



(86) Date de dépôt PCT/PCT Filing Date: 2001/10/12
(87) Date publication PCT/PCT Publication Date: 2002/04/18
(85) Entrée phase nationale/National Entry: 2003/03/18
(86) N° demande PCT/PCT Application No.: US 2001/031857
(87) N° publication PCT/PCT Publication No.: 2002/030984
(30) Priorité/Priority: 2000/10/13 (60/240,353) US

(51) Cl.Int.⁷/Int.Cl.⁷ C07K 16/00, A61K 39/395, A01N 43/04,
C07H 19/00, C12P 21/06
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(54) Titre : ANTICORPS EN CHAINE SIMPLE DE RECEPTEUR DE FACTEUR DE CROISSANCE ANTI-EPIDERMIQUE
HUMAIN
(54) Title: HUMAN ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR SINGLE-CHAIN ANTIBODIES

(57) **Abrégé/Abstract:**

Human anti-epidermal growth factor receptor (EGFR) single-chain antibodies (scFvs) were isolated from a human IgM phage display library using purified epidermal growth factor receptor as antigen. Two isolates with different amino acid sequences were identified by ELISA as epidermal growth factor receptor-specific. The scFvs bind to the full length epidermal growth factor receptor and the truncated and/or mutated epidermal growth factor receptor on human cells. These anti-EGFR-scFvs are useful as therapeutic and/or diagnostic agents.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 April 2002 (18.04.2002)

PCT

(10) International Publication Number
WO 02/30984 A1

- (51) International Patent Classification⁷: **C07K 16/00**, A61K 39/395, C07H 19/00, C12P 21/06, A01N 43/04
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- (21) International Application Number: PCT/US01/31857
- (81) Designated States (*national*): AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 12 October 2001 (12.10.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/240,353 13 October 2000 (13.10.2000) US
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
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Published:— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HUMAN ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR SINGLE-CHAIN ANTIBODIES

(57) Abstract: Human anti-epidermal growth factor receptor (EGFR) single-chain antibodies (scFvs) were isolated from a human IgM phage display library using purified epidermal growth factor receptor as antigen. Two isolates with different amino acid sequences were identified by ELISA as epidermal growth factor receptor-specific. The scFvs bind to the full length epidermal growth factor receptor and the truncated and/or mutated epidermal growth factor receptor on human cells. These anti-EGFR-scFvs are useful as therapeutic and/or diagnostic agents.



WO 02/30984 A1

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**HUMAN ANTI-EPIDERMAL GROWTH FACTOR RECEPTOR
SINGLE-CHAIN ANTIBODIES**

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BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This patent application claims benefit of patent application U.S. Serial number 60/240,353, filed October 13, 2000, now abandoned. _

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Field of the Invention

The present invention relates generally to the fields of molecular biology and monoclonal antibody technology. More specifically, the present invention relates to human single-chain antibodies that bind specifically to the epidermal growth factor receptor.

25

Description of the Related Art

The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein consisting of an extracellular ligand binding domain, a transmembrane region and an intracellular domain with tyrosine kinase activity. The binding of growth factors, EGF or TGF α , to the epidermal growth factor receptor results in receptor dimerization, auto-phosphorylation and induction of a tyrosine kinase cascade, leading ultimately to DNA synthesis and cell division.

The epidermal growth factor receptor gene (c-erb-1), located on chromosome 7, is homologous to the avian erythroblastosis virus oncogene (v-erbB) that induces malignancies in chickens. The v-erbB gene encodes for a truncated protein product that lacks the extracellular ligand binding domain. The tyrosine kinase domain of the epidermal growth factor receptor has been found to have 97% homology to the v-erbB transforming protein.

The epidermal growth factor receptor is overexpressed in a number of malignant human tissues when compared to their normal tissue counterparts. The gene for the receptor is both amplified and overexpressed in a number of cancer cells. Overexpression of the epidermal growth factor receptor is often accompanied by the co-expression of the growth factors, EGF and TGF α , suggesting that an autocrine pathway for control of growth may play a major part in the progression of tumors.

A high incidence of overexpression, amplification, deletion and structural rearrangement of the gene coding for the

epidermal growth factor receptor has been found in biopsies of brain tumors. In fact, the amplification of the epidermal growth factor receptor gene in glioblastoma multiforme tumors is one of the most consistent genetic alterations known, with the EGFR being overexpressed in approximately 40% of malignant gliomas. In addition to glioblastomas, abnormal epidermal growth factor receptor expression has also been reported in a number of squamous epidermoid cancers and breast cancers. Many patients with tumors that overexpress the epidermal growth factor receptor have a poorer prognosis than those who do not. Consequently, therapeutic strategies which can potentially inhibit or reduce the aberrant expression of the EGFR are of great interest as potential anti-cancer agents.

Since the advent of hybridoma technology to produce murine monoclonal antibodies (mAbs) developed by Milstein and Köhler in 1975 (1), the therapeutic potential of antibodies is beginning to come to fruition for cancer therapy. There are many reports describing a few antibodies which inhibit cell proliferation of epidermal growth factor receptor-overexpressing cell lines (2-6). One such mouse antibody, mAb 225, was shown to inhibit cell proliferation and block ligand-induced epidermal growth factor receptor tyrosine kinase activity (2-3, 7). Further analysis showed mAb 225 induced a G₁ growth arrest and activated an apoptotic pathway after a 24 h exposure to increasing concentrations of antibody (8).

Other monoclonal antibodies which bind to the epidermal growth factor receptor and block ligand binding also show promise for cancer therapy. One group of rat monoclonal

antibodies showed a dramatic antitumor effect in xenograft mouse models, with one antibody, ICR62 curing 4 out of 8 mice of the tumor (9). However, the problem with rat and mouse monoclonal antibodies or even the human-mouse chimeric antibody is the possibility of an immune or allergic response with prolonged treatment (10-13).

In order to avoid the human anti-murine antibody (HAMA) response in humans due to the repeated administration of murine mAbs, it is preferable to use human antibody in therapy or diagnostics. A 100% human monoclonal antibody against the epidermal growth factor receptor, E7.6.3, has been shown to completely eradicate human tumor xenografts in mice (4). This antibody is expected to elicit a minimal immune response in humans and shows promise for future cancer therapy. However due to the heterologous vascular structure around the tumor and the molecular size of the antibodies, monoclonal antibodies penetrate the tumor poorly and are unevenly distributed around the tumor.

In order to improve on the use of monoclonal antibodies, intact monoclonal antibodies have been reduced in size to antibody fragments or single-chain antibodies (scFvs). Therefore the development of human anti-EGFR scFvs will enhance its use as a diagnostic and/or therapeutic agent. One advantage of single-chain antibodies is their ability to penetrate deeper into the tumor (14). Thus, these molecules may potentially be more efficacious than intact antibodies for systemic administration. Also single-chain antibodies can be expressed intracellularly (intrabodies) and targeted to a subcellular

compartment of the tumor cell or be secreted by the tumor cell and bind in an autocrine/paracrine fashion.

The prior art is deficient in the lack of a 100% human single-chain antibody that binds to the epidermal growth factor receptor. The present invention fulfills this longstanding need and desire in the art.

10

SUMMARY OF THE INVENTION

The present invention provides a 100% human single-chain antibody (scFv) which binds to the epidermal growth factor receptor. Two single-chain antibodies were isolated from a human IgM phage display library using purified epidermal growth factor receptor as antigen, and identified by ELISA as epidermal growth factor receptor-specific. Sequence analysis confirmed the two isolates as individual clones based on differences in their nucleotide and putative amino acid sequences. One single-chain antibody was shown to bind to the native full length epidermal growth factor receptor and the truncated and/or mutated epidermal growth factor receptor on human cells.

The present invention is directed to a human anti-epidermal growth factor receptor single-chain antibody having a sequence of SEQ ID No. 1 (clone 6) or SEQ ID No. 2 (clone 63), as well as DNA molecules and expression vectors that encode for the

expression of the claimed human anti-epidermal growth factor receptor single-chain antibody.

The present invention is also drawn to a pharmaceutical composition comprising the disclosed human anti-epidermal growth factor receptor scFv and a therapeutic and/or diagnostic agent. Preferably, the therapeutic and/or diagnostic agent can be a toxin, a chemotherapeutic agent, a radioisotope, a transition metal or a gene therapy vector.

The present invention is also drawn to a method of treating or imaging a tumor, comprising the step of administering to a patient in need of such treatment or detection an effective amount of a radiolabeled anti-EGFR single-chain Fv of the present invention.

15

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows the phagemid, pSEX81, which is optimized for surface expression on the M13 bacteriophage. Depicted here is a single-chain antibody cloned into the multiple cloning site, in-frame with the pelB leader sequence and gene III
5 M13 protein.

Figure 2 shows the putative amino acid sequences of anti-EGFR scFv clones pSEX81-6 and pSEX81-63. Clones were sequenced both directions using primers from the pelB leader sequence, the gene III protein and two complementary primers
10 annealing to the alpha tubulin linker sequence. The heavy chain variable region (V_H) and light-chain variable region (V_L) are identified with their respective CDRs (bold) as described (16).
*identifies identical amino acids.

Figure 3 shows the eukaryotic secreting plasmid, pSECTAG/Bpu/neo which was modified from pSECTAG/Friendly
15 (Invitrogen). The neomycin gene replaced the zeomycin gene and a *Bpu* 1102I restriction enzyme site was added in-frame with the Ig leader sequence.

Figure 4 shows the analysis of secretory anti-EGFR-scFv from U87MG cell lines stably transfected with vector
20 (pSECTAG) or anti-EGFR scFv clone 6 (p6.34). **Figure 4A** shows immunoprecipitation of scFv from cell lysates of pSECTAG (lane 1) and p6.34 (lane 2) using anti-myc antibody. **Figure 4B** shows immunoprecipitation of secretory scFv from culture medium of pSECTAG (lane 1) and p6.34 (lane 2) using anti-alpha tubulin
25 antibody. Anti-myc antibody was used to detect scFv for both immunoblots in Figures 4A and 4B. **Figure 4C** shows ELISA using cell culture medium, or culture medium from pSECTAG,

p6.34 or anti-EGFR mAb (Sigma) to detect binding to the epidermal growth factor receptor antigen. ScFvs were detected using anti-myc-HRP antibody and anti-epidermal growth factor receptor mAb was detected with anti-mouse IgG-HRP antibody
5 and developed with OPD (Sigma).

Figure 5 shows FACS analysis for the detection of anti-epidermal growth factor receptor scFv bound to the extracellular domain of the epidermal growth factor receptor. The cells, U87MG, U87MG.wtEGFR and U87MG.ΔEGFR were incubated
10 with culture medium, culture medium from pSECTAG or p6.34 for 30 min at 4°C. The cells were washed, then incubated with rat anti-alpha tubulin followed by FITC-labeled anti-rat antibody.

Other and further aspects, features, and advantages of the present invention will be apparent from the following
15 description of the presently preferred embodiments of the invention given for the purpose of disclosure.

20 DETAILED DESCRIPTION OF THE INVENTION

Because the administration of murine monoclonal antibodies to humans resulted in human anti-murine antibody (HAMA) response, hindering any therapeutic and/or diagnostic
25 potential, the monoclonal antibodies had to be modified. Genetic engineering techniques were used to develop human-murine chimeric monoclonal antibodies. An alternative solution was to develop single-chain antibodies (scFvs). Initially murine scFv

were isolated and now technology has progressed to screening naive human phage display libraries for therapeutically and/or diagnostically useful single-chain antibodies of human origin (30-32).

5 Over the past 10 years, a variety of mouse and human single-chain antibodies have been isolated, including mouse scFvs which bind to the cell surface receptors, epidermal growth factor receptor and erbB-2 (23, 27, 33). Cell proliferation was inhibited when EGFR-overexpressing cell lines were transfected with a
10 plasmid encoding murine anti-EGFR scFvs targeted to the endoplasmic reticulum (ER) or secretory pathway (23, 27). Inhibition of cell proliferation was obtained when erbB-2-overexpressing cell lines were transfected with a plasmid resulting in the production of murine single-chain antibodies
15 which binds the erbB-2 receptor in the ER but no inhibition of cell proliferation was detected with a cytoplasm-targeted scFv (33). Even though both of the single-chain antibodies bound to the extracellular domain of their respective receptor they were expressed as an intracellular scFv (intrabody). The intrabodies
20 were directed to the lumen of the ER to bind the receptor as it was being processed for glycosylation, thereby decreasing the amount of receptor expressed on the plasma membrane and inhibiting cell proliferation. To obtain the greatest anti-proliferative effect, the optimal expression and targeting of anti-EGFR single-chain
25 antibodies to the subcellular components involved in epidermal growth factor receptor expression should be undertaken.

The ability of a single-chain antibody to inhibit tumor cell proliferation has considerable potential for cancer gene

therapy on its own merit. Moreover, the ability of a scFv to sensitize tumor cells to radiation or chemotherapy treatments will enhance their therapeutic potential. Tumor cells either transfected with scFv expressing plasmid DNA or transduced with viral vectors have shown an increased sensitivity to radiation and/or chemotherapy drugs *in vitro* and *in vivo* (34, 35). The anti-erbB-2 scFv, pGT21, was shown to sensitize ovarian cancer cells, SKOV3, to cis-DDP (34). The increased sensitization to cis-DDP was shown to be related to the down-modulation of the erbB-2 protein by targeting the scFv to the ER. The same scFv also sensitized tumor cells to radiation *in vitro* and *in vivo* (35). However, a major limitation for these scFvs is the fact that the majority are murine and therefore potentially immunogenic in human.

The present invention discloses a 100% human single-chain antibody (scFv) which binds to the epidermal growth factor receptor. Two scFvs were isolated from a human IgM phage display library using purified epidermal growth factor receptor as antigen, and identified by ELISA as epidermal growth factor receptor-specific. Sequence analysis confirmed the two isolates as individual clones based on differences in their nucleotide and putative amino acid sequences. One scFv was shown to bind to the native full length epidermal growth factor receptor and the truncated and/or mutated epidermal growth factor receptor on human cells.

As used herein, the term "monoclonal antibody" means an antibody composition recognizing a discrete antigen

determinant. It is not intended to be limited with regard to the source of the antibody or the manner in which it is made.

As used herein, single chain antibodies or scFvs are polypeptides which consist of the variable (V) region of an antibody heavy chain linked to the V region of an antibody light chain with or without an interconnecting linker. This comprises the entire antigen binding site, and is the minimal antigen binding site. These single-chain antibodies may be produced in bacteria, yeast or eukaryotic cells.

An "antigen-binding site" refers to the part of an immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable regions of the heavy and light chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions" or "FRs". In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen binding "surface". This surface mediates recognition and binding of the target antigen. The three hypervariable regions of each of the heavy and light chains are referred to as "complementarity determining regions" or "CDRs" and are characterized, for example by Kabat et al., Sequences of proteins of immunological interest, 4th ed., U.S. Dept. Health and Human Services, Public Health Services, Bethesda, Md. (1987).

As an agent by itself, the scFv disclosed herein may inhibit and/or block the growth of epidermal growth factor receptor-expressing human cells. The human anti-epidermal growth factor receptor scFv may also induce apoptosis and cell death in human cells that express epidermal growth factor receptor. A toxin, chemotherapeutic agent, a transition metal or radioisotope generally known in the art can be covalently or non-covalently conjugated to the scFv of the present invention, which would then target the agent to epidermal growth factor receptor-expressing human cells. The scFv disclosed herein may also be used as a part of a bi-specific scFv or some other combination with either itself or another scFv. Furthermore, all or portions of the scFv disclosed herein may be used to target viral or bacterial gene therapy vectors or other agents to bind to epidermal growth factor receptor-expressing human cells. The portions of the scFv could be as small as one complementarity determining region (CDR) or a combination of CDRs from one or both variable regions.

One object of the present invention is to target a scFv to a particular cellular process as a powerful therapeutic technique. Combining the targeted scFv with a gene-based therapeutic approach may enhance the efficacy of single-chain antibodies. The realization of the goals of the current invention will allow for the design of cancer gene therapy treatment using intratumoral injection of a viral vector for successful transduction of a therapeutic scFv to be used in combination with radiation and/or chemotherapy drugs.

A number of methods can be used to deliver the single-chain antibodies of the present invention to tumor cells. In

the *ex vivo* method, the scFv is expressed in bacterial cells (14, 36-37) or eukaryotic cells (24), then isolated and purified prior to administration to the tumor cell lines or tumors implanted in mice. The purified scFv may be administered directly or labeled
5 with a radioisotope, transition metal or toxin prior to administration (38-41). Also, the single-chain antibodies can be engineered to express a bacterial toxin protein on the C-terminal end to enhance the therapeutic potential of the scFv (37). The administration of an *ex vivo* produced scFv will rapidly localize
10 and penetrate the tumor before being quickly cleared from the circulatory system (14, 38-39).

The *in vivo* expression of single-chain antibodies can result from transiently or stably transfecting cells with DNA (24-25) or transducing cells with viral vectors. DNA transfer can be
15 accomplished by a variety of standard techniques, such as calcium phosphate, DEAE dextran, electroporation or lipophilic reagents or by using a viral vector to transport the DNA into the cells. Most DNA transfection methods work very well for *in vitro* experiments; however, viral vectors may be more advantageous
20 for *in vivo* protocols. Viral vectors commonly used for gene therapy include retrovirus, adenovirus, adeno-associated virus and herpesvirus.

The invention also includes biologically functional fragments of the single-chain antibodies described in this
25 specification. Biologically functional fragments are those fragments sufficient for binding of the antibody fragment to epidermal growth factor receptor. Functional fragments include polypeptides with amino acid sequences substantially the same as

the amino acid sequence of the variable or hypervariable regions of the antibodies of the present invention. "Substantially the same" amino acid sequence is defined herein as a sequence with at least 70% percent homology to an amino acid sequence of an antibody of the present invention.

Furthermore, other "substantially homologous" modified antibody polypeptides can be readily designed and manufactured utilizing various recombinant DNA techniques known to those skilled in the art. Modification of the genes may be readily accomplished by a variety of well-known techniques such as site-directed or random mutagenesis. These modifications can include amino acid additions, deletions, substitutions, preferably conservative, and other changes in the sequence of the polypeptide while retaining the appropriate property or biological activity.

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure and possessing binding and/or effector activities may be produced. Also because, like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes to produce fusion proteins (e.g. immunotoxins) having novel properties or novel combinations of properties.

The current invention is directed to a human anti-epidermal growth factor receptor single-chain antibody having a sequence of SEQ ID No. 1 (clone 6) or SEQ ID No. 2 (clone 63), as well as DNA molecules and expression vectors that encode for the

expression of the claimed human anti-epidermal growth factor receptor scFv.

The present invention is also drawn to a pharmaceutical composition comprising the disclosed human anti-epidermal growth factor receptor scFv and a therapeutic or diagnostic agent. Preferably, the therapeutic or diagnostic agent can be a toxin, a chemotherapeutic agent, a transition metal, a radioisotope or a gene therapy vector.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

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EXAMPLE 1

Isolating EGFR-Specific Human Single-Chain Antibody (scFv)

In the screening of the phage library for anti-epidermal growth factor receptor scFv, an IgM scFv display library with a calculated complexity of 2×10^7 independent clones was constructed in pSEX81 (Figure 1) as described (15) using peripheral leukocyte cDNA prepared from healthy donors. The IgM phage display library was screened for expression of scFvs which specifically bind the EGF receptor (EGFR). The phage library was suspended in 500 ml 2xYT-GA medium (17 g Tryptone, 10 g yeast extract, 100 mM glucose, 100 μ g/ml ampicillin and H₂O to 1 liter) to an initial OD₆₀₀ of 0.025. The cells were grown with shaking (240 rpm) at 37° C until an OD₆₀₀ of 0.1 at which point the

cells were superinfected with helper phage, M13K07 (Amersham Pharmacia Biotech), at an MOI of 20. After the addition of helper phage, the cells were mixed gently and left undisturbed for 15 min at 37° C followed by shaking (240 rpm) for 45 min. The medium was replaced by separating the bacteria at 1500 x g for 10 min at room temperature (RT), then the bacterial pellet was suspended in 500 ml 2xYT-AK medium (17 g Tryptone, 10 g yeast extract, 50 µg/ml kanamycin, 100 µg/ml ampicillin and H₂O to 1 liter). The culture was incubated at 37° C with shaking (240 rpm) for 7 h. The cells were separated from the medium by centrifugation at 6500 x g for 15 min at 4° C. The bacteriophage was precipitated out of the supernatant by adding 1/5 volume PEG/NaCl solution (200 g PEG-600, 146.1 g NaCl, up to 1 liter with H₂O) and incubating the medium overnight at 4° C.

During the PEG precipitation, affinity purified EGFR (Sigma) in 100 mM sodium carbonate, pH 9.6 was adsorbed to a MaxiSorb Immunotube (Nunc, Rochester, NY) at a concentration of 5-10 µg/ml for 18 h at 4° C. The next day, the immunotube was washed 3 times with PBS, then blocked with 2% skim milk in PBS-T (PBS with 0.05% Tween-20) for 2-3 h at room temperature (the immunotubes were blocked with 0.5% casein-PBS for the second round and 2% skim milk-PBS for the third round). The immunotube was washed 3 times with PBS-T, then stored at 4° C until ready to use.

The PEG precipitated bacteriophage was separated from the supernatant by high speed centrifugation (10,000 x g for 20 min at 4° C). The pellet was suspended in 4 ml ice-cold phage dilution buffer (10 mM Tris-HCl, pH 7.5, 20 mM NaCl, 2 mM

EDTA). The bacteriophage lysate was clarified at 12,000 x g for 5 min at 4° C. The supernatant was collected and stored at 4° C until the colony forming units (cfu) titer was determined.

To determine the cfu titer, an aliquot of the PEG-
5 concentrated bacteriophage was diluted 10-fold up to 10⁻¹⁰
dilution, then 10 µl of the 10⁻⁷ to 10⁻¹⁰ dilutions was added to 90
µl of an exponentially growing XL1-Blue culture in LB-tet broth
(10 g tryptone, 5 g yeast extract, 0.5 g NaCl, 15 µg/ml tetracycline
in 1 liter H₂O). The virus was allowed to adsorb for 20-30 min at
10 RT, then mixed with 3 ml of LB-amp (LB broth with 100 µg/ml
ampicillin) soft agar (0.5% agar) cooled to 45° C, and immediately
overlaid upon an LB-amp agar plate. The plates were inverted
and incubated overnight at 37° C. The titer was determined by
the number of ampicillin-resistant colonies that have formed at
15 each dilution.

EGFR-specific scFvs were recovered from the phage
library by absorbing 10¹¹ to 10¹² cfu in 4 ml PBS-T to the EGFR-
coated immunotubes with rocking for 2 hours at RT. The
immunotubes were washed 20 times with PBS-T followed by 20
20 times with PBS. The EGFR-specific virus was eluted in 1 ml 100
mM triethylamine (Sigma) for 5 min at room temperature, then
immediately neutralized with 1 ml 1 M Tris-HCl, pH 7.4 and
stored on ice until ready to infect XL1-Blue cells.

To amplify the EGFR-specific scFv bacteriophage, XL1-
25 Blue cells were grown in 20 ml LB-tet broth until an OD₆₀₀ of 0.4.
The neutralized-eluted phage was added to the culture, allowed to
adsorb undisturbed for 15 min at 37° C, followed by shaking (240
rpm) for 45 min. An aliquot was removed (200 µl) to determine

the cfu titer, by making 10-fold dilutions of the 200 μ l aliquot in SOB-GA broth (up to 10^{-4}), then 100 μ l from each dilution was spread onto an SOB-GA agar plate (100 mm²) and incubated overnight at 37° C. The cfu titer was determined by counting the number of ampicillin resistant colonies. The remaining cells were separated from the broth by centrifugation at 2000 x g for 5 min at RT. The cell pellet was suspended in 1000 μ l SOB-GA medium (20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 50 mM MgSO₄, 100 mM glucose, 100 μ g/ml ampicillin), then spread onto 3 SOB-GA agar plates (150 mm²). After the plates dried, they were inverted and incubated at 37° C for 18-24 h. The colonies grown on the 150 mm² plates were removed by scraping the bacteria into 10 ml SOB-GA broth per plate. The bacteria were pooled, then used to inoculate a 250 ml 2xYT-GA broth culture to an OD₆₀₀ of 0.025. Glycerol was added to a concentration of 20% to the remaining bacteria and stored at -80° C. To increase the specificity, the process for isolating EGFR-specific scFvs was repeated 2 additional times.

Putative anti-EGFR-specific scFv clones were isolated from the 2nd round and 3rd round of screening. All clones were stored at -80° C as a 20% glycerol stock of an overnight broth culture grown in 2x YT-GA broth.

Small Scale Phage Rescue

The scFv bacterial clones were used to inoculate 0.2 ml 2xYT-GA medium and grown overnight at 37° C. Ten μ l of the overnight culture was transferred to 1 ml 2xYT-GA medium and incubated with shaking (300 rpm) at 37° C for 3 hours. M13K07

helper phage (10^{10} cfu) was added to each culture, mixed gently and set undisturbed for 15 min at 37° C, then shaken for 45 min at 300 rpm. The cells were separated by centrifugation at 1000 x g for 5 min at room temperature and the supernatant removed, then 1 ml 2xYT-AK medium was added to the cell pellet and incubated with shaking for 7 hours. The cells were removed by centrifugation and the supernatant collected. The supernatant was stored at 4° C for up to 3 days.

10 Screening Clones by Phage ELISA

The 96-well MaxiSorb immunoplates (Nunc, Rochester, NY) were coated with 1 µg/ml affinity purified EGFR antigen (Sigma) in 100 mM sodium carbonate, pH 9.6 buffer. The antigen was allowed to adsorb overnight at 4° C, then the antigen was removed and the wells were washed 3 times with PBS-T. The wells were blocked with 2% skim milk-PBS for 2 hours at room temperature. The wells were washed 3 times with PBS-T, then 100 µl of rescued phage was added per well and incubated at room temperature for 2 hours. The wells were washed 3 times with PBS-T, then 100 µl of a 1/1000 dilution of anti-M13-HRP (Stratagene, LaJolla, CA) was added to each well and incubated for 1 h at room temperature. The wells were washed 5 times with PBS-T, then 200 µl of the TMB enzyme substrate (Sigma) was added per well. The ELISA plates were incubated at room temperature for 30 minutes, then read at 650 nM. Wells equal to and above $OD_{650} = 0.1$ were considered positive and below $OD_{650} = 0.1$ were considered negative.

EXAMPLE 2

Sequencing and Analysis of scFv Clones

5 After 3 rounds of phage panning, individual clones were identified by ELISA as described above. Plasmid DNA was isolated and sequenced according to standard manufacturer's protocol for the ABI DNA sequencer (UAB Automated DNA Sequencing Core Facility). Plasmid DNA was sequenced both
10 directions initially using pelB and gene III primers. Internal sequencing primers were determined from the initial sequence data and synthesized by Operon (Alameda, CA). After completion of the scFv sequence, the data was analyzed using SeqWeb software (Genetics Computer Group, Madison, WI) for alignment of
15 complementary-determining regions (CDRs) with known variable-chain sequence data.

 Two clones, pSEX81-6 and pSEX81-63, have been sequenced and their putative amino acid sequences are shown in Figure 2. The clones are in the order, variable heavy chain (V_H)-
20 linker-variable light chain (V_L), with both clones containing a lambda V_L chain.

 When comparing the two clones, there is a 48% amino acid identity in the V_H chain and an 87% amino acid identity in the V_L chain. The hypervariable or complementarity-determining
25 regions (as defined in ref. 16) are located at the tips of the Fabs in a 3-dimensional structure and have been shown to be primarily involved with antigen binding (17). The CDR1-L region is 100% identical between the two clones, whereas the other CDRs vary

from 2 amino acid differences in CDR3-L, CDR2-L and CDR1-H to
10 and 12 amino acid differences in CDR3-H and CDR2-H,
respectively. With the high variability between the CDRs of these
two clones, each clone may bind to a different antigenic site on the
5 EGFR.

EXAMPLE 3

10

Targeting the scFv To A Cellular Compartment and Expression of Secretory scFv

In eukaryotic cells, scFvs can be targeted to specific
subcellular compartments by engineering the nucleotide sequence
to express a protein with the appropriate signal sequences. In
15 this way the scFvs can be modified to be directed to a subcellular
compartment where the antibody might prove to be most
effective. Recently, Lotti *et al.* showed that the C-terminal
sequence KKXX from the adenovirus E19 protein would enhance
20 the localization of the protein to the cis-golgi complex with some
retention in the ER (18). To direct the scFv to the cytoplasm, the
hydrophobic amino acid core of the immunoglobulin secretory
signal sequence was removed (19). The addition of a nuclear
localization signal from the large T-antigen of SV40 virus,
25 PKKKRKV (SEQ ID No. 3), to the N-terminal end can target the scFv
to the nucleus (20).

To target mitochondria, the N-terminal presequence of
the subunit VIII of human cytochrome c oxidase was added to the

N-terminal end of the scFv (21). Other investigators have directed scFvs to the lumen of the endoplasmic reticulum by including the endoplasmic reticulum retention signal (SEKDEL, SEQ ID No. 4) at the C-terminus of the polypeptide (22-25). scFvs can also be
5 directed to the secretory pathway by the addition of an immunoglobulin signal peptide on the N-terminal end (23-27).

In order to express the secretory scFvs in eukaryotic cells, the insert encoding the scFvs must be cloned downstream of an Igk secretory leader sequence in a eukaryotic expression
10 vector. To this end, the eukaryotic expression vector, pSecTag (Invitrogen) was modified at the multiple cloning site to accept the restriction enzyme sites (Bpu1102I and NotI) in the correct orientation and in the proper reading frame between the leader sequence and the myc (mAB 9E10 epitope) and (His)₆ tags. Also,
15 the eukaryotic antibiotic resistance gene was changed from zeomycin to neomycin, thus the plasmid is named pSecTag/Bpu/neo (Figure 3).

Standard cloning techniques were employed to clone the scFv into pSecTag/Bpu/neo. Briefly, 20 ml LB-amp medium
20 were inoculated with XL1-Blue cells expressing the pSEX81-scFv. After an overnight incubation at 37°C, the bacteria were recovered by centrifugation at 4000 x g for 5 min. The plasmid DNA were isolated using a Wizard DNA Purification kit (Promega, Madison, WI). The pSEX81-scFv plasmid DNA were digested with
25 the restriction enzymes, Bpu1101I and NotI, and separated by agarose gel electrophoresis. The scFv fragment were recovered from the agarose gel using AgarACE Enzyme (Promega). T4 DNA ligase were used to ligate the scFv fragment into the

pSecTag/Bpu/neo plasmid which was digested with Bpu1101I and NotI and agarose gel purified. The ligated DNA was use to transform *E. coli* Top 10F' competent cells (Invitrogen) and plated onto LB-A (LB medium with 100 µg/ml ampicillin) agar plates. 5 After an overnight incubation at 37° C, individual colonies were selected and used to inoculate 5 ml LB-A broth cultures. Plasmid DNA was recovered using the Wizard DNA Purification kit and analyzed for pSecTag/Bpu/neo-scFv by PCR, amplifying the scFv clone between a T7 promoter primer and a myc tag primer. No 10 insert produced a 248 bp PCR product and a positive clone produced a PCR product between 800-1200 bp.

The positive clones were amplified in their bacterial host and the plasmid DNA was recovered using the Wizard PureFection Plasmid DNA Purification System (Promega). The low 15 EGFR expressing human glioma cell line U87MG was transiently transfected with insert positive plasmid DNA or vector alone using the Lipofectin Reagent (Life Technologies). Forty-eight hours after transfection, culture medium was collected and analyzed for secretory scFv to EGFR. Stable transfects were isolated by 20 selecting for antibiotic G-418 resistance with the scFv clones (U87MG-scFv) and vector clones (U87MG-pSecTag/Bpu/neo). The stable transfects were subcultured in 96-well plates at a density of less than 1 cell per well. The culture medium from the confluent wells were screened by ELISA testing for secreted anti- 25 EGFR-scFv. The positive subclones were subcultured in 96-well plates at a density of less than 1 cell per well and the wells grown to confluent monolayers were screened for secreting anti-EGFR-

scFv by ELISA. The positive subclones were expanded for further analysis.

5

EXAMPLE 4

Screening for Anti-EGFR-scFv by Immunoblot and ELISA

One of the stably transfected human glioma sublines, U87MG.6.34.A8 (referred to as clone p6.34) was tested for its ability to secrete a functional, anti-EGFR scFv. The anti-EGFR-scFvs were immunoprecipitated from cell lysates and culture medium. Briefly, the cells were placed on ice, washed three times with ice-cold PBS, lysed in ice-cold lysis buffer (containing 0.025 M Tris-HCl, pH 7.5; 0.25 M NaCl, 0.005 M EDTA, 1% v/v NP-40, 0.001 M phenylmethylsulfonylfluoride, 15 µg/ml aprotinin, 10 µg/ml leupeptin, 0.001 M Na-orthovanadate, 0.05 M Na-fluoride and 0.03 M Na-pyrophosphate) then clarified by centrifugation at 15,000 x g for 20 min at 4° C. Protein concentrations were determined using a BCA protein assay kit (Pierce). Equal amounts of protein were immunoprecipitated with mouse anti-myc antibody (9E10 epitope, Stratagene) using Protein A/G beads (Pierce). For immunoprecipitation of secretory scFvs from the culture medium, four-day-old cell culture supernatants were collected from the stably transfected cells. Equal volumes (1 ml) were immunoprecipitated with rat anti-tubulin antibody (Serotec Inc., Raleigh, NC) using Protein A/G beads.

Screening for the expression and secretion of the scFvs was by immunoblot, whereby the immunoprecipitated proteins

were denatured by boiling in sample buffer (0.125 M Tris-HCl, pH 6.8; 10% glycerol, 1% SDS, 0.7 M β -mercaptoethanol and 0.25% bromophenol blue) for 3 min and separated by SDS-PAGE then transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). Immunoblots were blocked in 10% milk-TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hour at room temperature. Primary antibody, mouse anti-myc (Stratagene) was incubated in 2% milk-TBS-T overnight at 4° C. Blots were washed three times in TBS-T followed by incubation with HRP (horseradish peroxidase) labeled secondary antibody, anti-mouse Ig-horseradish peroxidase antibody (Sigma) at room temperature for 1 hour. The blots were washed three times with TBS-T and once with TBS. The blots were developed by chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Results show that scFv was immunoprecipitated from the cell lysate (Figure 4A) and the culture medium (Figure 4B) of clone p6.34 but not from the control cell line pSECTAG that was stably transfected with the parent vector pSECTAG/Bpu/Neo. These data indicated that scFv was translated and processed into the secretory pathway. However, these data did not indicate whether clone p6.34 scFv binds to the EGFR antigen.

Therefore, culture medium from stably transfected cells was tested in an ELISA for the expression of secretory scFv against EGFR. The culture medium was clarified by low speed centrifugation (1000 x g for 5 min at 4° C) to remove any cells, then clarified at high speed centrifugation (10,000 x g for 15 min at 4° C) to remove any debris. Cell culture medium (100 μ l) was added to each well and incubated for 2h at 37° C in a CO₂

incubator. The wells were washed with PBS-Tween-20 (PBS-T). Secondary antibody, anti-myc (mAb 9E10, Invitrogen) at a 1:1000 dilution in PBS-T was added to each well and incubated for 1 hour at 22° C. After washing the wells, a tertiary antibody, anti-
5 mouse-HRP (Sigma) at 1:2000 dilution in PBS-T was added to each well, then incubated for 1 h at 22°C. The wells were washed and developed with o-phenylenediamine (OPD, Sigma) and read at 450 nm. The results shown in Figure 4C indicate that clone p6.34 secretes scFv that binds to the purified EGFR antigen.

10

EXAMPLE 5

15 Binding of Anti-EGFR-scFv To The Extracellular Domain of EGFR

The ELISA data shown above indicates that clone p6.34 scFv binds to denatured EGFR, however it does not provide any information as to which part of the receptor was recognized by the scFv. The EGFR has three major domains, intracellular,
20 transmembrane and extracellular, any of which may serve as the binding site for clone p6.34. To examine whether the scFv binds to the extracellular portion of the receptor, a FACS analysis was used for this determination. For this assay, culture medium collected from U87MG.pSECTAG or U87MG.pSECTAG.6.34.A8 was
25 allowed to interact with the cell surface of 3 different human glioma sublines; U87MG, U87MG.wtEGFR and U87MG.ΔEGFR (provided by Dr. H-J. Su Huang, UCSD).

The U87MG is the parent cell line into which the scFv clones were stably transfected as well as stably transfected with wild-type EGFR (U87MG.wtEGFR) or the truncated EGFR, EGFRvIII (U87MG.ΔEGFR) (28-29). U87MG has a very low number of EGFR, which is one reason why cell proliferation of the stably transfected cell line, clone p6.34, does not appear to be affected by the anti-EGFR scFv (data not shown). The U87MG.wtEGFR subline overexpresses a large number of EGFR/cell (estimated at $>3 \times 10^6$). The U87MG.ΔEGFR expresses the 135 kdal truncated EGFR which is constitutively phosphorylated (29).

The FACS results shown in Figure 5 indicates that the secretory scFv p6.34 binds to the extracellular domain of U87MG.wtEGFR and U87MG.ΔEGFR. The parent cell line, U87MG, does not have a significant number of receptors on its cell surface which results in no detectable scFv p6.34 binding. The data indicates that clone p6.34 produced a secretory scFv which bound to the cell surface of cells which overexpress EGFR and truncated EGFR (EGFRvIII). Since clone p6.34 bound to a common antigenic site on both prominent forms of cancer-related EGFRs, this scFv might block the surface expression of each receptor when presented in the proper subcellular compartment.

EXAMPLE 6**Radiolabelled Anti-EGFR-scFv For Early Detection of Breast Cancer**

Many tumor-specific antigens have been identified as
5 targets for imaging breast cancer. HER-2/neu is overexpressed on
25%-30% of breast cancer cells. The epidermal growth factor
receptor, a receptor in the same family as HER-2/neu, has been
found to be overexpressed in a high percentage of human
carcinomas. A compilation of the literature estimates that
10 approximately 30%-35% of the breast carcinomas have increased
levels of epidermal growth factor receptor protein and the
increase of epidermal growth factor receptor expression correlates
with the loss of estrogen receptor and a poor prognosis.

With the increasing data on the relationship between
15 the overexpression of the epidermal growth factor receptor and
poor prognosis, this receptor has become a target for breast cancer
imaging. Radiolabelled monoclonal antibodies (mAbs) have been
used for imaging epidermal growth factor receptor-
overexpressing breast tumors. However, due to the heterologous
20 vascular structure around the tumor and the molecular size of the
antibodies, the monoclonal antibodies penetrate the tumor poorly
and are unevenly distributed around the tumor making imaging
more difficult.

In order to improve tumor imaging, intact monoclonal
25 antibodies have been reduced to antibody fragments or single-
chain antibodies. The short plasma half-life for the scFv becomes
an advantage for tumor imaging because tumor-to-blood ratios
are higher than intact monoclonal antibodies and the rapidly

eliminated scFv does not accumulate in extravascular spaces and non-target organs. Isotopes used for imaging tumors include ^{111}In , ^{64}Cu , ^{131}I , ^{90}Y , and $^{99\text{m}}\text{Tc}$. Technetium-99m is used in the following protocol because the techniques are available for both direct and indirect scFv labelling and $^{99\text{m}}\text{Tc}$ is a low cost, readily available isotope with purely photon radiation, which is widely used in clinical imaging. The 6 h half-life of $^{99\text{m}}\text{Tc}$ is an excellent complement to the scFv which has a rapid clearance from the circulation system allowing for high-contrast imaging.

The radiolabelled scFv of the present invention will be tested for any loss of ability to bind to the epidermal growth factor receptor due to the radiolabelling process. Then, the radiolabelled scFv can be use in mouse xenograft models to determine its ability to bind to large or small xenografts of high, moderate or low epidermal growth factor receptor overexpressing breast cancer cells detected by a gamma camera with computer-enhanced imaging.

Although tissue biodistribution is currently a standard method to assess pharmacokinetics in mice, it suffers from several drawbacks. First, the concentrations of the radiolabelled peptides at each time point are measured from different mice and data are pooled for each separately evaluated group. Second, the number of time points to be sampled are often limited to 3-5 points, consequently, the fast phase of tissue uptake is ignored. Because of the small size of the anti-epidermal growth factor receptor-scFv, the uptake and clearance are expected to be fast in this study. Thus, the pharmacokinetics of the $^{99\text{m}}\text{Tc}$ -scFv will be determined by dynamic imaging using a pinhole gamma camera,

and the results are useful for the development of non-invasive imaging method for early detection of breast cancer.

Breast cancer cell lines

5 The following cell lines are purchased from ATCC (Manassas, VA): MDA-MB-468, MDA-MB-231 and MCF-7. These cell lines are reported to overexpress EGFR at levels ranging from low (10,000 receptors/cell, MCF-7), medium (100,000 receptors/cell, MDA-MB-231) and high (1,000,000 receptors/cell, 10 MDA-MB-468).

Radiolabelling of the anti-EGFR scFv

 The indirect radiolabelling procedure, with the trihydroxamat bifunctional chelating agent trisuccin (42), is used 15 for ^{99m}Tc labelling of the scFv. The protocol is in two steps consisting of conjugation of trisuccin to the scFv (43), followed by the radiolabelling of the conjugate.

 For trisuccin conjugation, the 50 μl solution of the scFv in DPBS is added to 250 μL of a 50 mM PBS buffer at pH 8.1. The 20 resulting solution is stirred at 0 $^{\circ}\text{C}$ and the solution of OHA-NHS (5.7 g, 23.5 nmol) in 50 μl of DMF is added. After 90 min at 0 $^{\circ}\text{C}$, the reaction is quenched by addition of 20 μl of 2 M glycine in the PBS buffer and the reaction mixture is purified by dialysis against 100 mM acetate buffer, pH 5.5. To this mixture, a solution of 25 trisuccin hydrazide (43) (0.12 mol - 1.2 mol) in water (5 μl - 20 μl) is added. The reaction mixture is stirred at room temperature for 90 min at which time 20 μl of NaCNBH_3 solution in water is added to a final concentration of 100 mM in NaCNBH_3 . The

resulting mixture is stirred gently at room temperature for 18 h followed by purification of the conjugate by size-exclusion HPLC (SEC-HPLC) and eluted with DPBS.

For ^{99m}Tc -labelling, the procedure reported previously is used (42, 43). Briefly, the ^{99m}Tc , eluted from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (Syncor, Birmingham, AL) is reduced with a solution of SnCl_2 in hydroxyisobutyric acid at room temperature. This solution is added to the trisuccin- scFv and incubated at 35 °C for 30 min. The labelled protein is purified by SEC-HPLC.

Alternately for direct ^{99m}Tc labelling, cysteine molecules on the purified scFv is radiolabeled with ^{99m}Tc by previously published methods using a ^{99m}Tc -D-glucarate transfer method (44, 45). Molecular techniques is used to add a cysteine molecule near the COOH-terminal end of the scFv which will be available for this site directed labelling method. The radiolabelled protein is purified by SEC-HPLC.

20 Testing the binding ability of the radiolabelled scFv

Whole cells or cell membranes from the three breast cancer cell lines were used in a standard competitive radioimmunoassay to determine if the radiolabelled scFv retains its ability to bind to the EGFR. The radiolabelled scFv used in competition with unlabelled scFv and assays were counted in a gamma counter.

Human breast cancer xenograft models

Initially, 3 groups of 3 female athymic nude mice, 6 to 8 weeks old, obtained from the National Cancer Institute Frederick Research Laboratory (Frederick, MD) are injected subcutaneously in the one flank with 2×10^7 human breast cancer cells. Each group is injected with one of the three breast cancer cell lines, MDA-MB-468, MDA-MB-231 and MCF-7. When the tumors are approximately 10 mm in diameter, the opposite flank are injected with the same cell line. When the second xenograft is approximately 1 mm in diameter, the mice are injected with the ^{99m}Tc -scFv and imaged. The xenograft model are repeated 2 additional times and modified if necessary to produce consistent, reproducible results.

15

In vivo detection of breast cancer tumors

Planar imaging of mice is performed using Picker Axis gamma camera equipped with a pinhole collimator with 2-mm or 4-mm aperture. The images are acquired with a matrix of 256 x 256 pixels and a zoom factor of 2. Studies are performed using a 20% energy window, centered on 140 keV photo-peak of ^{99m}Tc . Mice are imaged immediately after being anesthetized. Previous imaging studies suggest that most mice could be imaged for 45-60 minutes before they recover from the anesthesia. Each mouse receives 50 μCi ^{99m}Tc -scFv IV. Mice are imaged in a prone position as xenografts are planted subcutaneously in both ventral flanks. A distance of 7.5 cm from pinhole to mice is used for imaging a group of 3 mice simultaneously in one field of view.

25

Dynamic image is performed at 0-1 h, 60 frames of images are acquired at 1 min/frame rate to obtain fast phase for scFv uptake in normal tissue and the tumor. Static images are acquired at 2 h, 4 h, 6 h, respectively. Acquisition time of each image is 20-30 min, which is comparable to that of patient imaging using a gamma camera.

These images are used to determine the optimal imaging time and best tumor to non-tumor image contrast that can be used for future patient imaging. This imaging computerized kinetic model allows one to obtain pharmacokinetics information in same animals and provide direct evidence for the potential of using ^{99m}Tc -human anti-EGFR-scFv for early detection of breast cancer in patients.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

25

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

<110> Raisch, Kevin Paul
 Curiel, David T.
 Bonner, James Allen

<120> Human Anti-Epidermal Growth Factor Receptor
 Single-Chain Antibodies

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<141> 2001-10-12

<150> US 60/240,353

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				20					25					30
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				35					40					45
Glu	Tyr	Val	Ser	Ala	Ile	Ser	Ser	Asn	Gly	Gly	Ser	Thr	Tyr	Tyr
				50					55					60
Ala	Asp	Ser	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Ser
				65					70					75
Lys	Asn	Thr	Leu	Tyr	Leu	Gln	Met	Ser	Ser	Leu	Arg	Ala	Glu	Asp
				80					85					90

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Ser	Tyr	Ala	Ile	Ser	Trp	Val	Arg	Gln	Ala	Pro	Gly	Gln	Gly	Leu	35	40	45
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Ala	Gln	Lys	Phe	Gln	Gly	Arg	Val	Thr	Ile	Thr	Ala	Asp	Glu	Ser	65	70	75
Thr	Ser	Thr	Ala	Tyr	Met	Glu	Leu	Ser	Ser	Leu	Arg	Ser	Glu	Asp	80	85	90
Thr	Ala	Val	Tyr	Tyr	Cys	Ala	Arg	Asp	Pro	Asp	Tyr	Tyr	Gly	Ser	95	100	105
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Pro	Pro	Ser	Val	Ser	Val	Ser	Pro	Gly	Gln	Thr	Ala	Ser	Ile	Thr	155	160	165
Cys	Ser	Gly	Asp	Lys	Leu	Gly	Asp	Lys	Tyr	Ala	Ser	Trp	Tyr	Gln	170	175	180
Leu	Lys	Pro	Ala	Gln	Ser	Pro	Val	Trp	Val	Ile	Tyr	Gln	Asp	Thr	185	190	195
Lys	Arg	Ser	Ser	Gly	Ile	Pro	Glu	Arg	Ile	Ser	Gly	Ser	Asn	Ser	200	205	210
Gly	Asn	Thr	Ser	Thr	Leu	Thr	Ile	Thr	Gly	Thr	Gln	Ala	Met	Asp	215	220	225
Glu	Ala	Asp	Tyr	Tyr	Cys	Gln	Ala	Trp	Asp	Ser	Ser	Thr	Ala	Val	230	235	240
Val	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Thr	Val	Leu	Gly	Gln	Pro	Lys	245	250	255
Ala	Asn	Pro	Ser	Val	Thr	Leu	Phe	Pro	Pro	Ser	Ser	Ala	Ala	Ala	260	265	270

WHAT IS CLAIMED IS:

1. A human anti-epidermal growth factor receptor single-chain antibody having the sequence of SEQ ID No. 1 or
5 biologically functional fragment thereof.

2. The anti-epidermal growth factor receptor single-chain antibody of claim 1, wherein said antibody binds to
10 the extracellular domain of epidermal growth factor receptor.

3. A human anti-epidermal growth factor receptor single-chain antibody having the sequence of SEQ ID No. 2 or
15 biologically functional fragment thereof.

4. The anti-epidermal growth factor receptor single-chain antibody of claim 3, wherein said antibody binds to
20 the epidermal growth factor receptor.

5. An isolated DNA encoding the anti-epidermal growth factor receptor single-chain antibody of claim 1.
25

6. An isolated DNA encoding the anti-epidermal growth factor receptor single-chain antibody of claim 3.

7. An expression vector comprising the anti-epidermal growth factor receptor single-chain antibody of claim 1.

5

8. An expression vector comprising the anti-epidermal growth factor receptor single-chain antibody of claim 3.

10

9. A pharmaceutical composition comprising the anti-epidermal growth factor receptor single-chain antibody of claim 1 and a pharmaceutically acceptable carrier.

15

10. The pharmaceutical composition of claim 9, further comprising a therapeutic agent.

20

11. The pharmaceutical composition of claim 10, wherein said therapeutic agent is selected from the group consisting of a toxin, a chemotherapeutic agent, a radioisotope and gene therapy vector.

25

12. The pharmaceutical composition of claim 9, further comprising a diagnostic agent.

13. The pharmaceutical composition of claim 12, wherein said diagnostic agent is selected from the group consisting of radioisotopes and transition metals.

5

14. A pharmaceutical composition comprising the anti-epidermal growth factor receptor single-chain antibody of claim 3 and a pharmaceutically acceptable carrier.

10

15. The pharmaceutical composition of claim 14, further comprising a therapeutic agent.

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16. The pharmaceutical composition of claim 15, wherein said therapeutic agent is selected from the group consisting of a toxin, a chemotherapeutic agent, a radioisotope and gene therapy vector.

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17. The pharmaceutical composition of claim 14, further comprising a diagnostic agent.

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18. The pharmaceutical composition of claim 17, wherein said diagnostic agent is selected from the group consisting of radioisotopes and transition metals.

19. A method of treating a tumor, comprising administering to a patient in need of such treatment an effective amount of an anti-epidermal growth factor receptor single-chain antibody of claim 1.

20. A method of treating a tumor, comprising administering to a patient in need of such treatment an effective amount of an anti-epidermal growth factor receptor single-chain antibody of claim 3.

21. The anti-epidermal growth factor receptor single-chain antibody of claim 1, wherein said antibody is radiolabeled.

22.

22. The anti-epidermal growth factor receptor single-chain antibody of claim 3, wherein said antibody is radiolabeled.

23. A method for the diagnostic location and assessment of tumor growth, comprising administering to a patient in need of such diagnostic treatment an effective amount of an anti-epidermal growth factor receptor single-chain antibody of claim 21.

24. A method for the diagnostic location and assessment of tumor growth, comprising administering to a patient in need of such diagnostic treatment an effective amount of an anti-epidermal growth factor receptor single-chain antibody of claim 22.

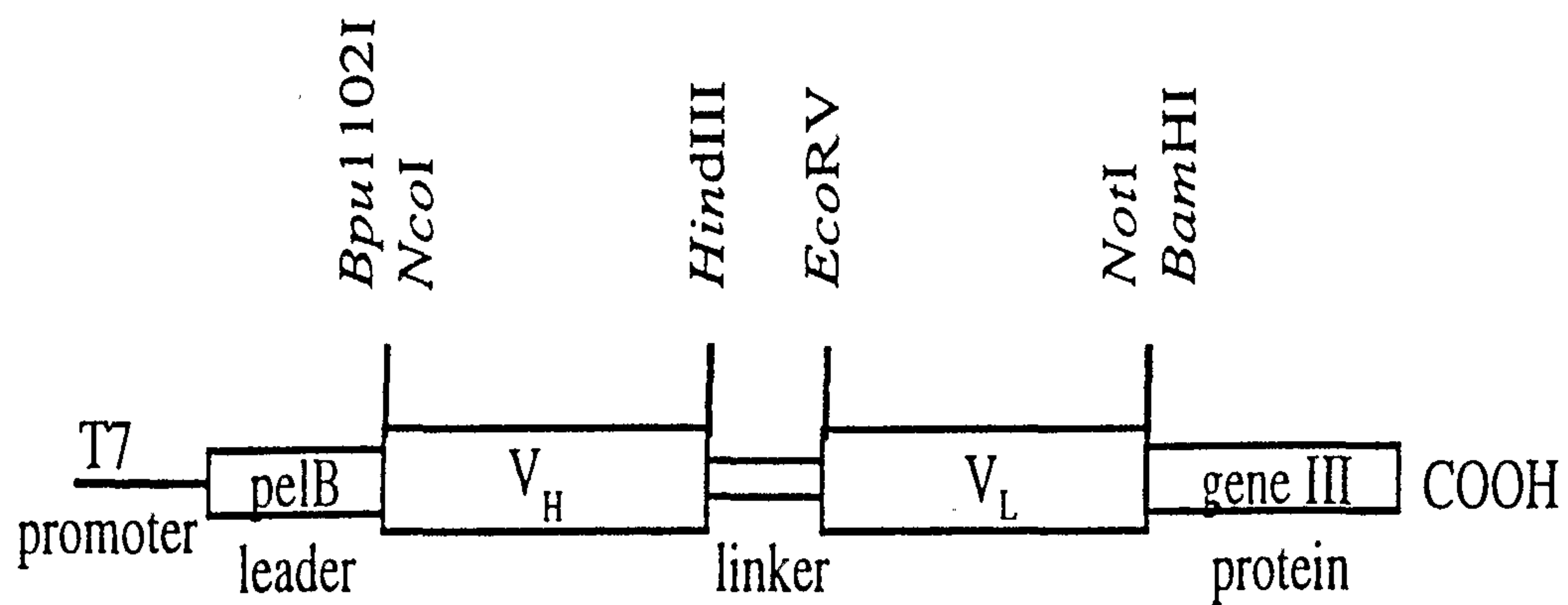


Fig. 1

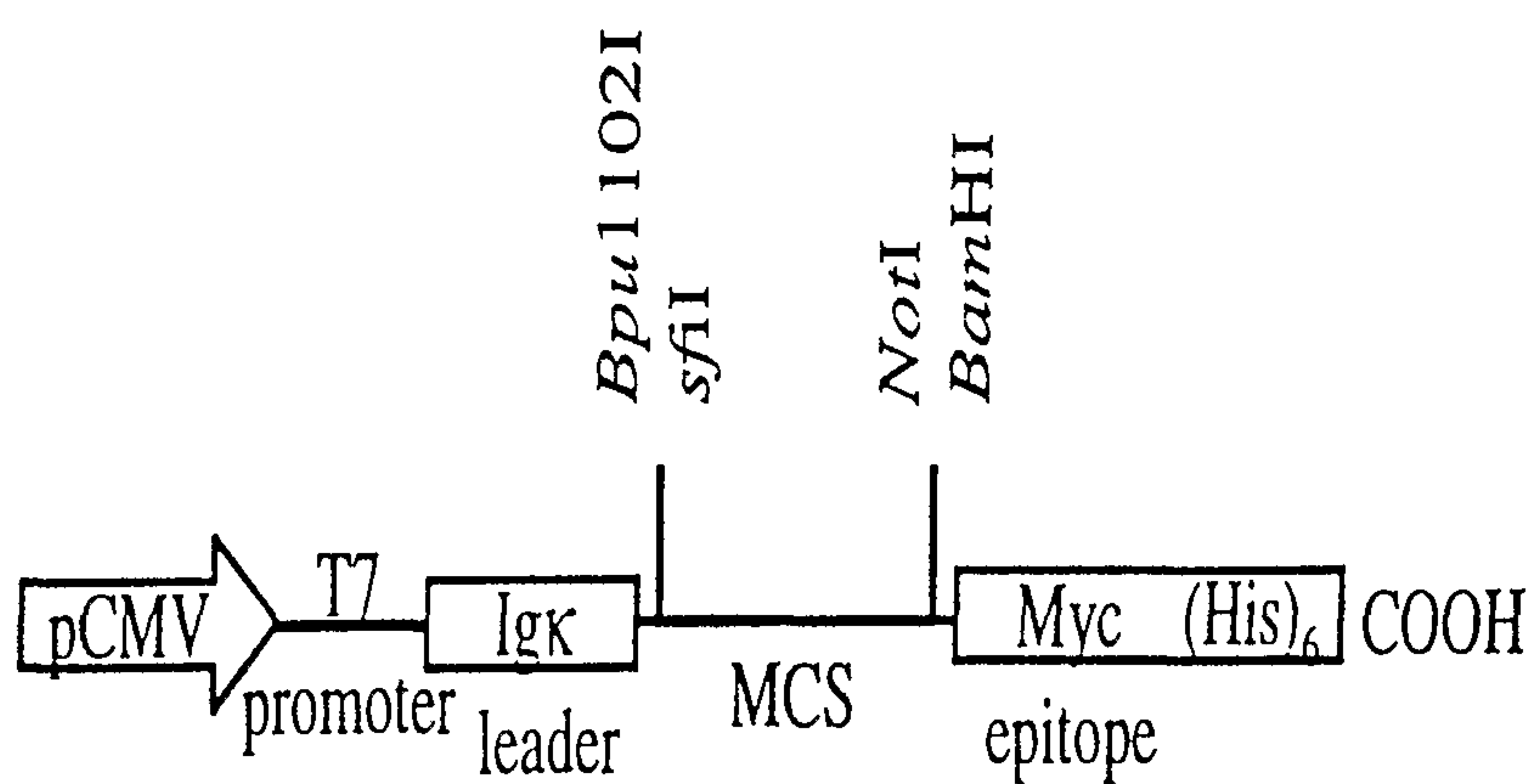


Fig. 3

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VH→
Clone      CDR1      CDR2      CDR3      VH
6          EVQLVESGGGLVQPGGSLRLSCSASGFTFSYAMHWVRQAPGKGLLEYVSAISSNGGSTYYADSVK
63         QVQLVQSGAEVKKPGSSVKVSKASGGTFSYAISWVRQAPGQGLEWMGGIIPFGTANYAQKFQ

          CDR1      CDR2      CDR3      VH
6          GRFTISRDNKNTLYLQMSLRAEDTAVYYCVKD.VGGSSWYWADYFDYWGQGLTVTVSSGSSASAP
63         GRVTITADESTSTAYMELSSLRSEDVAVYYCARDPDYVGGSSYYPNWFDPWGQGLTVTVSSGSSASAP

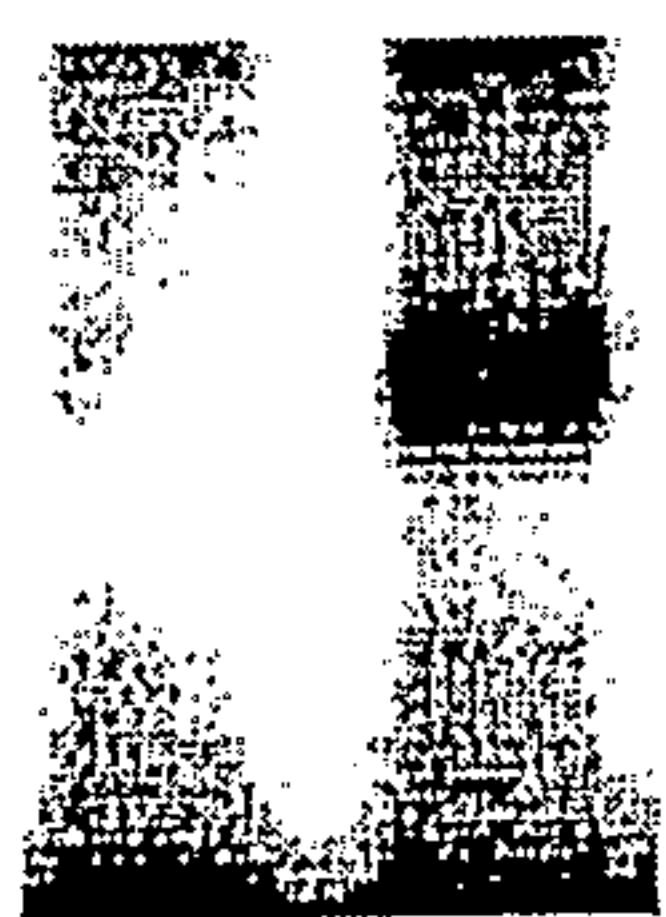
linker     VL→      CDR1      CDR2
6          KLEEGEFSEARVQSVLTQPPSLVSPGQTASITCSGDKLGDKYASWYQOKPQSPVLVIYQDRKRPSG
63         KLEEGEFSEARVQSALTQPPSVSVSPGQTASITCSGDKLGDKYASWYQLKPAQSPVWVIYQDTKRSSG

          CDR3      VL
6          IPERFSGNSGNTATLTIISGTQAMDEADYYCQAWDSSTFPYVFGTGTKVTVLGQPKANPT
63         IPERISGNSGNTSTLTIITGTQAMDEADYYCQAWDSSTAVVFGGGTKLTVLGQPKANPS

          VTLFPSSAAA (SEQ ID No. 1)
          VTLFPSSAAA (SEQ ID No. 2)

```

Fig. 2



1 2

Fig. 4A



1 2

Fig. 4B

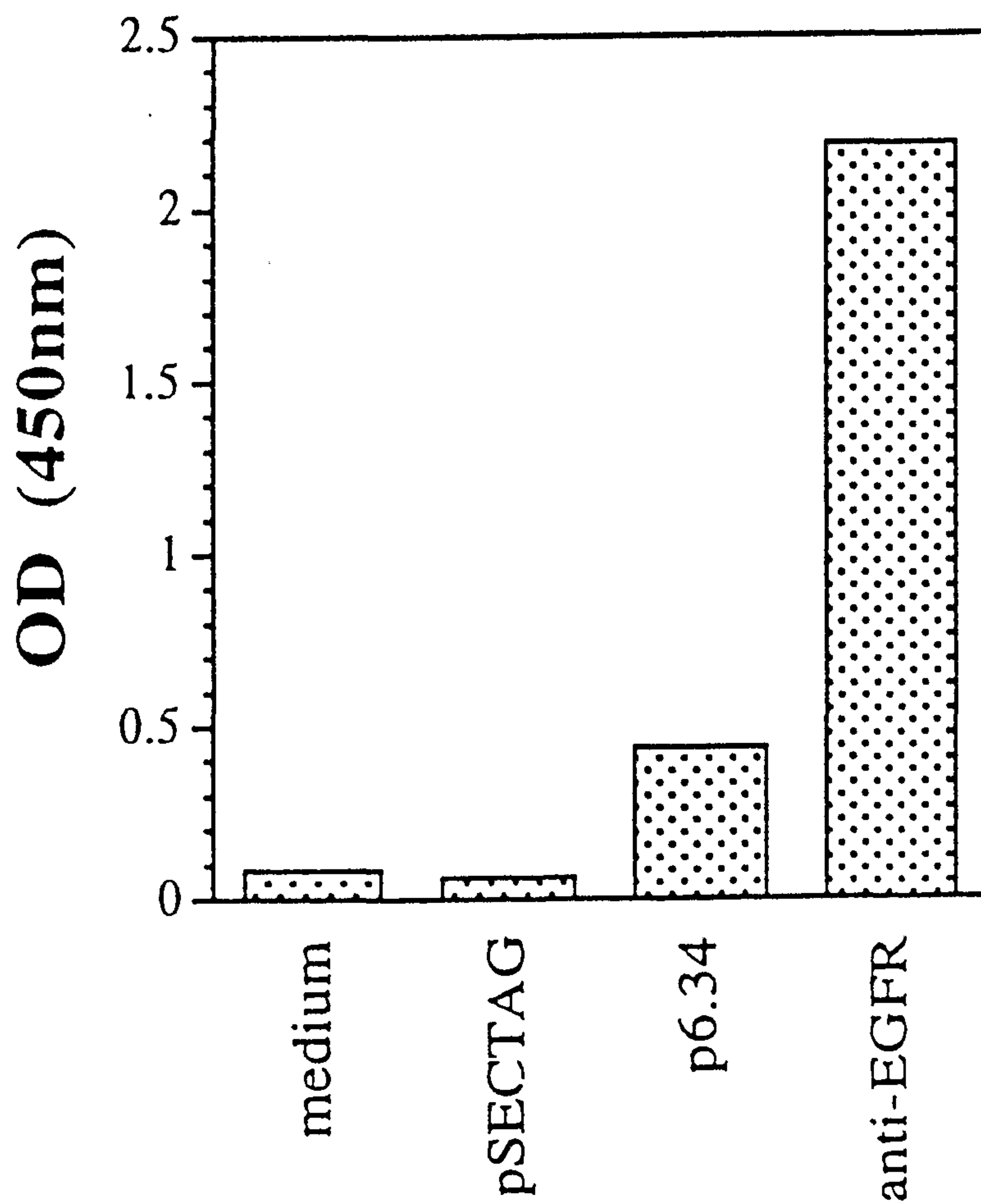


Fig. 4C

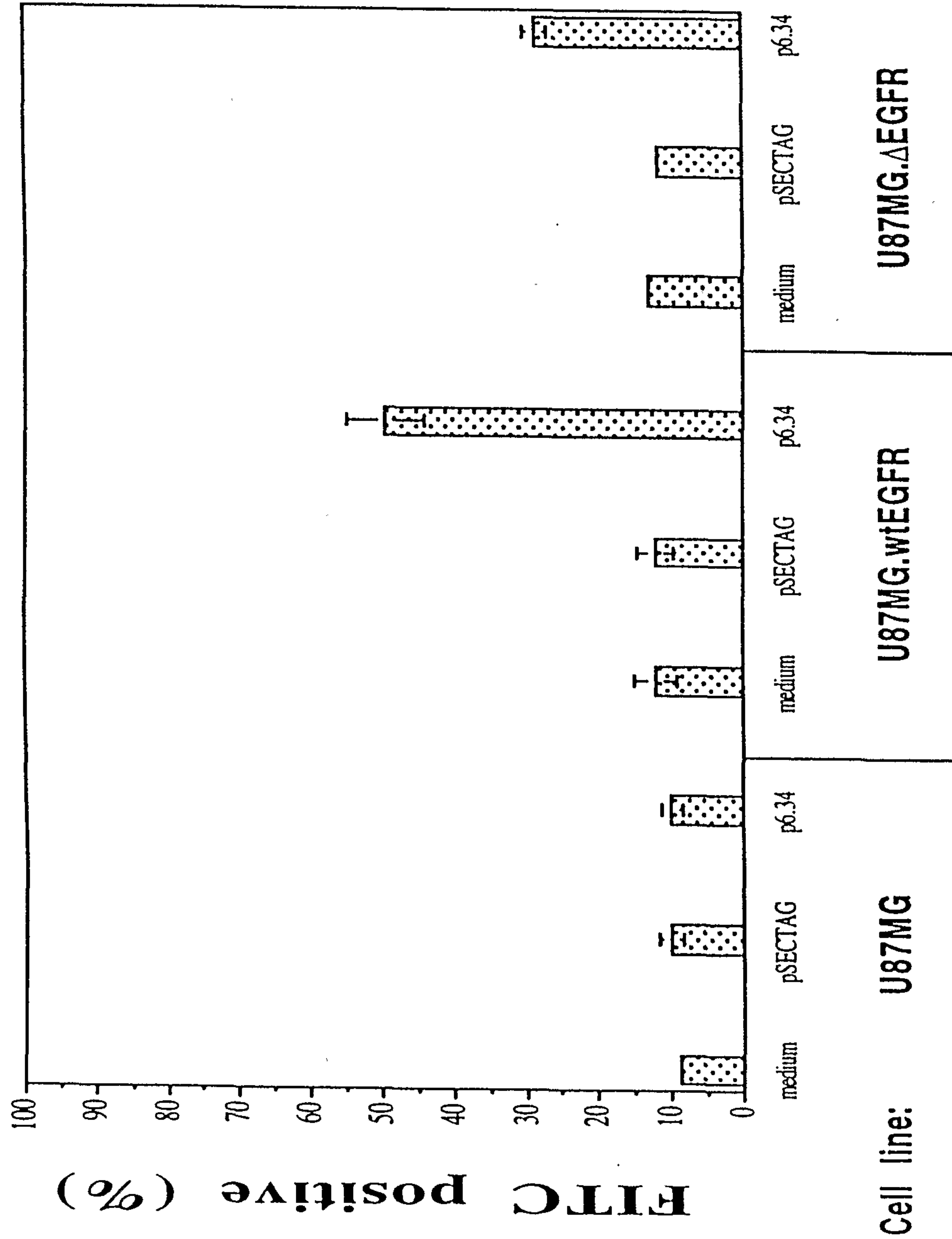


Fig. 5