syngeneic mouse strain (FVB mouse) are also used in addition to xenograft. Induction of IGF2-decoy secretion starts at the time of tumor inoculation first to confirm that IGF2-decoy is involved in tumorigenesis. When this is successful, the timing of IGF2-decoy induction (*e.g.*, when tumor is detectable, when the
20 tumors are fully established) can be changed. This can show whether IGF2 decoy suppresses well-established tumors. By monitoring the levels of angiogenesis, it can be determined if IGF2-decoy suppresses angiogenesis. The transfected cells are treated with doxycycline before inoculation suppress CSC population in cancer cells. The levels of CSC in vitro are tested as described above. Tumorigenicity of pre-treated cells in vivo (without further
25 doxycycline treatment) are monitored. Pre-treated cells show reduced tumorigenicity compared to the cells that had not been pre-treated.

[0179] **Statistical analysis and power analysis:** Tumor growth curves are compared across groups using standard repeated measures mixed models [24]. These models allow for possibly unequal spacing of measurements or unequal lengths of follow-up, as, for example,
30 if some mice develop unsustainable tumor burdens and are sacrificed early. These models are formulated to test specifically for IGF2-treated mice vs control, then test for the added impact of increasing doses, to identify an optimal dose level, on the rate of tumor growth. It was found that power analysis in the mouse breast cancer Met-1 orthograft is such that a 20%

difference between treatment and control groups can be detected with 8 mice in each group [26, 27]. Thus, 10-12 mice are typically used per group, unless pilot data suggest a much better than 20% effect, in which case 8 mice are used per group.

Results

5 [0180] IGF2-decoy is potent antagonists of IGF1R and IR-A and affect stemness and proliferation of CSC. IGF2-decoy will be more effective than IGF1-decoy in suppressing tumorigenesis. By comparing IGF2- decoy and IGF1-decoy it is possible to assess the effect of suppressing IR-A on CSC phenotype. IGF2 mutants can be further characterized in more detail to identify which is the most potent antagonist of IR-A and IGF1R. IGF2-decoy
10 suppresses the IR-A/IGF2 loop, an important therapeutic target in cancer. IGF2- decoy therefore can greatly benefit cancer patients.

Discussions

a) effect of IGF2-decoy on hybrid receptor

[0181] It is unclear at this point if IGF2-decoy effectively suppresses hybrid receptors that
15 contain IR-B (Table 1), IRB/IR-A and IR-B/IGF1R. To address this question, individual hybrid receptors are expressed on cells that lack IR (*e.g.*, CHO cells) and tested for specific hybrid receptors responding to IGF2-decoy.

b) Short half-life of IGF2-decoy

[0182] 1) IGFBP-binding defective IGF2-decoy: It is possible that IGF binding proteins (IGFBPs) may affect half-life of IGF2-decoy in vivo. Only a small fraction (0.5-2%,
20 depending on the literature) of total IGF2 is present in free form, and the rest are bound to IGFBP, with about 80% to IGFBP3. It is possible to suppress IGFBP binding to IGF2 by modifying the IGFBP-binding site in IGF2. Two amino acid residues of IGF2 (Fig. 1 in green) are critical for IGFBP binding. Additional mutations are introduced into IGF2-decoy
25 (*e.g.*, the E7R mutation) to test whether IGFBP binding affects availability of IGF2-decoy in vivo. It has been reported that the IGF1 E3R mutant that cannot bind to IGFBP is substantially more potent than WT IGF1 [28]. Thus, similar mutations in IGF2 can be more potent than IGF2.

[0183] 2) Continuous infusion of IGF2 decoy: It is possible that stabilization of IGF2 decoy
30 in vivo is difficult. To solve this problem one may use a small device (insulin pump), which has been used to continuously infuse insulin (this is programmable) to patients (Fig. 7). For the same purpose osmotic pumps can be used as well.

[0184] 3) PEGylation (the modification of biological molecules by covalent conjugation with polyethylene glycol (PEG), a non-toxic, non-immunogenic polymer) of IGF2 mutants:

IGF2 mutants that have site-specific PEGylation are generated. PEGylation has long been used to prolong half-life of biologics. Site-specific PEGylation of WT IGF1 (at Lys-68) has been reported to have much longer half-life (>140 h) than non-PEGylated version (20-30 min). PEGylation of IGF2 does not seriously affect efficacy in vivo [33]. Site-specific PEGylation at Lys-68 was accomplished by mutating two other Lys residues at position 25 and 60 to Arg, and by removing PEGylation at the N-terminus amine residue by proteolytic removal of N-terminal portion. IGF2 has only Lys residue at position 65. IGF2 (WT and mutant) are PEGylated and N-terminal PEG is then removed by proteolytic (thrombin) removal of N-terminal portion. Branched N-hydroxysuccinimidyl activated branched PEG (NHS-PEG) (molecular weight of 40 Kd) is used (commercially available). Conditions for cross-linking are in buffer pH 8-10 and incubated at 4-22°C for 1 h as described [33]. N-terminal His-tag and PEG are removed by thrombin digestion for 25°C overnight. Digested materials are further purified by gel filtration and ion-exchange chromatography.

[0185] 4) IGF2 WT and mutants as Fc fusion proteins: Fc fusion has been widely used to stabilize proteins in vitro and in vivo. TNF receptor 2 Fc-fusion protein (Etanercept/Enbrel) mimics the inhibitory effects of naturally occurring soluble TNF receptors, and has a greatly extended half-life in the bloodstream (70–132 hrs), and therefore a more profound and long-lasting biologic effect than a naturally occurring soluble TNF receptor. IGF2 decoy Fc-fusion protein (His-tagged) was synthesized in CHO cells and purified using NA-NTA affinity chromatography. The stability and efficacy of the IGF1 decoy Fc-fusion protein is studied. In the preliminary studies Fc-IGF1 was fully functional, and Fc-IGF2 is also functional.

Example 4 Study interaction of integrins and IGF1R/IR-A upon IGF1/IGF2 stimulation **Rationale**

[0186] In previous biochemical studies, it was shown that IGF1 induces ternary complex formation (integrin $\alpha\beta3$ and $\alpha6\beta4$ -IGF1-IGF1R) on the cell surface. IGF2 should also induce ternary complex formation (integrin $\alpha\beta3/\alpha6\beta4$ -IGF2-IGF1R). Since IGF2 binds to IR-A it is expected that IGF2 induced formation of the integrin-IGF2-IR-A complex. Two methods are used to detect ternary complex formation: co-precipitation and imaging. It is shown that co-precipitation detect association of integrins and IGF1R/IR-A and integrins.

Research design

a) Study interaction between integrins and IGF1R/IR-A by co-immunoprecipitation

[0187] MDA-MB231 or other cancer cells that express IR-A, IGF1R or hybrid receptors are used for this purpose. Cells with WT or mutant IGF2 are stimulated and integrin β 3 immunoprecipitated from cell lysates as described before for IGF1. IGF1R or IR-A is detected in the purified materials by western blotting using antibodies specific to IGF1R or IR. WT IGF2 induces integrin-IGF2-(IR-A/IGF1R) complex formation, but IGF2 mutants do not. This establishes that IGF1R/IR-A and integrins crosstalk in an IGF2-dependent manner. Co-immunoprecipitation technique has several limitations as it does not reflect interaction in the live cell context, deliver a high frequency of false-positives and quantitation is often hard to interpret, because of the artificial and harsh conditions[34]. Therefore, quantitative live cell imaging methods with better resolution in time and space are preferred.

b) Study interaction between integrins and IGF1R/IR-A by imaging and micro-patterning

[0188] **Micro-patterning based quantitation:** a technique to force membrane proteins into specific micro-patterns within the plasma membrane for studying protein-protein interactions has been published [35]. The idea is to laterally rearrange the bait directly in the live cell plasma membrane by growing cells on surfaces that are micro-patterned with a binding partner to the bait (*e.g.*, IGF1R). Bait-prey interactions are readout by quantifying the co-redistribution of the fluorescent prey (*e.g.*, integrins) (Fig. 8). Generation of the micro-structured surfaces will be performed by micro-contact printing as described [36]. For bait redistribution, antibodies or ligands including biotinylated hormones, toxins or purified proteins can be applied. Most importantly, this technique has been already successfully used to analyze receptor tyrosine kinase (RTK) downstream signaling events including EGFR, IR and IGF1R action [36].

[0189] **Localization of β 3-EGFP and IGF1R-RFP:** the β 3-GFP expression construct with GFP at the C-terminus of integrin β 3 was generated as described [37]. In the preliminary studies, β 3-GFP and IGF1R-RFP were expressed in Hela cells and studied for β 3 and IGF1R co-localized (Fig. 9). β 3 integrin was co-recruited into IGF1R-enriched regions, indicating the interaction between IGF1R and β 3 integrins. Since this experiment is performed in the presence of serum, it is likely that cells are exposed to WT IGF1/IGF2. Using medium that does not contain serum, the effect of dominant-negative IGF2 (IGF2 decoy) is studied. WT IGF2 supports ternary complex formation (integrin-IGF2-IGF1R), but IGF2 decoy (integrin-binding defective mutants) does not.

[0190] Interaction between integrin $\beta 3$ and IR-A: it is studies whether integrin $\beta 3$ and IR-A interact in an IGF2-dependent manner. IR-A-GFP is generated by removing 12 amino acid residues from IR-B-GFP (in pEGF-N, available from Addgene) by mutagenesis (IR-A is 12 amino acid shorter than IRB due to alternative splicing). The resulting IR-A cDNA is insert into RFP vector (pRFP-N). HeLa cells are transiently transfected with IR-ARFP and $\beta 3$ -integrin-GFP and grown on micro-biochips coated with anti-IGF-IR antibody. $\beta 3$ -integrin-GFP is co-recruited into IR-A-RFP enriched regions when WT IGF2 is present. When IGF2-decoy is present, $\beta 3$ -integrin-GFP is not co-recruited into IR-A-RFP, indicating interaction between IR-A and $\beta 3$ -integrin and the interaction requires IGF2-integrin interaction.

Results

[0191] IGF2 induces integrin-IGF1R/IR-A interaction through ternary complex formation. IGF2-decoy is a useful reagent for address this point. WT IGF2 induce ternary complex formation, but integrin-binding defective IGF2-decoy does not. In the preliminary studies, it was shown that integrin $\beta 3$ -EGFP is present in IGF1R-RFP-rich area (Fig. 9). It is likely that this co-localization is IGF1/IGF2-dependent (tested using IGF1 and IGF2-decoy). Upon establishing that IGF1R/IR-A interact with integrins in an IGF2-dependent manner, the interaction is studied in more detail using Fluorescence recovery after photobleaching (FRAP). FRAP is an optical technique capable of quantifying the two dimensional lateral diffusion of fluorescently labeled probes and is therefore a very useful tool in biological studies of protein binding. The FRAP experiments can confirm whether integrin $\beta 3$ and IGF1R dynamically interact.

Discussions

[0192] When antibodies of IGF1R are used, IGF1R signaling is inhibited on both normal and neoplastic IGF1R-positive cells, and also on IGF1R-positive cells in the hypothalamic-pituitary axis that are involved in the feedback inhibition of IGFI on growth hormone (GH) secretion. This results in substantial increases in GH, which stimulate the liver to increase IGF production and also cause insulin resistance in insulintarget tissues, which raise glucose levels, and thereby lead to increases in insulin production. The IGF1R tyrosine kinase inhibitors have similar effects, but they also block insulin receptors. When IGF2-decoy is used in vivo, it suppresses IGF1R and IR-A, which will reduce the effect of enhanced insulin production on normal and cancer cells. However, the effect of IGF2-decoy

on normal cells will be minimal since IR-B will not be affected by IGF2-decoy. This can be addressed by monitoring metabolic effects of IGF2-decoy in vitro and in vivo (*e.g.*, levels of serum glucose and insulin).

[0193] All patents, patent applications, and other publications, including GenBank

- 5 Accession Numbers, cited in this application are incorporated by reference in the entirety for all purposes.

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WHAT IS CLAIMED IS:

- 1 1. A method for inhibiting IGF2 signaling in a cell, comprising the step
2 of contacting the cell with an effective amount of an inhibitor of IGF2-integrin binding.
- 1 2. The method of claim 1, wherein the integrin is $\alpha v\beta 3$.
- 1 3. The method of claim 1, wherein the integrin is $\alpha 5\beta 1$ or $\alpha 6\beta 4$.
- 1 4. The method of claim 1, wherein the inhibitor is an IGF2 mutant
2 comprising two substitutions of R37E and R38E.
- 1 5. The method of claim 1, wherein the inhibitor is IGF2 mutant
2 R24E/R37E/R38E.
- 1 6. The method of claim 1, wherein the inhibitor is IGF2 mutant
2 R34E/R37E/R38E.
- 1 7. The method of claim 1, wherein the inhibitor is IGF2 mutant
2 R24E/R34E/R37E/R38E.
- 3 8. The method of claim 1, wherein the cell is within a patient's body.
- 1 9. The method of claim 1, wherein the contacting step is performed by
2 oral administration or intravenous, subcutaneous, intraperitoneal, or intratumor injection.
- 1 10. A method for identifying an inhibitor of IGF2-integrin binding,
2 comprising the steps of
3 (1) contacting an integrin and a polypeptide comprising an integrin-
4 binding sequence of an IGF2, in the presence of a test compound, under conditions
5 permissible for IGF2-integrin binding; and
6 (2) detecting the level of polypeptide-integrin binding, wherein a decrease
7 in the level of binding when compared with the level of binding in the absence of the test
8 compound indicates the compound as an inhibitor of IGF2-integrin binding.
- 1 11. The method of claim 10, wherein the integrin is $\alpha v\beta 3$.
- 1 12. The method of claim 10, wherein the integrin is $\alpha 5\beta 1$ or $\alpha 6\beta 4$.

- 2 **13.** The method of claim 11, wherein the polypeptide comprises the
3 sequence of C-domain of human IGF2 protein.
- 1 **14.** The method of claim 11, wherein the polypeptide comprises the full
2 length of human IGF2 protein.
- 1 **15.** The method of claim 11, wherein the polypeptide further comprises a
2 heterologous amino acid sequence.
- 1 **16.** The method of claim 15, wherein the heterologous amino acid
2 sequence is glutathione S-transferase (GST).
- 1 **17.** The method of claim 11, wherein the integrin is expressed on a cell
2 surface.
- 1 **18.** An isolated polypeptide comprising an amino acid sequence that (1)
2 has at least 95% sequence identity to a wild-type IGF2 protein sequence; (2) comprises
3 substitutions of at least two Arg residues at positions 37 and 38 of a wild-type human IGF2
4 protein; and (3) inhibits IGF2-integrin binding.
- 1 **19.** The polypeptide of claim 18, wherein the integrin is $\alpha v\beta 3$.
- 1 **20.** The polypeptide of claim 18, wherein the integrin is $\alpha 5\beta 1$ or $\alpha 6\beta 4$.
- 1 **21.** The polypeptide of claim 18, wherein at least 3 of the Arg residues at
2 positions 24, 34, 37, and 38 are substituted.
- 1 **22.** The polypeptide of claim 18, wherein the Arg residues at positions 24,
2 34, 37, and 38 are substituted.
- 1 **23.** The polypeptide of claim 21 or 22, wherein each of the Arg residues is
2 substituted with a Glu residue.
- 1 **24.** A composition comprising the polypeptide of claim 18 and a
2 pharmaceutically acceptable excipient.
- 1 **25.** The composition of claim 24, wherein the polypeptide is IGF2 mutant
2 R24E/R37E/R38E.

1 **26.** The composition of claim 24, wherein the polypeptide is IGF2 mutant
2 R34E/R37E/R38E.

1 **27.** The composition of claim 24, wherein the polypeptide is IGF2 mutant
2 R24E/R34E/R37E/R38E.

1 **28.** An isolated polynucleotide encoding the polypeptide of claim 18.

1 **29.** A recombinant expression cassette comprising the polynucleotide of
2 claim 28, optionally linked to a promoter.

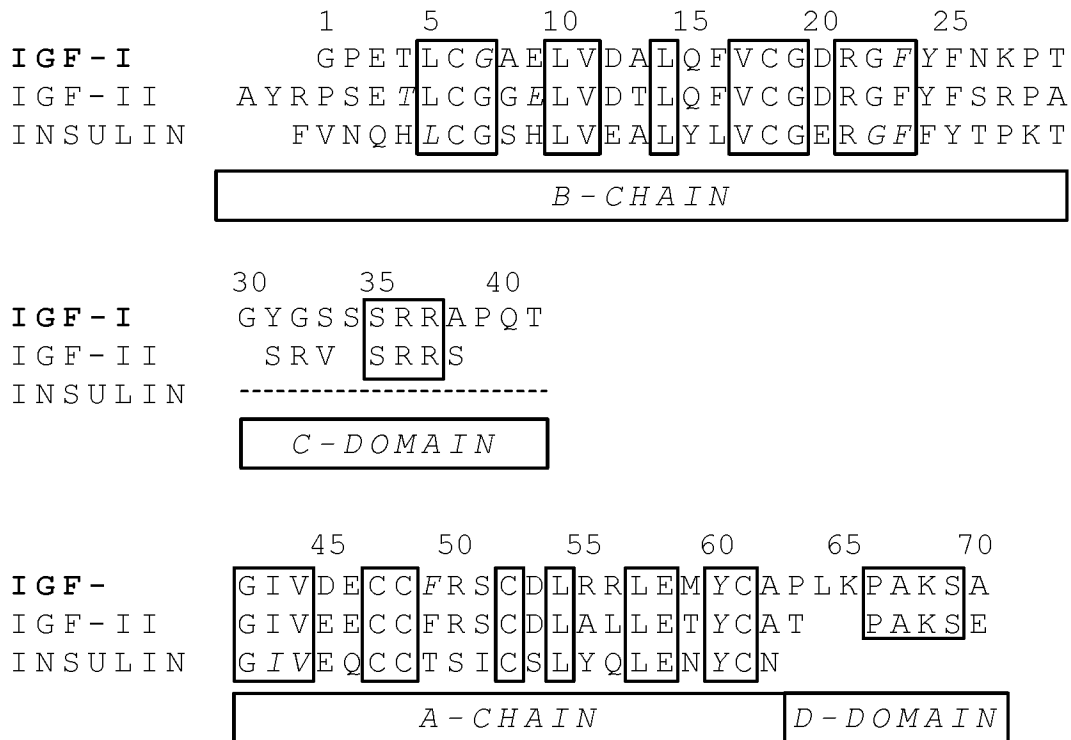
1 **30.** An isolated host cell comprising the expression cassette of claim 29.

1 **31.** A composition comprising the polynucleotide of claim 28 or the
2 expression cassette of claim 29, and a pharmaceutically acceptable excipient.

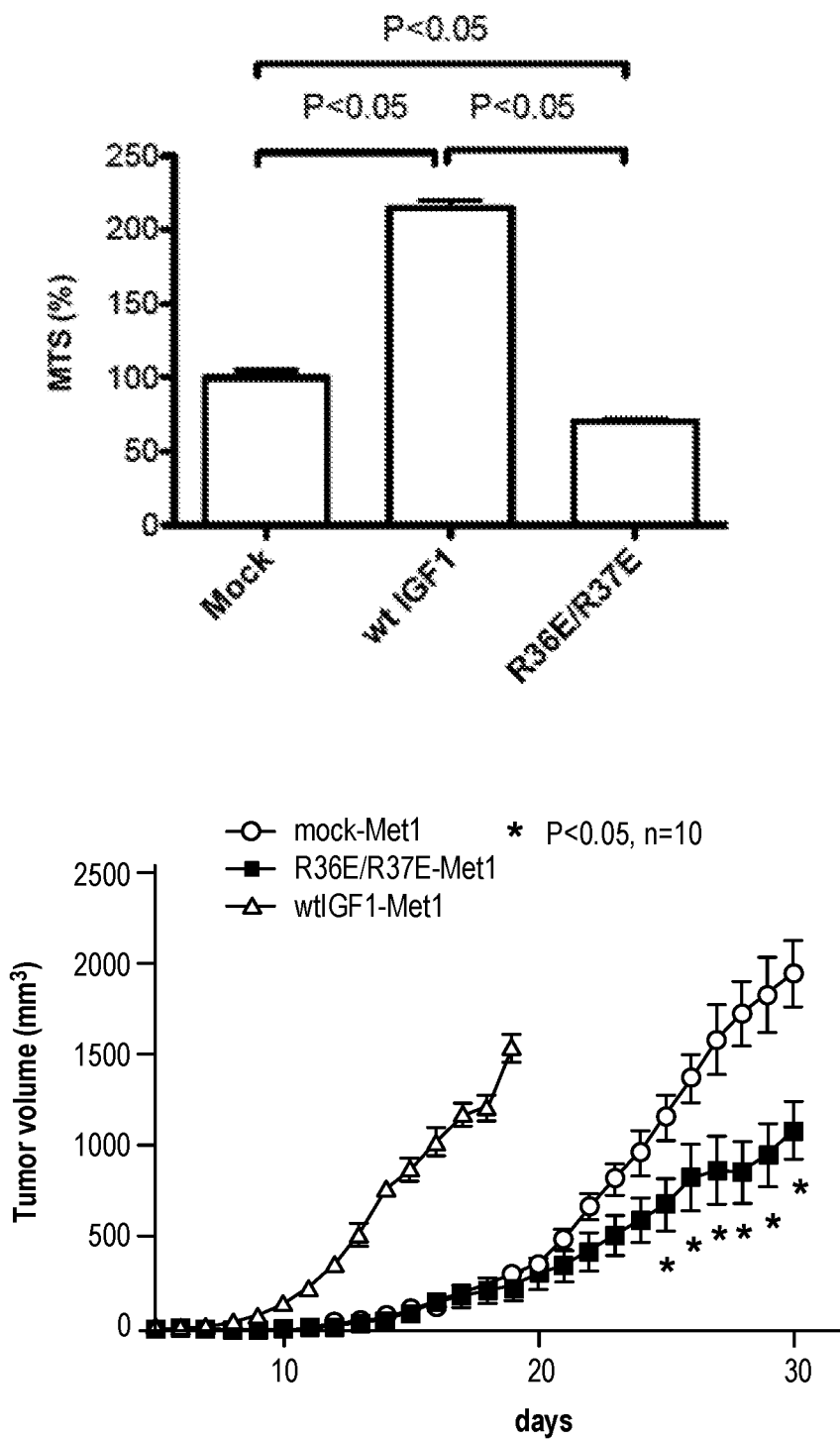
1 **32.** A kit for inhibiting IGF signaling, comprising the composition of claim
2 24 or 31.

1

1/9

**FIG. 1**

2/9

**FIG. 2**

3/9

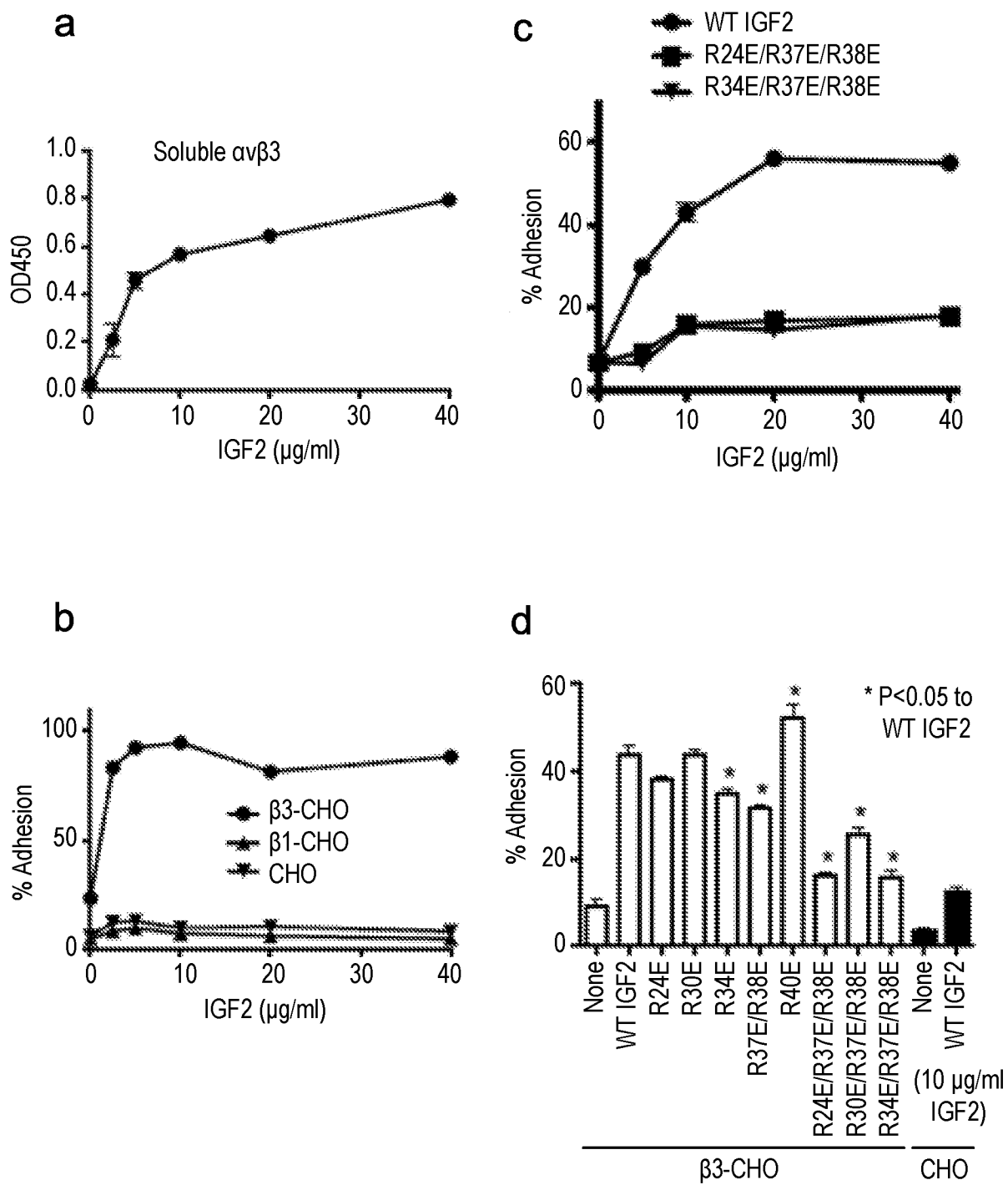
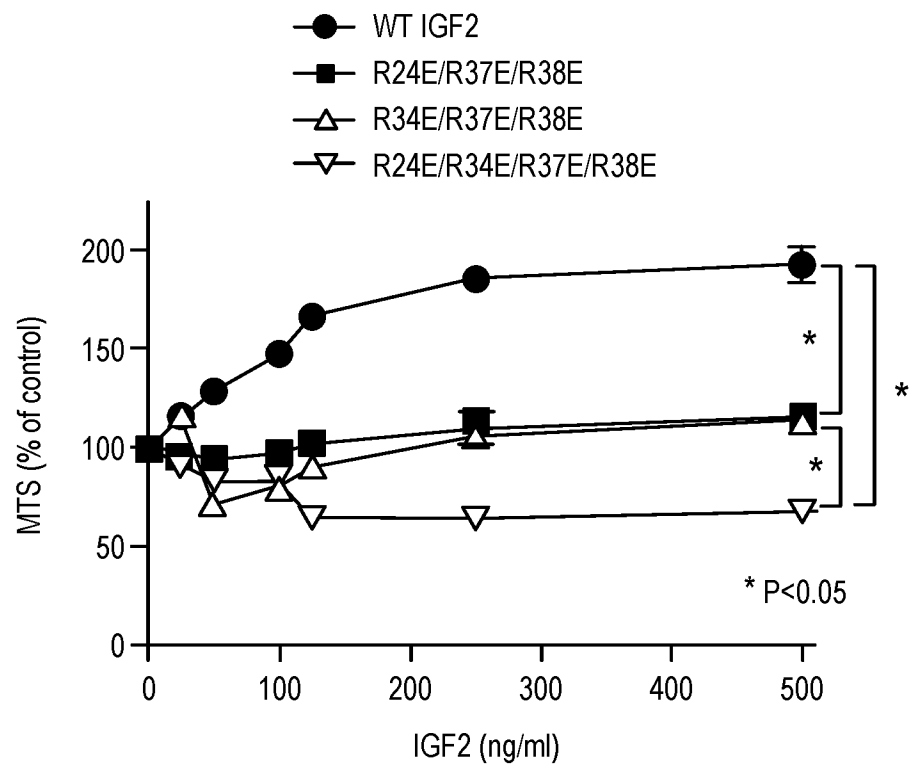
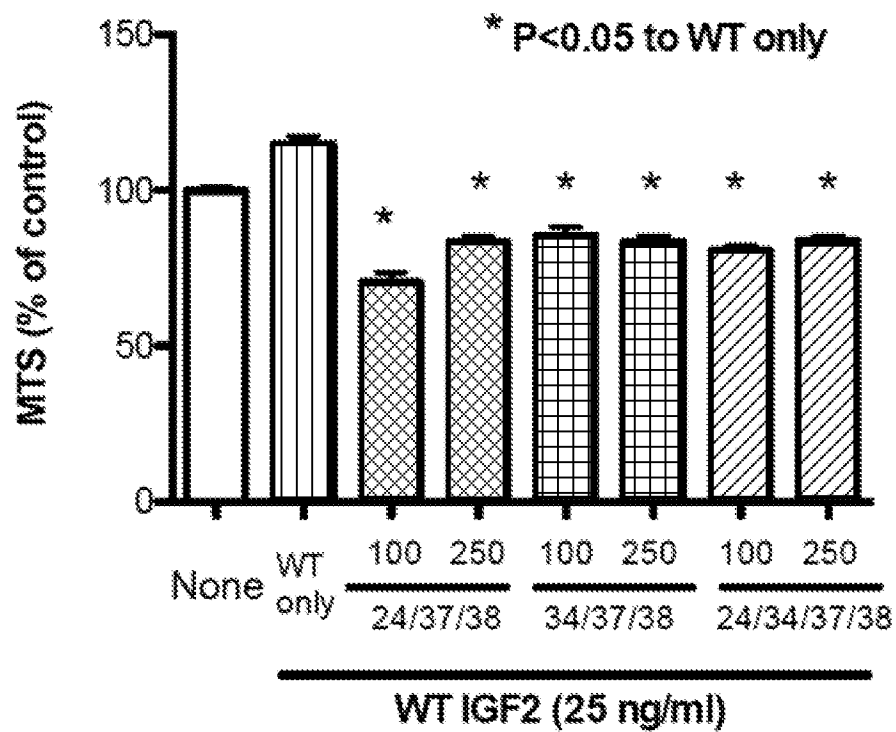


FIG. 3

4/9

**b****FIG. 4**

5/9

c IGF1R blockade

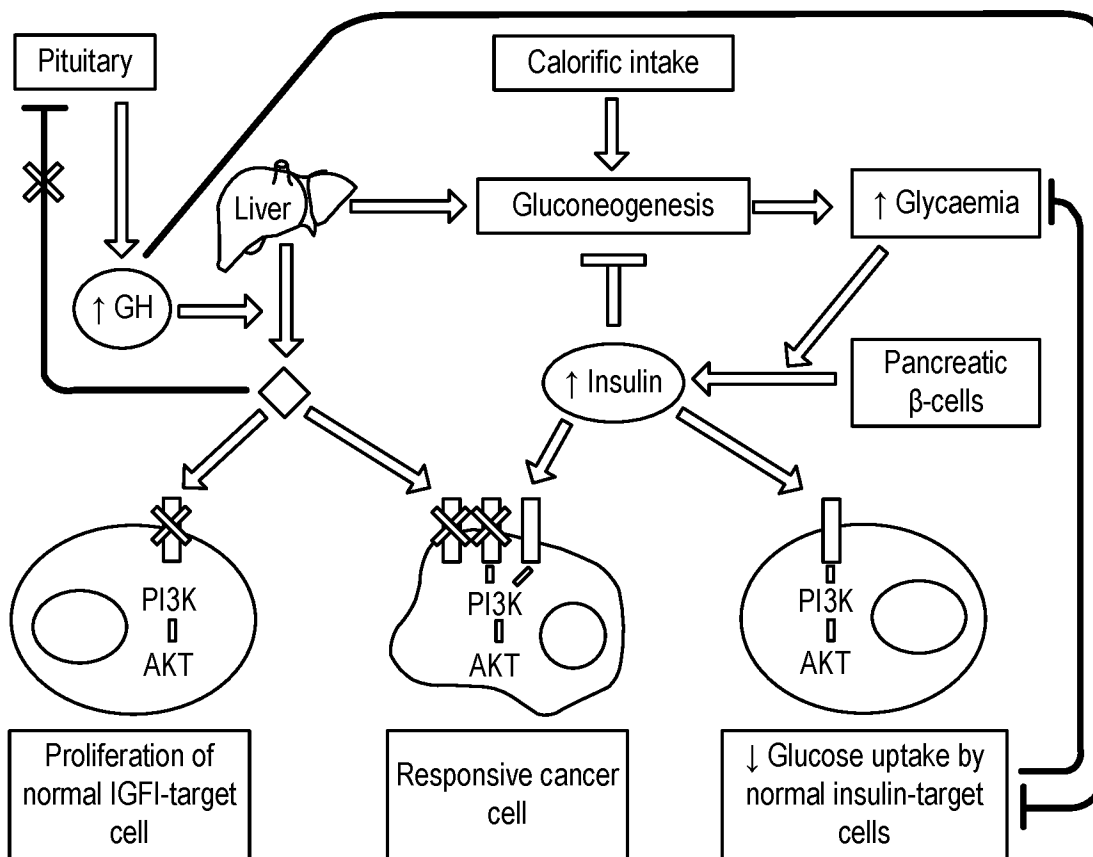
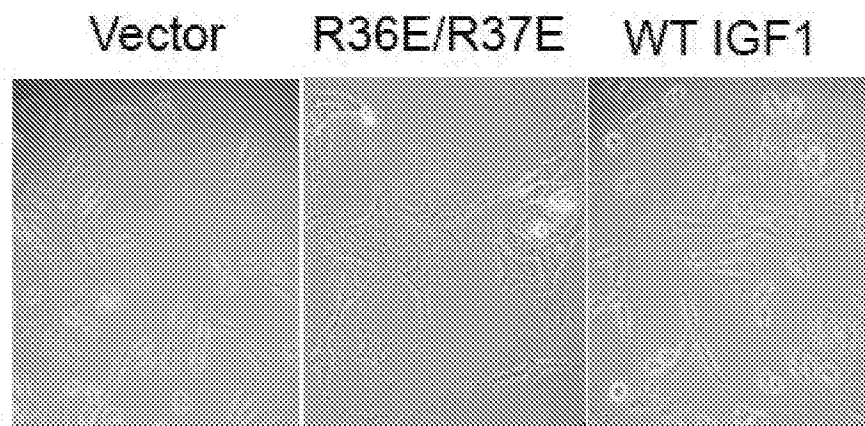


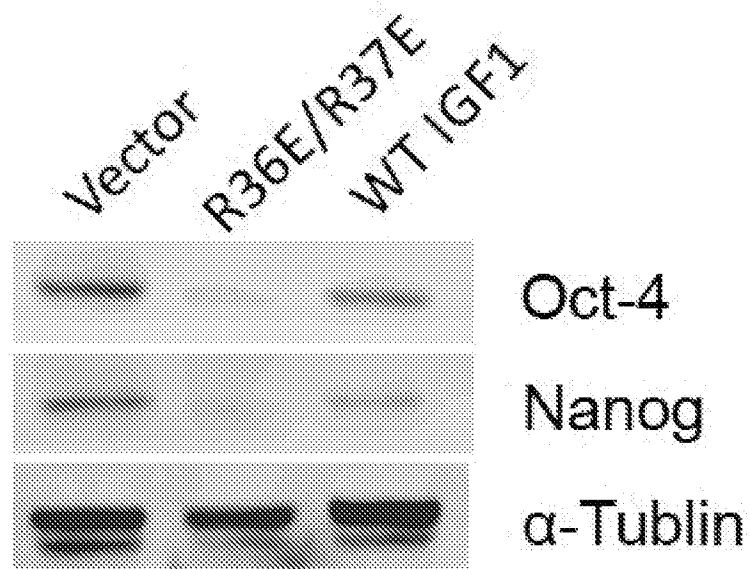
FIG. 5

6/9

a



b

**FIG. 6**

7/9

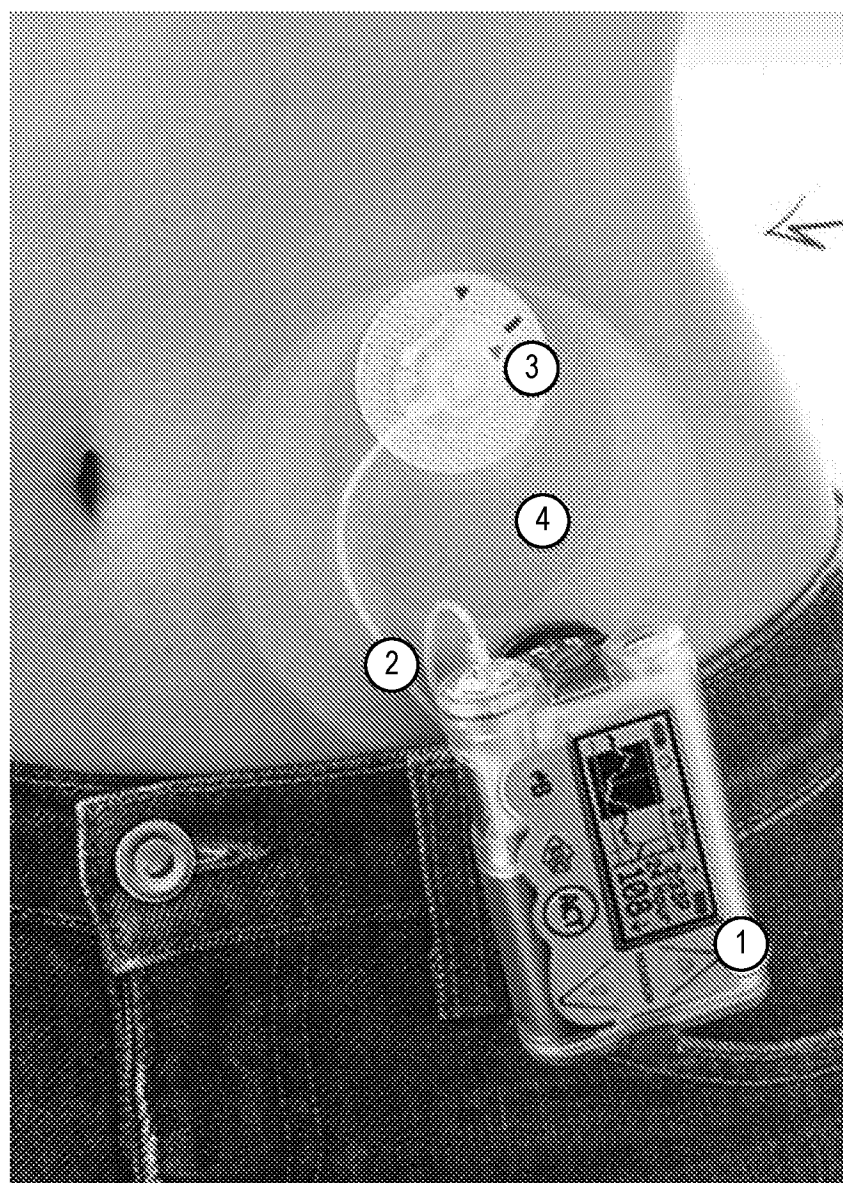


FIG. 7

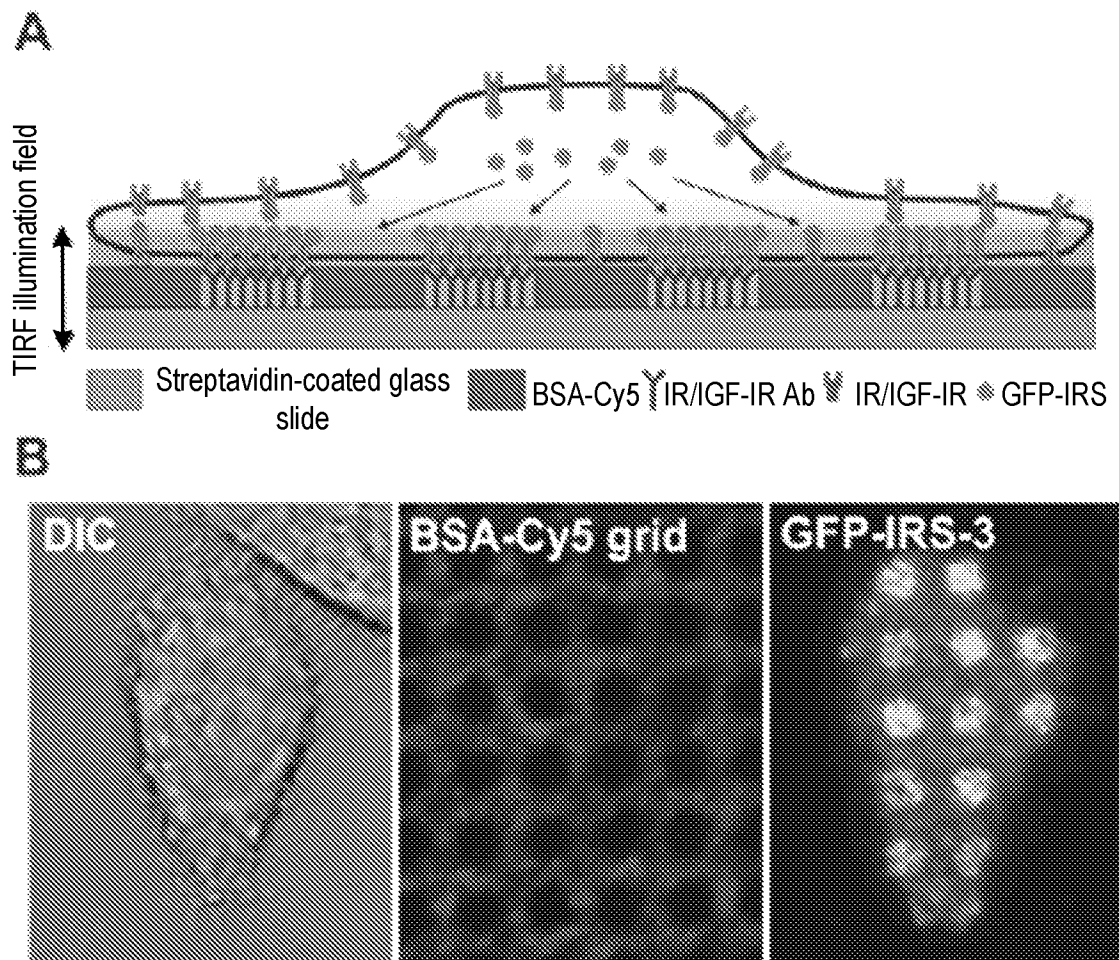
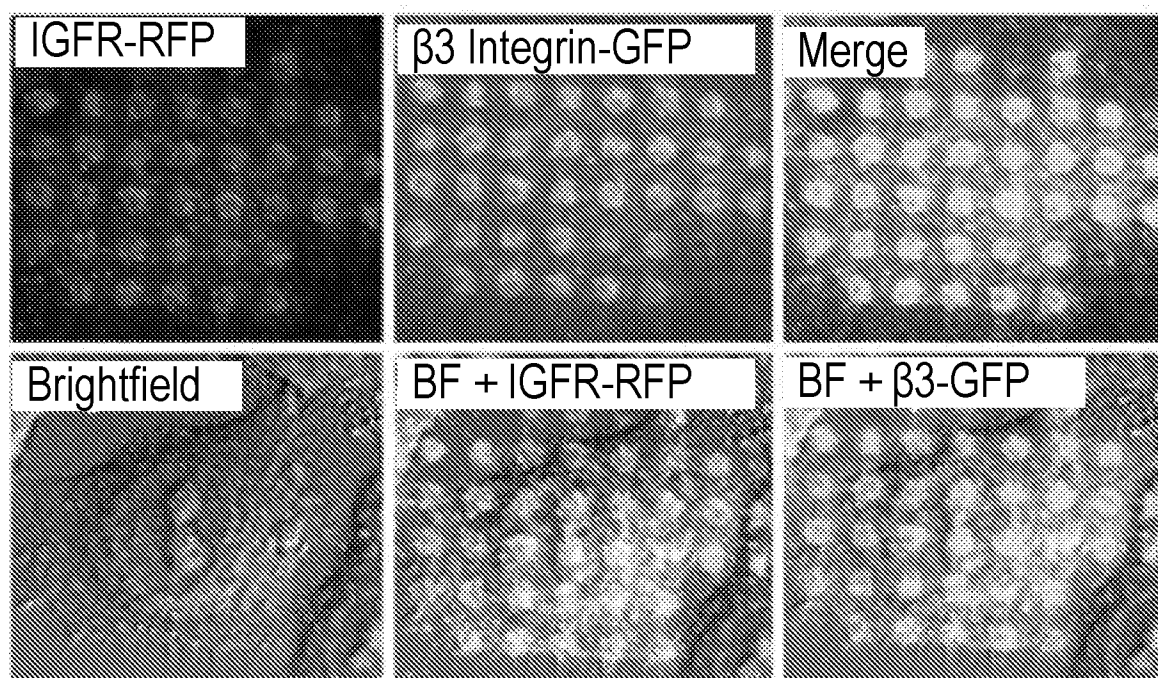


FIG. 8

**FIG. 9**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/013100

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/00; A61K 31/277; A61K 31/343 (2016.01)

CPC - A61K 31/00; A61K 31/704; C12Q 1/6886 (2016.05)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 31/00; A61K 31/277; A61K 31/343 (2016.01)

CPC - A61K 31/00; A61K 31/704; C12Q 1/6886 (2016.05)

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

USPC - 424/649; 424/145.1; 435/6 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Patbase, Google Patents, PubMed, Google.

Search terms used: IGF2 insulin like growth factor 2 integrin inhibit cell signaling Arg34 Arg24 Arg 37 Arg38 substitute

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0014204 A1 (FEINBERG et al) 20 January 2011 (20.01.2011) entire document	1-3, 8-17
Y	HAMIDOUCHE et al. "Crosstalks between integrin alpha 5 and IGF2/IGFBP2 signalling trigger human bone marrow-derived mesenchymal stromal osteogenic differentiation," BMC Cell Biology, 23 June 2010 (23.06.2010), Vol. 11, No. 44, Pgs. 1-9. entire document	1-3, 8-20, 24, 28-31
Y	US 2014/0065142 A1 (ZYNGENIA INC et al) 06 March 2014 (06.03.2014) entire document	2, 3, 11, 13-17, 19, 20
Y	WO 2011/159882 A2 (INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION et al) 22 December 2011 (22.12.2011) entire document	13
Y	US 5,854,025 A (EDWARDS et al) 29 December 1998 (29.12.1998) entire document	14
Y	JELINIC et al. "The Testis-Specific Factor CTCFL Cooperates with the Protein Methyltransferase PRMT7 in H19 Imprinting Control Region Methylation," PLoS Biology, 17 October 2006 (17.10.2006), Vol. 4, Iss. 11, Pgs. 1910-1922. entire document	15, 16
Y	US 2006/0166328 A1 (GLASS et al) 27 July 2006 (27.07.2006) entire document	18-20, 24, 28-31

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

22 May 2016

Date of mailing of the international search report

21 JUN 2016

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, VA 22313-1450

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/013100

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
 - ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/013100

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.