

expression of antibodies including bispecific antibodies in eukaryotic cells, particularly Chinese hamster (*Cricetulus griseus*) cell lines, by employing an expression-enhancing locus. The data in this document is presented in an anonymized way, thus, not allowing a conclusion what has actually been shown.

5 Rajendra, Y., et al. discloses that a single quad vector is a simple, yet effective, alternative approach for generation of stable CHO cell lines and may accelerate cell line generation for clinical hetero-mAb therapeutics (Biotechnol. Prog. 33 (2017) 469-477).

10 WO 2017/060144 discloses bispecific antibodies with tetra valency for a costimulatory TNF receptor. WO 2019/086497 discloses combination therapy with targeted Ox40 agonists.

Summary of the Invention

15 Herein is reported a recombinant mammalian cell expressing a multivalent, bispecific antibody. A multivalent, bispecific antibody is a heteromultimeric polypeptide not naturally expressed by said mammalian cell. More specifically, a multivalent, bispecific antibody is a heteromultimeric protein consisting of three polypeptides or polypeptide chains: one light chain, which is a full length light chain; one heavy chain, which is an extended heavy chain comprising an addition heavy chain Fab fragment at its N-terminus and an additional light chain variable domain
20 at its C-terminus; and a further heavy chain, which is an extended heavy chain comprising an addition heavy chain Fab fragment at its N-terminus and an additional heavy chain variable domain at its C-terminus. To achieve expression of a multivalent, bispecific antibody a recombinant nucleic acid comprising multiple different expression cassettes in a specific and defined sequence has been integrated
25 into the genome of a mammalian cell.

Herein is also reported a method for generating a recombinant mammalian cell expressing multivalent, bispecific antibody and a method for producing multivalent, bispecific antibody using said recombinant mammalian cell.

In one preferred **embodimentexample** the multivalent, bispecific antibody comprises

30 - a first heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a second copy of the first heavy chain

variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a first light chain variable domain,

- a second heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a second copy of the first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a second heavy chain variable domain, and
- a first light chain comprising from N- to C-terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In one preferred ~~embodiment~~example none of the first light chain and the second light chain of the multivalent, bispecific antibody is a common light chain or a universal light chain.

The current ~~invention~~disclosure is based, at least in part, on the finding that the sequence of the different expression cassettes required for the expression of the heteromultimeric multivalent, bispecific antibody, i.e. the expression cassette organization, as integrated into the genome of a mammalian cell influences the expression yield of multivalent, bispecific antibody.

The current ~~invention~~disclosure is based, at least in part, on the finding that by integrating a nucleic acid encoding the heteromultimeric multivalent, bispecific antibody that has a specific expression cassette organization into the genome of a mammalian cell efficient recombinant expression and production of the multivalent, bispecific antibody can be achieved.

It has been found that the defined expression cassette sequence can advantageously be integrated into the genome of a mammalian cell by a double recombinase mediated cassette exchange reaction.

~~One aspect according to the current invention is~~The present disclosure provides a method for producing multivalent, bispecific antibody comprising the steps of

- a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding multivalent, bispecific antibody optionally under conditions suitable for the expression of multivalent, bispecific antibody, and
- b) recovering multivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding multivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

In one aspect, the present disclosure provides a method for producing a multivalent, bispecific antibody comprising the steps of

a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the multivalent, bispecific antibody, and

b) recovering the multivalent, bispecific antibody from the cell or the cultivation medium.

wherein the deoxyribonucleic acid encoding the multivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

In one ~~embodiment~~example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

~~One aspect of the current invention is~~In another aspect, the present disclosure provides a deoxyribonucleic acid encoding a multivalent, bispecific antibody comprising in 5'- to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and

- a sixth expression cassette encoding the first light chain,
or (2)
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- 5 - a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- 10 - an eighth expression cassette encoding the first light chain.

~~One aspect of the current invention is~~ In another aspect, the present disclosure provides the use of a deoxyribonucleic acid comprising in 5' - to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- 15 - a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- 25 - a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain,

for the expression of multivalent, bispecific antibody in a mammalian cell.

30 In one embodimentexample of the use the deoxyribonucleic acid is integrated into the genome of the mammalian cell.

In one embodimentexample of the use exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

~~One aspect of the invention is~~In another aspect, the present disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a multivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding the multivalent, bispecific antibody comprises in 5' - to 3'-direction

either (1)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain,

or (2)

- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain, and
- an eighth expression cassette encoding the first light chain.

In one ~~embodiment~~example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

In one ~~embodiment~~example of ~~all previous aspects~~the above the deoxyribonucleic acid encoding multivalent, bispecific antibody further comprises

- a first recombination recognition sequence located 5' to the first (most 5') expression cassette,
- a second recombination recognition sequence located 3' to the sixth or eighth (most 3') expression cassette, and
- a third recombination recognition sequence located
 - between the first and the second recombination recognition sequence, and

- between two of the expression cassettes,

and

wherein all recombination recognition sequences are different.

In one ~~embodiment~~example the third recombination recognition sequence is located between the third and the fourth or the fourth and fifth expression cassette.

In one ~~embodiment~~example the deoxyribonucleic acid encoding multivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker and the expression cassette encoding for the selection marker is located partly 5' and partly 3' to the third recombination recognition sequence, wherein the 5'-located part of said expression cassette comprises the promoter and the start-codon and the 3'-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence.

~~One aspect of the current invention is~~The present disclosure provides a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction, either (1)
 - a first recombination recognition sequence,
 - a first expression cassette encoding the first heavy chain,
 - a second expression cassette encoding the first light chain,
 - a third expression cassette encoding the first light chain, and
 - a first copy of a third recombination recognition sequence,or (2)
 - a first recombination recognition sequence,
 - a first expression cassette encoding the first heavy chain,
 - a second expression cassette encoding the first light chain,
 - a third expression cassette encoding the first light chain,
 - a fourth expression cassette encoding the first light chain, and
 - a first copy of a third recombination recognition sequence,
- and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction

either (1)

- a second copy of the third recombination recognition sequence,
- a fourth expression cassette encoding the second heavy chain,
- a fifth expression cassette encoding the first light chain, and
- a sixth expression cassette encoding the first light chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain,
- an eighth expression cassette encoding the first light chain, and
- a second recombination recognition sequence.

In another aspect, the present disclosure provides a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and six or eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5' - to 3' -direction,
either (1)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first heavy chain,
- a second expression cassette encoding the first light chain,
- a third expression cassette encoding the first light chain,
- a fourth expression cassette encoding the first light chain, and

- a first copy of a third recombination recognition sequence,
and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction either (1)

- a second copy of the third recombination recognition sequence,

- a fourth expression cassette encoding the second heavy chain,

- a fifth expression cassette encoding the first light chain,

- a sixth expression cassette encoding the first light chain, and

- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,

- a fifth expression cassette encoding the second heavy chain,

- a sixth expression cassette encoding the first light chain,

- a seventh expression cassette encoding the first light chain,

- an eighth expression cassette encoding the first light chain, and

- a second recombination recognition sequence.

In one embodimentexample the first and the second deoxyribonucleic acid both comprises the organization according to (1); or the first and the second deoxyribonucleic acid both comprises the organization according to (2).

In one embodimentexample of ~~all previous aspects~~the above the deoxyribonucleic acid encoding multivalent, bispecific antibody further comprises a further expression cassette encoding for a selection marker.

In one embodimentexample the expression cassette encoding for a selection marker is located either

i) 5', or

ii) 3', or

iii) partly 5' and partly 3'

to the third recombination recognition sequence.

In one embodimentexample the expression cassette encoding for a selection marker is located partly 5' and partly 3' to the third recombination recognition sequences, wherein the 5'-located part of said expression cassette comprises the promoter and a start-codon and the 3'-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal.

In one ~~embodiment~~example the 5'-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by (i.e. is positioned downstream to) the third or fourth expression cassette, respectively, and the start-codon is flanked downstream by (i.e. is positioned upstream of) the third recombination recognition sequence; and the 3'-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked upstream by the third recombination recognition sequence and downstream by the fourth or fifth expression cassette, respectively.

In one ~~embodiment~~example the start-codon is a translation start-codon. In one ~~embodiment~~example the start-codon is ATG.

~~One aspect of the invention is~~The present disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding multivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding multivalent, bispecific antibody comprises the following elements:

a first, a second and a third recombination recognition sequence,

a first and a second selection marker, and

a first to sixth or first to eighth expression cassette,

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

RRS1-1st EC-2nd EC-3rd EC-RRS3-SM1-4th EC-5th EC-6th EC-RRS2

or

RRS1-1st EC-2nd EC-3rd EC-4th EC-RRS3-SM1-5th EC-6th EC-7th EC-8th
EC-RRS2

with

RRS = recombination recognition sequence,

EC = expression cassette,

SM = selection marker.

~~One aspect of the current invention is~~In another aspect, the present disclosure provides a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a multivalent,

bispecific antibody and secreting the multivalent, bispecific antibody comprising the following steps:

- 5 a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian 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 composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and six or eight expression cassettes, wherein
- the first deoxyribonucleic acid comprises in 5'- to 3'-direction, either (1)
 - 15 - a first recombination recognition sequence,
 - a first expression cassette encoding the first heavy chain,
 - a second expression cassette encoding the first light chain,
 - a third expression cassette encoding the first light chain, and
 - a first copy of a third recombination recognition sequence,
 - 20 or (2)
 - a first recombination recognition sequence,
 - a first expression cassette encoding the first heavy chain,
 - a second expression cassette encoding the first light chain,
 - a third expression cassette encoding the first light chain,
 - 25 - a fourth expression cassette encoding the first light chain, and
 - a first copy of a third recombination recognition sequence,
 - and
 - the second deoxyribonucleic acid comprises in 5'- to 3'-direction either (1)
 - 30 - a second copy of the third recombination recognition sequence,
 - a fourth expression cassette encoding the second heavy chain,
 - a fifth expression cassette encoding the first light chain,
 - a sixth expression cassette encoding the first light chain, and
 - a second recombination recognition sequence,
 - 35 or (2)
 - a second copy of the third recombination recognition sequence,

- a fifth expression cassette encoding the second heavy chain,
- a sixth expression cassette encoding the first light chain,
- a seventh expression cassette encoding the first light chain,
- an eighth expression cassette encoding the first light chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid 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 the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the multivalent, bispecific antibody,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the multivalent, bispecific antibody and secreting the multivalent, bispecific antibody.

In one embodimentexample the first and the second deoxyribonucleic acid both comprises the organization according to (1); or the first and the second deoxyribonucleic acid both comprises the organization according to (2).

In one embodimentexample the expression cassette encoding the one second selection marker is located partly 5' and partly 3' to the third recombination recognition sequences, wherein the 5'-located part of said expression cassette comprises the promoter and

the start-codon and said 3'-located part of the expression cassette comprises the coding sequence of the one second selection marker without a start-codon and a polyA signal.

5 In one ~~embodiment~~example the 5'-terminal part of the expression cassette encoding the one second selection marker comprises a promoter sequence operably linked to the start-codon, whereby the promoter sequence is flanked upstream by (i.e. is positioned downstream to) the third or fourth expression cassette, respectively, and the start-codon is flanked downstream by (i.e. is positioned upstream of) the third recombination recognition sequence; and the 3'-terminal part of the expression
10 cassette encoding the one second selection marker comprises the coding sequence of the one second selection marker lacking a start-codon flanked upstream by the third recombination recognition sequence and downstream by the fourth or fifth expression cassette, respectively.

15 In one ~~embodiment~~example the start-codon is a translation start-codon. In one ~~embodiment~~example the start-codon is ATG.

In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above the first deoxyribonucleic acid is integrated into a first vector and the second deoxyribonucleic acid is integrated into a second vector.

20 In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above
- the first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a first light chain variable domain,
- the second heavy chain comprises from N- to C-terminus a first heavy chain
25 variable domain, a CH1 domain, a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3 domain and a second heavy chain variable domain, and
- the first light chain comprises from N- to C-terminus a second light chain variable domain and a CL domain,

30 wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above each of the expression cassettes comprise in 5'-to-3' direction a promoter, a coding sequence and a polyadenylation signal sequence optionally followed by a terminator sequence.

5 In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above

each expression cassette for an antibody chain comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody chain, and a polyadenylation signal sequence and optionally a terminator sequence

and

10 each expression cassette encoding the selection marker comprises in 5'-to-3' direction a promoter, a nucleic acid encoding the selection marker, and a polyadenylation signal sequence and optionally a terminator sequence.

In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above the promoter is the human CMV promoter with or without intron A, the polyadenylation signal sequence is the bGH polyA site and the terminator is the hGT terminator.

15 A terminator sequence prevents the generation of very long RNA transcripts by RNA polymerase II, i.e. the read-through into the next expression cassette in the deoxyribonucleic acid according to the ~~invention~~disclosure and used in the methods according to the ~~invention~~disclosure. That is, the expression of one structural gene of interest is controlled by its own promoter.

20 Thus, by the combination of a polyadenylation signal and a terminator sequence efficient transcription termination is achieved. That is, read-through of the RNA polymerase II is prevented by the presence of double termination signals. The terminator sequence initiates complex resolution and promotes dissociation of RNA polymerase from the DNA template.

25 In one ~~embodiment~~example of ~~all previous aspects and embodiments~~the above the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

30

In one embodimentexample of ~~all previous aspects and embodiments~~the above all expression cassettes are arranged unidirectional.

In one embodimentexample of ~~all previous aspects and embodiments~~the above the mammalian cell is a CHO cell. In one embodimentexample the CHO cell is a CHO-K1 cell.

In one embodimentexample of ~~all aspects and embodiments~~the present disclosure the multivalent, bispecific antibody is an anti-FAP/Ox40 bispecific antibody. Such an antibody is reported in WO 2017/060144, which is incorporated herein by reference in its entirety.

In one embodimentexample of ~~all previous aspects and embodiments~~the above none of the first light chain and the second light chain of the multivalent, bispecific antibody is a common light chain or a universal light chain.

In one preferred embodimentexample of ~~all aspects and embodiments~~the present disclosure exactly two deoxyribonucleic acids are comprised or introduced.

The individual expression cassettes in the deoxyribonucleic acid according to the ~~invention~~disclosure are arranged sequentially. The distance between the end of one expression cassette and the start of the thereafter following expression cassette is only a few nucleotides, which were required for, i.e. result from, the cloning procedure.

In one embodimentexample of ~~all previous aspects and embodiments~~the above two directly following expression cassettes are spaced at most 100 bps apart (i.e. from the end of the poly A signal sequence or the terminator sequence, respectively, until the start of the following promoter element are at most 100 base pairs (bps)). In one embodimentexample two directly following expression cassettes are spaced at most 50 bps apart. In one preferred embodimentexample two directly following expression cassettes are spaced at most 25 bps apart.

Detailed Description of Embodiments of the Invention

The current invention is based, at least in part, on the finding that for the expression of a multivalent, bispecific antibody, which is a complex molecule comprising different polypeptides, i.e. which is a heteromultimer, the use of a defined and specific expression cassette organization results in efficient expression and production of the multivalent, bispecific antibody in mammalian cells, such as CHO cells.