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(54) Title: ANTIBODIES THAT BIND TGF-ALPHA AND EPIREGULIN

(57) Abstract: The present invention provides antibodies that bind human TGF-alpha and human Epiregulin and are characterized as having high affinity, selective, and strong neutralizing properties. The antibodies are useful in the treatment of diabetic nephropathy.
ANTIBODIES THAT BIND TGF-ALPHA AND EPIREGULIN

The present invention relates to antibodies that bind human TGF-alpha and Epiregulin and uses thereof.

TGF-alpha and Epiregulin are two of seven ligands of the Epidermal Growth Factor Receptor ("EGFR") that normally function in wound healing following injury. Diabetic nephropathy ("DN") is a major diabetic complication and is the leading cause of end stage renal disease ("ESRD"). Proteinuria is a clinical marker of renal functional decline accompanying DN and is associated with disease progression and increased cardiovascular risk, such as heart failure, vascular disease, dysrhythmia. The standard of care for DN includes ACE inhibitors and angiotensin receptor blockers ("ARBs") that only slow disease progression and leave considerable residual risk.

Blocking the EGFR attenuates not only proteinuria, but also renal pathology in preclinical animal models of renal disease. However, EGFR inhibitors, such as ERBITUX®, while approved for cancer, are associated with side effects such as a severe skin rash on the face and shoulders associated with target inhibition in the skin. Thus, there is still a need for alternative therapies for DN. In addition, there is a need for a more effective treatment therapy for DN.

Antibodies that bind TGF-alpha have been described (for example, see US 5190858). In addition, antibodies that bind Epiregulin have been described (for example, see US 2009/0324491).

The present invention provides antibodies against TGF-alpha and Epiregulin for the treatment of DN. Furthermore, the present invention provides antibodies against TGF-alpha and Epiregulin that engage the target in vivo and subsequently cause a reduction in proteinuria with a concomitant reduction in disease progression and cardiovascular risk.

The present invention provides therapeutically useful antibodies that bind both TGF-alpha and Epiregulin that possess a number of desirable properties. The antibodies of the present invention have high affinity and are selective with full neutralizing activity against human TGF-alpha and human Epiregulin. When administered, the antibodies of the present invention also result in a decrease in albuminuria and in renal pathology for tubular protein, interstitial fibrosis, mesangial matrix expansion, and pelvic dilation in
Furthermore, the preferred antibodies of the present invention cause no observed skin toxicity associated with complete EGFR inhibition.

The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the LCVR comprises amino acid sequences LCDRI, LCDR2, and LCDR3, and the HCVR comprises amino acid sequences HCDRI, HCDR2, and HCDR3, wherein LCDRI is SEQ ID NO:4, LCDR2 is SEQ ID NO:5, LCDR3 is SEQ ID NO:6, HCDRI is SEQ ID NO:1, HCDR2 is SEQ ID NO:2, and HCDR3 is SEQ ID NO:3.

The present invention also provides a pharmaceutical composition comprising an antibody of the present invention, as described herein, and at least one pharmaceutically acceptable carrier, diluent, or excipient.

The present invention provides an antibody of the present invention, as described herein, for use in the treatment of diabetic nephropathy.

Throughout this disclosure, an antibody of the present invention, as described herein, binds TGF-alpha and Epiregulin, and comprises a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the LCVR comprises amino acid sequences LCDRI, LCDR2, and LCDR3, and the HCVR comprises amino acid sequences HCDRI, HCDR2, and HCDR3, wherein LCDRI is SEQ ID NO:4, LCDR2 is SEQ ID NO:5, LCDR3 is SEQ ID NO:6, HCDRI is SEQ ID NO:1, HCDR2 is SEQ ID NO:2, and HCDR3 is SEQ ID NO:3.

The present invention provides an antibody, as described herein, wherein the antibody is selective to human TGF-alpha and human Epiregulin. Further, the present invention provides an antibody, as described herein, wherein the antibody has full neutralizing activity to human TGF-alpha and human Epiregulin. Further preferred, the present invention provides an antibody, as described herein, wherein the antibody is selective and has full neutralizing activity to human TGF-alpha and human Epiregulin.

The present invention provides an antibody, as described herein, wherein the antibody has a dissociation equilibrium constant, Kd, between 0.01 X 10⁻⁹ M and 1.0 X 10⁻⁹ M for human TGF-alpha (SEQ ID NO: 18). Further preferred, an antibody of the present invention, as described herein, has a dissociation equilibrium constant, Kd,
between 0.05 X 10⁻⁹ M and 0.8 X 10⁻⁹ M for human TFG-alpha. The Kd values are established by a binding equilibrium at 25°C as described in Example 2.

The present invention also provides an antibody, as described herein, wherein the antibody has a dissociation equilibrium constant, Kd, between 0.1 X 10⁻⁹ M and 30 X 10⁻⁹ M for met-human Epiregulin (SEQ ID NO: 22). Further preferred, an antibody of the present invention, as described herein, has a dissociation equilibrium constant, Kd, between 0.5 X 10⁻⁹ M and 10 X 10⁻⁹ M for human Epiregulin. The Kd values are established by a binding equilibrium at 25°C as described in Example 2.

The present invention provides an antibody, as described herein, wherein the antibody has a dissociation equilibrium constant, Kd, between 0.01 X 10⁻⁹ M and 1.0 X 10⁻⁹ M for human TGF-alpha (SEQ ID NO: 18) and a Kd between 0.1 X 10⁻⁹ M and 30 X 10⁻⁹ M for met-human Epiregulin (SEQ ID NO: 22). Further preferred, an antibody of the present invention, as described herein, has a dissociation equilibrium constant, Kd, between 0.05 X 10⁻⁹ M and 0.8 X 10⁻⁹ M for human TFG-alpha and a Kd between 0.5 X 10⁻⁹ M and 10 X 10⁻⁹ M for human Epiregulin. The Kd values are established by a binding equilibrium at 25°C as described in Example 2.

The present invention provides antibodies which bind human TGF-alpha and Epiregulin, and cause dose-dependent decrease in albuminuria, reduction in serum creatinine and blood urea nitrogen ("BUN") in vivo in a mouse remnant kidney model and a mouse uninephrectomy db/db model as described in Example 5 and Example 6, respectively.

The present invention provides antibodies which bind human TGF-alpha and Epiregulin, and cause reduction in renal pathology for tubular protein and interstitial fibrosis and a decrease in mesangial matrix expansion and pelvic dilation in vivo in a mouse remnant kidney model and a mouse uninephrectomy db/db model as described in Example 5 and Example 6, respectively.

The present invention provides antibodies which bind human TGF-alpha and Epiregulin, and are believed to cause a reduction in proteinuria with a concomitant reduction in disease progression and cardiovascular risk in humans. Further, the present invention provides antibodies which bind human TGF-alpha and Epiregulin, and are believed to be effective in the treatment of diabetic nephropathy in humans.
The present invention provides antibodies which bind human TGF-alpha and Epiregulin, and cause no observed skin toxicity in a toxicity study in cynomolgus monkeys as described in Example 7.

The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the LCVR comprises amino acid sequences LCDR1, LCDR2, and LCDR3, and the HCVR comprises amino acid sequences HCDR1, HCDR2, and HCDR3, wherein LCDR1 is SEQ ID NO:4, LCDR2 is SEQ ID NO:5, LCDR3 is SEQ ID NO:6, HCDR1 is SEQ ID NO:1, HCDR2 is SEQ ID NO:2, and HCDR3 is SEQ ID NO:3.

Furthermore, the present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the amino acid sequence of the LCVR is SEQ ID NO: 9 or SEQ ID NO: 10.

The present invention also provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the amino acid sequence of the HCVR is SEQ ID NO: 7.

The present invention also provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein an amino acid sequence of the LCVR and an amino acid sequence of the HCVR is selected from the group consisting of:

(i) the LCVR is SEQ ID NO: 9 and the HCVR is SEQ ID NO: 7; and
(ii) the LCVR is SEQ ID NO: 10 and the HCVR is SEQ ID NO: 7.

The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the amino acid sequence of the LCVR is SEQ ID NO: 9 and the amino acid sequence of the HCVR is SEQ ID NO: 7.
The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the amino acid sequence of the LCVR is SEQ ID NO: 10 and the amino acid sequence of the HCVR is SEQ ID NO: 7.

Furthermore, the present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the amino acid sequence of the light chain is SEQ ID NO: 13 or SEQ ID NO: 14.

The present invention also provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the amino acid sequence of the heavy chain is SEQ ID NO: 12.

Furthermore, the present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein an amino acid sequence of the heavy chain and an amino acid sequence of the light chain is selected from the group consisting of:

(i) the heavy chain is SEQ ID NO: 12 and the light chain is SEQ ID NO: 13, and

(ii) the heavy chain is SEQ ID NO: 12 and the light chain is SEQ ID NO: 14.

The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising two light chains wherein the amino acid sequence of each light chain is SEQ ID NO: 13, and two heavy chains wherein the amino acid sequence of each heavy chain is SEQ ID NO: 12.

The present invention provides an antibody that binds TGF-alpha and Epiregulin, comprising two light chains wherein the amino acid sequence of each light chain is SEQ ID NO: 14, and two heavy chains wherein the amino acid sequence of each heavy chain is SEQ ID NO: 12.

Furthermore, the present invention provides an antigen-binding fragment of an antibody, as described herein.

The present invention also provides a pharmaceutical composition comprising the antibody of the present invention, as described herein, and at least one pharmaceutically acceptable carrier, diluent, or excipient.
Furthermore, the present invention provides a pharmaceutical composition comprising the antibody of the present invention, as described herein, together with at least one pharmaceutically acceptable carrier, diluent, or excipient, and optionally other therapeutic ingredients.

The present invention also provides a method of treating diabetic nephropathy in a patient comprising administering to the patient the antibody of the present invention, as described herein.

Furthermore, the present invention provides an antibody of the present invention, as described herein, for use in therapy. Preferably, the present invention provides an antibody of the present invention, as described herein, for use in the treatment of diabetic nephropathy.

Furthermore, the present invention provides the use of an antibody of the present invention, as described herein, in the manufacture of a medicament for the treatment of diabetic nephropathy.

The present invention also provides a method of treating diabetic nephropathy in a patient comprising administering to the patient the antibody of the present invention, as described herein, in simultaneous or sequential combination with a standard of care.

Furthermore, the present invention provides an antibody of the present invention, as described herein, for use in therapy, wherein the antibody is to be administered in simultaneous or sequential combination with a standard of care. Preferably, the present invention provides an antibody of the present invention, as described herein, for use in the treatment of diabetic nephropathy, wherein the antibody is to be administered in simultaneous or sequential combination with a standard of care.

Furthermore, the present invention provides the use of an antibody of the present invention, as described herein, in the manufacture of a medicament for the treatment of diabetic nephropathy, wherein the antibody is to be administered in simultaneous or sequential combination with a standard of care.

The present invention also provides a pharmaceutical composition comprising the antigen-binding fragment of an antibody of the present invention, as described herein, and at least one pharmaceutically acceptable carrier, diluent, or excipient.

Furthermore, the present invention provides a pharmaceutical composition comprising the antigen-binding fragment of an antibody of the present invention, as
described herein, together with at least one pharmaceutically acceptable carrier, diluent, or excipient, and optionally other therapeutic ingredients.

The present invention also provides a method of treating diabetic nephropathy in a patient comprising administering to the patient the antigen-binding fragment of an antibody of the present invention, as described herein.

Furthermore, the present invention provides an antigen-binding fragment of an antibody of the present invention, as described herein, for use in therapy. Preferably, the present invention provides an antigen-binding fragment of an antibody of the present invention, as described herein, for use in the treatment of diabetic nephropathy.

Furthermore, the present invention provides the use of an antigen-binding fragment of an antibody of the present invention, as described herein, in the manufacture of a medicament for the treatment of diabetic nephropathy.

The present invention also provides a method of treating diabetic nephropathy in a patient comprising administering to the patient the antigen-binding fragment of an antibody of the present invention, as described herein, in simultaneous or sequential combination with a standard of care.

Furthermore, the present invention provides an antigen-binding fragment of an antibody of the present invention, as described herein, for use in therapy, wherein the antigen-binding fragment is to be administered in simultaneous or sequential combination with a standard of care. Preferably, the present invention provides an antigen-binding fragment of an antibody of the present invention, as described herein, for use in the treatment of diabetic nephropathy, wherein the antigen-binding fragment is to be administered in simultaneous or sequential combination with a standard of care.

Furthermore, the present invention provides the use of an antigen-binding fragment of an antibody of the present invention, as described herein, in the manufacture of a medicament for the treatment of diabetic nephropathy, wherein the antigen-binding fragment is to be administered in simultaneous or sequential combination with a standard of care.

The standard of care for DN includes, but is not limited to, ACE inhibitors and angiotensin receptor blockers (ARBs).

The general structure of an "antibody" is very well-known in the art. For an antibody of the IgG type, there are four amino acid chains (two "heavy" chains and two
"light" chains) that are cross-linked via intra- and inter-chain disulfide bonds. When expressed in certain biological systems, antibodies having unmodified human Fc sequences are glycosylated in the Fc region. Antibodies may be glycosylated at other positions as well. The subunit structures and three-dimensional configurations of antibodies are well known in the art. Each heavy chain is comprised of an N-terminal heavy chain variable region ("HCVR") and a heavy chain constant region ("HCCR"). The heavy chain constant region is comprised of three domains (CH1, CH2, and CH3) for IgG, IgD, and IgA; and 4 domains (CH1, CH2, CH3, and CH4) for IgM and IgE. Each light chain is comprised of a light chain variable region ("LCVR") and a light chain constant region ("LCCR").

The variable regions of each light/heavy chain pair form the antibody binding site. The HCVR and LCVR regions can be further subdivided into regions of hypervariability, termed complementarity determining regions ("CDRs"), interspersed with regions that are more conserved, termed framework regions ("FR"). Each HCVR and LCVR are composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. Herein, the 3 CDRs of the heavy chain are referred to as "CDRH1, CDRH2, and CDRH3" and the 3 CDRs of the light chain are referred to as "CDRL1, CDRL2 and CDRL3." The CDRs contain most of the residues which form specific interactions with the antigen. The assignment of amino acids to each domain is in accordance with well-known conventions [e.g., Kabat, "Sequences of Proteins of Immunological Interest," National Institutes of Health, Bethesda, Md. (1991)].

An antibody of the present invention may have a heavy chain constant region selected from any of the immunoglobulin classes (IgA, IgD, IgG, IgM, and IgE).

Furthermore, an antibody of the present invention contains an Fc portion which is derived from human IgG4 Fc region because of its reduced ability to bind complement factors as compared to other IgG sub-types.

An antibody may be derived from a single copy or clone, including e.g., any eukaryotic, prokaryotic, or phage clone. Preferably, an antibody of the present invention exists in a homogeneous or substantially homogeneous population of antibody molecules. An full-length antibody comprises full length or substantially full length constant regions, including the Fc region. An "antigen-binding fragment" of such an antibody is any
shortened form of a full length antibody that comprises the antigen-binding portion and retains antigen-binding capability. Such shortened forms include, e.g., a Fab fragment, Fab' fragment or F(ab') 2 fragment that includes the CDRs or the variable regions of the antibodies disclosed. Furthermore, such shortened antibody forms can be a single chain Fv fragment that may be produced by joining the DNA encoding the LCVR and HCVR with a linker sequence. (See, Pluckthun, The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp 269-315, 1994). The term "antibody" does not include such fragments unless otherwise indicated. An antibody of the present invention can be produced using techniques well known in the art, e.g., recombinant technologies, phage display technologies, synthetic technologies or combinations of such technologies or other technologies readily known in the art.

An antibody of the present invention is an engineered antibody that has been designed to have frameworks, hinge regions, and constant regions of human origin that are identical with or substantially identical (substantially human) with frameworks and constant regions derived from human genomic sequences. Fully human frameworks, hinge regions, and constant regions are those human germline sequences as well as sequences with naturally-occurring somatic mutations and those with engineered mutations. An antibody of the present invention may comprise framework, hinge, or constant regions derived from a fully human framework, hinge, or constant region containing one or more amino acid substitutions, deletions, or additions therein. Further, an antibody of the present invention is substantially non-immunogenic in humans.

A variety of different human framework sequences may be used singly or in combination as a basis for an antibody of the present invention. Preferably, the framework regions of an antibody of the present invention are of human origin or substantially human (at least 95%, 97% or 99% of human origin.) The sequences of framework regions of human origin may be obtained from The Immunoglobulin Factsbook, by Marie-Paule Lafranc, Gerard Lefranc, Academic Press 2001, ISBN 012441351.

The framework sequence for an antibody of the present invention serves as the "donor" variable framework region and can be used to create additional antibodies with the same CDRs specified herein using methodology known in the art. Furthermore, the framework sequence for an antibody of the present invention can be compared to other
known human framework sequences to generate additional antibodies. Thus, this information can be used to "back-mutate" another selected homologous human framework region to the donor amino acid residue at these positions. Further, any "rare" amino acids can be detected in additional human frameworks such that the consensus or donor amino acid residue can be used at the relevant position.

"TGF-alpha" or "human TGF-alpha" refers to human TGF-alpha protein (SEQ ID NO: 18).

"Epiregulin" or "human Epiregulin" refers to human Epiregulin protein (SEQ ID NO: 33). Met-human Epiregulin (SEQ ID NO: 22) is used in in vitro experiments herein.

References to the ability of the antibodies of the present invention, as described herein, to bind or to neutralize human Epiregulin pertain also to their ability to bind and to neutralize human met-Epiregulin in in vitro experiments.

A "patient" is a mammal, preferably a human.

The term "treating" (or "treat" or "treatment") means slowing, stopping, reducing, or reversing the progression or severity of a symptom, disorder, condition, or disease.

The term "therapeutically effective amount" refers to the amount or dose of an antibody of this invention which, upon single or multiple dose administration to a patient, provides the desired treatment.

The following examples may be performed essentially as described below.

EXAMPLES

Example 1: Production of Antibodies

Antibodies I and II can be made and purified as follows. An appropriate host cell, such as HEK 293 or CHO, is either transiently or stably transfected with an expression system for secreting antibodies using an optimal predetermined HC:LC vector ratio or a single vector system encoding both HC, such as SEQ ID NO: 15, and LC, such as SEQ ID NO: 16 or SEQ ID NO: 17. Clarified media, into which the antibody has been secreted, is purified using any of many commonly-used techniques. For example, the medium may be conveniently applied to a Protein A or G column that has been equilibrated with a compatible buffer, such as phosphate buffered saline (pH 7.4). The column is washed to remove nonspecific binding components. The bound antibody is
eluted, for example, by pH gradient (such as 0.1 M sodium phosphate buffer pH 6.8 to 0.1 M sodium citrate buffer pH 2.5). Antibody fractions are detected, such as by SDS-PAGE, and then are pooled. Further purification is optional, depending on the intended use. The antibody may be concentrated and/or sterile filtered using common techniques. Soluble aggregate and multimers may be effectively removed by common techniques, including size exclusion, hydrophobic interaction, ion exchange, or hydroxyapatite chromatography. The purity of the antibody after these chromatography steps is greater than 99%. The product may be immediately frozen at -70°C or may be lyophilized. The amino acid sequences for these antibodies are provided below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Heavy Chain</th>
<th>Light Chain</th>
<th>HCVR</th>
<th>LCVR</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>12</td>
<td>13</td>
<td>7</td>
<td>9</td>
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<td>II</td>
<td>12</td>
<td>14</td>
<td>7</td>
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<tr>
<td>III</td>
<td>31</td>
<td>32</td>
<td>8</td>
<td>11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HCDR1</th>
<th>HCDR2</th>
<th>HCDR3</th>
<th>LCDR1</th>
<th>LCDR2</th>
<th>LCDR3</th>
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<tr>
<td>I</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
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<td>2</td>
<td>3</td>
<td>4</td>
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<td>6</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

**Example 2: Affinity Binding Measurement by Surface Plasmon Reasonance (BIAcore) for Antibody I**

Biacore T2000 instrument (BIAcore® AB, Upsala, Sweden), reagents and Biacore T2000 Evaluation Software Ver 4.1 are used for the Surface Plasmon Resonance analysis.
A CM5 chip is prepared using manufacturer's EDC/NHS amine coupling method. The surfaces of all four flow cells are activated by injecting a 1:1 mixture of EDC/NHS for 7 minutes at 10 µL/min. Goat anti-human Fab γ specific antibody is diluted to 50 µg/ml in 10 mM acetate, pH 4.0 buffer and immobilized for approximately 10000 RU onto all four flow cells by 7 minute injection at a flow rate of 10 µL/min. Un-reacted sites are blocked with a 7 minute injection of ethanolamine at 10 µL/min. Injections of 3 x 20 seconds of glycine pH 1.5 at 30 µL/min are used to remove non-covalently associated protein. The running buffer is HBS-EP [10 mM HEPES, 150 mM Sodium Chloride, 3 mM EDTA, 0.005% Polysorbate 20].

In study 1, Antibody I is diluted to 50 µg/mL in running buffer, and approximately 400-600 RU is captured in flowcell 2. Human TGF-alpha (SEQ ID NO: 18), rat TGF-alpha (SEQ ID NO: 20), met-human Epiregulin (SEQ ID NO: 22), and cynomolgus Epiregulin (SEQ ID NO: 24) are diluted from 100 µg/mL to 200 nM in running buffer and then two-fold serially diluted in running buffer to 6.25 nM. Mouse Epiregulin (SEQ ID NO: 23) is diluted from 100 µg/mL to 4 µM in running buffer and then two-fold serially diluted in running buffer to 125 nM. Duplicate injections of each ligand concentration are injected at 30 µL/min for 300 seconds followed by a dissociation phase. The dissociation phase is 1800 seconds for human and rat TGF-alpha, 1200 seconds for human and cynomolgus Epiregulin, and 120 seconds for mouse Epiregulin. Regeneration is performed by injecting 10 mM glycine pH 1.5 for 3 x 20 seconds at 30 µL/min over all flowcell.

In study 2, Antibody III is diluted to 100 µg/mL in running buffer, and approximately 400-600 RU is captured in flowcell 2. Mouse TGF-alpha (SEQ ID NO: 19), is diluted from 100 µg/mL to 200 nM in running buffer and then two-fold serially diluted in running buffer to 6.25 nM. Mouse Epiregulin (SEQ ID NO: 23) is diluted from 100 µg/mL to 4 µM in running buffer and then two-fold serially diluted in running buffer to 125 nM. Duplicate injections of each ligand concentration are injected at 30 µL/min for 300 seconds followed by a dissociation phase. The dissociation phase is 1800 seconds for mouse TGF-alpha, and 120 seconds for mouse Epiregulin. Regeneration is performed by injecting 10 mM glycine pH 1.5 for 30 seconds at 30 µL/min over all flowcell.

Reference-subtracted data are collected as Fc2-Fc1. The measurements are obtained at 25°C. The on-rate (k_on) and off-rate (k_off) for each ligand are evaluated using a
"1:1 (Langmuir) Binding" binding model. The affinity (¾) is calculated from the binding kinetics according to the relationship: \( K_D = \frac{k_{off}}{k_{on}}. \)

Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Species</th>
<th>On Rate ( (k_{on}) ) ((\text{M}^{-1}\text{s}^{-1}) (\pm \text{SD}))</th>
<th>Off Rate ( (k_{off}) ) ((\text{s}^{-1}) (\pm \text{SD}))</th>
<th>Affinity ( (K_D) ) ((\pm \text{SD}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-alpha</td>
<td>Human</td>
<td>(4.18 \pm 0.28 \times 10^5)</td>
<td>(4.09 \pm 0.96 \times 10^5)</td>
<td>(97.6 \pm 20.6 \text{ pM})</td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>(3.78 \pm 0.39 \times 10^5)</td>
<td>(2.66 \pm 0.74 \times 10^5)</td>
<td>(70.5 \pm 19.4 \text{ pM})</td>
</tr>
<tr>
<td>Epiregulin</td>
<td>Human</td>
<td>(4.91 \pm 0.42 \times 10^5)</td>
<td>(6.31 \pm 0.55 \times 10^4)</td>
<td>(1.29 \pm 0.03 \text{ nM})</td>
</tr>
<tr>
<td></td>
<td>Cynomolgus</td>
<td>(6.73 \pm 0.71 \times 10^5)</td>
<td>(7.05 \pm 0.23 \times 10^4)</td>
<td>(1.05 \pm 0.09 \text{ nM})</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>(4.10 \pm 1.15 \times 10^4)</td>
<td>(1.33 \pm 0.16 \times 10^2)</td>
<td>(342 \pm 136 \text{ nM})</td>
</tr>
</tbody>
</table>

*Calculated as \(K_D = \frac{k_{off}}{k_{on}}.\)

Table 2

<table>
<thead>
<tr>
<th>Ligand</th>
<th>On Rate ( (k_{on}) ) ((\text{M}^{-1}\text{s}^{-1}) (\pm \text{SD}))</th>
<th>Off Rate ( (k_{off}) ) ((\text{s}^{-1}) (\pm \text{SD}))</th>
<th>Affinity ( (K_D) ) ((\pm \text{SD}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse TGF-alpha</td>
<td>(5.41 \pm 0.50 \times 10^5)</td>
<td>(2.02 \pm 0.54 \times 10^5)</td>
<td>(38.0 \pm 13.6 \text{ pM})</td>
</tr>
<tr>
<td>Mouse Epiregulin</td>
<td>(6.55 \pm 0.38 \times 10^5)</td>
<td>(1.41 \pm 0.09 \times 10^2)</td>
<td>(215 \pm 15 \text{ nM})</td>
</tr>
</tbody>
</table>

*Calculated as \(K_D = \frac{k_{off}}{k_{on}}.\)

Antibody I binds to human TGF-alpha and human Epiregulin with affinities of about 98 pM and 1.3 nM, respectively. Antibody I also binds to rat TGF-alpha and mouse Epiregulin with affinities of about 70 pM and 340 nM, respectively. Additionally, Antibody I binds to cynomolgus Epiregulin with an affinity of about 1 nM. Antibody III binds to mouse TGF-alpha and mouse Epiregulin with affinities of about 38 pM and 220 nM, respectively. Thus, Antibody I and Antibody III of the present invention have high affinity to human TGF-alpha and human Epiregulin.
Example 3; Internalization of EGF Target Ligands in the Human Colon Carcinoma Cell Line HT-29

Conjugation of Alexa Fluor® 488 to antibodies

Alexa Fluor® 488 is conjugated to Antibody I and Control IgG according to the manufacturer's protocol. Protein is diluted to 2 mg/mL in PBS. To 0.5 mL of this 2 mg/mL solution, 50 µL of 1M sodium bicarbonate pH 9 is added. The protein solution is then transferred to a vial of dye and stirred at room temperature for 1 hour. The labeled protein is purified using the Bio-Rad BioGel P-30 resin included with the labeling kit.

In vitro internalization assay

In study 1, 10,000 HT-29 cells, a colon adenocarcinoma cell line known to express TGF-alpha and Epiregulin, are seeded per well of a 96 well plate and allowed to incubate overnight in complete media [Dulbecco's Modified Eagle's Medium/F12 (Ham) Medium (1:1) ("DMEM/F12") containing L-glutamine, 10% heat-inactivated fetal bovine serum ("FBS"), 1x antibiotic, and 2.438 g/L sodium bicarbonate]. The next day, the cells are washed with PBS containing 0.1% BSA and then incubated with an Alexa Fluor® 488 conjugated Antibody I or Control IgG in PBS with 0.1% BSA at concentrations ranging from 0 to 88 ug/mL for 2 hours at 37 °C in a tissue culture incubator. Following the incubation period, the cells are washed in PBS with 0.1% BSA several times and then fixed with 4% formaldehyde for analysis. The quantitation of internalization is done as follows: 500 cells/well are collected with a Cellomics Arrayscan VTI (Thermo Scientific). Image analysis is performed with "Compartment al analysis" Bioapplications of the system. Cell nuclei are identified with a Hoechst stain (blue). Two regions of interest (ROD) are set to collect fluorescent signals from intracellular spots (red) and total green fluorescence (both red and blue) obtained from the masked image. The number, area and fluorescent intensity from each spot and cell are calculated. The mean spot total intensity of intracellular spots (red) is chosen for measuring Antibody I induced internalization.

In study 2, 10,000 HT-29 cells are prepared as previously described, and Alexa Fluor® 488 conjugated Antibody I or Control IgG in PBS containing 0.1% BSA is added to the cells at 40 ug/mL. Cells are incubated at 37°C in a tissue culture incubator for
various times ranging from 0-120 minutes, then washed with PBS containing 0.1% BSA several times and fixed with 4% formaldehyde for analysis. The quantification of signal is performed essentially as previously described.

Table 3a

<table>
<thead>
<tr>
<th>Dose (ug/ml)</th>
<th>88</th>
<th>44</th>
<th>22</th>
<th>11</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>2440 ± 199</td>
<td>1808 ± 207</td>
<td>1763 ± 68</td>
<td>1391 ± 76</td>
<td>1357 ± 63</td>
</tr>
<tr>
<td>Antibody I</td>
<td>24809 ± 4343</td>
<td>17451 ± 217</td>
<td>15135 ± 131</td>
<td>11516 ± 54</td>
<td>8474 ± 269</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3b

<table>
<thead>
<tr>
<th>Dose (ug/ml)</th>
<th>2.75</th>
<th>1.38</th>
<th>0.69</th>
<th>0.34</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>1570 ± 70</td>
<td>1473 ± 7</td>
<td>1483 ± 90</td>
<td>1407 ± 41</td>
<td>1630 ± 155</td>
</tr>
<tr>
<td>Antibody I</td>
<td>6503 ± 262</td>
<td>4349 ± 186</td>
<td>3440 ± 96</td>
<td>2432 ± 62</td>
<td>1460 ± 84</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results from the imaging analysis of study 1 determined that the fluorescence signal was internalized into the cell and was dose dependent with Antibody I, but not with the Control IgG (Table 3a and Table 3b).
The results from study 2 demonstrated that Antibody I was internalized rapidly and the internalization was complete by 2 hours post addition to cells (Table 4). Antibody I induced internalization of target on HT-29 cells in vitro in a time dependent manner (Table 4).

Example 4: Measurement of Neutralization of EGFR Ligand Stimulated Cell Proliferation in a Myofibroblast Cell Line

A clonal mouse myofibroblast cell line ("MFc7") is used to test the ability of the antibodies of the present invention to block the proliferative activity of EGFR ligands. The seven ligands that can activate the EGFR are TGF-alpha (TGFA), Epiregulin (EREG), EGF, Heparin-Binding EGF (HB-EGF), Epigen (EPGN), Amphiregulin (AREG) and Betacellulin (BTC). The EGFR ligands share a structural motif, the EGF-like domain, characterized by three intramolecular disulfide bonds that are formed by six similarly spaced conserved cysteine residues. Proliferative activity is determined by Bromodeoxyuridine ("BrDU") incorporation and is measured with a colorimetric BrDU ELISA kit according to the manufacturer's instructions.

First, 2,000 MFc7 cells/well are plated in a tissue culture treated 96 well microplate in 0.1 mL of Dulbecco's Modified Eagle's Medium/F12 (Ham) Medium (1:1) ("DMEM/F12") containing L-glutamine, 10% heat-inactivated FBS, 1x antibiotic, and

---

### Table 4

**Study 2 - Mean Ringspot Total Intensity of Fluorescence**

<table>
<thead>
<tr>
<th>Time post addition (min)</th>
<th>120</th>
<th>60</th>
<th>30</th>
<th>15</th>
<th>5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>177 ± 29</td>
<td>167 ± 23</td>
<td>124 ± 10</td>
<td>126 ± 18</td>
<td>116 ± 4</td>
<td>94 ± 11</td>
</tr>
<tr>
<td>Antibody I</td>
<td>4449 ± 866</td>
<td>4131 ± 1688</td>
<td>1494 ± 66</td>
<td>717 ± 72</td>
<td>261 ± 17</td>
<td>89 ± 1</td>
</tr>
</tbody>
</table>

Mean ± SEM
2.438 g/L sodium bicarbonate. Cells are allowed to attach for 6 hours, and then the medium is removed and replaced with 0.1 mL of serum free DMEM/F12 containing 0.1% BSA for serum starvation overnight. The next day, serial dilutions of the EGFR ligands are made with serum free media containing 0.1% BSA in 96 well polypropylene plates in a volume of 0.12 mL/well from concentrations ranging from 0.001 to 3000 ng/mL. Following dilutions, medium is removed from serum starved cells and then stimulated with EGFR ligand for 24 hrs. Following stimulation, the cells are pulsed with BrDU for 4 hrs and then analyzed with a colorimetric BrDU ELISA kit according to the manufacturer's instructions.

In testing the specificity of Antibody I to EGFR ligands, serial dilutions of 2X or 3X of the antibody are made in 96 well polypropylene plates in a volume of 0.06 mL/well from concentrations ranging from 3000 nM to 0.059 nM. Following serial dilutions of the antibody, 0.06 mL of the EGFR ligand is added per well. The plate is then incubated at 37°C in a humidified tissue culture incubator for 30 minutes. Following incubation, 0.1 mL of the solution is transferred per well to the cells. The cells are stimulated for 24 hours. Following stimulation, the cells are pulsed with BrDU for 4 hours and then analyzed with a colorimetric BrDU ELISA kit. Absorbance values (450 nM - 690 nM) are generated on a SpectraMax 190 plate reader (Molecular Devices) and data are analyzed.
Mouse Epiregulin and rat TGF-alpha, as well as all of the human EGFR ligands except for Epigen and Amphiregulin were found to be potent stimulators of cell proliferation in the assay (Table 5). Antibody I and Antibody III have high affinity to human and rat TGF-alpha and human Epiregulin activity (Table 5).

Table 5 summarizes the calculated EC50 values for the EGFR ligands tested and the absolute IC50 values for the antibodies to those ligands. The calculated average IC50 for Antibody I was 0.46 ± 0.03 nM to human TGF-alpha and 3.15 ± 1.04 nM to human Epiregulin. The calculated IC50 average for Antibody III was 0.52 ± 0.04 nM to human TGF-alpha and 1.12 ± 0.36 nM to human Epiregulin. The calculated average IC50 value for Antibody III was 0.13 ± 0.01 nM to rat TGF-alpha and 2.14 ± 0.49 nM to mouse Epiregulin. Thus, Antibody I and Antibody III have high affinity and are selective with full neutralizing activity against human TGF-alpha and human Epiregulin.
**Example 5: Renal Function and Pathology in a Mouse Remnant Kidney Model of Hypertensive Renal Disease**

A mouse remnant kidney model involving surgical reduction of 75% of the total renal mass is used as a preclinical model of hypertensive renal disease. [Ma LJ, Fogo AB. Kidney Int. 2003 Jul;64(l):350-5] Surgical reduction of renal mass or sham surgery is done in male 129 Svev mice at 9-10 weeks of age. Randomization into groups of 12 mice is done at 2 weeks post surgery, by urine albumin/creatinine ratio ("ACR") and body weight. An isotype Control IgG (10 mg/kg) or Antibody III (1 and 10 mg/kg) are dosed subcutaneously following randomization and continued once weekly out to week 16 post surgery. The endpoints for the study are survival, systolic blood pressure, albuminuria, serum creatinine, serum BUN, urine TGF-alpha, urine MIP-2 and renal pathology.

At the end of the study, there were 3 deaths in the Control IgG group (25% mortality) with no deaths in the Antibody III treatment groups.

**Measurement of systolic blood pressure**

Blood pressure is taken at 12 weeks post surgery by the tail cuff method. Selected mice from each group (N = 3-4 per group) are acclimated to the restraint by placing them in the mouse holder with the tail cuff attached for 5 minutes daily, 3-5 days prior to the actual measurement. The equipment room temperature is increased to 24°C to provide additional warmth during the blood pressure collection process. The mice are placed in a mouse restrainer and set on top of a warming pad unit (31-33°C) to provide dilation to the tail vasculature. The tail is placed through the tail cuff and each mouse is restrained for an approximate time of 30 minutes, not to exceed 45 minutes. This time includes the initial warming and pressure measurements followed by immediate return to general housing. No anesthesia is used. The tail cuff is inflated, compressing the tail tightly enough to momentarily interrupt arterial blood flow, and then is gradually loosened by deflation to observe the return of the arterial pulse. On return of arterial pulse, the cuff is fully deflated.
Measurement of albuminuria

Urine is collected every 4 weeks in Nalgene Metabolic cage units over a 24 hour time period. Each mouse (singly housed) receives food and water during the 24 hour collection process. At the end of the 24 hour period, the collected urine is placed on ice, centrifuged and subjected to albumin and creatinine analysis. Albuminuria is defined as the ratio of urine albumin to creatinine (ug/mg).

Serum creatinine and BUN

At study termination, serum obtained by cardiac puncture is analyzed for BUN and creatinine.

TGF-alpha and MIP-2 ELISA

Urine obtained by a 24 hour collection is concentrated 5-fold centrifugally using a 3K MW cutoff membrane spun at 14,000 x g for 30 minutes. A sandwich-type enzyme-linked immunosorbent assay ("ELISA") for mouse TGF alpha is established. Rat TGF-alpha is used as the standard. Polystyrene 96-well plates are coated with 3 µg/mL of Antibody III overnight at 4°C. Plates are washed, blocked with blocking buffer, washed again, and then the concentrated urine samples are added. After 2 hours at room temperature, plates are washed, and then secondary biotinylated polyclonal anti-hTGF alpha is added. After 2 hours at room temperature, plates are washed and incubated with streptavidin-HRP for 30 minutes. Signal is generated with TMB substrate, and the reaction is stopped with 2 N H2SO4. A commercial Quantikine® sandwich ELISA kit for mouse macrophage inflammatory protein 2 (MIP-2, the equivalent of human IL-8) is used to detect urine MIP-2 according to the manufacturer's instructions. Absorbance data for both ELISA assays are obtained on a SpectraMax 190 plate reader (Molecular Devices) and data are analyzed.

Renal Pathology

Remnant kidneys are removed at study termination, fixed in formalin and processed for paraffin sectioning according to standard methodology. Sections of kidney are evaluated for renal lesions by a pathologist. Tubular protein, increased mesangial matrix and interstitial fibrosis, are semi-quantitatively scored using the following scale:
none (0), minimal (1), slight (2), moderate (3), marked (4) and severe (5). Glomerular mesangial matrix expansion and basement membrane thickening are scored using hematoxylin and eosin ("H&E") and Periodic acid-Schiff ("PAS") stained sections. Masson's trichrome stained sections of kidney are evaluated to determine the degree of fibrosis (interstitial and glomerular).

**Statistical Methods**

All data are analyzed with JMP v.8.0 software (SAS Institute). Pathology scores are statistically evaluated by a contingency analysis and a Fishers exact test. All other data are evaluated by ANOVA with log transformed data and a Students unpaired t test. A P value of < 0.05 is considered statistically significant.

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Albuminuria progression over time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td>2</td>
</tr>
<tr>
<td>Control IgG (10 mg/kg)</td>
<td>1601 ± 269</td>
</tr>
<tr>
<td>Antibody III (1 mg/kg)</td>
<td>1665 ± 305</td>
</tr>
<tr>
<td>Antibody III (10 mg/kg)</td>
<td>1626 ± 273</td>
</tr>
</tbody>
</table>

Arithmetic mean ± SEM for the urine albumin to creatinine ratio (ug/mg)

<sup>a</sup> Statistically significant difference compared to the Control IgG (p < 0.05)

There was a dose dependent decrease in albuminuria relative to the Control IgG group with Antibody III (Table 6). Antibody III treatment at 10 mg/kg resulted in a significant reduction in albuminuria at weeks 8 and 12 post surgery relative to the Control IgG group, but not at weeks 2, 4, or 16 (Table 6).
Antibody III demonstrated no effect on the systolic blood pressure, as all groups demonstrated hypertension at 12 weeks post surgery (Table 7). Furthermore, Antibody III treatment at 10 mg/kg resulted in improvements in renal function as shown by significant reductions in serum creatinine and BUN relative to the Control IgG group (Table 7).

Table 7
Systolic blood pressure, Serum Creatinine and BUN

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Week 12 Systolic Blood Pressure (mm Hg)</th>
<th>Week 16 Serum Creatinine (mg/dL)</th>
<th>Week 16 Serum BUN (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>nd</td>
<td>0.17 ± 0.01</td>
<td>31.5 ± 2.5</td>
</tr>
<tr>
<td>Control IgG (10 mg/kg)</td>
<td>139.6 ± 4.0</td>
<td>0.31 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.0 ± 12.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Antibody III (1 mg/kg)</td>
<td>147.5 ± 8.2</td>
<td>0.29 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.6 ± 1.7</td>
</tr>
<tr>
<td>Antibody III (10 mg/kg)</td>
<td>157.3 ± 4.5</td>
<td>0.23 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.8 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Arithmetic mean ± SEM

<sup>a</sup> Statistically significant relative to the sham group (p < 0.05)

<sup>b</sup> Statistically significant difference compared to the Control IgG group (p < 0.05)

nd, not determined
There was a statistically significant reduction in urine TGF-alpha and urine MIP-2 at weeks 8 and 12 post surgery respectively with the 10 mg/kg Antibody III dose compared to the Control IgG group (Table 8). Furthermore, there were statistically significant reductions in renal pathology for tubular protein and interstitial fibrosis and a decrease in mesangial matrix expansion with the 10 mg/kg dose of Antibody III compared to the Control IgG (Table 8).

**Example 6; Albuminuria and renal pathology in a mouse uninephrectomy db/db model of diabetic renal disease**

uninephrectomized db/db model is used to determine the effects of Antibody III on renal disease parameters due to diabetes. The uninephrectomy (“UniNx”) surgery on db/db mice on a C57BLKS/J background is performed at 4 weeks of age with removal of the right kidney. Randomization into groups of 12 mice is done at 8 weeks of age, by urine ACR, blood glucose and body weight. All the mice are hyperglycemic at the beginning of each study. An isotype Control IgG or Antibody III are dosed subcutaneously starting at 9 weeks of age and continued once weekly out to 25 weeks of age. Study 1 is conducted with doses of 0.3 and 10 mg/kg of Antibody III and a 10 mg/kg dose of isotype Control IgG. The endpoints for study 1 are survival, % HbAlc, albuminuria, urine TGF-alpha, kidney weight and renal pathology. Study 2 contains dose groups of 30, 10, 3 and 0.3 mg/kg of Antibody III with a 30 mg/kg dose of an isotype Control IgG. The endpoints for study 2 are survival and albuminuria.

There was only one death in the Control IgG group in study 1. There were no deaths in study 2.

Urine collection and measurement of Albuminuria

Urine is collected by a spot collection method to collect urine over a 2-4 hour time period. An individual mouse is placed on top of a 96 well polypropylene microplate and then covered by a Plexiglas chamber with holes for breathing but no access to food or water. At the end of the time period, the urine is removed from the plate with a micropipette and placed on ice, centrifuged and subjected to albumin and creatinine analysis. Albuminuria is defined as the ratio of urine albumin to creatinine (ug/mg).

Determination of %HbAlc

The % HbAlc is used as a measure of hyperglycemia at the end of the study. EDTA plasma is obtained at necropsy by cardiac puncture. Blood samples are spun at 2000 g for 20 minutes to remove blood cells and obtain plasma. Plasma samples are analyzed for Hemoglobin Ale and Total Hemoglobin. From these data, the % HbAlc as calculated.

Kidney Weight

Kidneys are removed at necropsy to determine their weight.
Determination of Urine TGF-alpha by ELISA

Urine obtained by a spot collection is concentrated 5-fold with a 0.5 mL Amicon Ultra centrifugal filter containing an ultracel 3K MW cutoff membrane. The device is spun at 14,000 x g for 30 minutes, and then the concentrated urine samples are collected. A sandwich-type ELISA for mouse TGF alpha is established. Rat TGF-alpha is used as the standard for the TGF-alpha ELISA. Polystyrene 96-well plates are coated with 3 µg/mL of Antibody III overnight at 4°C. Plates are washed, blocked with blocking buffer, washed again, and then the concentrated urine samples are added. After 2 hours at room temperature, plates are washed, and then secondary biotinylated polyclonal anti-hTGF-alpha is added. After 2 hours at room temperature, plates are washed and incubated with streptavidin-HRP for 30 minutes. Signal is generated with TMB substrate, and the reaction is stopped with 2 N H2SO4. Absorbance data are obtained on a SpectraMax 190 plate reader (Molecular Devices) and data are imported into Microsoft Excel 2007 and Sigmaplot v.9.01 for analysis.

Renal Pathology

Kidneys are removed at study termination, capsules removed and then fixed in formalin and processed for paraffin sectioning according to standard methodology. Sections of kidney are evaluated for renal lesions by a pathologist. Mesangial matrix, pelvic dilation and glomerular fibrosis, are semi-quantitatively scored using the following scale: none (0), minimal (1), slight (2), moderate (3), marked (4) and severe (5). Glomerular mesangial matrix expansion and basement membrane thickening are scored using H&E and PAS stained sections. Masson's trichrome stained sections of kidney are evaluated to determine the degree of fibrosis (glomerular).

Statistical Methods

All data are analyzed with JMP v.8.0 software (SAS Institute). Pathology scores are statistically evaluated by a contingency analysis and a Fishers exact test. Statistical analysis of albuminuria (ACR) is done by a Fit model with nontransformed data and the baseline ACR at week 8 as a covariate. ACR progression is analyzed by comparing the week 24 data with the week 16 data within each group by ANOVA and a Student's
unpaired t test. The ACR change from week 16 to week 24 across groups is done by ANOVA and a student's unpaired t test. A P value of < 0.05 is considered statistically significant. All other data are evaluated by ANOVA with log transformed data and a Students unpaired t test.

Table 9
Study 1 - Albuminuria progression

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>Wk 16-24 ACR change (ug/mg)</th>
<th>Wk 16-24 ACR change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Lean</td>
<td>nd</td>
<td>15 ± 2</td>
<td>19 ± 3</td>
<td>13 ± 3</td>
<td>12 ± 2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Db/db Control IgG @ 10 mg/kg</td>
<td>273 ± 59a</td>
<td>903 ± 125b</td>
<td>1551 ± 180a</td>
<td>2384 ± 257b</td>
<td>3228 ± 488abc</td>
<td>1677 ± 419</td>
<td>108 ± 27</td>
</tr>
<tr>
<td>Db/db Antibody III @ 0.3 mg/kg</td>
<td>299 ± 174a</td>
<td>1573 ± 209a</td>
<td>1911 ± 222a</td>
<td>2248 ± 417ab</td>
<td>675 ± 332b</td>
<td>43 ± 21</td>
<td></td>
</tr>
<tr>
<td>Db/db Antibody III @ 10 mg/kg</td>
<td>291 ± 55a</td>
<td>1002 ± 107a</td>
<td>965 ± 141a</td>
<td>1433 ± 190ab</td>
<td>1426 ± 230ab</td>
<td>461 ± 219b</td>
<td>48 ± 23</td>
</tr>
</tbody>
</table>

Arithmetic mean ± SEM

a Statistically significant relative to the healthy lean group (p < 0.05)
b Statistically significant difference compared to the Control IgG group (p < 0.05)
c Statistically significant relative to the week 16 timepoint within that group (p < 0.05)

In Study 1, there was a dose dependent decrease in albuminuria relative to the Control IgG group with Antibody III (Table 9). There was less progression of albuminuria compared to the Control IgG group for both the Antibody III groups during the last two months. The change in albuminuria within the group over the last two months of the study indicated that the Control IgG group significantly increased from week 16 to week 24, while the Antibody III groups did not (Table 9). In Study 2, there was a dose dependent reduction in the albuminuria progression over time with Antibody III compared to the Control IgG (Table 9).
Table 10
Study 2 - Albuminuria progression

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>Wk 16-24 ACR change (µg/mg)</th>
<th>Wk 16-24 ACR change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Lean</td>
<td>nd</td>
<td>13 ± 0</td>
<td>15 ± 0</td>
<td>9 ± 0</td>
<td>9 ± 0</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Db/db Control IgG @ 30 mg/kg</td>
<td>358 ± 76(^a)</td>
<td>1325 ± 271(^a)</td>
<td>1621 ± 350(^a)</td>
<td>2219 ± 320(^a)</td>
<td>2397 ± 242(^a)</td>
<td>776 ± 379</td>
<td>48 ± 23</td>
</tr>
<tr>
<td>Db/db Antibody III @ 0.3 mg/kg</td>
<td>356 ± 60(^a)</td>
<td>1200 ± 213(^a)</td>
<td>2410 ± 393(^a)</td>
<td>2286 ± 416(^a)</td>
<td>2086 ± 394(^a)</td>
<td>-323 ± 279(^b)</td>
<td>-13 ± 12(^b)</td>
</tr>
<tr>
<td>Db/db Antibody III @ 3 mg/kg</td>
<td>367 ± 77(^a)</td>
<td>1122 ± 248(^a)</td>
<td>1670 ± 193(^a)</td>
<td>1427 ± 204(^a)</td>
<td>1544 ± 264(^ab)</td>
<td>-126 ± 208(^b)</td>
<td>-8 ± 12(^b)</td>
</tr>
<tr>
<td>Db/db Antibody III @ 10 mg/kg</td>
<td>326 ± 77(^a)</td>
<td>1107 ± 304(^a)</td>
<td>1659 ± 286(^ab)</td>
<td>1202 ± 189(^ab)</td>
<td>1171 ± 252(^ab)</td>
<td>-489 ± 275(^b)</td>
<td>-29 ± 17(^b)</td>
</tr>
<tr>
<td>Db/db Antibody III @ 30 mg/kg</td>
<td>308 ± 68(^a)</td>
<td>1155 ± 179(^a)</td>
<td>1669 ± 223(^a)</td>
<td>1334 ± 237(^a)</td>
<td>950 ± 132(^ab)</td>
<td>-719 ± 230(^b)</td>
<td>-43 ± 14(^b)</td>
</tr>
</tbody>
</table>

Arithmetic mean ± SEM

\(^a\) Statistically significant difference relative to the healthy lean group (p < 0.05)
\(^b\) Statistically significant difference compared to the Control IgG group (p < 0.05)
\(^c\) Statistically significant relative to the week 16 timepoint within a group (p < 0.05)

The change in albuminuria over the last two months of Study 2 indicated that 30 mg/kg Antibody III resulted in a significant reduction of albuminuria over the last two months of the study, while the Control IgG increased over the same time period (Table 10).
Table 11
HbAlc, Kidney weight, urine TGF alpha and renal pathology scores

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>HbAlc (%)</th>
<th>Kidney weight (mgs)</th>
<th>Wk8 Urine TGF alpha (pg/mg)</th>
<th>Wk24 Urine TGF alpha (pg/mg)</th>
<th>Pathology Mesangial Matrix Score (1-5)</th>
<th>Pathology Pelvic Dilation Score (1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Lean</td>
<td>4.1 ± 0.0</td>
<td>138 ± 4</td>
<td>nd</td>
<td>nd</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Db/db Control IgG @ 10 mg/kg</td>
<td>11.1 ± 0.3a</td>
<td>396 ± 13a</td>
<td>215 ± 17</td>
<td>199 ± 18</td>
<td>1.92 ± 0.08a</td>
<td>1.67 ± 0.14a</td>
</tr>
<tr>
<td>Db/db Antibody III @ 0.3 mg/kg</td>
<td>11.2 ± 0.4a</td>
<td>375 ± 14a</td>
<td>208 ± 17</td>
<td>145 ± 30b</td>
<td>1.64 ± 0.15a</td>
<td>0.45 ± 0.16ab</td>
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<tr>
<td>Db/db Antibody III @ 10 mg/kg</td>
<td>10.7 ± 0.4a</td>
<td>359 ± 12ab</td>
<td>193 ± 16</td>
<td>3 ± 1b</td>
<td>1.17 ± 0.11ab</td>
<td>0.25 ± 0.13ab</td>
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Arithmetic mean ± SEM

*Statistically significant difference relative to the healthy lean group (p < 0.05)

*Statistically significant difference compared to the Control IgG group (p < 0.05)

Left Kidney weight was significantly lower in the 10 mg/kg Antibody III group relative to the 10 mg/kg Control IgG and 0.3 mg/kg Antibody III groups (Table 11). There was a significant decrease in urine TGF-alpha over the course of the study in the Antibody III 10 mg/kg dose group (Table 11). Furthermore, the % HbAlc for all the treatment groups were significantly elevated over the Control lean mice (Table 11). Antibody III treatment did not affect the % HbAlc compared to the Control IgG group (Table 11). Furthermore, there were significant reductions in renal pathology scores for mesangial matrix expansion and pelvic dilation with 10 mg/kg of Antibody III compared to the Control IgG (Table 11).
Example 7: Toxicity and Toxicokinetic Study in Cynomolgus Monkeys Given Weekly Intravenous Bolus Injections for 6 Weeks

A 6-week toxicology study is conducted in monkeys to evaluate whether inhibition of TGF-alpha and Epiregulin would lead to skin toxicity. Monkeys are dosed with vehicle, 10 or 100 mg/kg of Antibody I intravenous injection (IV) on a weekly basis for 6 weeks. The injection site is alternated between the right and left saphenous veins. Feed is provided twice daily (once in the morning and once in the afternoon). The morning food ration is provided soon after dosing on dosing days. Supplements and treats high in calcium are not offered during the study. A children's multivitamin is offered once weekly on Saturdays (after the 96-hour post-dose blood collections, where applicable).

Monkeys are housed in "divided pair" stainless steel slat/mesh cages throughout the study. During the first three weeks, the animals are individually housed. For the remainder of the study, the animals are pair-housed within treatment groups, beginning each afternoon and continuing until the following morning, in order to provide additional opportunity for socialization.

The No-Observed-Adverse-Effect Level ("NOAEL") for this study was 100 mg/kg of Antibody I. No skin changes were observed in treated animals. There were no other pathology changes observed.
SEP ID Listing

**Heavy Chain CDRs**

SEQ ID NO:1  GYTFTDAYIN
SEQ ID NO:2  WIWPGPVITYYNPKFKG
SEQ ID NO:3  REVLSPFAY

**Light Chain CDRs**

SEQ ID NO:4  RSSQSIVHSTGNTYLE
SEQ ID NO:5  KVSNRFS
SEQ ID NO:6  FHGTHVPYT

**Heavy Chain Variable Regions**

SEQ ID NO: 7 (Antibody I and Antibody II)

QVQLVQSGAEVKKPGSSVKVSCKASGYTFDTDYINWVRQAPGQGLEWMGWIW
PGPVITYYNPKFKGRVTITADKSTSTAYMELELRLSEDTCAVYYCARREVLSPFAY
WGQGTTVTVSS

SEQ ID NO:8 (Antibody III)

QVQLQQSGPELVKPGASVKISCKASGYTFDTDYINWVKQRPGQGLEWIGWIPG
PVITYYNPKFKGKATLTVDKSSSTAYMLSLTSEDFAFYFCARREVLSPFAYWG
QGTLVTVSA

**Light Chain Variable Regions**

SEQ ID NO:9 (Antibody I)

DIVMTQSPDSLAVSLGERATINCRSSQSIVHSTGNTYLEWYQQPKPGQPPKLIYKV
SNRFSGVPDVFSGSRTDFLTLISSLQAEDVAVYYCFHGTVPYTFGGGTKVEIK
SEQ ID NO: 10 (Antibody II)

DIQMTQSPSSLASVGSJVRVTITCRSSQSIHVSTGNTYLEWYQQKPGKAPKLLIYKV
SNRSFGVPDRSFSGSGTDFTLTISLQPEDFATYYCFHGTHVPYTFGGGKVEIK

SEQ ID NO: 11 (Antibody III)

DVLMTQTPLSLPVSLGDQASISCRSSQSIHVSTGNTYLEWLQKGQSPKLLIYKV
SNRSFGVPDRSFSGSGTDFTLKITRVEAEDLGVYYCFHGTHVPYTFGGGKLEIK

**Complete Heavy Chain**

SEQ ID NO: 12 (Antibody I and Antibody II)

QVQLVQSGAEVKKPGSSVKVSCKASGYTFTTADYINWVRQAPGGLEGWMGWIGW
PGPVITYYNSKGRITARKSTSTAYMESSLRLSDTAAYYCARRELSPFAY
WGGQTVTVITSSASTKPGSFPLACSRSTSESTAALGCLVKDYFPEGPTVSVWNSG
ALTSGVHTFPAVLQSSGLSSVVTGPSLGLTKTYYCNVHPSNTKVDKRVE
SKYPCCCPAEAAAGPSVFLFPKPDILMISRTPEVTCVVDVSEQDEPVQF
WVYTGVEVHNAKTPEEQFNSTYRVSVLTVLHLDWSENDKVRKSCNSG
LPSSIEKTIKAKQPREQVYTLPSQIEMTKNQVSLTLCVGFYPSDIAVEWS
NGQPENNYKTPPVFLSDGFLYSLTVKSRWQEGNVFSCVMHEALHNHYT
KQLSLSLG

**Complete Light Chains**

SEQ ID NO: 13 (Antibody I)

DIVMTQSPDSLAVSLGERATINCRSSQSIHVSTGNTYLEWYQQKPGQPPLIIYKV
SNRSFGVPDRSFSGSGTDFTLTISLQAEVDAAYYYCFHGTHVPYTFGGGKVEIK
RTVAAVPVFIFPSSDEQLKSGTASVCLNNFYPREAKVQWKVDNALQSGNSQES
VTEQDSKDSTYSLSTTLKADYKHKVYSCEVTHQGLSSPVTKSFNREGC

SEQ ID NO: 14 (Antibody II)

DIQMTQSPSSLASVGSJVRVTITCRSSQSIHVSTGNTYLEWYQQKPGKAPKLLIYKV
SNRSFGVPDRSFSGSGTDFTLTISLQPEDFATYYCFHGTHVPYTFGGGKVEIKR
TVAAPSFIFFPQDEQLKSGTASVCLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDSTYSLSTTLKADYKHKVYSCEVTHQGLSSPVTKSFNREGC
Nucleotide Sequences

Heavy Chain Variable Region

SEQ ID NO: 15

5
CAGGTGCAGCTGTGCTTGGGCTGAGGTGAAGAAGCCTGGGTCCTCAG
TGAAAGTTTCTCTGCAAGCATCTGGCTACACCTTCACTGAGCGCTATATAAAC
TGCGTGGCACAGGCCTTGAGGATGTTGGGATGGGATGGGCT
CTGGACCCTGATTACTTACTACAATCCGGAAGTCAAGGGCAGAGTCACCATT
ACCGCGGACAAATCCACGAGCACAGCTACATGGAGCTGAGCAGCCTGAGAT
CTGAGGACACCGGCCGTTGTTACTGTGCGAGAAGGGAAGTACTATCCCTCC
TGCTTTACTGGGGGCGCAAGGAAAACCAGTGCTCAGGCTCCTCCT

Light Chain Variable Regions

SEQ ID NO: 16

20
GACATCGTGATGACCCAGTCTCCAGACTCCCTGGCTGTGTCTCTGGGCGAGAG
GGCCACCATCAACTGCAGATCTAGTCAGAGCATTGTACATAGTACTGGAAAC
ACCTATTTAAGATGCTACACGACAGAACCAGGGAAGTACTATCCCTCC
TGCCATTTACTGTGTTTCACGGCACTCATGTTCCGTACACGTTCGGCGGAG
GGGACCAAGGTGGAGATCAAA

SEQ ID NO: 17

30
GACATCCAGATGACCCAGTCTCCATCCTCTCTCTGTCTGCTATCTGTAAGAGACAG
AGTCACCATCACTTGCAAGATCTAGTCAAGCATTGTACATAGTACTGGAACC
ACCTATTTAAGATGCTACACGACAGAACCAGGGAAGTACTATCCCTCC
TGCTATAAGTCTTCAAGCAGAACCAGGGAAGTACTATCCCTCC
TGCAACTTACTTGTTTTCACGGCACTCATGTTCCGTACACGTTCGGCGGAG
GGGACCAAGGTGGAGATCAAA

Mature Human TGF alpha

SEQ ID NO: 18

VVSHFNDCPDSHTQFCFHGTCRFLVQEDKAPCVCWGXYVGRCEHADLLA
Mature Mouse (Mus musculus) TGF alpha

SEQ ID NO: 19

5 VVSHFNKCPDSHTQYCFHGTCRFLVQEEKPACVCHSGYVGVRCEHADLLA

Mature Rat (Rattus norvegicus) TGF alpha

10 SEQ ID NO:20

VVSHFNKCPDSHTQYCFHGTCRFLVQEEKPACVCHSGYVGVRCEHADLLA

Mature Cyno (Macaca fascicularis) TGF alpha

15 SEQ ID NO:21

VVSHFNDCPDSHTQFCFHGTCRFLVQEDKPACVCHSGYVGARCEHADLLA

Mature Human Epiregulin - addition of N-terminal methionine

20 SEQ ID NO:22

MVSITKCSSDMNGYCLHGQCIYLVDMSQNYCRCEVGYTGVRCEHFFL

Mature Mouse (Mus musculus) Epiregulin - addition of N-terminal methionine

25 SEQ ID NO:23

MVQITKCSSDMGDYCLHGQCIYLVDREKFCRCEVGYTGLRCEHFFL

Mature Cyno (Macaca fascicularis) Epiregulin

30 SEQ ID NO:24

35 VSITKCNSDMNGYCLHGQCIYLVDMSQNYCRCEVGYTGVRCEHFYL

40
Mature Human Epigen
SEQ ID NO:25
AVTVTPITAQQADNIEGPIALKFSHLCLEDHNSYCINGACAFHHELEKAICRCFT
GYTGERCEHLTLTSYA

Mature Mouse (Mus musculus) Epigen
SEQ ID NO:26
LKFSHPCLEDHNSYCINGACAFHHELKQAICRCFTGYTGQRCEHLTLTSYA

Mature Human EGF - addition of N-terminal methionine
SEQ ID NO:27
MNSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWE
LR

Mature Human HBEGF
SEQ ID NO:28
DLQEADLDLLRVTLSSKPQALATPNKEEHHGKRKKGKGLGKRDPCLRKYKDF
CIHGECKYVKERAPSCICHPGYHGERCHGLSL

Mature Human Betacellulin
SEQ ID NO:29
DGNSTRSPETNLGCGDEENCAATTQSRRGHFSRCPQYKHYCIKGRCRFV
VAEQTPSCVCDEGYIGARCERVDFLY

Mature Human Amphiregulin
SEQ ID NO:30
SVRVEQWKPPQNKTESENTSDKPKRKKGGKKNGKNRRNRRKKKNPCNAEFQNF
CIHGECKYIEHLEAVTCKCQHEYFGERCGEKSMKTHSMIDSSLSK
Complete Heavy Chain Antibody III - Mouse Antibody

SEQ ID NO:31

QSILQQSGPELVKPGASVKISCKASGYTFTDAYINWVKQRPGQGLEWIGWIPWPG
PVITYYNPKFGKATLTVDSSTTSTAMLSLTAEDSAFACARLEVLSFAYWG
QGTLVTVSAAKTTPSVYPLAPGSAATNSMVTLGCLVKGYFEPEPVTWNSGS
LSSGVHTFPALQLSLYLLSSVTSVPSTWPSKTVCNVAPASSTKVDKIVPRDCGCKPCICTTPEVSFVIFPPKDVLTIITLTPKTVTCCFVVDISKDDPEVQFSWFVDD
VEVHTAQTPREEQFNSTFRSVPSELPMHDWNLGKEFKCRVNSAFFAPIEKTISSKTGKRPKAPQVTIPPPKEQMAKDKVSLTCMTDFFFPETVEVWQWNGQPAENY
KNTQPIIMDTDGSYFVYSLNVQKSNEAGNTFTCSVLHEGLFTNHTEKSLSHSP

Complete Light Chain Antibody III - Mouse Antibody

SEQ ID NO:32

DVLMPTQTLPLSVSLGDQASISCRRSSQIVHSHTGNTYLEWLYQLPKQPGSPKLIYKV
SNRFSGVPRFSGGSGTDFTKLISRVSEAEDLVYFYCHVYPFGGKLEIK
RADAAPTTSIFPSSEQLTSGASGCFLNNFKPKDUVWKIDQSEQNGVNLNWTQDSDKSTYSMSSTLTLTKDEYERHNSYTCATHKTSTSPIVKSFRNEC

Mature Human Epiregulin

SEQ ID NO:33

VSITKCSSDMNGYCLHGGQCIYLVDMSQNYCRCVEVGYTGVRCEHFFL
WE CLAIM:

1. An antibody that binds TGF-alpha and Epiregulin, comprising a light chain and a heavy chain, wherein the light chain comprises a light chain variable region (LCVR) and the heavy chain comprises a heavy chain variable region (HCVR), wherein the LCVR comprises amino acid sequences LCDR1, LCDR2, and LCDR3, and the HCVR comprises amino acid sequences HCDR1, HCDR2, and HCDR3, wherein LCDR1 is SEQ ID NO:4, LCDR2 is SEQ ID NO:5, LCDR3 is SEQ ID NO:6, HCDR1 is SEQ ID NO:1, HCDR2 is SEQ ID NO:2, and HCDR3 is SEQ ID NO:3.

2. The antibody of Claim 1, wherein the amino acid sequence of the LCVR is SEQ ID NO:9 or SEQ ID NO:10.

3. The antibody of either Claim 1 or Claim 2, wherein the amino acid sequence of the HCVR is SEQ ID NO:7.

4. The antibody of any one of Claims 1 to 3, wherein the amino acid sequence of the LCVR is SEQ ID NO:9 and the amino acid sequence of the HCVR is SEQ ID NO:7.

5. The antibody of any one of Claims 1 to 4, wherein the amino acid sequence of the light chain is SEQ ID NO:13 or SEQ ID NO:14.

6. The antibody of any one of Claims 1 to 5, wherein the amino acid sequence of the heavy chain is SEQ ID NO:12.

7. The antibody of any one of Claims 1 to 6 comprising two light chains wherein the amino acid sequence of each light chain is SEQ ID NO:13, and two heavy chains wherein the amino acid sequence of each heavy chain is SEQ ID NO:12.

8. The antibody of any one of Claims 1 to 3 or Claims 5 to 6 comprising two light chains wherein the amino acid sequence of each light chain is SEQ ID NO:14, and two heavy chains wherein the amino acid sequence of each heavy chain is SEQ ID NO:12.

9. A pharmaceutical composition comprising the antibody of any one of Claims 1 to 8, and at least one pharmaceutically acceptable carrier, diluent, or excipient.
10. A method of treating diabetic nephropathy in a patient, comprising
administering to the patient the antibody of any one of Claims 1 to 8.

11. An antibody of any one of Claims 1 to 8 for use in therapy.

12. An antibody of any one of Claims 1 to 8 for use in the treatment of diabetic
nephropathy.

13. An antigen-binding fragment of any one of Claims 1 to 8.

14. A pharmaceutical composition comprising the antigen-binding fragment of
Claim 13, and at least one pharmaceutically acceptable carrier, diluent, or
excipient.

15. A method of treating diabetic nephropathy in a patient, comprising
administering to the patient the antigen binding fragment of Claim 13.


17. An antigen-binding fragment of Claim 13 for use in the treatment of diabetic
nephropathy.
A. CLASSIFICATION OF SUBJECT MATTER

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)

C07K

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search

4 June 2012

Date of mailing of the international search report

12/06/2012

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer

Gruber, Andreas

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<td>USHA PANCHARAPAKESAN ET AL: &quot;Renal epidermal growth factor receptor: Its role in sodium and water homeostasis in diabetic nephropathy&quot;. CLINICAL AND EXPERIMENTAL PHARMACOLOGY AND PHYSIOLOGY, vol. 38, no. 2, 1 February 2011 (2011-02-01), pages 84-88, XP55028105, ISSN: 0305-1870, DOI: 10.1111/j.1440-1681.2010.05472.x e.g. page 87, left-hand column, paragraph 3; the whole document</td>
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