

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) transfecting 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,

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

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- c) transfecting simultaneously with the first and second deoxyribonucleic acid of b) 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 wherein the one or more recombinases perform two recombinase mediated cassette exchanges;

and

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- 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, wherein the mammalian cell is a CHO cell.

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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 secrets the polypeptide of interest into the cultivation medium upon cultivation therein.

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.

Patent Claims

1. A method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a polypeptide and secreting the polypeptide 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
10 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;

15 b) transfecting 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),
- 20 - 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

- 25 - 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,

30 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,

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

c) transfecting simultaneously with the first and second deoxyribonucleic acid of b) 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 wherein
10 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,
15 thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the polypeptide and secreting the polypeptide, wherein the mammalian cell is a CHO cell.

2. The method according to claim 1, wherein the Cre mRNA encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 12.

20 3. The method according to claim 1, wherein the Cre mRNA comprises the nucleotide sequence of SEQ ID NO: 13 or a codon usage optimized variant thereof.

4. The method according to any one of claims 1 to 3, wherein exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the
25 mammalian cell at a single site or locus.

5. The method according to any one of claims 1 to 4, wherein the deoxyribonucleic acid encoding the polypeptide comprises at least 4 expression cassettes wherein

30 - 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, and
 - a third recombination recognition sequence is 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.
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6. The method according to any one of claims 1 to 5, wherein the deoxyribonucleic acid encoding the polypeptide comprises a further expression cassette encoding for a selection marker.
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7. The method according to any one of claims 1 to 6, wherein 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'-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.
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8. The method according to any one of claims 1 to 7, wherein the first and second deoxyribonucleic acid are in a first and second vector, and wherein the ratio by weight between Cre mRNA and mixture of first and second vector is in the range of from 1:3 to 2:1.
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9. The method according to any one of claims 1 to 8, wherein the first and second deoxyribonucleic acid are in a first and second vector, and wherein the ratio by weight between Cre mRNA and mixture of first and second vector is about 1:5.
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10. The method according to any one of claims 1 to 9, wherein each of the expression cassettes comprises in 5'-to-3' direction a promoter, a coding sequence and a polyadenylation signal sequence, wherein the promoter is the human CMV promoter with intron A and the polyadenylation signal sequence is the bGH polyadenylation signal sequence, 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.

11. The method according to claim 10, wherein each of the expression cassettes, except for the expression cassette of the selection marker, further comprises a terminator sequence following the polyadenylation signal sequence, and wherein the terminator sequence is the hGT terminator sequence.
- 5 12. The method according to any one of claims 1 to 11, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and
- 10 - the 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,
- the second heavy chain comprises from N- to C-terminus s first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- 15 - the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the 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.
- 25 13. The method according to any one of claims 1 to 11, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and
- the 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,

- the 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,
- the first light chain comprises from N- to C-terminus a second heavy chain variable domain and a CL domain, and
- the 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.

14. The method according to any one of claims 1 to 11, wherein the polypeptide is a heterotetramer comprising a first antibody heavy chain, a second antibody heavy chain, a first antibody light chain and a second antibody light chain, the deoxyribonucleic acid comprises four expression cassettes and

- the 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,
- the 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,
- the first light chain comprises from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- the second light chain comprises from N- to C- terminus a second light chain variable domain and a CL domain,

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.

15. The method according to any one of claims 1 to 14, wherein the first recombinase recognition sequence is L3, the second recombinase recognition sequence is 2L and the third recombinase recognition sequence is LoxFas.