

INDUCED PLURIPOTENT CELL COMPRISING A CONTROLLABLE TRANSGENE FOR CONDITIONAL IMMORTALISATION

Field of the Invention

5 This invention relates to induced pluripotent stem cells that are generated from cells, for example adult stem cells, that are conditionally-immortalisable. In particular, the invention relates to induced pluripotent stem cells generated from cells comprising a controllable transgene for conditional immortalisation, and the progeny of those induced pluripotent stem cells such as cells of the haematopoietic lineage.

Background of the Invention

10 Human Pluripotent Stem Cells (hPSCs) are defined by the property of *pluripotency*: the property of being capable of differentiation into any cell type found in the body. They include embryonic stem cells (hESCs), derived from the inner cell mass of the early-stage embryo (blastocyst, approximately day 6.5 post-fertilisation), and induced pluripotent cells (hiPSCs), generated by reprogramming of somatic cells to a pluripotent phenotype by the transduction and exogenous
15 expression of certain transcription factors.

The canonical set of such transcription factors capable of reprogramming to pluripotency is known as OKSM (OCT4, KLF4, SOX2, C-MYC), but other factors are known which may substitute for O, K, S or M, or modulate the efficiency with which reprogramming, a stochastic process, occurs.

Typically, low-passage primary cells are the preferred substrate for reprogramming to generate
20 induced pluripotent stem cells (iPSCs). Such cells have the advantages that they tend to divide faster than higher passage cells; non-dividing cells are refractory to reprogramming. They are also more likely to be euploid. Adult Stem Cells (ASCs) also provide a promising substrate for reprogramming to pluripotency, often requiring fewer transcription factors and a more modest reprogramming event, due to endogenous expression of reprogramming factors (e.g. SOX2,
25 KLF4) and a more open chromatin structure associated with (pluri-/multi-) potency.

There are some examples (mostly EBV-immortalised blood cells) of iPSCs being generated from immortal mammalian cells rather than primary cells. Since such cells are typically immortalised by the stable genomic integration of EBV or an oncogene such as the simian virus 40 large T antigen, their clinical utility is doubtful. The 293FT cell line, for example, stably expressed the
30 SV40 large T antigen and whilst phenotype changes upon transfection of reprogramming transcription factors, it generates anomalous colonies rather than true iPSCs. WO-A-2014/186766 describes another example of generating iPSCs from immortalised somatic

cells, wherein the somatic cells are immortalised by infection with CMV-hTERT and wherein the somatic cells were themselves differentiated from iPSCs. Similarly, CN-A-110628821 describes a method of creating iPSCs from fibroblasts taken from patients with the rapid ageing Werner Syndrome, wherein hTERT immortalisation of the sampled fibroblasts is used to generate a population of iPSCs from these patients for *in vitro* studies of the disease.

iPSC lines derived from such immortalised cells are thus typically limited to *in vitro* applications such as disease modelling, drug discovery and developmental studies.

Furthermore, a study by Skvortsova *et al* (Oncotarget, 2018, Vol.9 (No.81), pp35241-35250) reports that immortalised murine fibroblast cell lines are refractory to reprogramming to a pluripotent state, and that aneuploidies associated with immortalisation and *in vitro* selection are unlikely to be the cause of such refractoriness. There are therefore significant technical challenges when seeking to identify cells suitable for reprogramming to a pluripotent state, and at least some immortalised cells are refractory to reprogramming.

Pluripotent stem cells are stable and may be cultured indefinitely *in vitro*. However, there are challenges to their clinical application such as their capability of teratoma formation and the issue that most differentiation protocols result in less than 100% differentiation to the desired endpoint. There thus remains the formal risk of teratoma formation from residual pluripotent cells in the therapeutic population, and the issue of co-transfer of undesired cell types to the patient. Such undesired subpopulations may have either neutral or negative effects, even if only a reduction in the efficiency of the therapy. This is exacerbated by the fact that the desired therapeutic cell type is often not the terminally-differentiated cell type whose chronic or acute loss causes pathology in the patient, but rather a late tissue progenitor / adult stem cell population which gives rise to the final cell type in the appropriate tissues of the patient. Late progenitor populations are often not stable in *in vitro* culture, and thus in addition to the purity issues noted above their scalable production at acceptable levels of purity for clinical use is a non-trivial challenge, even if in theory their production from pluripotent cells is effectively unlimited.

Such progenitor cell populations are also typically very difficult to handle. If they are isolated from either the patient or another person prior to transplant (e.g., bone marrow cells), difficulties associated with the very limited availability of material, the requirement for both persons to be available for operations at the same time and place, availability of a suitable (e.g. immunocompatible) donor, the mixed population of cells, lack of transferred material purity and QC all serve to limit the generalisability of such treatments. Theoretically, the possibility of generating such cell populations by differentiating hPSCs such as hiPSCs would ameliorate such issues, but they present other challenges. Significantly, progenitor populations are difficult to handle *in vitro* and are not stable over time. As such, their homogenous, GMP-compliant scalable

production is typically not possible even if GMP (Good Manufacturing Practice) quality iPSCs are available. Most differentiation protocols are not 100% efficient, so that contaminating subpopulations are likely to be present in the therapeutic cell population.

5 Cells of the haematopoietic lineage are of particular interest, in particular for therapeutic applications. The haematopoietic system consists of a large group of cell types with a variety of functions including both innate and acquired immunity to infection or cancer, blood clotting and the production of red blood cells. All of these cell types comprise a single lineage, derived ultimately from the haematopoietic stem cell (HSC), a multipotent adult stem cell type (ASC) present in the bone marrow. Haematopoietic lineage cells have utility as therapeutics for a variety
10 of conditions, including immunotherapy for cancer and treatment of autoimmune diseases, anaemias, and trauma.

Cells of the haematopoietic lineage are used as therapies for a variety of conditions. These include the use of killer cells such as CD8+ T cells or Natural Killer (NK) cells as anti-tumour therapeutics, perhaps carrying genetically-engineered receptors that target tumour cells
15 specifically (e.g. Chimeric Antigen Receptor-T cells), T-Regs for the treatment of autoimmune disorders, and platelets, e.g. for patients with clotting disorders such as certain cancer patients or trauma patients, erythrocytes, e.g. for the treatment of anaemias and battlefield or trauma medicine, and B-lymphocytes for the treatment of various conditions including cancer, or for the production of antibodies, dendritic cells for the treatment of cancers eg Provenge (sipuleucel-T)
20 and others. All of these cell types comprise part of the haematopoietic lineage, and are ultimately derived from haematopoietic stem cells, HSCs. HSCs are a rare cell type present in a niche in the bone marrow, and are defined by their ability to reconstitute the immune system of a lethally-irradiated animal (which entirely ablates the immune system), for example post-radiotherapy treatment for cancer. They can be identified by the presence of protein markers, such as the
25 surface receptors CD34 and KIT (CD117), and concurrent absence of mature haematopoietic markers such as the T cell receptor or rearranged genetic loci capable of producing mature antigen-specific antibody molecules by B lymphocytes.

The clinical application of HSCs is non-trivial. They are rare in adults, difficult to isolate *in vitro* and typically have a slow growth rate. There are known to be several subtypes of HSCs, which
30 vary in their potency; not all are apparently capable of complete reconstitution of the immune system of a compromised organism.

HSCs can therefore generate a large range of haematopoietic-lineage cells that are useful for the treatment of a range of diseases, are useful as mediators to generate products useful in therapy, and as research tools. HSCs can be generated from patient-specific sources to generate
35 autologous therapies or may be generated from allogeneic induced Pluripotent Cells (iPSCs) in

the case where single cell lines are to be developed for use as allogeneic cell therapies (for treating any or a wide range of patients). However to date HSCs have been difficult to isolate from patients in significant numbers and purity and thereafter to harvest and maintain in stable form outside of the organism. It would be desirable to maintain stable and expandable HSCs or lineage dependent differentiated progeny from such HSCs as a single stable and expandable cell line or cell line. This would be commercially and clinically valuable for generating immunotherapies and as producer cells for biologics for use in treatments and as research tools. To date such cell lines have proven elusive despite extensive efforts to generate such from iPSCs.

There remains a need to generate stem cells with clinical utility, in particular of the haematopoietic lineage

Summary of the Invention

The present invention is based on the surprising realisation that induced Pluripotent Stem Cell (iPSC) technology can be improved by generating iPSCs from conditionally-immortalised cells, in particular conditionally-immortalised stem cells. In particular aspects of the invention, the iPSCs are subsequently directed down the haematopoietic lineage. Some aspects therefore relate to methods of generating Haematopoietic Stem Cells from mammalian iPSCs carrying a conditional immortalisation cassette, such as CTX-iPSCs, and optionally further differentiating these HSCs further down the lineage towards a particular fate, for example B cells, T cells, Dendritic cells, NK cells or neutrophils.

A first aspect of the invention provides an induced pluripotent stem cell comprising a controllable transgene for conditional immortalisation. The controllable transgene will typically be able to conditionally-immortalise downstream (more differentiated) cells that are derived from the induced pluripotent stem cell. These downstream cells are typically cells of the haematopoietic lineage. These downstream cells may otherwise be difficult to handle, so the presence of the conditionally-immortalising transgene is an improvement.

In certain embodiments, the cells of the haematopoietic lineage may be a CD34⁺ CD43⁺ haematopoietic stem cell, a CD4⁺ T cell, a CD8⁺ T cell, a regulatory T cell, a CD56^{high}CD16[±] Natural Killer cell, a CD56^{low}CD16^{high} Natural Killer cell, a CD19⁺ B cell, a myeloid dendritic cell, a plasmacytoid dendritic cell, or a neutrophil.

In some embodiments, the cells of the haematopoietic lineage may be CD34⁺ cells that are also positive for CD49F and CD90, and negative for markers CD38 and CD45RA.

In some embodiments, the cells of the haematopoietic lineage may be Long Term Haematopoietic Stem Cells ("LT-HSCs").

5 A second aspect of the invention provides a pluripotent stem cell that is obtainable or obtained from a conditionally-immortalised cell, typically a conditionally-immortalised stem cell. This pluripotent stem cell is a useful source of other cells, including cells of the haematopoietic lineage.

10 A third aspect of the invention provides a method of producing a pluripotent stem cell, comprising the step of reprogramming a conditionally-immortalised cell, typically a conditionally-immortalised stem cell. The method may further comprise subsequent steps to generate different cell types from the pluripotent stem cell, typically to generate cells of the haematopoietic lineage. Example 6 below demonstrates in particular the generation of Haematopoietic Stem Cells from induced pluripotent stem cells, and then the differentiation of the HSCs further down the haematopoietic lineage, for example towards a T cell fate.

15 In certain embodiments, the method of the third aspect comprises a step of differentiating the pluripotent cell to an HSC and optionally further down the haematopoietic lineage. In certain embodiments, this method may comprise the pluripotent cell being differentiated into an HSC by (i) culturing in a medium comprising activin A, VEGF, SCF and BMP4 to form mesodermal cells and then (ii) culturing the mesodermal cells in the presence of FLT3, SCF, BMP-4, and interleukins 3 and 6, to form the HSCs.

20 The resulting HSCs may then, in some embodiments, be differentiated towards a T lymphocyte fate by (i) providing DLL-1 or DLL-4 protein in the culture to activate NOTCH signalling in the HSCs; or (ii) co-culturing the HSCs with stromal cells, optionally engineered to express the Notch ligand DLL1 or DLL4, or (iii) culturing the HSCs on a monolayer of bound VCAM and DLL4 proteins. The culture period for differentiation may be at least 7 days, at least 14 days or at least 21 days, for example 25 days or longer. Differentiation towards other cell fates are described herein and will also be apparent to the skilled person.

30 In one embodiment, a method of generating progenitor T cells from CTX-HSCs is provided by culturing the HSCs for a period of 14 days on a layer of bound chimeric proteins presenting VCAM and DLL4 to the HSCs. At the end of the 14 day period, a heterogeneous population of adherent and suspension cells is obtained. This cell population may typically express CD3 (T-cell receptor associated protein), CD43 (leucocyte marker), CD5 (lymphocyte, predominantly early T-cell marker), CD7 (immature T cell marker and NK cell marker) and CD25 (interleukin 2 receptor). Consistent with the interpretation of a T-progenitor phenotype rather than their being mature T

cells, in addition to their expression of CD5 and CD7, the cells of this embodiment do not express the T cell receptor itself or the associated molecules CD4 or CD8.

5 Culturing the progenitor T lymphocyte cells on bound Fc-DLL4 and Fc-VCAM proteins for longer periods can result in the cell population becoming more homogenous and with a more mature phenotype. Such cells may be more uniform (e.g. over 60% of them expressed the leucocyte marker CD43) and consistent with the interpretation that they represent a more mature lymphocyte population, also expressed CD8 in addition to CD3, but lost their expression of CD5 and CD7.

10 Alternative methods of differentiation have also been used to create more mature lymphocyte populations from conditionally-immortalised hiPSC-derived HSCs. Coculturing the CTX-HSCs with murine MS5 stromal cells engineered to express the human NOTCH ligand DLL1 or on a monolayer of MS5-DLL1 cells induced strong growth in the small non adherent cell population.
15 This population matured during the course of differentiation, losing its CD34 expression, but CD8 expression was not observed, although the early markers CD5 and CD7 expression levels fell. Thus, T cell progenitors produced by this method probably represent an earlier stage of T cell development than that produced by the bound proteins approach (described above). These results can be seen in Figures 17, 18, 20 and 21, for example.

20 Further aspects of the invention relate to cells produced by any method of the invention, in particular cells of the haematopoietic lineage, and extracellular vesicles produced by any of those cells.

25 The inventors have found that reprogramming conditionally-immortalised cells such as stem cells, for example cells from the CTX0E03 or STROC05 cell line, to pluripotency will allow the generation of other adult stem cell or tissue progenitor populations. Once the pluripotent cell is generated, conventional differentiation protocols can be used to provide cells of any desired lineage, for example the ectoderm, endoderm or mesoderm lineage. The mesodermal lineage gives rise to
30 haematopoietic cells, and is typical according to the present invention. In certain embodiments, the pluripotent cells are directed down a lineage that is different from the lineage of the original conditionally-immortalised stem cell. In certain embodiments, the induced pluripotent cell can be differentiated into a mesenchymal stem cell or a neural stem cell.

35 Typically, the induced pluripotent cell is differentiated into a haematopoietic stem cell, including further differentiation to cells of the immune system such as T lymphocytes, B-Lymphocytes, NK cells, neutrophils and dendritic cells. These cells are of mesodermal origin.

In certain embodiments, the induced pluripotent cells may be differentiated into a somatic (adult) stem cell, a multipotent cell, an oligopotent cell or a unipotent cell; or a terminally-differentiated cell. All of these cells are typically of the haematopoietic lineage.

5 Examples 1 to 5 below demonstrate that iPSCs, generated according to the invention from different conditionally-immortalised cells, are pluripotent and are able to enter the endoderm, mesoderm and ectoderm lineages. The Examples further show that adult stem cells (MSCs) can be generated from the iPSCs of the invention. These MSCs are shown to be multipotent, and able to differentiate into cartilage, fat and bone cells.

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Differentiation of the induced pluripotent cells of the invention into functional cells of the immune system, is also described and exemplified. These immune cells include T cells, B cells, Natural Killer (NK) cells and Dendritic cells. As will be understood by the skilled person, these cells will usually be arrived at via the haematopoietic lineage. T cells can include CD4+ T cells (often broadly referred to as "T helper cells"), Regulatory T cells (Tregs) often characterised by the marker FoxP3, and CD8+ T cells (for example CD8+ Cytotoxic T Lymphocytes). Another cell derived from the haematopoietic lineage is the neutrophil. Yet another cell derived from the haematopoietic lineage is a macrophage. Each of these cells can optionally be genetically engineered or otherwise modified. In particular, the cells can be modified to express a Chimeric Antigen Receptor to form a CAR-T cell, a CAR-NK cell, or any other CAR-modified immune cell. The Chimeric Antigen Receptor is typically directed to a protein or other marker on a target cell, usually a tumour cell. An example is CD19, which is targeted by CAR-cells (e.g. CART-T cells) to treat B- cell malignancies such as lymphoid leukemias (acute (ALL) and chronic (CLL)) and lymphomas. Other cells of interest, of the haematopoietic lineage according to the invention, are Tumour- Infiltrating Lymphocytes (TILs). When used in therapy, each of these cells is typically allogeneic to the patient. Nonetheless, in some circumstances the cells may be autologous to the patient, for example where patient cells are extracted, engineered and re-administered, such as in CAR-T or CAR-NK therapies. One CAR-NK cell type is the scFv-NKG2D-2B4-CD3 ζ cell described by Li *et al.*, 2018, *Cell Stem Cell* **23** 181-92.

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Other cells of the haematopoietic lineage that can be produced according to the invention, are red blood cells (erythrocytes). There remains a constant need for donor blood, of which a significant proportion of the need is for the red blood cell component. The ability to produce a large supply of red blood cells that can act as donor blood, simply by seeding a bioreactor with a standard vial of progenitors is highly advantageous. As the word "donor" indicates, these red blood cells are allogeneic to the recipient.

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A cell, e.g. a stem cell, can be rendered conditionally-immortalisable through the c-myc-ER^{TAM} transgene. This transgene has already been shown to permit the stable, scalable production of stem cell lines, such as the neural stem cell lines CTX0E03 and STR0C05, by the addition of 4-hydroxytamoxifen to the cell culture medium, which promotes growth and cell division without any change in phenotype.

The conditionally-immortalised stem cell is typically an adult stem cell, also referred to as a somatic stem cell. For example, it could be a neural stem cell, such as the CTX0E03 stem cell line. The CTX0E03 neural stem cell line has been deposited by the applicant (ReNeuron Limited) at the European Collection of Authenticated Cell Cultures (ECACC), Porton Down, UK and having ECACC Accession No. 04091601. In other embodiments, the neural stem cell line may be the "STR0C05" cell line, the "HPC0A07" cell line (also deposited by the applicant at ECACC) or the neural stem cell line disclosed in Miljan *et al* Stem Cells Dev. 2009.

The conditionally-immortalised stem cell is reprogrammed to pluripotency. Inducing the pluripotent phenotype typically involves introducing products of specific sets of pluripotency-associated genes, or "reprogramming factors", into a given cell type. The original set of reprogramming factors (also dubbed Yamanaka factors) are the transcription factors Oct4, Sox2, cMyc, and Klf4. The reprogramming factors are typically introduced into the cell using viral or episomal vectors, as is well-known in the art.

Viral vectors suitable for introducing reprogramming factors into a cell include lentivirus, retrovirus and Sendai-virus. Other techniques for introducing reprogramming factors include mRNA transfection.

It has been surprisingly observed that only one transcription factor is required to reprogram certain conditionally-immortalised stem cells, such as CTX0E03, to pluripotency. For example, Figures 2B-D show that OCT4 alone can induce pluripotency of CTX0E03. Combinations of transcription factors that were observed to achieve pluripotency include: OCT4 and SOX2; OCT, KLF4 and SOX2; OCT4, KLF4, SOX2 and MYC. Accordingly, reprogramming factors that comprise or consist of these combinations are provided for use in the present invention. Each of the combinations of factors that successfully induce pluripotency in Figure 2C is provided as a separate embodiment of the invention. Reprogramming factors for use in inducing conditionally-immortalised stem cells to pluripotency may comprise or consist of an exemplified combination.

Other immortalisation factors are known in the art and will be apparent to the skilled person. Any suitable combination of factors can be used, which is well within the ability of one skilled in the art. For example, reprogramming with small molecule inhibitors is known in the art, while NANOG

and TET1 are known as other suitable reprogramming factors. By way of example, Thomson and colleagues used NANOG, KLF4, SOX 2 and LIN28 as an alternative to OKSM. In another example, TET1 has been shown to be capable of substituting for OCT4.

- 5 As the activity of the c-myc-ER^{TAM} transgene recapitulates cellular MYC activity, vectors expressing the MYC oncogene are dispensable when reprogramming c-myc-ER^{TAM} inducibly-immortalised stem cells, because if required this activity can be provided by the provision of 4-OHT to the medium to activate the c-myc-ER^{TAM} fusion protein. Accordingly, in some embodiments, MYC (for example a MYC reprogramming vector) is not used as a separate reprogramming factor. The skilled person will of course be aware that conditional immortalisation systems using genes other than MYC, may require exogenous MYC to reprogram them.

The induced pluripotent cells can be differentiated into any desired cell type. Techniques for determining a cell lineage or cell type are well known in the art. Typically, these techniques involve the determination of markers of differentiation on either the cell surface (and/or the absence of markers of pluripotency such as Oct4) or internally, such as the presence of lineage-specific transcription factors, cell morphology and function. For example, pluripotent stem cells typically are positive for the canonical pluripotent transcription factor OCT4, and the cell surface antigens TRA-1-60 and SSEA-4, but do not express the early differentiation marker SSEA-1.

20 Markers of the endoderm lineage include GATA6, AFP or HNF-alpha. Other endoderm markers can include one or more of Claudin-6, Cytokeratin 19, EOMES, SOX7 and SOX17.

25 Markers of the mesoderm lineage include BMP2, Brachyury or VEGF. Other mesoderm markers can include one or more of Activin A, GDF-1, GDF-3, and TGF-beta.

Markers of the ectoderm lineage include PAX6, Nestin or TubIII. Other ectoderm markers can include one or more of Noggin, PAX2 and chordin.

- 30 Differentiation into different lineages is shown for example in Figure 3D. Differentiation of CTX-iPSCs and STR0C-iPSCs into endoderm, mesoderm and ectoderm lineages is also demonstrated in Example 2 (Figure 7) and Example 3 (Figure 9). Example 2 uses the following markers:

Lineage	Marker
Endoderm	SOX17
	FOXA2
Mesoderm	BRACHYURY
	CXCR4

Ectoderm	PAX6
	NESTIN

The pluripotent cells can be differentiated into any desired cell type. This can include a mesenchymal stem cell, a neural stem cell, or a haematopoietic stem cell. In another embodiment, a somatic (adult) stem cell results from differentiation of the induced pluripotent cell of the invention. In other embodiments, the cell that is provided by the method is a multipotent cell, an oligopotent cell or a unipotent cell. An example of this embodiment would be the production of progenitor cells, for example neuronal progenitor cells. Neuronal progenitor cells have been described as being potentially of use in treatment of neurodegenerative diseases, by Nistor and colleagues in *PloS One* (2011) vol. 6 e20692. The cell that results can also be fully differentiated, using known techniques for differentiation, into a terminally-differentiated cell. An example of this embodiment is the differentiation to and scalable production of medium spiny neurons, as are lost in Huntington's disease, as described by Carri and colleagues (2013); see *Stem Cell Review and Reports*, DOI 10.1007/s12015-013-9441-8. In a particular embodiment, the haematopoietic stem cell can be differentiated into a T cell, an NK cell and/or a dendritic cell. T cells are therefore provided as one embodiment. Natural Killer cells are provided as another embodiment. Dendritic cells are further provided.

Differentiation of CTX-iPSCs into mesenchymal stem cells is demonstrated in Example 1 and Figure 5. In this example the MSC phenotype is identified by the presence of the markers CD73, CD90 and CD105, but not CD14, CD20, CD34 or CD45.

Differentiation of the CTX-iPSC-MSCs into cartilage, fat and bone cells is demonstrated in Example 3 and Figure 10.

Reactivation (if necessary) of the c-myc-ER^{TAM} transgene followed by addition of 4-OHT to the medium should permit indefinite growth of the derived cell population. By analogy with CTX0E03 itself, this is expected to allow the scalable production of effectively unlimited quantities of a therapeutically useful cell population in vitro which may be used as an "off-the-shelf" therapy for any condition characterised by acute or chronic cell loss for which cell therapies either do not exist, or are limited by tissue donor availability or technical limitations of differentiation protocols from hPSCs.

The methods of the invention can involve further processing, culture or formulation steps that may be necessary to provide the desired product. In certain embodiments, a method of the invention can include one or more of the following steps, typically at the end of the method:

culturing the cells that result from the method;

passaging the cells that result from the method;
harvesting or collecting the cells that result from the method;
packaging the cells that result from the method into one or more containers; and/or
formulating the cells that result from the method with one or more excipients,
5 stabilisers or preservatives.

The conditionally-immortalised stem cells, the pluripotent cells that result from reprogramming, and the more differentiated cells that can be obtained from the pluripotent cells, are typically isolated or purified. The extracellular vesicles produced by any of these cells, for example
10 exosomes, are also typically isolated or purified.

The cells that are provided following differentiation, and the extracellular vesicles produced by them, can be used in therapy. Therapy will typically be of a disease or disorder in an individual in need thereof. The patient will typically be human.
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In a further aspect, the invention provides a composition comprising: conditionally-immortalised stem cells; the pluripotent cells that result from reprogramming; the more differentiated cells that can be obtained from the pluripotent cells; or the extracellular vesicles for example exosomes produced by any of these cells; and a pharmaceutically acceptable excipient, carrier or diluent.

20 **Brief Description of the Drawings**

Figure 1: Reprogramming of CTX cells to a pluripotent phenotype. (A) Schematic of CTX reprogramming process (HPSC medium: E8 / Stemflex; hPSC substrate: LN-521 / vitronectin-XF) and episomal plasmids driving OKSML expression which may be used for reprogramming (Epi5
25 kit, Invitrogen). CTX culture medium may be supplemented with 4-OHT or not, to provide MYC activity through the c-myc-ER^{TAM} transgene, as desired. Transfection may be achieved in a variety of ways, such as by lipofection, nucleofection or electroporation. (B) EGFP signal indicates transfection efficiency at 24 hours post-transfection. (C) Example young colonies of reprogrammed CTX cells with hPSC phenotype at day 15 post-transfection, showing very different cell and colony morphology from the parental CTX cells surrounding the colony. (D) Example 6-
30 well plate showing hPSC-phenotypic (alkaline phosphatase-positive, red stained) colonies at day 21 endpoint.

Figure 2 shows that CTX0E03 cells are reprogrammable with fewer factors. (A) Vectors expressing single factors, pCE-OCT3/4, pCE-SOX2 and pCE-KLF4; 4-OHT provision mimics MYC via c-myc-ER^{TAM}. (B) Inset: example AP-stained plate for colony counting. Main image: colony reprogrammed with transcription factor OCT4 alone. (C) Colony numbers obtained with
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different factor combinations (S-K: pCE-SK, M-L: pCE-UL, S: pCE-SOX2, K: pCE-KLF4, M: 4-OHT → d 14). (D) Venn diagram showing combination effects (numbers: x colonies obtained; zeroes: no colonies).

5 Figure 3 shows the pluripotent phenotype of CTX-iPSCs:

(A) Cell and colony morphology of CTX-iPSCs, wherein (ii, iii) are two examples of induced pluripotent CTX cell lines that recapitulate the dense colonies of small, closely-packed cells with prominent nucleoli characteristic of hPSCs, differing markedly from the neuronal phenotype of the parental CTX cells (i);

10 (B) CTX-iPSC lines express the enzymatic marker alkaline phosphatase (pink stain). Alkaline-phosphatase staining of established CTX-derived hPSC lines, wherein Pink coloration indicates the cells are positive for the pluripotency marker TNAP and are thus capable of performing the enzyme-catalysed colour change reaction *in vitro*;

(C) Flow cytometry shows that CTX-iPSC lines express pluripotency-associated markers including the transcription factor OCT4, and the cell surface antigens SSEA-4 and TRA-1-60, but do not express the early differentiation marker SSEA-1. (i) Summary table for several CTX-iPSC lines, (ii) example data for one CTX-iPSC line: upper row, left to right: SSEA-1, OCT4, SSEA-4; lower row, left to right: TRA-1-60, forward v. side scatter, SSEA-4;

(D) RT-qPCR showing upregulation of lineage-specific markers upon *in vitro* differentiation to endoderm, mesoderm and ectoderm (individual CTX-iPSC lines indicated by colour).

Figure 4 assesses the transgene locus in CTX-iPSCs. (A) Giemsa staining of parental CTX0E03 cells (top, 4 days, 2nd row, 10 days) and five CTX-iPSC lines at 4 days in G418 (3rd-7th rows) indicates expression activity of the c-myc-ER^{TAM}-associated *NeoR* gene. (B) Bisulphite-conversion of the CMV-IE promoter driving the c-myc-ER^{TAM} transgene shows the cytosine methylation state at the locus (white circle, unmethylated CpG; black circle, methylated CpG; comma, indeterminate read).

Figure 5 shows the production of an exemplary therapeutic cell population derived from CTX-iPSCs. (A) Pluripotent CTX-iPSCs on Laminin-521 in mTeSR1 medium (standard culture conditions for preserving pluripotency *in vitro*). (B) Plastic-adherent candidate mesenchymal stem cells (MSCs) derived from cells in (A) in MSC medium (α -MEM, 10% FCS, 25 mM HEPES). (C) Flow cytometry of the CTX-iPSC-MSCs shows they express the MSC markers CD73, CD90 and CD105, but not CD14, CD20, CD34 or CD45, in accordance with ISCT criteria (blue, staining; red, isotype controls).

Figure 6: Cellular reprogramming of CTX to pluripotency results in dramatic genome-wide changes in gene expression, shown here by examples of expression modulation in genes with

significant roles in pluripotency and neural development. Single cell RNA sequence (transcriptome) data are shown for (see key, top left hand panel) three samples of CTX (green), three CTX-iPSC cell lines (blue) and the same CTX-iPSC lines having undergone differentiation along a cortical lineage (red). In the latter case, differentiation was halted at a point most closely recapitulating CTX itself, as defined by RT-qPCR analysis of a select set of neuroectodermal gene expression. Each panel is a "tSNE plot" of single cell gene expression data, with each dot in a "cloud" representing a single cell. Grey: no expression, orange: moderate expression; red: high expression. The plots show that pluripotency genes inactive in CTX have been activated in the reprogrammed cells: POU5F1, NANOG, UTF1, TET1, DPP4, TDGF1, ZSCAN10 and GAL. Importantly, of these genes only POU5F1 was provided exogenously during reprogramming, unequivocally confirming activation of the endogenous gene upon reprogramming. Conversely, several neural genes expressed by CTX are downregulated upon reprogramming to pluripotency (NOGGIN, ADAM12, NTRK3, PAX6), as is OCIAD2, a gene strongly expressed by CTX cells. Some genes important in neuroectodermal development, such as GLI3 and PAX6, are upregulated upon cortical differentiation of the pluripotent cells as they select a neuroectodermal fate.

Figure 7 provides additional confirmation that CTX-iPSCs are pluripotent. Immunostaining for protein markers such as transcription factors identifying the three primary germ cell lineages, endoderm, mesoderm and ectoderm.

Figure 8 shows the reprogramming of another conditionally immortalised adult stem cell type. The Figure shows successful reprogramming of another conditionally-immortalised adult stem cell (ASC) line, STR0C05, derived from fetal striatal cells. Panels: A, a colony of reprogrammed STR0C05 cells 24 days post-transfection with reprogramming factors; B, alkaline phosphatase (red)-stained STR0C05 cells early in reprogramming, showing some cells beginning to express the pluripotency marker alkaline phosphatase; C, an established STR0C05-iPSC line; D, AP-positive colonies appear at different frequencies in wells subjected to different transfection conditions; well 1 with no positive colonies was transfected with a GFP (non-reprogramming) plasmid as a control and had no reprogrammed cells, whereas wells 4 and 6 had few surviving cells; E, an established STR0C05-iPSC line is alkaline phosphatase positive, and F, is also positive for the pluripotency marker SSEA4 but negative for the early differentiation marker SSEA1.

Figure 9: Confirmation of pluripotency of the STR0C05-iPSCs. Differentiation to endoderm, mesoderm and ectoderm, shown by immunostaining confirming coexpression of protein markers (mostly transcription factors) identifying the three primary germ cell lineages.

Figure 10: Confirmation of multipotency of adult stem cells derived from CTX-iPSCs. Candidate CTX-iPSC-derived mesenchymal stem cells (CTX-iPSC-MSCs) are multipotent. In addition to expression of a defined panel of cell surface marker proteins and adherence to tissue culture plastic (see main body of the Application), this Figure confirms their capacity to differentiate into cartilage (shown by alcian blue staining of glycosaminoglycans), fat (shown by staining of intracellular lipid droplets with oil red O) and bone (shown by alizarin red staining of deposited calcium).

Figure 11: Function of the conditional immortalisation transgene in adult stem cells differentiated from iPSCs created in turn by reprogramming of conditionally-immortalised cells. Flow cytometric profiles of CTX-iPSC-MSCs cultured to high passage (20 passages) in the presence or absence of 4-hydroxytamoxifen (4-OHT). This cell line is one that DNA methylation data suggest has a demethylated C-MYC-ER^{TAM} promoter, in turn implying that the promoter is active. This cell line appears to better maintain its cell surface marker profile when cell cycling is induced through the 4-OHT / C-MYC-ER^{TAM} system. CD90 and CD105 expression are more uniform and more highly expressed, and the negative markers CD14, 20, 34 and 45 are more consistently low. In the second panel, the 4-OHT-treated cells appear to be more efficient at generating bone upon differentiation, suggesting that 4-OHT / C-MYC-ER^{TAM}-driven cell cycling somewhat inhibits differentiation and associated loss of potency that might otherwise occur upon exit from the cell cycle.

Figure 12: An example of CTX-iPSC-MSC lines cultured in the absence or presence of 4-OHT, showing improved and more consistent growth behaviour long-term when the C-MYC-ERTAM transgene is active (presence of 4-OHT).

Figure 13: A second example of CTX-iPSC-MSC lines cultured in the absence or presence of 4-OHT, showing improved and more consistent growth behaviour long-term when the C-MYC-ER^{TAM} transgene is active (presence of 4-OHT).

Figure 14: Generation of mesoderm. This shows the first essential step in the creation of haematopoietic lineage cells from hPSCs *in vitro*, whereby commercially-available media supplemented with activin A, VEGF, SCF and BMP4 induce CTX-iPSC differentiation to mesoderm.

Figure 15: CTX-iPSC-Derived Mesoderm Differentiation to Haematopoietic Stem Cells (HSCs). This shows that HSCs were generated from mesodermal cells derived from the CTX-iPSCs of Figure 14B.

Figure 16: *In vitro* generation of T cells. This shows that CTX-iPSC-HSCs have been differentiated towards a T-lymphocyte cell fate using two coculture methods.

Figure 17: Development of T-cell progenitors on DLL4/VCAM coating. Figure 17A shows the method of generating progenitor T cells from CTX-HSCs by culturing them for a period of 14 days on a layer of bound chimeric proteins presenting VCAM and DLL4 to the HSCs. At the end of the 14 day period, a heterogeneous population of adherent and suspension cells was obtained. These cells could be distinguished by flow cytometry (Figure 17B), with the smaller, suspension cells ("Single Cells 2" population, Figure 17) comprising the pre-lymphocyte population.

Figure 18: Development of T-cell progenitors on DLL4/VCAM coating. Culturing the progenitor T lymphocyte cells on bound Fc-DLL4 and Fc-VCAM proteins for longer periods (Figure 18A, 25 days, as opposed to 14 days) resulted in the cell population becoming somewhat more homogenous (Figure 18B) and their achieving a more mature phenotype (Figure 18C-E).

Figure 19: Development of T-cells using organoids. Coculturing the CTX-HSCs with murine MS5 stromal cells engineered to express the human NOTCH ligand DLL1.

Figure 20: Development of T-cells on a monolayer of MS5 cells expressing human DLL-1. Coculturing the CTX-HSCs on a monolayer of MS5-DLL1 cells induced strong growth in the small non adherent cell population (Figure 20B). This population matured during the course of differentiation, losing its CD34 expression (Figure 20C), but CD8 expression was not observed, although the early markers CD5 and CD7 expression levels fell (Figure 20D-F).

Figure 21: T cell development – Chimeric protein vs MS5-hDLL-1 monolayer T cell progenitors produced by the method of Figure 20 probably represent an earlier stage of T cell development than that produced by the bound proteins approach described above. This shows that the development of T cells during 25 days of culture was more efficient on a DLL4/VCAM coating than using a monolayer of MS5 expressing hDLL-1

Figure 22: NK Cell Potential: Expression of the NK cell marker CD56 (NCAM) on iPSCs, Mesoderm cells and HSCs generated according to the invention.

Figure 23: Overview of Cells of the Haematopoietic lineage.

Figure 24: Schematic of L-MYC-ER^{TAM} virus, an alternative to the C-MYC-ER^{TAM} system.

Figure 25: Hematopoietic differentiation of CTX-iPSCs produces HSCs, lymphoid progenitors and effectors. (A) Embryoid bodies were formed from CTX-iPSCs by plating a single cell suspension in non-adherent microwell plates. (B) The EBs were cultured in the presence of mesoderm-promoting medium (to day 3), and then in haematopoietic specification medium (to day 10) to (C) generate CD34⁺ cells, approximately 5% of which were CD34⁺CD49f⁺CD90⁺CD38⁺CD45RA⁺LT-HSCs. (D) CD34⁺ cells derived in this way were isolated with anti-CD34 magnetic beads and then differentiated for a further 14 days to generate (E) CD7⁺ lymphoid progenitors, which retained some reduced multipotency and could in turn be differentiated for 14 or 21 days respectively to produce Natural Killer or CD4-CD8⁺TCR $\alpha\beta$ cytotoxic T-cells.

10 Detailed Description of the Invention

The present inventors have surprisingly identified that conditionally-immortalised cells can be reprogrammed into a pluripotent stem cell phenotype. This provides advantages over existing induced Pluripotent Stem Cells. In particular, despite *in vitro* immortalisation and long-term culture, it has surprisingly been shown that the neural stem cell lines CTX0E03 and STR0C05 can be reprogrammed by exogenous transcription factors. Furthermore, it is also surprising that the conditionally controlled gene remains able to be activated and silenced after the reprogramming, as it was before so endowing the reprogrammed cell with the same functional conditionally immortalised gene, C-MYC-ER^{TAM} which can be transcribed upon the addition of hydroxytamoxifen (4-OHT). The advantage of this is that the reprogrammed cell can also be controlled to stably proliferate for longer in cell culture than otherwise would be possible so making the reprogrammed cell amenable to industrial scale up for treating more patients with a single batch or to increase the yields of any by-product of the cell.

Advantageously, the reprogramming of conditionally-immortalised cells can often be achieved with fewer reprogramming factors than in standard (non conditionally-immortalised) cells. The use of Adult Stem Cells in certain embodiments provides similar advantages.

Furthermore, the conditionally-immortalised nature of the cells provides beneficial controllability over the cells and over the immortalisation system. In some embodiments, these benefits are provided by the C-MYC-ER^{TAM} conditional-immortalisation system. Without wishing to be bound by theory, the use of conditionally-immortalised cells as the source for reprogramming to pluripotency is thought to contribute to the observed benefits over previous attempts using immortalised (i.e. permanently immortalised) cells.

hiPSCs have been shown (Heo et al., 2018, Cell Death Dis 9 1090. “*Reprogramming method influences efficiency of generating haematopoietic progenitors by differentiation*”) to be less

efficient than hESCs derived from nuclear transfer embryos. This presents a problem for applying conventional iPSC technology to haematopoietic lineage applications, that is avoided by the cells of the present invention because even quite poor (inefficient) differentiation systems can be useful in their ability to expand more- or less-committed progenitors and adult stem cells (e.g. “partially differentiated” cells, which will be expanded as a homogenous population by the MYC-ERTAM/4-OHT system) prior to banking and patient transfer.

The induced Pluripotent cells of the invention, such as the CTX-iPSCs and STR0C-iPSCs in the Examples, represent a very useful clinical resource. They may be differentiated along a desired lineage to generate a target population such as a tissue progenitor cell type or adult stem cell population. Typically, the iPSCs described herein are differentiated into the haematopoietic lineage. Then, provision of the immortalising agent (e.g. 4-OHT) to promote continuous growth and prevent cell cycle exit and associated further differentiation could allow the routine and scalable production of previously-unattainable clinically-relevant subpopulations without repeated cell isolation from primary material or repeating a differentiation protocol *de novo* from induced pluripotent stem cells each time a new batch of cell therapy product is required. This provides for the possibility of an off-the-shelf cell resource, for example for allogeneic cell therapy such as an allogeneic T cell therapy, CAR-T therapy, NK therapy or CAR-NK cell therapy, or B-cells for the production of antibodies, or neutrophils or dendritic cells for the generation of therapeutic vaccines.

The ability to re-apply inducible immortalisation to produce at scale an allogeneic, off-the-shelf, adult stem cell therapeutic population, in particular of the haematopoietic lineage, is expected to be particularly beneficial.

In certain aspects, the invention advantageously provides conditionally immortalised iPS cells from which stable haematopoietic lineage cells are generated that are able to be cultured at commercially or clinically-relevant scale.

The provision of allogeneic cell therapies where previously autologous therapies have been the only available approved treatments, such as treating cancer using CAR-T therapies, is a particular advantage. The clonal expandable banks of cells according to the invention improve industrial manufacture and clinical application compared to autologous cell therapies. This includes larger batches during production, wider application to any number of patients rather than a single patient, improved distribution and availability, shorter lead times to treat the patient, and lower costs and the ability to generate better consistency, quality and safety profiles than autologous therapies made anew on a patient-by-patient basis.

Cloning or purification steps can be used to generate pure populations of the desired therapeutic types from more- or less-heterogeneous differentiation cultures for large-scale production of off-the-shelf treatments for conditions for which the original conditionally-immortalised cell (e.g. CTX0E03) itself is unsuitable, avoiding the drawbacks seen in the art with incomplete efficiency of differentiation protocols. This applies to both the cells themselves or extracellular vesicle (e.g. exosomal) fractions produced by different cell types with alternative repertoires of payload molecules to those produced by CTX cells themselves.

Furthermore, as these CTX-iPSC-derivative sublines are derived from a cell line which has already passed clinical phase safety trials (CTX), their entry into clinical trials for efficacy in new indications is likely to be accelerated.

In certain aspects, the invention relates to induced pluripotent stem cells generated from different neural stem cells comprising a controllable transgene for conditional immortalisation such as the CTX0E03 or STR0C05 neural stem cell lines derived from cortical and striatal tissue respectively and each from a different human donor, and the progeny of those induced pluripotent stem cells.

Cells of the Haematopoietic lineage

The invention relates, in particular, to the production of cells of the haematopoietic lineage from pluripotent cells. The haematopoietic lineage derives from the mesoderm germ layer which can be identified as described in detail elsewhere herein.

The haematopoietic system consists of a large group of cell types with a variety of functions including both innate and acquired immunity to infection or cancer, blood clotting and the production of red blood cells. All of these cell types comprise a single lineage, derived ultimately from the haematopoietic stem cell (HSC), a multipotent adult stem cell type (ASC) present in the bone marrow. Haematopoietic lineage cells have utility as therapeutics for a variety of conditions, including immunotherapy for cancer and treatment of autoimmune diseases, anaemias, trauma, and infection.

Therapeutic applications of cells from this lineage include the use of killer cells such as CD8+ T cells or Natural Killer (NK) cells as anti-tumour therapeutics, perhaps carrying genetically-engineered receptors that target tumour cells specifically (e.g. Chimeric Antigen Receptor-T cells), T-Regs for the treatment of autoimmune disorders, and platelets, e.g. for patients with clotting disorders such as certain cancer patients or trauma patients, erythrocytes, e.g. for the treatment of anaemias and battlefield or trauma medicine, and B-lymphocytes for the treatment of various conditions including cancer, or for the production of antibodies, dendritic cells for the treatment of cancers eg Provenge (sipuleucel-T), and others. All of these cell types comprise

part of the haematopoietic lineage, and are ultimately derived from haematopoietic stem cells, HSCs. HSCs are a rare cell type present in a niche in the bone marrow, and are defined by their ability to reconstitute the immune system of a lethally-irradiated animal (which entirely ablates the immune system), for example post-radiotherapy treatment for cancer. They can be identified by the presence of protein markers, such as the surface receptors CD34 and c-KIT (CD117), and concurrent absence of mature haematopoietic markers such as the T cell receptor or rearranged genetic loci capable of producing mature antigen-specific antibody molecules by B lymphocytes.

Figure 23 summarises the Cells of the Haematopoietic lineage. Every cell type mentioned in this figure is explicitly provided as part of the invention and can be produced according to the methods described herein.

Cells or their progeny of the haematopoietic lineage comprise at least: multipotent haematopoietic stem cells ("HSCs", also known as Haemocytoblasts); common myeloid progenitors (which can give rise to myeloblasts, monoblasts, erythroblasts and megakaryoblasts); Myeloblasts (which will develop into granulocytes (neutrophil, eosinophil, basophil)); Monoblasts (which will develop into monocytes, that can be differentiated into macrophages and dendritic cells in tissues); common lymphoid progenitors; Lymphoblasts; Megakaryocytes; thrombocytes; Erythroblasts; Erythrocytes; Mast cells; Myeloblast-derived cells including basophils, neutrophils, eosinophils; Monoblast-derived cells such as monocytes and macrophages; Lymphoblast-derived cells; large granular lymphocytes such as Natural Killer cells (typically CD56+); small lymphocytes such as T lymphocytes and B lymphocytes; plasma B cells; and dendritic cells (DCs) such as myeloid DCs (e.g. mDC-1 or the rarer mDC-2) or plasmacytoid DCs.

In certain embodiments, cells of the haematopoietic lineage are optionally selected from Myeloblasts, Lymphoblasts, Megakaryocytes, Thrombocytes, Erythrocytes, Mast cells, Basophils, Neutrophils, Eosinophils, Monocytes, Macrophages, CD56^{DIM} Natural Killer cells, CD56^{BRIGHT} Natural Killer Cells, CD56^{high}CD16[±] Natural Killer Cells, CD56^{low}CD16^{high} Natural Killer cells, Natural Killer T (NKT) cells, NKT cells expressing CD161, CD4⁺ T cells, CD8⁺ T cells, memory T cells, B-2 cells, B-1 cells, memory B cells, plasma B cells, myeloid Dendritic Cells, or plasmacytoid DCs.

CD4⁺ T cells may be of the Th1 type, Th2 type, Th17 type, Th9 type, Th22 type, or Tfh type. Regulatory T cells are also typically CD4⁺.

FoxP3⁺ Regulatory T cells are provided in one embodiment.

In certain embodiments, the cells of the haematopoietic lineage are Long Term repopulating Haematopoietic Stem Cells (LT-HSCs), for example as exemplified in Example 7. These LT-HSCs may be CD34+CD49f+CD90+CD38-CD45RA- LT-HSCs.

- 5 In some embodiments, the cells of the haematopoietic lineage are CD7+ lymphoid progenitors. These progenitors may in turn be differentiated for 14 or 21 days respectively to produce Natural Killer or CD3+CD4-CD8+TCR+ cytotoxic T-cells, which are also provided in certain embodiments.

- 10 CD8+ T cells are provided in some embodiments. These can be CD8+ effector cells or CD8+ memory cells. An example of a CD8+ Effector T cell is a CD8+ CD45RA+ effector cells, for example a CD8⁺ CD45RA⁺ CD62L⁻ CCR7⁻ CD45RO⁻ cell. An example of a memory CD8+ T cell is a CD8⁺ CD45RA⁺ CD62L⁺ CCR7⁺ CD45RO⁺ cell.

- 15 In some embodiments, the cells of the haematopoietic lineage are CD3+CD8+TCR+ cytotoxic T-cells.

- 20 Natural killer cells may be in certain embodiments CD56+ Natural Killer cells, CD56^{DIM} Natural Killer cells, CD56^{BRIGHT} Natural Killer Cells, CD56^{high}CD16[±] Natural Killer Cells, or CD56^{low}CD16^{high} Natural Killer cells. In some embodiments the NK cell is positive for CD56, CD16, IL2-R beta and CD94.

- 25 B cells may be immature B cells or mature B cells. Immature B cells express CD19, CD20, CD34, CD38, and CD45R, but not IgM. For most mature B cells the key markers include IgM and CD19. Activated B cells express CD30, a regulator of apoptosis. Plasma B cells lose CD19 expression, but gain CD78, which is used to quantify these cells. Memory B cells can be immunophenotyped using CD20 and CD40 expression. The cells can be further categorized using CD80 and PDL-2 regardless of the type of immunoglobulin present on the cell surface (Zuccarino-Catania GV et al. Nat Immunol. 2014.).

- 30 B cells may be B-2 cells, B-1 cells, memory B cells or plasma B cells. B cells (except plasma cells) are typically IgM+ CD19+. Activated B cells are typically CD19+ CD25+ CD30+. Some plasma cells are positive for IgG, CD27, CD38, CD78, CD138 and CD319, while other plasma cells are positive for IL-6, or may be characterised as positive for CD138. In a further embodiment, the cells may be follicular B cells (e.g. positive for IgG, CD21, CD22 and CD23). In yet a further
35 embodiment, the B cell is a Regulatory B cell, which may be positive for: IgD, CD1, CD5, CD21, CD24, TLR4; or may be positive for IL-10 and TGF-β. In some embodiments, the B cell is a memory cell, for example one that is positive for IgA, IgG, IgE, CD20, CD27, CD40, CD80, PDL-2, or a memory B cell that is positive for CXCR3, CXCR4, CXCR5 and CXCR6.

A typical cell of the haematopoietic lineage is a haematopoietic stem cell (HSC). These cells are usually defined as being CD34+ multipotent cells. CD34+ CD43+ HSCs are also provided. Cells of the invention can be characterised as HSCs and cells that can be differentiated from HSCs.

5 Cells of the invention may be myeloid cells or lymphoid cells.

The differentiation of HSCs to myeloblasts or lymphoblasts occurs in the bone marrow.

Common myeloid progenitors will originate myeloid cells including monocyte (peripheral blood), macrophages (tissues), and Myeloblasts and resulting granulocytes (neutrophils, eosinophils, basophils in blood while mast cells reside in tissues).

10

Lymphoblasts (common lymphoid progenitors) will generate lymphocytes (T and B cells) and NK cells. CD7 is not a normal marker for myeloblasts but it is found during the development of T cells. It is found on myeloid leukemias, so its expression is aberrant on myeloid cells. Among the common myeloid progenitor markers are CD36, CD163 and CCR2.

15

Functional cells of the immune system are of the haematopoietic lineage. These immune cells include T cells, B cells, Natural Killer (NK) cells, Dendritic cells, macrophages, monocytes and granulocytes. T cells can include CD4+ T cells (often broadly referred to as "T helper cells"), Regulatory T cells (Tregs) often characterised by the marker FoxP3, CD8+ T cells (for example CD8+ Cytotoxic T Lymphocytes). Another cell derived from the haematopoietic lineage is the neutrophil. Yet another cell derived from the haematopoietic lineage is a macrophage. Each of these cells can optionally be genetically engineered or otherwise modified. In particular, the cells can be modified to express a Chimeric Antigen Receptor to form a CAR-T cell, a CAR-NK cell, or any other CAR-modified immune cell. The Chimeric Antigen Receptor is typically directed to a protein or other marker on a target cell, usually a tumour cell. A typical target protein is CD19, which targets the CAR- cells (e.g. CAR-T cells) to treat leukaemias. Other cells of interest, of the haematopoietic lineage according to the inventing, are Tumour- Infiltrating Lymphocytes (TILs). When used in therapy, each of these cells is typically allogeneic to the patient. Nonetheless, in some circumstances the cells may be autologous to the patient, for example where patient cells are extracted, engineered and re-administered, such as in CAR-T or CAR-NK therapies.

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Red blood cells (erythrocytes) are also cells of the haematopoietic lineage.

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Dendritic cells (DCs) may be myeloid DCs, such as mDC-1 or the rarer mDC-2. DCs may also be plasmacytoid DCs. The markers BDCA-2, BDCA-3, and BDCA-4 can be used to discriminate

among the DC types. Lymphoid and myeloid DCs evolve from lymphoid and myeloid precursors, respectively, and thus are of hematopoietic origin.

5 In one embodiment a cell of the invention is a CD34⁺ cell with Erythroid/Myeloid and T-lymphoid potential. In another embodiment, the cell is a CD43⁺ cell that is an Erythroid/Myeloid Progenitor. In a further embodiment, the cells are CD45⁺ leukocytes.

10 In one embodiment, the cells are T-cells that do not express CD5 and/or CD7. In other embodiments, the T cells are T cell progenitors and do express CD5 and/or CD7.

Cells of the haematopoietic lineage are useful in therapy. For example, CD8⁺ CTLs and NK cells are known (when particular modified with a CAR) for use in cancer therapy. Neutrophils have utility in a number of therapies, including in cancer treatment and in treatment of infectious disease. Tregs are in development for autoimmune therapy. Immune cells such as CD8 cells,
15 NK and B cells (and the antibodies produced by them) are of course also useful for treating infectious diseases, including bacterial infection, fungal infection and viral infection.

Because T-cell immunity contributes to the control of many viral pathogens, adoptive immunotherapy with virus-specific T cells (VSTs) has been a logical and effective way of
20 combating severe viral disease, in particular in immunocompromised patients. In some embodiments a virus such as a Corona virus or other virus such as cytomegalovirus (CMV), adenovirus (AdV), Epstein-Barr virus (EBV), human herpes virus 6 (HHV6) and BK virus, is treated using adoptive T cell therapy, as described by Riddell and Greenberg "Principles for adoptive T cell therapy of human viral diseases" Annu Rev Immunol. 1995;13:545-86, and more
25 recently by Ottaviano, Giorgio *et al.* "Adoptive T Cell Therapy Strategies for Viral Infections in Patients Receiving Haematopoietic Stem Cell Transplantation." Cells vol. 8,1 47. 14 Jan. 2019.

Restoration of virus-specific immunity by virus-specific T lymphocytes administration has offered an attractive alternative to conventional drugs characterized by substantial toxicities or ineffective
30 long term protection. Most studies have used Viral Specific T Cells (VSTs) derived from allogenic stem cell donors, the use of banked VSTs derived from partially HLA-matched donors has shown efficacy in multicentre settings. Hence, this approach could shorten the time for patients to receive VST therapy thus improving accessibility. To improve survival outcomes anti-virus specific T cell therapies are currently in clinical development.

35 An anti-viral T cell therapy generated according to the methods of the present invention is therefore provided. This may comprise CD4⁺ and/or CD8⁺ cells according to the invention,

typically that have been stimulated with one or more viral antigens or that have been engineered to recognise the target virus, typically using a chimeric antigen receptor targeted to a viral antigen.

5 The T cells prepared according to the invention may have specificity for multiple different viruses, which may optionally include a coronavirus. Multivirus-specific T cells are known in the art and have, for example, been produced using direct isolation via the cytokine-capture technique (Kallay *et al*, "Early experience with CliniMACS prodigy CCS (IFN-gamma) system in selection of virus-specific T cells from third-party donors for pediatric patients with severe viral infections after hematopoietic stem cell transplantation". J Immunother. 2018;41(3):158–63) and also in a
10 protocol where multipathogen-specific T cells expressing CD154 were directly isolated via magnetic cell separation (Khanna *et al* "Generation of a multipathogen-specific T-cell product for adoptive immunotherapy based on activation-dependent expression of CD154". Blood. 2011;118(4):1121–31).

15 The anti-viral T cells will typically be allogeneic and be able to be produced at large scale and stored, optionally stored long-term for example when frozen, which is particularly useful for providing a large number of treatments in an outbreak of transmissible disease, such as an epidemic or pandemic.

20 In some embodiments, the treatment is for a Coronavirus-mediated disease ("Covid"), such as Covid-19. The CD4+ and/or CD8+ T cells will typically recognise, and bind to, a peptide derived from the coronavirus bound to a major histocompatibility antigen (e.g MHC class I or II). This peptide could be an external peptide such as a spike protein, or an internal protein such as an RNA-binding protein. This embodiment therefore provides a virus-specific T cell population for
25 therapeutic use, in particular in treating Covid-19 or another disease caused by a coronavirus.

As discussed by Ruella and Kenderian (BioDrugs. 2017 Dec;31(6):473–481), the successful track record of using allogeneic virus specific T cells provides a compelling rationale for the development of allogeneic off the shelf CAR-T cells. The application of third party, off the shelf
30 virus specific T cells has been proven to be an effective strategy in the prophylaxis and treatment of viral infections, especially post allogeneic transplantation. CAR-T cells of the invention may thus also be used as antiviral therapies.

35 In other embodiments, NK cells of the invention are able to treat a viral disease, for example a coronavirus-mediated disease such as Covid-19. These NK cells may optionally be CAR-NK cells comprising a receptor targeted to a virus such as coronavirus. The NK cells may be further engineered, for example to express IL-15.

In some embodiments, the following cells are produced from a HSC that is derived from an iPSC according to the invention:

- T-cell, optionally CD8+, CD4+ or a Regulatory T cell (Treg) such as a FoxP3+ Treg;
- B-cell;
- 5 NK cell;
- Red blood cell;
- Neutrophil;
- Dendritic cell; or
- Macrophage.

10

In some embodiments, platelets are generated in a process involving HSCs of the invention.

In one embodiment, a CAR-T cell therapeutic product is produced. This may be allogeneic. Here, the T cells generated as a product of the present methods are subsequently modified. Another
15 embodiment provides the generation of NK cells, for example for allogeneic therapy. The NK cells produced according to the methods described herein are optionally modified, for example by genetic engineering. Typically, selection of suitable T cell or NK cell clones with the desired modification is followed by further expansion of the cell number using the addition of the conditionally-immortalising agent (e.g. Tamoxifen or 4-OHT in the case of c-Myc-ER) to expand
20 the clones at scale to produce industrial sized batches. Such cell modifications include:

- a. A chimeric antigen receptor eg including but not limited to CD19, CD20, CD1 or MR1, which is expressed via transfection of the T-cell generated of the invention, optionally using a vector encoding the gene coding for the CAR under the control of a suitable promoter
- 25 b. A modification to control expression of the CAR protein
- c. A modification to reduce toxicity to the patient
- d. A modification to introduce a suicide gene in the case where the patient suffers a serious adverse event after administration eg cytokine storm.

30 In another embodiment, a B cell is generated from an HSC of the invention, as described herein. The B cell can then be used to produce an antibody (or antibody fragment) and kept in the immortalised state by maintaining the immortalising factor (e.g. 4-OHT) in the culture medium.

35 In some embodiments, the cells of haematopoietic lineage produced according to the invention are used to produce a protein, glycoprotein, or peptide of interest. This can be a biologic therapeutic.

In some embodiments, the cells of haematopoietic lineage produced according to the invention are used to produce extracellular vesicles, typically exosomes.

5 In some embodiments, the cells of haematopoietic lineage produced according to the invention are used to produce a nucleic acid drug, for example an siRNA or mRNA.

Combinations of any of the therapies described herein are provided. Each of the therapies, alone and in combination with each other, can be combined with other therapeutic agents. In one embodiment, a therapeutic cell produced according to the invention is combined with a different
10 immunotherapy, which may for example be a checkpoint inhibitor such as an anti-PD1, anti-PD-L1, anti-TIM3, anti-LAG3, or anti-CTLA4 antibody.

In some embodiments, a combination therapy for treating cancer comprises a T cell (e.g. a CD8+ T cell) produced according to the invention and a checkpoint inhibitor such as an anti-PD1, anti-
15 PD-L1, anti-TIM3, anti-LAG3, or anti-CTLA4 antibody. Certain combination therapies include: a CAR-T cell and an anti-PD1 antibody; a CAR-T cell and an anti-PDL1 antibody; a CAR-T cell and an anti-CTLA4 antibody. Other combination therapies may include other immune cells described herein, for example: a CAR-NK cell and an anti-PD1 antibody; a CAR-NK cell and an anti-PDL1 antibody; a CAR-NK cell and an anti-CTLA4 antibody; a T cell and an anti-PD1 antibody; an NK
20 cell and an anti-PD1 antibody.

In one embodiment, a Treg according to the invention can be combined with another therapeutic agent. The Treg is typically a FoxP3+ Treg. An example of a combination therapy is a Treg combined with an immunosuppressive or anti-inflammatory drug.

25

A number of other cell types of the haematopoietic lineage that are producible by this invention are described below.

Myeloid progenitors, e.g. Myeloid-erythroid progenitors (MEPs, precursors of erythrocytes and monocytes, below), and erythroblasts, the unipotent precursors of erythrocytes.
30 Erythroblasts are difficult to expand in vitro. Scalable expansion and cryopreservation of erythroblasts followed by differentiation to red blood cells (erythrocytes) for transfusion would have wide medical applicability, ameliorating many of the problems associated with current blood donation systems (reviewed by Focosi and Amabile, 2018, in Cells v7 2; Bernecker et al., 2019 Stem Cells Dev v28 1540–51).

35 **Megakaryocytes** for platelets. Platelets are small, enucleated blood structures derived from megakaryocytes with an essential role in haemostasis. Presently, they are harvested for treatment of bleeding complications caused by conditions such as cancer chemo- or radiotherapy or major trauma from blood donations. However, they are characterised by a very short shelf life

(5 days) and this, together with the large scale multi-donor system of supply, can lead to issues with availability and safety. Megakaryocytes (MKs), the unipotent progenitors of platelets, are an example of an adult progenitor cell type which is a rare subpopulation in the bone marrow. Megakaryocytes can be created from iPSCs (Eto et al., 2010, J Exp Med, 207 2817–30) and may be cryopreserved, but issues are observed with a low level of cell expansion. The capacity to scalably expand hiPSC-derived MKs as well as cryopreserve them for on-demand platelet production would represent a significant benefit.

Myeloblasts give origin to neutrophils.

a. Neutrophils (and the progenitors between them and myeloblasts).

Neutrophils may be produced by differentiation of iPSCs to CD34+ HSCs (see below), and then culturing the HSCs on OP9 stromal cells in the presence of SCF, IL-6, Thrombopoietin, IL-3, Flt-3 ligand, and then maturing them on OP9 cells in the presence of G-CDF (Brault et al, 2012, BioResearch Open Access 3 311-26).

Monoblasts give origin to monocytes.

b. Monocytes. Monocytes (precursors of macrophages) have been shown to be differentiated from iPSCs, using a multistep protocol whereby mesoderm generated from pluripotent cells through the action of activin-A, BMP4 and CHIR99021, was further differentiated towards hemogenic epithelium by addition of SB431542, VEGF, bFGF and SCF. Subsequent treatment TPO and with interleukin-3, interleukin-6, and M-CSF induce further differentiation to myeloid progenitors and then to mature macrophages (Cao et al., 2019, Stem Cell Reports 12 1282-97).

i. Macrophages; for example by the generation of embryoid bodies containing mesoderm from iPSCs, followed by multistep in vitro culture in the presence of M-CSF and Interleukin-3, as described by Mukherjee and colleagues (2018) in Meth Mol Biol 1784 13-28, or similar methods involving cocultures (Brault et al, 2012, BioResearch Open Access 3 311-26) or bioreactors (Ackermann et al., 2018 Nature Comms 9 5088).

ii. Microglia (brain macrophages).

Lymphoid progenitors to produce NK cells and T- or B-lymphocytes (see Examples below).

A summary of exemplary methods to produce various cells of the haematopoietic lineage is provided in the Table below. The information in the method column provided in each row of this table is explicitly provided as an embodiment of the invention, for producing the respective cell types. The Reference in the final column provides additional guidance.

For example, NK cells can be differentiated from iPSCs of the invention by culturing with SCF, VEGF, BMP4 to create CD34+/CD43+ cells, then culturing with IL-3, IL-15, SCF. FLT3-ligand and expansion on artificial APCs.

- 5 In another example, T cells can be derived from iPSCs of the invention by Coculture on stromal (OP9) cells, then transfer to OP9-DLL1 stromal cells in the presence of FLT3-L, IL-7, SCF.

In a further example, B cells can be derived from iPSCs of the invention by culturing in the presence of IL-7, IL-3, FLT3-L, SCF in the absence of a Notch ligand.

10

In yet another example taken from the table below, erythrocytes can be derived from iPSCs of the invention by culturing in the presence of IL-3, SCF, IGF-1, EPO, Dexamethasone.

15

In other embodiments described in the table below, erythroid, megakaryocytes and myeloid cells of the invention can be derived from HSCs of the invention by culturing the HSCs in the presence of EPO, IL-1 β or G-CSF (or GM-CSF), respectively.

20

In some embodiments, iPSCs of the invention are differentiated by culturing in the presence of 1, 2, 3, 4, or all of FLT3-L, IL-3, IL-7, SCF, TPO, for example to differentiate into Myeloid cells.

In some embodiments of the invention, CD31+/34+ HE cells are provided. These may be provided by inhibiting GSK3 in iPSCs. These CD31+/34+ HE cells can be differentiated (e.g. to myeloid cells) by culturing with FLT3-L, IL-3, IL-7, SCF, TPO or (e.g. to lymphoid cells) by coculture with DLL-4 expressing stromal cells, SCF, FLT3-L, IL-3 and IL-7.

25

CELL TYPE	Start Cell	Method	Reference
Natural Killer Cells	hiPSCs	SCF, VEGF, BMP4 → CD34+/CD43+;	Hermanson <i>et al.</i> , 2018, <i>Stem Cells</i> 34 93–101
Natural Killer Cells	hiPSCs	Then IL-3, IL-15, SCF, FLT3-Lig Expansion on artificial antigen presenting cells.	Knorr <i>et al.</i> , <i>Stem Cells Transl Med</i> 2 274–283
T cells	hiPSCs	Coculture on OP9 cells, transfer to OP9-DLL1 stromal cells + FLT3-L, IL-7, SCF	Timmermans <i>et al.</i> , 2009, <i>J Immunol</i> 182 6879-88
T cells	ucHSC	Coculture on OP9-DLL1 cells + FLT3-L & IL-7 → IL-2, α -CD3 mAb and α -CD28 mAb	La Motte-Mohs <i>et al.</i> , 2005, <i>Blood</i> 105 1431-9

T cells	hiPSCs	Coculture on feeder cells + FLT3-L, SCF & VEGF → CD34+ HSCs, transfer to OP9-DLL1 stromal cells + FLT3-L, IL-7	Nishimura <i>et al.</i> , 2013, <i>Cell Stem Cell</i> 12 114-26.
T cells	hiPSCs	Coculture on OP9-DLL1 cells + SCF, FLT3-L & IL-7	Vizcardo <i>et al.</i> , 2013, <i>Cell Stem Cell</i> 12 31-6
T cells	hiPSCs	Coculture on feeder cells + FLT3-L, SCF & VEGF → CD34+ HSCs, then coculture on OP9-DLL1 cells + SCF, FLT3-L & IL-7	Wakao <i>et al.</i> , 2013, <i>Cell Stem Cell</i> 12 546-558
Erythroid	iPSCs	VEGF, BMP4, EPO	Hansen <i>et al.</i> , 2018, <i>Stem Cell Res</i> 29 232-44
Megakaryocytes	iPSCs	bFGF → IL-1β	
Myeloid	iPSCs	Mesoderm, then IL-3, IL-6, TPO, SCF → CD34+/CD43+ HSCs	
B lymphocytes	iPSCs	IL-7, IL-3, FLT3-L, SCF	Yang <i>et al.</i> , 2014, <i>Brit J Haemat</i> 166 435-48
Erythrocytes	iPSCs	IL-3, SCF, IGF-1, EPO, Dexamethasone	
Myeloid & Lymphoid cells (NK, T, B)	iPSCs	GSK3 inhibition → CD31+/34+ HE cells	Galat <i>et al.</i> , 2017, <i>Stem Cell Research & Therapy</i> 8 (1)
		Coculture with DLL-4 expressing stromal cells; SCF, FLT3-L, IL-3, IL-7	
Hemagenic progenitors and various haematopoietic lineages	iPSCs	Coculture with DLL-1 expressing stromal cells in 3D hydrogel matrix, & sequential factors including BMP4, SCF, FLT3L, TPO, VEGF, PGE2, IL-3, IL-6, GM-CSF, G-CSF, EPO	Xu <i>et al.</i> , 2016, <i>J Hematol Oncol</i> 9 1
Hematogenic progenitors and various	iPSCs	GSK3 inhibition, and sequential factors including	Kitajima <i>et al.</i> , 2016 <i>Exp Hematol</i> 44 68-74.e10

haematopoietic lineages		BMP4, VEGF, SCF, FLT3-L, IL-3, IL-6, G-CDF	
CAR		Reference	
NK-CAR4 (α -hMesothelin, scFv-NKG2D-2B4-CD3 ζ)		Li <i>et al.</i> , 2018, <i>Cell Stem Cell</i> 23 181-92	

Abbreviations

	HE	Hempgenic endothelium
5	FLT3-L	fms-like tyrosine kinase 3 receptor ligand (FLT3 ligand)
	SCF	Stem Cell Factor
	bFGF	Basic fibroblast growth factor, (aka FGF2)
	TPO	Thrombopoietin
	EPO	Erythropoietin
10	IL α	Interleukin, e.g. IL6: Interleukin-6
	ucHSC	Umbilical Cord blood Haematopoietic Stem Cell
	HSC	Haematopoietic Stem Cell
	mAb	Monoclonal antibody
	α	Anti, e.g. α -CD3 mAb = anti-CD3 monoclonal antibody
15		

Induction of Pluripotency and iPS Cells

The generation of Induced Pluripotent Cells is known in the art, since Takahashi and Yamanaka showed that stem cells with properties similar to Embryonic Stem Cells could be generated from mouse fibroblasts by simultaneously introducing four genes (Cell. 2006; 126: 663-676). The principle was applied to human cells in 2007 (Takahashi *et al* Cell. 2007; 131: 861-872; Yu *et al* Science. 2007; 318: 1917-1920). A recent review is provided by Shi *et al*, Nature Reviews Drug Discovery volume 16, pages 115–130 (2017).

iPSCs are typically derived by introducing products of specific sets of pluripotency-associated genes, or "reprogramming factors", into a given cell type. The original set of reprogramming factors (also dubbed Yamanaka factors) are the transcription factors Oct4, Sox2, cMyc, and Klf4.

The generation of iPS cells depends on the transcription factors used for the induction. Oct-3/4 and certain products of the Sox gene family (Sox1, Sox2, Sox3, and Sox15) have been identified as crucial transcriptional regulators involved in the induction process whose absence makes induction impossible. Additional genes, however, including certain members of the Klf family (Klf1, Klf2, Klf4, and Klf5), the Myc family (c-myc, L-myc, and N-myc), Nanog, and LIN28, have been identified to increase the induction efficiency.

“POU5F1”, “OCT4” and “OCT3/4” are synonyms for the same transcription factor. This is the transcription factor commonly referred to as OCT4 in the art, but more recently re-named POU5F1 (POU class 5 homeobox 1). These names are used interchangeably herein, as will be apparent to the skilled person.

- 5 The reprogramming factors are typically introduced into the cell using viral or episomal vectors, as is well-known in the art. Viral vectors suitable for introducing reprogramming factors into a cell include lentivirus, retrovirus and Sendai-virus. Other techniques for introducing reprogramming factors include mRNA transfection.

10 Non-integrating reprogramming methods are known in the art, for example as reviewed by Schlaeger *et al* Nat Biotechnol. 2015 Jan; 33(1): 58–63. In Sendai-virus reprogramming, Sendai-viral particles are typically used to transduce target cells with replication-competent RNAs that encode the set of reprogramming factors. In Episomal reprogramming, prolonged reprogramming factor expression is typically achieved by Epstein-Barr virus–derived sequences that facilitate episomal plasmid DNA replication in dividing cells. In mRNA reprogramming, cells are typically
15 transfected with *in vitro*–transcribed mRNAs that encode the reprogramming factors, and chemical measures are often employed to limit activation of the innate immune system by foreign nucleic acids. Owing to the very short half-life of mRNAs, daily transfections are often required to induce hiPSCs.

- 20 Transfection of reprogramming factors may be achieved in a variety of ways known in the art, such as by lipofection, nucleofection or electroporation.

In one Example below, conditionally-immortal CTX0E03 cells were reprogrammed to pluripotency using standard non-integrating episomal vectors encoding the “Yamanaka Factors” OCT4, L-MYC, KLF4 and SOX2, and LIN28. In another Example, OCT4 alone is shown to induce
25 pluripotency of CTX0E03. Combinations of transcription factors that were also observed to achieve pluripotency include: OCT4 and SOX2; OCT, KLF4 and SOX2; OCT4, KLF4, SOX2 and MYC.

In certain embodiments, one, two, three or four of OCT4, L-MYC, KLF4 and SOX2, and LIN28
30 are used to reprogram conditionally-immortalised cells to pluripotency. In certain embodiments, OCT4 and one or more of L-MYC, KLF4 and SOX2, and LIN28 are used. In some embodiments, these factors are used in combination with a cMYC-ER^{TAM} conditional immortalisation system.

In another Example below (Example 3), STR0C05 cells were reprogrammed with the reprogramming plasmids pCE-hOCT3/4, pCE-hSK, pCE-hUL and pCEmp53DD, expressing the
35 transcription factors POU5F1, SOX2, KLF4, L-MYC, LIN28 and a dominant negative inhibitor of

p53. Therefore, in certain embodiments the transcription factors for use according to the invention may comprise or consist of POU5F1, SOX2, KLF4, L-MYC, LIN28 and a dominant negative inhibitor of p53. One, two, three or more of these may be removed or replaced as will be apparent to the skilled person. In certain embodiments, one, two, three, four or more of POU5F1, SOX2, KLF4, L-MYC, LIN28 and a dominant negative inhibitor of p53 are used to reprogram conditionally-immortalised cells to pluripotency. In some embodiments, these factors are used in combination with a c-myc-ER^{TAM} conditional immortalisation system.

In some embodiments, MYC activity is provided to promote the reprogramming process by the provision of 4-OHT in the medium to activate a c-myc-ER^{TAM} transgene in the stem cell to be reprogrammed. In certain embodiments, therefore, separately added MYC is not required.

Conditionally-immortalised Cells

The invention takes conditionally-immortalised cells and induces them to have a pluripotent phenotype. The conditionally-immortalised cells are typically conditionally-immortalised stem cells, for example conditionally-immortalised adult stem cells.

The conditionally-immortalised cells are typically mammalian, more typically human.

Stem cells are known in the art. Stem cells are cells with the ability to proliferate, exhibit self-maintenance or renewal over the lifetime of the organism and to generate clonally related progeny. The stem cells that are re-programmed according to the invention are typically multipotent cells. The stem cells that are re-programmed according to the invention are typically adult (somatic) stem cells.

The stem cells for use in the invention are isolated. The term "isolated" indicates that the cell or cell population to which it refers is not within its natural environment. The cell or cell population has been substantially separated from surrounding tissue. In some embodiments, the cell or cell population is substantially separated from surrounding tissue if the sample contains at least about 75%, in some embodiments at least about 85%, in some embodiments at least about 90%, and in some embodiments at least about 95% stem cells. In other words, the sample is substantially separated from the surrounding tissue if the sample contains less than about 25%, in some embodiments less than about 15%, and in some embodiments less than about 5% of materials other than the stem cells. Such percentage values refer to percentage by weight. The term encompasses cells which have been removed from the organism from which they originated, and exist in culture. The term also encompasses cells which have been removed from the organism from which they originated, and subsequently re-inserted into an organism. The organism which contains the re-inserted cells may be the same organism from which the cells were removed, or it may be a different organism.

The stem cells are typically allogeneic to any future recipient of the progeny cells produced according to the invention.

5 The invention uses conditionally-immortalised stem cells, such as a stem cell line, in which the expression of an immortalisation factor can be regulated without adversely affecting the production of therapeutically effective stem cells. This may be achieved by introducing an immortalisation factor which is inactive unless the cell is supplied with an activating agent. Such an immortalisation factor may be a gene such as c-mycER. The c-MycER gene product is a fusion protein comprising a c-Myc variant fused to the ligand-binding domain of a mutant estrogen receptor. C-MycER only drives cell proliferation in the presence of the synthetic steroid 4-hydroxytamoxifen (4-OHT) (Littlewood *et al.* 1995). This approach allows for controlled expansion of neural stem cells *in vitro*, while avoiding undesired *in vivo* effects on host cell proliferation (e.g. tumour formation) due to the presence of c-Myc or the gene encoding it in the neural stem cell line.

15 Other members of the Myc oncogene family can be used as conditionally-immortalising agents in an equivalent manner to c-Myc. Accordingly, the immortalising factor may comprise L-Myc, N-Myc or V-Myc. The Myc oncogene will typically be fused to the ligand-binding domain of a mutant estrogen receptor, to form L-MycER, N-MycER or V-MycER. The inventors have successfully created an L-MYC-ER^{TAM} construct, depicted in Figure 24.

A particular advantage about the MYC-ER^{TAM} constructs is their controllability and associated safety features.

25 Another gene that can be used for conditional immortalisation is TERT (telomerase reverse transcriptase). Conditional immortalisation has also been successfully achieved using the SV40 Large T antigen and temperature-sensitive variants thereof. These approaches are known in the art, for example as described in WO-A-01/21790 (ReNeuron Limited).

30 The immortalising gene can optionally be incorporated at a safe harbour site within the genome of the cell that is engineered to be conditionally-immortalised. A safe harbour genomic is a site where transgenes can be inserted and expressed without causing significant alternations in the expression of other genetic elements. An example of a known safe harbour site is AAVS1, also known as PPP1R12C on human chromosome 19. Another example of a safe harbour is the insertion site for the c-MycER^{TAM} transgene in the CTX0E03 cell line described herein, which is within the SPATA13 gene on human chromosome 13q12.12. The exact location of the insertion in the CTX0E03 cells is on (GRCh38) chromosome 13q12.12 between nucleotides 24,083,331 -

35

- 332 bp from the P-terminus. However, it is expected that equivalent results can be obtained if a site in that general area is targeted according to the invention, for example within 10kb, or within 5kb, within 2.5kb, for example within 1000bp or within 500bp of that specific site. In certain embodiments, the locus targeted for modification can be within an intron of the SPATA13 gene.
- 5 In further embodiments, the locus is within the third intron of the SPATA13 gene. Typically, the locus is within the third intron of a cDNA clone with Genbank accession number BX648244. More specifically, the locus may be on chromosome 13q12.12 anywhere between nucleotides 24,083,250 - 400 bp from the P-terminus, anywhere between nucleotides 24,083,300 - 350 bp from the P-terminus, or anywhere between nucleotides 24,083,325 - 335 bp from the P-terminus.
- 10 The insertion site is referred to as "GRCh38:13: 24083331-24083332". GRCh38 refers to the version of the human genome reference currently used by UCSC browser, as will be apparent to the skilled person.

- In certain embodiments, the conditionally-immortalised stem cell may be:
- 15 a mesenchymal stem cell, optionally selected from a bone marrow derived stem cell, an endometrial regenerative cell, a mesenchymal progenitor cell or a multipotent adult progenitor cell;
- a neural stem cell;
- a haematopoietic stem cell, optionally a CD34+ cell and/or isolated from umbilical cord blood, or optionally a CD34+/CXCR4+ cell;
- 20 a non-haematopoietic umbilical cord blood stem cell; or
- a mesenchymal stem cell derived from adipose tissue.

- In each of these embodiments, the cell is typically mammalian, more typically human.
- 25 Typically, the conditionally-immortalised stem cell is a neural stem cell, for example a human neural stem cell.

- Neural stem cells give rise to neurons, astrocytes and oligodendrocytes during development and can replace a number of neural cells in the adult brain. Typical neural stem cells for use in certain aspects according to the present invention cells that exhibit one or more of the neural phenotypic
- 30 markers Musashi-1, Nestin, NeuN, class III β -tubulin, GFAP, NF-L, NF-M, microtubule associated protein (MAP2), S100, CNPase, glypican, (especially glypican 4), neuronal pentraxin II, neuronal PAS 1, neuronal growth associated protein 43, neurite outgrowth extension protein, vimentin, Hu, internexin, 04, myelin basic protein and pleiotrophin, among others.

- The neural stem cell may be from a stem cell line, i.e. a culture of stably dividing stem cells. A
- 35 stem cell line can to be grown in large quantities using a single, defined source.

Preferred conditionally-immortalised neural stem cell lines include the CTX0E03, STR0C05 and HPC0A07 neural stem cell lines, which have been deposited by the applicant of this patent application, ReNeuron Limited, at the European Collection of Animal Cultures (ECACC), Vaccine Research and Production laboratories, Public Health Laboratory Services, Porton Down, Salisbury, Wiltshire, SP4 0JG, with Accession No. 04091601 (CTX0E03); Accession No.04110301 (STR0C05); and Accession No.04092302 (HPC0A07). The derivation and provenance of these cells is described in EP1645626 B1 and United States Patent 7416888, both incorporated herein by reference in their entirety.

10 CTX0E03 (ECACC Deposit #04091601)

CTX0E03 is a neural stem cell line in clinical trials as a therapy for ischemic stroke and limb damage. It is controllably immortalised by the integration of a C-MYC-ER^{TAM} fusion protein, which upon binding of the ER^{TAM} domain to the synthetic estrogen derivative 4-hydroxytamoxifen (4-OHT) translocates to the nucleus where the C-MYC domain promotes indefinite cell cycling. Expression of the C-MYC-ER^{TAM} does not apparently affect cell phenotype. Thus an indefinitely-large number of patients may be treated with CTX as an “off-the-shelf” allogeneic therapy. The transgene has been shown to be silenced upon removal of 4-OHT and/or transfer to a patient.

The cells of the CTX0E03 cell line may be cultured in the following culture conditions:

- Human Serum Albumin 0.03%
- Transferrin, Human 5µg/ml
- Putrescine Dihydrochloride 16.2 µg/ml
- Insulin Human recombinant 5 µ/ml
- Progesterone 60 ng/ml
- L-Glutamine 2 mM
- Sodium Selenite (selenium) 40 ng/ml

Plus basic Fibroblast Growth Factor (10 ng/ml), epidermal growth factor (20 ng/ml) and 4-hydroxytamoxifen (100nM) for cell expansion. The cells can be differentiated by removal of the 4-hydroxytamoxifen. Typically, the cells can either be cultured at 5% CO₂/37°C or under hypoxic conditions of 5%, 4%, 3%, 2% or 1% O₂. These cell lines do not require serum to be cultured successfully. Serum is required for the successful culture of many cell lines, but contains many contaminants. A further advantage of the CTX0E03, STR0C05 or HPC0A07 neural stem cell lines, or any other cell line that does not require serum, is that the contamination by serum is avoided. In some embodiments of the present invention, absence of serum in the system can be maintained, for example by the use of E8 medium for the steps of reprogramming and culture of induced pluripotent stem cells.

CTX culture medium may be supplemented with 4-OHT or not, to provide MYC activity through the c-myc-ER^{TAM} transgene, as desired.

5 The cells of the CTX0E03 cell line are multipotent cells originally derived from 12 week human fetal cortex. The isolation, manufacture and protocols for the CTX0E03 cell line is described in detail by Sinden, et al. (U.S. Pat. 7,416,888 and EP1645626 B1). The CTX0E03 cells are not “embryonic stem cells”, i.e. they are not pluripotent cells derived from the inner cell mass of a blastocyst; isolation of the original cells did not result in the destruction of an embryo. In growth
10 medium CTX0E03 cells are nestin-positive with a low percentage of GFAP positive cells (i.e. the population is negative for GFAP).

CTX0E03 is a clonal cell line that contains a single copy of the c-mycER transgene that was delivered by retroviral infection and is conditionally regulated by 4-OHT (4-hydroxytamoxifen).
15 The C-mycER transgene expresses a fusion protein that stimulates cell proliferation in the presence of 4-OHT and therefore allows controlled expansion when cultured in the presence of 4-OHT. This cell line is clonal, expands rapidly in culture (doubling time 50-60 hours) and has a normal human karyotype (46 XY). It is genetically stable and can be grown in large numbers. The cells are safe and non-tumorigenic. In the absence of growth factors and 4-OHT, the cells
20 undergo growth arrest and differentiate into neurons and astrocytes. Once implanted into an ischemia-damaged brain, these cells migrate only to areas of tissue damage.

The development of the CTX0E03 cell line has allowed the scale-up of a consistent product for clinical use. Production of cells from banked materials allows for the generation of cells in
25 quantities for commercial application (Hodges et al, 2007).

The CTX0E03 drug product can be provided as a fresh (as was the case for the PISCES trial) or frozen suspension of living cells, as described in US9265795 and used in the PISCES II trial. The drug product typically comprises CTX0E03 cells at a passage of ≤ 37 .

30 The CTX clinical drug product is typically formulated as an “off the shelf” cryopreserved product in a solvent-free excipient (e.g. as described in US Patent 9265795) with a shelf life of many months. This formulation typically comprises Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, H₂PO₄⁻, HEPES, lactobionate, sucrose, mannitol, glucose, dextran-40, adenosine and glutathione. One or more, for example two, three, or four, of
35 these excipients may optionally be removed or replaced. Typically, the formulation does not comprise a dipolar aprotic solvent, in particular DMSO.

Clinical release criteria for stem cell products typically include measures of sterility, purity (cell number, cell viability), and a number of other tests of identity, stability, and potency that are required for clinical product release or for information, as requested by regulatory authorities. The tests employed for CTX0E03 are summarised in Table 1, below.

Test	Outcome
PCR Sequencing of cDNA	Sequence of insert conforms to transgene identity. No insertions, deletions, or mutations from expected sequence
Determination of Flanking Nucleotide Sequence	Consistent with published sequence
PCR across integration site	PCR across integration site confirms cell line identity
Karyology	Comparable with published normal chromosome, male XY
Viability and growth	≥70% viability on recovery. Viable cell numbers at least double within 7 days
c-mycER ^{TAM} gene copy number (PCR)	Modal ~1 (range 0.87–3.46)
Phenotypic marker (Nestin)	At least 95% of cells are Nestin positive
Position, sequence, and indication of number of integrated target gene by fluorescent in situ hybridization	Chromosomal (Chr 13) localization of integrated c-mycER ^{TAM} sequences
Potency	Cell dose-dependent IL-10 production in co-culture with U937 monocyte cell line
Neural differentiation	Upregulation of Tub-β3, GFAP, and GAL-C marker expression by qPCR after seeding into Alvetex [®] three-dimensional cell matrix

Table 1: Identity, Stability, and Potency Tests That Are Employed to Characterize CTX Cell Banks and/or Drug Products (for Phase II Trial)

The CTX0E03 cell line has been previously demonstrated, using a human PBMC assay, not to be immunogenic. The lack of immunogenicity allows the cells to avoid clearance by the host/patient immune system and thereby exert their therapeutic effect without a deleterious immune and inflammatory response.

Pollock *et al* 2006 describes that transplantation of CTX0E03 in a rat model of stroke (MCAo) caused statistically significant improvements in both sensorimotor function and gross motor asymmetry at 6–12 weeks post-grafting. These data indicate that CTX0E03 has the appropriate biological and manufacturing characteristics necessary for development as a therapeutic cell line.

Stevanato *et al* 2009 confirms that CTX0E03 cells downregulated c-mycERTAM transgene expression both *in vitro* following EGF, bFGF and 4-OHT withdrawal and *in vivo* following

implantation in MCAo rat brain. The silencing of the c-mycER^{TAM} transgene in vivo provides an additional safety feature of CTX0E03 cells for potential clinical application.

Smith *et al* 2012 describe preclinical efficacy testing of CTX0E03 in a rat model of stroke (transient middle cerebral artery occlusion). The results indicate that CTX0E03 implants robustly recover behavioural dysfunction over a 3 month time frame and that this effect is specific to their site of implantation. Lesion topology is potentially an important factor in the recovery, with a stroke confined to the striatum showing a better outcome compared to a larger area of damage.

STR0C05 (ECACC Deposit #04110301)

This c-MycER^{TAM} transduced-neural stem cell line was derived from 12 week fetal striatum. The line is maintained on laminin coated culture flasks using defined serum free "Human Media" in the presence of bFGF, EGF and 4-hydroxy tamoxifen. In routine culture the cell line has a doubling time of 3-4 days although in short term culture a doubling time of 20-30h was seen.

In growth medium the cells are nestin-positive, beta-III tubulin-negative with a low percentage of GFAP positive cells. Following differentiation for 7 days there is down regulation of nestin with low-level expression of beta III tubulin and strong expression of GFAP suggesting that the cell line becomes predominantly astrocytic.

This cell line is genetically normal, male XY, and stable over 50 population doublings.

The lines described here were derived under Quality Assured conditions suitable for progressing designated lines for clinical use. As source material, human neural stem cells were isolated post mortem from the striatum of a 12-week gestation fetus GS006 by enzymatic digestion with trypsin in combination with mechanical trituration. Once established in culture these primary neural cells were transformed by retroviral transduction with the c-MycERTAM oncogene (as described for the CTX0E03 cell line above) and a range of clonal and mixed population cell lines isolated. All lines in this series were derived on laminin coated culture-ware and using Human Media (HM); DMEM:F12 plus designated supplements as described below.

Human Media (HM)

DMEM:F12 supplemented with the components listed below:

Human Serum Albumin 0.03%.

Transferrin, Human 100 µg/ml.

- Putrescine Dihydrochloride 16.2 µg/ml.
Insulin, Human recombinant 5 µg/ml.
L-Thyroxine (T4) 400 ng/ml.
Tri-Iodo-Thyronine (T3) 337 ng/ml.
- 5 Progesterone 60ng/ml.
L-Glutamine 2 mM.
Sodium Selenite (selenium) 40 ng/ml.
Heparin, sodium salt 10 Units/ml.
Corticosterone 40ng/ml.
- 10 Plus basic Fibroblast Growth Factor (10 ng/ml) and epidermal growth factor (20 ng/ml) for cell expansion.

STR0C05 Growth Characteristics

- 15 Under routine culture conditions cells are expanded from frozen stocks, usually 2-4 million cells in T180 culture flasks. After several media changes the cells are passaged when confluent. From process records, population doubling times for STR0C05 have been estimated at 3-4 days as shown on the graph below. This doubling time is slower than for log phase growth and also includes cell loss during the passaging.
- 20 As a more representative assessment of log phase growth for STR0C05, a cell proliferation assay was set up using the Cyquant fluorescent dye (Molecular Probes). Cell number is measured using a Tecan Magellan fluorescence plate reader; ex..480nm; em 520nm.
- 25 STR0C05 cells were passaged, resuspended in HM plus growth factors and seeded on laminin coated 96 well strip-well plates at 5000 cells/well. A time course study was carried out by removing strips from the plate on a daily basis, n=16 wells per time point, removing the media and freezing the cells at -70°C.
- 30 At the end of the time course all the frozen strips were put back together on the plate and analysed with the Cyquant assay. Briefly cells are lysed in lysis buffer then Cyquant reagent added and placed in dark for 5 minutes. A 150ul sample of each well was then transferred to black, Optilux plates for reading on a Tecan Magellan plate reader. Data was exported to an Excel spreadsheet for numerical averaging and further exported to GraphPad Prism for analysis.
- 35 The results showed that the cells grew steadily over 7 days with an estimated doubling time of 20-30 hours.

STROC05 Phenotype

The phenotype of the STROC05 has been profiled using immunocytochemistry to stain for the neural stem cell marker nestin and to stain for mature markers of differentiation, beta-III tubulin (neuronal) and GFAP (astrocytic).

5

STROC05 phenotype was determined in the presence and absence of growth factors plus 4-OHT. Cells were originally sourced from STROC05 working stock. Cells were passaged and seeded in 96 well plates.

10 Cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature, washed with PBS and permeabilised with 0.1 % Triton X100/PBS for 15 minutes. Non-specific binding was then blocked with 10% Normal Goat Serum (NGS) in PBS for 1 hour at room temperature. Cells were then probed with antibodies to Nestin (1:200; Chemicon), Beta-III Tubulin (1:500; Sigma) and GFAP (1:5000; DAKO) at room temperature overnight. After washing with PBS, they were then

15 processed with filtered Alexa Goat α Mouse 488 (1:200; Molecular Probes) and Alexa Goat α Rabbit 568 (1:2500; Molecular Probes) dissolved in 1% NGS/PBS for 1 hour at room temperature. They were then washed with PBS and counterstained with Hoechst 33342 (Sigma) for 2 minutes before being analysed on a fluorescent microscope.

20 Removal of growth factors and 4-OHT from the medium induces a morphological and phenotypic change in the cells that is accompanied by down regulation of nestin. Specifically a small proportion of the cells become positive for the neuronal marker beta-III tubulin and acquire a neuronal morphology with rounded cell bodies extending into dendritic/ axonal outgrowths. The more dominant phenotypic change however is the up-regulation of GFAP suggesting a

25 predominance of an astrocytic lineage.

Clonality**Southern Blot for STROC05**

30 In two separate experiments there is no evidence of probe hybridisation in contrast to clear bands seen with other cell lines.

Cell populations

The invention uses and relates to a population of isolated stem cells, wherein the population essentially comprises only stem cells of the invention, i.e. the stem cell population is substantially

35 pure. In many aspects, the stem cell population comprises at least about 75%, or at least 80% (in other aspects at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% or 100%) of the stem cells of the invention, with respect to other cells that make up a total

cell population. For example, with respect to neural stem cell populations, this term means that there are at least about 75%, in some embodiments at least about 85%, in some embodiments at least about 90%, and in some embodiments at least about 95% pure, neural stem cells compared to other cells that make up a total cell population. The term “substantially pure”
5 therefore refers to a population of stem cells of the present invention that contain fewer than about 25%, in some embodiments fewer than about 15%, and in some embodiments fewer than about 5%, of cells that are not neural stem cells.

Isolated stem cells can be characterised by a distinctive expression profile for certain markers and is distinguished from stem cells of other cell types. When a marker is described herein, its
10 presence or absence may be used to distinguish the neural stem cell.

A neural stem cell population may in some embodiments be characterised in that the cells of the population express one, two, three, four, five or more, for example all, of the markers Nestin, Sox2, GFAP, β III tubulin, DCX, GALC, TUBB3, GDNF and IDO.
15

Typically, neural stem cells are nestin positive.

A “Marker” refers to a biological molecule whose presence, concentration, activity, or phosphorylation state may be detected and used to identify the phenotype of a cell.
20

A cell of the invention is typically considered to carry a marker if at least about 70% of the cells of the population show a detectable level of the marker. In other aspects, at least about 80%, at least about 90% or at least about 95% or at least about 97% or at least about 98% or more of the population show a detectable level of the marker. In certain aspects, at least about 99% or 100%
25 of the population show detectable level of the markers. Quantification of the marker may be detected through the use of a quantitative RT-PCR (qRT-PCR) or through fluorescence activated cell sorting (FACS). It should be appreciated that this list is provided by way of example only, and is not intended to be limiting. Typically, a neural stem cell of the invention is considered to carry a marker if at least about 90% of the cells of the population show a detectable level of the marker
30 as detected by FACS.

The term “expressed” is used to describe the presence of a marker within a cell. In order to be considered as being expressed, a marker must be present at a detectable level. By “detectable level” is meant that the marker can be detected using one of the standard laboratory
35 methodologies such as qRT-PCR, or RT-PCR, blotting, Mass Spectrometry or FACS analysis. A gene is considered to be expressed by a cell of the population of the invention if expression can be reasonably detected at a crossing point (cp) values below or equal 35 (standard cut off on a

qRT-PCR array). The C_p represents the point where the amplification curve crosses the detection threshold, and can also be reported as crossing threshold (ct).

The terms “express” and “expression” have corresponding meanings. At an expression level below this c_p value, a marker is considered not to be expressed. The comparison between the expression level of a marker in a stem cell of the invention, and the expression level of the same marker in another cell, such as for example an mesenchymal stem cell, may preferably be conducted by comparing the two cell types that have been isolated from the same species. Preferably this species is a mammal, and more preferably this species is human. Such comparison may conveniently be conducted using a reverse transcriptase polymerase chain reaction (RT-PCR) experiment.

As used herein, the term “significant expression” or its equivalent terms “positive” and “+” when used in regard to a marker shall be taken to mean that, in a cell population, more than 20%, preferably more than, 30%, 40%, 50%, 60%, 70%, 80%, 90% 95%, 98%, 99% or even all of the cells of the cells express said marker.

As used herein, “negative” or “-” as used with respect to markers shall be taken to mean that, in a cell population, fewer than 20%, 10%, preferably fewer than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1 % or none of the cells express said marker.

Expression of cell surface markers may be determined, for example, by means of flow cytometry and/or Fluorescence activated cell sorting (FACS) for a specific cell surface marker using conventional methods and apparatus (for example a Beckman Coulter Epics XL FACS system used with commercially available antibodies and standard protocols known in the art) to determine whether the signal for a specific cell surface marker is greater than a background signal. The background signal is defined as the signal intensity generated by a non-specific antibody of the same isotype as the specific antibody used to detect each surface marker. For a marker to be considered positive the specific signal observed is typically more than 20%, preferably stronger than 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 500%, 1000%, 5000%, 10000% or above, greater relative to the background signal intensity. Alternative methods for analysing expression of cell surface markers of interest include visual analysis by electron microscopy using antibodies against cell-surface markers of interest.

Stem cell culture and production

Simple bioreactors for stem cell culture are single compartment flasks, such as the commonly-used T-175 flask (e.g. the BD Falcon™ 175 cm² Cell Culture Flask, 750 ml, tissue-culture treated polystyrene, straight neck, blue plug-seal screw cap, BD product code 353028).

The conditionally-immortalised stem cells may typically be taken from proliferating stem cells cultured in T-175 or T-500 flasks.

Bioreactors can also have multiple compartments, as is known in the art. These multi-compartment bioreactors typically contain at least two compartments separated by one or more membranes or barriers that separate the compartment containing the cells from one or more compartments containing gas and/or culture medium. Multi-compartment bioreactors are well-known in the art. An example of a multi-compartment bioreactor is the Integra CeLLine bioreactor, which contains a medium compartment and a cell compartment separated by means of a 10 kDa semi-permeable membrane; this membrane allows a continuous diffusion of nutrients into the cell compartment with a concurrent removal of any inhibitory waste product. The individual accessibility of the compartments allows to supply the cells with fresh medium without mechanically interfering with the culture. A silicone membrane forms the cell compartment base and provides an optimal oxygen supply and control of carbon dioxide levels by providing a short diffusion pathway to the cell compartment. Any multi-compartment bioreactor may be used according to the invention.

The term “culture medium” or “medium” is recognized in the art, and refers generally to any substance or preparation used for the cultivation of living cells. The term “medium”, as used in reference to a cell culture, includes the components of the environment surrounding the cells. Media may be solid, liquid, gaseous or a mixture of phases and materials. Media include liquid growth media as well as liquid media that do not sustain cell growth. Media also include gelatinous media such as agar, agarose, gelatin and collagen matrices. Exemplary gaseous media include the gaseous phase to which cells growing on a petri dish or other solid or semisolid support are exposed. The term “medium” also refers to material that is intended for use in a cell culture, even if it has not yet been contacted with cells. In other words, a nutrient rich liquid prepared for culture is a medium. Similarly, a powder mixture that when mixed with water or other liquid becomes suitable for cell culture may be termed a “powdered medium”. “Defined medium” refers to media that are made of chemically defined (usually purified) components. “Defined media” do not contain poorly characterized biological extracts such as yeast extract and beef broth. “Rich medium” includes media that are designed to support growth of most or all viable forms of a particular species. Rich media often include complex biological extracts. A “medium suitable for growth of a high density culture” is any medium that allows a cell culture to reach an OD600 of 3 or greater when other conditions (such as temperature and oxygen transfer rate) permit such growth. The term “basal medium” refers to a medium which promotes the growth of many types of microorganisms which do not require any special nutrient supplements. Most basal media generally comprise of four basic chemical groups: amino acids, carbohydrates, inorganic salts, and vitamins. A basal medium generally serves as the basis for a more complex medium,

to which supplements such as serum, buffers, growth factors, lipids, and the like are added. In one aspect, the growth medium may be a complex medium with the necessary growth factors to support the growth and expansion of the cells of the invention while maintaining their self-renewal capability. Examples of basal media include, but are not limited to, Eagles Basal Medium, Minimum Essential Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Nutrient Mixtures Ham's F-10 and Ham's F-12, McCoy's 5A, Dulbecco's MEM/F-12, RPMI 1640, and Iscove's Modified Dulbecco's Medium (IMDM).

Extracellular Vesicles produced by the pluripotent cells of the invention and their progeny

10

The pluripotent stem cells of the invention, and the differentiated cells generated from those cells, will produce extracellular vesicles. The invention provides, in one aspect, extracellular vesicles obtainable from the induced pluripotent stem cells of the invention, or from differentiated cells generated from those iPS cells. These extracellular vesicles can be used in therapy.

15

The extracellular vesicles obtained from cells of the invention can also be used as delivery vehicles for exogenous cargo. The cargo may, in some embodiments, be exogenous nucleic acid (e.g. DNA or RNA, in particular an RNAi agent such as siRNA or chemically-modified siRNA), exogenous protein (e.g. an antibody or antibody fragment, a signalling protein, or a protein drug).

20

It is known in that art that cargo can be directly loaded into extracellular vesicles, for example by transfection or electroporation. It is also known that manipulating the cell that produces the extracellular vesicle can change the content of the extracellular vesicle.

The nature, content and characteristics of extracellular vesicles are influenced by the cell that produces them. Therefore, the invention advantageously provides for a diverse range of extracellular vesicles to be produced from a single well-characterised starting material (i.e. the conditionally-immortalised cell). For example, extracellular vesicles can be isolated from the iPS cell or any more differentiated cell derived from that cell, such as a cell that has entered the endoderm, mesoderm or ectoderm lineage. This allows for the provision of many different extracellular vesicles from a single, known starting cell.

An "extracellular vesicle" (sometimes referred to in older publications by the general term "microparticle") is a lipid bilayer particle of 30 to 1000 nm diameter that is released from a cell. It is limited by a lipid bilayer that encloses biological molecules. The term "extracellular vesicle" is known in the art and encompasses a number of different species of extracellular vesicle, including a membrane particle, membrane vesicle, microvesicle, exosome-like vesicle, exosome, ectosome-like vesicle, ectosome or exovesicle. The different types of extracellular vesicle are distinguished based on diameter, subcellular origin, their density in sucrose, shape,

sedimentation rate, lipid composition, protein markers and mode of secretion (i.e. following a signal (inducible) or spontaneously (constitutive)). Three main types of extracellular vesicles are now generally recognised based on the biogenesis and size of the vesicles: 1) Exosomes, 2) Microvesicles (also sometimes known as Microparticles) and 3) Apoptotic bodies.

5

Common extracellular vesicles and their distinguishing features are described in Table 1, below. In certain embodiments, the extracellular vesicle is an exosome.

Table 1: Various Extracellular vesicles

Extracellular vesicle	Typical Size	Shape	Markers	Lipids	Origin
Microvesicles	100-1000nm	Irregular	Integrins, selectins, CD40 ligand	Phosphatidylserine	Plasma membrane
Exosomes	30-100nm; (<200nm)	Cup shaped	Tetraspanins (e.g. CD63, CD9), Alix, TSG101, ESCRT	Cholesterol, sphingomyelin, ceramide, lipid rafts, phosphatidylserine	Multivesicular bodies, endosomes
Apoptotic Bodies	500-1000nm	Irregular		Phosphatidylserine	Plasma membrane

10

Extracellular vesicles are thought to play a role in intercellular communication by acting as vehicles between a donor and recipient cell through direct and indirect mechanisms. Direct mechanisms include the uptake of the extracellular vesicle and its donor cell-derived components (such as proteins, lipids or nucleic acids) by the recipient cell, the components having a biological activity in the recipient cell. Indirect mechanisms include vesicle-recipient cell surface interaction, and causing modulation of intracellular signalling of the recipient cell. Hence, extracellular vesicles may mediate the acquisition of one or more donor cell-derived properties by the recipient cell. It has been observed that, despite the efficacy of stem cell therapies in animal models, the stem cells do not appear to engraft into the host. Accordingly, the mechanism by which stem cell therapies are effective is not clear. Without wishing to be bound by theory, the inventors believe that the extracellular vesicles secreted by neural stem cells play a role in the therapeutic utility of these cells and are therefore therapeutically useful themselves.

15

20

The extracellular vesicles of the invention are isolated, as defined herein for the cells.

25

The invention provides a population of isolated stem cell extracellular vesicles produced by a cell of the invention, wherein the population essentially comprises only extracellular vesicles of the invention, i.e. the extracellular vesicle population is pure. In many aspects, the extracellular vesicle population comprises at least about 80% (in other aspects at least 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.9% or 100%) of the extracellular vesicles of the invention.

In certain embodiments, the extracellular vesicles are exosomes. The lipid bilayer of an exosome is typically enriched with cholesterol, sphingomyelin and ceramide. Exosomes also express one or more tetraspanin marker proteins. Tetraspanins include CD81, CD63, CD9, CD53, CD82 and CD37. CD63 is a typical exosome marker. Exosomes can also include growth factors, cytokines and RNA, in particular miRNA. Exosomes typically express one or more of the markers TSG101, Alix, CD109, thy-1 and CD133. Alix (Uniprot accession No. Q8WUM4), TSG101 (Uniprot accession No. Q99816) and the tetraspanin proteins CD81 (Uniprot accession No. P60033) and CD9 (Uniprot accession No. P21926) are characteristic exosome markers.

Alix is an endosomal pathway marker. Exosomes of the invention are typically positive for Alix. Microvesicles are typically negative for Alix.

In some embodiments, the extracellular vesicles such as exosomes can be loaded with exogenous cargo. The exogenous cargo can be a protein (for example an antibody), peptide, drug, prodrug, hormone, diagnostic agent, nucleic acid (e.g. RNAi agent such as miRNA, siRNA or shRNA, or a DNA or RNA vector), carbohydrate or other molecule of interest. The cargo can be loaded directly into the exosomes, for example by electroporation or transfection, or can be loaded into the exosome by engineering the cell that produces the exosome such that the cell encapsulates the cargo into the exosome before exosome release. The loading of cargo into extracellular vesicles such as exosomes is known in the art.

Pharmaceutical Compositions

The pluripotent stem cells of the invention can be differentiated to generate cells that are useful in therapy, typically of the haematopoietic lineage, and can therefore be formulated as a pharmaceutical composition. The pluripotent stem cells of the invention, and the differentiated cells generated from those cells, will produce extracellular vesicles as described elsewhere herein, that may also be useful in therapy and can therefore be formulated as a pharmaceutical composition. In particular, the scalable production of effectively unlimited quantities of cells of the haematopoietic lineage, in particular the immune system cells described herein, allows for formulation of these cells into an off-the-shelf pharmaceutical product. For example, the cells can be cryopreserved in aliquots (e.g. individual dosages) for rapid "off-the-shelf" allogeneic treatment

of large numbers of patients. Particularly suitable cells for this application include both terminally differentiated cells, for example CAR-T cells carrying particular engineered tumour receptors, or HSCs or lineage progenitors with varying ranges of potency and expansion potential.

In certain embodiments, the pharmaceutical composition is frozen.

5

In certain embodiments, the pharmaceutical composition is cryopreserved .

In certain embodiments, the pharmaceutical composition is lyophilised.

10 When the pharmaceutical composition is frozen, cryopreserved or lyophilised, it is typically thawed, or reconstituted as appropriate, prior to administration to the patient.

In some embodiments, a non-terminally differentiated population of cells is stored, for example frozen. In one embodiment this could be myeloblasts, which when needed are thawed and cultured (e.g. in the hospital or clinic) for a short period with suitable provided reagents to generate
15 neutrophils that are then transferred to the patient. Some myoblasts express CD7 and CD34, as demonstrated in the Examples below. Neutrophils have utility in a number of therapies, including in cancer treatment and in treatment of infectious disease.

The scalable expansion of adult stem cells or tissue progenitor types that are often hard to produce in quantity, is a particular advantage of the present invention. For example, Myeloblasts
20 are an oligopotent ASC type downstream of the multipotent HSCs, that are capable of generating the entire haematopoietic lineage by themselves and as such are envisaged to be particularly useful.

With regard to neutrophils, the granules within these cells are delicate and the potential problem of degranulation after freezing/thawing neutrophils can be avoided by thawing and differentiating
25 a frozen myeloblast shortly before administration to a patient for treatment. Neutrophils can be differentiated *in vitro* from conditional Hoxb8 -immortalized precursors using SCF and G-CSF.

A pharmaceutically acceptable composition typically includes at least one pharmaceutically acceptable carrier, diluent, vehicle and/or excipient in addition to the therapeutic cells or extracellular vesicles. An example of a suitable carrier is Ringer's Lactate solution. A thorough
30 discussion of such components is provided in Gennaro (2000) Remington: The Science and Practice of Pharmacy, 20th edition, ISBN: 0683306472.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without

excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

5 The composition, if desired, can also contain minor amounts of pH buffering agents. The composition may comprise storage media such as Hypothermosol®, commercially available from BioLife Solutions Inc., USA. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E W Martin. Such compositions will contain a prophylactically or therapeutically effective amount of a prophylactic or therapeutic stem cell preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the subject. The formulation should suit the mode of administration. In a preferred embodiment, the pharmaceutical compositions are sterile and in suitable form for administration to a subject, preferably an animal subject, more preferably a mammalian subject, and most preferably a human subject.

15 The pharmaceutical composition of the invention may be in a variety of forms. These include, for example, semi-solid, and liquid dosage forms, such as lyophilized preparations, frozen preparations, liquid solutions or suspensions, injectable and infusible solutions. The pharmaceutical composition is preferably injectable.

20 Pharmaceutical compositions will generally be in aqueous form. Compositions may include a preservative and/or an antioxidant.

To control tonicity, the pharmaceutical composition can comprise a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride and calcium chloride.

30 Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer. Buffers will typically be included at a concentration in the 5-20mM range. The pH of a composition will generally be between 5 and 8, and more typically between 6 and 8 e.g. between 6.5 and 7.5, or between 7.0 and 7.8.

35 The composition is preferably sterile. The composition is preferably non-pyrogenic.

In a typical embodiment, the cells or extracellular vesicles are suspended in a composition comprising 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more excipients selected from 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, H₂P0₄⁻, HEPES,

lactobionate, sucrose, mannitol, glucose, dextran-40, adenosine and glutathione. In one embodiment the composition comprises all of these excipients. Typically, the composition will not include a dipolar aprotic solvent, e.g. DMSO. Suitable compositions are available commercially, e.g. HypoThermasol®-FRS. Such compositions are advantageous as they allow the cells to be stored at 4°C to 25°C for extended periods (hours to days) or preserved at cryothermic temperatures, i.e. temperatures below -20°C. The stem cells may then be administered in this composition after thawing.

Although the invention has been described in detail for purposes of clarity of understanding, certain modifications may be practiced within the scope of the appended claims. All publications, accession numbers, and patent documents cited in this application are hereby incorporated by reference in their entirety for all purposes to the same extent as if each were so individually denoted. To the extent more than one sequence is associated with an accession number at different times, the sequences associated with the accession number as of the effective filing date of this application is meant. The effective filing date is the date of the earliest priority application disclosing the accession number in question. Unless otherwise apparent from the context any element, embodiment, step, feature or aspect of the invention can be performed in combination with any other.

The invention is further described with reference to the following non-limiting examples. In these examples, the inventors first demonstrate that conditional immortalised neural stem cells (CTX0E03; deposited on 16 September 2004 by the applicant of this patent application, ReNeuron Limited, at the European Collection of Animal Cultures (ECACC) with Accession No. 04091601) can be reprogrammed to pluripotency, on the basis of several independent replicates. These iPSCs are then differentiated into Mesenchymal stem cells (MSCs). The genetic reprogramming and pluripotency of the CTX-iPSCs is also confirmed.

The inventors then demonstrate the successful reprogramming of another conditionally immortalised adult stem cell type. This line is STR0C05, derived from fetal striatal cells (and deposited on 3rd November 2004 by the applicant of this patent application, ReNeuron Limited, at the European Collection of Animal Cultures (ECACC) with Accession No. 04110301). Generation of iPSCs from STR0C05 and subsequent differentiation of these STR0C-iPSCs to endoderm, mesoderm and ectoderm lineages is shown. These data confirm that the benefits afforded by the invention are not limited to the CTX cell line in which we have first demonstrated it, but apply widely, to any conditionally-immortalised adult cell type.

The Examples then provide a further characterisation of the MSC cells derived from the reprogrammed iPSCs, reinforcing the finding that it is possible to expand an adult stem cell type

derived from these iPSCs beyond the normal limits for such cells, thereby permitting the treatment of large numbers of patients from such a line. These CTX-iPSC-MSCs are shown (Figure 10) to differentiate into cartilage (shown by alcian blue staining of sialoglycans), fat (shown by staining of intracellular lipid droplets with oil red O) and bone (shown by alizarin red staining of deposited calcium) cells.

Finally, yet further detailed characterisation of the CTX-iPSC cells is provided, with detailed exemplification in Example 6 of haematopoietic lineages including HSCs and terminally-differentiated haematopoietic cells, derived from conditionally-immortalisable hiPSCs.

10 **Examples**

Example 1: iPSCs derived from inducibly-immortalised adult stem cells as a source for clinical-scale manufacture of allogeneic cell therapies

Introduction

- 15 • Induced pluripotent stem cells (iPSCs) have great potential as a source material for cell therapies
- Candidate therapeutic populations are typically adult stem cells or tissue progenitors (ASCs / TPs) rather than terminally-differentiated cells
- ASCs / TPs are often difficult to culture and purify
- 20 • Conditional immortalisation of ASCs / TPs would be beneficial for the scalable production of cells for allogeneic cell therapy
- CTX is a neural stem cell line in clinical trials for ischemic stroke. It is immortalised with a c-myc-ER^{TAM} transgene, controllable by the addition of 4- hydroxytamoxifen (4-OHT) to the culture medium

25

Reprogramming CTX0E03 to pluripotency

CTX0E03 cells were reprogrammed to pluripotency using standard non-integrating episomal vectors encoding the “Yamanaka Factors” (OCT4, L-MYC, KLF4 and SOX2, “OKSM”, and LIN28) (Figure 1).

30

The CTX cells were successfully reprogrammed, independently, several times.

CTX-iPSCs share many features characteristic of human iPSCs and ESCs. After reprogramming, cell morphology changes dramatically from the neuronal phenotype with extended processes characteristic of CTX cells to one of small, rounded, undifferentiated cells with prominent nucleoli and difficult-to-distinguish divisions between cells densely packed into “islands” characteristic of human pluripotent stem cells (Figure 1C, Figure 2). CTX-iPSCs express the tissue non-specific alkaline phosphatase enzymatic marker at day 21 endpoint (Figure 1D, Figure 3).

Varying transcription factor combinations to dissect CTX reprogramming requirements.

Figure 2 shows that CTX0E03 cells are reprogrammable with fewer factors. (A) Vectors expressing single factors, pCE-OCT3/4, pCE-SOX2 and pCE-KLF4; 4-OHT provision mimics MYC via c-myc-ER^{TAM}. (B) Inset: example AP-stained plate for colony counting. Main image: colony reprogrammed with transcription factor OCT4 alone. (C) Colony numbers obtained with different factor combinations (S-K: pCE-SK, M-L: pCE-UL, S: pCE-SOX2, K: pCE-KLF4, M: 4-OHT → d 14). (D) Venn diagram showing combination effects (numbers: x colonies obtained; zeroes: no colonies).

CTX-iPSCs share many features with classical hPSCs

The pluripotent phenotype of the CTX-iPSCs is shown in Figure 3.

(A) Cell and colony morphology assessment in of CTX-iPSCs on two different cell lines (ii, iii) derived from CTX cells by reprogramming to pluripotency by transfection of the OKSML transcription factor set, shows that these reprogrammed cell lines recapitulate the dense colonies of small, closely-packed cells with prominent nucleoli characteristic of hPSCs, differing markedly from the neuronal phenotype of the parental CTX cells (i).

CTX-iPSC lines express the enzymatic marker alkaline phosphatase (pink stain), as shown in Figure 3B.

As expected for human pluripotent stem cells, flow cytometry shows that CTX-iPSCs are positive for the canonical pluripotent transcription factor OCT4, and the cell surface antigens TRA-1-60 and SSEA-4, but do not express the early differentiation marker SSEA-1. (Figure 3C).

(D) RT-qPCR showing upregulation of lineage-specific markers upon in vitro differentiation to endoderm, mesoderm and ectoderm (individual CTX-iPSC lines indicated by shade).

Status of the c-myc-ER^{TAM} Transgene in CTX-iPSCs

Assessment of the transgene locus in CTX-iPSCs is shown in Figure 4.

(A) Giemsa staining of parental CTX0E03 cells (top, 4 days, 2nd row, 10 days) and five CTX-iPSC lines at 4 days in G418 (3rd-7th rows) indicates expression activity of the c-myc-ER^{TAM}-associated *NeoR* gene.

(B) Bisulphite-conversion of the CMV-IE promoter driving the c-myc-ER^{TAM} transgene shows the cytosine methylation state at the locus (white circle, unmethylated CpG; black circle, methylated CpG; comma, indeterminate read).

5 Derivation of therapeutic cell populations from CTX-iPSCs

It can be shown using RT-qPCR that differentiation along the three germline lineages (endoderm, mesoderm, ectoderm) is achieved. Differentiation of CTX-iPSCs to therapeutically-relevant cell types can also be confirmed. This has been demonstrated for adult stem cell types (mesenchymal stem cells). Other cell types can be generated by appropriate culture conditions, as will be apparent to the skilled person. In particular, cells of the immune system such as T lymphocytes, NK cells and dendritic cells can be differentiated by the methods disclosed in Themeli *et al.* (2013) Nature Biotechnology (31), 928–933.

15 Figure 5 shows the production of a therapeutic cell population derived from CTX-iPSCs. (A) CTX-iPSCs on Laminin-521 in mTeSR1 medium. (B) Plastic-adherent candidate mesenchymal stem cells (MSCs) derived from cells in (A) in MSC medium (α -MEM, 10% FCS, 25 mM HEPES). (C) Flow cytometry of the CTX-iPSC-MSCs shows they express the MSC markers CD73, CD90 and CD105, but not CD14, CD20, CD34 or CD45, in accordance with ISCT criteria (blue, staining; red, isotype controls).

Conclusion

Despite *in vitro* immortalisation and long term culture, it has surprisingly been shown that the neural stem cell line CTX0E03 can be reprogrammed by exogenous transcription factors.

CTX-iPSCs are apparently indistinguishable from conventional iPSCs generated from low passage primary cells, as defined by cellular morphology, expression of cell surface, transcription factor and enzymatic markers, and pluripotency.

30 The c-myc-ER^{TAM} locus in CTX-iPSCs remains active in at least some lines.

Clinically-relevant cell types (e.g. MSCs, immune cells such as T cells, NK cells and dendritic cells) may be generated from CTX-iPSCs

35 Induction of cell cycling via the 4-OHT / c-myc-ERTAM system in CTX-iPSC-MSCs could permit their scalable production for allogeneic therapy.

The CTX-iPSCs therefore represent a very useful clinical resource. They may be differentiated along a desired lineage to generate a target population such as a tissue progenitor cell type or adult stem cell population, and then provision of 4-OHT to promote continuous growth and prevent cell cycle exit and associated further differentiation could allow the routine and scalable production of previously-unattainable clinically-relevant subpopulations without repeated cell isolation from primary material.

Cloning or purification steps can be used to generate pure populations of the desired therapeutic types from more- or less-heterogeneous differentiation cultures for large-scale production of off-the-shelf treatments for conditions for which CTX itself is unsuitable, obviating the drawbacks seen on the art with incomplete efficiency of differentiation protocols. This applies to both the cells themselves or exosomal fractions produced by different cell types with alternative repertoires of payload molecules to those produced by CTX cells themselves.

Furthermore, as these CTX-iPSC-derivative sublines are derived from a cell line which has already passed clinical phase safety trials (CTX), their entry into clinical trials for efficacy in new indications is likely to be accelerated.

Example 2: Characterisation of the reprogrammed CTX-iPSCs

Reprogramming-induced modulation of expression of significant genes is shown, confirming that the CTX cells have been properly reprogrammed.

The results are provided in Figure 6. Each panel is a "tSNE" plot of single cell transcriptome data created from CTX. The key in the top left indicates that the green "cloud" is CTX, CTX-iPSCs are blue and CTX-iPSCs that have been subjected to a cortical differentiation protocol and then their transcriptome has been analysed when they are closest as possible to CTX itself are in red. Each cloud consists of dots representing a single cell. Grey: no expression, orange: moderate expression; red: high expression. The plots show that the pluripotency genes inactive in CTX have been activated in the reprogrammed cells: POU5F1, NANOG, UTF1, TET1, DPP4, TDGF1, ZSCAN10 and GAL. Importantly, of these genes only POU5F1 was provided exogeneously during reprogramming. Conversely, several neural genes expressed by CTX are downregulated upon reprogramming to pluripotency (NOGGIN, ADAM12, OCIAD2, NTRK3, PAX6. Finally, GLI3 (and to a great extent PAX6) are upregulated upon cortical differentiation of the pluripotent cells.

Germ lineage Differentiation and Staining Thereof of Induced Pluripotent Stem Cells (Figure 7 and Figure 9)

Methods

1. CTX-iPSCs or STR0C05-iPSCs as appropriate were plated on human laminin-521-coated 8 well chamber slides. They were then treated with appropriate differentiation media (StemCell Technologies, cat. no. 05230) for 5-7 days as appropriate before fixation in 4% formaldehyde in phosphate buffered saline (PBS) and storage at 4°C until immunostaining.
2. Wells were immunostained as follows:
 1. Blocked with normal goat serum (NGS) by incubation in 10% NGS/PBS for 30 minutes at room temperature.
 2. Wells were incubated with primary antibodies: Mouse anti-x and rabbit anti-y diluted as appropriate (see table below) in 0.1% PBST (0.1% Triton-X-100/PBS) for 2-4 hours at room temperature or overnight at 4°C.
 3. Wells were washed 3 times with PBS for 10 minutes, or kept overnight at 4°C in PBS.
 4. Wells were incubated with secondary antibodies: Goat anti-mouse IgG-Alexafluor-488 (diluted 1:300) and / or Goat anti-rabbit IgG Alexafluor-568 (diluted 1:2000) in PBS for 2 hours at room temperature.
 5. Wells were washed 3 times with PBS at room temperature.
 6. Wells were stained with Hoechst 33342, diluted 1:10,000 in PBS, for 5 minutes.
 7. Wells were washed 3 times with PBS, for 5 minutes.
 8. The wells were removed from the slides, 2 drops of Vectashield was added and a glass cover slip placed on top, followed by examination by fluorescent microscopy.
3. The antibodies employed are shown in the table below.

Lineage	Marker	Co.	Cat No.	Species	Isotype	Clone	Dilution
Endoderm	SOX17	Abcam	ab84990	Mouse	IgG1	OT13B10	1:100
	FOXA2	Abcam	ab108422	Rabbit	IgG	EPR4466	1:500
Mesoderm	BRACHYURY	Insightbio	sc-374321-AF488	Mouse	IgG2b	A-4	1:150
	CXCR4	Abcam	ab124824	Rabbit	IgG	UMB2	1:500
Ectoderm	PAX6	Abcam	ab5790	Rabbit	IgG	Polyclonal	1:50
	NESTIN	Abcam	ab22035	Mouse	IgG1	10C2	1:100

Results:

- 25 Additional confirmation of the pluripotency of the CTX-iPSCs is provided by evidence of differentiation to endoderm, mesoderm and ectoderm, shown by coexpression of protein markers (mostly transcription factors) identifying the three primary germ layers. These data, in Figure 7, are a complement to the RT-qPCR data previously shown.

Example 3: Reprogramming of fetal striatal cells

Another conditionally immortalised adult stem cell type was successfully reprogrammed. This line is STR0C05, derived from fetal striatal cells.

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Methods - Reprogramming of STR0C05 cells to Pluripotency

1. An optimal range of transfection conditions specific for STR0C05 cells was identified, using the Neon electroporation instrument offered by Thermofisher.com. The frequency of live and green cells obtained was evaluated when a GFP expression plasmid was transfected into the cells using a range of different parameters such as voltage, pulse duration, etc., as suggested by the instrument manufacturer, to identify suitable transfection conditions for this cell line.
2. STR0C05 cells were then electroporated with the plasmids of the Epi5 reprogramming kit (Thermofisher cat. no. A15960; contains the reprogramming plasmids pCE-hOCT3/4, pCE-hSK, pCE-hUL and pCEmP53DD, expressing the transcription factors POU5F1, SOX2, KLF4, L-MYC, LIN28 and an dominant negative inhibitor of p53) using the conditions identified in (1), and plated onto human laminin-521. Wells were monitored daily with an Incucyte Zoom automated phase contrast microscope running inside the incubator.
3. After one week, the cells were either replated or remained in the same well, and medium was changed to mTeSR1 (StemCell Technologies cat. no. 85850).
4. Wells were monitored until pluripotent phenotypic colonies arose.
5. Once large enough, individual colonies were picked with a pipette tip into a well of a 24 well plate, also coated with hLn-521, and expanded until freezing or analysis.
6. As with previous work, alkaline phosphatase staining was performed with the Stemgent alkaline phosphatase staining kit (cat. no. 00-0055) and flow cytometry for pluripotent stem cell markers such as SSEA1 and SSEA4 was performed with the Becton Dickinson Stemflow antibody kit (cat. no. 560477) supplemented with FITC-conjugated mouse anti-human TRA-1-60 antibody (BD cat. no. 560380), both according to the manufacturer's instructions. Flow cytometry samples were analysed on a Miltenyi MACSQuant 10 flow cytometer.

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Results

The results are shown in Figure 8, wherein:

Panel A shows a colony of reprogrammed STR0C05 cells 24 days post-transfection with reprogramming factors;

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Panel B shows alkaline phosphatase (red)-stained STR0C05 cells at an early stage of reprogramming, showing some express the pluripotency marker alkaline phosphatase;

Panel C shows the established STR0C05-iPSC line;

Panel D shows that AP-positive colonies appear at different frequencies in wells subjected to different transfection conditions; well 1 with no colonies was transfected with a GFP non-reprogramming plasmid as a control and had no reprogrammed cells, wells 4 and 6 had few surviving cells;

5 Panel E shows that established STROC05-iPSC lines is alkaline phosphatase positive; and

Panel F shows that it is also positive for the pluripotency marker SSEA4 but negative for the early differentiation marker SSEA1.

10 The pluripotency of the STROC05-iPSCs is also confirmed, using the Germ lineage Differentiation method described in Example 2 above and with the results shown in Figure 9. Differentiation is demonstrated to endoderm, mesoderm and ectoderm, shown by coexpression of protein markers (mostly transcription factors) identifying the three primary germ layers, as figure 7 for CTX.

15 **Example 4: Adult stem cells derived from the reprogrammed iPSCs are multipotent**

The multipotency of adult stem cells derived from CTX-iPSCs was confirmed. Previously we have shown example flow cytometry profiles showing appropriate marker expression and the ability to adhere to plastic for candidate CTX-iPSC-MSCs (mesenchymal stem cells). This experiment
20 confirms the ability of the CTX-iPSC-MSCs to differentiate into several different cell types.

Methods - Differentiation of CTX-iPSC-MSCs to Confirm Multipotency

1. For assessment of fat and bone cell formation, CTX-iPSC-MSCs were plated in 6 well tissue
25 culture-treated plates and incubated for up to 28 days with commercially-available media promoting adipogenesis and osteogenesis (adipogenesis: StemCell Technologies cat. no. 05412, osteogenesis: StemCell Technologies cat. no. 05465 or R&D systems cat. nos. CCMN007 and CCM008), prior to fixation and staining. For assessment of cartilage formation, CTX-iPSC-MSCs were pelleted as clumps in the bottom of a 15ml tube and cultured with
30 chondrogenic medium (StemCell Technologies cat. no. 05455) followed by formaldehyde fixation, paraffin embedding and sectioning using standard methods.
2. Alcian Blue Staining (chondrogenesis): Sections on slides were hydrated to distilled water, treated with 3% acetic acid for 3 minutes and then stained with 1% Alcian blue in 3% acetic acid, pH 2.5, for 30 minutes. The slides were then washed in running water for 5 minutes, rinsed in
35 distilled water and counterstained for 5 minutes with 0.1% nuclear fast red in 5% aluminium sulphate solution prior to imaging.
3. Oil Red O Staining (adipogenesis): Cells in the 6 well plate were washed with PBS, fixed with 10% formaldehyde for 10 minutes at room temperature and washed twice with PBS. They were

stained for 15 minutes in 0.3% oil red O in 60% isopropanol/40% water and washed with double distilled water prior to imaging.

4. Alizarin Red S Staining (osteogenesis): Cells in the 6 well plate were washed with PBS, fixed with 10% formaldehyde for 10 minutes at room temperature and washed twice with PBS. They were stained for 15 minutes at room temperature with 2% alizarin red S solution, pH 4.2, washed with water and imaged.

Results

Figure 10 shows the capacity of the iPSC-derived MSCs to differentiate into cartilage (shown by alcian blue staining of sialoglycans), fat (shown by staining of intracellular lipid droplets with oil red O) and bone (shown by alizarin red staining of deposited calcium).

Flow cytometric profiles were then obtained for CTX-iPSC-MSCs cultured to high passage (20 passages) in the presence or absence of 4-OHT. The results are shown in Figure 11. The tested line is one for which the previously generated bisulphite data indicated had a demethylated C-MYC-ER^{TAM} promoter, in turn suggesting the promoter should still be active in these cells. Interestingly, this line appears to better maintain its marker profile when 4-OHT is inducing cell cycling: CD90 and CD105 expression are more uniform and higher, and the negative markers CD14, 20, 34 and 45 are more tightly "off". (This line always shows lower CD73 expression, possibly an antibody artefact.) In the second panel, the 4-OHT-treated cells appear to be more efficient at generating bone upon differentiation, suggesting that 4-OHT-mediated forcing of cell cycling ameliorates exit from the cycle and loss of potency.

Example 5: Further Characterisation of the reprogrammed CTX-iPSC-MSCs

CTX-iPSC-MSC lines were cultured in the absence or presence of 4-OHT. The results from experiments with two different CTX-iPSC-MSC cell cultures in Figures 12 and 13 show improved and more consistent growth long-term with 4-OHT/C-MYC-ERTAM present and active.

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This Example shows that conditionally-immortalised iPSC-ASCs may be propagated more reliably and for longer.

Example 6: Haematopoietic Lineages Derived from Conditionally-Immortalisable hiPSCs for Scalable Production of Allogeneic Immunotherapy

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Methods and Supporting Data

This Example shows that CTX-iPSCs are capable of generating mesodermal cells, HSCs and terminally-differentiated haematopoietic cells (for example, killer T cells). We have used a variety

of methods, including both commercially-available systems such as proprietary media and protocols published in the literature. In both cases, we have made our own modifications to the established art where necessary, since the established systems are typically designed with alternative donor cell types in mind, such as HSCs from bone marrow or cord blood, rather than hPSCs.

Mesoderm Figure 14 shows the first essential step in the creation of haematopoietic lineage cells from hPSCs *in vitro*, whereby commercially-available media supplemented with activin A, VEGF, SCF and BMP4 induce CTX-iPSC differentiation to mesoderm (Jung M *et al.* [2018] *Blood Advances* 2 3553).

Haematopoietic Stem Cells HSCs were generated from mesodermal cells derived from CTX-iPSCs (Figure 14B) as shown in Figure 15A. CTX-iPSC-mesodermal cells were cultured in the presence of FLT3, SCF, BMP-4, and interleukins 3 and 6 for 14 days. We observe up to approximately 60% of cells positive for CD34 at this point. A significant proportion of these cells (Figure 15B) were also positive for CD43. This is noteworthy as CD43⁺ HSCs appear to have wider potency than cells positive for CD34 alone, apparently also capable of generating cells of the erythroid (Kessel *et al.*, 2017, *Transfus Med Haemother* 44 143-50) and myeloid lineages as well as those of the lymphoid. Although the leucocyte marker CD45 is expressed at very low levels at this stage (Figure 15B), consistent with the cells' immaturity and hence low expression of mature markers, we have observed cells expressing the NK marker CD56 in cells from this differentiation stage, suggesting that CTX-HSCs also have the potential to produce natural killer cells.

Lymphocytes CTX-iPSC-HSCs have been differentiated towards a T-lymphocyte cell fate using both coculture methods (Montel-Hagen *et al.*, 2019 *Cell Stem Cell* 24 1-14), and an adaptation of a method whereby cord blood HSCs were cultured on a monolayer of bound VCAM and DLL4 proteins (Shukla *et al.*, 2017 *Nature Methods* 14 531-538) (Figure 16). In both cases, one of the related proteins DLL-1 or DLL-4 was provided to activate NOTCH signalling in the HSCs and induce differentiation towards a T-lymphocyte fate.

Figure 17A shows the method of generating progenitor T cells from CTX-HSCs by culturing them for a period of 14 days on a layer of bound chimeric proteins presenting VCAM and DLL4 to the HSCs. At the end of the 14 day period, a heterogeneous population of adherent and suspension cells was obtained. These cells could be distinguished by flow cytometry (Figure 17B), with the smaller, suspension cells ("Single Cells 2" population, Figure 17) comprising the pre-lymphocyte population. This cell population expressed CD3 (T-cell receptor associated protein), CD43 (leucocyte marker), CD5 (lymphocyte, predominantly early T-cell marker), CD7 (immature T cell

marker and NK cell marker) and CD25 (interleukin 2 receptor). However, consistent with the interpretation of their T-progenitor phenotype rather than their being mature T cells, in addition to their expression of CD5 and CD7, they did not express the T cell receptor itself or the associated molecules CD4 or CD8. This suggests the intriguing possibility of isolating early lymphoid or lymphocyte (e.g. pre-T and / or pre-B cells specifically), using the conditional immortalisation system to induce cycling to expanding the cell population whilst maintaining its progenitor cell phenotype.

Culturing the progenitor T lymphocyte cells on bound Fc-DLL4 and Fc-VCAM proteins for longer periods (Figure 18A, 25 days, as opposed to 14 days) resulted in the cell population becoming somewhat more homogenous (Figure 18B) and their achieving a more mature phenotype (Figure 18C-E). These cells were more uniform (e.g. over 60% of them expressed the leucocyte marker CD43) and consistent with the interpretation that they represent a more mature lymphocyte population, also expressed CD8 in addition to CD3, but lost their expression of CD5 and CD7. This protocol thus generated a population of lymphocytes most similar to cytotoxic T cells such as form the basis of CAR-T antitumour therapeutics at present.

Alternative methods of differentiation have also been used to create more mature lymphocyte populations from conditionally-immortalised hiPSC-derived HSCs. Coculturing the CTX-HSCs with murine MS5 stromal cells engineered to express the human NOTCH ligand DLL1 (Figure 19) or on a monolayer of MS5-DLL1 cells induced strong growth in the small non adherent cell population (Figure 20B). This population matured during the course of differentiation, losing its CD34 expression (Figure 20C), but CD8 expression was not observed, although the early markers CD5 and CD7 expression levels fell (Figure 20D-F). Thus, T cell progenitors produced by this method probably represent an earlier stage of T cell development than that produced by the bound proteins approach described above (Figure 21).

Figure 22 indicates increased expression of CD56 in the HSCs of the invention, and so highlights the potential of the HSCs of the invention to produce non-antigen specific lymphocytes such as NK cells.

This ability to “fine tune” the stage a differentiation pathway reaches prior to the scalable expansion of the population using conditional immortalisation represents a very powerful system providing an exquisite level of control over the cells one might provide to a patient.

Conclusion

The CTX-iPSC-HSCs and their differentiated derivatives potentially represent a very useful clinical resource. They may be expanded to generate large cell banks using the conditional immortalisation system for cryopreservation, or further differentiated, perhaps with genetic

modification, to generate target populations of pure, GMP-standard cells for therapy. This could allow the routine and scalable production of previously-unattainable clinically-relevant subpopulations without the need for identification of immunocompatible donors for each patient, and cell isolation of primary material from them. Furthermore, as these CTX-iPSC-HSCs and their derivative subtypes are derived from a cell line which has already passed clinical phase safety trials (CTX), their entry into clinical trials for efficacy in new indications is likely to be accelerated.

Example 7: Hematopoietic differentiation of CTX-iPSCs produces HSCs, lymphoid progenitors and effectors

A number of additional experiments confirmed the ability of the CTX-iPSC-HSCs to differentiate into a variety of cells of the haematopoietic lineage. The results of these additional experiments are provided in Figure 25.

Briefly, these experiments demonstrate the following:

- Confirmation of CTX-iPSC differentiation to CD34+ cells (hemoendothelial progenitors and stem cells).
- Confirmation that some of the CD34+ cells are also positive for CD49F and CD90, and negative for markers CD38 and CD45RA. Taken together, these results strongly suggest that the created cells are true, long term repopulating haematopoietic stem cells (LT-HSCs) which are the cells capable of reconstituting the entire immune system and the progenitors of all effector cell types of this lineage. This is a significant result.
- Production of lymphoid progenitors (LPs) by further differentiation of the CTX-iPSC-derived CD34+ cells (likely the CD34+CD49F+CD45RA-CD90+CD38- LT-HSCs above).
- Production of CD3+CD8+TCR+ cytotoxic T-cells from the above CTX-LPs.
- Further evidence of production of natural killer cells from the CTX-LPs.

More specifically, Figure 25 shows that hematopoietic differentiation of CTX-iPSCs produces HSCs, lymphoid progenitors and effectors.

Panel A shows that embryoid bodies were formed from CTX-iPSCs by plating a single cell suspension in non-adherent microwell plates.

In panel B the EBs were cultured in the presence of mesoderm-promoting medium (to day 3), and then in haematopoietic specification medium (to day 10) to (panel C) generate CD34⁺ cells, approximately 5% of which were CD34⁺CD49f⁺CD90⁺CD38⁺CD45RA⁻ LT-HSCs. Panel D depicts that CD34⁺ cells derived in this way were isolated with anti-CD34 magnetic beads and then differentiated for a further 14 days to generate (E) CD7⁺ lymphoid progenitors, which retained some reduced multipotency and could in turn be differentiated for 14 or 21 days respectively to produce Natural Killer or CD4-CD8⁺TCR $\alpha\beta$ cytotoxic T-cells.

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