

expression-enhancing locus. The data in this document is presented in an anonymized way, thus, not allowing a conclusion what has actually been done. When Cre-recombinase was used, it was co-transfected on an additional plasmid but this plasmid has not been described with respect to its composition or origin.

5 Gurumurthy, C.B. and Kent Lloyd, K.C., disclosed mouse models for biomedical research (Dis. Mod. Mech. 12 (2019)). They discuss how conventional gene targeting by homologous recombination in embryonic stem cells has given way to more refined methods that enable allele-specific manipulation in zygotes.

10 Bahr, S., et al. disclosed the development of a platform expression system using targeted integration in Chinese hamster ovary cells (proceedings of Cell Culture Engineering XVI, 2018).

Summary

15 Herein is reported a method for generating a recombinant mammalian cell expressing a heterologous polypeptide and a method for producing a heterologous polypeptide using said recombinant mammalian cell.

20 The current disclosure is based, at least in part, on the finding that the number of clones obtained by targeted integration can be improved if Cre-recombinase mRNA (Cre mRNA) is used instead of e.g. Cre-recombinase DNA (Cre DNA). In more detail, it has been found that after the selection period, the absolute number of clones in Cre mRNA-generated recombinant cell pools is higher than in CRE plasmid-generated recombinant cell pools. Thus, by using Cre mRNA instead of e.g. a Cre-recombinase encoding plasmid (Cre plasmid), a recombinant cell pool with increased clone number and heterogeneity can be obtained. Without being bound by this theory it is assumed that thereby the probability of finding a recombinant cell clone with
25 high titer and good product quality is increased. In addition, it has been found that an increased number of recombinant cell clones from Cre mRNA-generated pools are stable compared to Cre plasmid-generated cell pools.

30 It has to be pointed out that the Cre mRNA introduced for the recombinase reaction is isolated Cre mRNA as well as the only source of Cre-recombinase in the method according to the current disclosure.

The current disclosure provides a method for producing a polypeptide comprising the steps of

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

5 wherein the deoxyribonucleic acid encoding the polypeptide has been stably integrated into the genome of the mammalian cell by Cre-recombinase mediated cassette exchange using Cre mRNA.

10 The current disclosure provides a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a polypeptide and secreting the polypeptide, wherein the method comprises the following steps:

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
15 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 composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and one to eight expression cassettes, wherein
20 the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

- a first recombination recognition sequence,
- one or more expression cassette(s),
- a 5'-terminal part of an expression cassette encoding one second selection marker, and
- 25 - a first copy of a third recombination recognition sequence,

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

- a second copy of the third recombination recognition sequence,
- 30 - a 3'-terminal part of an expression cassette encoding the one second selection marker,
- one or more expression cassette(s), 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,

5 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

10 i) either simultaneously with the first and second deoxyribonucleic acid of b); or

ii) sequentially thereafter

Cre-recombinase mRNA,

15 wherein the Cre-recombinase recognizes the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the recombinase performs two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the polypeptide,

20 thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the polypeptide and secreting the polypeptide.

One aspect of the current disclosure is a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a polypeptide and secreting the polypeptide comprising the following steps:

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, wherein the mammalian cell is free of Cre-recombinase encoding DNA;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and one to eight expression cassettes, wherein

the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

- a first recombination recognition sequence,
- one or more expression cassette(s),
- a 5'-terminal part of an expression cassette encoding one second selection marker, and
- a first copy of a third recombination recognition sequence,

and

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

- a second copy of the third recombination recognition sequence,
- a 3'-terminal part of an expression cassette encoding the one second selection marker,
- one or more expression cassette(s), 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,

wherein the deoxyribonucleic acids are free of Cre-recombinase encoding DNA;

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b); or

ii) sequentially thereafter

Cre-recombinase mRNA as sole source of Cre-recombinase,

wherein the Cre-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 polypeptide,

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the polypeptide and secreting the polypeptide.

The current disclosure provides the use of Cre-recombinase mRNA for increasing the number of recombinant mammalian cells comprising (exactly one copy of) a (heterologous and/or transgenic) deoxyribonucleic acid encoding a (heterologous) polypeptide of interest stably integrated at a single site in the genome of said cell by targeted integration, In one example the recombinant cell also secretes the polypeptide of interest into the cultivation medium upon cultivation therein.

Another aspect of the current disclosure is the use of Cre-recombinase mRNA for increasing the number of recombinant mammalian cells comprising a deoxyribonucleic acid encoding a polypeptide or protein of interest stably integrated at a single site in the genome of said cell by targeted integration.

- 5 In one example of the current disclosure the mammalian cell and/or the introduced Cre-recombinase mRNA is free of Cre-recombinase encoding deoxyribonucleic acid.

In one example of the current disclosure the Cre-recombinase mRNA is isolated Cre-recombinase mRNA.

In one example of the current disclosure the Cre mRNA encodes a polypeptide that has the amino acid sequence of SEQ ID NO: 12.

5 In one example of the current disclosure the Cre mRNA encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 12 and that further comprises at its N- or C-terminus or at both a nuclear localization sequence. In one example the Cre mRNA encodes a polypeptide that has the amino acid sequence of SEQ ID NO: 12 and further comprises at its N- or C-terminus or at both independently of each
10 other one to five nuclear localization sequences.

In one example of the current disclosure the Cre mRNA comprises the nucleotide sequence of SEQ ID NO: 13 or a codon usage optimized variant thereof. In one example of the current disclosure the Cre mRNA comprises the nucleotide sequence of SEQ ID NO: 13 or a codon usage optimized variant thereof and further comprises
15 at its 5'- or 3'-end or at both a further nucleic acid encoding a nuclear localization sequence. In one example of the current disclosure the Cre mRNA comprises the nucleotide sequence of SEQ ID NO: 13 or a codon usage optimized variant thereof and further comprises at its 5'- or 3'-end or at both independently of each other one to five nucleic acids encoding nuclear localization sequences.

20 In one example of the current disclosure 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 example of the current disclosure the deoxyribonucleic acid encoding the polypeptide comprises one to eight expression cassettes.

25 In one example of the current disclosure the deoxyribonucleic acid encoding the polypeptide comprises at least 4 expression cassettes wherein

- a first recombination recognition sequence is located 5' to the most 5' (i.e. first) expression cassette,
 - a second recombination recognition sequence is located 3' to the most 3' expression cassette (i.e. the last expression cassette), and
 - 5 - a third recombination recognition sequence is located
 - between the first and the second recombination recognition sequence, and
 - between two of the expression cassettes,
- and
- 10 wherein all recombination recognition sequences are different.

In one example of the current disclosure the third recombination recognition sequence is located between the second and the third, or the third and the fourth, or the fourth and the fifth expression cassette.

15 In one example of the current disclosure the deoxyribonucleic acid encoding the polypeptide comprises a further expression cassette encoding for a selection marker.

In one example of the current disclosure the deoxyribonucleic acid encoding the polypeptide 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'-

20 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.

25 In one example of the current disclosure 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.

5 In one example of the current disclosure 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 second, third or fourth, respectively, expression cassette 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 third, fourth or fifth, respectively, expression cassette.

In one example of the current disclosure the start-codon is a transcription start-codon. In one example the start-codon is ATG.

15 In one example of the current disclosure the first deoxyribonucleic acid is integrated into a first vector and the second deoxyribonucleic acid is integrated into a second vector.

20 In one example of the current disclosure 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.

In one example of the current disclosure 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.

25 In one example of the current disclosure 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.

In one example of the current disclosure the mammalian cell is a CHO cell. In one example the CHO cell is a CHO-K1 cell.

5 In one example of the current disclosure the polypeptide is selected from the group of polypeptides consisting of a bivalent, monospecific antibody, a bivalent, bispecific antibody, a bivalent, bispecific antibody comprising at least one domain exchange, and a trivalent, bispecific antibody comprising at least one domain exchange.

In one example of the current disclosure the polypeptide is a heterotetrameric polypeptide comprising

- 10 - a first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- 15 - a first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- a second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,
- 20 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 example of the current disclosure the polypeptide is a heterotetrameric polypeptide comprising

- 25 - a first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a second heavy chain variable domain, a CL domain, a hinge region, a CH2 domain and a CH3 domain,

- a second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- a second light chain comprises 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 example of the current disclosure the polypeptide is a heterotetrameric polypeptide comprising

- a first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a second heavy chain comprises from N- to C-terminus a first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- a second light chain comprises 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 example of the current disclosure the polypeptide is a heterotetrameric polypeptide comprising

- a first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,

- a second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CL domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- a second light chain comprises 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 example of the current disclosure the polypeptide is a heteromultimeric polypeptide comprising

- a 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,
- a second 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 second heavy chain variable domain, and
- a first light chain comprises 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 example of the current disclosure the polypeptide is a heterotetrameric polypeptide comprising

- a first heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain, a CH3

domain, a peptidic linker, a second heavy chain variable domain and a CL domain,

- 5 - a second heavy chain comprises from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- a second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

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

 In one example of the current disclosure the polypeptide is a therapeutic antibody. In one preferred example the therapeutic antibody is a bispecific (therapeutic) antibody. In one example the bispecific (therapeutic) antibody is a TCB.

15 In one example of the current disclosure the polypeptide is a bispecific (therapeutic) antibody (TCB) comprising

- a first and a second Fab fragment, wherein each binding site of the first and the second Fab fragment specifically bind to the second antigen,
- 20 - a third Fab fragment, wherein the binding site of the third Fab fragment specifically binds to the first antigen, and wherein the third Fab fragment comprises a domain crossover such that the variable light chain domain (VL) and the variable heavy chain domain (VH) are replaced by each other, and
- 25 - an Fc-region comprising a first Fc-region polypeptide and a second Fc-region polypeptide,

 wherein the first and the second Fab fragment each comprise a heavy chain fragment and a full length light chain,

wherein the C-terminus of the heavy chain fragment of the first Fab fragment is fused to the N-terminus of the first Fc-region polypeptide,

5 wherein the C-terminus of the heavy chain fragment of the second Fab fragment is fused to the N-terminus of the variable light chain domain of the third Fab fragment and the C-terminus of the heavy chain constant domain 1 of the third Fab fragment is fused to the N-terminus of the second Fc-region polypeptide.

10 In one example of the current disclosure the polypeptide is an anti-CD3/CD20 bispecific antibody. In one example the anti-CD3/CD20 bispecific antibody is a TCB with CD20 being the second antigen. In one example the bispecific anti-CD3/CD20 antibody is RG6026.

15 The individual expression cassettes in the deoxyribonucleic acid according to the 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.

Detailed Description of Embodiments of the Invention

Herein is reported a method for generating a recombinant mammalian cell expressing a heterologous polypeptide and a method for producing a heterologous polypeptide using said recombinant mammalian cell.

20 The current invention is based, at least in part, on the finding that the number of clones obtained by targeted integration can be improved if as sole source of Cre-recombinase Cre mRNA is used compared e.g. with the use of Cre DNA (Cre plasmid). In more detail, it has been found that after the selection period, the absolute number of clones in the CRE mRNA-generated recombinant cell pools is higher than
25 in the CRE plasmid-generated recombinant cell pools (see Example 6 and Figures 2, 3 and 4). Thus, by using CRE mRNA instead of a CRE plasmid, a recombinant cell pool with greater size and heterogeneity is produced. Without being bound by this theory it is assumed that thereby the probability of identifying a recombinant cell clone with high titer and good product quality is increased. In addition, an increased
30 number of recombinant cell clones from CRE mRNA-generated pools are stable compared to CRE plasmid-generated cell pools.