

unspecific integration events and generate puromycin-resistant colonies only in the case of correct recombination with the RMCE acceptor locus.

5 Kawabe, Y., et al. (Cytotechnol. 64 (2011) 267-279) disclosed repeated integration of antibody genes into a pre-selected chromosomal locus of CHO cells using an accumulative site-specific gene integration system. A method for preparing bispecific antibodies in E. coli cells is disclosed in KR 2011 0068814. WO 00/31246 disclosed methods for the preparation by in vivo recombination of nucleic acid and polypeptide libraries and uses thereof. Transposition-mediated identification of specific binding or functional proteins are disclosed in EP 2 692 10 865 B1.

Summary

The disclosure provides an RMCE-base method for generating combinatorial expression libraries for bispecific antibodies in CHO cells in which each cell expresses exactly one type of bispecific molecule.

15 The disclosure provides the vector library as such, especially its small size.

The disclosure provides the vectors useful for generating the libraries themselves.

The disclosure provides a method for preparing a recombinant host cell library expressing a library of multispecific antibodies comprising:

20 a) providing a targeted integration host cell comprising an exogenous nucleotide sequence integrated at a site within a locus of the genome of the host cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all 25 the recombination recognition sequences are different;

b) introducing into the cell provided in a) a library of first vectors each comprising two recombination recognition sequences that are matching the first and the third recombination recognition sequence on the integrated exogenous nucleotide sequence, wherein the two recombination recognition 30 sequences are flanking two or more exogenous nucleotide sequences and at

least (a part of) one second selection marker, and a library of second vectors each comprising two recombination recognition sequences that are matching the second and the third recombination recognition sequence on the integrated exogenous nucleotide sequence, wherein the two recombination recognition sequences are flanking two or more (further) exogenous nucleotide sequences;

c) introducing i) either simultaneously with the libraries of first and second vectors of b); or ii) sequentially thereafter one or more recombinases, wherein the one or more recombinases recognize the recombination recognition sequences of the first and second vectors; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for recombinant host cells expressing the second selection marker and secreting a bispecific antibody,

thereby preparing a recombinant host cell library expressing a library of bispecific antibodies.

In one example the multispecific antibody is a bispecific antibody.

One aspect of the disclosure is a method for preparing a recombinant host cell library expressing a library of bispecific antibodies comprising:

a) providing a targeted integration host cell comprising an exogenous nucleotide sequence integrated at a site within a locus of the genome of the host cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a library of first vectors each comprising two recombination recognition sequences matching the first and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking two exogenous nucleotide sequences and at least one second selection marker, and a library of second vectors each comprising two recombination recognition sequences matching the second and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking at least two further exogenous nucleotide sequences; wherein one of the four exogenous nucleotide sequences encode a first light chain, one a second light chain, one a first heavy chain and one a second heavy chain of the bispecific antibody;

c) introducing i) either simultaneous with the libraries of first and second vectors of b); or ii) sequentially thereafter one or more recombinases, wherein the one or more recombinases recognize the recombination recognition sequences of the first and second vectors; wherein the one or more recombinases recognize the recombination recognition sequences and perform two recombinase mediated cassette exchanges;

and

d) selecting for recombinant host cells expressing the second selection marker and secreting a bispecific antibody,

thereby preparing a recombinant host cell library expressing a library of bispecific antibodies.

In one example the two or more exogenous nucleotide sequences are independently of each other two to six, or two to four, or three, or four, or five, or six exogenous nucleotide sequences. In one example the two or more exogenous nucleotide sequences are independently of each other two to three, or two to four. In one example the two or more exogenous nucleotide sequences are two exogenous nucleotide sequences.

5 In one example wherein the first vector and the second vector comprise in total four exogenous nucleotide sequences, wherein one of the four exogenous nucleotide sequences encodes the first light chain, one encodes the second light chain, one encodes the first heavy chain and one encodes the second heavy chain of the bispecific antibody.

In one example each of the first and the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain.

In one example each of the first and the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding the cognate antibody heavy chain.

5 In one example the first or the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain and the other vector
10 comprises the exogenous nucleotide sequence encoding the antibody heavy chain located upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain.

15 In one example the first and the second vector each comprise one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein in each vector the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain.

20 In one example the first and the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody heavy chain located upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain.

25 In one example the first or the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover.

30 In one example the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover.

In one preferred example the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the

antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody light chain with domain crossover is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain with domain crossover.

5 In one preferred example the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody heavy chain with domain crossover is located
10 upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain with domain crossover.

In one example the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody light chain with domain crossover is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain with domain crossover; and the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding
15 an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain, or vice versa.

In one preferred example the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody heavy chain with domain crossover is located upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain with domain crossover; and the second vector comprises one exogenous
25 nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain.

The disclosure provides a method for preparing a recombinant host cell expressing a bispecific antibody with domain crossover, comprising:

- 5 a) providing a targeted integration host cell comprising an exogenous nucleotide sequence integrated at a site within a locus of the genome of the host cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;
- 10 b) introducing into the cell provided in a) a first vector each comprising two recombination recognition sequences matching the first and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking two exogenous nucleotide sequences and at least (a part of) one second selection marker coding sequence, and a second vector
- 15 each comprising two recombination recognition sequences matching the second and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking at least two further exogenous nucleotide sequences; wherein the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous
- 20 nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody heavy chain with domain crossover is located upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain with domain crossover; and the
- 25 second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain.
- 30 c) introducing i) either simultaneously with the first and second vectors of b); or ii) sequentially thereafter one or more recombinases, wherein the one or more recombinases recognize the recombination recognition sequences of the first and second vectors; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

- d) selecting for recombinant host cells expressing the second selection marker and secreting a bispecific antibody,

5 thereby preparing a recombinant host cell expressing a bispecific antibody with domain crossover.

Another aspect of the disclosure is a method for preparing a recombinant host cell expressing a bispecific antibody with domain crossover, comprising:

- 10 a) providing a targeted integration host cell comprising an exogenous nucleotide sequence integrated at a site within a locus of the genome of the host cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

- 15 b) introducing into the cell provided in a) a first vector each comprising two recombination recognition sequences matching the first and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking two exogenous nucleotide sequences and at least one second selection marker, and a second vector each comprising two recombination recognition sequences matching the second and the third recombination recognition sequence on the integrated exogenous nucleotide sequence and flanking at least two further exogenous nucleotide sequences; wherein the first vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the antibody light chain and the antibody heavy chain have a domain crossover and the exogenous nucleotide sequence encoding the antibody heavy chain with domain

crossover is located upstream (5') to the exogenous nucleotide sequence encoding the antibody light chain with domain crossover; and the second vector comprises one exogenous nucleotide sequence encoding an antibody light chain and one exogenous nucleotide sequence encoding an antibody heavy chain, wherein the exogenous nucleotide sequence encoding the antibody light chain is located upstream (5') to the exogenous nucleotide sequence encoding the antibody heavy chain.

c) introducing i) either simultaneous with the first and second vectors of b); or ii) sequentially thereafter one or more recombinases, wherein the one or more recombinases recognize the recombination recognition sequences of the first and second vectors;

and

d) selecting for recombinant host cells expressing the second selection marker and secreting a bispecific antibody,

thereby preparing a recombinant host cell expressing a bispecific antibody with domain crossover.

In one example the first vector comprises a promoter sequence operably linked to the codon ATG, whereby the promoter sequence is flanked upstream by (i.e. positioned downstream to) the (two) exogenous nucleotide sequences and the ATG codon is flanked downstream by (i.e. positioned upstream to) a recombination recognition sequence; and the second vector comprises a selection marker lacking an ATG transcription start codon flanked upstream by a recombination recognition sequence and downstream by the (two) exogenous nucleotide sequences.

The disclosure provides a recombinant cell comprising an exogenous nucleotide sequence integrated at a site within a locus of the genome of the host cell,

wherein the exogenous nucleotide sequence comprises the following elements: a first, a second and a third recombination recognition sequence, a first and a second selectable marker, and a first to fourth expression cassette,

wherein the 5'-to-3' sequences of said elements is

5 RRS1-1st EC-2nd EC-RRS3-SM1-3rd EC-4th EC-RRS2-SM2

with

RRS = recombination recognition sequence, EC = expression cassette, SM = selection marker.

10 In one example each of the expression cassettes comprise in 5'-to-3' direction a promoter, a gene of interest and a polyA-site and optionally a terminator sequence.

In one example

- i) the first expression cassette comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody heavy chain with domain crossover, and a polyA-site and optionally a terminator sequence,

ii) the second expression cassette comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody light chain with domain crossover, and a polyA-site and optionally a terminator sequence,

5 iii) the third expression cassette comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody light chain (without domain crossover), and a polyA-site and optionally a terminator sequence, and

iv) the fourth expression cassette comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody heavy chain (without domain crossover), and a polyA-site and optionally a terminator sequence.

10 In one example the promoter is the human CMV promoter with intron A, the polyA-site is the BGH polyA site and the terminator is the hGT terminator.

In one example the domain crossover is a CH1-CL-crossover, or a VH-VL-crossover, or a VH/CH1-VL/CL-crossover.

15 In one example i) the variable domains of the light chain with domain crossover and the heavy chain with domain crossover form a binding site specifically binding to a first antigen, and ii) the variable domains of the light chain without domain crossover and the heavy chain without domain crossover form a binding site specifically binding to a second antigen. In one example the first antigen is a first epitope on an antigen and the second antigen is a second epitope on the antigen that
20 is different from the first epitope. In one example the first antigen and the second antigen are different antigens (polypeptides).

The disclosure provides a method of producing a bispecific antibody comprising:

a) providing a recombinant host cell according to the disclosure or prepared with a method according to the disclosure;

25 b) culturing the recombinant host cell in a) and recovering the bispecific antibody from the cell or the cultivation medium.

Another aspect of the disclosure is a method of producing a bispecific antibody comprising:

- a) providing a recombinant host cell prepared with a method according to the disclosure;
- 5 b) culturing the recombinant host cell in a) and recovering the bispecific antibody from the cell or the cultivation medium.

Detailed Description of Embodiments of the Invention

The invention is based, at least in part, on the finding that recombinase mediated cassette exchange (RMCE) can be used for generating combinatorial expression