

Summary:

The method disclosed herein describes a novel technology offering unparalleled efficiency, flexibility, utility and speed for the stable integration of transgenes into lymphocytes and other mammalian cells. The novel method is based on the use of an mRNA-encoded transposase (e.g. sleeping beauty transposase) in combination with a minicircle DNA-encoded transposable element. The novel method enables higher gene-transfer rates and is at the same time less toxic than the conventional approach, which is the use of plasmid DNA-encoded transposase in combination with a plasmid DNA-encoded transposable element.

The following effects will contribute to the higher gene-transfer rates achieved by the minicircles according to the disclosure:

- the longer half-life of minicircles as compared to plasmids,
- the easier migration of minicircles through cytoplasm and into the nucleus as compared to plasmids,
- the easier mobilization of the transposon from small supercoiled MCs compared to large circular plasmids.

According to the disclosure, these effects are not limited to minicircles but also apply to any other DNA encoding a transposable element containing an expression cassette for a transgene, provided that such DNA has a smaller size than a conventional plasmid which is suitable as a donor plasmid for transposable elements. Thus, according to the disclosure, any DNA encoding a transposable element containing an expression cassette for a transgene can also be used, provided that the DNA encoding the transposable element is a DNA encoding the transposable element as defined below.

Due to the higher gene-transfer rates achieved according to the disclosure, the implementation of the methods and uses of the disclosure under good manufacturing practice (GMP) will be facilitated. For instance, when the disclosure is used to generate CAR T cells, CD3/CD28 stimulation can be used to activate T cells prior to transfection, and unlike state of the art methods, the present disclosure does not require the use of feeder cells to expand the CAR T cells to achieve therapeutically relevant doses of the CAR T cells.

According to the disclosure, the lower amounts of transfected minicircle DNA (as compared to plasmid DNA) contribute to the reduction in toxicity achieved by the minicircles. Again, this effect is not limited to minicircles but also applies to any other DNA encoding a transposable

element containing an expression cassette for a transgene, provided that such DNA has a smaller size than a conventional plasmid which is suitable as a donor plasmid for transposable elements. Thus, according to the disclosure, any DNA encoding a transposable element containing an expression cassette for a transgene can also be used, provided that the DNA encoding the transposable element is a DNA encoding the transposable element as defined below.

A further advantage of the disclosure is that due to the lack of antibiotic resistance genes in minicircles, horizontal gene transfer of the antibiotic resistance genes to host bacteria and unintended integration of the antibiotic resistance genes into the host genome is excluded.

According to the disclosure, it was found that mRNA can be used as a source of the transposase. This finding was unexpected, because it was not known whether mRNA, which is short-lived, would be a suitable source to supply sufficient amounts of the transposase for the disclosure. According to the disclosure, the use of mRNA as a source of the transposase has two advantages: Firstly, because the transposase supplied by the mRNA is short-lived, there is a lower risk that already integrated transposons are re-mobilized. Secondly, the supply of the transposase as mRNA eliminates the risk of unintentional integration of a transposase expression cassette into the host genome, which could lead to uncontrollable, continuous transposition of genomically integrated transposons.

The present disclosure is also advantageous in that it provides a close-to-random integration profile of the transposons carrying the transgene, without preference for highly expressed or cancer related genes. Additionally, when using the disclosure, a significantly higher proportion of transgene integrations occurs in genomic safe harbors compared to LV integrations, close to the perfect score expected for random integration. Accordingly, the disclosure can be used to manufacture recombinant mammalian cells such as lymphocytes (e.g. CAR T cells) using virus-free transposition. The superior safety profile, high level stable transposition rate and ease-of-handling of the vectors of the disclosure make the disclosure a preferred gene-transfer strategy, e.g. in advanced cellular and gene-therapy.

Applications of the disclosure include but are not limited to the stable integration of a transgene encoding an immune receptor (e.g. a T-cell receptor or synthetic chimeric antigen receptor) into human T lymphocytes, with the immune receptor conferring specificity for a molecule expressed by a tumor cell. The transposase mRNA and transposon minicircle DNA may be introduced into lymphocytes by methods including but not limited to electrotransfer such as electroporation and nucleofection.

In one aspect, the present disclosure refers to an *in vitro* use of
a combination of a minicircle DNA encoding a transposable element and
a source of a transposase
to stably integrate the transposable element into the genome of a mammalian cell,
wherein the source of the transposase is an mRNA encoding the transposase or wherein the
source of the transposase is a transposase polypeptide, and
wherein the mammalian cell is a human lymphocyte.

In another aspect, the present disclosure refers to an *in vitro* use of a combination of:
a DNA encoding a transposable element containing an expression cassette for a
transgene and
a source of a transposase
to stably integrate the transposable element into the genome of a mammalian cell,
wherein the DNA encoding the transposable element lacks an origin of replication and/or
lacks an antibiotic resistance gene,
wherein the source of the transposase is an mRNA encoding the transposase or wherein the
source of the transposase is a transposase polypeptide,
and wherein the mammalian cell is a human lymphocyte.

In another aspect, the present disclosure refers to an *in vitro* method for obtaining a
recombinant mammalian cell containing a stably integrated transposable element, the
method comprising:

introducing a combination of a minicircle DNA encoding the transposable element
and a nucleic acid encoding a transposase into a mammalian cell,
thereby obtaining the recombinant mammalian cell, wherein the nucleic acid encoding the
transposase is an mRNA encoding the transposase; and/or the transposase is SB100X and
is optionally delivered as a polypeptide,
and wherein the mammalian cell is a human lymphocyte.

In another aspect, the present disclosure refers to an *in vitro* method for obtaining a
recombinant mammalian cell containing a stably integrated transposable element, the
method comprising:

introducing a combination of:

a DNA encoding a transposable element containing an expression cassette
for a transgene and
a nucleic acid encoding a transposase
into a mammalian cell,
thereby obtaining the recombinant mammalian cell,
wherein the DNA encoding the transposable element lacks an origin of replication and/or
lacks an antibiotic resistance gene,
wherein the nucleic acid encoding the transposase is an mRNA encoding the transposase,
and wherein the mammalian cell is a human lymphocyte.

In another aspect, the present disclosure refers to a recombinant mammalian cell obtained
by the *in vitro* method as disclosed herein, wherein the copy number of the transposable
element in the chromosomal genome of the recombinant cell is at least 5 and wherein the
mammalian cell is a human lymphocyte.

In another aspect, the present disclosure refers to use of the recombinant cell as disclosed
herein in the manufacture of a medicament for the treatment of cancer.

In another aspect, the present disclosure refers to use of the recombinant cell as disclosed
herein in the manufacture of a medicament for the treatment of an autoimmune disease or an
infectious disease, preferably wherein the infectious disease is a bacterial infection, a viral
infection or a fungal infection.

In another aspect, the present disclosure refers to a composition comprising a minicircle
DNA encoding a transposable element and a nucleic acid encoding the transposase, wherein
the nucleic acid encoding the transposase is as defined herein; and wherein the
transposable element is as defined herein.

Claims

1. *An in vitro* use of
a combination of a minicircle DNA encoding a transposable element and
a source of a transposase
to stably integrate the transposable element into the genome of a mammalian cell,
wherein the source of the transposase is an mRNA encoding the transposase or wherein the
source of the transposase is a transposase polypeptide, and
wherein the mammalian cell is a human lymphocyte.
2. The *in vitro* use according to claim 1, wherein the transposase is SB100X.
3. The *in vitro* use according to claim 1 or claim 2, wherein the transposable element contains the
genetic information for the expression of a T-cell receptor or chimeric antigen receptor, and
wherein the mammalian cell is a human T lymphocyte, preferably wherein the T-cell receptor or
chimeric antigen receptor is tumor-reactive, and wherein the human T lymphocyte obtained by
the use is a tumor-reactive human T lymphocyte suitable for use in the adoptive immunotherapy
of cancer, and/or wherein the transposable element contains the genetic information for a
chimeric antigen receptor, preferably, wherein the chimeric antigen receptor is specific for
CD19, CD20, CD22, CD33, CD44v6, CD123, CD135, EpCAM, EGFR, an EGFR variant, GD2,
ROR1, ROR2, CD269, CD319, CD38, or CD138.
4. The *in vitro* use according to any one of claims 1 to 3, wherein
 - I) the minicircle DNA encoding the transposable element encodes an a/b or g/d T-cell
receptor, a cytokine, a suicide gene, or a transduction marker; and/or
 - II) the nucleic acid encoding the transposase and the DNA minicircle encoding the
transposable element are introduced into the cell by electrotransfer, such as
electroporation, nucleofection; chemotransfer, calcium phosphate; or nanoparticles;
and/or
 - III) the transposase mediating transposition of the transposable element into the genome is
Sleeping Beauty, PiggyBac, Frog Prince, Himarl, Passport, Minos, hAT, Tol1, Tol2,
AciDs, PIF, Harbinger, Harbinger3-DR, and Hsmar1, or a derivative thereof having
transposition activity.
5. *An in vitro* use of a combination of:
a DNA encoding a transposable element containing an expression cassette for a
transgene and
a source of a transposase
to stably integrate the transposable element into the genome of a mammalian cell,

wherein the DNA encoding the transposable element lacks an origin of replication and/or lacks an antibiotic resistance gene,

wherein the source of the transposase is an mRNA encoding the transposase or wherein the source of the transposase is a transposase polypeptide,

and wherein the mammalian cell is a human lymphocyte.

6. The *in vitro* use according to claim 5, wherein:

I) the DNA encoding the transposable element is obtainable by deleting said origin of replication and/or said antibiotic resistance gene from a plasmid selected from the group consisting of:

pT;

pT2;

a plasmid having a DNA sequence which is at least 90% identical to the DNA sequence of pT;

a plasmid having a DNA sequence which is at least 90% identical to the DNA sequence of pT2; and

any other plasmid which is suitable as a donor plasmid for transposable elements; and/or

II) the total length of said DNA encoding the transposable element is not more than 3.0 kb, preferably not more than 2.0 kb greater than the length of said expression cassette; and/or

III) the total length of said DNA encoding the transposable element is not more than 1.5 kb greater than the length of said expression cassette; and/or

IV) the total length of said DNA encoding the transposable element is not more than 1.0 kb greater than the length of said expression cassette; and/or

V) the transgene is a T-cell receptor or chimeric antigen receptor as defined in claim 3, and wherein the mammalian cell is a human T lymphocyte; and/or

VI) the transgene is an α/β or γ/δ T-cell receptor, a cytokine, a suicide gene, or a transduction marker; and/or

VII) the use is a non-viral use.

7. An *in vitro* method for obtaining a recombinant mammalian cell containing a stably integrated transposable element, the method comprising:

introducing a combination of a minicircle DNA encoding the transposable element and a nucleic acid encoding a transposase into a mammalian cell,

thereby obtaining the recombinant mammalian cell, wherein the nucleic acid encoding the transposase is an mRNA encoding the transposase; and/or the transposase is SB100X and is optionally delivered as a polypeptide,
and wherein the mammalian cell is a human lymphocyte.

8. The *in vitro* method according to claim 7, wherein the transposable element contains the genetic information for the expression of a T-cell receptor or chimeric antigen receptor, and wherein the mammalian cell is a human T lymphocyte, preferably wherein the T-cell receptor or chimeric antigen receptor is tumor-reactive, and wherein the human T lymphocyte obtained by the method is a tumor-reactive human T lymphocyte suitable for use in the adoptive immunotherapy of cancer.
9. The *in vitro* method according to claim 7 or claim 8, wherein the transposable element contains the genetic information for a chimeric antigen receptor, preferably wherein the chimeric antigen receptor is specific for CD19, CD20, CD22, CD33, CD44v6, CD123, CD135, EpCAM, EGFR, an EGFR variant, GD2, ROR1, ROR2, CD269, CD319, CD38, or CD138.
10. The *in vitro* method according to any one of claims 7 to 9, wherein:
 - I) the minicircle DNA encoding the transposable element encodes an a/b or g/d T-cell receptor, a cytokine, a suicide gene, or a transduction marker; and/or
 - II) the nucleic acid encoding the transposase and the DNA minicircle encoding the transposable element are introduced into the cell by electrotransfer, such as electroporation, nucleofection; chemotransfer, calcium phosphate; or nanoparticles; and/or
 - III) the transposase mediating transposition of the transposable element into the genome is Sleeping Beauty, PiggyBac, Frog Prince, Himarl, Passport, Minos, hAT, Tol1, Tol2, AciDs, PIF, Harbinger, Harbinger3-DR, and Hsmar1, or a derivative thereof having transposition activity; and/or
 - IV) the combination of the minicircle DNA encoding the transposable element and the nucleic acid encoding the transposase are introduced together into the mammalian cell; and/or
 - V) the nucleic acid encoding the transposase and the minicircle DNA encoding the transposable element are introduced into the mammalian cell in a molar ratio of 1:1 or more, preferably in a molar ratio of 2:1 to 10:1, more preferably in a molar ratio of 3:1 to 9:1, still more preferably in a molar ratio of 4:1 to 8:1.
11. An *in vitro* method for obtaining a recombinant mammalian cell containing a stably integrated transposable element, the method comprising:

introducing a combination of:

a DNA encoding a transposable element containing an expression cassette for a transgene and

a nucleic acid encoding a transposase

into a mammalian cell,

thereby obtaining the recombinant mammalian cell,

wherein the DNA encoding the transposable element lacks an origin of replication and/or lacks an antibiotic resistance gene,

wherein the nucleic acid encoding the transposase is an mRNA encoding the transposase,

and wherein the mammalian cell is a human lymphocyte.

12. The *in vitro* method according to claim 11, wherein the DNA encoding the transposable element is obtainable by deleting said origin of replication and/or said antibiotic resistance gene from a plasmid selected from the group consisting of:

pT;

pT2;

a plasmid having a DNA sequence which is at least 90% identical to the DNA sequence of pT;

a plasmid having a DNA sequence which is at least 90% identical to the DNA sequence of pT2;

and

any other plasmid which is suitable as a donor plasmid for transposable elements.

13. The *in vitro* method according to claim 12, wherein:

- I) the total length of said DNA encoding the transposable element is not more than 3.0 kb, preferably not more than 2.0 kb greater than the length of said expression cassette; and/or
- II) the total length of said DNA encoding the transposable element is not more than 1.5 kb greater than the length of said expression cassette; and/or
- III) the total length of said DNA encoding the transposable element is not more than 1.0 kb greater than the length of said expression cassette; and/or
- IV) the DNA encoding the transposable element is a minicircle DNA as used in any one of the preceding claims; and/or
- V) the transgene is a T-cell receptor or chimeric antigen receptor as defined in any one of claims 8 or 9, and wherein the mammalian cell is a human T lymphocyte or the transgene is an a/b or g/d T-cell receptor, a cytokine, a suicide gene, or a transduction marker; and/or
- VI) the method is a non-viral method.

14. The *in vitro* method according to any one of claims 7 to 13, wherein said combination is introduced by introducing
- said nucleic acid encoding the transposase
 - and said DNA or minicircle DNA encoding the transposable element
- simultaneously; or
- said combination is introduced by introducing
- said DNA or minicircle DNA encoding the transposable element; and
 - said nucleic acid encoding the transposase sequentially.
15. The *in vitro* method according to any one of claims 7 to 14 or the *in vitro* use according to any one of claims 1 to 6, wherein:
- I) the minicircle DNA encoding the transposable element is a linearized DNA or a circular DNA; and/or
 - II) the nucleic acid encoding the transposase and the DNA or minicircle DNA encoding the transposable element are introduced into the mammalian cell in a weight ratio of 1:1 or more, preferably in a weight ratio of 2:1 to 10:1, more preferably in a weight ratio of 3:1 to 9:1, still more preferably in a weight ratio of 4:1 to 8:1.
16. A recombinant mammalian cell obtained by the *in vitro* method according to any one of claims 7 to 15, wherein the copy number of the transposable element in the chromosomal genome of the recombinant cell is at least 5 and wherein the mammalian cell is a human lymphocyte.
17. The recombinant cell of claim 16, wherein the copy number of the transposable element in the chromosomal genome of the recombinant cell is at least 6, at least 7, at least 8, at least 9 or at least 10.
18. The recombinant cell according to claim 16 or claim 17, wherein:
- A) 0% to 5%; or
 - B) at least 5%; or
 - C) at least 10%; or
 - D) at least 15%; or
 - E) at least 20%;
- of the copies of the transposable element in the chromosomal genome of the recombinant cell are integrated in genomic chromosomal regions which satisfy all of the following criteria:
- (i) not ultraconserved,
 - (ii) more than 300 kb away from miRNA genes,
 - (iii) more than 50 kb away from transcriptional start sites,
 - (iv) more than 300 kb away from genes involved in cancer, and

- (v) outside transcription units.
19. The recombinant cell according to any one of claims 16 to 18, wherein at least 40% of the copies of the transposable element in the chromosomal genome of the recombinant cell are integrated in genomic chromosomal regions which satisfy the following criterion:
- (v) outside transcription units.
20. The recombinant cell according to any one of claims 16 to 19, wherein at least one, preferably all copies of the transposable element in the chromosomal genome of the recombinant cell are integrated in genomic chromosomal regions which satisfy at least:
- A) any one; or
 - B) at least any two; or
 - C) at least any three; or
 - D) at least any four of the following criteria:
 - (i) not ultraconserved,
 - (ii) more than 300 kb away from miRNA genes,
 - (iii) more than 50 kb away from transcriptional start sites,
 - (iv) more than 300 kb away from genes involved in cancer, and
 - (v) outside transcription units.
21. Use of the recombinant cell according to any one of claims 16 to 20 in the manufacture of a medicament for the treatment of cancer.
22. Use of the recombinant cell according to any one of claims 16 to 20 in the manufacture of a medicament for the treatment of an autoimmune disease or an infectious disease, preferably wherein the infectious disease is a bacterial infection, a viral infection or a fungal infection.
23. A composition comprising a minicircle DNA encoding a transposable element and a nucleic acid encoding the transposase, wherein the nucleic acid encoding the transposase is as defined in claim 1; and wherein the transposable element is as defined in claims 3 or 4.
24. The *in vitro* use of any one of claims 1 to 6, the *in vitro* method of any one of claims 7 to 15, or the recombinant cell of any one of claims 16 to 20,
- wherein the cell is a human T lymphocyte; or
- wherein the cell is a CD8+ killer T cell, a CD4+ helper T cell, a naive T cell, a memory T cell, a central memory T cell, an effector memory T cell, a memory stem T cell, an invariant T cell, an NKT cell, a cytokine induced killer T cell, a g/d T cell, a B lymphocyte, or a natural killer cell.