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(54) Title: TRANSGENIC RABBIT WITH COMMON LIGHT CHAIN

(57) Abstract: Herein is reported a transgenic vector comprising a humanized light chain locus, wherein said humanized light chain locus comprises (a) a V gene segment derived from human light chain V segment IGKV1-39-01, (b) 3' proximal to said light chain gene segment a promoter, and (c) 5' proximal to said light chain gene segment at least a fragment of the human IGKJ4 J-element.

Transgenic rabbit with common light chain

Herein is reported a common light chain locus useful for the generation of transgenic rabbits producing human antibodies. Also reported herein is a common light chain variable domain amino acid sequence, multispecific antibodies comprising the common light chain variable domain and transgenic rabbits comprising the respective common light chain locus.

Background of the Invention

The production of multispecific antibodies is hampered by the problem of chain mispairing resulting in un-paired and mispaired by-product formation. Depending on the chosen format a not neglectable number and amount of these by-products can be formed.

Different approaches for addressing this problem have been developed.

To reduce heavy chain mispairing the knobs-into-hole technology (see e.g. Ridgway, J.B., et al. Prot. Eng. 9 (1996) 617-621) or the CrossMab format (see e.g. Schaefer, W., et al. Proc. Natl. Acad. Sci USA 108 (2011) 11187-11192) have been reported.

To reduce light chain mispairing a common light chain can be employed. This approach inherently requires that for both binding sites each formed by a pair of an antibody heavy chain variable domain and an antibody light chain variable domain the same antibody light chain variable domain has to be used.

Non-human animals comprising a human immunoglobulin locus can be used to produce monospecific antibodies having a common light chain. The human immunoglobulin locus in such animals generally comprises a reduced and limited number of heavy chain germline genes, rearranged germline heavy chain genes or heavy chain V gene segments and a single light chain gene. When such a non-human animal is immunized in order to produce antibodies the elicited immune response comprises antibodies with a plurality of different heavy chain variable domains but only a single light chain variable domain.

The design and development of a new common light chain suitable for fitting to de-novo generated antibodies is demanding. Thus, this approach is not deemed the

first choice for developing recombinant, multispecific antibodies, as it is very likely that further optimization is required and sequence modifications have to be made.

Common light chains and methods to generate such common light chains are reported, e.g., in WO 98/50431, WO 2010/084197, US 2013/045492, WO 2011/097603 and WO 2012/148873.

In WO 2004/009618 a common VL is reported in SEQ ID NO: 1 (comprised in UBS54 and K53). In SEQ ID NO: 18 a common light chain obtained from phages directed against CD22 (clone B28), CD72 (clone II-2) and HLA-DR (class II; clone I-2) is reported.

In US 2007/098712 common VL sequences of anti-Ob-R antibody clone 26 and anti-HER3 antibody clone 18 were used to construct a bispecific antibody. Also reported is that the anti-Mpl scFv 12B5 (GenBank accession number AF048775) and the anti-HER3 scFv clone H6 (GenBank accession number AF048774) utilize identical VL sequences and substantially different VH sequences.

In WO 2010/84197 a recombinant antibody comprising a heavy chain and a light chain, wherein the light chain comprises the sequence as set forth in SEQ ID NO: 8 is reported. SEQ ID NO: 8 is the amino acid sequence of V-segment VKVI-2-l-(l)-A14 (IGKV6D-41*01). Further amino acid sequences of common light chains are reported in SEQ ID NO: 12 to 14.

Another common light chain approach is reported in US 2010/0331527, wherein two antibodies of different specificity use the same light chain.

In WO 2011/097603 engineered human Vkappa and Vlambda common light chains based on the human Vkappa 1-39Jkappa 5 locus, the human Vkappa 3-20Jkappa 1 locus and the human VpreBJlambda 5 locus are reported.

Common light chains and methods for making them are reported in US 2012/0192300, US 2012/021409, US 2011/0195454, and US 2013/0045492.

In WO 2012/018764 genetically modified mice and methods for making and using them are reported, wherein the mice comprise a replacement of all or substantially all immunoglobulin heavy chain V gene segments, D gene segments, and J gene segments with at least one light chain V gene segment and at least one light chain J gene segment.

In WO 2013/157953 a germline-like common light chain derived from the rearranged germline human kappa light chain IgVK1-39/JK or IGVK3-20/JK is reported.

In WO 2014/22540 it is outlined that a universal light chain can be a κ light chain selected from a VK1-39 and a VK3-20 light chain or a λ light chain selected from a VL1-40 and a VL2-14 light chain. In a specific embodiment the human VL gene segment is a human VK1-39JK5 gene segment or a human VK3-20JK1 gene segment.

In WO 2014/51433 the common light chain 012 is reported, which is the human rearranged kappa light chain IgVK1-39*01/IgJK1*01. This sequence is a germline sequence that is frequently used in the human repertoire and has superior ability to pair with many different VH regions, and has good thermodynamic stability, yield and solubility.

In US 2015/037337 it is reported that human JH6*02 is a common, conserved variant in humans, and thus a good candidate for construction of a transgenic IgH locus.

In WO 2015/052230 in SEQ ID NO: 6 the amino acid sequence of modified heavy chain CH3-CH2-CH1-VL, wherein VL is a variable domain of a common light chain (CLC-Fc cross-MAb), is reported.

In WO 2015/153765 common light chains are reported in the N-term - VL - CK - C-term fusion polypeptides of SEQ ID NO: 78 and 79.

Transgenic rabbits comprising a human immunoglobulin locus are reported in WO 2000/46251, WO 2002/12437, WO 2005/007696, WO 2006/047367, US 2007/0033661, and WO 2008/027986.

Summary of the Invention

One aspect as reported herein is a common antibody light chain variable domain that has the amino acid sequence

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP
GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP
EDFATYYCQQ SYSTPLTFGG GTKVEIK (SEQ ID NO: 01)

or a variant thereof.

One aspect as reported herein is a common antibody light chain comprising a light chain variable domain that has the amino acid sequence

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP
GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP
EDFATYYCQQ SYSTPLTFGG GTKVEIK (SEQ ID NO: 01)

or a variant thereof.

In one embodiment the common light chain comprises up to 13 amino acid mutations. In one preferred embodiment the common light chain comprises up to 13 amino acid mutations, whereof at most 11 are in the HVRs.

In one embodiment the common light chain comprises up to 11 amino acid mutations.

In one embodiment the common light chain comprises 1 to 11 amino acid mutations within the amino acid sequence of SEQ ID NO: 01. In one preferred embodiment the common light chain comprises 1 to 13 amino acid mutations within the amino acid sequence of SEQ ID NO: 01, whereof at most 11 mutations are in the HVRs.

In one embodiment a variant of the common antibody light chain as reported herein comprises a light chain variable domain that has a sequence identity to SEQ ID NO: 01 of 90 % or more (i.e. comprises up to 11 mutations). In one embodiment the sequence identity is 95 % or more. In one embodiment the sequence identity is 98 % or more.

One aspect as reported herein is an antibody comprising a light chain as reported herein.

One aspect as reported herein is a multispecific antibody comprising two or more different heavy chain variable domains and two or more common light chain variable domains as reported herein.

In one embodiment the multispecific antibody is a bispecific full-length antibody comprising two different heavy chains and two common light chain variable domains or two common antibody light chains as reported herein.

In one embodiment the multispecific antibody is a trispecific antibody comprising three different heavy chain variable domains and three common light chain variable domains as reported herein.

In one embodiment the multispecific antibody is a tetraspecific antibody comprising four different heavy chain variable domains and four common light chain variable domains as reported herein.

One aspect as reported herein is the use of a common antibody light chain as reported herein for the generation of bispecific antibodies.

In one embodiment the use is by combining two common antibody light chains with a first antibody heavy chain and a second antibody heavy chain, wherein the first antibody heavy chain together with a common antibody light chain forms a first antigen binding site and the second antibody heavy chain together with a common antibody light chain forms a second antigen binding site.

One aspect as reported herein is a transgenic vector comprising a humanized immunoglobulin light chain locus, wherein said humanized immunoglobulin light chain locus comprises

(a) a V gene segment derived from human light chain V segment IGKV1-39-01,

(b) 3' proximal to said light chain gene segment a promoter, and

(c) 5' proximal to said light chain gene segment at least a fragment of the human IGKJ4 J-element.

In one embodiment transgenic vector comprises a humanized light chain locus, wherein said humanized light chain locus comprises

(a) as V gene segment the human light chain V segment IGKV1-39-01,

(b) 3' proximal to said light chain gene segment a promoter, and

(c) 5' proximal to said light chain gene segment the human IGKJ4 J-element or a functional fragment thereof.

One aspect as reported herein is a transgenic rabbit comprising the humanized immunoglobulin light chain locus present in the transgenic vector as reported herein. In one embodiment the transgenic rabbit has an essentially intact endogenous regulatory and antibody production machinery.

In one embodiment the transgenic rabbit further comprises

(1) a transgene derived from the rabbit immunoglobulin heavy chain locus, substituted with 8 human VH elements, human JH1-JH6 elements, human Cμ-coding regions fused to human bcl2 coding sequence, and human Cγ coding regions;

(2) a transgene derived from the rabbit immunoglobulin light chain locus, comprising the human Vκ element IGKV1-39-01 and the human IgKJ4 J-element;

(3) transgenes derived from the human CD79α and CD79β loci; and

(4) loss-of-function mutations within the rabbit Cμ and rabbit Cκ loci.

One aspect as reported herein is a B-cell from the transgenic rabbit as reported herein, comprising the humanized immunoglobulin light chain locus present in the transgenic vector as reported herein.

One aspect as reported herein is a method for producing a human immunoglobulin using the transgenic rabbit as reported herein.

In one embodiment the human immunoglobulin is an antibody. In one embodiment the human immunoglobulin is a polyclonal antibody. In one preferred embodiment the human immunoglobulin is a monoclonal antibody.

Detailed Description of the Invention

DEFINITIONS

The term "common light chain variable domain" as used herein denotes a specific antibody light chain variable domain amino acid sequence that can pair with different antibody heavy chain variable domain amino acid sequence to form a functional antigen binding site of different specificities, i.e. bind to different epitopes either on the same antigen or on different antigens. The common light chain variable domain has in one embodiment an amino acid sequence identity of at least 80%, or at least 90%, or at least 95%, or in a preferred embodiment more than 98% to SEQ ID NO: 01. The amino acid residue differences normally have only little or even no effect on antigen binding. Thus, the term „common light chain variable domain“ also encompasses antibody light chain variable domains which have some minor amino acid sequence differences but which when paired with the same heavy chain of an antibody form a binding site of the same specificity and comparable affinity.

It is possible to identify common light chain variable domains which on the one hand are not identical but on the other hand are functionally equivalent. This is possible, for example, by introducing and testing conservative amino acid mutations, changes of amino acids residues in parts of the common light chain that do not or only slightly influence the binding specificity of the binding site when the common light chain is paired with an antibody heavy chain variable domain.

"Operably linked" refers to a juxtaposition of two or more components, wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a promoter and/or enhancer are operably linked to a coding sequence, if it acts in cis to control or modulate the transcription of the linked sequence. Generally, but not necessarily, the DNA sequences that are "operably linked" are contiguous and, where necessary to join two protein encoding regions such as a secretory leader and a polypeptide, contiguous and in (reading) frame. However, although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. Enhancers do not have to be contiguous. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within or downstream of coding sequences and at considerable distance from the promoter. A polyadenylation site is operably

linked to a coding sequence if it is located at the downstream end of the coding sequence such that transcription proceeds through the coding sequence into the polyadenylation sequence. A translation stop codon is operably linked to an exonic nucleic acid sequence if it is located at the downstream end (3' end) of the coding sequence such that translation proceeds through the coding sequence to the stop codon and is terminated there. Linking is accomplished by recombinant methods known in the art, e.g., using PCR methodology and/or by ligation at convenient restriction sites. If convenient restriction sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "isolated" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman, S. et al., J. Chromatogr. B 848 (2007) 79-87.

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and

methods utilizing transgenic animals containing all or part of the human immunoglobulin loci, such methods and other exemplary methods for making monoclonal antibodies being described herein.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B,

and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

The term "pharmaceutical formulation" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

ANTIBODY GENERATION IN MAMMALS

Antibody gene generation (see Molecular Biology of the Cell. 4th edition. Alberts B, Johnson A, Lewis J, et al. New York: Garland Science; 2002; and Immunobiology: The Immune System in Health and Disease. 5th edition. Janeway, C.A. Jr, Travers P, Walport M, et al. New York: Garland Science; 2001):

The genetic locus for the λ light chain (chromosome 22) has about 30 functional Vλ gene segments and four pairs of functional Jλ gene segments and Cλ genes. The κ locus (chromosome 2) is organized in a similar way, with about 40 functional Vκ gene segments accompanied by a cluster of five Jκ gene segments but with a single Cκ gene. In approximately 50% of individuals, the entire cluster of κ V gene segments has undergone an increase by duplication. The heavy-chain locus (chromosome 14) has about 65 functional VH gene segments and a cluster of around 27 D segments lying between these VH gene segments and six JH gene segments. The heavy-chain locus also contains a large cluster of CH genes. The total length of the heavy-chain locus is over 2 megabases (2 million bases), whereas some of the D segments are only six bases long.

The V region, or V domain, of an immunoglobulin heavy or light chain is encoded by more than one gene segment. For the light chain, the V domain is encoded by two separate DNA segments. The first segment encodes the first 95–101 amino acids of the light chain and is termed a V gene segment because it encodes most of the V domain. The second segment encodes the remainder of the V domain (up to 13 amino acids) and is termed a joining or J gene segment. Thus, of the three hypervariable loops in the variable domains of immunoglobulins, two are encoded within the V gene segment DNA, whereas the third (HV3 or CDR3) falls at the

joint between the V gene segment and the J gene segment, and in the heavy chain is partially encoded by the D gene segment. In both heavy and light chains, the diversity of CDR3 is significantly increased by the addition and deletion of nucleotides at two steps in the formation of the junctions between gene segments. The added nucleotides are known as P-nucleotides and N-nucleotides.

During B-cell development, the V and J gene segments (for the light chain) and the V, D, and J gene segments (for the heavy chain) are joined together to form a functional VL- or VH-region coding sequence by a process of site-specific recombination called V(D)J joining. Conserved DNA sequences flank each gene segment and serve as recognition sites for the joining process, ensuring that only appropriate gene segments recombine. Thus, for example, a V segment will always join to a J or D segment but not to another V segment. Joining is mediated by an enzyme complex called the V(D)J recombinase. This complex contains two proteins that are specific to developing lymphocytes, as well as enzymes that help repair damaged DNA in all our cells.

Any of the 40 V segments in the human κ light-chain gene-segment pool, for example, can be joined to any of the 5 J segments, so that at least 200 (40 × 5) different κ-chain V regions can be encoded by this pool. Similarly, any of the 51 V segments in the human heavy-chain pool can be joined to any of the 6 J segments and any of the 27 D segments to encode at least 8262 (51 × 6 × 27) different heavy-chain V regions.

The combinatorial diversification resulting from the assembly of different combinations of inherited V, J, and D gene segments just discussed is an important mechanism for diversifying the antigen-binding sites of antibodies. By this mechanism alone, a human can produce 287 different VL regions (200 κ and 116 λ) and 8262 different VH regions.

In most cases of site-specific recombination, DNA joining is precise. But during the joining of antibody (and T cell receptor) gene segments, a variable number of nucleotides are often lost from the ends of the recombining gene segments, and one or more randomly chosen nucleotides may also be inserted. This random loss and gain of nucleotides at joining sites is called junctional diversification, and it enormously increases the diversity of V-region coding sequences created by recombination, specifically in the third hypervariable region.

THE COMMON LIGHT CHAIN AS REPORTED HEREIN

Herein is reported a humanized light chain locus.

The invention is based at least in part on the finding that a humanized light chain immunoglobulin locus comprising multiple V gene elements but only single V gene element combined with a promoter can be used as common light chain locus in a transgenic rabbit.

The humanized light chain locus as reported herein comprises

(a) a V gene segment derived from human light chain V segment IGKV1-39-01,

(b) 3' proximal to said light chain gene segment a promoter, and

(c) 5' proximal to said light chain gene segment at least a fragment of the human IGKJ4 J-element.

The complete light chain V gene segment IGKV1-39-01 has the following nucleic acid sequence (see e.g. GenBank X93627, Homo sapiens germline immunoglobulin kappa light chain, variable region (DPK9); 287 bp; SEQ ID NO: 02):

```
gacatccaga tgacccagtc tccatcctcc ctgtctgcat ctgtaggaga
cagagtcacc atcacttgcc gggcaagtca gagcattagc agctatttaa
attggtatca gcagaaacca gggaaagccc ctaagctcct gatctatgct
gcatccagtt tgcaaagtgg ggtcccatca aggttcagtg gcagtggatc
tgggacagat ttcactctca ccatcagcag tctgcaacct gaagattttg
caacttacta ctgtcaacag agttacagta cccctcc.
```

The corresponding amino acid sequence is (SEQ ID NO: 03):

```
DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA
ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTP
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The full-length human IgKJ4*01/02 has the following nucleic acid (SEQ ID NO: 04) and amino acid (SEQ ID NO: 05) sequences:

```
nucleic acid:    ctcactttcggcggagggaccaaggtggagatcaaa
amino acid:      L  T  F  G  G  G  T  K  V  E  I  K
```

The use of a common light chain enables the generation of multispecific antibodies (e.g. bispecific full length antibodies) by combining different heavy chain variable domains, each binding to a different epitope/antigen/target with the same light chain variable domain or the same variant thereof and thereby reducing the side-product complexity.

In one embodiment the humanized light chain locus comprises 25 to 30 human Vκ elements and a human Cκ coding region, wherein

(a) the 3' proximal Vκ element is a V gene segment derived from human light chain V segment IGKV1-39-01,

(b) to said 3' proximal light chain gene segment (3' proximal) a promoter is operably linked, and

(c) 5' proximal to said light chain gene segment at least a fragment of the human IGKJ4 J-element is operably linked.

In one embodiment the promoter is a human kappa variable region promoter (subgroup V kappa I).

In one embodiment the V gene segment comprises a human kappa immunoglobulin light chain leader peptide encoding nucleic acid. In one embodiment the leader peptide has the amino acid sequence of SEQ ID NO: 15.

In one embodiment the V gene segment comprises a human kappa immunoglobulin leader peptide encoding nucleic acid and a chicken derived spacer sequence between the leader peptide encoding nucleic acid sequence and the V gene segment. In one embodiment the chicken derived spacer sequence is SEQ ID NO: 16.

The light chain immunoglobulin locus encodes the following light chain V-segment (SEQ ID NO: 03, HVRs underlined):

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTP

and the following human J-element (SEQ ID NO: 05, part of the HVR-L3 is underlined):

LTFGG GTKVEIK.

Thus, one aspect as reported herein is an antibody light chain that comprises a light chain variable domain with the amino acid sequence

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG GTKVEIK

or a variant thereof.

Also encompassed herein are variants of this amino acid sequence that arise due to gene conversion and hypermutation in the rabbit during B-cell maturation.

In one embodiment the (mature) light chain comprises 1 to 4 amino acid mutations with respect to the light chain encoded by the light chain immunoglobulin locus outside the HVRs.

In one embodiment the (mature) light chain comprises 1 to 15 amino acid mutations with respect to the light chain encoded by the light chain immunoglobulin locus.

In one embodiment the (mature) light chain comprises 1 to 11 amino acid mutations with respect to the light chain encoded by the light chain immunoglobulin locus.

In one embodiment the (mature) light chain comprises 1 to 15 amino acid mutations with respect to the light chain encoded by the light chain immunoglobulin locus, whereof at most 11 are in the HVRs.

One aspect as reported herein is a bispecific full-length antibody comprising two different heavy chains and two light chains, whereby the light chains are identical and the variable domains have an amino acid sequence as reported herein.

TRANSGENIC RABBITS

The light chain locus as reported herein can be used in the generation of human immunoglobulin producing transgenic rabbits.

Thus, one aspect as reported herein is a light chain transgenic rabbit with a humanized immunoglobulin light chain locus as reported herein.

The transgenic rabbit has a humanized immunoglobulin locus and still has the antibody maturation process of a wild-type rabbit, using e.g. gene conversion in order to generate antibody diversity. Therefore the heavy chain and light chain loci of a wild-type rabbit have been inactivated and respective humanized immunoglobulin transgene loci have been introduced into the genome of the rabbit enabling the rabbit to produce human(ized)/human-like antibodies. The genotype of the transgenic rabbit can be described as follows:

the transgenic rabbit comprises

(1) a transgene derived from the rabbit immunoglobulin heavy chain locus, substituted with 8 human VH elements, human JH1-JH6 elements, human Cμ-coding regions fused to human bcl2 coding sequence, and human Cγ coding regions;

(2) a transgene derived from the rabbit immunoglobulin light chain locus, comprising the human Vκ element IGKV1-39-01 and the human IgKJ4 J-element;

(3) transgenes derived from the human CD79α and CD79β loci; and

(4) loss-of-function mutations within the rabbit Cμ and rabbit Cκ loci.

Herein is reported a transgenic rabbit comprising a humanized immunoglobulin heavy chain locus and a humanized immunoglobulin light chain locus, wherein

i) the humanized heavy chain immunoglobulin locus is derived from an immunoglobulin locus or a portion of an immunoglobulin locus of a rabbit, and comprises multiple immunoglobulin heavy chain gene segments wherein

(a) at least one of said heavy chain gene segments is a human heavy chain V segment of the VH3 family as 3' proximal V gene segment flanked by nucleotide sequences comprising (between 20 and 1000 contiguous nucleotides from) a rabbit spacer sequence of SEQ ID NO: 06,

(b) said gene segments are juxtaposed in an unrearranged, or partially rearranged, or fully rearranged configuration, and

(c) said humanized immunoglobulin locus is capable of undergoing gene rearrangement, if necessary, and gene conversion and/or

hypermutation, and producing a repertoire of humanized immunoglobulins in said rabbit,

and

ii) the humanized light chain immunoglobulin locus comprises

(a) a V gene segment derived from human light chain V segment IGKV1-39-01,

(b) 3' proximal to said light chain gene segment a promoter, and

(c) 5' proximal to said light chain gene segment at least a fragment of the human IGKJ4 J-element.

In one embodiment the transgenic rabbit is homozygous for the humanized heavy chain locus and the humanized light chain locus.

In one embodiment the transgenic rabbit is heterozygous for the humanized heavy chain locus and the humanized light chain locus.

In one embodiment the transgenic rabbit is inactivated for endogenous antibody heavy chain expression and/or endogenous antibody light chain expression.

One aspect as reported herein is a B-cell from the transgenic rabbit as reported herein comprising the humanized light chain immunoglobulin locus as reported herein.

One aspect as reported herein is an isolated B-cell comprising the humanized light chain immunoglobulin locus as reported herein.

In one embodiment the B-cell further comprises a humanized heavy chain immunoglobulin locus that is derived from an immunoglobulin locus or a portion of an immunoglobulin locus of a rabbit, comprising multiple immunoglobulin heavy chain gene segments wherein

(a) at least one of said heavy chain gene segments is a human heavy chain V segment of the VH3 family flanked by nucleotide sequences comprising (between 20 and 1000 contiguous nucleotides from) a rabbit spacer sequence of SEQ ID NO: 06,

(b) said gene segments are juxtaposed in an unrearranged, partially rearranged or fully rearranged configuration, and

(c) said humanized immunoglobulin locus is capable of undergoing gene rearrangement, if necessary, and gene conversion and/or hypermutation, and producing a repertoire of human immunoglobulins in said rabbit.

Also an aspect as reported herein is a method for producing a human immunoglobulin using the transgenic rabbit as reported herein.

In one embodiment the human immunoglobulin is obtained from the blood of the rabbit.

Herein is reported a rabbit having a genome comprising a modification of the heavy chain immunoglobulin locus and the light chain immunoglobulin locus, wherein the modification is the inactivation of the endogenous rabbit immunoglobulin loci and the introduction of humanized immunoglobulin loci, resulting in a transgenic rabbit. The genome of the transgenic rabbit, thus, comprises exogenous nucleic acid sequences encoding different human immunoglobulin heavy chain variable domains and a (single functional) human immunoglobulin light chain variable domain.

The humanized immunoglobulin loci, i.e. the respective nucleic acid sequences, are integrated into the rabbit genome. The modification of the immunoglobulin loci is an insertion of one or more transgenic human immunoglobulin gene segments sequences with the concomitant inactivation of the respective one or more endogenous rabbit immunoglobulin gene segments.

The term “humanized immunoglobulin locus” denotes an isolated immunoglobulin locus comprising one or more human elements, such as one or more V-regions and/or none, and/or one or more J-elements. These are combined with exogeneous elements, i.e. combined with genetic elements not combined therewith in nature, such as promoters and/or regulatory elements from non-human organisms.

The transgenic rabbit as reported herein can be used for the generation of human antibodies. Thus, one aspect as reported herein is an (isolated) B-cell or (isolated) tissue from a transgenic rabbit as reported herein.

Also an aspect as reported herein is the use of a transgenic rabbit as reported herein for the generation of either (i) a chimeric antibody comprising human heavy chain and light chain variable regions and rabbit constant regions, or (ii) a fully human antibody.

An aspect as reported herein is a method for producing an antibody specifically binding to an antigen comprising the steps of:

(a) immunizing a transgenic rabbit as reported herein (with the antigen),

(b) isolating at least one cell from the immunized transgenic rabbit producing an antibody specifically binding the antigen,

(c) culturing the at least one cell of step (b) as single deposited cell to produce the antibody.

In one embodiment the at least one cell obtained in step b) is a splenocyte. In one embodiment the at least one cell obtained in step b) is a B-cell.

Also an aspect as reported herein is a method for producing an antibody specifically binding to an antigen (of interest) comprising the steps of:

(a) providing one or more B-cell(s) from a transgenic rabbit as reported herein, which had been immunized with the antigen (of interest),

(b) culturing the at least one or more B-cell(s) of step (a) as single deposited cell to produce the antibody.

Also an aspect as reported herein is a method for producing an antibody specifically binding to an antigen comprising the steps of:

(a) cultivating a mammalian cell comprising a nucleic acid encoding an antibody specifically binding to the antigen, wherein at least the nucleic acid encoding the variable domains as the antibody had been obtained from a transgenic rabbit as reported herein that had been immunized with the antigen,

(b) recovering the antibody from the mammalian cell or the cultivation medium.

In one embodiment the antibody is a monoclonal antibody.

In one embodiment the immunizing is with the antigen, with DNA encoding the antigen, with the antigen and DNA encoding the antigen, or with cells expressing the antigen.

In one embodiment the immunizing is performed by administering the antigen, DNA encoding the antigen, the antigen together with DNA encoding the antigen, or cells expressing the antigen to the transgenic rabbit as reported herein.

The following examples and sequences are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Example 1

Immunization of rabbits

The transgenic rabbits used for immunization contained (1) a transgene derived from the rabbit immunoglobulin heavy chain locus, substituted with 8 human VH elements, human JH1-JH6 elements, human Cμ-coding regions fused to human bcl2 coding sequence, and human Cγ coding regions; (2) a transgene derived from the rabbit immunoglobulin light chain locus, substituted with 25 human Vκ elements, the proximal Vκ element fused to human Jκ4, and a human Cκ coding region; (3) transgenes derived from the human CD79a and CD79b loci; and (4) loss-of-function mutations within the rabbit Cμ and rabbit Cκ loci.

Protein immunization

Rabbits were immunized with 400 μg recombinant soluble antigen, emulsified with complete Freund's adjuvant, at day 0 by intradermal application, and with 200 μg each of antigen, emulsified with complete Freund's adjuvant, at days 7, 14, 42, 70 and 84 or 98, by alternating intramuscular and subcutaneous applications. Blood (10% of estimated total blood volume) was taken at around days 20-21, 34-48, 62-76 and 90-104. Serum was prepared, which was used for titer determination by ELISA, and peripheral mononuclear cells were isolated, which were used as a source of antigen-specific B-cells in the B-cell cloning process. Accordingly human antibodies were obtained.

DNA immunization

Rabbits were immunized genetically, using a plasmid expression vector coding for full-length antigen, by intradermal application of 400 µg vector DNA, followed by electroporation (5 square pulses of 750 V/cm, duration 10 ms, interval 1 s). Rabbits received 7 consecutive immunizations at days 0, 14, 28, 49, 70, 98 and 126. Blood (10% of estimated total blood volume) was taken at days 35, 77, 105 and 133. Serum was prepared, which was used for titer determination by ELISA, and peripheral mononuclear cells were isolated, which were used as a source of antigen-specific B-cells in the B-cell cloning process.

Example 2

Determination of serum titers

Antigen was immobilized on a 96-well NUNC Maxisorb plate at 1.75-2 µg/ml, 100 µl/well, in PBS, followed by: blocking of the plate with 2% CroteinC in PBS, 200 µl/well; application of serial dilutions of antisera, in duplicates, in 0.5% CroteinC in PBS, 100 µl/well; detection with either (1) HRP-conjugated donkey anti-rabbit IgG antibody (Jackson Immunoresearch), or (2) HRP-conjugated rabbit anti-human IgG antibody (Pierce/Thermo Scientific; 1/5000), or (3) biotinylated goat anti-human kappa antibody (Southern Biotech/Biozol; 1/5000) and streptavidin-HRP; each diluted in 0.5% CroteinC in PBS, 100 µl/well. For all steps, plates were incubated for 1 h at 37°C. Between all steps, plates were washed 3-times with 0.05% Tween 20 in PBS. Signal was developed by addition of BM Blue POD Substrate soluble (Roche), 100 µl/well; and stopped by addition of 1 M HCl, 100 µl/well. Absorbance was read out at 450 nm, against 690 nm as reference. Titer was defined as dilution of antisera resulting in half-maximal signal.

Example 3

B-cell cloning and sorting

Isolation of rabbit peripheral blood mononuclear cells (PBMC)

Transgenic rabbits of Example 1 were used as a source of blood. EDTA containing whole blood was diluted two-fold with 1x PBS before density centrifugation on lympholyte mammal (Cedarlane Laboratories, Burlington, Ontario, Canada) according to the specifications of the manufacturer. PBMCs were washed twice with 1x PBS before staining with antibodies.

EL-4 B5 medium

RPMI 1640 (Pan Biotech, Aidenbach, Germany) supplemented with 10% FCS (Hyclone, Logan, UT, USA), 2 mM Glutamine, 1% penicillin/streptomycin solution (PAA, Pasching, Austria), 2 mM sodium pyruvate, 10 mM HEPES (PAN Biotech, Aidenbach, Germany) and 0.05 mM β-mercaptoethanol (Gibco, Paisley, Scotland).

Depletion of macrophages/monocytes

Sterile 6-well plates (cell culture grade) were used to deplete macrophages and monocytes through unspecific adhesion. Each well was filled at maximum with 4 ml media and up to $6x10^6$ peripheral blood mononuclear cells from the immunized rabbit and allowed to bind for 1 h at 37°C and 5% CO_2 in the incubator. The cells in the supernatant were used for the antigen panning step.

Coating of plates

Sterile cell culture 6-well plates were coated with 2 µg/ml antigen protein, or sterile streptavidin coated 6-well plates (Microcoat, Bernried, Germany) were coated with 2 µg/ml biotinylated antigen for 3 hours at room temperature or overnight at 4 °C. Plates were washed in sterile PBS three times before use.

Enrichment of B cells on the antigen protein

6-well tissue culture plates coated with antigen protein were seeded with up to $6x10^6$ cells per 4 ml medium and allowed to bind for 1 h at 37 °C and 5% CO_2 in the incubator. After the enrichment step on antigen protein non-adherent cells were removed by carefully washing the wells 1-2 times with 1x PBS. The remaining sticky cells were detached by trypsin for 10 min. at 37 °C in the incubator. Trypsination was stopped with EL-4 B5 medium. Then the cells were washed twice in media. The cells were kept on ice until the immune fluorescence staining.

Immune fluorescence staining and Flow Cytometry

Anti-IgG FITC antibody (AbD Serotec, Düsseldorf, Germany) was used for single cell sorting. For surface staining, cells from the depletion and enrichment step were incubated with the anti-IgG FITC antibody in PBS for 30-45 min. rolling in the cold room at 4°C in the dark. Following centrifugation, the supernatants were removed by aspiration. The PBMCs were subjected to 2 cycles of centrifugation

and washing with ice cold PBS. Finally the PBMCs were resuspended in ice cold PBS and immediately subjected to the FACS analyses. Propidium iodide in a concentration of 5 µg/ml (BD Pharmingen, San Diego, CA, USA) was added prior to the FACS analyses to discriminate between dead and live cells.

A Becton Dickinson FACSAria equipped with a computer and the FACSDiva software (BD Biosciences, USA) were used for single cell sort.

B-cell cultivation

The cultivation of the rabbit B-cells was done by a method described by Seeber, S., et al., PLoS One 9 (2014) e86184. Briefly, single sorted rabbit B-cells were incubated in 96-well plates with 200 µl/well EL-4 B5 medium containing Pansorbin cells (1:100,000) (Calbiochem (Merck), Darmstadt, Deutschland), 5% rabbit thymocyte supernatant (MicroCoat, Bernried, Germany) and gamma-irradiated murine EL-4 B5 thymoma cells (2.5 × $10e^4$ cells/well) for 7 days at 37 °C in the incubator. The supernatants of the B-cell cultivation were removed for screening and the remaining cells were harvested immediately and were frozen at -80 °C in 100 µl RLT buffer (Qiagen, Hilden, Germany).

Example 4

B-cell PCR

Total RNA was prepared from B-cells lysate (resuspended in RLT buffer) using the NucleoSpin 8/96 RNA kit (Macherey&Nagel) according to manufacturer's protocol. RNA was eluted with 60 µl RNase free water. 6 µl of RNA was used to generate cDNA by reverse transcriptase reaction using the Superscript III First-Strand Synthesis SuperMix (Invitrogen) and an oligo dT-primer according to the manufacturer's instructions. All steps were performed on a Hamilton ML Star System. 4 µl of cDNA were used to amplify the immunoglobulin heavy and light chain variable regions (VH and VL) with the AccuPrime SuperMix (Invitrogen) in a final volume of 50 µl using the primers rbHC.up and rbHC.do for the heavy chain and BcPCR_FHLC_leader.fw and BcPCR_huCkappa.rev for the light chain. All forward primers were specific for the signal peptide (of respectively VH and VL) whereas the reverse primers were specific for the constant regions (of respectively VH and VL). The PCR conditions for the RbVH+RbVL were as follows: Hot start at 94°C for 5 min.; 35 cycles of 20 sec. at 94°C, 20 sec. at 70°C, 45 sec. at 68 °C, and a final extension at 68°C for 7 min. The PCR conditions for the HuVL were as

follows: Hot start at 94°C for 5 min.; 40 cycles of 20 sec. at 94°C, 20 sec. at 52°C, 45 sec. at 68 °C, and a final extension at 68°C for 7 min.

Primer sequences:

rbHC.up (SEQ ID NO: 07)	AAGCTTGCCACCATGGAGACTGGGCTGCGCTGGCTTC
rbHCf.do (SEQ ID NO: 08)	CCATTGGTGAGGGTGCCCGAG
BcPCR_FHLC_leader.fw (SEQ ID NO: 09)	ATGGACATGAGGGTCCCCGC
BcPCR_huCkappa.rev (SEQ ID NO: 10)	GATTTCAACTGCTCATCAGATGGC

8µl of 50µl PCR solution were loaded on a 48 E-Gel 2% (Invitrogen G8008-02). Positive PCR reactions were cleaned using the NucleoSpin Extract II kit (Macherey&Nagel; 740609250) according to manufacturer's protocol and eluted in 50 µl elution buffer. All cleaning steps were performed on a Hamilton ML Starlet System.

The used antigen was the extracellular domain of TPBG (trophoblast glycoprotein, SEQ ID NO: 11).

The resulting antibodies for the extracellular domain of TPBG have the following light chain variable domains:

051 (SEQ ID NO: 12): DIQMTQSPSS VSASVGDRVT ITCRASQGIY SWLAWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SDSPPYTFGQ GTKLEIK,

091 (SEQ ID NO: 13): DIQMTQSPSS LSASVGDRVT ITCQASQDIS NYLNWYQQKP GKAPKLLIYA ASTLQIGVPS RFSGSGSGTD FTFTISSLQP EDFATYYCQQ ANSFPLTFGG GTKVEIK,

097 (SEQ ID NO: 14): DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SDSFPLTFGG GTKVEIK.

Example 5

Binding of TPBG-specific Fab fragments to TPBG

To assess binding of recombinant TPBG, Nunc Maxisorb streptavidin coated plates (MicroCoat #11974998001) were coated with 25 µl/well biotinylated human TPBG-AviHis at a concentration of 100 ng/ml. Plates were incubated at 4°C overnight. After washing (3x90 µl/well with PBST-buffer) anti-TPBG samples were added in a 1:2 dilution series starting at 2 µg/ml and incubated 1 h at RT. After washing (3x90 µl/well with PBST-buffer) 25µl/well goat anti c-myc HRP (Bethyl, # A190-104P) or goat anti hu kappa HRP (Millipore, # AP502P) was added in a 1:7000 or 1:4000 dilution, respectively and incubated at RT for 1 h on a shaker. After washing (3x90 µl/well with PBST-buffer) 25 µl/well TMB substrate (Calbiochem, #CL07) was added and incubated 2 min. Measurement took place at 370/492 nm on a Safire2 reader (Tecan).

To assess cellular binding of human TPBG, the human breast cancer tumor cell line MFC7 endogenously expressing TPBG was seeded at a concentration of 21000 cells/well in 384-well cellcoat Poly-D-Lysine plates (Greiner, #781940). Cells were allowed to attach over night at 37°C. After removing the supernatant, 25 µl/well of supernatant containing anti-TPBG antibodies were added in a 1:2 dilution series starting at 5 µg/ml and incubated 1 h at 4°C. Upon washing (2 x 50 µl/well PBST) cells were fixed by adding 50 µl/well 0.05% Glutaraldehyde (Sigma, 25%) diluted in 1xPBS-buffer and incubated for 10 min at RT. After washing (3 times; 90 µl/well PBS-T), 25 µl/well secondary antibody was added for detection: goat anti c-myc HRP (1:5000, Bethyl) followed by 1 h incubation at room temperature on a shaker. After washing (3 times; 90 µl/well PBS-T) 25 µl/well TMB substrate solution (Calbiochem) was added. After 10 min at room temperature, measurement took place at 370/492 nm on a Safire2 reader (Tecan).

Table: Binding of anti-TPBG Fab fragments to human TPBG

EC50 [ng/ml]	recombinant TPBG	MCF7
051	18.1	57.5
091	27.7	14.0
097	15.2	451.3

Fab fragments of 051, 091, and 097 were found to bind to human TPBG or recombinant source or expressed on cells of a human breast cancer cell line.

Patent Claims

1. Use of a common antibody light chain comprising a variable domain that has the amino acid sequence of SEQ ID NO: 01 or is a variant thereof for the generation of bispecific antibodies.

2. The use according to claim 1 wherein the use is by combining two common antibody light chains with a first antibody heavy chain and a second antibody heavy chain, wherein the first antibody heavy chain together with a common antibody light chain forms a first antigen binding site and the second antibody heavy chain together with a common antibody light chain forms a second antigen binding site.

3. The use according to any one of claims 1 to 2 wherein the common light chain comprises 1 to 11 amino acid mutations within the amino acid sequence of SEQ ID NO: 01.

4. The use according to any one of claims 1 to 3, wherein the common light chain comprises 1 to 13 amino acid mutations within the amino acid sequence of SEQ ID NO: 01, whereof at most 11 mutations are in the HVRs.

5. A bispecific full-length antibody comprising two heavy chains and two common light chains each comprising a variable domain that has the amino acid sequence of SEQ ID NO: 01 or is a variant thereof.

6. A transgenic vector comprising a humanized light chain locus, wherein said humanized light chain locus comprises

   (a) as V gene segment the human light chain V segment IGKV1-39-01,

   (b) 3' proximal to said light chain gene segment a promoter, and

   (c) 5' proximal to said light chain gene segment the human IGKJ4 J-element or a functional fragment thereof.

7. A transgenic rabbit, comprising the humanized immunoglobulin locus present in the transgenic vector according to claim 5.

8. The transgenic rabbit according to claim 7, wherein the transgenic rabbit further comprises

(1) a transgene derived from the rabbit immunoglobulin heavy chain locus, substituted with 8 human VH elements, human JH1-JH6 elements, human Cμ-coding regions fused to human bcl2 coding sequence, and human Cγ coding regions;

(2) a transgene derived from the rabbit immunoglobulin light chain locus, comprising the human Vκ element IGKV1-39-01 and the human IgKJ4 J-element;

(3) transgenes derived from the human CD79α and CD79β loci; and

(4) loss-of-function mutations within the rabbit Cμ and rabbit Cκ loci.

9. A B-cell from the transgenic rabbit according to any one of claims 7 to 8, comprising the humanized immunoglobulin locus present in the transgenic vector according to claim 6.

10. A method for producing a human immunoglobulin using the transgenic rabbit of any one of claims 7 or 8.

11. The method according to claim 10, characterized in that the human immunoglobulin is an antibody.

12. The method according to any one of claims 10 to 11, characterized in that the human immunoglobulin is a polyclonal antibody.

13. The method according to any one of claims 10 to 11, characterized in that the human immunoglobulin is a monoclonal antibody.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/075882

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/075882

A. CLASSIFICATION OF SUBJECT MATTER
INV. A01K67/027 C07K16/00 C07K16/28
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A01K C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/157953 A1 (MERUS B V [NL]) 24 October 2013 (2013-10-24) cited in the application	1-6
Y	Examples	7-13

X	WO 2014/022540 A1 (REGENERON PHARMA [US]) 6 February 2014 (2014-02-06) cited in the application	1-6
Y	Examples	7-13

X	WO 2014/051433 A1 (MERUS B V [NL]) 3 April 2014 (2014-04-03) cited in the application	1-6
Y	Examples	7-13

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 January 2017	30/01/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fellows, Edward

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/080926 A2 (WYETH LLC [US]; GELES KENNETH G [US]; ZHOU BIN-BING STEPHEN [US]; TCHI) 21 June 2012 (2012-06-21)	1-6
Y	The query sequence SEQ ID NO:1 has 100.00 % identity (100.00 % similarity) over 107 positions in a common overlap (range (q:s): 1-107:1-107) with subject GSP:AZX42155 (length: 107) from WO2012080926-A2 published on 2012-06-21.	7-13

X	WO 2010/032059 A2 (MEDIMMUNE LLC [US]; MEDIMMUNE LTD [GB]; BABCOOK JOHN [CA]; BARRY SIMON) 25 March 2010 (2010-03-25)	1-5
Y	The query sequence SEQ ID NO:1 has 100.00 % identity (100.00 % similarity) over 107 positions in a common overlap (range (q:s): 1-107:1-107) with subject GSP:AXX25532 (length: 107) from WO2010032059-A2 published on 2010-03-25.	6-11

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/075882

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(54)发明名称

具有共同轻链的转基因兔

(57)摘要

本文报道了包含人源化轻链基因座的转基因载体，其中所述人源化轻链基因座包含(a)衍生自人轻链V区段IGKV1-39-01的V基因区段，(b)启动子，其3'近端连接有所述轻链基因区段，和(c)至少一个人IGKJ4 J元件的片段，其5'近端连接有所述轻链基因区段。

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1.包含具有SEQ ID NO:01的氨基酸序列的可变结构域或其变体的共同抗体轻链用于产生双特异性抗体的用途。

2.根据权利要求1所述的用途,其中所述用途是通过将两个共同抗体轻链与第一抗体重链和第二抗体重链组合,其中所述第一抗体重链与共同抗体轻链一起形成第一抗原结合位点并且第二抗体重链与共同抗体轻链一起形成第二抗原结合位点。

3.根据权利要求1或2所述的用途,其中所述共同轻链在SEQ ID NO:01的氨基酸序列内包含1至11个氨基酸突变。

4.根据权利要求1至3中任一项所述的用途,其中所述共同轻链在SEQ ID NO:01的氨基酸序列内包含1至13个氨基酸突变,其中至多11个突变位于HVR中。

5.一种双特异性全长抗体,包含两条重链和两条共同轻链,每条轻链包含具有SEQ ID NO:01的氨基酸序列或者包含其变体的可变结构域。

6.一种包含人源化轻链基因座的转基因载体,其中所述人源化轻链基因座包含

(a)作为V基因区段的人轻链V区段IGKV1-39-01,

(b)启动子,其3'近端连接有所述轻链基因区段,和

(c)人IGKJ4J元件或其功能片段,其5'近端连接有所述轻链基因区段。

7.一种转基因兔,包含存在于权利要求5的转基因载体中的人源化免疫球蛋白基因座。

8.根据权利要求7所述的转基因兔,其中所述转基因兔进一步包含

(1)衍生自用8个人VH元件、人JH1-JH6元件、与人bcl2编码序列融合的人Cμ编码区和人Cγ编码区取代的、兔免疫球蛋白重链基因座的转基因;

(2)衍生自包含人Vκ元件IGKV1-39-01和人IgKJ4J元件的、兔免疫球蛋白轻链基因座的转基因;

(3)衍生自人CD79α和CD79β基因座的转基因;和

(4)兔Cμ和兔Cκ基因座内的功能丧失的突变。

9.来自权利要求7或8所述的转基因兔的B细胞,包含存在于根据权利要求6所述的转基因载体中的人源化免疫球蛋白基因座。

10.使用权利要求7或8的转基因兔制备人免疫球蛋白的方法。

11.根据权利要求10所述的方法,其特征在于所述人免疫球蛋白是抗体。

12.根据权利要求10或11所述的方法,其特征在于所述人免疫球蛋白是多克隆抗体。

13.根据权利要求10或11所述的方法,其特征在于所述人免疫球蛋白是单克隆抗体。

具有共同轻链的转基因兔

[0001] 本文报道了可用于产生生产人抗体的转基因兔的共同轻链基因座。本文还报道了共同轻链可变结构域氨基酸序列，包含共同轻链可变结构域的多特异性抗体和包含相应共同轻链基因座的转基因兔。

背景技术

[0002] 多特异性抗体的产生受到链错配导致未配对和错配副产物形成问题的阻碍。根据所选择的形式，可以形成这些副产物的不可忽略的数目和量。

[0003] 已经开发了解决这个问题的不同方法。

[0004] 为了减少重链错配，已经报道了结入扣(knobs-into-hole)技术(参见例如Ridgway，JB等人，Prot.Eng.9(1996)617-621)或CrossMab形式(参见例如Schaefer，W等人，Natl.Acad.Sci USA 108(2011)11187-11192)。

[0005] 为了减少轻链错配，可以使用共同轻链。这种方法固有地要求对于每个都由一对抗体重链可变结构域和抗体轻链可变结构域形成的两个结合位点，必须使用相同的抗体轻链可变结构域。

[0006] 包含人免疫球蛋白基因座的非人动物可以用于产生具有共同轻链的单特异性抗体。这些动物中的人免疫球蛋白基因座通常包含减少且有限数量的重链种系基因，重排的种系重链基因或重链V基因区段和单个轻链基因。当为了产生抗体而免疫这种非人动物时，引发的免疫应答包含具有多种不同重链可变结构域但仅有单一轻链可变结构域的抗体。

[0007] 需要设计并开发适合于配合从头产生抗体的新型共同轻链。因此，这种方法不被认为是开发重组多特异性抗体的首选，因为很可能需要进一步优化并且不得不进行序列修饰。

[0008] 例如在WO 98/50431，WO 2010/084197，US2013/045492，WO2011/097603和WO2012/148873中报道了共同轻链和产生这种共同轻链的方法。

[0009] 在WO 2004/009618中，在SEQ ID NO:1中报道了共同VL(包含在UBS54和K53中)。在SEQ ID NO:18中报道了从针对CD22(克隆B28)、CD72(克隆II-2)和HLA-DR(II类;克隆I-2)的噬菌体获得的共同轻链。

[0010] 在US 2007/098712中，使用抗Ob-R抗体克隆26和抗HER3抗体克隆18的共同VL序列构建双特异性抗体。还报道了抗Mpl scFv 12B5(GenBank登录号AF048775)和抗HER3scFv克隆H6(GenBank登录号AF048774)利用相同的VL序列和基本上不同的VH序列。

[0011] 在WO2010/84197中报道了包含重链和轻链的重组抗体，其中轻链包含如SEQ ID NO:8所示的序列。SEQ ID NO:8是V区段VKVI-2-1-(1)-A14(IGKV6D-41*01)的氨基酸序列。共同轻链的其他氨基酸序列在SEQ ID NO:12-14中报道。

[0012] 在US2010/0331527中报道了另一种共同轻链方法，其中不同特异性的两种抗体使用相同的轻链。

[0013] 在WO2011/097603中报道了基于人Vκ1-39Jκ5基因座，人Vκ3-20Jκ1基因座和人VpreBJλ5基因座的工程化人Vκ和Vλ共同轻链。

[0014] 在US2012/0192300,US2012/021409,US2011/0195454和US2013/0045492中报道了共同轻链及其制备方法。

[0015] 在WO2012/018764中报道了遗传修饰的小鼠以及制备和使用它们的方法,其中小鼠包含用至少一个轻链V基因区段和至少一个轻链J基因区段替代全部或基本上全部的免疫球蛋白重链V基因区段、D基因区段和J基因区段。

[0016] 在WO 2013/157953中报道了衍生自重排的种系人κ轻链IgVK1-39/Jκ或IGVK3-20/JK的种系样共同轻链。

[0017] 在WO2014/22540中概述了通用轻链可以是选自Vκ1-39和Vκ3-20轻链的κ轻链或选自VL1-40和VL2-14轻链的λ轻链。在一个具体实施方案中,人VL基因区段是人Vκ1-39Jκ5基因区段或人Vκ3-20Jκ1基因区段。

[0018] 在WO2014/51433中报道了共同轻链012,其是人重排κ轻链IgVK1-39*01/IgJK1*01。该序列是在人库中经常使用的种系序列,并且具有与许多不同VH区配对的优越能力,并且具有良好的热力学稳定性、产量和溶解性。

[0019] 在US2015/037337中报道了人JH6*02是人中共同的保守变体,并因此是构建转基因IgH基因座的良好候选者。

[0020] 在WO 2015/052230的SEQ ID NO:6中报道了修饰的重链CH3-CH2-CH1-VL的氨基酸序列,其中VL是共同轻链(CLC-Fc cross-MAb)的可变结构域。

[0021] 在WO 2015/153765中,报道了在SEQ ID NO:78和79的N-term-VL-CK-C-term融合多肽中的共同轻链。

[0022] 在WO 2000/46251,WO 2002/12437,WO 2005/007696,WO 2006/047367,US 2007/0033661和WO 2008/027986中报道了包含人免疫球蛋白基因座的转基因兔。

[0023] 发明概述

[0024] 本文报道的一个方面是具有以下氨基酸序列的共同抗体轻链可变结构域:

[0025]

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG GTKVEIK (SEQ ID NO: 01)

[0026] 或其变体。

[0027] 本文报道的一个方面是包含具有以下氨基酸序列的轻链可变结构域的共同抗体轻链

[0028]

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG GTKVEIK (SEQ ID NO: 01)

[0029] 或其变体。

[0030] 在一个实施方案中,共同轻链包含多达13个氨基酸突变。在一个优选实施方案中,共同轻链包含多达13个氨基酸突变,其中至多11个突变位于HVR中。

[0031] 在一个实施方案中,共同轻链包含多达11个氨基酸突变。

[0032] 在一个实施方案中,所述共同轻链在SEQ ID NO:01的氨基酸序列内包含1至11个

氨基酸突变。在一个优选实施方案中，共同轻链在SEQ ID NO:01的氨基酸序列内包含1至13个氨基酸突变，其中至多11个突变位于HVR中。

[0033] 在一个实施方案中，如本文报道的共同抗体轻链的变体包含与SEQ ID NO:01具有90％或更多的序列同一性(即包含多达11个突变)的轻链可变结构域。在一个实施方案中，序列同一性为95％或更高。在一个实施方案中，序列同一性为98％或更高。

[0034] 本文报道的一个方面是包含本文报道的轻链的抗体。

[0035] 本文报道的一个方面是包含如本文报道的两个或更多个不同重链可变结构域和两个或更多个共同轻链可变结构域的多特异性抗体。

[0036] 在一个实施方案中，多特异性抗体是双特异性全长抗体，其包含本文报道的两个不同重链和两个共同轻链可变结构域或两个共同抗体轻链。

[0037] 在一个实施方案中，多特异性抗体是三特异性抗体，其包含本文报道的三个不同的重链可变结构域和三个共同的轻链可变结构域。

[0038] 在一个实施方案中，多特异性抗体是四特异性抗体，其包含本文报道的四个不同重链可变结构域和四个共同轻链可变结构域。

[0039] 本文报道的一个方面是使用如本文报道的共同抗体轻链来产生双特异性抗体。

[0040] 在一个实施方案中，所述用途是通过将两个共同抗体轻链与第一抗体重链和第二抗体重链组合，其中第一抗体重链与共同抗体轻链一起形成第一抗原结合位点并且第二抗体重链与共同抗体轻链一起形成第二抗原结合位点。

[0041] 本文报道的一个方面是包含人源化免疫球蛋白轻链基因座的转基因载体，其中所述人源化免疫球蛋白轻链基因座包含

[0042] (a)衍生自人轻链V区段IGKV1-39-01的V基因区段，

[0043] (b)启动子，其3’近端连接有所述轻链基因区段，和

[0044] (c)至少一个人IGKJ4J元件的片段，其5’近端连接有所述轻链基因区段。

[0045] 在一个实施方案中，转基因载体包含人源化轻链基因座，其中所述人源化轻链基因座包含

[0046] (a)作为V基因区段的人轻链V区段IGKV1-39-01，

[0047] (b)启动子，其3’近端连接有所述轻链基因区段，和

[0048] (c)人IGKJ4J元件或其功能片段，其5’近端连接有所述轻链基因区段。

[0049] 本文报道的一个方面是转基因兔，其包含本文报道的转基因载体中存在的人源化免疫球蛋白轻链基因座。在一个实施方案中，转基因兔具有基本完整的内源调控和抗体生产机制。

[0050] 在一个实施方案中，转基因兔还包含

[0051] (1)衍生自用8个人VH元件、人JH1-JH6元件、与人bcl2编码序列融合的人Cμ编码区和人Cγ编码区取代的、兔免疫球蛋白重链基因座的转基因；

[0052] (2)衍生自包含人Vκ元件IGKV1-39-01和人IgKJ4J元件的、兔免疫球蛋白轻链基因座的转基因；

[0053] (3)衍生自人CD79α和CD79β基因座的转基因；和

[0054] (4)兔Cμ和兔Cκ基因座内的功能丧失的突变。

[0055] 本文报道的一个方面是如本文报道的来自转基因兔的B细胞，其包含存在于本文

报道的转基因载体中的人源化免疫球蛋白轻链基因座。

[0056] 本文报道的一个方面是使用如本文报道的转基因兔产生人免疫球蛋白的方法。

[0057] 在一个实施方案中，人免疫球蛋白是抗体。在一个实施方案中，人免疫球蛋白是多克隆抗体。在一个优选的实施方案中，人免疫球蛋白是单克隆抗体。

[0058] 本发明的详细描述

[0059] 定义

[0060] 如本文所用的术语“共同轻链可变结构域”表示特异性抗体轻链可变结构域氨基酸序列，其可以与不同抗体重链可变结构域氨基酸序列配对以形成不同特异性的功能性抗原结合位点，即结合到相同抗原或不同抗原上的不同表位。在一个实施方案中，共同轻链可变结构域与SEQ ID NO:01具有至少80％，或至少90％，或至少95％，或在一个优选的实施方案中大于98％的氨基酸序列同一性。氨基酸残基差异通常对抗原结合仅具有很小甚至没有影响。因此，术语“共同轻链可变结构域”还包括抗体轻链可变结构域，其具有一些次要氨基酸序列差异，但当与抗体的相同重链配对时形成具有相同特异性和类似亲和力的结合位点。

[0061] 能够鉴定一方面不相同但另一方面在功能上等同的多个共同轻链可变结构域。例如，这通过引入并测试保守氨基酸突变，当共同轻链与抗体重链可变结构域配对时共同轻链部分中不影响或仅略微影响结合位点的结合特异性的氨基酸残基的改变是可能的。

[0062] ″有效连接的″是指两个或更多个组分的并列，其中如此描述的组分处于一种允许它们以其预期方式发挥功能的关系。例如，如果启动子和/或增强子顺式控制或调节所连接的编码序列的转录的话，则启动子和/或增强子有效连接到编码序列。通常但不必″有效连接″的DNA序列是邻接的，并在需要连接两个蛋白编码区域(例如分泌前导序列和多肽)时，是邻接的且在读码框内。然而，虽然有效连接的启动子通常位于编码序列的上游，它不必与编码序列邻接。增强子不必是邻接的。如果增强子增加了编码序列的转录，那么增强子有效连接到编码序列。有效连接的增强子可以位于编码序列的上游、之内或下游，并距启动子相当的距离。如果多聚腺苷酸化位点位于编码序列的下游使得转录进行通过编码序列到聚腺苷酸化序列，那么多聚腺苷酸化位点有效连接到编码序列。如果翻译终止密码子位于编码序列的下游末端(3’末端)使得翻译进行通过编码序列到终止密码子并在那里终止，那么翻译终止密码子有效连接到外显子核酸序列。连接是通过本领域中已知的重组方法，例如，使用PCR方法和/或通过在方便的限制性位点连接而完成的。如果不存在方便的限制性位点，那么根据常规实践使用合成的寡核苷酸衔接头或接头。

[0063] ″分离的″抗体是已经与其天然环境的组分分离的抗体。在一些实施方案中，将抗体纯化至超过95％或99％纯度，如由例如电泳(例如，SDS-PAGE，等电聚焦(IEF)，毛细管电泳(或色谱(例如，离子交换或反相HPLC)测定的。对于用于评估抗体纯度的方法的综述，见例如，Flatman等人，J.Chromatogr.B848(2007)79-87。

[0064] “分离的”核酸分子是这样的核酸分子，其已经与其天然环境的组分分离。分离的核酸包括包含在通常含有核酸分子的细胞中的核酸分子，但是核酸分子存在于染色体外或存在于与其天然的染色体位置不同的染色体位置处。

[0065] 术语“单克隆抗体”在用于本文时指由基本上同质的抗体群获得的抗体，即构成群体的各个抗体相同和/或结合相同表位，还有生产单克隆抗体的过程中可能产生的变体，这

种变体通常以少量存在。与典型的包含针对不同决定簇(表位)的不同抗体的多克隆抗体制备物不同,单克隆抗体制备物的每种单克隆抗体针对抗原上的单一决定簇。因此修饰语“单克隆”指示由基本上同质的抗体群获得的抗体的特征,并不解释为需要通过任何特定方法来生产抗体。例如,依照本发明使用的单克隆抗体可以通过多种技术来制备,所述技术包括但不限于杂交瘤方法、重组DNA方法、噬菌体展示方法和利用含有全部或部分人免疫球蛋白基因座的转基因动物的方法,本文描述了用于制备单克隆抗体的此类方法和其他示例性方法。

[0066] 关于参照多肽序列的"氨基酸序列同一性百分数(%)"定义为对比序列并在必要时引入缺口以获取最大百分比序列同一性后,且不将任何保守替代视为序列同一性的一部分时,候选序列中与参照多肽序列中的氨基酸残基相同的氨基酸残基的百分率。可以本领域技术范围内的多种方式进行测定氨基酸序列同一性百分数目的的序列比对,例如使用公众可得到的计算机软件,诸如BLAST、BLAST-2、ALIGN或Megalign(DNASTAR)软件。本领域技术人员可决定用于比对序列的适宜参数,包括对所比较序列全长获得最大对比所需的任何算法。然而,为了本发明,氨基酸序列同一性%值是使用序列比较计算机程序ALIGN-2获得的。ALIGN-2序列比较计算机程序由Genentech公司编写,源代码已经连同用户文档一起提交给美国版权局(US Copyright Office,Washington D.C.,20559),并以美国版权注册号TXU510087注册。公众从Genentech公司(South San Francisco,California)可得到ALIGN-2程序或可从源代码编译。ALIGN2程序应当编译成在UNIX操作系统,包括数码UNIX V4.0D上使用。所有序列比较参数由ALIGN-2程序设定且不变。

[0067] 在采用ALIGN-2用于氨基酸序列比较的情况中,给定氨基酸序列A相对于、与、或针对给定氨基酸序列B的%氨基酸序列同一性(或者可表述为具有或包含相对于、与、或针对给定氨基酸序列B的某一%氨基酸序列同一性的给定氨基酸序列A)如下计算:

[0068] 分数X/Y乘100

[0069] 其中X是由序列比对程序ALIGN-2在该程序的A和B对比中评分为相同匹配的氨基酸残基数,且其中Y是B中的氨基酸残基总数。可以领会,若氨基酸序列A的长度与氨基酸序列B的长度不相等,则A相对于B的%氨基酸序列同一性将不等于B相对于A的%氨基酸序列同一性。除非另外特别说明,否则所有本文使用的氨基酸序列同一性%值是使用ALIGN-2计算机程序如前面段落中所述获得的。

[0070] 术语“药物制剂”是指这样一种制备物,其形式允许其中含有的活性成分的生物活性是有效的,并且其不含有对制剂将给予的受试者不可接受的毒性的额外组分。

[0071] 哺乳动物中的抗体产生

[0072] 抗体基因产生(参见Molecular Biology of the Cell.第四版,Alberts B,Johnson A,Lewis J等人,New York:Garland Science;2002;和Immunobiology:The Immune System in Health and Disease.第5版,Janeway,CA Jr,Travers P,Walport M等人,New York:Garland Science;2001):

[0073] λ轻链(染色体22)的遗传基因座具有约30个功能性Vλ基因区段和四对功能性Jλ基因区段和Cλ基因。κ基因座(染色体2)以相似的方式组织,具有约40个功能性Vκ基因片段,伴随有5个Jκ基因区段的簇,但具有单个Cκ基因。在大约50%的个体中,整个κV基因区段簇经历了重复增加。重链基因座(14号染色体)具有约65个功能性VH基因区段和位于这些VH基因

区段和6个JH基因区段之间的约27个D区段的簇。重链基因座还含有大的CH基因簇。重链基因座的总长度超过2兆碱基(200万个碱基),而一些D链段只有6个碱基长。

[0074] 免疫球蛋白重链或轻链的V区或V结构域由多于一个基因区段编码。对于轻链,V结构域由两个独立的DNA区段编码。第一区段编码轻链的前95-101个氨基酸,并被称为V基因区段,因为它编码大部分V结构域。第二区段编码V结构域的其余部分(多达13个氨基酸)并被称为连接或J基因区段。因此,在免疫球蛋白的可变结构域中的三个高变环中,两个在V基因区段DNA内编码,而第三个(HV3或CDR3)在V基因区段和J基因区段之间的连接处,并且在重链中由D基因区段部分编码。在重链和轻链中,通过在基因区段之间形成连接的两个步骤添加和缺失核苷酸,CDR3的多样性显著增加。添加的核苷酸也称为P-核苷酸和N-核苷酸。

[0075] 在B细胞发育期间,V和J基因区段(对于轻链)和V,D和J基因区段(对于重链)通过称为V(D)J连接的位点特异性重组过程连接在一起以形成功能性VL-或VH-区编码序列。保守的DNA序列位于每个基因区段的侧翼,且用作连接过程的识别位点,确保只有合适的基因区段发生重组。因此,例如,V区段将总是连接至J或D区段,但不连接到另一V区段。连接由称为V(D)J重组酶的酶复合物介导。该复合物含有两种对发育淋巴细胞特异的蛋白质,以及帮助修复本文所有细胞中受损DNA的酶。

[0076] 例如,人κ轻链基因区段池中的任何40V区段都可以连接至5个J区段中的任何区段,从而至少200个(40x5)不同的κ链V区可以是由此池编码。类似地,人重链池中的51V区段的任一个可以连接至6个J区段中的任一个和27个D区段中的任一个以编码至少8262(51×6×27)个不同的重链V区。

[0077] 由刚刚讨论的遗传性V,J和D基因区段的不同组合的组装产生的组合多样化是使抗体的抗原结合位点多样化的重要机制。仅通过这种机制,人可以产生287个不同的VL区(200种κ和116种λ)和8262个不同的VH区。

[0078] 在大多数位点特异性重组的情况下,DNA连接是精确的。但是在连接抗体(和T细胞受体)基因区段期间,可变数量的核苷酸常常从重组基因区段的末端丢失,并且还可以插入一个或多个随机选择的核苷酸。这种在连接位点的核苷酸的随机丢失和获得被称为连接多样化,并且它极大地增加了通过重组产生的V区编码序列的多样性,特别是在第三高变区中的多样性。

[0079] 如本文报道的共同轻链

[0080] 这里报道了人源化轻链基因座。

[0081] 本发明至少部分基于如下发现:包含多个V基因元件但仅包含与启动子组合的单个V基因元件的人源化轻链免疫球蛋白基因座可以用作转基因兔的共同轻链基因座。

[0082] 如本文报道的人源化轻链基因座包含

[0083] (a)衍生自人轻链V区段IGKV1-39-01的V基因区段,

[0084] (b)启动子,其3'近端连接有所述轻链基因区段,和

[0085] (c)至少一个人IGKJ4J元件的片段,其5'近端连接有所述轻链基因区段。

[0086] 完整的轻链V基因区段IGKV1-39-01具有以下核酸序列(参见例如GenBank X93627,智人种系免疫球蛋白κ轻链,可变区(DPK9);287bp;SEQ ID NO:02):

[0087]

gacatccaga tgacccagtc tccatcctcc ctgtctgcat ctgtaggaga
cagagtcacc atcacttgcc gggcaagtca gagcattagc agctatttaa
attggtatca gcagaaacca gggaaagccc ctaagctcct gatctatgct
gcatccagtt tgcaaagtgg ggtcccatca aggttcagtg gcagtggatc
tgggacagat ttcactctca ccatcagcag tctgcaacct gaagattttg
caacttacta ctgtcaacag agttacagta cccctcc.

[0088] 相应的氨基酸序列是(SEQ ID NO:03)：

[0089]

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA
ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTP

[0090] 全长人IgKJ4*01/02具有以下核酸(SEQ ID NO:04)和氨基酸(SEQ ID NO:05)序列：

[0091] 核酸： ctcactttcggcggagggaccaaggtggagatcaaa

[0092] 氨基酸：L T F G G G T K V E I K

[0093] 使用共同轻链能够通过组合不同的重链可变结构域来产生多特异性抗体(例如双特异性全长抗体)，每个重链可变结构域与相同轻链可变结构域或其相同变体结合不同表位/抗原/靶标从而降低副产品的复杂性。

[0094] 在一个实施方案中，人源化轻链基因座包含25至30个人Vκ元件和人Cκ编码区，其中

[0095] (a)3'近端Vκ元件是衍生自人轻链V区段IGKV1-39-01的V基因区段，

[0096] (b)启动子(3'近侧)有效连接至所述3'近端轻链基因区段，和

[0097] (c)人IGKJ4J元件的至少一个片段5'近端有效连接至所述轻链基因区段。

[0098] 在一个实施方案中，启动子是人κ可变区启动子(亚组Vκ1)。

[0099] 在一个实施方案中，V基因区段包含人κ免疫球蛋白轻链前导肽编码核酸。在一个实施方案中，前导肽具有SEQ ID NO:15的氨基酸序列。

[0100] 在一个实施方案中，V基因区段包含人κ免疫球蛋白前导肽编码核酸和前导肽编码核酸序列与V基因区段之间的鸡衍生的间隔序列。在一个实施方案中，鸡衍生的间隔区序列是SEQ ID NO:16。

[0101] 轻链免疫球蛋白基因座编码下列轻链V区段(SEQ ID NO:03，加下划线的HVR)：

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP

[0102] GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ SYSTP

[0103] 和下列人J元件(SEQ ID NO:05，HVR-L3的一部分加下划线)：

[0104] LTFGG GTKVEIK。

[0105] 因此，本文报道的一个方面是包含具有下述氨基酸序列或其变体的轻链可变结构域的抗体轻链

[0106]

DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SYSTPLTFGG GTKVEIK。

[0107] 本文还涵盖由于B细胞成熟期间兔中的基因转变和超变而产生的该氨基酸序列的变体。

[0108] 在一个实施方案中，(成熟的)轻链相对于由HVR外部的轻链免疫球蛋白基因座编码的轻链包含1至4个氨基酸突变。

[0109] 在一个实施方案中，(成熟的)轻链相对于由轻链免疫球蛋白基因座编码的轻链包含1至15个氨基酸突变。

[0110] 在一个实施方案中，(成熟的)轻链相对于由轻链免疫球蛋白基因座编码的轻链包含1至11个氨基酸突变。

[0111] 在一个实施方案中，(成熟的)轻链相对于由轻链免疫球蛋白基因座编码的轻链包含1至15个氨基酸突变，其中至多11个在HVR中。

[0112] 如本文报道的一个方面是包含两个不同重链和两个轻链的双特异性全长抗体，其中轻链相同且可变结构域具有本文报道的氨基酸序列。

[0113] 转基因兔

[0114] 如本文报道的轻链基因座可以用于产生生产人免疫球蛋白的转基因兔。

[0115] 因此，本文报道的一个方面是具有如本文报道的人源化免疫球蛋白轻链基因座的轻链转基因兔。

[0116] 使用例如基因转化的转基因兔具有人源化免疫球蛋白基因座，并且仍然具有野生型兔的抗体成熟过程，以产生抗体多样性。因此，野生型兔的重链和轻链基因座已经失活并且各自的人源化免疫球蛋白转基因基因座已被引入兔的基因组中，使兔能够产生人(源化的)/人样抗体。转基因兔的基因型可以描述如下：

[0117] 转基因兔包含

[0118] (1)衍生自用8个人VH元件、人JH1-JH6元件、与人bcl2编码序列融合的人Cμ编码区和人Cγ编码区取代的、兔免疫球蛋白重链基因座的转基因；

[0119] (2)衍生自包含人Vκ元件IGKV1-39-01和人IgKJ4J元件的、兔免疫球蛋白轻链基因座的转基因；

[0120] (3)衍生自人CD79α和CD79β基因座的转基因；和

[0121] (4)兔Cμ和兔Cκ基因座内的功能丧失的突变。

[0122] 本文报道了包含人源化免疫球蛋白重链基因座和人源化免疫球蛋白轻链基因座的转基因兔，其中

[0123] i)人源化重链免疫球蛋白基因座衍生自兔免疫球蛋白基因座或一部分的免疫球蛋白基因座，并且包含多个免疫球蛋白重链基因区段，其中

[0124] (a)所述重链基因区段中的至少一个基因区段是VH3家族的人重链V区段，作为3'近端V基因区段，侧翼为包含(介于20个和1000个连续核苷酸之间的来自)SEQ ID:06的兔间隔序列的核苷酸序列，

[0125] (b)所述基因区段以未重排、或部分重排或完全重排的构型并置，和(c)所述人源化免疫球蛋白基因座能够进行基因重排，根据需要，以及能够进行基因转变和/或超变，并且在所述兔中产生人源化免疫球蛋白的库，和

[0126] ii)人源化轻链免疫球蛋白基因座包含

[0127] (a)衍生自人轻链V区段IGKV1-39-01的V基因区段，

[0128] (b)启动子，其3'近端连接有所述轻链基因区段，和

[0129] (c)至少一个人IGKJ4J元件的片段，其5'近端连接有所述轻链基因区段。

[0130] 在一个实施方案中，转基因兔对于人源化重链基因座和人源化轻链基因座是纯合的。

[0131] 在一个实施方案中，转基因兔对于人源化重链基因座和人源化轻链基因座是杂合的。

[0132] 在一个实施方案中，转基因兔对于内源性抗体重链表达和/或内源性抗体轻链表达失活。

[0133] 如本文报道的一个方面是如本文报道的来自转基因兔的B细胞，其包含本文报道的人源化轻链免疫球蛋白基因座。

[0134] 如本文报道的一个方面是包含本文报道的人源化轻链免疫球蛋白基因座的分离的B细胞。

[0135] 在一个实施方案中，B细胞还包含衍生自兔免疫球蛋白基因座或一部分的免疫球蛋白基因座的人源化重链免疫球蛋白基因座，其包含多个免疫球蛋白重链基因区段，其中

[0136] (a)至少一个所述重链基因区段是VH3家族的人重链V区段，侧翼为包含(介于20个和1000个连续核苷酸之间的来自)SEQ ID:06的兔间隔序列的核苷酸序列，

[0137] (b)所述基因区段以未重排、部分重排或完全重排的构型并置，和

[0138] (c)所述人源化免疫球蛋白基因座能够进行基因重排，根据需要，以及能够进行基因转变和/或高变，并且在所述兔中产生人免疫球蛋白的库。

[0139] 本文报道的一个方面也是使用本文报道的转基因兔产生人免疫球蛋白的方法。

[0140] 在一个实施方案中，人免疫球蛋白获自兔的血液。

[0141] 本文报道了具有包含重链免疫球蛋白基因座和轻链免疫球蛋白基因座的修饰的基因组的兔，其中所述修饰是内源性兔免疫球蛋白基因座的失活和人源化免疫球蛋白基因座的引入，产生转基因兔。因此，转基因兔的基因组包含编码不同人免疫球蛋白重链可变结构域和(单功能)人免疫球蛋白轻链可变结构域的外源核酸序列。

[0142] 人源化免疫球蛋白基因座，即相应的核酸序列被整合到兔基因组中。免疫球蛋白基因座的修饰是插入一个或多个转基因人免疫球蛋白基因区段序列，伴随相应的一个或多个内源兔免疫球蛋白基因区段的失活。

[0143] 术语“人源化免疫球蛋白基因座”表示包含一个或多个人元件，例如一个或多个V区和/或无和/或一个或多个J元件，的分离的免疫球蛋白基因座。这些与外源元件结合，即与自然界中未与其组合的遗传元件(例如来自非人生物体的启动子和/或调控元件)组合。

[0144] 如本文报道的转基因兔可以用于产生人抗体。因此，本文报道的一个方面是来自如本文报道的转基因兔的(分离的)B细胞或(分离的)组织。

[0145] 本文报道的又一个方面是如本文报道的转基因兔的用途，用于产生(i)包含人重

链可变区和轻链可变区和兔恒定区的嵌合抗体，或(ii)完全人抗体。

[0146] 本文报道的一个方面是用于产生特异性结合抗原的抗体的方法，其包括以下步骤：

[0147] (a)(用抗原)免疫本文报道的转基因兔，

[0148] (b)从免疫的转基因兔中分离产生特异性结合抗原的抗体的至少一个细胞，

[0149] (c)将步骤(b)的至少一个细胞作为单个保藏细胞培养以产生抗体。

[0150] 在一个实施方案中，步骤b)中获得的至少一个细胞是脾细胞。在一个实施方案中，步骤b)中获得的至少一个细胞是B细胞。

[0151] 本文报道的一个方面是用于产生特异性结合(目的)抗原的抗体的方法，其包括以下步骤：

[0152] (a)提供来自本文报道的转基因兔的一个或多个B细胞，其中所述转基因兔已经用(目的)抗原免疫，

[0153] (b)将步骤(a)的至少一个或多个B细胞作为单个保藏细胞培养以产生抗体。

[0154] 本文报道的一个方面是用于产生特异性结合抗原的抗体的方法，包括以下步骤：

[0155] (a)培养包含编码特异性结合抗原的抗体的核酸的哺乳动物细胞，其中至少作为抗体的编码可变结构域的核酸已从本文报道的已用抗原免疫的转基因兔获得，

[0156] (b)从哺乳动物细胞或培养基中回收抗体。

[0157] 在一个实施方案中，抗体是单克隆抗体。

[0158] 在一个实施方案中，用抗原、用编码抗原的DNA、用抗原和编码抗原的DNA、或用表达抗原的细胞进行免疫。

[0159] 在一个实施方案中，通过将本文所报道的抗原、编码抗原的DNA、抗原连同编码抗原的DNA、或表达抗原的细胞给予转基因兔来进行免疫。

[0160] 提供以下实施例和序列以帮助理解本发明，其真实范围在所附权利要求中阐述。应该理解，可以在不背离本发明精神的情况下对所述程序进行修改。

[0161] 实施例1

[0162] 兔的免疫

[0163] 用于免疫的转基因兔含有(1)衍生自用8个人VH元件、人JH1-JH6元件、与人bcl2编码序列融合的人Cμ编码区和人Cγ编码区取代的、兔免疫球蛋白重链基因座的转基因；(2)衍生自用25个人Vκ元件、近端Vκ元件融合至人Jκ4、和人Cκ编码区取代的、兔免疫球蛋白轻链基因座的转基因；(3)衍生自人CD79a和CD79b基因座的转基因；和(4)兔Cμ和兔Cκ基因座内的功能丧失的突变。

[0164] 蛋白质免疫

[0165] 在第0天用400μg重组可溶性抗原(用弗氏完全佐剂乳化)通过皮内施用以及在第7、14、42、70和84或98天用200μg各种抗原(用完全弗氏佐剂乳化)通过交替肌内和皮下施用免疫兔。在20-21、34-48、62-76和90-104天左右采集血液(估计总血量的10％)。制备血清，其用于通过ELISA测定滴度，并分离外周单个核细胞，其在B细胞克隆过程中用作抗原特异性B细胞的来源。因此获得人抗体。

[0166] DNA免疫

[0167] 使用编码全长抗原的质粒表达载体，通过皮内施用400μg载体DNA，然后电穿孔

(750V/cm的5个方形脉冲,持续时间10ms,间隔1s)对兔进行基因免疫。兔在第0、14、28、49、70、98和126天接受7次连续免疫。在第35、77、105和133天采集血液(估计总血量的10%)。制备血清,将其用于通过ELISA测定滴度,并分离外周单个核细胞,其在B细胞克隆过程中用作抗原特异性B细胞的来源。

[0168] 实施例2

[0169] 血清滴度的测定

[0170] 将抗原以1.75-2μg/ml在PBS中100μl/孔固定在96孔NUNC Maxisorb板上,随后:用PBS溶液中的2%CroteinC 200μl/孔封闭板;以100μl/孔施用在PBS中的0.5%CroteinC中连续稀释的抗血清,一式两份;用(1)HRP缀合的驴抗兔IgG抗体(Jackson Immunoresearch)或(2)HRP缀合的兔抗人IgG抗体(Pierce/Thermo Scientific;1/5000)或(3)生物素化山羊抗人κ抗体(Southern Biotech/Biozol;1/5000)和链霉亲和素-HRP检测;其每种在PBS中的0.5%CroteinC中稀释,100μl/孔。对于所有步骤,将板在37℃孵育1h。在所有步骤之间,用PBS中的0.05%Tween 20洗涤板3次。通过加入100μL/孔的BM Blue POD Substrate溶液(Roche)使信号显色;并通过加入1M HCl,100μl/孔停止。在450nm处读取吸光度,相对于690nm作为参照。滴度定义为导致半数最大信号的抗血清的稀释。

[0171] 实施例3

[0172] B细胞克隆和分选

[0173] 兔外周血单个核细胞(PBMC)的分离

[0174] 使用实施例1的转基因兔作为血源。根据制造商的说明书,在哺乳动物淋巴细胞分离液(lympholyte mammal)(Cedarlane Laboratories,Burlington,Ontario,Canada)上密度离心之前,用1x PBS将含EDTA的全血稀释2倍。在用抗体染色之前用1x PBS洗涤PBMC两次。

[0175] EL-4B5培养基

[0176] RPMI 1640(Pan Biotech,Aidenbach,Germany)补充有10%FCS(Hyclone,Logan,UT,USA),2mM谷氨酰胺,1%青霉素/链霉素溶液(PAA,Pasching,Austria),2mM丙酮酸钠,10mM HEPES(PAN Biotech,Aidenbach,Germany)和0.05mMβ-巯基乙醇(Gibco,Paisley,Scotland)。

[0177] 巨噬细胞/单核细胞的消耗

[0178] 使用无菌6孔板(细胞培养级)通过非特异性粘附来消耗巨噬细胞和单核细胞。每个孔最多装填4ml培养基和多达$6x10^6$个来自免疫兔的外周血单个核细胞,并使其在37℃和5%CO_2的培养箱中结合1h。上清液中的细胞用于抗原淘选步骤。

[0179] 板的包被

[0180] 室温用2μg/ml抗原蛋白包被无菌细胞培养6孔板或用2μg/ml生物素化抗原包被无菌链霉抗生物素蛋白包被的6孔板(Microcoat,Bernried,德国)3小时,或者在4℃过夜。使用前将板在无菌PBS中洗涤三次。

[0181] B细胞在抗原蛋白上富集

[0182] 用抗原蛋白包被的6孔组织培养板接种每4ml培养基高达6×10^6个细胞,并使其在37℃和5%CO_2的培养箱中结合1h。在富集步骤后,通过用1x PBS小心洗涤孔1-2次去除抗原蛋白非粘附细胞的。剩余的粘附细胞用胰蛋白酶在37℃的培养箱中去粘附10分钟。用EL-

4B5培养基终止胰蛋白酶消化。然后将细胞在培养基中洗涤两次。将细胞保持在冰上直至免疫荧光染色。

[0183] 免疫荧光染色和流式细胞术

[0184] 使用抗IgG FITC抗体(AbD Serotec,Düsseldorf,Germany)进行单细胞分选。对于表面染色,将来自消耗和富集步骤的细胞与PBS中的抗IgG FITC抗体孵育30-45分钟。在冷的房间里,在4℃的黑暗中滚动。离心后,通过抽吸取出上清液。将PBMC进行2轮离心并用冰冷的PBS洗涤。最后将PBMC重悬于冰冷的PBS中并立即进行FACS分析。在FACS分析之前加入浓度为5μg/ml的碘化丙啶(BD Pharmingen,San Diego,CA,USA)以区分死细胞和活细胞。

[0185] 装配有计算机和FACSDiva软件(BD Biosciences,USA)的Becton Dickinson FACSAria用于单细胞分选。

[0186] B细胞培养

[0187] 通过Seeber,S等人,PLoS One 9(2014)e86184所述的方法进行兔B细胞的培养。简言之,将单个分选的兔B细胞在含有Pansorbin细胞(1:100000)(Calbiochem(Merck),Darmstadt,Deutschland)的200μl/孔EL-4B5培养基,5%兔胸腺细胞上清液MicroCoat,Bernried,Germany)和γ辐照的鼠EL-4B5胸腺瘤细胞(2.5×10e^{4}个细胞/孔)中在37℃培养箱中孵育7天。移出B细胞培养物的上清液用于筛选,并立即收获剩余的细胞,并在100μl RLT缓冲液(Qiagen,Hilden,Germany)中于-80℃冷冻。

[0188] 实施例4

[0189] B细胞PCR

[0190] 根据制造商的方案使用NucleoSpin 8/96RNA试剂盒(Macherey&Nagel)从B细胞裂解物(重悬于RLT缓冲液)中制备总RNA。RNA用60μl无RNA酶的水洗脱。根据制造商的说明,使用6μl的RNA通过使用Superscript III First-Strand Synthesis SuperMix(Invitrogen)和寡dT-引物的逆转录酶反应来产生cDNA。所有步骤均在Hamilton ML Star系统上进行。使用4μl的cDNA用AccuPrime SuperMix(Invitrogen)以终体积50μl使用用于重链的引物rbHC.up和rbHC.do和用于轻链的引物BcPCR_FHLC_leader.fw和BcPCR_huCkappa.rev来扩增免疫球蛋白重链可变区和轻链可变区(VH和VL)。所有正向引物对(分别为VH和VL的)信号肽都是特异性的,而反向引物对(分别为VH和VL的)恒定区是特异性的。RbVH+RbVL的PCR条件如下:94℃热启动5分钟;94℃20秒,70℃20秒,68℃45秒,35个循环,最后在68℃延伸7分钟。HuVL的PCR条件如下:94℃热启动5分钟;94℃20秒,52℃20秒,68℃45秒,40个循环。最后在68℃延伸7分钟。

[0191] 引物序列:

[0192]

rbHC.up (SEQ ID NO: 07)	AAGCTTGCCACCATGGAGACTGGGCT GCGCTGGCTTC
rbHCf.do (SEQ ID NO: 08)	CCATTGGTGAGGGTGCCCGAG
BcPCR_FHLC_leader.fw (SEQ ID NO: 09)	ATGGACATGAGGGTCCCCGC
BcPCR_huCkappa.rev (SEQ ID NO: 10)	GATTTCAACTGCTCATCAGATGGC

[0193] 将8μl的50μl PCR溶液加载到48E-Gel 2%(Invitrogen G8008-02)上。使用NucleoSpin Extract II试剂盒(Macherey&Nagel;740609250)根据制造商的方案清洁阳性PCR反应物,并在50μl洗脱缓冲液中洗脱。所有清洁步骤均在Hamilton ML Starlet系统上进行。

[0194] 使用的抗原是TPBG(滋养层糖蛋白,SEQ ID NO:11)的胞外结构域。

[0195] 产生的TPBG胞外结构域的抗体具有以下轻链可变结构域:

[0196]

051 (SEQ ID NO: 12): DIQMTQSPSS VSASVGDRVT ITCRASQGIY SWLAWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SDSPPYTFGQ GTKLEIK,

091 (SEQ ID NO: 13): DIQMTQSPSS LSASVGDRVT ITCQASQDIS NYLNWYQQKP GKAPKLLIYA ASTLQIGVPS RFSGSGSGTD FTFTISSLQP EDFATYYCQQ ANSFPLTFGG GTKVEIK,

[0197]

097 (SEQ ID NO: 14): DIQMTQSPSS LSASVGDRVT ITCRASQSIS SYLNWYQQKP GKAPKLLIYA ASSLQSGVPS RFSGSGSGTD FTLTISSLQP EDFATYYCQQ SDSFPLTFGG GTKVEIK.。

[0198] 实施例5

[0199] TPBG特异性Fab片段与TPBG的结合

[0200] 为了评估重组TPBG的结合,用浓度为100ng/ml的25μl/孔生物素化的人TPBG-AviHis包被Nunc Maxisorb链霉亲和素包被的板(MicroCoat#11974998001)。将板在4℃孵育过夜。洗涤后(用PBST缓冲液3×90μl/孔)从2μg/ml开始以1:2稀释系列添加抗TPBG样品并在室温孵育1h。洗涤后(用PBST缓冲液3×90μl/孔)分别以1:7000或1:4000稀释加入25μl/孔山羊抗c-myc HRP(Bethyl,#A190-104P)或山羊抗huκHRP(Millipore,#AP502P),并在室温在振荡器上孵育1h。洗涤后(用PBST缓冲液3×90μl/孔)加入25μl/孔TMB底物(Calbiochem,#CL07)并孵育2分钟。在Safire2读数器(Tecan)上于370/492nm处测量。

[0201] 为了评估人TPBG的细胞结合,将内源表达TPBG的人乳腺癌肿瘤细胞系MFC7以21000个细胞/孔的浓度接种于384孔包被有聚-D-赖氨酸的板(Greiner,#781940)中。使细胞在37℃贴壁过夜。除去上清后,以从5μg/ml开始的1:2稀释系列加入25μl/孔含有抗TPBG抗体的上清液并在4℃孵育1h。洗涤(2×50μl/孔PBST)后,通过加入50μl/孔在1xPBS缓冲液

中稀释的0.05％戊二醛(Sigma,25％)固定细胞,并在室温孵育10分钟。洗涤(3次;90μl/孔PBS-T)后,加入25μl/孔第二抗体用于检测:山羊抗c-myc HRP(1:5000,Bethyl),然后在室温在振荡器上孵育1小时。洗涤(3次;90μl/孔PBS-T)后,加入25μl/孔的TMB底物溶液(Calbiochem)。在室温10分钟后,在Safire2读数器(Tecan)上于370/492nm处测量。

[0202] 表:抗TPBG Fab片段与人TPBG的结合

[0203]

EC50 [ng/ml]	重组 TPBG	MCF7
051	18.1	57.5
091	27.7	14.0

[0204]

EC50 [ng/ml]	重组 TPBG	MCF7
097	15.2	451.3

[0205] 发现051、091和097的Fab片段结合人TPBG或重组来源或在人乳腺癌细胞系的细胞上表达物。

序列表

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<120> 具有共同轻链的转基因兔
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<150> EP 15192002.2
<151> 2015-10-29
<150> EP 16162580.1
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[0001]

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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Ser Tyr
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35 40 45
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

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<212> DNA
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35 40 45
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
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[0002]

[0003]

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[0004]

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[0005]

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    50                  55                  60
Leu Cys Glu Cys Ser Glu Ala Ala Arg Thr Val Lys Cys Val Asn Arg
65                  70                  75                  80
Asn Leu Thr Glu Val Pro Thr Asp Leu Pro Ala Tyr Val Arg Asn Leu
                85                  90                  95
Phe Leu Thr Gly Asn Gln Leu Ala Val Leu Pro Ala Gly Ala Phe Ala
            100                 105                 110
Arg Arg Pro Pro Leu Ala Glu Leu Ala Ala Leu Asn Leu Ser Gly Ser
        115                 120                 125
Arg Leu Asp Glu Val Arg Ala Gly Ala Phe Glu His Leu Pro Ser Leu
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Arg Gln Leu Asp Leu Ser His Asn Pro Leu Ala Asp Leu Ser Pro Phe
145                 150                 155                 160
Ala Phe Ser Gly Ser Asn Ala Ser Val Ser Ala Pro Ser Pro Leu Val
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Glu Leu Ile Leu Asn His Ile Val Pro Pro Glu Asp Glu Arg Gln Asn
            180                 185                 190
Arg Ser Phe Glu Gly Met Val Val Ala Ala Leu Leu Ala Gly Arg Ala
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Leu Gln Gly Leu Arg Arg Leu Glu Leu Ala Ser Asn His Phe Leu Tyr
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Leu Thr His Leu Glu Ser Leu His Leu Glu Asp Asn Ala Leu Lys Val
260 265 270
Leu His Asn Gly Thr Leu Ala Glu Leu Gln Gly Leu Pro His Ile Arg
275 280 285
Val Phe Leu Asp Asn Asn Pro Trp Val Cys Asp Cys His Met Ala Asp
290 295 300
Met Val Thr Trp Leu Lys Glu Thr Glu Val Val Gln Gly Lys Asp Arg
305 310 315 320
Leu Thr Cys Ala Tyr Pro Glu Lys Met Arg Asn Arg Val Leu Leu Glu
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[0006]

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35 40 45
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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35 40 45
Tyr Ala Ala Ser Thr Leu Gln Ile Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro
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[0007]

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35 40 45
Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
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Leu Arg

[0008]

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tcca 124