meganucleases with new nuclease specificity are described. Nuclease-directed library generation is not described.

US2014/0113375 A1 describes a transient expression system for generation single-stranded DNA sequences homologous to a target genomic sequence, which can be transported to the nucleus to alter the genetic information of the target genomic sequence via DNA repair pathways or homologous recombination. It is suggested that a "library" of mutations could be created by low fidelity reverse transcription of the introduced (non-library) DNA. Mammalian display and selection of molecules with binding activity is not described.

US2012/0277120 describes methods and compositions for the simultaneous integration of a plurality of exogenous nucleic acids is in a single transformation reaction using the native homologous recombination machinery in yeast, which recombination may be further enhanced by inducing targeted double-strand breaks in the host cell's genome at the intended sites of integration. The methods are intended to overcome the need for multiple rounds of engineering to integrate multiple DNA assemblies, for example, for the construction of functional metabolic pathways in industrial microbes, such as yeast. The display or expression of libraries of binding molecules, the use of higher eukaryotes and the selection of molecules with binding activity is not described.

To fully realize the potential for antibody display on mammalian cells and other higher eukaryotes there is a need for a system to create large libraries which combine accurate integration into a pre-defined site with an efficiency that allows construction of large libraries.

Summary of the Invention

We have overcome the problem of creating large libraries of binders encompassing one or two binder genes per cell by using nuclease-directed integration of populations of genes encoding binders. The disclosure thus allows preparation of populations of eukaryotic cells wherein a repertoire of binder-encoding is integrated into a fixed locus in the genome allowing expression of the encoded binding molecule, thereby creating a population of cells expressing different binders.

The present disclosure relates to methods of producing eukaryotic cell libraries encoding a repertoire of binding molecules ("binders"), wherein the methods use a site-specific nuclease for targeted cleavage of cellular DNA to enhance site-specific integration of binder genes

through endogenous cellular repair mechanisms. Site–specific nucleases permit the accurate introduction of donor DNA encoding binder molecules into one or more defined loci within the eukaryotic genome or other eukaryotic cell DNA. The disclosure provides methods of preparing populations of eukaryotic cells in which a repertoire of genes encoding binders are integrated into a desired locus in cellular DNA *(e.g.*, a genomic locus) allowing expression of the encoded *binding molecule, thereby creating a population of cells expressing different binders.*

Construction of libraries of binders within eukaryotic cells according to the present disclosure has advantages over recombinase–directed approaches for site-directed incorporation of expression constructs. The present disclosure uses cellular DNA cleavage by site-specific nucleases to solve problems previously associated with construction of large repertoires of binder genes in eukaryotic cells and particularly higher eukaryotes. This disclosure allows the efficient creation of large populations of cell clones each expressing individual binders integrated at a fixed locus in cellular DNA. From these libraries of cellular clones it becomes possible to isolate genes encoding novel binding or function-modifying proteins and peptides.

Rather than recombinase-directed exchange of DNA, the approach of the present disclosure utilises site-specific cleavage of cellular (*e.g.*, genomic) DNA followed by the use of natural repair mechanisms to integrate binder-encoding donor DNA. Following cleavage of the cellular DNA at a sequence recognised by the site-specific nuclease ("recognition sequence"), breaks in the cellular DNA are repaired using mechanisms such as homologous recombination or non-homologous end joining (NHEJ). Creation of site-specific breaks in the cellular DNA enhances incorporation of exogenous donor DNA allowing the construction of large populations of cells with binder genes integrated at a fixed locus.

To date, site-specific nucleases such as meganucleases, ZFNs, TALE nucleases and CRISPR/Cas systems have been directed towards the efficient creation of cells with modifications to endogenous genes or for introduction of reporter genes for the study of cell function. There are also instances where nuclease-directed genomic targeting has been used to integrate genes encoding single secreted antibodies for antibody production (by purification from culture medium) [21, i,].

The disclosure simplifies construction of large libraries while directing integration to a single or limited number of defined genetic loci. Integration of donor DNA at one or more fixed loci normalises transcription compared with random integration of variable numbers of transgenes, and allows selection of antibody clones on the basis of translational and stability properties of the binder itself. Faithful integration of donor DNA at a pre-determined location or locations in the cellular DNA results in relatively uniform levels of transcription of binders in the library, and high efficiency of donor DNA introduction, make cell populations created by the methods of the disclosure particularly useful as libraries for display and selection of binders.

Methods of the disclosure thus produce high quality libraries of binders in eukaryotic cells, which can be screened to identify cells encoding and expressing a specific binder for a target of interest.

Also disclosed are new and improved methods of preparing eukaryotic cell libraries, the libraries themselves, isolation of desired binders, encoding nucleic acid and cells from the libraries, and uses of the libraries such as for expression and screening of binding molecules and for screening for the effects of binding molecules. Various methods will be described for producing libraries *in vitro* and using libraries *in vitro* or *in vivo*.

The disclosure provides a method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of binders, the method comprising using a site-specific nuclease to target cleavage of eukaryotic cell DNA to enhance site-specific integration of binder genes into the cellular DNA through endogenous cellular DNA repair mechanisms.

A method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of binders may comprise:

providing donor DNA molecules encoding the binders, and eukaryotic cells,

introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells.

For multimeric binders comprising at least a first and second subunit (i.e., separate polypeptide chains, such as antibody VH and VL domains presented within a Fab or IgG format), the multiple subunits may be encoded on the same molecule of donor DNA. However, it may be desirable to integrate the different subunits into separate loci, in which case the subunits can be provided on separate donor DNA molecules. These could be integrated within the same cycle of nuclease-directed integration or they may be integrated sequentially using nuclease-directed integration for one or both integration steps.

In one aspect, the present invention provides a method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of binders, comprising providing donor DNA molecules encoding the binders, and eukaryotic cells, introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating recombinant cells containing donor DNA integrated in the cellular DNA, and culturing the recombinant cells to produce clones,

thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of binders.

Methods of producing libraries of eukaryotic cell clones encoding multimeric binders may comprise:

providing eukaryotic cells containing DNA encoding the first subunit, and providing donor DNA molecules encoding the second binder subunit,

introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating recombinant cells which contain donor DNA integrated in the cellular DNA. These recombinant cells will contain DNA encoding the first and second subunits of the multimeric binder, and may

be cultured to express both subunits. Multimeric binders are obtained by expression and assembly of the separately encoded subunits.

In the above example, nuclease-directed integration is used to integrate DNA encoding a second subunit into cells already containing DNA encoding a first subunit. The first subunit could be previously introduced using the techniques of the present disclosure or any other suitable DNA integration method. An alternative approach is to use nuclease-directed integration in a first cycle of introducing donor DNA, to integrate a first subunit, followed by introducing the second subunit either by the same approach or any other suitable method. If the nuclease-directed approach is used in multiple cycles of integration, different site-specific nucleases may optionally be used to drive nuclease-directed donor DNA integration at different recognition sites. A method of generating the library may comprise:

providing first donor DNA molecules encoding the first subunit, and providing eukaryotic cells,

introducing the first donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating a first set of recombinant cells containing first donor DNA integrated in the cellular DNA,

culturing the first set of recombinant cells to produce a first set of clones containing DNA encoding the first subunit,

introducing second donor DNA molecules encoding the second subunit into cells of the first set of clones, wherein the second donor DNA is integrated into cellular DNA of the first set of clones, thereby creating a second set of recombinant cells containing first and second donor DNA integrated into the cellular DNA, and

culturing the second set of recombinant cells to produce a second set of clones, these clones containing DNA encoding the first and second subunits of the multimeric binder,

thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of multimeric binders.

In another aspect, the present invention provides a method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of multimeric binders, each binder comprising a first and a second subunit, wherein the method comprises providing eukaryotic cells containing DNA encoding the first subunit and providing donor DNA molecules encoding the second binder subunit, introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating recombinant cells which contain donor DNA integrated in the cellular DNA, and

culturing the recombinant cells to produce clones containing DNA encoding the first and second subunits of the multimeric binder, thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of multimeric binders.

In yet another aspect, the present invention provides a method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of multimeric binders, each binder comprising at least a first and a second subunit, wherein the method comprises providing first donor DNA molecules encoding the first subunit, and providing eukaryotic cells, introducing the first donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating a first set of recombinant cells containing first donor DNA integrated in the cellular DNA, culturing the first set of recombinant cells to produce a first set of clones containing DNA encoding the first subunit, introducing second donor DNA molecules encoding the second subunit into cells of the first set of clones, wherein the second donor DNA is integrated into cellular DNA of the first set of clones, thereby creating a second set of recombinant cells containing first and second donor DNA integrated into the cellular DNA, and culturing the second set of recombinant cells to produce a second set of clones, these clones containing DNA encoding the first and second subunits of the multimeric binder, thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of multimeric binders

Site-specific integration of donor DNA into cellular DNA creates recombinant cells, which can be cultured to produce clones. Individual recombinant cells into which the donor DNA has been integrated are thus replicated to generate clonal populations of cells – "clones" – each clone being derived from one original recombinant cell. Thus, the method generates a number of clones corresponding to the number of cells into which the donor DNA was successfully integrated. The collection of clones form a library encoding the repertoire of binders (or, at an intermediate stage where binder subunits are integrated in separate rounds, the clones may

encode a set of binder subunits). Methods of the disclosure can thus provide a library of eukaryotic cell clones containing donor DNA encoding the repertoire of binders.

Methods of the disclosure can generate libraries of clones containing donor DNA integrated at a fixed locus, or at multiple fixed loci, in the cellular DNA. By "fixed" it is meant that the locus is the same between cells. Cells used for creation of the library may therefore contain a nuclease recognition sequence at a fixed locus, representing a universal landing site in the cellular DNA at which the donor DNA can integrate. The recognition sequence for the site-specific nuclease may be present at one or more than one position in the cellular DNA.

Libraries produced according to the present disclosure may be employed in a variety of ways. A library may be cultured to express the binders, thereby producing a diverse repertoire of binders. A library may be screened for a cell of a desired phenotype, wherein the phenotype results from expression of a binder by a cell. Phenotype screening is possible in which library cells are cultured to express the binders, followed by detecting whether the desired phenotype is exhibited in clones of the library. Cellular read-outs can be based on alteration in cell behaviour such as altered expression of endogenous or exogenous reporter genes, differentiation status, proliferation, survival, cell size, metabolism or altered interactions with other cells. When the desired phenotype is detected, cells of a clone that exhibits the desired phenotype may then be recovered. Optionally, DNA encoding the binder is then isolated from the recovered clone, providing DNA encoding a binder which produces the desired phenotype when expressed in the cell.

A key purpose for which eukaryotic cell libraries have been used is in methods of screening for binders that recognise a target of interest. In such methods a library is cultured to express the binders, and the binders are exposed to the target to allow recognition of the target by one or more cognate binders, if present, and detecting whether the target is recognised by a cognate binder. In such methods, binders may be displayed on the cell surface and those clones of the library that display binders with desired properties can be isolated. Thus cells incorporating genes encoding binders with desired functional or binding characteristics could be identified within the library. The genes can be recovered and used for production of the binder or used for further engineering to create derivative libraries of binders to yield binders with improved properties.

In yet another aspect, the present invention provides a method of screening for a binder that recognises a target, comprising: producing a library by the method disclosed herein, culturing cells of the library to express the binders, exposing the binders to the target, allowing recognition of the target by one or more cognate binders, if present, and detecting whether the target is recognised by a cognate binder and optionally; further comprising recovering cells of a clone containing DNA encoding the cognate binder.

In yet another aspect, the present invention provides a method of screening for a cell of a desired phenotype, wherein the phenotype results from expression of a binder by the cell, the method comprising producing a library by the method disclosed herein, culturing the library cells to express the binders, and detecting whether the desired phenotype is exhibited, and optionally further comprising: recovering cells of a clone that expresses a binder that produces the desired phenotype, and/or: isolating DNA encoding the binder from the recovered clone, thereby obtaining DNA encoding a binder which produces the desired phenotype.

The present disclosure offers advantages over previous approaches for construction of libraries in higher eukaryotes. Some studies have used lentiviral infection to introduce antibody genes into mammalian reporter cells [106]. This has the advantage that large libraries can be generated but there is no control over the site of integration and copy number is controlled by using a low multiplicity of infection (as discussed above). In an alternative approach antibody genes were introduced via homologous recombination, without the benefit of nuclease-directed

integration and using homology arms of 10 kb but the efficiency of targeting was relatively low meaning that the potential library size was limited [105]. In contrast the use of sequence-directed nucleases retains the benefits of targeted integration to one or a few loci of choice while allowing efficient construction of large libraries. Nuclease-directed integration has the advantage that transgenes are targeted to a fixed locus or fixed loci within the cellular DNA. This means that promoter activity driving transcription of binder genes in all clones will be the same and the functionality of each binder will be a reflection of its inherent potency, translational efficiency and stability rather than being due to variation related to the integration site. Targeting to a single or limited number of loci will also enable better control of expression if required e.g., using inducible promoters.

In yet another aspect, the present invention provides use of a site-specific nuclease for targeted cleavage of cellular DNA in the construction of a library of eukaryotic cells containing DNA encoding a repertoire of binders, wherein nuclease-mediated DNA cleavage enhances site-specific integration of binder genes through endogenous cellular DNA repair mechanisms.

In yet another aspect, the present invention provides use of the library produced by a method disclosed herein as a display library for selecting binders to a desired target or for screening for cells displaying a desired cellular phenotype, wherein the phenotype results from expression of a binder by a cell.

Various features of the disclosure are further described below. It is noted that headings used throughout this specification are to assist navigation only and should not be interpreted as definitive, and that embodiments described in different sections may be combined as appropriate.

Detailed Description

Eukaryotic cells

The potential of populations of eukaryotic cells expressing a diverse repertoire of binders is exemplified and discussed in the Examples herein in relation to expression of antibody repertoires on the surface of mammalian cells. The benefits of the invention are not limited to mammalian cells and include all eukaryotes.

Yeast (*e.g.*, *Saccharomyces cerevisiae*) has a smaller genome than mammalian cells and homologous recombination directed by homology arms (in the absence of nuclease-directed cleavage) is an effective way of introducing foreign DNA compared to higher eukaryotes. Thus, a particular benefit of nuclease-directed integration of the present invention relates to integration of binder genes into higher eukaryotic cells with larger genomes where homologous recombination in the absence of nuclease cleavage is less effective. Nuclease-

directed integration has been used in yeast cells to solve the problem of efficient integration of multiple genes into individual yeast cells, *e.g.*, for engineering of metabolic pathways (US2012/0277120), but this work does not incorporate introduction of libraries of binders nor does it address the problems of library construction in higher eukaryotes.

Libraries of eukaryotic cells according to the present invention are preferably higher eukaryotic cells, defined here as cells with a genome greater than that of *Saccharomyces cerevisiae* which has a genome size of 12×10^6 base pairs (bp). The higher eukaryotic cells may for example have a genome size of greater than 2×10^7 base pairs. This includes, for

Claims

What is claimed is:

1. A method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of binders, comprising

providing donor DNA molecules encoding the binders, and eukaryotic cells,

introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating recombinant cells containing donor DNA integrated in the cellular DNA, and

culturing the recombinant cells to produce clones,

thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of binders.

2. A method according to claim 1, wherein the binders are antibody molecules; T cell receptors; and/or wherein the binders are multimeric, comprising at least a first and a second subunit.

3. A method according to claim 2, wherein the binders are antibody molecules and the antibody molecules are full length immunoglobulins, IgG, Fab, scFv-Fc or scFv.

4. A method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of multimeric binders, each binder comprising a first and a second subunit, wherein the method comprises

providing eukaryotic cells containing DNA encoding the first subunit and providing donor DNA molecules encoding the second binder subunit,

introducing the donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating recombinant cells which contain donor DNA integrated in the cellular DNA, and

culturing the recombinant cells to produce clones containing DNA encoding the first and second subunits of the multimeric binder,

thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of multimeric binders.

5. A method of producing a library of eukaryotic cell clones containing DNA encoding a diverse repertoire of multimeric binders, each binder comprising at least a first and a second subunit, wherein the method comprises

providing first donor DNA molecules encoding the first subunit, and providing eukaryotic cells,

introducing the first donor DNA into the cells and providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells, thereby creating a first set of recombinant cells containing first donor DNA integrated in the cellular DNA,

culturing the first set of recombinant cells to produce a first set of clones containing DNA encoding the first subunit,

introducing second donor DNA molecules encoding the second subunit into cells of the first set of clones, wherein the second donor DNA is integrated into cellular DNA of the first set of clones, thereby creating a second set of recombinant cells containing first and second donor DNA integrated into the cellular DNA, and

culturing the second set of recombinant cells to produce a second set of clones, these clones containing DNA encoding the first and second subunits of the multimeric binder,

thereby providing a library of eukaryotic cell clones containing donor DNA encoding the repertoire of multimeric binders.

6. A method according to claim 5, wherein the second donor DNA molecules are integrated by a method comprising providing a site-specific nuclease within the cells, wherein the nuclease cleaves a recognition sequence in cellular DNA to create an integration site at which the donor DNA becomes integrated into the cellular DNA, integration occurring through DNA repair mechanisms endogenous to the cells.

7. A method according to any of claims 3 to 6 wherein the multimeric binders are antibody molecules comprising a heavy chain variable (VH) domain and a light chain variable (VL) domain as separate subunits.

8. A method according to claim 7 wherein the antibody molecule further comprises one or more additional subunits, which may be introduced on the same donor DNA as the first or second subunit or which may be integrated at separate sites in the cellular DNA.

9. A method according to any of the preceding claims, wherein the cells are higher eukaryotic cells with a genome size of greater than 2×10^7 base pairs.

10. A method according to any of the preceding claims, wherein the recognition sequence is in genomic DNA of the cells or wherein the recognition sequence is in episomal DNA within the cells.

11. A method according to any of the preceding claims, wherein the recognition sequence for the site-specific nuclease occurs only once or twice in the cellular DNA.

12. A method according to any of the preceding claims, wherein the library contains at least 100, 10^3, 10^4, 10^5 or 10^6 clones, each clone being derived from an individual recombinant cell produced by integration of donor DNA.

13. A method according to any of the preceding claims, wherein each clone contains integrated donor DNA encoding only one or two members of the repertoire of binders, or wherein each clone contains integrated donor DNA encoding a single member of the repertoire of binders.

14. A method according to any of the preceding claims, further comprising:

culturing the library to express the binders,

recovering one or more clones expressing a binder of interest, and

generating a derivative library from the one or more recovered clones, wherein the derivative library contains DNA encoding a second repertoire of binders optionally wherein generating the derivative library comprises isolating donor DNA from the one or more recovered clones, introducing mutation into the DNA to provide a derivative population of donor DNA molecules encoding a second repertoire of binders, and introducing the derivative population of donor DNA molecules into cells to create a derivative library of cells containing DNA encoding the second repertoire of binders or wherein generating the derivative library comprises introducing mutation into the donor DNA in the one or more recovered clones by inducing mutation of the DNA within the clones.

15. A method of screening for a binder that recognises a target, comprising:

producing a library by the method of any of claims 1 to 14,

culturing cells of the library to express the binders,

exposing the binders to the target, allowing recognition of the target by one or more cognate binders, if present, and

detecting whether the target is recognised by a cognate binder and optionally;

further comprising recovering cells of a clone containing DNA encoding the cognate binder.

16. A method of screening for a cell of a desired phenotype, wherein the phenotype results from expression of a binder by the cell, the method comprising

producing a library by the method of any of claims 1 to 14,

culturing the library cells to express the binders, and

detecting whether the desired phenotype is exhibited,

and optionally further comprising:

recovering cells of a clone that expresses a binder that produces the desired phenotype, and/or:

isolating DNA encoding the binder from the recovered clone, thereby obtaining DNA encoding a binder which produces the desired phenotype.

17. Use of a site-specific nuclease for targeted cleavage of cellular DNA in the construction of a library of eukaryotic cells containing DNA encoding a repertoire of binders, wherein nuclease-mediated DNA cleavage enhances site-specific integration of binder genes through endogenous cellular DNA repair mechanisms.

18. Use of the library produced by a method according to any of claims 1 to 14 as a display library for selecting binders to a desired target or for screening for cells displaying a desired cellular phenotype, wherein the phenotype results from expression of a binder by a cell.