able to promote iPSC reprogramming. Remarkably, the inventors have found that closed linear DNA vectors have the capability of maintaining long-term expression in reprogramming without the need for any mechanism of chromosomal attachment, retention or segregation. Surprisingly, the closed linear DNA molecules are able to maintain long-term expression and effect reprogramming without p53 suppression. Further, the cells derived using a transfection of closed linear DNA appear to be more stable than cells transfected with a comparable circular plasmid carrying OriP/EBNA1, since the closed linear DNA constructs are naturally lost from the cells much more rapidly than plasmids carrying OriP/EBNA1. This is supported by the data presented on Figures 14A and 14B. These results are surprising, because most vectors without the use of chromosomal scaffold attachment and/or p53 suppression are not maintained for a sufficient period to express the necessary reprogramming factors. Further, the closed linear DNA vectors appear to effect reprogramming without the requirement for accessory sequences, elements or expression cassettes. The inventors have also found that closed linear DNA vectors were able to effect reprogramming of cells that were intransigent to reprogramming using standard OriP/EBNA1 based methods, see Example 5 and Figure 11. Surprisingly, pluripotent stem cells developed in this manner are stable and closer in phenotype to natural stem cells such as embryonic stem cells.

Summary

In one aspect, the present disclosure refers to an *in vitro* use of a closed linear DNA vector encoding one or more reprogramming factors for producing induced pluripotent stem cells from somatic cells, wherein said vector lacks sequences for chromosomal scaffold attachment.

In another aspect, the present disclosure refers to an *in vitro* method of producing induced pluripotent stem cells (iPSCs) comprising introducing one or more closed linear vector(s) encoding one or more reprogramming factors into a population of somatic cells, and culturing said cells to effect expression of the one or more reprogramming factor(s), wherein said vector(s) lacks sequences for chromosomal scaffold attachment.

In a further aspect, the present disclosure refers to a population of pluripotent stem cells induced from somatic cells with a closed linear DNA vector encoding at least one reprogramming factor, wherein said vector lacks sequences for chromosomal scaffold attachment.

The inventors have found that it is possible to reprogram a somatic cell using a one or more closed linear DNA vectors encoding reprogramming factors. If a somatic cell is transfected in this fashion and cultured for a sufficient period of time it is possible to obtain a stable and homogenous population of induced pluripotent stem cells.

A sufficient period of time/sufficient time is usually about 30 days, i.e. a period of 25 to 35 days, any one of 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 days.

The present disclosure thus relates to the use of a closed linear DNA vector encoding one or more reprogramming factors for producing induced pluripotent stem cells.

The present disclosure further relates to a method of producing induced pluripotent stem cells (iPSCs) comprising introducing one or more closed linear vector(s) comprising one or more reprogramming factors into a population of somatic cells, and culturing said cells to effect expression of the one or more reprogramming factor(s). Said introduction may be via transfection/nucleofection. The cells may be cultured for sufficient time, for example about 15-30 days to induce the reprogramming of the cells to pluripotency.

The required reprogramming factors for any part of this present disclosure can all be encoded on one closed linear DNA vector, or the required reprogramming factors can be divided amongst two or more closed linear DNA vectors. Any number of closed linear DNA vectors can be used. Two or more closed linear DNA vectors may be used, or 3, 4, 5, 6, 7, 8 or more.

The closed linear DNA vector(s) may lack sequences for chromosomal scaffold attachment and replication, such as OriP/EBNA1. Further or alternatively, the closed linear DNA vectors may lack sequences for the suppression of p53, for example by RNA interference.

The reprogramming factors expressed by the closed linear DNA vector(s) may be any reprogramming factor required in order to reprogram a mature cell into an iPSC. The reprogramming vectors may be natural, modified or synthetic factors. They may be one or more Yamanaka factors. The closed linear DNA vector(s) may be used to transfect a somatic cell in order to produce an induced pluripotent stem cell. Somatic is generally used to refer to the cells of the body in contrast to the germ line cells, and encompasses all cell types of the body other than egg or sperm cells.

In order to effect reprogramming, the closed linear DNA vector(s) are transfected into the somatic cell. Any method of transfection may be used, and may depend upon the type of donor cell undergoing transfection. Nucleofection may be required in some instances.

The transfected cells are then cultured using appropriate conditions to permit expression of the reprogramming factors from the closed linear DNA vectors. Conditions for permitting reprogramming are generally known in the art, and any appropriate conditions may be selected. Ideally, for human therapeutic use, the culturing may be performed in a feeder-free system, such that the cells obtained are free of any animal component contamination (xenofree). This culturing step is performed for a sufficient time in order for the cells to be induced into pluripotency. Sufficient time is described previously.

Closed linear DNA is generally understood to be double-stranded DNA covalently closed at each end. There is, therefore, no free 3' or 5' ends to the DNA. The double stranded DNA in the linear section is complementary in sequence. When denatured, closed linear DNA may form a single stranded circle. The DNA may be closed at each end by any suitable sequence, forming any secondary structure, such as a hairpin or a hairpin loop, or more complex structures such as cruciform. The sequence at the closed ends of the linear DNA may be complementary or non-complementary. The closed linear DNA may be made by any suitable method. Given the use of the DNA vector in human cells, it may be preferred to ensure that the closed linear DNA is free from any prokaryotic DNA sequences, such as antibiotic resistance genes or origins of replication.

The present disclosure further relates to the cells made using the method of the present disclosure, since the present inventors have found that these are of a higher grade or are more stable compared to those transfected using alternative episomal vectors.

The present disclosure therefore further relates to a population of induced pluripotent stem cells, wherein said population of cells is made using any use or method as described here. Said population of cells may no longer harbour the closed linear DNA vectors, since these are naturally lost during cell maintenance, making these much safer than cells induced by current methods, including retroviral methods where the reprogramming factors are not removed. Said present disclosure therefore includes a therapeutic and/or Good Manufacturing Practice

(GMP) grade population of stable, pluripotent stem cells induced with a closed linear DNA vector encoding at least one reprogramming factor.

The cells of the present disclosure are preferably human.

When cultured, pluripotent stem cells are generally densely packed. When differentiated, the cells are less densely packed, and therefore in a mixed cell population (pluripotent and differentiated) a small proportion of differentiated cells can appear to be more numerous. Pluripotent stem cell colonies are graded by those skilled in the art. Colonies in grade A are clear pluripotent stem cell colonies with clean edges and borders, the cells are densely packed and no differentiation is seen, nor disperse cells around the colony edge. Colonies in grade B have spontaneously differentiated cells at the edge, which are identified as they are more disperse. Colonies in grade C have cells which have mainly spontaneously differentiated, leaving only pockets of pluripotent stems in the disperse cells.

The inventors have identified that many more of the colonies obtained using closed linear DNA vectors are classified as category A, particularly when compared to cells obtained using the current state of the art techniques.

Indicative data for stability is the generation of colonies which are classified as Grade A. Such analysis and classification includes the visual (including by microscope) observation of the relevant cell colonies, looking in particular at the borders of the colonies to examine for spontaneous differentiation. Since differentiated cells are less densely packed, this leads the colony to have more disperse edges. Colonies with clean edges or borders are placed into grade A, and are therefore defined as more stable, since they are retaining a pluripotent phenotype. Cells that are not stable in the pluripotent state spontaneously differentiate. Thus, visual inspection allows the stability of the cells in the colony to be determined.

Spontaneous differentiation may also be determined using the presence of either a marker for differentiation, or a marker for pluripotency. For example, SSEA-1 is a marker of very early differentiation/exit from the pluripotent state, and TRA-1-60 and TRA-1-81 are human markers of pluripotency. A colony may be extracted and subjected to analysis using fluorescence activated cell sorting (FACS). The cells are dissociated from the culture and individual cells are labelled using fluorophore-conjugated antibodies to the particular cell marker (i.e. SSEA-1 or TRA-18-1). The cells are then separated depending on whether they are labelled with the fluorophore or not. It is now possible to perform FACS on live cells and continue culturing after sorting.

Additionally, alkaline phosphatase (AP) activity is associated with the pluripotent state and can be quantified using a colorimetric substrate assay which may be applied to individual cells or cell colonies. It is possible to stain cells for AP activity and visually determine that the cells in a colony are pluripotent. This stain is sensitive enough for the cells to be inspected on a plate. Additionally, it is possible to use technology to determine the levels of AP staining in a colony to provide a quantitative result. This quantitative data allows comparison between methodologies. AP may allow the skilled person to grade a colony, if further confirmation is required.

These are all examples of ways in which the stability of a pluripotent stem cell colony may be determined.

The present disclosure therefore includes a population of stable pluripotent stem cells induced with a closed linear DNA vector, in particular a population of stable pluripotent stem cells induced with a closed linear DNA vector which does not contain EBNA1, or a functional variant or derivative thereof. Preferably the closed linear DNA vector also lacks the OriP sequence or functional variants or derivatives thereof. Optionally the closed linear DNA vector(s) lack genes for chromosomal scaffold attachment. Further or alternatively, the closed linear DNA vectors may lack sequences for the suppression of p53.

The stable cells or reprogrammed cells of the present disclosure are less likely to undergo spontaneous differentiation, a common issue with other methods of reprogramming. Indeed, in the examples it is shown that cells reprogrammed using reprogramming vectors encoded by closed linear DNA are more stable than those expressed by standard vectors (Figure 5). In reprogramming, colonies are commonly observed with cells at the periphery which have lost their pluripotency and have differentiated. To deal with this issue, commonly cells are mechanically cut away from the colonies. The present inventors have observed that cells reprogrammed using closed linear DNA vectors encoding the reprogramming factors do not differentiate at the periphery and do not need to be excised. More colonies are classified as grade A. The stability of the iPS cells generated using reprogramming factors expressed by closed linear DNA vectors can be confirmed using FACs™ (fluorescence activated cell sorting) at various passage numbers (i.e. Passage 10, 15 etc.). The cells can be interrogated using early differentiation markers and/or pluripotency markers to enable the cell sorting. Stable iPS cells will have few, if any of the former markers, and more of the latter. Ideally, the iPSCs induced according to the methods of the present disclosure will have low to no expression of the cell surface marker SSEA1 and express on their surface the cell surface marker SSEA3, TRA -1 -60 or TRA-1-81.

When using FACS analysis to determine stability, it will be important to use an optimal gating strategy when assessing cell samples. Those skilled in the art will be aware of the factors that need to be considered when determining an appropriate gating strategy, in order to exclude debris, include appropriate negative controls, exclude non-viable cells, staining with shared markers if appropriate and setting up the appropriate fluorophore analysis plots. The results obtained will depend on the success of the gating strategy.

The cells of the present disclosure preferably lack the closed linear DNA vector(s) used to induce their production.

Since the methods and uses of the present disclosure result in a more genetically and phenotypically stable and safer iPSC population, these are therefore more likely to be able to be used clinically and/or therapeutically, the use of a closed linear DNA vector to obtain these cells is new. The present disclosure therefore further relates to a composition for preparing therapeutically acceptable induced pluripotent stem cell comprising a closed linear DNA vector encoding at least one reprogramming factor. The present disclosure therefore further includes a pharmaceutical composition comprising a closed linear DNA vector encoding at least one reprogramming factor and at least one pharmaceutically acceptable excipient.

Claims

1. An *in vitro* use of a closed linear DNA vector encoding one or more reprogramming factors for producing induced pluripotent stem cells from somatic cells, wherein said vector lacks sequences for chromosomal scaffold attachment.

2. An *in vitro* method of producing induced pluripotent stem cells (iPSCs) comprising introducing one or more closed linear vector(s) encoding one or more reprogramming factors into a population of somatic cells, and culturing said cells to effect expression of the one or more reprogramming factor(s), wherein said vector(s) lacks sequences for chromosomal scaffold attachment.

3. The use of claim 1 or the method of claim 2 wherein two or more closed linear DNA vectors are provided, each encoding one or more different reprogramming factors.

4. The use or method of any preceding claim wherein said reprogramming factor is selected from any one or more of: Oct 3/4, Sox2, Sox1, Sox3, Sox15, Sox18, Klf1, Klf2, Klf4, Klf5, c-myc, L-myc, and N-myc, NANOG, and/or LIN28.

5. The use or method of any preceding claim wherein said closed linear DNA vector lacks EBNA1, or functional derivatives or variants of EBNA1 and/or OriP, or functional derivatives or variants of OriP.

6. The use or method of any preceding claim wherein said closed linear DNA vector lacks any sequence for the knockdown of p53.

7. The use or method of any preceding claim wherein said reprogramming factors are operably linked to one or more promoters, preferably operably linked to the same promoter on the closed linear DNA vector.

8. The use or method of any preceding claim wherein said closed linear DNA vector lacks one or more of:

(i) bacterial CpG motifs;

(ii) a bacterial origin of replication; and/or

(iii) antibiotic resistance genes.

9. The use or method of any preceding claim wherein the closed linear DNA vector is introduced into a mature somatic cell.

10. The use or method of any preceding claim wherein said closed linear DNA vector is introduced via transfection, optionally via nucleofection.

11. The use or method of claim 10 wherein the cell is cultured for around 30 days after transfection or nucleofection prior to reprogramming being complete, preferably 28 to 32 days.

12. A population of pluripotent stem cells induced from somatic cells with a closed linear DNA vector encoding at least one reprogramming factor, wherein said vector lacks sequences for chromosomal scaffold attachment.

13. A population of cells as claimed in claim 12 wherein said cell is induced with two or more closed linear DNA vectors are provided, each encoding one or more different reprogramming factors.

14. A population of cells as claimed in claim 13 wherein said closed DNA vector:

(i) encodes one or more reprogramming factors, selected from any one or more of: Oct 3/4, Sox2, Sox1, Sox3, Sox15, Sox18, Klf1, Klf2, Klf4, Klf5, c-myc, L-myc, and N-myc, NANOG, and/or LIN28;

(ii) lacks EBNA1, or functional derivatives or variants of EBNA1 and/or OriP, or functional derivatives or variants of OriP;

(iii) lacks any sequence for the knockdown of p53; and/or

(iv) lacks one or more of:

(a) bacterial CpG motifs;

(b) bacterial origin of replication; and/or

(c) antibiotic resistance genes.

15. A population of cells as claimed in claim 12 wherein said cells are prepared according to the use or method of any one of claims 1 to 10.

16. A population of cells as claimed in any one of claims 12 to 15 wherein said population of cells are therapeutic and/or GMP grade.

17. A population of cells as claimed in any one of claims 12 to 16 wherein said population of cells lack the closed linear DNA vector used to induce them.

18. A population of cells as claimed in any one of claims 12 to 17 which are closer in phenotype to embryonic stem cells than to pluripotent stem cells induced with OriP/EBNA1 vectors.

19. A population of cells as claimed in claim 18 wherein the phenotype may be selected from:

(i) markers for differentiation;

(ii) markers for pluripotency;

(iii) immune system gene expression;

(iv) cytokine signalling;

(v) interferon signalling; and/or

(vi) inflammatory response.

20. A population of cells as claimed in any one of claims 12 to 19, wherein said cells are stable and optionally resist or have delayed spontaneous differentiation during passaging.

21. The use, method or population of cells of any preceding claim wherein said cells are human.