COMPOSITIONS AND METHODS FOR ANTIBODIES TARGETING EPO

BACKGROUND OF THE INVENTION

Diabetic retinopathy (DR) is the most common complication in patients with diabetes. Diabetic macular edema (DME) can occur in any stage of DR and is the main cause of vision loss in patients with DR. The incidence of DME after 10 years of follow-up has been reported to be 20.1% in Type 1 diabetes, 25.4% in Type 2 insulin-dependent diabetes, and 13.9% in Type 2 non-insulin-dependent diabetes (Klein et al. (1995) Ophthalmology *102*, 7-16). The ETDRS trial ((1985) Photocoagulation for Diabetic Macular Edema - Early Treatment Diabetic- Retinopathy Study Report .1. Archives of Ophthalmology *103*, 1796-1806), a pioneering study in DR, demonstrated that although laser photocoagulation therapy reduces the risk of moderate visual loss in DME eyes by ~50% at 3 years , only a few eyes gain vision, and some eyes continue to experience vision loss even after intensive treatment. In recent years, advances in pharmacotherapy and ocular drug delivery have shown promise in the treatment of DME. The RESTORE study, one of two pivotal Phase III trials in DME (Mitchell et al. (2011) Ophthalmology *118*, 615-625) demonstrated that Lucentis ® was superior to laser monotherapy. The mean change in best-corrected visual acuity (BCVA), which was the primary clinical endpoint, was significantly improved in the Lucentis ® group (+6.1 letters for the Lucentis ® group vs. +0.8 letters for the laser group; $p < 0.0001$). Similar beneficial effects have been demonstrated with VEGF Trap-Eye (Regeneron Inc. NY, USA) and Ozurdex® (dexamethasone intravitreal implant; Allergan Inc., CA, USA)(Do et al. (2011) Ophthalmology *118*, 1819-1826; Haller et al. (2010) Archives of Ophthalmology *128*, 289-296). However, 16% of Ozurdex treated eyes developed increased intra-ocular pressure, a risk for glaucoma.

Despite these new treatment options for DME, there remains a substantial unmet medical need. ~25% of eyes in the Lucentis ® pivotal trials did not gain any visual acuity after 12 months of treatment and ~50% of eyes are left with visual acuity of 20/40 or worse. Genome-wide association studies indicated that diabetics who are homozygous for an erythropoietin (Epo) promoter polymorphism (T) have a 2.17-fold higher risk of developing proliferative DR (Tong et al. (2008) Proc. Natl. Acad. Sci. U. S. A *105*, 6998-7003). Interestingly, people with the T promoter allele for Epo have approximately 7.5-fold higher vitreal concentration of Epo compared to people with the G allele (Tong et al. (2008) Proc. Natl. Acad. Sci. U. S. A *105*, 6998-7003).

There remains a need to develop an effective treatment for diabetic retinopathy, particularly DME to replace or supplement current treatments.

SUMMARY OF THE INVENTION

The invention relates to antibodies, or antigen binding fragments, as described herein which bind Epo and/or Darbepoietin.

The isolated antibodies, or antigen binding fragments, described herein bind Epo and/or Darbepoietin, with a K_D of less than or equal to 100 pM. For example, the isolated antibodies or antigen binding fragments described herein may bind to human Epo and/or Darbepoietin with a K_D of less than or equal to 50 pM, less than or equal to 40 pM, less than or equal to 35 pM, less than or equal to 30 pM, less than or equal to 25 pM, less than or equal to 20 pM, less than or equal to 15 pM, less than or equal to 14 pM, less than or equal to 13 pM, less than or equal to 12 pM, less than or equal to 11 pM, less than or equal to 10 pM. More specifically, the isolated antibodies or antigen binding fragments described herein may also bind human Epo with a K_D of less than or equal to 35pM, as measured by Biacore, or less than or equal to 6pM, as measured by Solution Equilibrium Titration (SET). More specifically, the isolated antibodies or antigen binding fragments described herein may also bind Darbepoietin with a K_D of less than or equal to 24pM, as measured by Biacore, or less than or equal to 4pM, as measured by SET.

The present invention relates to an isolated antibody, or antigen binding fragment thereof, that binds to human, cynomolgus, mouse and/or rat Epo. The invention also relates to an isolated antibody, or antigen binding fragment thereof, that binds a conformational epitope comprising amino acids selected from human Epo Helix D and Loop A-B. The invention further relates to an isolated antibody, or antigen binding fragment thereof, that binds a conformational epitope comprising amino acids selected from human Epo Helix D, Loop A-B and Helix A. In particular aspects of the invention, the isolated antibodies, or antigen binding fragments thereof, may bind to the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88). In other aspects, the isolated antibodies, or antigen binding fragments described herein may bind the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89). In other aspects the isolated antibodies, or antigen binding fragments described herein may bind the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89) and Helix A (amino acids 4-26 of Human Epo; SEQ ID NO: 86). In further aspects of the invention, the isolated antibodies, or antigen binding fragments described herein may bind the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88), and the Loop A-B domain

(amino acids 27-55 of Human Epo; SEQ ID NO: 89). In still further aspects of the invention, the isolated antibodies, or antigen binding fragments described herein may bind the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88), and Helix A (amino acids 4-26 of Human Epo; SEQ ID NO: 86). In still further aspects of the invention, the isolated antibodies, or antigen binding fragments described herein may bind the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88), the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89) and Helix A (amino acids 4-26 of Human Epo; SEQ ID NO: 86).

The present invention also relates to an isolated antibody, or antigen binding fragment thereof, that binds a confirmational epitope on Epo comprising amino acid residues Thr44, Lys45, Val46, Asn47, Phe48, Tyr49, Ala50, Lys52, Arg53, Asn147, Arg150, Gly151, Lys154, Leu155, Glu159, and Arg162 of Human Epo (SEQ ID NO.81). The present invention further relates to an isolated antibody, or antigen binding fragment thereof, that binds a confirmational epitope on Epo comprising amino acids residues Ser9, Gln13, Thr44, Lys45, Val46, Asn47, Phe48, Tyr49, Ala50, Lys52, Arg53, Asn147, Arg150, Gly151, Lys154, Leu155, Gly158, Glu159, and Arg162, of Human Epo (SEQ ID NO.81). The present invention still further relates to relates to an isolated antibody, or antigen binding fragment thereof, that binds a confirmational epitope on Epo comprising amino acid residues Glu23, Asp43, Thr44, Lys45, Val46, Asn47, Phe48, Tyr49, Ala50, Lys52, Arg53, Arg131, Arg143, Asn147, Arg150, Gly151, Lys154, Leu155, Glu159, and Arg162 of Human Epo (SEQ ID NO.81).

The present invention also relates to an isolated antibody, or antigen binding fragment thereof, that binds Epo and further competes for binding with an antibody as described in Table 1. The present invention also further relates to an isolated antibody, or antigen binding fragment thereof, that binds the same epitope as an antibody as described in Table 1.

The present invention also relates to an isolated antibody, or antigen binding fragment thereof, that binds Epo and has an isoelectric point (pI) greater than 8.2, greater than 8.3, greater than 8.4, greater than 8.5 or greater than 9.0.

The isolated antibodies or antigen binding fragments described herein may also bind cynomolgus Epo, mouse Epo and/or rat Epo with a K_D of less than or equal to 100 pM, less than or equal to 80 pM, less than or equal to 70 pM, less than or equal to 60 pM, less than or equal to 50 pM, less than or equal to 40 pM, less than or equal to 35 pM, less than or equal to 30 pM, less than or equal to 25 pM, less than or equal to 20

pM, less than or equal to 15 pM, less than or equal to 10 pM, less than or equal to 5 pM, less than or equal to 4 pM, less than or equal to 3 pM, less than or equal to 2 pM, less than or equal to 1 pM. More specifically, the isolated antibodies or antigen binding fragments described herein may also bind cynomolgus Epo, mouse Epo and/or rat Epo with a K_D of less than or equal to 80pM, as measured by Biacore, or less than or equal to 40pM, as measured by SET. More specifically, the isolated antibodies or antigen binding fragments described herein may also bind Cynomolgus Epo with a K_D of less than or equal to 80pM, as measured by Biacore, or less than or equal to 8pM, as measured by SET. More specifically, the isolated antibodies or antigen binding fragments described herein may also bind mouse Epo with a K_D of less than or equal to 45pM, as measured by Biacore, or less than or equal to 37pM, as measured by SET. More specifically, the isolated antibodies or antigen binding fragments described herein may also bind rat Epo with a K_D of less than or equal to 57pM, as measured by Biacore, or less than or equal to 13pM, as measured by SET.

The binding affinity of isolated antibodies and antigen binding fragments described herein can be determined by SET. Methods for SET are known in the art and are described in further detail below. Alternatively, binding affinity of the isolated antibodies, or fragments, described herein can be determined by Biacore assay. Methods for Biacore kinetic assays are known in the art and are described in further detail below.

The isolated antibodies and antigen binding fragments described herein can be used to inhibit Epo-dependent cell proliferation with an IC_{50} of less than or equal to 350 pM, less than or equal to 300 pM, less than or equal to 250 pM, less than or equal to 200 pM, less than or equal to 190 pM, less than or equal to 180 pM, less than or equal to 175 pM, less than or equal to 170 pM, less than or equal to 160 pM, less than or equal to 150 pM, less than or equal to 125 pM, less than or equal to 115 pM, less than or equal to 110 pM, less than or equal to 100 pM, less than or equal to 90 pM, or less than or equal to 80 pM. More specifically, an isolated antibody or antigen binding fragment thereof as described herein can inhibit Epo-dependent cell proliferation as measured by an *in vitro* Ba/F3-EpoR cell proliferation assay with an IC_{50} of less than or equal to 338 pM, less than or equal to 183 pM, less than or equal to 175 pM, less than or equal to 174 pM, less than or equal to 145 pM, less than or equal to 112 pM, less than or equal to 89 pM, or less than or equal to 74 pM.

The isolated antibodies and antigen binding fragments described herein can be used to inhibit Epo-dependent cell proliferation in B-cells. More specifically, the isolated

antibodies and antigen binding fragments described herein can be used to inhibit Epo-dependent cell proliferation in mouse B-cells. For example, an isolated antibody or antigen binding fragment thereof can inhibit Epo-dependent cell proliferation as measured by an *in vitro* Ba/F3-EpoR cell proliferation assay with an IC_{50} of less than or equal to 350 pM, less than or equal to 300 pM, less than or equal to 250 pM, less than or equal to 200 pM, less than or equal to 175 pM, less than or equal to 150 pM, less than or equal to 125 pM, less than or equal to 115 pM, less than or equal to 110 pM, less than or equal to 100 pM, less than or equal to 90 pM, or less than or equal to 80 pM. More specifically, an isolated antibody or antigen binding fragment thereof as described herein can inhibit Epo-dependent cell proliferation as measured by an *in vitro* Ba/F3-EpoR cell proliferation assay with an IC_{50} of less than or equal to 338 pM, less than or equal to 174 pM, less than or equal to 112 pM, or less than or equal to 74 pM.

The isolated antibodies and antigen binding fragments described herein can be used to inhibit Epo-dependent cell proliferation of human B-cells. For example, an isolated antibody or antigen binding fragment thereof can inhibit Epo-dependent cell proliferation, as measured by an *in vitro* F36E cell proliferation assay, with an IC_{50} of less than or equal to 200 pM, less than or equal to 190 pM, less than or equal to 180 pM, less than or equal to 170 pM, less than or equal to 160 pM, less than or equal to 150 pM, less than or equal to 125 pM, less than or equal to 100 pM, or less than or equal to 90 pM. More specifically, an isolated antibody or antigen binding fragment thereof as described herein can inhibit Epo-dependent cell proliferation as measured by an *in vitro* F36E cell proliferation assay with an IC_{50} of less than or equal to 183 pM, less than or equal to 175 pM, less than or equal to 145 pM, or less than or equal to 89 pM.

The isolated antibodies, or antigen binding fragments thereof, may also block Epo binding to the Epo receptor and/or prevent Epo binding to a cell surface.

Another aspect of the invention includes an isolated antibody, or antigen binding fragment thereof, that specifically binds to human, cynomolgus, mouse and/or rat Epo. In a further aspect, the isolated antibody, or antigen binding fragment, competes for binding with an antibody, or antigen binding fragment, described in Table 1.

The isolated antibodies, or antigen binding fragments thereof, as described herein can be monoclonal antibodies, human or humanized antibodies, chimeric antibodies, single chain antibodies, Fab fragments, Fv fragments, F(ab')2 fragments, or ScFv fragments, and/or IgG isotypes.

The isolated antibodies, or antigen binding fragments thereof, as described herein can also include a framework in which an amino acid has been substituted into the antibody framework from the respective human VH or VL germline sequences.

Another aspect of the invention includes an isolated antibody or antigen binding fragment thereof having the full heavy and light chain sequences of Fabs described in Table 1. More specifically, the isolated antibody or antigen binding fragment thereof can have the heavy and light chain sequences of Fab NVS1, NVS2, NVS3, or NVS4.

A further aspect of the invention includes an isolated antibody or antigen binding fragment thereof having the heavy and light chain variable domain sequences of Fabs described in Table 1. More specifically, the isolated antibody or antigen binding fragment thereof can have the heavy and light chain variable domain sequence of Fab NVS1 (SEQ ID NOs 13 and 14, respectively), NVS2 (SEQ ID NOs 33 and 34, respectively), NVS3 (SEQ ID NOs 53 and 54, respectively), or NVS4 (SEQ ID NOs 73 and 74, respectively).

The invention also relates to an isolated antibody or antigen binding fragment thereof that includes a heavy chain CDR1 selected from the group consisting of SEQ ID NOs 1, 21, 41, and 61; a heavy chain CDR2 selected from the group consisting of SEQ ID NOs: 2, 22, 42, and 62; and a heavy chain CDR3 selected from the group consisting of SEQ ID NOs: 3, 23, 43, and 63, wherein the isolated antibody or antigen binding fragment thereof binds to human Epo. In another aspect, such isolated antibody or antigen binding fragment thereof further includes a light chain CDR1 selected from the group consisting of SEQ ID NOs: 4, 24, 44, and 64; a light chain CDR2 selected from the group consisting of SEQ ID NOs 5, 25, 45, and 65; and a light chain CDR3 selected from the group consisting of SEQ ID NOs 6, 26, 46, and 66.

The invention also relates to an isolated antibody or antigen binding fragment thereof that includes a light chain CDR1 selected from the group consisting of SEQ ID NOs: 4, 24, 44, and 64; a light chain CDR2 selected from the group consisting of SEQ ID NOs 5, 25, 45, and 65; and a light chain CDR3 selected from the group consisting of SEQ ID NOs 6, 26, 46, and 66, wherein the isolated antibody or antigen binding fragment thereof binds to human Epo.

The invention also relates to an isolated antibody or antigen binding fragment thereof that binds Epo having HCDR1, HCDR2, HCDR3 and LCDR1, LCDR2, LCDR3, wherein HCDR1, HCDR2, HCDR3 comprises SEQ ID NOs: 1, 2, 3, and LCDR1, LCDR2, LCDR3 comprises SEQ ID NOs: 4, 5, 6; or HCDR1, HCDR2, HCDR3 comprises SEQ ID

NOs: 21, 22, 23, and LCDR1, LCDR2, LCDR3 comprises SEQ ID NOs: 24, 25, 26; or HCDR1, HCDR2, HCDR3 comprises SEQ ID NOs: 41, 42, 43, and LCDR1, LCDR2, LCDR3 comprises SEQ ID NOs: 44, 45, 46; or HCDR1, HCDR2, HCDR3 comprises SEQ ID NOs: 61, 62, 63, and LCDR1, LCDR2, LCDR3 comprises SEQ ID NOs: 64, 65, 66.

The invention also relates to an antibody or antigen binding fragment having HCDR1, HCDR2, and HCDR3 of the variable heavy chain of SEQ ID NO: 13, 33, 53 or 73, and the LCDR1, LCDR2 and LCDR3 of the variable light chain of SEQ ID NO: 14, 34, 54 or 74, as defined by Chothia. In another aspect of the invention the antibody or antigen binding fragment may have the HCDR1, HCDR2, and HCDR3 of the heavy chain variable domain sequence of SEQ ID NO: 13, 33, 53 or 73, and the LCDR1, LCDR2 and LCDR3 of the light chain variable domain sequence of SEQ ID NO: 14, 34, 54 or 74, as defined by Kabat.

In one aspect of the invention the isolated antibody or antigen binding fragment thereof includes a heavy chain variable domain (VH) sequence selected from the group consisting of SEQ ID NOs: 13, 33, 53 and 73. The isolated antibody or antigen binding fragment further can comprise a light chain variable domain (VL) sequence wherein the heavy chain variable domain and light chain variable domain combine to form an antigen binding site for Epo. In particular the light chain variable domain sequence can be selected from SEQ ID NOs: 14, 34, 54 and 74 wherein said isolated antibody or antigen binding fragment thereof binds Epo.

The invention also relates to an isolated antibody or antigen binding fragment thereof that includes a light chain variable domain sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54 and 74, wherein said isolated antibody or antigen binding fragment thereof binds to human Epo. The isolated antibody or antigen binding fragment may further comprise a heavy chain variable domain sequence wherein the light chain variable domain and heavy chain variable domain combine to form and antigen binding site for Epo.

In particular, the isolated antibody or antigen binding fragment thereof that binds Epo, may have heavy and light chain variable domains comprising the sequences of SEQ ID NOs: 13 and 14; 33 and 34; 53 and 54; or 73 and 74, respectively.

The invention further relates to an isolated antibody or antigen binding fragment thereof, that includes a heavy chain variable domain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group

consisting of SEQ ID NOs:13, 33, 53, and 73, wherein said antibody binds to Epo. In one aspect, the isolated antibody or antigen binding fragment thereof also includes a light chain variable domain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54, and 74. In a further aspect of the invention, the isolated antibody or antigen binding fragment has an HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 as defined by Kabat and as described in Table 1. It is also contemplated that the HCDR1, HCDR2, HCDR3, LCDR1, LCDR2 and LCDR3 may be defined by Chothia and as described in Table 1.

The invention also relates to an isolated antibody or antigen binding fragment thereof, having a light chain variable domain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54, and 74, wherein said antibody binds Epo.

In another aspect of the invention, the isolated antibody, or antigen binding fragment thereof, that binds to Epo may have a heavy chain comprising the sequence of SEQ ID NO: 15, 35, 55, or 75. The isolated antibody can also include a light chain that can combine with the heavy chain to form an antigen binding site to human Epo. In particular, the light chain may have a sequence comprising SEQ ID NO: 16, 36, 56, or 76. In particular, the isolated antibody or antigen binding fragment thereof that binds Epo, may have a heavy chain and a light chain comprising the sequences of SEQ ID NOs: 15 and 16; 35 and 36; 55 and 56; or 75 and 76, respectively.

The invention still further relates to an isolated antibody or antigen binding fragment thereof that includes a heavy chain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 15, 35, 55, and 75, wherein said antibody binds to Epo. In one aspect, the isolated antibody or antigen binding fragment thereof also includes a light chain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs 16, 36, 56, and 76.

The invention still further relates to an isolated antibody or antigen binding fragment thereof that includes a light chain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs 16, 36, 56, and 76, wherein said antibody binds Epo.

The invention also relates to compositions comprising the isolated antibody, or antigen binding fragment thereof, as described herein. As well as, antibody

compositions in combination with a pharmaceutically acceptable carrier. Specifically, the invention further includes pharmaceutical compositions comprising an antibody or antigen binding fragment thereof of Table 1, such as, for example antibody NVS1, NVS2, NVS3 or NVS4. The invention also relates to pharmaceutical compositions comprising a combination of two or more of the isolated antibodies or antigen binding fragments thereof of Table 1.

The invention also relates to an isolated nucleic acid sequence encoding the variable heavy chain having a sequence selected from SEQ ID NO: 13, 33, 53 and 73. In particular the nucleic acid has a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 17, 37, 57, and 77. In a further aspect of the invention the sequence is SEQ ID NOs: 17, 37, 57, or 77.

The invention also relates to an isolated nucleic acid sequence encoding the variable light chain having a sequence selected from SEQ ID NO: 14, 34, 54 and 74. In particular the nucleic acid has a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 18, 38, 58, and 78. In a further aspect of the invention the sequence is SEQ ID NOs: 18, 38, 58, or 78.

The invention also relates to an isolated nucleic acid comprising a sequence encoding a polypeptide that includes a light chain variable domain having at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence selected from the group consisting of SEQ ID NOs: 18, 38, 58, and 78.

The invention also relates to a vector that includes one or more of the nucleic acid molecules described herein.

The invention also relates to an isolated host cell that includes one or more of the nucleic acid molecules or vectors described herein. The invention also relates to an isolated host cell that includes a recombinant DNA sequence encoding a heavy chain of the antibody described above, and a second recombinant DNA sequence encoding a light chain of the antibody described above, wherein said DNA sequences are operably linked to a promoter and are capable of being expressed in the host cell. It is contemplated that the antibody can be a human monoclonal antibody. It is also contemplated that the host cell is a non-human mammalian cell, for example a CHO cell.

The invention also relates to a method of inhibiting Epo-dependent cell proliferation wherein the method includes the step of contacting Epo (e.g., contacting Epo in a subject) with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. In one aspect, the method comprises contacting a cell (e.g., a cell comprising Epo) with a composition coprising the isolated antibody or antigen binding fragment thereof as described herein. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo-dependent cell proliferation in a subject. It is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

The invention also relates to a method of inhibiting Epo-dependent cell signalling wherein the method includes the step of contacting Epo with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein to prevent Epo from interacting with a receptor on a cell surface. In one aspect, the method comprises contacting a cell comprising Epo with a composition coprising the isolated antibody or antigen binding fragment thereof as described herein. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo-dependent cell signalling in a subject. It is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

The invention also relates to a method of inhibiting Epo-dependent cell proliferation or signalling wherein the method includes the step of contacting Epo with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein to prevent Epo from interacting with a receptor on a cell surface. It is contemplated that the cell is a B cell. It is contemplated that the cell is a human cell.

The invention also relates to a method of inhibiting Epo binding to the Epo receptor wherein the method includes the step of contacting Epo (e.g., contacting Epo in a subject) with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as

described herein for use to inhibit Epo binding to the Epo receptor on a cell of a subject; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. It is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

The invention still further relates to a method of inhibiting Epo binding to a cell wherein the method includes the step of contacting Epo (e.g., in a subject) with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. In one aspect, the method comprises contacting a cell (e.g., a cell comprising Epo) with a composition coprising the isolated antibody or antigen binding fragment thereof as described herein. The invention still further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo binding to a cell in a subject. In one aspect, it is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

The invention also relates to a method of treating macular edema in a subject, wherein the method includes the step of administering to the subject an effective amount of a composition comprising the antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an antibody or antigen binding fragment thereof as described herein to treat macular edema in a subject. In one aspect, macular edema is associated with retinal vascular disease. It is contemplated that the retinal vascular disease associated with the macular edema can include diabetic retinopathy, diabetic macular edema, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, retinal vein occlusion, multifocal choroiditis, myopic choroidal neovascularization, or retinopathy of prematurity. It is also contemplated that the subject is human.

The invention also relates to a method of treating a condition or disorder associated with retinal vascular disease in a subject, wherein the method includes the step of administering to the subject an effective amount of a composition comprising the antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an antibody or antigen binding fragment thereof

as described herein to treat a condition or disorder associated with retinal vascular disease in a subject. In one aspect, it is contemplated that the condition or disorder associated with retinal vascular disease is diabetic retinopathy. In another aspect, it is contemplated that the condition or disorder is age-related macular degeneration. It is still further contemplated that the condition or disorder associated with retinal vascular disease can be retinal vein occlusion, multifocal choroiditis, myopic choroidal neovascularization, or retinopathy of prematurity. It is also contemplated that the subject is human.

The invention also relates to a method of treating a condition or disorder associated with diabetic retinopathy in a subject, wherein the method includes the step of administering to the subject an effective amount of a composition comprising the antibody or antigen binding fragments thereof as described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an antibody or antigen binding fragment thereof as described herein to treat a condition or disorder associated with diabetic retinopathy in a subject. It is contemplated that the subject is human.

The invention also relates to a method of treating a condition or disorder associated with macular edema in a subject, wherein the method includes the step of administering to the subject an effective amount of a composition comprising the antibody or antigen binding fragments thereof as described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an antibody or antigen binding fragment thereof as described herein to treat a condition or disorder associated with macular edema in a subject. It is further contemplated that the condition or disorder associated with macular edema is diabetic macular edema. It is further contemplated that the subject is human.

The invention also relates to a method of treating proliferative diabetic retinopathy in a subject, wherein the method includes the step of administering to the subject an effective amount of a composition comprising the antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an antibody or antigen binding fragment thereof as described herein to treat proliferative diabetic retinopathy in a subject. It is further contemplated that the composition is administered to the eye of the subject wherein the composition decreases retinal vein dilation, decreases vascular leakage and/or increases blood flow in the eye. It is further contemplated that the subject is human.

Any of the foregoing isolated antibodies or antigen binding fragments thereof may be a monoclonal antibody or antigen binding fragment thereof.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains.

The term "antibody" as used herein means a whole antibody and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain thereof. A whole antibody is a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is 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. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The term "antigen binding portion" or “antigen binding fragment” of an antibody, as used herein, refers to one or more fragments of an intact antibody that retain the ability to specifically bind to a given antigen (*e.g.*, Erythropoietin: Epo). Antigen binding functions of an antibody can be performed by fragments of an intact antibody. Examples of binding fragments encompassed within the term antigen binding portion or antigen binding fragment of an antibody include a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; a $F(ab)_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; an Fd fragment consisting of the VH and CH1 domains; an Fv fragment consisting of the VL and VH domains of a single arm of an antibody; a single domain antibody (dAb) fragment

(Ward *et al.*, 1989 Nature 341:544-546), which consists of a VH domain or a VL domain; and an isolated complementarity determining region (CDR).

Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by an artificial peptide linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see, *e.g.*, Bird *et al.*, 1988 Science 242:423-426; and Huston *et al.*, 1988 Proc. Natl. Acad. Sci. 85:5879-5883). Such single chain antibodies include one or more antigen binding portions or fragments of an antibody. These antibody fragments are obtained using conventional techniques known to those of skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

Antigen binding fragments can also be incorporated into single domain antibodies, maxibodies, minibodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, *e.g.*, Hollinger and Hudson, 2005, Nature Biotechnology, 23, 9, 1126-1136). Antigen binding portions of antibodies can be grafted into scaffolds based on polypeptides such as Fibronectin type III (Fn3) (see U.S. Pat. No. 6,703,199, which describes fibronectin polypeptide monobodies).

Antigen binding fragments can be incorporated into single chain molecules comprising a pair of tandem Fv segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata *et al.*, 1995 Protein Eng. 8(10):1057-1062; and U.S. Pat. No. 5,641,870).

As used herein, the term "affinity" refers to the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody “arm” interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, the stronger the affinity. As used herein, the term "high affinity" for an antibody or antigen binding fragment thereof (e.g.: a Fab fragment) generally refers to an antibody, or antigen binding fragment, having a KD of 10^{-9}M or less.

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino

acid, *i.e.*, an alpha carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

The term “binding specificity” as used herein refers to the ability of an individual antibody combining site to react with only one antigenic determinant.

The phrase “specifically (or selectively) binds” to an antibody (*e.g.*, an Epo-binding antibody) refers to a binding reaction that is determinative of the presence of a cognate antigen (*e.g.*, a human Epo or cynomolgus Epo) in a heterogeneous population of proteins and other biologics. The phrases “an antibody recognizing an antigen” and “an antibody specific for an antigen” are used interchangeably herein with the term “an antibody which binds specifically to an antigen”.

The term “condition or disorder associated with retinal vascular disease” refers to conditions, disorders or diseases in which the retina degenerates or becomes dysfunctional. This includes diabetic retinopathy (DR), diabetic macular edema (DME), proliferative diabetic retinopathy (PDR), non-proliferative diabetic retinopathy (NPDR), age-related macular degeneration (AMD), retinal vein occlusion (RVO), multifocal choroiditis, myopic choroidal neovascularization, or retinopathy of prematurity. Anatomic characteristics of retinal vascular disease that may be treated by Epo inhibition include macular edema, venous dilation, vessel tortuosity, vascular leakage as measured by fluorescein angiography, retinal hemorrhage, and microvascular anomalies (e.g. microaneurysm, cotton-wool spots, IRMA), capillary dropout, leukocyte adhesion, retinal ischemia, neovascularization of the optic disk, neovascularization of the posterior pole, iris neovascularization, intraretinal hemorrhage, vitreous hemorrhage, macular scar, subretinal fibrosis, and retinal fibrosis.

The term "condition or disorder associated with diabetic retinopathy" refers to conditions in which the retina degenerates or becomes dysfunctional, as a consequence of effects of diabetes mellitus (Type 1 or Type 2) on retinal vasculature, retinal metabolism, retinal pigment epithelium, the blood-retinal barrier, or ocular levels of advanced glycation end products (AGEs), aldose reductase activity, glycosylated hemoglobin, and protein kinase C. Visual loss in patients with diabetic retinopathy can be

a result of retinal ischemia, macular edema, vascular leakage, vitreous hemorrhage, or direct effects of elevated glucose levels on retinal neurons. Anatomic characteristics of diabetic retinopathy that may be treated by Epo inhibition include microaneurysm, cotton wool spots, venous dilation, macular edema, intra-retinal microvascular abnormalities (IRMA), intra-retinal hemorrhage, vascular proliferation, neovascularization of the disk, rubeosis, and retinal ischemia. "Diabetic macular edema" occurs in a subject with diabetic retinopathy and can occur at any stage of the disease.

The term "condition or disorder associated with macular edema", refers to conditions or disorders in which swelling or thickening of the macula occurs as a result of retinal blood vessels leaking fluid, "macular edema". Macular edema occurs in, and is often a complication of, retinal vascular disease. Specific conditions or disorders associated with macular edema include, diabetic retinopathy, diabetic macular edema, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, retinal vein occlusion, multifocal choroiditis, myopic choroidal neovascularization, or retinopathy of prematurity. Treatment of macular edema by the inhibition of Epo can be determined by funduscopic examination, optical coherence tomography, and improved visual acuity.

The term "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, *e.g.*, an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity. For example, a mouse antibody can be modified by replacing its constant region with the constant region from a human immunoglobulin. Due to the replacement with a human constant region, the chimeric antibody can retain its specificity in recognizing the antigen while having reduced antigenicity in human as compared to the original mouse antibody.

The terms "Epo protein" or "Epo antigen" or "EPO" or "Epo" are used interchangeably, and refer to the erythropoietin protein in different species. For example, human Epo has the sequence as set out in Table 1: SEQ ID NO: 81. Examples of Epo proteins from other species are provided in Table 1, SEQ ID NOs: 82, 83, 84 or 85. The protein sequences for human, cynomolgus, mouse, rat, and rabbit Epo are publicly available and described in Table 1. Human Epo can also be hyperglycosylated.

Hyperglycosylated Epo is also know in the art as “darbepoietin” and can be obtained from various sources including, LEK Pharmacueticals.

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

For polypeptide sequences, “conservatively modified variants” include individual substitutions, deletions or additions to a polypeptide sequence which result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention. The following eight groups contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, *e.g.*, Creighton, Proteins (1984)). In some embodiments, the term "conservative sequence modifications" are used to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence.

The term “epitope” means a protein determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules

such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term "human antibody", as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from sequences of human origin. Furthermore, if the antibody contains a constant region, the constant region also is derived from such human sequences, *e.g.*, human germline sequences, or mutated versions of human germline sequences. The human antibodies of the invention may include amino acid residues not encoded by human sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*).

The term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable regions in which both the framework and CDR regions are derived from human sequences. In one embodiment, the human monoclonal antibodies are produced by hybridomas which include (i) a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene (ii) fused to an immortalized cell.

A "humanized" antibody is an antibody that retains the reactivity of a non-human antibody while being less immunogenic in humans. This can be achieved, for instance, by retaining the non-human CDR regions and replacing the remaining parts of the antibody with their human counterparts (*i.e.*, the constant region as well as the framework portions of the variable region). See, *e.g.*, Morrison *et al.*, Proc. Natl. Acad. Sci. USA, 81:6851-6855, 1984; Morrison and Oi, Adv. Immunol., 44:65-92, 1988; Verhoeyen *et al.*, Science, 239:1534-1536, 1988; Padlan, Molec. Immun., 28:489-498, 1991; and Padlan, Molec. Immun., 31:169-217, 1994. Other examples of human engineering technology include, but are not limited to Xoma technology disclosed in US 5,766,886.

The terms “identical” or 100% percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%

identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol. 48:443, 1970, by the search for similarity method of Pearson and Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444, 1988, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, *e.g.*, Brent *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou ed., 2003)).

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, Nuc. Acids Res. 25:3389-3402, 1977; and Altschul *et al.*, J. Mol. Biol. 215:403-410, 1990, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying

short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915, 1989) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul, Proc. Natl. Acad. Sci. USA 90:5873-5787, 1993). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller (Comput. Appl. Biosci., 4:11-17, 1988) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (J. Mol, Biol. 48:444-453, 1970) algorithm which has been incorporated into the GAP program in the GCG software package (available on the world

wide web at gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

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

The term “inhibit (or inhibits) Epo-dependent cell proliferation” refers to the ability of an anti-Epo antibody to interfere with cell activation (e.g., cell signaling), replication and/or proliferation stimulated and/or induced by Epo. Specifically, “inhibit” refers to a statistically significant decrease (i.e., $p<0.05$) in Epo-dependent cell proliferation, or other parameter (e.g., Epo dependent cell signaling, angiogenesis), in a subject following contact with an anti-Epo antibody or fragment thereof as described herein relative to a control. As used herein, “inhibit (or inhibits) Epo-dependent cell proliferation” can also refer to a clinically relevant improvement in visual function or retinal anatomy following treatment with an anti-Epo antibody described herein in a patient diagnosed with a condition or disorder associated with retinal vascular disease as described below.

As used herein, “inhibit (or inhibits) Epo dependent cell signaling” refers to the ability of an anti-Epo antibody described herein to produce a statistically significant (i.e., $p<0.05$) decrease in the activation of the intracellular signaling pathways stimulated or induced by Epo.

The term "isolated antibody" refers to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds Epo is substantially free of antibodies that specifically bind antigens other than Epo). An isolated antibody that specifically binds Epo may, however, have cross-reactivity to other antigens. Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

The term "isotype" refers to the antibody class (*e.g.*, IgM, IgE, IgG such as IgG1 or IgG4) that is provided by the heavy chain constant region genes. Isotype also includes modified versions of one of these classes, where modifications have been made to alter the Fc function, for example, to enhance or reduce effector functions or binding to Fc receptors. Isotype also refers to the antibody class (e.g., kappa, lambda) that is provided by the light-chain constant regions.

The term "Kassoc" or "Ka", as used herein, is intended to refer to the association rate of a particular antibody-antigen interaction, whereas the term "Kdis" or "Kd," as used herein, is intended to refer to the dissociation rate of a particular antibody-antigen interaction. The term "K_D", as used herein, is intended to refer to the dissociation constant, which is obtained from the ratio of Kd to Ka (*i.e.* Kd/Ka) and is expressed as a molar concentration (M). K_D values for antibodies can be determined using methods well established in the art. Methods for determining the K_D of an antibody include measuring surface plasmon resonance using a biosensor system such as a Biacore® system, or measuring affinity in solution by solution equilibrium titration (SET).

The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

The term "nucleic acid" is used herein interchangeably with the term “polynucleotide” and refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, as detailed below, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*,

Nucleic Acid Res. 19:5081, 1991; Ohtsuka *et al.*, J. Biol. Chem. 260:2605-2608, 1985; and Rossolini *et al.*, Mol. Cell. Probes 8:91-98, 1994).

The term "operably linked" refers to a functional relationship between two or more polynucleotide (*e.g.*, DNA) segments. Typically, the term refers to the functional relationship of a transcriptional regulatory sequence to a transcribed sequence. For example, a promoter or enhancer sequence is operably linked to a coding sequence if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, *i.e.*, they are *cis*-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

As used herein, the term, "optimized" means that a nucleotide sequence has been altered to encode an amino acid sequence using codons that are preferred in the production cell or organism, generally a eukaryotic cell, for example, a cell of Pichia, a Chinese Hamster Ovary cell (CHO) or a human cell. The optimized nucleotide sequence is engineered to retain completely or as much as possible the amino acid sequence originally encoded by the starting nucleotide sequence, which is also known as the "parental" sequence. The optimized sequences herein have been engineered to have codons that are preferred in mammalian cells. However, optimized expression of these sequences in other eukaryotic cells or prokaryotic cells is also envisioned herein. The amino acid sequences encoded by optimized nucleotide sequences are also referred to as optimized.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. Unless otherwise indicated, a particular polypeptide sequence also implicitly encompasses conservatively modified variants thereof.

The term "recombinant human antibody", as used herein, includes all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for human immunoglobulin genes or a hybridoma prepared therefrom,

antibodies isolated from a host cell transformed to express the human antibody, *e.g.*, from a transfectoma, antibodies isolated from a recombinant, combinatorial human antibody library, and antibodies prepared, expressed, created or isolated by any other means that involve splicing of all or a portion of a human immunoglobulin gene, sequences to other DNA sequences. Such recombinant human antibodies have variable regions in which the framework and CDR regions are derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term “recombinant host cell” (or simply “host cell”) refers to a cell into which a recombinant expression vector has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term “host cell” as used herein.

The term “subject” includes human and non-human animals. Non-human animals include all vertebrates (e.g.: mammals and non-mammals) such as, non-human primates (e.g.: cynomolgus monkey), sheep, dog, cow, chickens, amphibians, and reptiles. Except when noted, the terms “patient” or “subject” are used herein interchangeably. As used herein, the terms “cyno” or “cynomolgus” refer to the cynomolgus monkey (*Macaca fascicularis*).

As used herein, the term “treating" or "treatment" of any disease or disorder (e.g., retinal vascular disease, diabetic retinopathy, macular edema) refers in one embodiment, to ameliorating the disease or disorder (i.e., slowing or arresting or reducing the development of the disease or at least one of the clinical symptoms thereof). In another embodiment "treating" or "treatment" refers to alleviating or ameliorating at least one physical parameter including those which may not be discernible by the patient. In yet another embodiment, "treating" or "treatment" refers to modulating the disease or disorder, either physically, (*e.g.*, stabilization of a discernible symptom), physiologically, (*e.g.*, stabilization of a physical parameter), or both. In yet another embodiment, "treating" or "treatment" refers to preventing or delaying the onset or development or

progression of the disease or disorder. "Prevention" as it relates to indications described herein, including, conditions or disorders associated with retinal vascular disease, conditions or disorders associated with diabetic retinopathy, and/or conditions or disorders associated with macular edema, means any action that prevents or slows a worsening in visual function, retinal anatomy, retinal vascular disease parameter, diabetic retinopathy disease parameter, and/or macular edema disease parameter, as described below, in a patient at risk for said worsening. More specifically, "treatment" of conditions or disorders associated with retinal vascular disease, conditions or disorders associated with diabetic retinopathy, and/or conditions or disorders associated with macular edema means any action that results in, or is contemplated to result in, the improvement or preservation of visual function and/or retinal anatomy. Methods for assessing treatment and/or prevention of disease are known in the art and described herein below.

The term "vector" is intended to refer to a polynucleotide molecule capable of transporting another polynucleotide to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, such as an adeno-associated viral vector (AAV, or AAV2), wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Shows that EPO induces vessel dilation in the central retina.

Figure 2. Shows that an anti-EPO Fab neutralizes EPO in rabbit eyes.

Figure 3. Shows that an anti-EPO Fab neutralizes EPO in rabbit eyes.

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery of antibody molecules that specifically bind to Epo. The invention relates to both full IgG format antibodies as well as antigen binding fragments thereof, such as Fab fragments (e.g., see antibodies NVS1, NVS2, NVS3 and NVS4).

Accordingly, the present invention provides antibodies that specifically bind to Epo (*e.g.*, human Epo, cynomolgus Epo, rat Epo, and mouse Epo), pharmaceutical compositions, production methods, and methods of use of such antibodies and compositions.

Epo Antibodies & Antigen Binding Fragments

The present invention provides antibodies that specifically bind to Epo. In some embodiments, the present invention provides antibodies that specifically bind to human, cynomolgus, rat and/or mouse Epo, as well as human-hyperglycosylated Epo (darbepoietin). Antibodies of the invention include, but are not limited to, the human monoclonal antibodies and Fabs, isolated as described in the Examples.

The present invention provides antibodies that specifically bind an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo), wherein the antibodies comprise a VH domain having an amino acid sequence of SEQ ID NO: 13, 33, 53 or 73. The present invention also provides antibodies that specifically bind to an Epo protein, wherein the antibodies comprise a VH CDR having an amino acid sequence of any one of the VH CDRs listed in Table 1, *infra*. In particular, the invention provides antibodies that specifically bind to an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo), wherein the antibodies comprise (or alternatively, consist of) one, two, three, or more VH CDRs having an amino acid sequence of any of the VH CDRs listed in Table 1, *infra*.

The present invention provides antibodies that specifically bind to an Epo protein, said antibodies comprising a VL domain having an amino acid sequence of SEQ ID NO:14, 34, 54 or 74. The present invention also provides antibodies that specifically bind to an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo), said antibodies comprising a VL CDR having an amino acid sequence of any one of the VL CDRs listed in Table 1, *infra*. In particular, the invention provides antibodies that specifically bind to

an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo), said antibodies comprising (or alternatively, consisting of) one, two, three or more VL CDRs having an amino acid sequence of any of the VL CDRs listed in Table 1, *infra*.

Other antibodies of the invention include amino acids that have been mutated, yet have at least 80, 85, 90, 95, 96, 97, 98, or 99 percent identity in the CDR regions with the CDR regions depicted in the sequences described in Table 1. In some embodiments, it includes mutant amino acid sequences wherein no more than 1, 2, 3, 4 or 5 amino acids have been mutated in the CDR regions when compared with the CDR regions depicted in the sequence described in Table 1.

The present invention also provides nucleic acid sequences that encode VH, VL, the full length heavy chain, and the full length light chain of the antibodies that specifically bind to an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo). Such nucleic acid sequences can be optimized for expression in mammalian cells (for example, Table 1 shows the optimized nucleic acid sequences for the heavy chain and light chain of antibodies of the invention).

Table 1 Examples of Epo Antibodies, Fabs and Epo Proteins

Amino acid sequence or polynucleotide (PN)	Sequence Identifier (SEQ.I.D.NO:)and sequence
NVS1	
CDRH1 Kabat	1 SYAIS
CDRH2 Kabat	2 GIDPISGFADYAQKFQG
CDRH3 Kabat	3 ELYYPGTWMAVMAY
CDRL1 Kabat	4 SGDNIPEYYVH
CDRL2 Kabat	5 RDNERPS
CDRL3 Kabat	6 QVFDESSWHWV
CDRH1 Chothia	7 GGTFRSY
CDRH2 Chothia	8 DPISGF
CDRH3 Chothia	9 ELYYPGTWMAVMAY
CDRL1 Chothia	10 DNIPEYY
CDRL2 Chothia	11 RDN
CDRL3 Chothia	12 FDESSWHW
VH	13 QVQLVQSGAEVKKPGSSVKVSCKASGGTFRSYAISWVRQAPGQGLEWMGGID PISGFADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARELYYPG TWMAVMAYWGRGTLVTVSS

VL	14 SYVLTQPPSVSVAPGKTARITCSGDNIPEYYVHWYQQKPGQAPVLVIYRDNE RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCQVFDESSWHWVFGGGTK LTVL
Heavy chain	15 QVQLVQSGAEVKKPGSSVKVSCKASGGTFRSYAISWVRQAPGQGLEWMGGID PISGFADYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYCARELYYPG TWMAVMAYWGRGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNV NHKPSNTKVDKRVEPKSC
Light chain	16 SYVLTQPPSVSVAPGKTARITCSGDNIPEYYVHWYQQKPGQAPVLVIYRDNE RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCQVFDESSWHWVFGGGTK LTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSP VKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTV APTECS
PN encoding SEQ.I.D.NO:13	17 caggtgcagctggtgcagtcaggcgccgaagtgaagaaacccggctctagcg tgaaggtgtcctgtaaagctagtggcggcacctttagatcctacgctattag ctgggtgcgacaggctccaggccagggcctcgaatggatgggcggcatcgac cctattagcggcttcgccgactacgctcagaaatttcagggcagagtgacta tcaccgccgacgagtctactagcaccgcctacatggaactgtctagcctgag atcagaggacaccgccgtgtactactgcgctagagagctgtactaccccggc acctggatggccgtgatggcctattggggcagaggcaccctggtgacagtgt cttct
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PN encoding SEQ.I.D.NO:15	19 caggtgcagctggtgcagtcaggcgccgaagtgaagaaacccggctctagcg tgaaggtgtcctgtaaagctagtggcggcacctttagatcctacgctattag ctgggtgcgacaggctccaggccagggcctcgaatggatgggcggcatcgac cctattagcggcttcgccgactacgctcagaaatttcagggcagagtgacta tcaccgccgacgagtctactagcaccgcctacatggaactgtctagcctgag atcagaggacaccgccgtgtactactgcgctagagagctgtactaccccggc acctggatggccgtgatggcctattggggcagaggcaccctggtgacagtgt cttctgctagcactaagggcccctccgtgttccctctggccccttccagcaa gtctacctctggcggcaccgctgctctgggctgcctggtgaaggactacttc cctgagcctgtgacagtgtcctggaactctggcgccctgacctccggcgtgc acaccttccctgccgtgctgcagtcctccggcctgtactccctgtcctccgt ggtgacagtgccttcctccagcctgggcacccagacctatatctgcaacgtg aaccacaagccttccaacaccaaggtggacaagcgggtggagcctaagtcat gc
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NVS2	
CDRH1_Kabat	21SYWIG
CDRH2_Kabat	22 WIDPYRSEIRYSPSFQG
CDRH3_Kabat	23 VSSEPFDS
CDRL1_Kabat	24 SGDKLGDHYAY
CDRL2_Kabat	25 DDSKRPS
CDRL3_Kabat	26 ATWTFEGDYV
CDRH1 Chothia	27 GYSFTSY
CDRH2 Chothia	28 DPYRSE
CDRH3 Chothia	29 VSSEPFDS
CDRL1 Chothia	30 DKLGDHY
CDRL2 Chothia	31 DDS
CDRL3 Chothia	32 WTFEGDY
VH	33 EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGWID PYRSEIRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARVSSEPF DSWGQGTLVTVSS
VL	34 SYVLTQPPSVSVAPGKTARITCSGDKLGDHYAYWYQQKPGQAPVLVIYDDSK RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCATWTFEGDYVFGGGTKL TVL
Heavy chain	35 EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGWID PYRSEIRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARVSSEPF DSWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTV SWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSN TKVDKRVEPKSC
Light chain	36 SYVLTQPPSVSVAPGKTARITCSGDKLGDHYAYWYQQKPGQAPVLVIYDDSK RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCATWTFEGDYVFGGGTKL TVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPV KAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVA PTECS
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CDRH1_Kabat	41 SNTAAWN
CDRH2_Kabat	42 VIYYRSKWYNDYAVSVKS
CDRH3_Kabat	43 SVPGGDPGLEHAFAY
CDRL1_Kabat	44 SGDNLGTYYVE
CDRL2_Kabat	45 DDSDRPS
CDRL3_Kabat	46 ASFASWSDSV
CDRH1 Chothia	47 GDSVSSNTA
CDRH2 Chothia	48 YYRSKWY
CDRH3 Chothia	49 SVPGGDPGLEHAFAY

CDRL1 Chothia	50 DNLGTYY
CDRL2 Chothia	51 DDS
CDRL3 Chothia	52 FASWSDS
VH	53 QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNTAAWNWIRQSPSRGLEWLGV IYYRSKWYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARSVP GGDPGLEHAFAYWGRGTLVTVSS
VL	54 SYVLTQPPSVSVAPGKTARITCSGDNLGTYYVEWYQQKPGQAPVLVIYDDSD RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCASFASWSDSVFGGGTKL TVL
Heavy chain	55 QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNTAAWNWIRQSPSRGLEWLGV IYYRSKWYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARSVP GGDPGLEHAFAYWGRGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY ICNVNHKPSNTKVDKRVEPKSC
Light chain	56 SYVLTQPPSVSVAPGKTARITCSGDNLGTYYVEWYQQKPGQAPVLVIYDDSD RPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCASFASWSDSVFGGGTKL TVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPV KAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVA PTECS
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NVS4	
CDRH1_Kabat	61 SYYMS
CDRH2_Kabat	62 WINPLKGNTNYAQKFQG
CDRH3_Kabat	63 EGMYFDI
CDRL1_Kabat	64 SGDSIGDKYVY
CDRL2_Kabat	65 DTNKRPS
CDRL3_Kabat	66 QSWDLDFNTYV
CDRH1 Chothia	67 GYTFTSY
CDRH2 Chothia	68 NPLKGN
CDRH3 Chothia	69 EGMYFDI
CDRL1 Chothia	70 DSIGDKY
CDRL2 Chothia	71 DTN
CDRL3 Chothia	72 WDLDFNTY
VH	73 QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYYMSWVRQAPGQGLEWMGWIN PLKGNTNYAQKFQGRVTMTRDTSISTAYMELSRLRSEDTAVYYCAREGMYFD IWGQGTLVTVSS
VL	74 SYELTQPLSVSVALGQTARITCSGDSIGDKYVYWYQQKPGQAPVLVIYDTNK RPSGIPERFSGSNSGNTATLTISRAQAGDEADYYCQSWDLDFNTYVFGGGTK LTVL
Heavy chain	75 qvqlvqsgaevkkpgasvkvsckasgytftsyymswvrqapgqglewmgwin plkgntnyaqkfqgrvtmtrdtsistaymelsrlrsedtavyycaregmyfd iwgqgtlvtvssastkgpsvfplapsskstsggtaalgclvkdyfpepvtvs wnsgaltsgvhtfpavlqssglyslssvvtvpssslgtqtyicnvnhkpsnt kvdkrvepksc
Light chain	76 SYELTQPLSVSVALGQTARITCSGDSIGDKYVYWYQQKPGQAPVLVIYDTNK RPSGIPERFSGSNSGNTATLTISRAQAGDEADYYCQSWDLDFNTYVFGGGTK LTVLGQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSP VKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTV

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Human Epo NP_000790.2	81 apprlicdsrvlerylleakeaenittgcaehcslnenitvpdtkvnfyawk rmevgqqavevwqglallseavlrgqallvnssqpweplqlhvdkavsglrs lttllralgaqkeaisppdaasaaplrtitadtfrklfrvysnflrgklkly tgeacrtgdr
Cynomolgus Epo Uniprot: P07865	82 apprlicdsrvlerylleakeaenvtmgcsescslnenitvpdtkvnfyawk rmevgqqavevwqglallseavlrgqavlanssqpfeplqlhmdkaisglrs ittllralgaqeaislpdaasaaplrtitadtfcklfrvysnflrgklklyt geacrrgdr
Mouse Epo	83

NP_031968.1	apprlicdsrvleryileakeaenvtmgcaegprlsenitvpdtkvnfyawk rmeveeqaievwqglsllseailqaqallanssqppetlqlhidkaisglrs ltsllrvlgaqkelmsppdttppaplrtltvdtfcklfrvyanflrgklkly tgevcrrgdr
Rat Epo NP_ 058697.1	84 apprlicdsrvleryileakeaenvtmgcaegprlsenitvpdtkvnfyawk rmkveeqavevwqglsllseailqaqalqanssqppeslqlhidkaisglrs ltsllrvlgaqkelmsppdatqaaplrtltadtfcklfrvysnflrgklkly tgeacrrgdr
Rabbit Epo NP_ 001075559.1	85 Klatmgvrgrlallplallcllvlalglpvlgaparlicdsrvleryileak eaenvtmgcaegcslgenitvpdtkvnfhhwkkseagrhavevwqglallse amlrsqallanssqlpetlqvhvdkavsglrsltsllralgvqkeavsppea assaaplrtvaadtlcklfriysnflrgklklytgeacrrgdr
Epo Helix A, amino acids 4-26 of SEQ ID NO: 81	86 rlicdsrvlerylleakeaenit
Epo Helix B, amino acids 56-83 of SEQ ID NO: 81	87 vgqqavevwqglallseavlrgqallvn
Epo Helix D, amino acids 138-162 of SEQ ID NO: 81	88 frklfrvysnflrgklklytgeacr
Epo Loop A-B, amino acids 27-55 of SEQ ID NO: 81	89 tgcaehcslnenitvpdtkvnfawkrme

Other antibodies of the invention include those where the amino acids or nucleic acids encoding the amino acids have been mutated, yet have at least 60, 65, 70, 75, 80, 85, 90, or 95 percent identity to the sequences described in Table 1. Some embodiments include mutant amino acid sequences wherein no more than 1, 2, 3, 4 or 5 amino acids have been mutated in the variable regions when compared with the variable regions depicted in the sequence described in Table 1, while retaining substantially the same antigen binding activity.

Since each of these antibodies can bind to Epo, the VH, VL, full length light chain, and full length heavy chain sequences (amino acid sequences and the nucleotide sequences encoding the amino acid sequences) can be "mixed and matched" to create other Epo-binding antibodies of the invention. Such "mixed and matched" Epo-binding antibodies can be tested using the binding assays known in the art (*e.g.*, ELISAs, and other assays described in the Example section). When these chains are mixed and matched, a VH sequence from a particular VH/VL pairing should be replaced with a structurally similar VH sequence. Likewise a full length heavy chain sequence from a particular full length heavy chain / full length light chain pairing should be replaced with a

structurally similar full length heavy chain sequence. Likewise, a VL sequence from a particular VH/VL pairing should be replaced with a structurally similar VL sequence. Likewise a full length light chain sequence from a particular full length heavy chain / full length light chain pairing should be replaced with a structurally similar full length light chain sequence. Accordingly, in one aspect, the invention provides an isolated antibody or antigen binding region thereof having: a heavy chain variable domain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 33, 53, and 73, and a light chain variable domain comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54 and 74 wherein the antibody specifically binds to Epo (*e.g.*, human, cynomolgus, rat and/or mouse Epo). More specifically, in certain aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain variable domain and a light chain variable domain comprising amino acid sequences selected from SEQ ID NOs: 13 and 14, respectively. In other specific aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain variable domain and a light chain variable domain comprising amino acid sequences selected from SEQ ID NOs: 33 and 34, respectively. In still other aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain variable domain and a light chain variable domain comprising amino acid sequences selected from SEQ ID NOs: 53 and 54, respectively. In still other aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain variable domain and a light chain variable domain comprising amino acid sequences selected from SEQ ID NOs: 73 and 74, respectively.

In another aspect, the invention provides (i) an isolated antibody having: a full length heavy chain comprising an amino acid sequence that has been optimized for expression in a mammalian cell selected from the group consisting of SEQ ID NOs: 15, 35, 55 and 75, and a full length light chain comprising an amino acid sequence that has been optimized for expression in a mammalian cell selected from the group consisting of SEQ ID NOs: 16, 36, 56, and 76; or (ii) a functional protein comprising an antigen binding portion thereof. More specifically, in certain aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain and a light chain comprising amino acid sequences selected from SEQ ID NOs: 15 and 16, respectively. In other specific aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain and a light chain comprising amino acid sequences selected from SEQ ID NOs: 35 and 36, respectively. In still other aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain and a light chain comprising amino acid sequences selected from SEQ ID NOs: 55 and 56,

respectively. In still other aspects, the invention provides an isolated antibody or antigen binding region thereof having a heavy chain and a light chain comprising amino acid sequences selected from SEQ ID NOs: 75 and 76, respectively.

The terms "complementarity determining region," and "CDR," as used herein refer to the sequences of amino acids within antibody variable regions which confer antigen specificity and binding affinity. In general, there are three CDRs in each heavy chain variable region (HCDR1, HCDR2, HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, LCDR3).

The precise amino acid sequence boundaries of a given CDR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD ("Kabat" numbering scheme), Al-Lazikani et al., (1997) JMB 273,927-948 ("Chothia" numbering scheme).

For example, under Kabat, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under Chothia the CDR amino acids in the VH are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the amino acid residues in VL are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3). By combining the CDR definitions of both Kabat and Chothia, the CDRs consist of amino acid residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in human VH and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in human VL.

In another aspect, the present invention provides Epo binding antibodies that comprise the heavy chain and light chain CDR1s, CDR2s, and CDR3s as described in Table 1, or combinations thereof. The amino acid sequences of the VH CDR1s of the antibodies are shown in SEQ ID NOs: 1, 21, 41 or 61. The amino acid sequences of the VH CDR2s of the antibodies and are shown in SEQ ID NOs: 2, 22, 42 or 62. The amino acid sequences of the VH CDR3s of the antibodies are shown in SEQ ID NOs: 3, 23, 43, or 63. The amino acid sequences of the VL CDR1s of the antibodies are shown in SEQ ID NOs: 4, 24, 44, or 64. The amino acid sequences of the VL CDR2s of the antibodies are shown in SEQ ID NOs: 5, 25, 45, or 65. The amino acid sequences of the VL CDR3s of the antibodies are shown in SEQ ID NOs: 6, 26, 46, or 66. These CDR regions are delineated using the Kabat system.

Alternatively, as defined using the Chothia system (Al-Lazikani et al., (1997) JMB 273,927-948) the amino acid sequences of the VH CDR1s of the antibodies are shown in SEQ ID NOs: 7, 27, 47, or 67. The amino acid sequences of the VH CDR2s of the antibodies and are shown in SEQ ID NOs: 8, 28, 48, or 68. The amino acid sequences of the VH CDR3s of the antibodies are shown in SEQ ID NOs: 9, 29, 49, or 69. The amino acid sequences of the VL CDR1s of the antibodies are shown in SEQ ID NOs: 10, 30, 50, or 70. The amino acid sequences of the VL CDR2s of the antibodies are shown in SEQ ID NOs: 11, 31, 51, or 71. The amino acid sequences of the VL CDR3s of the antibodies are shown in SEQ ID NOs: 12, 32, 52, or 72.

Given that each of these antibodies can bind to Epo and that antigen-binding specificity is provided primarily by the CDR1, 2 and 3 regions, the VH CDR1, 2 and 3 sequences and VL CDR1, 2 and 3 sequences can be "mixed and matched" (*i.e.*, CDRs from different antibodies can be mixed and matched, although each antibody preferably contains a VH CDR1, 2 and 3 and a VL CDR1, 2 and 3 to create other Epo binding molecules of the invention. Such "mixed and matched" Epo binding antibodies can be tested using the binding assays known in the art and those described in the Examples (*e.g.*, ELISAs, SET, Biacore). When VH CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular VH sequence should be replaced with a structurally similar CDR sequence(s). Likewise, when VL CDR sequences are mixed and matched, the CDR1, CDR2 and/or CDR3 sequence from a particular VL sequence should be replaced with a structurally similar CDR sequence(s). It will be readily apparent to the ordinarily skilled artisan that novel VH and VL sequences can be created by substituting one or more VH and/or VL CDR region sequences with structurally similar sequences from the CDR sequences shown herein for monoclonal antibodies of the present invention. In addition to the foregoing, in one embodiment, the antigen binding fragments of the antibodies described herein can comprise a VH CDR1, 2, and 3, or a VL CDR 1, 2, and 3, wherein the fragment binds to Epo as a single variable domain.

In certain embodiments of the invention, the antibodies or antigen binding fragments thereof may have the heavy and light chain sequences of the Fabs described in Table 1. More specifically, the antibody or antigen binding fragment thereof may have the heavy and light sequence of Fab NVS1, NVS2, NVS3 or NVS4.

In other embodiments of the invention the antibody or antigen binding fragment that specifically binds Epo comprises a heavy chain variable region CDR1, a heavy chain variable region CDR2, a heavy chain variable region CDR3, a light chain variable region

CDR1, a light chain variable region CDR2, and a light chain variable region CDR3 as defined by Kabat and described in Table 1. In still other embodiments of the invention the antibody or antigen binding fragment in that specifically binds Epo comprises a heavy chain variable region CDR1, a heavy chain variable region CDR2, a heavy chain variable region CDR3, a light chain variable region CDR1, a light chain variable region CDR2, and a light chain variable region CDR3 as defined by Chothia and described in Table 1.

In a specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO:1; a heavy chain variable region CDR2 of SEQ ID NO: 2; a heavy chain variable region CDR3 of SEQ ID NO: 3; a light chain variable region CDR1 of SEQ ID NO: 4; a light chain variable region CDR2 of SEQ ID NO: 5; and a light chain variable region CDR3 of SEQ ID NO: 6. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 21; a heavy chain variable region CDR2 of SEQ ID NO: 22; a heavy chain variable region CDR3 of SEQ ID NO: 23; a light chain variable region CDR1 of SEQ ID NO: 24; a light chain variable region CDR2 of SEQ ID NO: 25; and a light chain variable region CDR3 of SEQ ID NO: 26. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 41; a heavy chain variable region CDR2 of SEQ ID NO: 42; a heavy chain variable region CDR3 of SEQ ID NO: 43; a light chain variable region CDR1 of SEQ ID NO: 44; a light chain variable region CDR2 of SEQ ID NO: 45; and a light chain variable region CDR3 of SEQ ID NO: 46. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 61; a heavy chain variable region CDR2 of SEQ ID NO: 62; a heavy chain variable region CDR3 of SEQ ID NO: 63; a light chain variable region CDR1 of SEQ ID NO: 64; a light chain variable region CDR2 of SEQ ID NO: 65; and a light chain variable region CDR3 of SEQ ID NO: 66.

In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 7; a heavy chain variable region CDR2 of SEQ ID NO: 8; a heavy chain variable region CDR3 of SEQ ID NO: 9; a light chain variable region CDR1 of SEQ ID NO: 10; a light chain variable region CDR2 of SEQ ID NO: 11; and a light chain variable region CDR3 of SEQ ID NO: 12. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 27; a heavy chain variable region CDR2 of SEQ ID NO: 28; a heavy chain variable region CDR3 of SEQ ID NO: 29; a light chain variable region CDR1 of SEQ ID NO: 30; a

light chain variable region CDR2 of SEQ ID NO: 31; and a light chain variable region CDR3 of SEQ ID NO: 32. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 47; a heavy chain variable region CDR2 of SEQ ID NO: 48; a heavy chain variable region CDR3 of SEQ ID NO: 49; a light chain variable region CDR1 of SEQ ID NO: 50; a light chain variable region CDR2 of SEQ ID NO: 51; and a light chain variable region CDR3 of SEQ ID NO: 52. In another specific embodiment, the invention includes an antibody that specifically binds to Epo comprising a heavy chain variable region CDR1 of SEQ ID NO: 67; a heavy chain variable region CDR2 of SEQ ID NO: 68; a heavy chain variable region CDR3 of SEQ ID NO: 69; a light chain variable region CDR1 of SEQ ID NO: 70; a light chain variable region CDR2 of SEQ ID NO: 71; and a light chain variable region CDR3 of SEQ ID NO: 72.

In certain embodiments, the invention includes antibodies or antigen binding fragments that specifically bind to Epo as described in Table 1. In a preferred embodiment, the antibody, or antigen binding fragment, that binds Epo is Fab NVS1, NVS2, NVS3, or NVS4.

As used herein, a human antibody comprises heavy or light chain variable regions or full length heavy or light chains that are "the product of" or "derived from" a particular germline sequence if the variable regions or full length chains of the antibody are obtained from a system that uses human germline immunoglobulin genes. Such systems include immunizing a transgenic mouse carrying human immunoglobulin genes with the antigen of interest or screening a human immunoglobulin gene library displayed on phage with the antigen of interest. A human antibody that is "the product of" or "derived from" a human germline immunoglobulin sequence can be identified as such by comparing the amino acid sequence of the human antibody to the amino acid sequences of human germline immunoglobulins and selecting the human germline immunoglobulin sequence that is closest in sequence (*i.e.*, greatest % identity) to the sequence of the human antibody. A human antibody that is "the product of" or "derived from" a particular human germline immunoglobulin sequence may contain amino acid differences as compared to the germline sequence, due to, for example, naturally occurring somatic mutations or intentional introduction of site-directed mutations. However, in the VH or VL framework regions, a selected human antibody typically is at least 90% identical in amino acids sequence to an amino acid sequence encoded by a human germline immunoglobulin gene and contains amino acid residues that identify the human antibody as being human when compared to the germline immunoglobulin amino acid sequences of other species (*e.g.*, murine germline sequences). In certain cases, a human antibody

may be at least 60%, 70%, 80%, 90%, or at least 95%, or even at least 96%, 97%, 98%, or 99% identical in amino acid sequence to the amino acid sequence encoded by the germline immunoglobulin gene. Typically, a recombinant human antibody will display no more than 10 amino acid differences from the amino acid sequence encoded by the human germline immunoglobulin gene in the VH or VL framework regions. In certain cases, the human antibody may display no more than 5, or even no more than 4, 3, 2, or 1 amino acid difference from the amino acid sequence encoded by the germline immunoglobulin gene. Examples of human germline immunoglobulin genes include, but are not limited to the variable domain germline fragments described below, as well as DP47 and DPK9.

Homologous antibodies

In yet another embodiment, the present invention provides an antibody, or an antigen binding fragment thereof, comprising amino acid sequences that are homologous to the sequences described in Table 1, and the antibody binds to an Epo protein (*e.g.*, human, cynomolgus, rat and/or mouse Epo), and retains the desired functional properties of those antibodies described in Table 1.

For example, the invention provides an isolated antibody, or a functional antigen binding fragment thereof, comprising a heavy chain variable domain and a light chain variable domain, wherein the heavy chain variable domain comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 33, 53, and 73; the light chain variable domain comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54, and 74; and the antibody specifically binds to Epo (*e.g.*, human, cynomolgus, rat and/or mouse Epo). In certain aspects of the invention the heavy and light chain sequences further comprise HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 sequences as defined by Kabat, for example SEQ ID NOs: 1, 2, 3, 4, 5, and 6, respectively; SEQ ID NOs: 21, 22, 23, 24, 25, and 26, respectively; SEQ ID NOs: 41, 42, 43, 44, 45, and 46, respectively; or SEQ ID NOs: 61, 62, 63, 64, 65, and 66, respectively. In certain other aspects of the invention the heavy and light chain sequences further comprise HCDR1, HCDR2, HCDR3, LCDR1, LCDR2, and LCDR3 sequences as defined by Chothia, for example SEQ ID NOs: 7, 8, 9, 10, 11, and 12, respectively; SEQ ID NOs: 27, 28, 29, 30, 31, and 32, respectively; SEQ ID NOs: 47, 48, 49, 50, 51, and 52, respectively; or SEQ ID NOs: 67, 68, 69, 70, 71, and 72, respectively.

In other embodiments, the VH and/or VL amino acid sequences may be 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences set forth in Table 1. In other embodiments, the VH and/or VL amino acid sequences may be identical except for an amino acid substitution in no more than 1,2,3,4 or 5 amino acid positions. An antibody having VH and VL regions having high (*i. e.*, 80% or greater) identity to the VH and VL regions of those described in Table 1 can be obtained by mutagenesis (*e.g.*, site-directed or PCR-mediated mutagenesis) of nucleic acid molecules encoding SEQ ID NOs: 13, 33, 53 or 73 and SEQ ID NOs: 14, 34, 54, or 74, respectively, followed by testing of the encoded altered antibody for retained function using the functional assays described herein.

In other embodiments, the full length heavy chain and/or full length light chain amino acid sequences may be 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences set forth in Table 1. An antibody having a full length heavy chain and full length light chain having high (*i.e.*, 80% or greater) identity to the full length heavy chains of any of SEQ ID NOs : 15, 35, 55, or 75, and full length light chains of any of SEQ ID NOs: 16, 36, 56, or 76, can be obtained by mutagenesis (*e.g.*, site-directed or PCR-mediated mutagenesis) of nucleic acid molecules encoding such polypeptides, followed by testing of the encoded altered antibody for retained function using the functional assays described herein.

In other embodiments, the full length heavy chain and/or full length light chain nucleotide sequences may be 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences set forth in Table 1.

In other embodiments, the variable regions of heavy chain and/or the variable regions of light chain nucleotide sequences may be 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences set forth in Table 1.

As used herein, the percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity equals number of identical positions/total number of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

Additionally or alternatively, the protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to,

for example, identify related sequences. For example, such searches can be performed using the BLAST program (version 2.0) of Altschul, *et al.*, 1990 J.Mol. Biol. 215:403-10.

Antibodies with Conservative Modifications

In certain embodiments, an antibody of the invention has a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein one or more of these CDR sequences have specified amino acid sequences based on the antibodies described herein or conservative modifications thereof, and wherein the antibodies retain the desired functional properties of the Epo-binding antibodies of the invention. Accordingly, the invention provides an isolated antibody, or an antigen binding fragment thereof, comprising of a heavy chain variable region comprising CDR1, CDR2, and CDR3 sequences and a light chain variable region comprising CDR1, CDR2, and CDR3 sequences, wherein: the heavy chain variable region CDR1 amino acid sequences are selected from the group consisting of SEQ ID NOs: 1, 21, 41, and 61, and conservative modifications thereof; the heavy chain variable region CDR2 amino acid sequences are selected from the group consisting of SEQ ID NOs: 2, 22, 42 and 62, and conservative modifications thereof; the heavy chain variable region CDR3 amino acid sequences are selected from the group consisting of SEQ ID NOs: 3, 23, 43, and 63, and conservative modifications thereof; the light chain variable regions CDR1 amino acid sequences are selected from the group consisting of SEQ ID NOs: 4, 24, 44 and 64, and conservative modifications thereof; the light chain variable regions CDR2 amino acid sequences are selected from the group consisting of SEQ ID NOs: 5, 25, 45 and 65, and conservative modifications thereof; the light chain variable regions of CDR3 amino acid sequences are selected from the group consisting of SEQ ID NOs: 6, 26, 46, and 66, and conservative modifications thereof; and the antibody or antigen binding fragment thereof specifically binds to Epo.

In other embodiments, the antibody of the invention is optimized for expression in a mammalian cell and has a full length heavy chain sequence and a full length light chain sequence, wherein one or more of these sequences have specified amino acid sequences based on the antibodies described herein or conservative modifications thereof, and wherein the antibodies retain the desired functional properties of the Epo binding antibodies of the invention. Accordingly, the invention provides an isolated antibody optimized for expression in a mammalian cell consisting of a full length heavy chain and a full length light chain wherein the full length heavy chain has amino acid sequences selected from the group of SEQ ID NOs: 15, 35, 55 and 75, and conservative

modifications thereof; and the full length light chain has amino acid sequences selected from the group of SEQ ID NOs: 16, 36, 56, and 76, and conservative modifications thereof; and the antibody specifically binds to Epo (*e.g.*, human, cynomolgus, rat and/or mouse Epo).

Antibodies That Bind to the Same Epitope

The present invention provides antibodies that bind to the same epitope as the Epo binding antibodies described in Table 1. Additional antibodies can therefore be identified based on their ability to compete (*e.g.*, to competitively inhibit the binding of, in a statistically significant manner) with other antibodies of the invention in Epo binding assays (such as those described in the Examples). The ability of a test antibody to inhibit the binding of antibodies of the present invention to an Epo protein demonstrates that the test antibody can compete with that antibody for binding to Epo; such an antibody may, according to non-limiting theory, bind to the same or a related (*e.g.*, a structurally similar or spatially proximal) epitope on the Epo protein as the antibody with which it competes. In a certain embodiment, the antibody that binds to the same epitope on Epo as the antibodies of the present invention is a human monoclonal antibody. Such human monoclonal antibodies can be prepared and isolated as described herein. As used herein, an antibody "competes" for binding when the competing antibody inhibits Epo binding of an antibody or antigen binding fragment of the invention by more than 50%, in the presence of an equimolar concentration of competing antibody.

In other embodiments the antibodies or antigen binding fragments of the invention bind the Helix D domain of Epo (amino acids 138-162 of the Epo protein; SEQ ID NO: 88). In other embodiments the antibodies or antigen binding fragments of the invention bind the Helix A (amino acids 4-26 of the Epo protein; SEQ ID NO: 86) and Loop A-B of Epo (amino acids 27-55 of the Epo protein; SEQ ID NO: 89).

In other embodiments the antibodies, or antigen binding fragments of the invention bind to the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88). In other embodiments, the isolated antibodies, or antigen binding fragments bind the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89). In other embodiments the isolated antibodies, or antigen binding fragments bind the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89) and Helix A (amino acids 4-26 of Human Epo; SEQ ID NO: 86). In still other embodiments the isolated antibodies, or antigen binding fragments bind the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88), and the Loop A-B domain (amino acids 27-55 of Human

Epo; SEQ ID NO: 89). In other embodiments the isolated antibodies, or antigen binding fragments bind the D Helix domain of Epo (amino acids 138-162 of Human Epo; SEQ ID NO: 88), the Loop A-B domain (amino acids 27-55 of Human Epo; SEQ ID NO: 89) and Helix A (amino acids 4-26 of Human Epo; SEQ ID NO: 86).

In other aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids at positions, 44-50, 52, 53, 147, 150, 151, 154, 155, 159, and 162 of Human Epo (SEQ ID NO.81). In other aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids at positions 9, 13, 44-53, 147, 150, 151, 154, 155, 158, 159, and 162 of Human Epo (SEQ ID NO.81). In other aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids at positions 23, 43-50, 52, 53, 131, 143, 147, 150, 151, 154, 155, 159, and 162 of Human Epo (SEQ ID NO.81). In particular aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids Thr-Lys-Val-Asn-Phe-Tyr-Ala (at positions 44-50), Lys-Arg (at positions 52-53), Asn (at position 147), Arg-Gly (at positions 150-151), Lys-Leu (at positions 154-155), Glu (at position 159), and Arg (at position 162) of Human Epo (SEQ ID NO.81). In other particular aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids Ser (at position 9), Glu (at position 13), Thr-Lys-Val-Asn-Phe-Tyr-Ala (at positions 44-50), Lys-Arg (at positions 52-53), Asn (at position 147), Arg-Gly (at positions 150-151), Lys-Leu (at positions 154-155), Gly (at position 158), Glu (at position 159), and Arg (at position 162) of Human Epo (SEQ ID NO.81). In still further aspects of the invention the isolated antibodies or antigen binding fragments bind an epitope comprising amino acids Glu (at positions 23), Asp-Thr-Lys-Val-Asn-Phe-Tyr-Ala (at positions 43-50), Lys-Arg (at positions 52-53), Arg (at position 131), Arg (at position 143), Asn (at position 147), Arg-Gly (at positions 150-151), Lys-Leu (at positions 154-155), Glu (at position 159), and Arg (at position 162) of Human Epo (SEQ ID NO.81).

The invention also includes a conformational epitope on human Epo, the epitope comprising amino acid residues Thr44, Lys45, Val46, Asn47, Phe48, Tyr49, Ala50, Lys52, Arg53, Asn147, Arg150, Gly151, Lys154, Leu155, Glu159, and Arg162, wherein an antibody binding to the epitope will inhibit Epo binding to the Epo receptor. It is also contemplated that an antibody binding to the epitope of the invention will further inhibit Epo-dependent cell proliferation.

The invention further includes a conformational epitope on human Epo, the epitope comprising amino acid residues Ser9, Glu13, Thr44, Lys45, Val46, Asn47,

Phe48, Tyr49, Ala50, Lys52, Arg53, Asn147, Arg150, Gly151, Lys154, Leu155, Gly158, Glu159, and Arg162, wherein an antibody binding to the epitope will inhibit Epo binding to the Epo receptor. It is also contemplated that an antibody binding to the epitope of the invention will further inhibit Epo-dependent cell proliferation.

The present invention still further includes a conformational epitope on human Epo, the epitope comprising amino acid residues Glu23, Asp43, Thr44, Lys45, Val46, Asn47, Phe48, Tyr49, Ala50, Lys52, Arg53, Arg131, Arg143, Asn147, Arg150, Gly151, Lys154, Leu155, Glu159, and Arg162, wherein an antibody binding to the epitope will inhibit Epo binding to the Epo receptor. It is also contemplated that an antibody binding to the epitope of the invention will further inhibit Epo-dependent cell proliferation.

Engineered and Modified Antibodies

An antibody of the invention further can be prepared using an antibody having one or more of the VH and/or VL sequences shown herein as starting material to engineer a modified antibody, which modified antibody may have altered properties from the starting antibody. An antibody can be engineered by modifying one or more residues within one or both variable regions (*i. e.*, VH and/or VL), for example within one or more CDR regions and/or within one or more framework regions. Additionally or alternatively, an antibody can be engineered by modifying residues within the constant region(s), for example to alter the effector function(s) of the antibody.

One type of variable region engineering that can be performed is CDR grafting. Antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.*, 1998 Nature 332:323-327; Jones, P. *et al.*, 1986 Nature 321:522-525; Queen, C. *et al.*, 1989 Proc. Natl. Acad., U.S.A. 86:10029-10033; U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*)

Accordingly, another embodiment of the invention pertains to an isolated antibody, or an antigen binding fragment thereof, comprising a heavy chain variable

region comprising CDR1 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 1, 21, 41, and 61; CDR2 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 22, 42, and 62; CDR3 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 23, 43 and 63, respectively; and a light chain variable region having CDR1 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 24, 44 and 64; CDR2 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 25, 45, and 65; and CDR3 sequences consisting of an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 26, 46, and 66, respectively. Thus, such antibodies contain the VH and VL CDR sequences of monoclonal antibodies, yet may contain different framework sequences from these antibodies.

Alternatively, another embodiment of the invention pertains to an isolated antibody, or an antigen binding fragment thereof, comprising a heavy chain variable region comprising CDR1 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 7, 27, 47, and 67; CDR2 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 28, 48, and 68; CDR3 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 9, 29, 49, and 69, respectively; and a light chain variable region having CDR1 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 30, 50, and 70; CDR2 sequences having an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 31, 51, and 71; and CDR3 sequences consisting of an amino acid sequence selected from the group consisting of SEQ ID NOs: 12, 32, 52, and 72, respectively. Thus, such antibodies contain the VH and VL CDR sequences of monoclonal antibodies, yet may contain different framework sequences from these antibodies.

Such framework sequences can be obtained from public DNA databases or published references that include germline antibody gene sequences. For example, germline DNA sequences for human heavy and light chain variable region genes can be found in the "VBase" human germline sequence database (available on the world wide web at mrc- cpe.cam.ac.uk/vbase), as well as in Kabat, E. A., *et al.*, 1991 Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I. M., *et al.*, 1992 J. Mol. Biol. 227:776-798; and Cox, J. P. L. *et al.*, 1994 Eur. J Immunol. 24:827-836; the contents of each of which are expressly incorporated herein by reference.

An example of framework sequences for use in the antibodies of the invention are those that are structurally similar to the framework sequences used by selected antibodies of the invention, *e.g.*, consensus sequences and/or framework sequences used by monoclonal antibodies of the invention. The VH CDR1, 2 and 3 sequences, and the VL CDR1, 2 and 3 sequences, can be grafted onto framework regions that have the identical sequence as that found in the germline immunoglobulin gene from which the framework sequence derive, or the CDR sequences can be grafted onto framework regions that contain one or more mutations as compared to the germline sequences. For example, it has been found that in certain instances it is beneficial to mutate residues within the framework regions to maintain or enhance the antigen binding ability of the antibody (see *e.g.*, U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen et al). Frameworks that can be utilized as scaffolds on which to build the antibodies and antigen binding fragments described herein include, but are not limited to VH1A, VH1B, VH3, Vk1, Vl2, and Vk2. Additional frameworks are known in the art and may be found, for example, in the vBase data base on the world wide web at vbase.mrc-cpe.cam.ac.uk/index.php?&MMN_position=1:1.

Accordingly, an embodiment of the invention relates to isolated Epo binding antibodies, or antigen binding fragments thereof, comprising a heavy chain variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 13, 33, 53, and 73, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions in the framework region of such sequences, and further comprising a light chain variable region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 14, 34, 54, and 74, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions in the framework region of such sequences.

Another type of variable region modification is to mutate amino acid residues within the VH and/or VL CDR1, CDR2 and/or CDR3 regions to thereby improve one or more binding properties (*e.g.*, affinity) of the antibody of interest, known as "affinity maturation." Site-directed mutagenesis or PCR-mediated mutagenesis can be performed to introduce the mutation(s) and the effect on antibody binding, or other functional property of interest, can be evaluated in *in vitro* or *in vivo* assays as described herein and provided in the Examples. Conservative modifications (as discussed above) can be introduced. The mutations may be amino acid substitutions, additions or deletions. Moreover, typically no more than one, two, three, four or five residues within a CDR region are altered.

Accordingly, in another embodiment, the invention provides isolated Epo-binding antibodies, or antigen binding fragments thereof, consisting of a heavy chain variable region having a VH CDR1 region consisting of an amino acid sequence selected from the group having SEQ ID NOs: 1, 21, 41, and 61 or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 1, 21, 41, or 61; a VH CDR2 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 2, 22, 42, and 62 or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 2, 22, 42, or 62; a VH CDR3 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 3, 23, 43, and 63, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 3, 23, 43, or 63; a VL CDR1 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 4, 24, 44, and 64, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 4, 24, 44, or 64; a VL CDR2 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 25, 45, and 65, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 5, 25, 45, or 65; and a VL CDR3 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 6, 26, 46, and 66, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 6, 26, 46, or 66.

Accordingly, in another embodiment, the invention provides isolated Epo-binding antibodies, or antigen binding fragments thereof, consisting of a heavy chain variable region having a VH CDR1 region consisting of an amino acid sequence selected from the group having SEQ ID NOs: 7, 27, 47, and 67 or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs:7, 27, 47, or 67; a VH CDR2 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 28, 48, and 68 or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 8, 28, 48, or 68; a VH CDR3 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 9, 29, 49, and 69, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 9, 29, 49, or 69; a VL CDR1 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 10, 30, 50, and 70, or an amino acid sequence having one, two, three, four or

five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 10, 30 50, or 70; a VL CDR2 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 11, 31, 51, and 71, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 11, 31, 51, or 71; and a VL CDR3 region having an amino acid sequence selected from the group consisting of SEQ ID NOs: 12, 32, 52, and 72, or an amino acid sequence having one, two, three, four or five amino acid substitutions, deletions or additions as compared to SEQ ID NOs: 12, 32, 52, or 72.

Grafting Antigen-binding Domains Into Alternative Frameworks or Scaffolds

A wide variety of antibody/ immunoglobulin frameworks or scaffolds can be employed so long as the resulting polypeptide includes at least one binding region which specifically binds to Epo. Such frameworks or scaffolds include the 5 main idiotypes of human immunoglobulins, or fragments thereof, and include immunoglobulins of other animal species, preferably having humanized aspects. Single heavy-chain antibodies such as those identified in camelids are of particular interest in this regard. Novel frameworks, scaffolds and fragments continue to be discovered and developed by those skilled in the art.

In one aspect, the invention pertains to generating non-immunoglobulin based antibodies using non-immunoglobulin scaffolds onto which CDRs of the invention can be grafted. Known or future non-immunoglobulin frameworks and scaffolds may be employed, as long as they comprise a binding region specific for the target Epo protein. Known non-immunoglobulin frameworks or scaffolds include, but are not limited to, fibronectin (Compound Therapeutics, Inc., Waltham, MA), ankyrin (Molecular Partners AG, Zurich, Switzerland), domain antibodies (Domantis, Ltd., Cambridge, MA, and Ablynx nv, Zwijnaarde, Belgium), lipocalin (Pieris Proteolab AG, Freising, Germany), small modular immuno-pharmaceuticals (Trubion Pharmaceuticals Inc., Seattle, WA), maxybodies (Avidia, Inc., Mountain View, CA), Protein A (Affibody AG, Sweden), and affilin (gamma-crystallin or ubiquitin) (Scil Proteins GmbH, Halle, Germany).

The fibronectin scaffolds are based on fibronectin type III domain (*e.g.*, the tenth module of the fibronectin type III (10 Fn3 domain)). The fibronectin type III domain has 7 or 8 beta strands which are distributed between two beta sheets, which themselves pack against each other to form the core of the protein, and further containing loops (analogous to CDRs) which connect the beta strands to each other and are solvent exposed. There are at least three such loops at each edge of the beta sheet sandwich,

where the edge is the boundary of the protein perpendicular to the direction of the beta strands (see US 6,818,418). These fibronectin-based scaffolds are not an immunoglobulin, although the overall fold is closely related to that of the smallest functional antibody fragment, the variable region of the heavy chain, which comprises the entire antigen recognition unit in camel and llama IgG. Because of this structure, the non-immunoglobulin antibody mimics antigen binding properties that are similar in nature and affinity to those of antibodies. These scaffolds can be used in a loop randomization and shuffling strategy *in vitro* that is similar to the process of affinity maturation of antibodies *in vivo*. These fibronectin-based molecules can be used as scaffolds where the loop regions of the molecule can be replaced with CDRs of the invention using standard cloning techniques.

The ankyrin technology is based on using proteins with ankyrin derived repeat modules as scaffolds for bearing variable regions which can be used for binding to different targets. The ankyrin repeat module is a 33 amino acid polypeptide consisting of two anti-parallel α-helices and a β-turn. Binding of the variable regions is mostly optimized by using ribosome display.

Avimers are derived from natural A-domain containing protein such as LRP-1. These domains are used by nature for protein-protein interactions and in human over 250 proteins are structurally based on A-domains. Avimers consist of a number of different "A-domain" monomers (2-10) linked via amino acid linkers. Avimers can be created that can bind to the target antigen using the methodology described in, for example, U.S. Patent Application Publication Nos. 20040175756; 20050053973; 20050048512; and 20060008844.

Affibody affinity ligands are small, simple proteins composed of a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A. Protein A is a surface protein from the bacterium *Staphylococcus aureus*. This scaffold domain consists of 58 amino acids, 13 of which are randomized to generate affibody libraries with a large number of ligand variants (See *e.g.*, US 5,831,012). Affibody molecules mimic antibodies, they have a molecular weight of 6 kDa, compared to the molecular weight of antibodies, which is 150 kDa. In spite of its small size, the binding site of affibody molecules is similar to that of an antibody.

Anticalins are products developed by the company Pieris ProteoLab AG. They are derived from lipocalins, a widespread group of small and robust proteins that are usually involved in the physiological transport or storage of chemically sensitive or

insoluble compounds. Several natural lipocalins occur in human tissues or body liquids. The protein architecture is reminiscent of immunoglobulins, with hypervariable loops on top of a rigid framework. However, in contrast with antibodies or their recombinant fragments, lipocalins are composed of a single polypeptide chain with 160 to 180 amino acid residues, being just marginally bigger than a single immunoglobulin domain. The set of four loops, which makes up the binding pocket, shows pronounced structural plasticity and tolerates a variety of side chains. The binding site can thus be reshaped in a proprietary process in order to recognize prescribed target molecules of different shape with high affinity and specificity. One protein of lipocalin family, the bilin-binding protein (BBP) of *Pieris brassicae* has been used to develop anticalins by mutagenizing the set of four loops. One example of a patent application describing anticalins is in PCT Publication No. WO 1999/16873.

Affilin molecules are small non-immunoglobulin proteins which are designed for specific affinities towards proteins and small molecules. New affilin molecules can be very quickly selected from two libraries, each of which is based on a different human derived scaffold protein. Affilin molecules do not show any structural homology to immunoglobulin proteins. Currently, two affilin scaffolds are employed, one of which is gamma crystalline, a human structural eye lens protein and the other is "ubiquitin" superfamily proteins. Both human scaffolds are very small, show high temperature stability and are almost resistant to pH changes and denaturing agents. This high stability is mainly due to the expanded beta sheet structure of the proteins. Examples of gamma crystalline derived proteins are described in WO 2001/04144 and examples of "ubiquitin-like" proteins are described in WO 2004/106368.

Protein epitope mimetics (PEM) are medium-sized, cyclic, peptide-like molecules (MW 1-2kDa) mimicking beta-hairpin secondary structures of proteins, the major secondary structure involved in protein-protein interactions.

The present invention provides fully human antibodies that specifically bind to an Epo protein. Compared to the chimeric or humanized antibodies, the human Epo-binding antibodies of the invention have further reduced antigenicity when administered to human subjects.

Camelid antibodies

Antibody proteins obtained from members of the camel and dromedary (*Camelus bactrianus* and *Camelus dromaderius*) family including new world members such as llama species (*Lama pacos*, *Lama glama* and *Lama vicugna*) have been characterized

with respect to size, structural complexity and antigenicity for human subjects. Certain IgG antibodies from this family of mammals as found in nature lack light chains, and are thus structurally distinct from the typical four chain quaternary structure having two heavy and two light chains, for antibodies from other animals. See PCT/EP93/02214 (WO 94/04678 published 3 March 1994).

A region of the camelid antibody which is the small single variable domain identified as VHH can be obtained by genetic engineering to yield a small protein having high affinity for a target, resulting in a low molecular weight antibody-derived protein known as a "camelid nanobody". See U.S. patent number 5,759,808 issued June 2, 1998; see also Stijlemans, B. *et al.*, 2004 J Biol Chem 279: 1256-1261; Dumoulin, M. *et al.*, 2003 Nature 424: 783-788; Pleschberger, M. *et al.* 2003 Bioconjugate Chem 14: 440-448; Cortez-Retamozo, V. *et al.* 2002 Int J Cancer 89: 456-62; and Lauwereys, M. *et al.* 1998 EMBO J 17: 3512-3520. Engineered libraries of camelid antibodies and antibody fragments are commercially available, for example, from Ablynx, Ghent, Belgium. As with other antibodies of non-human origin, an amino acid sequence of a camelid antibody can be altered recombinantly to obtain a sequence that more closely resembles a human sequence, *i.e.*, the nanobody can be "humanized". Thus the natural low antigenicity of camelid antibodies to humans can be further reduced.

The camelid nanobody has a molecular weight approximately one-tenth that of a human IgG molecule, and the protein has a physical diameter of only a few nanometers. One consequence of the small size is the ability of camelid nanobodies to bind to antigenic sites that are functionally invisible to larger antibody proteins, *i.e.*, camelid nanobodies are useful as reagents detect antigens that are otherwise cryptic using classical immunological techniques, and as possible therapeutic agents. Thus yet another consequence of small size is that a camelid nanobody can inhibit as a result of binding to a specific site in a groove or narrow cleft of a target protein, and hence can serve in a capacity that more closely resembles the function of a classical low molecular weight drug than that of a classical antibody.

The low molecular weight and compact size further result in camelid nanobodies being extremely thermostable, stable to extreme pH and to proteolytic digestion, and poorly antigenic. Another consequence is that camelid nanobodies readily move from the circulatory system into tissues, and even cross the blood-brain barrier and can treat disorders that affect nervous tissue. Nanobodies can further facilitated drug transport across the blood brain barrier. See U.S. patent application 20040161738 published August 19, 2004. These features combined with the low antigenicity to humans indicate

great therapeutic potential. Further, these molecules can be fully expressed in prokaryotic cells such as *E. coli* and are expressed as fusion proteins with bacteriophage and are functional.

Accordingly, a feature of the present invention is a camelid antibody or nanobody having high affinity for Epo. In certain embodiments herein, the camelid antibody or nanobody is naturally produced in the camelid animal, *i.e.*, is produced by the camelid following immunization with Epo or a peptide fragment thereof, using techniques described herein for other antibodies. Alternatively, the Epo-binding camelid nanobody is engineered, *i.e.*, produced by selection for example from a library of phage displaying appropriately mutagenized camelid nanobody proteins using panning procedures with Epo as a target as described in the examples herein. Engineered nanobodies can further be customized by genetic engineering to have a half-life in a recipient subject of from 45 minutes to two weeks. In a specific embodiment, the camelid antibody or nanobody is obtained by grafting the CDRs sequences of the heavy or light chain of the human antibodies of the invention into nanobody or single domain antibody framework sequences, as described for example in WO 1994/004678.

Bispecific Molecules and Multivalent Antibodies

In another aspect, the present invention features bispecific or multispecific molecules comprising an Epo-binding antibody, or a fragment thereof, of the invention. An antibody of the invention, or antigen-binding regions thereof, can be derivatized or linked to another functional molecule, *e.g.*, another peptide or protein (*e.g.*, another antibody or ligand for a receptor) to generate a bispecific molecule that binds to at least two different binding sites or target molecules. The antibody of the invention may in fact be derivatized or linked to more than one other functional molecule to generate multi-specific molecules that bind to more than two different binding sites and/or target molecules; such multi-specific molecules are also intended to be encompassed by the term "bispecific molecule" as used herein. To create a bispecific molecule of the invention, an antibody of the invention can be functionally linked (*e.g.*, by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other binding molecules, such as another antibody, antibody fragment, peptide or binding mimetic, such that a bispecific molecule results.

Accordingly, the present invention includes bispecific molecules comprising at least one first binding specificity for Epo and a second binding specificity for a second

target epitope. For example, the second target epitope is another epitope of Epo different from the first target epitope.

Additionally, for the invention in which the bispecific molecule is multi-specific, the molecule can further include a third binding specificity, in addition to the first and second target epitope.

In one embodiment, the bispecific molecules of the invention comprise as a binding specificity at least one antibody, or an antibody fragment thereof, including, *e.g.*, a Fab, Fab', F(ab')2, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner *et al.* U.S. Patent No. 4,946,778.

Diabodies are bivalent, bispecific molecules in which VH and VL domains are expressed on a single polypeptide chain, connected by a linker that is too short to allow for pairing between the two domains on the same chain. The VH and VL domains pair with complementary domains of another chain, thereby creating two antigen binding sites (see e.g., Holliger *et al.*, 1993 Proc. Natl. Acad. Sci. USA 90:6444-6448; Poljak *et al.*, 1994 Structure 2:1121-1123). Diabodies can be produced by expressing two polypeptide chains with either the structure VHA-VLB and VHB-VLA (VH-VL configuration), or VLA-VHB and VLB-VHA (VL-VH configuration) within the same cell. Most of them can be expressed in soluble form in bacteria. Single chain diabodies (scDb) are produced by connecting the two diabody-forming polypeptide chains with linker of approximately 15 amino acid residues (see Holliger and Winter, 1997 Cancer Immunol. Immunother., 45(3-4):128-30; Wu *et al.*, 1996 Immunotechnology, 2(1):21-36). scDb can be expressed in bacteria in soluble, active monomeric form (see Holliger and Winter, 1997 Cancer Immunol. Immunother., 45(34): 128-30; Wu *et al.*, 1996 Immunotechnology, 2(1):21-36; Pluckthun and Pack, 1997 Immunotechnology, 3(2): 83-105; Ridgway *et al.*, 1996 Protein Eng., 9(7):617-21). A diabody can be fused to Fc to generate a “di-diabody” (see Lu *et al.*, 2004 J. Biol. Chem., 279(4):2856-65).

Other antibodies which can be employed in the bispecific molecules of the invention are murine, chimeric and humanized monoclonal antibodies.

Bispecific molecules can be prepared by conjugating the constituent binding specificities, using methods known in the art. For example, each binding specificity of the bispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents

include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), o-phenylenedimaleimide (oPDM), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohaxane-l-carboxylate (sulfo-SMCC) (see *e.g.*, Karpovsky *et al.*, 1984 J. Exp. Med. 160:1686; Liu, MA *et al.*, 1985 Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described in Paulus, 1985 Behring Ins. Mitt. No. 78,118-132; Brennan *et al.*, 1985 Science 229:81-83), and Glennie *et al.*, 1987 J. Immunol. 139: 2367-2375). Conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies, they can be conjugated by sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, for example one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific molecule is a mAb x mAb, mAb x Fab, Fab x F(ab')2 or ligand x Fab fusion protein. A bispecific molecule of the invention can be a single chain molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific molecules may comprise at least two single chain molecules. Methods for preparing bispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the bispecific molecules to their specific targets can be confirmed by, for example, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (REA), FACS analysis, bioassay (*e.g.*, growth inhibition), or Western Blot assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (*e.g.*, an antibody) specific for the complex of interest.

In another aspect, the present invention provides multivalent compounds comprising at least two identical or different antigen-binding portions of the antibodies of the invention binding to Epo. The antigen-binding portions can be linked together via protein fusion or covalent or noncovalent linkage. Alternatively, methods of linkage have

been described for the bispecific molecules. Tetravalent compounds can be obtained for example by cross-linking antibodies of the antibodies of the invention with an antibody that binds to the constant regions of the antibodies of the invention, for example the Fc or hinge region.

Trimerizing domain are described for example in Borean patent EP 1 012 280B1. Pentamerizing modules are described for example in WO 1998/018943.

Antibodies with Extended Half Life

The present invention provides for antibodies that specifically bind to Epo protein which have an extended half-life *in vivo*.

Many factors may affect a protein's half-life *in vivo,* for example, kidney filtration, metabolism in the liver, degradation by proteolytic enzymes (proteases), and immunogenic responses (*e.g.*, protein neutralization by antibodies and uptake by macrophages and dendritic cells). A variety of strategies can be used to extend the half-life of the antibodies of the present invention. For example, by chemical linkage to polyethyleneglycol (PEG), reCODE PEG, antibody scaffold, polysialic acid (PSA), hydroxyethyl starch (HES), albumin-binding ligands, and carbohydrate shields; by genetic fusion to proteins binding to serum proteins, such as albumin, IgG, FcRn, and transferring; by coupling (genetically or chemically) to other binding moieties that bind to serum proteins, such as nanobodies, Fabs, DARPins, avimers, affibodies, and anticalins; by genetic fusion to rPEG, albumin, domain of albumin, albumin-binding proteins, and Fc; or by incorporation into nanocarriers, slow release formulations, or medical devices.

To prolong the serum circulation of antibodies *in vivo*, inert polymer molecules such as high molecular weight polyethylene glycol (PEG) can be attached to the antibodies or a fragment thereof with or without a multifunctional linker either through site-specific conjugation of the PEG to the N- or C-terminus of the antibodies or via epsilon-amino groups present on lysine residues. To pegylate an antibody, the antibody, or fragment thereof, typically is reacted with PEG, such as a reactive ester or aldehyde derivative of PEG, under conditions in which one or more PEG groups become attached to the antibody or antibody fragment. The pegylation can be carried out by an acylation reaction or an alkylation reaction with a reactive PEG molecule (or an analogous reactive water-soluble polymer). As used herein, the terms "polyethylene glycol" and "PEG" are intended to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (C1-C10) alkoxy- or aryloxy-polyethylene glycol or polyethylene glycol-maleimide. In certain embodiments, the antibody to be pegylated is an

aglycosylated antibody. Linear or branched polymer derivatization that results in minimal loss of biological activity will be used. The degree of conjugation can be closely monitored by SDS-PAGE and mass spectrometry to ensure proper conjugation of PEG molecules to the antibodies. Unreacted PEG can be separated from antibody-PEG conjugates by size-exclusion or by ion-exchange chromatography. PEG-derivatized antibodies can be tested for binding activity as well as for *in vivo* efficacy using methods well-known to those of skill in the art, for example, by immunoassays described herein. Methods for pegylating proteins are known in the art and can be applied to the antibodies of the invention. See for example, EP 0 154 316 by Nishimura *et al.* and EP 0 401 384 by Ishikawa *et al.*

Other modified pegylation technologies include reconstituting chemically orthogonal directed engineering technology (ReCODE PEG), which incorporates chemically specified side chains into biosynthetic proteins via a reconstituted system that includes tRNA synthetase and tRNA. This technology enables incorporation of more than 30 new amino acids into biosynthetic proteins in *E. coli*, yeast, and mammalian cells. The tRNA incorporates a nonnative amino acid any place an amber codon is positioned, converting the amber from a stop codon to one that signals incorporation of the chemically specified amino acid.

Recombinant pegylation technology (rPEG) can also be used for serum half-life extension. This technology involves genetically fusing a 300-600 amino acid unstructured protein tail to an existing pharmaceutical protein. Because the apparent molecular weight of such an unstructured protein chain is about 15-fold larger than its actual molecular weight, the serum half-life of the protein is greatly increased. In contrast to traditional PEGylation, which requires chemical conjugation and repurification, the manufacturing process is greatly simplified and the product is homogeneous.

Polysialytion is another technology, which uses the natural polymer polysialic acid (PSA) to prolong the active life and improve the stability of therapeutic peptides and proteins. PSA is a polymer of sialic acid (a sugar). When used for protein and therapeutic peptide drug delivery, polysialic acid provides a protective microenvironment on conjugation. This increases the active life of the therapeutic protein in the circulation and prevents it from being recognized by the immune system. The PSA polymer is naturally found in the human body. It was adopted by certain bacteria which evolved over millions of years to coat their walls with it. These naturally polysialylated bacteria were then able, by virtue of molecular mimicry, to foil the body's defense system. PSA, nature's ultimate stealth technology, can be easily produced from such bacteria in large

quantities and with predetermined physical characteristics. Bacterial PSA is completely non-immunogenic, even when coupled to proteins, as it is chemically identical to PSA in the human body.

Another technology includes the use of hydroxyethyl starch ("HES") derivatives linked to antibodies. HES is a modified natural polymer derived from waxy maize starch and can be metabolized by the body's enzymes. HES solutions are usually administered to substitute deficient blood volume and to improve the rheological properties of the blood. Hesylation of an antibody enables the prolongation of the circulation half-life by increasing the stability of the molecule, as well as by reducing renal clearance, resulting in an increased biological activity. By varying different parameters, such as the molecular weight of HES, a wide range of HES antibody conjugates can be customized.

Antibodies having an increased half-life *in vivo* can also be generated introducing one or more amino acid modifications (*i.e.*, substitutions, insertions or deletions) into an IgG constant domain, or FcRn binding fragment thereof (preferably a Fc or hinge Fc domain fragment). See, *e.g.*, International Publication No. WO 98/23289; International Publication No. WO 97/34631; and U.S. Patent No. 6,277,375.

Further, antibodies can be conjugated to albumin (e.g., human serum albumin; HSA) in order to make the antibody or antibody fragment more stable *in vivo* or have a longer half-life *in vivo*. The techniques are well-known in the art, see, *e.g.*, International Publication Nos. WO 93/15199, WO 93/15200, and WO 01/77137; and European Patent No. EP0 413622. In addition, in the context of a bispecific antibody as described above, the specificities of the antibody can be designed such that one binding domain of the antibody binds to Epo while a second binding domain of the antibody binds to serum albumin, preferably HSA.

The strategies for increasing half-life are especially useful in nanobodies, fibronectin-based binders, and other antibodies or proteins for which increased *in vivo* half-life is desired.

Antibody Conjugates

The present invention provides antibodies or fragments thereof that specifically bind to an Epo protein recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide (or fragment thereof, preferably to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino

acids) to generate fusion proteins. In particular, the invention provides fusion proteins comprising an antigen-binding fragment of an antibody described herein (*e.g.*, a Fab fragment, Fd fragment, Fv fragment, F(ab)2 fragment, a VH domain, a VH CDR, a VL domain or a VL CDR) and a heterologous protein, polypeptide, or peptide. Methods for fusing or conjugating proteins, polypeptides, or peptides to an antibody or an antibody fragment are known in the art. See, *e.g.*, U.S. Patent Nos. 5,336,603, 5,622,929, 5,359,046, 5,349,053, 5,447,851, and 5,112,946; European Patent Nos. EP 0307434 and EP 0367166; International Publication Nos. WO 96/04388 and WO 91/06570; Ashkenazi *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88: 10535-10539; Zheng *et al.*, 1995, J. Immunol. 154:5590-5600; and Vil *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:11337-11341.

Additional fusion proteins may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to alter the activities of antibodies of the invention or fragments thereof (*e.g.*, antibodies or fragments thereof with higher affinities and lower dissociation rates). See, generally, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458; Patten *et al.*, 1997, Curr. Opinion Biotechnol. 8:724-33; Harayama, 1998, Trends Biotechnol. 16(2):76-82; Hansson, *et al.*, 1999, J. Mol. Biol. 287:265-76; and Lorenzo and Blasco, 1998, Biotechniques 24(2):308-313 (each of these patents and publications are hereby incorporated by reference in its entirety). Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. A polynucleotide encoding an antibody or fragment thereof that specifically binds to an Epo protein may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

Moreover, the antibodies or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin ("HA") tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, 1984, Cell 37:767), and the "flag" tag.

In other embodiments, antibodies of the present invention or fragments thereof conjugated to a diagnostic or detectable agent. Such antibodies can be useful for monitoring or prognosing the onset, development, progression and/or severity of a disease or disorder as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can be accomplished by coupling the antibody to detectable substances including, but not limited to, various enzymes, such as, but not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as, but not limited to, streptavidin/biotin and avidin/biotin; fluorescent materials, such as, but not limited to, umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as, but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as, but not limited to, iodine (131I, 125I, 123I, and 121I,), carbon (14C), sulfur (35S), tritium (3H), indium (115In, 113In, 112In, and 111In,), technetium (99Tc), thallium (201Ti), gallium (68Ga, 67Ga), palladium (103Pd), molybdenum (99Mo), xenon (133Xe), fluorine (18F), 153Sm, 177Lu, 159Gd, 149Pm, 140La, 175Yb, 166Ho, 90Y, 47Sc, 186Re, 188Re,142 Pr, 105Rh, 97Ru, 68Ge, 57Co, 65Zn, 85Sr, 32P, 153Gd, 169Yb, 51Cr, 54Mn, 75Se, 113Sn, and 117Tin; and positron emitting metals using various positron emission tomographies, and non-radioactive paramagnetic metal ions.

The present invention further encompasses uses of antibodies or fragments thereof conjugated to a therapeutic moiety. An antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, *e.g.*, a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, *e.g.*, alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells.

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety or drug moiety that modifies a given biological response. Therapeutic moieties or drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein, peptide, or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, cholera toxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, an anti-angiogenic agent; or, a biological response modifier such as, for example, a lymphokine.

Moreover, an antibody can be conjugated to therapeutic moieties such as a radioactive metal ion, such as alpha-emitters such as 213Bi or macrocyclic chelators useful for conjugating radiometal ions, including but not limited to, 131In, 131LU, 131Y, 131Ho, 131Sm, to polypeptides. In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo *et al.*, 1998, Clin Cancer Res. 4(10):2483-90; Peterson *et al.*, 1999, Bioconjug. Chem. 10(4):553-7; and Zimmerman *et al.*, 1999, Nucl. Med. Biol. 26(8):943-50, each incorporated by reference in their entireties.

Techniques for conjugating therapeutic moieties to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies 84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, 1982, Immunol. Rev. 62:119-58.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Methods of Producing Antibodies of the Invention

Nucleic Acids Encoding the Antibodies

The invention provides substantially purified nucleic acid molecules which encode polypeptides comprising segments or domains of the Epo-binding antibody chains described above. Some of the nucleic acids of the invention comprise the nucleotide sequence encoding the heavy chain variable region shown in SEQ ID NO: 13, 33, 53, or 73, and/or the nucleotide sequence encoding the light chain variable region shown in SEQ ID NO: 14, 34, 54, or 74. In a specific embodiment, the nucleic acid molecules are those identified in Table 1. Some other nucleic acid molecules of the invention comprise nucleotide sequences that are substantially identical (*e.g.*, at least 65, 80%, 95%, or

99%) to the nucleotide sequences of those identified in Table 1. When expressed from appropriate expression vectors, polypeptides encoded by these polynucleotides are capable of exhibiting Epo antigen binding capacity.

Also provided in the invention are polynucleotides which encode at least one CDR region and usually all three CDR regions from the heavy or light chain of the Epo-binding antibody set forth above. Some other polynucleotides encode all or substantially all of the variable region sequence of the heavy chain and/or the light chain of the Epo-binding antibody set forth above. Because of the degeneracy of the code, a variety of nucleic acid sequences will encode each of the immunoglobulin amino acid sequences.

The nucleic acid molecules of the invention can encode both a variable region and a constant region of the antibody. Some of nucleic acid sequences of the invention comprise nucleotides encoding a mature heavy chain sequence that is substantially identical (*e.g.*, at least 80%, 85% 90%, 95%, 96%, 97%, 98% or 99%) to the mature heavy chain sequence set forth in SEQ ID NO: 15, 35, 55, or 75. Some other nucleic acid sequences comprising nucleotide encoding a mature light chain sequence that is substantially identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%) to the mature light chain sequence set forth in SEQ ID NO: 16, 36, 56, or 76.

The polynucleotide sequences can be produced by de novo solid-phase DNA synthesis or by PCR mutagenesis of an existing sequence (*e.g.*, sequences as described in the Examples below) encoding an Epo-binding antibody or its binding fragment. Direct chemical synthesis of nucleic acids can be accomplished by methods known in the art, such as the phosphotriester method of Narang *et al.*, 1979, Meth. Enzymol. 68:90; the phosphodiester method of Brown *et al.*, Meth. Enzymol. 68:109, 1979; the diethylphosphoramidite method of Beaucage *et al.*, Tetra. Lett., 22:1859, 1981; and the solid support method of U.S. Patent No. 4,458,066. Introducing mutations to a polynucleotide sequence by PCR can be performed as described in, *e.g.*, PCR Technology: Principles and Applications for DNA Amplification, H.A. Erlich (Ed.), Freeman Press, NY, NY, 1992; PCR Protocols: A Guide to Methods and Applications, Innis *et al.* (Ed.), Academic Press, San Diego, CA, 1990; Mattila *et al.*, Nucleic Acids Res. 19:967, 1991; and Eckert *et al.*, PCR Methods and Applications 1:17, 1991.

Also provided in the invention are expression vectors and host cells for producing the Epo-binding antibodies described above. Various expression vectors can be employed to express the polynucleotides encoding the Epo-binding antibody chains or binding fragments. Both viral-based and non-viral expression vectors can be used to

produce the antibodies in a mammalian host cell. Non-viral vectors and systems include plasmids, episomal vectors, typically with an expression cassette for expressing a protein or RNA, and human artificial chromosomes (see, *e.g.*, Harrington *et al.*, Nat Genet 15:345, 1997). For example, non-viral vectors useful for expression of the Epo-binding polynucleotides and polypeptides in mammalian (*e.g.*, human) cells include pThioHis A, B & C, pcDNA3.1/His, pEBVHis A, B & C, (Invitrogen, San Diego, CA), MPSV vectors, and numerous other vectors known in the art for expressing other proteins. Useful viral vectors include vectors based on retroviruses, adenoviruses, adenoassociated viruses, herpes viruses, vectors based on SV40, papilloma virus, HBP Epstein Barr virus, vaccinia virus vectors and Semliki Forest virus (SFV). See, Brent *et al.*, supra; Smith, Annu. Rev. Microbiol. 49:807, 1995; and Rosenfeld *et al.*, Cell 68:143, 1992.

The choice of expression vector depends on the intended host cells in which the vector is to be expressed. Typically, the expression vectors contain a promoter and other regulatory sequences (*e.g.*, enhancers) that are operably linked to the polynucleotides encoding an Epo-binding antibody chain or fragment. In some embodiments, an inducible promoter is employed to prevent expression of inserted sequences except under inducing conditions. Inducible promoters include, *e.g.*, arabinose, lacZ, metallothionein promoter or a heat shock promoter. Cultures of transformed organisms can be expanded under non-inducing conditions without biasing the population for coding sequences whose expression products are better tolerated by the host cells. In addition to promoters, other regulatory elements may also be required or desired for efficient expression of an Epo-binding antibody chain or fragment. These elements typically include an ATG initiation codon and adjacent ribosome binding site or other sequences. In addition, the efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (see, *e.g.*, Scharf *et al.*, Results Probl. Cell Differ. 20:125, 1994; and Bittner *et al.*, Meth. Enzymol., 153:516, 1987). For example, the SV40 enhancer or CMV enhancer may be used to increase expression in mammalian host cells.

The expression vectors may also provide a secretion signal sequence position to form a fusion protein with polypeptides encoded by inserted Epo-binding antibody sequences. More often, the inserted Epo-binding antibody sequences are linked to a signal sequences before inclusion in the vector. Vectors to be used to receive sequences encoding Epo-binding antibody light and heavy chain variable domains sometimes also encode constant regions or parts thereof. Such vectors allow expression of the variable regions as fusion proteins with the constant regions thereby

leading to production of intact antibodies or fragments thereof. Typically, such constant regions are human.

The host cells for harboring and expressing the Epo-binding antibody chains can be either prokaryotic or eukaryotic. E. coli is one prokaryotic host useful for cloning and expressing the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which typically contain expression control sequences compatible with the host cell (*e.g.*, an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation. Other microbes, such as yeast, can also be employed to express Epo-binding polypeptides of the invention. Insect cells in combination with baculovirus vectors can also be used.

In some preferred embodiments, mammalian host cells are used to express and produce the Epo -binding polypeptides of the present invention. For example, they can be either a hybridoma cell line expressing endogenous immunoglobulin genes (*e.g.*, the 1D6.C9 myeloma hybridoma clone as described in the Examples) or a mammalian cell line harboring an exogenous expression vector (*e.g.*, the SP2/0 myeloma cells exemplified below). These include any normal mortal or normal or abnormal immortal animal or human cell. For example, a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed including the CHO cell lines, various Cos cell lines, HeLa cells, myeloma cell lines, transformed B-cells and hybridomas. The use of mammalian tissue cell culture to express polypeptides is discussed generally in, *e.g.*, Winnacker, FROM GENES TO CLONES, VCH Publishers, N.Y., N.Y., 1987. Expression vectors for mammalian host cells can include expression control sequences, such as an origin of replication, a promoter, and an enhancer (see, *e.g.*, Queen, *et al.*, Immunol. Rev. 89:49-68, 1986), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. These expression vectors usually contain promoters derived from mammalian genes or from mammalian viruses. Suitable promoters may be constitutive, cell type-specific, stage-specific, and/or modulatable or regulatable. Useful promoters include, but are not limited to, the metallothionein promoter, the constitutive adenovirus major late promoter, the dexamethasone-inducible

MMTV promoter, the SV40 promoter, the MRP polIII promoter, the constitutive MPSV promoter, the tetracycline-inducible CMV promoter (such as the human immediate-early CMV promoter), the constitutive CMV promoter, and promoter-enhancer combinations known in the art.

Methods for introducing expression vectors containing the polynucleotide sequences of interest vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See generally Sambrook, *et al.*, supra). Other methods include, *e.g.*, electroporation, calcium phosphate treatment, liposome-mediated transformation, injection and microinjection, ballistic methods, virosomes, immunoliposomes, polycation:nucleic acid conjugates, naked DNA, artificial virions, fusion to the herpes virus structural protein VP22 (Elliot and O'Hare, Cell 88:223, 1997), agent-enhanced uptake of DNA, and ex vivo transduction. For long-term, high-yield production of recombinant proteins, stable expression will often be desired. For example, cell lines which stably express Epo-binding antibody chains or binding fragments can be prepared using expression vectors of the invention which contain viral origins of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth of cells which successfully express the introduced sequences in selective media. Resistant, stably transfected cells can be proliferated using tissue culture techniques appropriate to the cell type.

Generation of monoclonal antibodies of the invention

Monoclonal antibodies (mAbs) can be produced by a variety of techniques, including conventional monoclonal antibody methodology *e.g.*, the standard somatic cell hybridization technique of Kohler and Milstein, 1975 Nature 256: 495. Many techniques for producing monoclonal antibody can be employed *e.g.*, viral or oncogenic transformation of B lymphocytes.

Animal systems for preparing hybridomas include the murine, rat and rabbit systems. Hybridoma production in the mouse is a well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (*e.g.*, murine myeloma cells) and fusion procedures are also known.

Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody prepared as described above. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*,. human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art. See *e.g.*, U.S. Patent No. 5225539 to Winter, and U.S. Patent Nos. 5530101; 5585089; 5693762 and 6180370 to Queen *et al.*

In a certain embodiment, the antibodies of the invention are human monoclonal antibodies. Such human monoclonal antibodies directed against Epo can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system. These transgenic and transchromosomic mice include mice referred to herein as HuMAb mice and KM mice, respectively, and are collectively referred to herein as "human Ig mice."

The HuMAb mouse® (Medarex, Inc.) contains human immunoglobulin gene miniloci that encode un-rearranged human heavy (μ and γ) and κ light chain immunoglobulin sequences, together with targeted mutations that inactivate the endogenous μ and κ chain loci (see *e.g.*, Lonberg, *et al.*, 1994 Nature 368(6474): 856-859). Accordingly, the mice exhibit reduced expression of mouse IgM or κ, and in response to immunization, the introduced human heavy and light chain transgenes undergo class switching and somatic mutation to generate high affinity human IgGκ monoclonal (Lonberg, N. *et al.*, 1994 supra; reviewed in Lonberg, N., 1994 Handbook of Experimental Pharmacology 113:49-101; Lonberg, N. and Huszar, D., 1995 Intern. Rev. Immunol.13: 65-93, and Harding, F. and Lonberg, N., 1995 Ann. N. Y. Acad. Sci. 764:536-546). The preparation and use of HuMAb mice, and the genomic modifications carried by such mice, is further described in Taylor, L. *et al.*, 1992 Nucleic Acids Research 20:6287-6295; Chen, J. et at., 1993 International Immunology 5: 647-656; Tuaillon *et al.*, 1993 Proc. Natl. Acad. Sci. USA 94:3720-3724; Choi *et al.*, 1993 Nature Genetics 4:117-123; Chen, J. *et al.*, 1993 EMBO J. 12: 821-830; Tuaillon *et al.*, 1994 J. Immunol. 152:2912-2920; Taylor, L. *et al.*, 1994 International Immunology 579-591; and Fishwild, D. *et al.*, 1996 Nature Biotechnology 14: 845-851, the contents of all of which are hereby specifically incorporated by reference in their entirety. See further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397;

5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92103918, WO 93/12227, WO 94/25585, WO 97113852, WO 98/24884 and WO 99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*

In another embodiment, human antibodies of the invention can be raised using a mouse that carries human immunoglobulin sequences on transgenes and transchromosomes such as a mouse that carries a human heavy chain transgene and a human light chain transchromosome. Such mice, referred to herein as "KM mice", are described in detail in PCT Publication WO 02/43478 to Ishida *et al.*

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise Epo -binding antibodies of the invention. For example, an alternative transgenic system referred to as the Xenomouse (Abgenix, Inc.) can be used. Such mice are described in, *e.g.*, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise Epo-binding antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome, referred to as "TC mice" can be used; such mice are described in Tomizuka *et al.*, 2000 Proc. Natl. Acad. Sci. USA 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art (Kuroiwa *et al.*, 2002 Nature Biotechnology 20:889-894) and can be used to raise Epo-binding antibodies of the invention.

Human monoclonal antibodies of the invention can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies are established in the art or described in the examples below. See for example: U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*

Human monoclonal antibodies of the invention can also be prepared using SCID mice into which human immune cells have been reconstituted such that a human

antibody response can be generated upon immunization. Such mice are described in, for example, U.S. Patent Nos. 5,476,996 and 5,698,767 to Wilson *et al.*

Framework or Fc engineering

Engineered antibodies of the invention include those in which modifications have been made to framework residues within VH and/or VL, *e.g.* to improve the properties of the antibody. Typically such framework modifications are made to decrease the immunogenicity of the antibody. For example, one approach is to "backmutate" one or more framework residues to the corresponding germline sequence. More specifically, an antibody that has undergone somatic mutation may contain framework residues that differ from the germline sequence from which the antibody is derived. Such residues can be identified by comparing the antibody framework sequences to the germline sequences from which the antibody is derived. To return the framework region sequences to their germline configuration, the somatic mutations can be "backmutated" to the germline sequence by, for example, site-directed mutagenesis. Such "backmutated" antibodies are also intended to be encompassed by the invention.

Another type of framework modification involves mutating one or more residues within the framework region, or even within one or more CDR regions, to remove T cell - epitopes to thereby reduce the potential immunogenicity of the antibody. This approach is also referred to as "deimmunization" and is described in further detail in U.S. Patent Publication No. 20030153043 by Carr *et al.*

In addition or alternative to modifications made within the framework or CDR regions, antibodies of the invention may be engineered to include modifications within the Fc region, typically to alter one or more functional properties of the antibody, such as serum half-life, complement fixation, Fc receptor binding, and/or antigen-dependent cellular cytotoxicity. Furthermore, an antibody of the invention may be chemically modified (*e.g.*, one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody. Each of these embodiments is described in further detail below. The numbering of residues in the Fc region is that of the EU index of Kabat.

In one embodiment, the hinge region of CH1 is modified such that the number of cysteine residues in the hinge region is altered, *e.g.*, increased or decreased. This approach is described further in U.S. Patent No. 5,677,425 by Bodmer *et al.* The number of cysteine residues in the hinge region of CH1 is altered to, for example,

facilitate assembly of the light and heavy chains or to increase or decrease the stability of the antibody.

In another embodiment, the Fc hinge region of an antibody is mutated to decrease the biological half-life of the antibody. More specifically, one or more amino acid mutations are introduced into the CH2-CH3 domain interface region of the Fc-hinge fragment such that the antibody has impaired Staphylococcyl protein A (SpA) binding relative to native Fc-hinge domain SpA binding. This approach is described in further detail in U.S. Patent No. 6,165,745 by Ward *et al*.

In another embodiment, the antibody is modified to increase its biological half-life. Various approaches are possible. For example, one or more of the following mutations can be introduced: T252L, T254S, T256F, as described in U.S. Patent No. 6,277,375 to Ward. Alternatively, to increase the biological half-life, the antibody can be altered within the CH1 or CL region to contain a salvage receptor binding epitope taken from two loops of a CH2 domain of an Fc region of an IgG, as described in U.S. Patent Nos. 5,869,046 and 6,121,022 by Presta *et al.*

In yet other embodiments, the Fc region is altered by replacing at least one amino acid residue with a different amino acid residue to alter the effector functions of the antibody. For example, one or more amino acids can be replaced with a different amino acid residue such that the antibody has an altered affinity for an effector ligand but retains the antigen-binding ability of the parent antibody. The effector ligand to which affinity is altered can be, for example, an Fc receptor or the C1 component of complement. This approach is described in further detail in U.S. Patent Nos. 5,624,821 and 5,648,260, both by Winter *et al.*

In another embodiment, one or more amino acids selected from amino acid residues can be replaced with a different amino acid residue such that the antibody has altered C1q binding and/or reduced or abolished complement dependent cytotoxicity (CDC). This approach is described in further detail in U.S. Patent Nos. 6,194,551 by Idusogie *et al*.

In another embodiment, one or more amino acid residues are altered to thereby alter the ability of the antibody to fix complement. This approach is described further in PCT Publication WO 94/29351 by Bodmer *et al*.

In yet another embodiment, the Fc region is modified to increase the ability of the antibody to mediate antibody dependent cellular cytotoxicity (ADCC) and/or to increase

the affinity of the antibody for an Fcγ receptor by modifying one or more amino acids. This approach is described further in PCT Publication WO 00/42072 by Presta. Moreover, the binding sites on human IgG1 for FcγRI, FcγRII, FcγRIII and FcRn have been mapped and variants with improved binding have been described (see Shields, R.L. *et al.*, 2001 J. Biol. Chen. 276:6591-6604).

In still another embodiment, the glycosylation of an antibody is modified. For example, an aglycoslated antibody can be made (*i.e.*, the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for "antigen'. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Patent Nos. 5,714,350 and 6,350,861 by Co *et al.*

Additionally or alternatively, an antibody can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNac structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. For example, EP 1,176,195 by Hang *et al.* describes a cell line with a functionally disrupted FUT8 gene, which encodes a fucosyl transferase, such that antibodies expressed in such a cell line exhibit hypofucosylation. PCT Publication WO 03/035835 by Presta describes a variant CHO cell line, Lecl3 cells, with reduced ability to attach fucose to Asn(297)-linked carbohydrates, also resulting in hypofucosylation of antibodies expressed in that host cell (see also Shields, R.L. *et al.*, 2002 J. Biol. Chem. 277:26733-26740). PCT Publication WO 99/54342 by Umana *et al.* describes cell lines engineered to express glycoprotein-modifying glycosyl transferases (*e.g.*, beta(1,4)-N acetylglucosaminyltransferase III (GnTIII)) such that antibodies expressed in the engineered cell lines exhibit increased bisecting GlcNac structures which results in increased ADCC activity of the antibodies (see also Umana *et al.*, 1999 Nat. Biotech. 17:176-180).

Methods of Engineering Altered Antibodies

As discussed above, the Epo-binding antibodies having VH and VL sequences or full length heavy and light chain sequences shown herein can be used to create new Epo-binding antibodies by modifying full length heavy chain and/or light chain sequences, VH and/or VL sequences, or the constant region(s) attached thereto. Thus, in another aspect of the invention, the structural features of an Epo-binding antibody of the invention are used to create structurally related Epo-binding antibodies that retain at least one functional property of the antibodies of the invention, such as binding to human Epo and also inhibiting one or more functional properties of Epo (*e.g.*, inhibit Epo binding to the Epo receptor, inhibit Epo-dependent cell proliferation).

For example, one or more CDR regions of the antibodies of the present invention, or mutations thereof, can be combined recombinantly with known framework regions and/or other CDRs to create additional, recombinantly-engineered, Epo-binding antibodies of the invention, as discussed above. Other types of modifications include those described in the previous section. The starting material for the engineering method is one or more of the VH and/or VL sequences provided herein, or one or more CDR regions thereof. To create the engineered antibody, it is not necessary to actually prepare (*i.e.*, express as a protein) an antibody having one or more of the VH and/or VL sequences provided herein, or one or more CDR regions thereof. Rather, the information contained in the sequence(s) is used as the starting material to create a "second generation" sequence(s) derived from the original sequence(s) and then the "second generation" sequence(s) is prepared and expressed as a protein.

Accordingly, in another embodiment, the invention provides a method for preparing a modified Epo-binding antibody comprising the steps of: a) producing and Epo-binding antibody comprising a heavy chain variable region antibody sequence having a CDR1 sequence selected from the group consisting of SEQ ID NOs: 1, 21, 41, and 61, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 2, 22, 42, and 62, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 3, 23, 43, and 63; and a light chain variable region antibody sequence having a CDR1 sequence selected from the group consisting of SEQ ID NOs: 4, 24, 44, and 64, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 5, 25, 45, and 65, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 6, 26, 46, and 66; b) altering at least one amino acid residue within the heavy chain variable region antibody sequence and/or the light chain variable region antibody sequence to create at least one

altered antibody sequence; and c) expressing the altered antibody sequence as a protein.

Accordingly, in another embodiment, the invention provides a method for preparing an Epo-binding antibody consisting of a heavy chain variable region antibody sequence having a CDR1 sequence selected from the group consisting of SEQ ID NOs: 7, 27, 47, and 67, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 8, 28, 48, and 68, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 9, 29, 49, and 69; and a light chain variable region antibody sequence having a CDR1 sequence selected from the group consisting of SEQ ID NOs: 10, 30, 50, and 70, a CDR2 sequence selected from the group consisting of SEQ ID NOs: 11, 31, 51, and 71, and/or a CDR3 sequence selected from the group consisting of SEQ ID NOs: 12, 32, 52, and 72; altering at least one amino acid residue within the heavy chain variable region antibody sequence and/or the light chain variable region antibody sequence to create at least one altered antibody sequence; and expressing the altered antibody sequence as a protein.

Accordingly, in another embodiment, the invention provides a method for preparing an Epo-binding antibody optimized for expression in a mammalian cell consisting of: a full length heavy chain antibody sequence having a sequence selected from the group of SEQ ID NOs: 15, 35, 55 and 75; and a full length light chain antibody sequence having a sequence selected from the group of SEQ ID NOs: 16, 36, 56, and 76; altering at least one amino acid residue within the full length heavy chain antibody sequence and/or the full length light chain antibody sequence to create at least one altered antibody sequence; and expressing the altered antibody sequence as a protein. In one embodiment, the alteration of the heavy or light chain is in the framework region of the heavy or light chain.

The altered antibody sequence can also be prepared by screening antibody libraries having fixed CDR3 sequences or minimal essential binding determinants as described in US20050255552 and diversity on CDR1 and CDR2 sequences. The screening can be performed according to any screening technology appropriate for screening antibodies from antibody libraries, such as phage display technology.

Standard molecular biology techniques can be used to prepare and express the altered antibody sequence. The antibody encoded by the altered antibody sequence(s) is one that retains one, some or all of the functional properties of the Epo-binding antibodies described herein, which functional properties include, but are not limited to,

specifically binding to human, cynomolgus, rat, and/or mouse Epo; and the antibody inhibit Epo-dependent cell proliferation in a F36E and/or Ba/F3-EpoR cell proliferation assay.

In certain embodiments of the methods of engineering antibodies of the invention, mutations can be introduced randomly or selectively along all or part of an Epo-binding antibody coding sequence and the resulting modified Epo-binding antibodies can be screened for binding activity and/or other functional properties as described herein. Mutational methods have been described in the art. For example, PCT Publication WO 02/092780 by Short describes methods for creating and screening antibody mutations using saturation mutagenesis, synthetic ligation assembly, or a combination thereof. Alternatively, PCT Publication WO 03/074679 by Lazar *et al.* describes methods of using computational screening methods to optimize physiochemical properties of antibodies.

In certain embodiments of the invention antibodies have been engineered to remove sites of deamidation. Deamidation is known to cause structural and functional changes in a peptide or protein. Deamidation can result in decreased bioactivity, as well as alterations in pharmacokinetics and antigenicity of the protein pharmaceutical. (*Anal Chem*. 2005 Mar 1;77(5):1432-9).

In certain embodiments of the invention the antibodies have been engineered to increase pI and improve their drug-like properties. The pI of a protein is a key determinant of the overall biophysical properties of a molecule. Antibodies that have low pIs have been known to be less soluble, less stable, and prone to aggregation. Further, the purification of antibodies with low pI is challenging and can be problematic especially during scale-up for clinical use. Increasing the pI of the anti-Epo antibodies, or Fabs, of the invention improved their solubility, enabling the antibodies to be formulated at higher concentrations (>100 mg/ml). Formulation of the antibodies at high concentrations (e.g. >100mg/ml) offers the advantage of being able to administer higher doses of the antibodies into eyes of patients via intravitreal injections, which in turn may enable reduced dosing frequency, a significant advantage for treatment of chronic diseases including retinal vascular diseases. Higher pIs may also increase the FcRn-mediated recycling of the IgG version of the antibody thus enabling the drug to persist in the body for a longer duration, requiring fewer injections. Finally, the overall stability of the antibodies is significantly improved due to the higher pI resulting in longer shelf-life and bioactivity *in vivo*. Preferably, the pI is greater than or equal to 8.2.

The functional properties of the altered antibodies can be assessed using standard assays available in the art and/or described herein, such as those set forth in the Examples (*e.g.*, ELISAs).

Prophylactic and Therapeutic Uses

Antibodies that binds Epo as described herein, can be used at a therapeutically useful concentration for the treatment of a disease or disorder associated with increased Epo levels and/or activity by administering to a subject in need thereof an effective amount of the antibodies or antigen binding fragments of the invention. The present invention provides a method of treating conditions or disorders associated with retinal vascular disease by administering to a subject in need thereof an effective amount of the antibodies of the invention. The present invention provides a method of treating conditions or disorders associated with diabetic retinopathy (DR) by administering to a subject in need thereof an effective amount of the antibodies of the invention. The present invention provides a method of treating conditions or disorders associated with macular edema administering to a subject in need thereof an effective amount of the antibodies of the invention. The invention also provides a method of treating diabetic macular edema (DME) by administering to a subject in need thereof an effective amount of the antibodies of the invention. The present invention further provides a method of treating proliferative diabetic retinopathy (PDR) by administering to a subject in need thereof an effective amount of the antibodies of the invention. Still further, the present invention provides methods for treating age-related macular edema (AMD), retinal vein occlusion (RVO), angioedema, multifocal choroiditis, myopic choroidal neovascularization, and/or retinopathy of prematurity, by administering to a subject in need thereof an effective amount of the antibodies of the invention. The invention also provides methods of treating beta thelassemia and/or cancer.

The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use in treating a disease or disorder associated with increased Epo levels and/or activity. The invention further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use in treating conditions or disorders associated with retinal vascular disease. The invention further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use in treating conditions or disorders associated with diabetic retinopathy (DR). The invention further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use in treating conditions or disorders

associated with macular edema, diabetic macular edema (DME), and/or proliferative diabetic retinopathy (PDR). The invention still further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use age-related macular edema (AMD), retinal vein occlusion (RVO), angioedema, multifocal choroiditis, myopic choroidal neovascularization, and/or retinopathy of prematurity. The invention further relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use in treating beta thelassemia and/or cancer. More specifically, the isolated antibody or antigen binding fragment thereof as described herein for use in treating a disease or disorder associated with increased Epo levels and/or activity, may be any one of the antibodies or antigen binding fragments described herein, in addition to those described in Table 1. Still further, the isolated antibody or antigen binding fragment thereof as described herein for use in treating conditions or disorders associated with retinal vascular disease, may be any one of the antibodies or antigen binding fragments described herein, in addition to those described in Table 1.The antibodies of the invention can be used, *inter alia*, to prevent progression of conditions or disorders associated with retinal vascular disease (for example, DR, DME, NPDR, PDR, age-related macular degeneration (AMD), retinal vein occlusion (RVO), angioedema, multifocal choroiditis, myopic choroidal neovascularization, and/or retinopathy of prematurity), to treat or prevent macular edema associated with retinal vascular disease, to reduce the frequency of Lucentis® (RTM) injection, and to improve vision lost due to retinal vascular disease progression. The antibodies of the invention can also be used in combination with anti-VEGF therapies for the treatment of patients with retinal vascular disease.

In one aspect, the invention relates to a method of inhibiting Epo-dependent cell proliferation wherein the method includes the step of contacting Epo (e.g., contacting Epo in a subject) with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. In one aspect, the method comprises contacting a cell (e.g., a cell comprising Epo) with a composition coprising the isolated antibody or antigen binding fragment thereof as described herein. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo-dependent cell proliferation in a subject. It is contemplated that the cell is a human cell. The cell could be a B cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

Cell proliferation can be measured by, for example, slit-lamp bio-microscopt, optical coherence tomography, color fundus photography, and fluorescein angiography (Heng et al. Diabet. Med. 2013 Jun;30(6):640-50). In addition, the ability of an antibody or antigen binding fragment described herein to inhibit Epo-dependent cell proliferation can be measured using an assay such as the F36E, or Ba/F3-EpoR cell proliferation assay described below.

The invention also relates to a method of inhibiting Epo-dependent cell signalling wherein the method includes the step of contacting Epo with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein to prevent Epo from interacting with a receptor on a cell surface. In one aspect, the method comprises contacting a cell comprising Epo with a composition coprising the isolated antibody or antigen binding fragment thereof as described herein. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo-dependent cell signalling in a subject. It is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human.

Binding of Epo to the EpoR induces signaling via JAK2 kinases that lead to activation of downstream signaling pathways that include phosphatidyl-inositol 3-kinase (PI-3K)/Akt, MAP kinase, STAT5 and protein kinase C (Jelkmann, 2007; Jelkmann, 2004). Epo or the Epo receptor (EpoR) have been reported to be produced endogenously by different cell types such as endothelial cells, smooth muscle cells, and CNS cells (Ogunshola and Bogdanova, 2013). Activation of EpoR upon binding of Epo can trigger downstream signalling pathways leading to different activities such as calcium transport (Korbel et al., 2004), cell survival (Velly et al., 2010), neuroprotection (Grimm et al., 2002), and angiogenesis (Ribatti, 2010; Ribatti et al., 2003). Accordingly, inhibition of Epo-dependent cell signaling can be determined by measuring the activity of one or more of these signaling pathways. For example, inhibition of Epo-dependent cell signaling can be determined by measuring JAK2 kinase, PI-3K/Akt, MAP kinase, STAT5 or protein kinase C. Methods for measuring these signaling pathways are known in the art and kits for measuring such pathway activity are commercially available. In addition, inhibition of Epo-dependent cell signaling can be determined by measuring cell proliferation as described above. Cell proliferation can be in a subject (e.g., angiogenesis), or can be measured using an assay such as the F36E, or Ba/F3-EpoR cell proliferation assay described below. In one aspect, Epo-dependent cell signaling is

statistically significantly ($p<0.05$) decreased in the presence of an antibody described herein, relative to control.

The invention also relates to a method of inhibiting Epo-dependent cell proliferation or signalling wherein the method includes the step of contacting Epo with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein to prevent Epo from interacting with a receptor on a cell surface. It is contemplated that the cell is a B cell. It is contemplated that the cell is a human cell.

The invention also relates to a method of inhibiting Epo binding to the Epo receptor wherein the method includes the step of contacting Epo (e.g., contacting Epo in a subject) with an effective amount of a composition comprising the isolated antibody or antigen binding fragments thereof described herein; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. The invention also relates to a composition comprising an isolated antibody or antigen binding fragment thereof as described herein for use to inhibit Epo binding to the Epo receptor on a cell of a subject; in particular, the composition can comprise the antibody NVS1, NVS2, NVS3, or NVS4. It is contemplated that the cell is a human cell. It is further contemplated that the cell is in a subject. It is also contemplated that the cell is in the eye of the subject. It is still further contemplated that the subject is human. Inhibition of Epo binding to the Epo receptor can be measured as described by Khankin et al. PLoS ONE, 2010 5:e9246

Treatment and/or prevention of retinal vascular disease and macular edema associated with retinal vascular disease can be determined by an ophthalmologist or health care professional using clinically relevant measurements of visual function and/or retinal anatomy. Treatment of conditions or disorders associated with retinal vascular disease means any action (e.g., administration of an anti-Epo antibody described herein) that results in, or is contemplated to result in, the improvement or preservation of visual function and/or retinal anatomy. In addition, prevention as it relates to conditions or disorders associated with retinal vascular disease means any action (e.g., administration of an anti-Epo antibody described herein) that prevents or slows a worsening in visual function, retinal anatomy, and/or a retinal vascular disease parameter, as defined herein, in a patient at risk for said worsening.

Visual function may include, for example, visual acuity, visual acuity with low illumination, visual field, central visual field, peripheral vision, contrast sensitivity, dark adaptation, photostress recovery, color discrimination, reading speed, dependence on

assistive devices (e.g., large typeface, magnifying devices, telescopes), facial recognition, proficiency at operating a motor vehicle, ability to perform one or more activities of daily living, and/or patient-reported satisfaction related to visual function.

Exemplary measures of visual function include Snellen visual acuity, ETDRS visual acuity, low-luminance visual acuity, Amsler grid, Goldmann visual field, Humphrey visual field, microperimetry, Pelli-Robson charts, SKILL card, Ishihara color plates, Farnsworth D15 or D100 color test, standard electroretinography, multifocal electroretinography, validated tests for reading speed, facial recognition, driving simulations, and patient reported satisfaction. Thus, treatment of vascular disease and/or macular edema can be said to be achieved upon a gain of or failure to lose 2 or more lines (or 10 letters) of vision on an ETDRS scale. In addition, treatment of vascular disease and/or macular edema can be said to occur where a subject exhibits at least a 10% an increase or lack of 10% decrease in reading speed (words per minute). In addition, treatment of vascular disease and/or macular edema can be said to occur where a subject exhibits at least a 20% increase or lack of a 20% decrease in the proportion of correctly identified plates on an Ishihara test or correctly sequenced disks on a Farnsworth test. Further, treatment of retinal vascular disease and/or macular edema, can be said to occur if a subject has, for example, at least 10% decrease or lack of a 10% or more increase in time to a pre-specified degree of dark adaptation. In addition, treatment of retinal vascular disease and/or macular edema can be said to occur where a subject exhibits, for example, at least a 10% reduction or lack of a 10% or more increase in total area of visual scotoma expressed as a visual angle determined by a qualified health care professional (i.e., ophthalmologist).

Undesirable aspects of retinal anatomy that may be treated or prevented include, for example, microaneurysm, macular edema, cotton-wool spot, intraretinal microvascular abnormality (IRMA), capillary dropout, leukocyte adhesion, retinal ischemia, neovascularization of the optic disk, neovascularization of the posterior pole, iris neovascularization, intraretinal hemorrhage, vitreous hemorrhage, macular scar, subretinal fibrosis, and retinal fibrosis, venous dilation, vascular tortuosity, vascular leakage. Thus, treatment of, for example, macular edema can be determined by a 20% or more reduction in thickness of the central retinal sub-field as measured by optical coherence tomography.

Exemplary means of assessing retinal anatomy include funduscopy, fundus photography, fluorescein angiography, indocyanine green angiography, optical coherence tomography (OCT), spectral domain optical coherence tomography, scanning

laser ophthalmoscopy, confocal microscopy, adaptive optics, fundus autofluorescence, biopsy, necropsy, and immunohistochemistry. Thus, vascular disease and/or macular edema can be said to be treated in a subject upon a 10% reduction in leakage area as determined by fluorescein angiography.

Subjects to be treated with therapeutic agents of the present invention can also be administered other therapeutic agents with known methods of treating conditions associated with diabetes mellitus, such as all forms of insulin and anti-hypertensive medications.

Treatment and/or prevention of ocular disease such as age-related macular degeneration (AMD), retinal vein occlusion (RVO), angioedema, multifocal choroiditis, myopic choroidal neovascularization, and/or retinopathy of prematurity can be determined by an ophthalmologist or health care professional using clinically relevant measurements of visual function and/or retinal anatomy by any of the measures described above. Although the measures described herein don't apply to each and every ocular disease herein, one of skill in the art would recognize the clinically relevant measurement of visual function and/or retinal anatomy that could be used to treat the given ocular disease.

When the therapeutic agents of the present invention are administered together with another agent, the two can be administered sequentially in either order or simultaneously. In some aspects, an antibody of the present invention is administered to a subject who is also receiving therapy with a second agent (e.g., Lucentis®). In other aspects, the binding molecule is administered in conjunction with surgical treatments.

Suitable agents for combination treatment with Epo binding antibodies include agents known in the art that are able to modulate the activities of VEGF, VEGF receptors, other receptor tyrosine kinase inhibitors, or other entities that modulate HIF-1 mediated pathways. Other agents have been reported to inhibit these pathways include ranibizumab, bevicizumab, pegaptanib, aflibercept, pazopanib, sorafinib, sunitinib, and rapamycin. Combination treatments with anti-inflammatory agents such as corticosteroids, NSAIDS, and TNF-α inhibitors could also be beneficial in the treatment of retinal vascular disease and macular edema, for example, diabetic retinopathy and DME.

A combination therapy regimen may be additive, or it may produce synergistic results (*e.g.*, reductions in retinopathy severity more than expected for the combined use of the two agents). In some embodiments, the present invention provide a combination therapy for preventing and/or treating retinal vascular diseases and macular edema,

specifically, diabetic retinopathy, including DME and/or PDR as described above with an Epo binding antibody of the invention and an anti-angiogenic, such as anti-VEGF agent.

Pharmaceutical Compositions

The invention provides pharmaceutical compositions comprising the Epo-binding antibodies (intact or binding fragments) formulated together with a pharmaceutically acceptable carrier. The compositions can additionally contain one or more other therapeutic agents that are suitable for treating or preventing, for example, diabetic retinopathy. Pharmaceutically acceptable carriers enhance or stabilize the composition, or can be used to facilitate preparation of the composition. Pharmaceutically acceptable carriers include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible.

A pharmaceutical composition of the present invention can be administered by a variety of methods known in the art. The route and/or mode of administration vary depending upon the desired results. It is preferred that administration be intravitreal, intravenous, intramuscular, intraperitoneal, or subcutaneous, or administered proximal to the site of the target. The pharmaceutically acceptable carrier should be suitable for intravitreal, intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

The composition should be sterile and fluid. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

Pharmaceutical compositions of the invention can be prepared in accordance with methods well known and routinely practiced in the art. See, *e.g.*, Remington: The Science and Practice of Pharmacy, Mack Publishing Co., 20th ed., 2000; and Sustained and Controlled Release Drug Delivery Systems, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978. Pharmaceutical compositions are preferably manufactured under GMP conditions. Typically, a therapeutically effective dose or efficacious dose of the Epo-

binding antibody is employed in the pharmaceutical compositions of the invention. The Epo-binding antibodies are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art. Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention can be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level depends upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors.

A physician or veterinarian can start doses of the antibodies of the invention employed in the pharmaceutical composition at levels lower than that required to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, effective doses of the compositions of the present invention, for the treatment of a retinal vascular disease described herein vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Treatment dosages need to be titrated to optimize safety and efficacy. For systemic administration with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 15 mg/kg, of the host body weight. For intravitreal administration with an antibody, the dosage may range from 0.1 mg/eye to 10 mg/eye. More specifically, the dose may range from 1 mg/eye to 9 mg/eye, 2 mg/eye to 8 mg/eye, 3 mg/eye to 7 mg/eye, 4 mg/eye to 6 mg/eye, or 4.5

mg/eye to 5.5 mg/eye. In certain instances the does may be 0.1 mg/eye, 0.2 mg/eye, 0.3 mg/eye, 0.4 mg/eye, 0.5 mg/eye, 0.6 mg/eye, 0.7 mg/eye, 0.8 mg/eye, 0.9 mg/eye, 1 mg/eye, 2 mg/eye, 3 mg/eye, 4 mg/eye, 5 mg/eye, 6 mg/eye, 7 mg/eye, 8 mg/eye, 9 mg/eye, or 10 mg/eye. An exemplary treatment regime entails systemic administration once per every two weeks or once a month or once every 3 to 6 months. An exemplary treatment regime entails systemic administration once per every two weeks or once a month or once every 3 to 6 months, or as needed (PRN).

Antibody is usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of Epo-binding antibody in the patient. In addition alternative dosing intervals can be determined by a physician and administered monthly or as necessary to be efficacious. Efficacy is based on lesion growth, rate of Lucentis® rescue, retinal thickness as determined by Optical Coherence Tomography (OCT), and visual acuity. In some methods of systemic administration, dosage is adjusted to achieve a plasma antibody concentration of 1–1000 µg/ml and in some methods 25–500 µg/ml. Alternatively, antibody can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the antibody in the patient. In general, humanized antibodies show longer half-life than that of chimeric antibodies and nonhuman antibodies. The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, a relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives. In therapeutic applications, a relatively high dosage at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

EXAMPLES

The following examples are provided to further illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

Example 1: Generation of Affinity Matured Epo Antibodies

A fully human phage display library was used to generate the Epo binding antibodies described herein.

Biotinylated and non-biotinylated human and cynomolgus Epo were used in solution and solid phase pannings. Standard panning were performed as well as RapMAT approaches (Prassler *et al.,* (2009) Immunotherapy 1(4):571-583). Following secondary screening and RapMAT panning, clones were selected for sequence analysis and a set of 8 antibodies were selected for conversion to a FabCys format, germlining, pI optimization and removal of deamidation sites. FabCys generation was accomplished with a proprietary RapCLONE®method. RapCLONE® was performed as a two-step method for convenient and efficient conversion of a large amount of Fab clones into the IgG and FabCys format. In a first cloning step, a eukaryotic expression cassette was introduced into the expression vectors pMORPH®x11 (for HuCAL PLATINUM®) via *BsiWI/MfeI* (for κ pools) or *HpaI/MfeI* (for λ pools) digestion and subsequent ligation. This was followed by a second cloning step, in which the Fab pools containing the expression cassette were digested using *EcoRV/BlpI* (κ and λ pools) and subsequently cloned into the pMorph®4_IgG1f or pMorph®4_h_FabCys acceptor vector for expression in mammalian cells. For this project, RapCLONE® was applied only on unique, sequenced and characterized Fab. Therefore all clones were recovered after RapCLONE®.

Low pIs (<8.2) are generally associated with poor biophysical properties including stability and aggregation. 8 final candidates (HCDR3 unique clones) were selected for germlining, pI optimization and removal of de-amidation sites leading to a total of 12 germlined variants. 12 VL-genes were synthesized (two for 11317, 11324, 11331 and 11345) and one VH (11324). Possibly due to early de-selection of candidates with PTMs, only 11317 (VL), 11332 (VL) and 11380 (VH) contained de-amidation sites that were removed with the germlining. Germlining was in general done to the closest germline. To increase the pI, the lambda germline 3h was chosen for 6 of the candidates instead of or in addition to the closest germline 3r. Additionally lambda 3j variants were constructed for three candidates to minimize risk (11317, 11331 and 11345).

Initial Antibody	PTM-modifications*	pI modifications**	Final FabCys	Final pI of FabCys
11317	3j, S96T	NA	NVS4	9.4
11319	3h	Q105R	NVS1	8.3
11331	3h	Q1E	NVS2	8.8
11380	3h	Q105R, S33T	NVS3	8.3

NA, Not applicable

* All PTM-modification occured in VL, **pI modifications occured in VH

As mentioned above, the pI of a protein is a key determinant of the overall biophysical properties of a molecule. The anti-Epo Fabs identified from the phage display library had pIs lower than 8.2. To improve the manufacturing properties, the antibodies were specifically engineered to increase their pI and improve their drug-like properties. Increasing the pI of the anti-Epo Fabs improved their solubility, enabling the Fabs to be formulated at higher concentrations (>100 mg/ml). Formulation of the Fabs at high concentrations (e.g. >100mg/ml) offers the advantage of being able to administer higher doses of the Fabs into eyes of patients via intravitreal injections, which in turn may enable reduced dosing frequency, a significant advantage for treatment of chronic ocular diseases including, but not limited to wet AMD and diabetic retinopathy.

The resulting Fabs are shown in Table 1 (NVS1, NVS2, NVS3, and NVS4).

Example 2: Characterization of Optimized Antibodies

The following example describes methods that may be used to measure antibody affinity. These and other methods of measuring binding affinity are known in the art.

Affinity Determination

Antibody affinity for Epo was measured by surface plasmon resonance (SPR) using a Biacore® T200 (Biacore) and solution equilibrium titration (SET). Explanations of each technology and corresponding mean results for Epo binding are described below. Modelling assumptions take into account concentrations of Epo in the system, kinetics of Epo biosynthesis and half-life, as well as the desired dosing schedule, and suggest that a Fab with an affinity of less than 50pM for Epo is sufficient to lower levels of free Epo.

Biacore Determination

The kinetics of an interaction, i.e. the rates of complex formation (k_a) and dissociation (k_d), can be determined from the information in a sensorgram. If binding occurs as sample passes over a prepared sensor surface, the response in the sensorgram increases. If equilibrium is reached a constant signal will be seen. Replacing sample with buffer causes the bound molecules to dissociate and the response decreases. Biacore evaluation software generates the values of k_a and k_d by fitting the data to interaction models.

Three flow cells were utilized for the method run. Flow cell 1 (fc1) served as the reference, where no Epo Fab was captured, to assess for non-specific binding of the Epo to the antibody coated chip surface. Both capture and binding steps were carried out on flow cells 2-4.

Capture step: In order to achieve an Rmax of 20, the capture level of anti-hu Fab on fc2-4 was approximately 50RL. Anti-hu Fab at a concentration of 1ug/ul, flowed over Fc2-4 at a flow rate of 10 µl/min.

The calculations for the relative Rmax is as follows:

Fabs: $R_{max} = R_L*(MW_{analyte} / MW_{ligand})*stoichiometry$ 20=RL*(21.4/50)*1 = 50 RL

The analyte started at concentrations of 20nM and included 8 1:2 dilutions with a duplicate at 2.5nM for the long and short dissociation. The analyte was run at a flow rate of 60µl/min for 240 seconds. Dissociation times were set at 4000 seconds and 600 seconds. Dissociation time was set at 4000 seconds for 10nM, 2.5nM and 0.3125nM analyte concentrations for NVS2 and NVS4. After the sample injection, there was a wash step with the regeneration buffer.

Regeneration was performed at the end of each cycle on all flow cells. Regeneration condition for this method was 1% Phosphoric acid with 10% sodium Hydroxide at 60ul/min for 100 seconds.

All other running conditions were carried out at 25°C in 1x HBS-EP+ buffer (Biacore cat# BR-1006-69). The resulting signals were adjusted by double referencing, thus subtracting the refraction index values from the reference flow cell and the binding step with no analyte. Data was collected at 10 Hz and analyzed using the Biacore® T100 Evaluation Software Version 1.1 (GE Healthcare). This program uses a global fitting analysis method for the determination of rate and affinity constants for each interaction.

The results of the Biacore binding kinetics determination are shown in Table 2. As shown the antibodies described herein exhibited high affinity binding to human Epo, with K_D values typically less than or equal to 40 pM.

Table 2: Affinity Binding of Epo Antibodies (Biacore)

	K_D (pM)			
Epo	**NVS2**	**NVS3**	**NVS4**	**NVS1***
Human	34.2	37	27.1	11
Human-darbapoetin	23.5	ND	18.1	ND
Cyno	78.7	49	76.0	31
Mouse	44.9	1	30.5	22
Rat	56.6	38	34.4	41
Rabbit	5160	674	ND	661

ND: not determined

*Data shown for NVS1 is single datapoint

SET Determination

In contrast to kinetic assays using sensor surfaces, such as SPR, SET is a method which determines affinities in solution. It is an equilibrium measurement that does not deliver kinetic data.

In SET, a constant amount of antibody is incubated with different concentrations of antigen until equilibrium is reached. The concentration of free antibody in the equilibrated solution is determined by applying the solution on an antigen coated MSD™ plate (Meso Scale Discovery™) followed by incubation with an ECL-labeled secondary antibody and measurement of signal intensity. At low antigen concentrations, a strong signal is achieved (high concentration of free antibody which binds to the antigen on the plate) whereas for high antigen concentration, the antibody is completely antigen-captured, resulting in a low signal. If a sufficient number of antigen concentrations in a matching range are available, the titration curve allows for a reasonable determination of the affinity, using the appropriate fit model. For a complete titration, antigen concentrations of at least 10-fold higher than the anticipated K_D have to be applied. The constant concentration of antibody applied in the assay should be in the range of, or below, the K_D (Table 3).

For K_D determination by SET, monomer fractions of antibody protein were used (at least 90% monomer content, analyzed by analytical SEC; Superdex75 (Amersham Pharmacia) for Fab, or Tosoh G3000SWXL (TOSOH BIOSCIENCE) for IgG, respectively).

Affinity determination in solution was basically performed as described in the literature (Friguet et al. 305-19). In order to improve the sensitivity and accuracy of the SET method, it was transferred from classical ELISA to ECL based technology (Haenel et al., 2005).

Epo antibodies were diluted to a fixed concentration in incubation buffer (PBS with 2% BSA (Sigma cat#A4503) and 1% Tween20 and 1% Triton-X (Sigma cat#234729)), and added to a serial dilution (1:5) of Epo in incubation buffer.

<u>Final highest concentration of Epo:</u>

Human, Hu-darbapoetin, cynomolgus, mouse, rat = 10nM

Rabbit= 100nM

<u>Final concentrations of Fabs:</u>

NVS2: 2pM, except Rabbit = 30pM

NVS3: 2pM, except Rabbit = 5pM

NVS4: 2pM, except Rabbit = 10nM

NVS1: 2pM

Samples were allowed to reach equilibrium by incubation at RT overnight.

Streptavidin-coated standard MSD plates (Meso-Scale Discovery, 384-well: MSD cat#L11SA) were blocked with 25µl incubation buffer at RT for 1hr. Plates were washed 3x in TBST buffer (25mM TBS with 0.05% Tween20), and 0.1µg/ml of biotinylated-Epo was added in 25µl incubation buffer and incubated at RT for 1hr. Plates were washed 3x in TBST buffer. Samples containing Fabs and Epo titration were added to the plate (25µl), and incubated at RT for 15min. Plates were washed 3x in TBST buffer. 25µl detection antibody was added (Anti-Human (Goat) Sulfo-TAG, 1:1000 in incubation buffer, MSD cat#R32AJ-1), and incubated at RT for 60min. Plates were washed 3x in wash buffer, and 50µl of 1x MSD Read buffer T was added (with surfactant, MSD cat#R92TC-1). Plates were read on a MSD Spector Imager 6000.

Three experiments were performed on separate days, each data point in triplicate.

Data was analyzed using GraphPad Prism software v4, with background (an average of wells containing no Fab) subtracted from each value. X-axis values (concentration of Epo in solution) were transformed into log10x.

KD values (KD) were fitted from the following model:

Y=(Top-((Top/(2×Fab))×((((10^x)+Fab)+KD)-((((((10^x)+Fab)+KD)×(((10^x)+Fab)+KD)) - ((4×(10^x))×Fab))^0.5))))

Top= signal at antigen concentration = 0

x= concentration of Epo in solution

Fab= constraint for Fab concentration was set to 1pM

Affinities of Epo Fabs were determined using the SET assay and resulting K_D values ([pM] concentrations) are summarized in Table 3. NVS2 bound human, human-darbepeotin and cynomolgus Epo with a K_D less than 10pM. NVS2 also bound mouse Epo with a K_D less than 50pM and rat Epo with a K_D less than 20pM. NVS3 bound human, human-darbepeotin, cynomolgus, mouse and rat Epo with a K_D less than 5pM. NVS4 bound human, human-darbepoetin, cynomolgus, mouse and rat Epo with a K_D less than 10pM.

Table 3: Affinity Binding of Epo Antibodies (SET)

	K_D (pM)			
Epo	**NVS2**	**NVS3**	**NVS4**	**NVS1***
Human	5.4	0.9	2.5	1.2
Darbepoietin	3.7	0.5	1.3	ND
Cyno	7.3	0.8	7.3	4.4
Mouse	37.0	2.5	7.8	16.1
Rat	12.7	1.2	12.7	5.4
Rabbit	3864.7	39.9	28670	ND

ND: not determined

*Data shown for NVS1 is single datapoint

Example 3: Inhibition of Epo Induced Cell Proliferation

Cells which are dependent on erythropoietin for growth and survival can be utilized to measure the potency of anti-Epo therapeutics by means of Epo-dependent proliferation inhibition (Chiba et al., 1991).

Example 3a: Ba/F3-EpoR Cell Proliferation Assay

This assay demonstrates the ability of Epo antibodies to inhibit Epo induced cell proliferation in mouse Ba/F3 cells expressing the Epo receptor (Ba/F3-EpoR cells). Ba/F3 cells are IL-3 dependent for growth and survival and have been shown to grow in an IL-3 independent manner upon transformation with various oncogenic tyrosine kinases. Upon stable transfection with EpoR, Ba/F3-EpoR cells became IL-3 independent. The mammalian expression plasmid pcDNA3.1 carrying human EpoR was transfected into Ba/F3 cells using the Amaxa nucleofection system (catalogue number VCA-1003, Amaxa GmbH) according to the manufacturer's instructions using the Nucleofector device (Amaxa, Nucleofactor™ II).

Materials

Materials	Description	Source	Catalog #
384-well plate	Matrix 384-well microplate	ThermoScientific	50823639
384-well plate	uClear-Plate Black, 384 well TC w/Lid	Greiner Bio-One	7881091

Materials	Description	Source	Catalog #
RPMI 1640		Invitrogen	11875
FBS		Hyclone	SH30071.03
Pen/Strep		Invitrogen	15140
Hygromycin B		Invitrogen	10687010
Epo		Genway	10-663-45072
Darbepoietin		Sandoz	CAS #: 209810-58-2
Ba/F3-EpoR cells		Described herein	
Cell Titer Blue		Promega	G8081

Cell maintenance

Growth Medium: RPMI1640/10% FBS/1% Pen-Strep/100µg/ml Hygromycin B/1U/ml Epo
Assay Medium: RPMI1640/10% FBS/1% Pen-Strep/100µg/ml Hygromycin B

Ba/F3-EpoR cells were maintained in growth medium (RPMI1640/10%FBS/1%Pen-Strep/100µg/ml Hygromycin B/1U/ml Epo). Cells were split at ~1e6cells/ml (every 3-4 days) down to 0.4-0.6e5 cells/ml.

Epo Induced Cell Proliferation assay

1. A day before the experiment, Ba/F3-EpoR cells were prepared by centrifugation to remove growth medium, following which the cells were resuspended in assay medium (RPMI1640/10% FBS/1% Pen-Strep/100µg/mL Hygromycin B) which does not contain Epo.
2. On the day of experiment, cells were washed 2-3 times in assay medium (centrifuge 1000rpm, 5min) and resuspended in assay medium at 1.25x 10^5 cells/ml.
3. 2500 cells were added to each assay well in a 384-well black plate (clear bottom, TC treated).
4. Epo was serially diluted in a 384-well microplate with assay media such that the final concentration of Epo was two-fold higher than desired final concentration.
5. 20µl of serially diluted Epo (in triplicate) was added in triplicate to sample wells containing Ba/F3-EpoR cells of 384-well black plate.
6. The plate was spun in a centrifuge at 1000rpm for 30-60 seconds and incubated for 48hrs at 37°C, 5% CO_2.

7. Four hours prior to endpoint, 8µl Cell Titer Blue was added to all wells and re-incubated at 37^0C, 5% CO_2.
8. Four hours later, the plate was read on a Beckman Coulter Paradigm with Paradigm Multimode SW, or comparable scanner.
9. Epo stimulated proliferation of Ba/F3-EpoR cells 4-fold over baseline. Epo stimulated Ba/F3-EpoR with an average EC_{50} of 11.2 pM and range of 10pM and 26pM.
10. Anti-Epo antibodies were serially diluted in triplicate in a 384-well microplate containing 4ng/ml Epo in assay medium and incubated for 30 minutes at room temperature.
11. 20µl/well of the above Epo/anti-Epo antibody mixture was added to the 384-well black walled plate previously seeded with 2500 BaF3/EpoR cells per well.
12. Post-incubation plates were processed as outlined in steps 7-9 above

Results

Epo antibodies inhibited Ba/F3-EpoR cells proliferation in the presence of 1ng/ml Epo after 48hrs. Antibodies inhibited Ba/F3-EpoR cell proliferation with an IC_{50} less than or equal to 350pM.

Table 4:

	IC_{50} (pM)				
Assay	**NVS2**	**NVS3**	**NVS4**	**NVS1**	**Epo26**
Ba/F3 Assay	112.0	76.3	173.1	338	590

Example 3b: F36E Cell Proliferation Assay

F36E cells are highly dependent on Epo for proliferation. Stimulation with Epo using the methods described above typically results in a greater then 6-fold signal over baseline. The EC50 of this curve is 7 pM.

Protocol for Neutralization of Epo Induced F36E Cell Proliferation Assay

A proliferation assay using the F36E cell line, an Epo-dependent lymphocyte-like immortalized cell line derived from a parental bone marrow cell line, was used for screening anti-Epo therapeutic antibodies and to select candidates for development.

Materials

Materials/reagents	Source	Catalog #
384 well polystyrene cell culture microplates, black	Greiner Bio One	781091
384 well polypropylene microplate without lid	Greiner Bio One	781280
RPMI 1640	Invitrogen	11875
FBS	Hyclon	SH30071.03
Pen/Strep	Invitrogen	15140
Darbepoietin	Sandoz	CAS #: 209810-58-2
F36E cells	Riken Cell Bank	RCD0776
Cell Titer Blue	Promega	G8081
Epo26 anti-human Epo monoclonal antibody	Stem Cell Tech	01350

Cell maintenance

Darbepoietin, a recombinant hyperglycosylated human Epo, was used for cell maintenance and proliferation assays described herein. Darbepoietin stimulates proliferation in F36E cells with a comparable EC50 to recombinant human Epo (63.2 pg/ml darbepoietin and 81.25 pg/ml erythropoietin; see LU-15432, pg. 44). F36E cells were maintained in growth media (RPMI1640/5% FBS/1% Pen-Strep/5.2U/ml dEpo) at minimum density 0.25e6 cells per ml to maximum density 1.0e6 cells per ml up to 10 passages.

Epo Induced Proliferation Assay Protocol

1. Epo was diluted in assay media (RPMI1640/5% FBS/1% Pen-Strep) to 4ng/ml, 4x-fold desired final concentration.
2. Anti-Epo antibody was diluted in assay media to 200nM, 4x final concentration, and this concentration was serially diluted in assay media for six points. Dilution was repeated for a positive reference antibody (e.g.: Epo26) and a negative reference antibody (e.g.: anti-chicken lysozyme monoclonal antibody).

3. 7.5ul diluted dEpo and 7.5ul anti-Epo antibody serial dilutions were mixed in 384-well polypropylene microplate, in triplicate, and incubated at room temperature for 30 minutes.
4. F36E cells (2e6 per 384-well plate) were pelleted, growth media was aspirated and cells were washed once in assay media (centrifuge 1200rpm, 5min), then resuspended in assay media to 3.33e5 cells/ml.
5. 15µl/well cells (5,000 cells/well) were added to all wells in 384-well polystyrene cell culture plate.
6. 15ul antibody-Epo mixture was added to cells.
7. Incubated 68hrs at 37°C, 5% CO2.
8. 8µl Cell Titer Blue was added per well and incubated at 37°C, 5% CO2 for 4 hours .
9. Fluorescence was meadured at 560(20)Ex/590(10)Em on a Fluoroskan Ascent Microplate Fluorometer or comparable scanner.
10. The average RFU +/- standard deviation vs. nM antibody was plotted and IC50 determined by non-linear regression curve fit in Graph Pad Prizm software.

Results

Anti-Epo antibodies inhibited F36E cell proliferation with an IC_{50} less than or equal to 200pM.

Table 5:

	IC_{50} (pM)				
Assay	**NVS2**	**NVS3**	**NVS4**	**NVS1**	**Epo26**
F36E Assay	144.1	88.7	182.7	175	590

Example 4: *Epitope Binding*

Synthetic Peptide & Peptide Trunctation Studies

Synthetic peptides corresponding to structural domains of human Epo (hEpo), domain truncations of hEpo, or chimeric molecules containing portions of hEpo and human thrombopoietin (TPO) were synthesized or expressed recombinantly. Positive binding to the synthetic peptides indicated that residues contained in that domain of Epo

were involved in binding to the anti-Epo antibody. For the truncated proteins, loss of binding indicated the involvement of the truncated portion in binding to the anti-Epo antibody. However, the loss of binding did not preclude the possibility that the truncation altered the structure of the remaining protein significantly so as to affect binding to the anti-Epo antibodies. The human Epo-human TPO chimeras enabled maintenance of structure while still allowing epitope mapping. Loss of binding to a variant that contained a portion of hTPO indicated that the homologous region in hEpo was important for binding to the anti-Epo antibody.

Peptide Epitope Mapping of Anti-Erythropoietin Antibodies

The following six peptides (Table 6), corresponding to the helices of erythropoietin were synthesized.

Table 6:

Peptide	Sequence	EPO domain
1	SEQ ID NO: 86 RLICDSRVLERYLLEAKEAENITTG	Helix A
2	SEQ ID NO: 89 ITVPDTKVNFYAWKRM	Loop A-B
3	SEQ ID NO: 87 EVGQQAVEVWQGLALLSEAVLRGQALLVNS	Helix B
4	SEQ ID NO: 90 EPLQLHVDKAVSGLRSLTTLLRALGAQKEAISPPD	Helix C
5	SEQ ID NO: 91 DKAVSGLRSLTTLLRAL	Helix C
6	SEQ ID NO: 88 TFRKLFRVYSNFLRGKLKLYTGEACR	Helix D

Assay Set Up

1. 25ul of peptide in PBS (5ug/ml) was coated on 384 well MSD standard plate (Mesoscale Discovery, Cat. No.L21XA-4) overnight.
2. The plate was blocked with 90ul of PBS+5%BSA/0.1%Tween-20/0.1% TritonX-100 for 4 hours.
3. 500nM Morphosys Epo Fab in diluents of PBS+2%BSA/0.1%Tween-20/0.1% TritonX-100 was added to plate and incubated for 1 hour.

4. The plate was washed and incubated with Sulfo-tag anti-human IgG (Meso Scale Discovery, Cat. No. R32AJ-1) for Epo Fabs or species appropriate for reference proteins/antibodies (1 hr)

5. Plate was washed and MSD Read Solution (Meso Scale Discovery, Cat. R92TC-1) was added.

6. Read plate

Epitope Mapping of Anti-Erythropoietin Antibodies with Truncated Variants of Erythropoietin

Epo Variant 1: Helix A
Epo Variant 2: Helix A, Loop A-B
Epo Variant 3: Helix A, Loop A-B, Helix B
Epo Variant 4: Helix A, Loop A-B, Helix B, Loop B-C, Helix C
Epo Variant 5: Full length erythropoietin

Assay Set Up

1. Plate was coated with biotinylated HEK293 expressed Epo variants on standard streptavidin 384 well plate plate (Mesoscale Discovery, Cat. No.L21SA-1) overnight at 4°C.

2. The plate was blocked with 90ul of PBS+5%BSA/0.1%Tween-20/0.1% TritonX-100 for 4 hours.

3. The place was washed and 500nM Morphosys Epo Fab was added to the plate and incubated for 1 hour

4. The plate was washed and incubated with Sulfo-tag anti-human IgG (Meso Scale Discovery, Cat. No. R32AJ-1) for Epo Fabs or species appropriate for reference proteins/antibodies (1 hr)

5. The plate was washed and MSD Read Solution (Meso Scale Discovery, Cat. R92TC-1) was added.

6. Read plate

Epitope Mapping of Anti-Erythropoietin Antibodies with Epo/Thrombopoietin (Tpo) and Rabbit/Human Epo Chimerics

Epo/Tpo Chimerics

Epo/Tpo Variant 1: Human Epo with Tpo Helix A
Epo/Tpo Variant 2: Human Epo with Tpo Loop A-B
Epo/Tpo Variant 3: Human Epo with Tpo Helix B
Epo/Tpo Variant 4: Human Epo with Tpo Helix C
Epo/Tpo Variant 5: Human Epo with Tpo Helix D

Rabbit/Human Epo Chimerics

Rb/Hu Epo Variant 1: Rabbit Epo with Human Helix A
Rb/Hu Epo Variant 2: Rabbit Epo with Human Loop A-B
Rb/Hu Epo Variant 3: Rabbit Epo with Human Helix B
Rb/Hu Epo Variant 4: Rabbit Epo with Human Loop B-C and Helix C

Rb/Hu Epo Variant 5: Rabbit Epo with Human Loop C-D
Rb/Hu Epo Variant 6: Rabbit Epo with Human Helix D

Assay Set Up

1. 25ul of Epo chimerics in PBS (2ug/ml) were coated on a 384 well MSD standard plate (Mesoscale Discovery, Cat. No.L21XA-4) overnight at 4°C.
2. The plate was blocked with 90ul of PBS+5%BSA/0.1%Tween-20/0.1% TritonX-100 for 4 hours.
3. 500nM Morphosys Epo Fab in diluents of PBS+2%BSA/0.1%Tween-20/0.1% TritonX-100 was added to plate and incubated for 1 hour.
4. The plate was washed and incubated with Sulfo-tag anti-human IgG (Meso Scale Discovery, Cat. No. R32AJ-1) for Epo Fabs or species appropriate for reference proteins/antibodies (1 hr)
5. The plate was washed and MSD Read Solution (Meso Scale Discovery, Cat. R92TC-1) was added.
6. Read plate

General Protocol

Standard capture 384-well MSD plates (Meso Scale Discovery) were coated with peptide (5ug/ml in PBS, New England Peptide LLC) or Epo chimerics (2ug/ml in PBS) and incubated overnight at 4°C. Biotinylated truncated Epo variants (2ug/ml in PBS) were coated on standard streptavidin capture 384-well MSD plates overnight. After washing the plates 1X with TBST (Thermo Scientific, Cat. No. Cat. No. 28360), the plates were blocked in diluent (PBS, 5% BSA, 0.1% Tween-20, 0.1% TritonX-100) for 4 hours at room temperature. Plates were washed 3X in TBST. Five hundred nanomolar of anti-erythropoietin fabs were added to the peptide/Epo variants precoated MSD plates for 1 hour. Plates were washed 3X in TBST and anti-Human IgG-Sulfotag (1ug/ml, Meso Scale Discovery, Cat. No.R32AJ-1) was added and incubated for 60 minutes. Plates were washed 3X in TBST and 1X Read Buffer T (Meso Scale Discovery, Cat. No.R92TC-1) was added. The plates were read on a MSD Spector Imager 6000 and data was analyzed using GraphPad Prism software v4.

Results:

Results indicated that the antibodies minimally bound to the following domains (Table 7). No antibodies bound to Helix C.

Table 7:

	Helix A	Loop A-B	Helix D
NVS1	++		+
NVS2		++	+
NVS3	+	+	++
NVS4			++

(++) Dominant epitope; (+) observed binding

Crystal Structure of Antibodies in Complex with Epo

Glycosylated, recombinant human Erythropoeitin (Epo) was received from LEK Pharmaceuticals, Inc.

Epo was de-glycosylated using Protein De-glycosylation Mix (New England Biolabs, cat #P6039S). 30 mg of hEpo was combined with 1 ml of Protein Deglycosylation Mix and incubated at 37°C for 1 hour at which point de-glycosylation was incomplete as determined by SDS-PAGE. An additional 0.5 ml of Protein De-glycosylation Mix was then added to Epo and incubated for a further 1 hour at 37°C. Gel analysis showed near complete deglycosylation of Epo. This protein was then further purified using a 120 ml Superdex75 column (GE Healthcare, cat #28-9893-33) equilibrated in 25 mM HEPES pH 7.5, 150 mM NaCl. Elution fractions containing the highest level of de-glycoslation of hEpo were pooled. Protein complexes were formed by combining 5mg of de-glycosylated Epo with 7 mg of NVS3, followed by incubation on ice for 1 hour. The protein complex mix was then concentrated and applied to a 120 ml Superdex 75, equilibrated in 25mM HEPES pH 7.5, 150mM NaCl. Fractions containing SDS-gel evaluated stoichiometric ratios of Epo:NVS3 were pooled and concentrated to 19 mg/ml (concentration estimated by LCUV) (PRONOVA #27SN). Crystallization screens were set up using this concentrated Epo:NVS3 complex. Crystals were grown by the technique of sitting-drop vapor diffusion, with the drops containing equal volumes of protein and reservoir solution. Crystals formed at 4°C with the following reservoir condition: 0.1M Hepes pH7.0, 12%PEG3350, 50mM zinc acetate dehydrate. Crystals were frozen using the following cryoprotection solution: : 0.1M Hepes pH7.0, 15%PEG3350, 50mM zinc acetate dehydrate, 22% glycerol.

Epo:NVS3 complex crystal diffraction data were collected at beamline 17-ID at the Advanced Photon Source (Argonne National Laboratory, USA). Data were processed and scaled at 2.6Å using autoPROC (Global Phasing, LTD) in space group C2 with cell dimensions a=125.57Å, b=150.15 Å, c=163.84Å, alpha=90°, beta=110.81°, gamma=90°.

The Epo:NVS3 structure was solved by molecular replacement using Phaser (McCoy *et al.*, (2007) J. Appl. Cryst. 40:658-674). The Fab from 3H0T structure in the PDB database (Berman 2000) was split into variable and constant domains, and the human erythropoietin structure Syed *et. al.,* Nature. 1998 Oct 1;395(6701):511-6, PDB code 1EER, were used as search models.

The final model, which contains 3 molecule of the Epo:NVS3 complex per asymmetric unit, was built in COOT (Emsley & Cowtan (2004) Acta Cryst. 60:2126-2132) and refined to R and R_{free} values of 23.0% and 26.7%, respectively, with an rmsd of 0.010 Å and 1.34° for bond lengths and bond angles, respectively, using PHENIX (Adams *et al.*, Acta Cryst. D66, 213-221 (2010)).

The crystal structure of Epo:NVS3 was solved and refined to 2.6Å. It revealed an asymmetric unit composed of three Epo:NVS2 protein complexes, each composed of one Fab bound to one Epo protein. Two of these complexes form a zinc mediated dimer and the third exhibits higher b-factors and weaker density. Interactions from the Fab to Epo were mediated by the complementarity determining region (CDR) loops from both the heavy and light chains of NVS3. Conformational changes of Epo when compared to 1EER were limited to loops distal from the Fab binding epitope, with an RMSD of 0.5Å for all 144 aligned amino acids. The heavy and light chains of Fab NVS3 show typical immunoglobulin-like folds for the domains.

The crystals structure of Epo:NVS3 was used to identify the Epo epitope of the fragment antigen binding of NVS3. The interaction surface on Epo was formed primarily by residues comprising residue Ser^{9}, Glu^{13}, residues Thr^{44} through Arg^{53} and residues Asn^{147} through Arg^{162}. These correspond to the secondary structure elements of Epo denoted as α-helix A, loop βA-αB and α-helix D. These residues formed the three-dimensional surface that is recognized by NVS3. Interactions included backbone interactions, solvent mediated interactions, and direct side-chain interactions.

Table 8: Epo interacting residues to NVS3

Amino Acid	Residue number	Contact Area (A2	Exposed Area (A2)	Percentage buried (%)
Ser	9	11.79	93.23	13
Glu	13	14.89	103.00	14
Thr	44	22.47	61.60	36
Lys	45	39.98	172.59	23
Val	46	34.38	55.22	62
Asn	47	48.17	81.92	59

Phe	48	83.76	129.76	65
Tyr	49	96.12	137.70	70
Ala	50	12.68	57.03	22
Trp	51	0.63	43.74	1
Lys	52	23.97	135.25	18
Arg	53	49.96	181.86	27
Asn	147	34.39	48.65	71
Arg	150	73.32	140.37	52
Gly	151	18.97	24.63	77
Lys	154	73.59	127.20	58
Leu	155	34.85	75.06	46
Gly	158	18.59	43.31	43
Glu	159	32.95	122.17	27
Arg	162	36.72	185.91	20

Epo residues that contain atoms in contact with NVS3 are listed in Table 9. Contact is defined to be within 5 Å of NVS3 to account for potential water mediated interactions.

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Table 9:

Protein	Amino acid	Sequence position*	Epo Domain
Epo	S	9	Helix A
Epo	E	13	Helix A
Epo	T	44	Loop A-B
Epo	K	45	Loop A-B
Epo	V	46	Loop A-B
Epo	N	47	Loop A-B
Epo	F	48	Loop A-B
Epo	Y	49	Loop A-B
Epo	A	50	Loop A-B
Epo	W	51	Loop A-B
Epo	K	52	Loop A-B
Epo	R	53	Loop A-B
Epo	N	147	Helix D
Epo	R	150	Helix D
Epo	G	151	Helix D

Epo	K	154	Helix D
Epo	L	155	Helix D
Epo	G	158	Helix D
Epo	E	159	Helix D
Epo	R	162	Helix D

*Sequence Position relative to SEQ ID NO: 81

Epo residues that contain atoms in contact with NVS2 are listed. Contact is defined to be within 5 Å of protein partner to account for potential water mediated interactions.

Table 10:

Protein	Amino acid	Sequence position*	Epo Domain
Epo	E	23	Helix A
Epo	D	43	Loop A-B
Epo	T	44	Loop A-B
Epo	K	45	Loop A-B
Epo	V	46	Loop A-B
Epo	N	47	Loop A-B
Epo	F	48	Loop A-B
Epo	Y	49	Loop A-B
Epo	A	50	Loop A-B
Epo	K	52	Loop A-B
Epo	R	53	Loop A-B
Epo	R	131	Helix D
Epo	R	143	Helix D
Epo	N	147	Helix D
Epo	R	150	Helix D
Epo	G	151	Helix D
Epo	K	154	Helix D
Epo	L	155	Helix D
Epo	E	159	Helix D
Epo	R	162	Helix D

*Sequence Position relative to SEQ ID NO: 81

Example 5: *In vivo Model*

Example 5a: Mouse model of ocular edema

C57/Bl6 mice (Taconic) were sub-retinally injected with ssAAV2-EPO-eGFP (DR005) and ssAAV2-EGFP (TM003) (control). Mice were sacrificed three weeks (21 days) post-injection. The retinas were flat-mounted and the vessel caliber was measured.

Methods:

Subretinal injection of ssAAV2-EPO-eGFP and ssAAV2-eGFP

8-week old C57/Bl6 mice were divided into two groups (10 mice each, 20 eyes/group) and sub-retinally injected with 1 µl ssAAV2 at $2x10^9$ DRP/µl. The first group (control) received sub-retinal ssAAV2-EPO (TM003), and the second (experimental) ssAAV2- eGFP (DR005). The effect of mouse Epo on the retinal vascular changes was examined in retinal flat-mounts at 21 days post injection.

The AAV (adeno-associated virus) tested were: ssAAV2-EPO-eGFP [(AAV2-CMV-mEPO-IRES-eGFP) from Gene Therapy Center Virus Vector Core Facility, The University of North Carolina at Chapel Hill, Lot# AV3782] and ssAAV2-GFP [(AAV2-eGFP) from Gene Therapy Center Virus Vector Core Facility, The University of North Carolina at Chapel Hill: Lot# AV3725].

Procedure:

AAV vectors were delivered via sub-retinal injection on both eyes of the mice tested. All procedures described were performed under aseptic conditions, using sterile reagents, syringes and appropriate PPE.

1. The mice were immobilized, and their pupils dilated with a drop of cyclopentolate (1%), followed by a drop of 2.5% phenylephrine.
2. Next, the animal was anesthetized with Avertin (250 mg/kg) i.p. The cornea was topically anesthetized with a drop of 0.5% proparacaine.
3. After placing the animal under a surgical microscope, a micro-scalpel was used to make a 0.5 mm nasal incision, posterior to the limbus.
4. A blunt needle attached to a 10 µl Hamilton syringe was tangentially inserted through the scleral incision toward the temporal retina. The needle was advanced until resistance was felt.

5. 1 µl of ssAAV2 vector (either ssAAV2-EPO-eGFP or ssAAV2-GFP, both containing fluorescein diluted 1:50 to visualize delivery) was slowly injected into the sub-retinal space.
6. The eye was examined under the surgical microscope. A successful sub-retinal injection was confirmed by visualizing a fluorescein containing retinal detachment.
7. The injection was scored depending on the degree of retinal damage (visualized by hemorrhage size) and damage to the lens.
8. The animal was turned to the other side and the procedure was repeated.
9. Antibiotic ointment was applied to both eyes after injection.

Retinal Dissection, Imaging and Quantification on Retinal Flatmount:

1. 0.1 ml Concavalin-A (Con-A) was injected (i.v., tail vein) 1 to 5 minutes before euthanasia (CO_2)
2. The eyes were enucleated and fixed in paraformaldehyde (4% in PBS) for two hours. They were subsequently maintained at $4^{O}C$ in PBS buffer for 1-3 days until dissection
3. The cornea and lens were removed, and the retina was dissected from the posterior eye cup (retinal pigmented epithelium/choroid)
4. Four radial incisions were made to the retina and flat-mounted in Vectashield mounting media with the photoreceptor layer face down.
5. Once mounted, the flat-mounts were centered on the central retina (using the optic nerve head as a reference) and the Con-A labeled retinal vessels were captured at 20X using the Zeiss Imaging System (AxioVision)
6. The AxioVision software was used to measure the diameter of central retinal vessels that are 200 µm away from optical nerve head.
7. Data obtained was analyzed with GraphPad Prism.

Results and Conclusion:

Quantification of vessel diameter revealed that ssAAV2-EPO induced significant (* $p<0.001$) vessel dilation in the central retina compared to ssAAV2-GFP and naïve eyes (6 eyes) (Figure 1). No significant difference was found comparing ssAAV2-GFP vs. naïve group. Samples were analyzed using a one-way ANOVA with Dunnet's post-test (C) Representative flat-mounts for each group. Long-term delivery of Epo by AAV2-Epo-eGFP resulted in a statistically significant increase in venous caliber (Figure 1), a key hallmark of diabetic macular edema in humans. Accordingly, in one aspect, the

invention relates to a method of decreasing venous caliber in the eye by administering an anti-EPO antibody described herein to a subject in a therapeutically effective amount.

Example 5b: In vivo Efficacy of anti-Epo antibodies

The *in vivo* activity, and therapeutic efficacy, of the anti-Epo antibodies described herein can be assessed in the mouse model of ocular edema described above.

In vivo Challenge in the mouse Model

C57B6 mice aged 8 weeks old are injected subretinally with one of the following. Groups:

Group 1: AAV2-eGFP @ titer 2 x 10^9 DRP @ titer, 1ul/eye, n = 20 eyes of 10 mice

Group 2: AAV2-Epo-eGFP @ titer 2 x 10^9 DRP , 1ul/eye, n = 20 eyes of 10 mice

Group 3: AAV2-Epo-eGFP @ titer 2 x 10^9 , 1ul/eye, + anti-Epo Fab, 100ug/eye, 1 weekly, n = 20 eyes of 10 mice

The effect of anti-Epo antibodies dosed appropriately are examined in the mouse model by measuring vessel diameter 2 weeks post injection.

AAV-GFP (AAV2-eGFP) and AAV2-Epo-eGFP (AAV2-CMV-mEpo-IRES-eGFP) from Gene Therapy Center Virus Vector Core Facility, The University of North Carolina at Chapel Hill.

Intraocular injection of the anti-Epo antibodies will inhibit retinal vessel dilation anti-Epo antibodies to ameliorate the effects of Epo on decrease blood flow and hypoxic conditions in the retina. Thus, the anti-Epo antibodies are expected to reduce the retinal pathology that is also seen in patients with vascular retinal diseases such as wet AMD and diabetic retinopathy.

Example 6: In vivo neutralization of free EPO

Example 6a: In vivo neutralization of free EPO using an anti-Epo Fab

The *in vivo* activity and therapeutic efficacy of anti-EPO antibodies were assessed in rabbit eyes as follows. Rabbits were dosed intravitreally with an anti-EPO Fab, NVS2 (1mg/eye) and challenged with an intravitreal dose of EPO (3 ug/eye) four days later. Animals were sacrificed and ocular tissues including vitreous was extracted. The amount of free EPO and total EPO in the vitreous was determined as described below.

Group	[Anti-EPO Fab] mg/eye	EPO ug/eye
1	1	-
2	1	3

Total/Free EPO levels:

Assays were performed using standard binding MSD plates (Meso-Scale Discovery, 384-well: MSD cat#L21XA), using coating buffer (PBS) and incubation buffer (PBS with 2% BSA (Sigma cat#A4503) and 0.1% Tween20 and 0.1% Triton-X).

Capture antibodies were coated at 1µg/ml in PBS (25µl), and incubated overnight at 4°C. Plates were washed 3x in wash buffer (PBS with 0.05% Tween20), and blocked with 25µl incubation buffer at RT for 2hrs. Plates were washed 3x in wash buffer. Vitreous dilutions in incubation buffer were added to the plate (25µl), and incubated for 60min at RT. Human recombinant Darbepoietin was used as a standard (A000123, starting at 2µg/ml). Plates were washed 3x in wash buffer. 25µl primary antibody was added (1µg/ml in incubation buffer), and incubated at RT for 60min. Plates were washed 3x in wash buffer. 25µl of anti-species secondary Sulfo-TAG antibodies were added (1:1000 in incubation buffer), and incubated at RT for 60min. Plates were washed 3x in wash buffer, and 25µl of 1x MSD Read buffer T was added (with surfactant, MSD cat#R92TC-1). Plates were read on a MSD Spector Imager 6000.

Total EPO	Coat Antibody	Epo-26 Clone 26G9C10
	Primary Antibody	Anti-EPO Fab
	Secondary Antibody	anti-human R32AJ-1
	Vitreous Dilution	1:20-1:25
	Sensitivity	0.03ng/ml
Free EPO	Coat Antibody	Anti-EPO Fab
	Primary Antibody	Epo-26 Clone 26G9C10
	Secondary Antibody	anti-mouse R32AC-1
	Vitreous Dilution	1:75-1:500
	Sensitivity	1.6ng/ml

Results and Conclusion:

The total EPO levels measured in the vitreous of animals injected with anti-EPO or vehicle was similar as expected (Figure 2). In contrast, no free EPO was measured in the vitreous of rabbits injected with anti-EPO Fab, but average ~ 100ng/ml free EPO was

measured in the vitreous of rabbits injected with vehicle. Anti-EPO Fab administered intravitreally completely neutralized free EPO levels as expected.

Example 6b: In vivo neutralization of free EPO using a anti-Epo Fab

The *in vivo* activity and therapeutic efficacy of anti-EPO antibodies were assessed in rabbit eyes as follows. Rabbits were dosed intravitreally with a pre-mixed solution of an anti-EPO Fab, NVS2 (1mg/eye) and EPO (3ug/eye). Animals were sacrificed and ocular tissues including vitreous was extracted. The amount of free EPO and total EPO in the vitreous was determined as described below. Note: Some eyes received a pre-mix solution of an anti-EPO Fab, EPO, and VEGF.

Group	[Anti-EPO Fab] mg/eye	EPO ug/eye	VEGF ng/eye
1	-	-	200
2	-	3	200
3	1	3	200
4	1	3	-

Total/Free EPO levels:

Assays were performed using standard binding MSD plates (Meso-Scale Discovery, 384-well: MSD cat#L21XA), using coating buffer (PBS) and incubation buffer (PBS with 2% BSA (Sigma cat#A4503) and 0.1% Tween20 and 0.1% Triton-X).

Capture antibodies were coated at 1µg/ml in PBS (25µl), and incubated overnight at 4°C. Plates were washed 3x in wash buffer (PBS with 0.05% Tween20), and blocked with 25µl incubation buffer at RT for 2hrs. Plates were washed 3x in wash buffer. Vitreous dilutions in incubation buffer were added to the plate (25µl), and incubated for 60min at RT. Human recombinant Darbepoietin was used as a standard (A000123, starting at 2µg/ml). Plates were washed 3x in wash buffer. 25µl primary antibody was added (1µg/ml in incubation buffer), and incubated at RT for 60min. Plates were washed 3x in wash buffer. 25µl of anti-species secondary Sulfo-TAG antibodies were added (1:1000 in incubation buffer), and incubated at RT for 60min. Plates were washed 3x in wash buffer, and 25µl of 1x MSD Read buffer T was added (with surfactant, MSD cat#R92TC-1). Plates were read on a MSD Spector Imager 6000.

Total EPO	Coat Antibody	Epo-26 Clone 26G9C10
	Primary Antibody	Anti-EPO Fab
	Secondary Antibody	anti-human R32AJ-1

	Vitreous Dilution	1:20-1:25
	Sensitivity	0.03ng/ml
Free EPO	Coat Antibody	Anti-EPO Fab
	Primary Antibody	Epo-26 Clone 26G9C10
	Secondary Antibody	anti-mouse R32AC-1
	Vitreous Dilution	1:75-1:500
	Sensitivity	1.6ng/ml

Results and Conclusion:

The total EPO levels measured in the vitreous of animals injected with anti-EPO or vehicle were similar as expected (Figure 3). In contrast, no free EPO was measured in the vitreous of rabbits injected with an anti-EPO Fab, while on an average ~ 200ng/ml free EPO was measured in the vitreous of rabbits injected with vehicle (Figure 3). Presence of VEGF did not appear to have any effect on either free or total EPO levels measured. Anti-EPO Fab administered intravitreally completely neutralized free EPO levels as expected.

Claims

1. An isolated antibody, or antigen binding fragment thereof, that binds Epo with a K_D less than or equal to 50pM.
2. The isolated antibody or antigen binding fragment of claim 1 that binds the Helix D domain of Epo.
3. The isolated antibody or antigen binding fragment of claim 1 or claim 2 that further binds the Loop A-B domain of Epo.
4. The isolated antibody or antigen binding fragment of any of claim 2 or claim 3 that further binds the Helix A domain of Epo.
6. The isolated antibody or antigen binding fragment of any of the preceding claims wherein said antibody binds human Epo.
7. The isolated antibody, or antigen binding fragment, of claim 1, that binds a conformational epitope comprising amino acids within Epo Helix D and Loop A-B.
8. The antibody, or antigen binding fragment, of claim 7, that binds a conformational epitope further comprising amino acids within Epo Helix A.
9. The isolated antibody, or antigen binding fragment of claim 1 that binds a conformational epitope comprising amino acids at positions, 44-50, 52, 53, 147, 150, 151, 154, 155, 159, and 162 of SEQ ID NO.81.
10. The isolated antibody, or antigen binding fragment, of any of the preceding claims, that binds a conformational epitope comprising amino acids at positions, 9, 13, 44-53, 147, 150, 151, 154, 155, 158, 159, and 162 of SEQ ID NO.81.
11. The isolated antibody, or antigen binding fragment, of any of the preceding claims, that binds a conformational epitope comprising amino acids at positions, 23, 43-50, 52, 53, 131, 143, 147, 150, 151, 154, 155, 159, and 162 of SEQ ID NO.81.
12. The isolated antibody, or antigen binding fragment, of any of the preceding claims that also binds cynomologous, mouse or rat Epo.
13. The isolated antibody, or antigen binding fragment, of any of the preceding claims that further inhibits EPO-dependent cell proliferation with an EC_{50} less than or equal to 350 pM.
14. The isolated antibody, or antigen binding fragment, of any of the preceding claims that inhibits EPO-dependent cell proliferation in B-cells expressing the EPO receptor.
15. An isolated antibody, or antigen binding fragment thereof, that binds the same epitope as an antibody described in Table 1 or competes with an antibody described in Table 1.

16. An isolated antibody, or antigen binding fragment, of any of the preceding claims that binds EPO, and comprises:
a) heavy chain variable region HCDR1, HCDR2 and HCDR3 as set forth in SEQ ID NOs: 1, 2, and 3, respectively, and light chain variable region LCDR1, LCDR2, and LCDR3 as set forth in SEQ ID NOs: 4, 5, and 6, respectively;
b) heavy chain variable region HCDR1, HCDR2 and HCDR3 as set forth in SEQ ID NOs: 21, 22, and 23, respectively, and light chain variable region LCDR1, LCDR2, and LCDR3 as set forth in SEQ ID NOs: 24, 25, and 26, respectively;
c) heavy chain variable region HCDR1, HCDR2 and HCDR3 as set forth in SEQ ID NOs: 41, 42, and 43, respectively, and light chain variable region LCDR1, LCDR2, and LCDR3 as set forth in SEQ ID NOs: 44, 45, and 46, respectively; or
d) heavy chain variable region HCDR1, HCDR2 and HCDR3 as set forth in SEQ ID NOs: 61, 62, and 63, respectively, and light chain variable region LCDR1, LCDR2, and LCDR3 as set forth in SEQ ID NOs: 64, 65, and 66, respectively.

17. The isolated antibody, or antigen binding fragment, of any of the preceding lcaims comprising heavy and light chain variable regions having amino acid sequences at least 90% identical to SEQ ID NOs: 13 and 14; SEQ ID NOs: 33 and 34; SEQ ID NOs: 53 and 54; or SEQ ID NOs: 73 and 74, respectively.

18. The isolated antibody, or antigen binding fragment, of any of the preceding claims that binds Epo comprising a heavy chain and a light chain with an amino acid sequence having at least 90% sequence identity to SEQ ID NO: 15 and 16, 35 and 36, 55 and 56, or 75 and 76.

19. The antibody or antigen binding fragment of any of the preceding claims, wherein said antibody is a human antibody, a chimeric antibody, a monoclonal antibody, a single chain antibody, Fab, Fab', $F(ab')_2$, Fv or scFv.

20. The antibody or antigen binding fragment of any of the preceding claims, wherein said antibody is an IgG isotype.

21. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the antibody or fragment as claimed in any of the preceding claims.

22. The nucleic acid molecule of claim 21, wherein said nucleic acid comprises a sequence encoding a heavy chain variable domain and said sequence has at least 95% sequence identity to a sequence selected from SEQ ID NOs: 17, 37, 57, and 77.

23. The nucleic acid molecule of claim 21 wherein said nucleic acid comprises a sequence encoding a light chain variable domain and said sequence has at least 95% sequence identity to a sequence selected from SEQ ID NO: 18, 38, 58, and 78.

24. A vector comprising the nucleic acid molecule of any of claims 21 to 23.

25. An isolated host cell comprising the nucleic acid as claimed in any of claims 21 to 23 or the vector of claim 24.

26. A composition comprising the antibody or antigen binding fragment of any of claims 1 to 20 and a pharmaceutically acceptable diluent or carrier.

27. A method of treating retinal vascular disease in a subject comprising administering to said subject, an effective amount of a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

28. The method of claim 27, wherein the retinal vascular disease in the subject is associated with a disease or condition selected from: diabetic retinopathy, diabetic macular edema, proliferative diabetic retinopathy, non-proliferative diabetic retinopathy, age-related macular degeneration, retinal vein occlusion, multifocal choroiditis, myopic choroidal neovascularization, and retinopathy of prematurity.

29. A method of treating macular edema in a subject comprising administering to said subject, an effective amount of a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

30. A method of treating diabetic retinopathy in a subject comprising administering to said subject, an effective amount of a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

31. A method of treating diabetic macular edema in a subject comprising administering to said subject an effective amount of a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

32. A method of treating proliferative diabetic retinopathy, comprising contacting EPO with a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

33. A method of inhibiting EPO-dependent cell proliferation, comprising contacting EPO with a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

34. A method of inhibiting EPO-dependent cell proliferation in a cell expressing an Epo receptor, comprising contacting said cell with a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

35. The method of claim 34 wherein the cell is a B-cell.

36. A method of inhibiting diabetic macular edema, comprising administering to the eye of a subject a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20, wherein administration of said composition decreases retinal vein dilation, decreases vascular leakage and/or increases blood flow in the eye.

37. A method of inhibiting EPO binding to the EPO receptor on a cell, comprising contacting EPO with a composition comprising the antibody or antigen binding fragment of any of claims 1 to 20.

38. The method of claim 37 wherein the cell is in a subject.