



(51) International Patent Classification:

C12N 5/0775 (2010.01) A61K 35/28 (2015.01)

(21) International Application Number:

PCT/SG2019/050454

(22) International Filing Date:

10 September 2019 (10.09.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

10201808423W 26 September 2018 (26.09.2018) SG

(71) Applicant: NATIONAL UNIVERSITY OF SINGAPORE [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: WANG, Shu; c/o National University of Singapore, Faculty of Science/Department of Biological Sciences, 21 Lower Kent Ridge Road, Singapore 119077 (SG). ZHA, Shijun; c/o National University of Singapore, Faculty of Science/Department of Biological Sciences, 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(74) Agent: YUSARN AUDREY; 24 Raffles Place #27-01 Clifford Centre, Singapore 048621 (SG).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,

UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

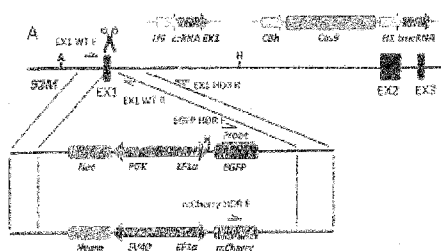
- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ENGINEERED HUMAN MESENCHYMAL STROMAL CELLS WITH LOW IMMUNOGENICITY, METHODS AND KITS OF GENERATING THE SAME

Figure 1



(57) Abstract: Methods and kits for generating human mesenchymal stromal cells with reduced immunogenicity comprising one or more plasmid capable of mutating both alleles of a beta-2-microglobulin gene in a human pluripotent stem cell that is differentiated into a genetically modified human mesenchymal stromal cell expressing no beta-2-microglobulin and expressing reduced or no HLA class I molecules on the cell surface.



Engineered human mesenchymal stromal cells with low immunogenicity, methods and kits of generating the same.

Cross reference to related applications

[0001] This application claims the priority to Singapore application No. 10201808423W, filed 26 September 2018, the contents of which are incorporated herein by reference.

Field

[0002] The present invention relates to methods and kits for generating human mesenchymal stromal cells, preferably mesenchymal stromal cells with low immunogenicity and genetically modified human mesenchymal stromal cells.

Background

[0003] Mesenchymal stem cells have great potential for use in regenerative medicine due to their plasticity, immunomodulatory and anti-inflammatory properties. It has many advantages for clinical application which includes high plasticity, ability to mediate inflammation and promotes cell growth, cell differentiation and tissue repair by immunomodulation and immunosuppression.

[0004] Naturally occurring Human mesenchymal stem cells (MSC) are a rare subset of non-hematopoietic stem cells localized around the vasculature and trabeculae in the bone marrow (BM), representing 0.01-0.001 % of total BM cells. They are rare and difficult to obtain. In recent years techniques have been developed to generate mesenchymal stroma cells (MSCs) from Human iPS cells that display stem cell properties and immune regulatory functions. These iPS cell-derived MSCs are equivalently effective as primary adult mesenchymal stromal cells in treating numerous diseases from tissue damage to immune disorders.

[0005] Although MSCs are reported to be immune privileged and suitable for allogenic usage without provoking harmful immunity reactions, self-immunity is still a concern to evoke host rejection, such as humoral and cellular immune responses *in vivo*. Generally, both naturally occurring MSC and iPS-derived MSCs have the risk of inducing rejection from hosts by antibodies against the grafts or cellular immune memory against infused cells. Most cell types express HLA-I genes (HLA-A, HLA-B, and HLA-C) and these function to present "non-self" antigen-processed peptides to cytolytic CD8+ T cells to mediate immune rejection.

Summary

[0006] An object of the invention is to ameliorate some of the above mentioned difficulties preferably by using a reliable, unlimited and standardizable starting cell source of human pluripotent stem cells such as induced pluripotent stem cells (iPSCs) to generate mesenchymal stromal cells with low immunogenicity, derived from B2M knockout human pluripotent stem cells.

[0007] Accordingly, a first aspect of the invention includes a method of generating human mesenchymal stromal cells with reduced immunogenicity, the method comprising:

(a) mutating a human pluripotent stem cell at both alleles of a beta-2-microglobulin gene;

(b) differentiating the mutated human pluripotent stem cell into a derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene;

wherein the derived human mesenchymal stromal cell expresses no beta-2-microglobulin and express reduced or no HLA class I molecules on the cell surface.

[0008] Another aspect of the invention includes genetically modified human mesenchymal stromal cells comprising cells expressing no beta-2-microglobulin and expressing reduced or no HLA class I molecules on the cell surface.

[0009] Another aspect of the invention includes a kit for generating human mesenchymal stromal cells with reduced immunogenicity, the kit comprising:

(a) one or more plasmid capable of mutating both alleles of a beta-2-microglobulin gene in a human pluripotent stem cell;

(b) a mesenchymal stromal cell medium for differentiating human pluripotent stem cells into human mesenchymal stromal cells.

[0010] Other aspects and features of the present invention will become apparent to those of ordinary skill in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying figures.

Brief Description of the Drawings

[0011] In the figures, which illustrate, by way of example only, embodiments of the present invention,

[0012] Figure 1: Generation of B2M knockout hP-iPS cells with CRISPR/Cas9 and double colour selection. **(A)** Schematic of a CRISPR/Cas9 system targeting B2M exon 1 (EX1) with two selection donor templates for homologous recombination. The system was used for genetic modification in hP-iPS cells by electroporation. Arrows showed the binding sites of PCR primers for genotyping. The restriction enzyme site, HindIII, was indicated as H. **(B)** Representative images of B2M biallelic knockout hP-iPS single cell clone. The scale bar was 200 μ m and the exposure time was one second for fluorescence. **(C)** PCR analysis of wild type (WT) and homology-direct recombination (HDR) alleles of B2M gene from B2M monoallelic and biallelic knockout hP-iPS single cell clones. WT hP-iPS cells were included as a control. **(D)** Western blot analysis of B2M expression in B2M monoallelic and biallelic knockout hP-iPS single cell clones. Sample cells were treated by IFN- γ for 48 hours before analysis. WT clone was used as a control and β -actin was detected as housekeeping expression. **(E)** Representative flow cytometry diagrams of surface B2M and HLA-A,B,C expression on WT, B2M monoallelic and biallelic knockout hP-iPS single cell clones.

[0013] Figure 2: Analysis of the 18 mono-allelic clones selected **(A)** Western blot analysis of EGFP expression in B2M monoallelic knockout hP-iPS single cell clones. **(B)** Western blot analysis of B2M expression in B2M monoallelic knockout hP-iPS single cell clones. **(C)** sequencing analysis of the target region.

[0014] Figure 3: **(A)** Southern blot with EGFP probe with gDNA cut by HindIII. **(B)** RT-PCR analysis of pluripotent marker Oct4, Sox2 and Nanog expression in WT, B2M monoallelic and biallelic knockout hP-iPS single cell clones. The expression of β -actin was included as a housekeeping control. **(C)** Representative images of embryoid bodies (EBs) derived from WT, B2M monoallelic and biallelic knockout hP-iPS single cell clones. The scale bar was 200 μ m and the exposure time was one second for fluorescence. **(D)** RT-PCR analysis of markers' expression of three germ layers in WT, B2M monoallelic and biallelic knockout hP-iPS cells derived EBs. Pax6 was for ectoderm; MHC- α was for mesoderm and AFP was for endoderm. The expression of β -actin was included as a housekeeping control.

[0015] Figure 4: Generation of B2M knockout hP-iPS cells with CRISPR/Cas9 and puromycin selection. **(A)** Schematic of a CRISPR/Cas9 system targeting B2M exon 1 (EX1). The system was used for genetic modification in hP-iPS cells by electroporation. Arrows showed the binding sites of PCR primers for genotyping. **(B)** Representative flow cytometry diagrams of surface B2M expression on, B2M knockout (B2MKO) hP-iPS single cell clones.

[0016] Figure 5: One step generation of B2M knockout hP-iPS cells by CRISPR/Cas9 technology. **(A)** Representative flow cytometry diagrams of surface B2M and HLA-A,B,C expression on WT, B2M knockout (B2MKO) #3 and #8 hP-iPS single cell clones. **(B)** Sanger Sequencing analysis of B2M exon1 in B2MKO #3 and #8 hP-iPS single cell clones. CRISPR targeting sequences were shown in orange and target site in B2M gene was shown in green. The insertion was marked by red while deletion was marked by "-". The genotypes of B2MKO #3 and #8 hP-iPS single cell clones were summarized. **(C)** karyotyping of chromosomes in the B2M knockout clone #8.

[0017] Figure 6: Pluripotency of B2M knockout hP-iPS cells. **(A)** RT-PCR analysis of pluripotent marker Oct4, Sox2 and Nanog expression in WT, B2M monoallelic and biallelic knockout hP-iPS single cell clones. The expression of β -actin was included as a housekeeping control. **(B)** Representative images of embryoid bodies (EBs) derived from WT, B2M monoallelic and biallelic knockout hP-iPS single cell clones. The scale bar was 200 μ m and the exposure time was one second for fluorescence. **(C)** RT-PCR analysis of markers' expression of three germ layers in WT, B2M monoallelic and biallelic knockout hP-iPS cells derived EBs. Pax6 was for ectoderm; MHC- α was for mesoderm and AFP was for endoderm. The expression of β -actin was included as a housekeeping control.

[0018] Figure 7: Generation of mesenchymal stromal cells from B2M knockout hP-iPS cells. **(A)** Representative images of WT and B2MKO hP-iPSC-derived mesenchymal stromal cells (iMSCs). The scale bar was 200 μ m. **(B)** Representative flow cytometry diagrams of surface B2M and HLA-A,B,C expression on WT and B2MKO iMSCs. **(C)** Phenotyping of WT and B2MKO iMSCs by surface markers. The expression of MSC negative markers (CD14, CD24, CD34, CD45 and HLA-DR) and MSC positive markers (CD29, CD44, CD73, CD90, CD105 and CD166) was shown as representative flow cytometry diagrams.

[0019] Figure 8: Multipotency of B2M knockout hP-iPSC-derived mesenchymal stromal cells. Expression of specific markers was detected by RT-PCR for both WT and B2MKO iMSCs and differentiated cells. **(A)** Lipoprotein lipase (LPL) was for adipogenesis; **(C)** Bone relevant alkaline phosphatase (ALP) was for osteogenesis and **(E)** collagen type II alpha 1 (COL2a) was for chondrogenesis. **(B)** Adipogenesis, **(D)** Osteogenesis and **(F)** Chondrogenesis of B2MKO iMSCs. Lipid content was stained in red by Oil Red O for adipocytes; Calcific deposition was stained in red by Alizarin Red S for osteocytes and acidic polysaccharides were stained in blue by Alcian blue 8GX for chondrocytes. The scale bar for all the images was 80 μ m.

[0020] Figure 9: Hypoimmunogenicity of B2M knockout hP-iPSC-derived mesenchymal stromal cells. **(A, B)** Immunogenicity of both WT and B2MKO iMSCs when they were challenged by iMSC-primed peripheral blood mononuclear cells (PBMCs). Phenotyping of iMSC-primed PBMCs were shown as flow cytometry diagrams for CD3, CD56 and CD8 expression. The immunogenicity of WT and B2MKO iMSCs was examined with DELFIA EuTDA cytotoxicity assays (2 hours Eu-ligand release) as target cells challenged by iMSC-primed PBMCs. **(C)** The susceptibility of WT and B2MKO iMSC to NK lysis. Primary NK cells were used as effectors to target WT and B2MKO iMSCs in DELFIA EuTDA cytotoxicity assays (2 hours Eu-ligand release). For cell cytotoxicity assays, three independent assays from three individual donors were performed. Shown is percentage lysis of target cells at varying E:T ratios in one representative experiment (Mean $\pm$ SD of triplicate samples). ***: $p < 0.001$.

[0021] Figure 10: Advanced immunosuppressive property of B2MKO iMSCs compared to WT and PBMC's. **(B)** OKT-3-induced hPBMC proliferation in the presence of MSCs hPBMC proliferation was evaluated on day 3 and is expressed as the percentage of CFSE/Far Red stained cells. Data are expressed as the percentage of hPBMC proliferation in the absence of MSCs and represent the mean $\pm$ SD of three separate experiments. * $P < 0.05$, ** $P < 0.01$.

Detailed Description

[0022] Described is a method to generate human leukocyte antigen (HLA) class I negative mesenchymal stromal cells (MSCs). Human PBMC-derived IPS cells (hP-iPSCs) were first generated and genetically modified by knocking out the B2M gene. The selected clones were then differentiated into B2M knockout iPS-derived MSCs (iMSCs) that express relevant MSC markers without HLA class I expression and display multipotency to differentiate into osteoblasts, chondrocytes, and adipocytes. Importantly, iMSCs display lower immunogenicity to allogenic immune cells as compared to wild type iMSCs. Hence, the B2M knockout hP-iPSCs as the "off-the-shelf" cell resources to generate MSCs with low immunogenicity. The iMSCs generated have a great potential in regenerative medicine. However, hiPSCs are notoriously difficult to transfect, and optimized experimental design considerations are often necessary.

[0023] The technology provides a novel type of mesenchymal stromal cells with low or reduced immunogenicity, which are derived from B2M knockout human PBMC-derived induced pluripotent stem cells (hP-iPSCs). The B2M knockout iMSCs are HLA class I

negative and display low immunogenicity, thus reducing the above risk of allogenic rejection and providing prolonged survival and therapeutic function after allogenic transplantation. In this case, the B2M knockout iMSCs have the potential to be the universal therapeutic cell resources for MSC-based cellular therapy due to its reduced immunogenicity.

[0024] Accordingly, a first aspect of the invention includes a method of generating human mesenchymal stromal cells with reduced immunogenicity, the method comprising:

(a) mutating a human pluripotent stem cell at both alleles of a beta-2-microglobulin gene;

(b) differentiating the mutated human pluripotent stem cell into a derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene;

wherein the derived human mesenchymal stromal cell expresses no beta-2-microglobulin and express reduced or no HLA class I molecules on the cell surface.

[0025] As used herein the term 'reduced immunogenicity' refers to cells with a lower or reduced measurable reaction to primed T cells expressing CD8+ compared to the reaction of natural or wild type (WT) MSCs that have not been genetically modified. In various embodiments the reduced reaction is measured as a percentage of cell lysis whereby genetically modified MSC cells that do not express beta-2-microglobulin (B2M) have a lower percentage of cell lysis than natural or wild type (WT) MSCs that have not been genetically modified when the cells are challenged with CD8+ T cells. In various embodiments the reduced reaction is measured as relative percentage of proliferation whereby genetically modified MSC cells that do not express beta-2-microglobulin (B2M) have a lower percentage of proliferation than natural or wild type (WT) MSCs that have not been genetically modified when the cells are challenged with CD8+ T cells. In various embodiments the reduced reaction is measured as cytokine secretions such as interleukins (IL), tissue necrotic factors (TNF) interferons (IFN) among other known cytokines in the genetically modified MSC cells that do not express beta-2-microglobulin (B2M) are compared to the same cytokine secretions in natural or wild type (WT) MSCs.

[0026] As used herein, the term "mutating" or "mutated" or "mutation" refers to any change in the genome of a cell. In the context of the method, mutations may include, but are not limited to, insertion, or deletion or substitution. Mutations may result in a loss or removal of the function of a beta-2-microglobulin (B2M) gene. In various embodiments the B2M gene comprises the nucleic acid sequence set out in SEQ ID NO. 1. In various embodiments the mutation in the B2M gene is present in exon 1. In various embodiments the target sequence

in exon 1 of the B2M gene comprises nucleobases 12 to 57 of SEQ ID NO. 1. In various embodiments the target sequence comprises nucleic acid sequence as set out in SEQ ID NO. 2 (TGACAGCATTCGGGCCGAGATGTCTCGCTCCGTGGCCTTAGCTGTG). In various embodiments the target sequence comprises nucleic acid sequence as set out in SEQ ID NO. 3. In various embodiments the target sequence comprises nucleic acid sequence as set out in SEQ ID NO. 4. A mutation anywhere in the B2M gene is likely to stop expression and reduce immunogenicity, however, advantageously, a mutation in exon 1 results in a high number of clones with no expression of B2M. As used herein the term "expresses no beta-2-microglobulin" refers to a knock-out or refers to the elimination of the beta-2-microglobulin (B2M) gene or the expression of a B2M gene. For example, a B2M gene can be knocked out by either a deletion or an addition of a nucleotide sequence that leads to a disruption of the reading frame. As another example, a gene may be knocked out by replacing or substituting a part of the gene with an irrelevant sequence. The term "express reduced or no HLA class I molecules" as used herein refers to a knockdown or reduction in the expression of a gene or its gene product(s). The beta-2 microglobulin (B2M) genes that encodes the common subunit required for surface expression of all HLA class I heterodimers (HLA-A, B, C, E, F and G). As a result of a B2M gene knockout, the protein activity or function may be attenuated or the protein levels of HLA class I complexes may be reduced or eliminated.

[0027] As used herein, the term "insertion" refers to an addition of one or more nucleotides in a DNA sequence. Insertions can range from small insertions of a one nucleotide to insertions of large segments such as a cDNA or a gene. In various embodiments the insertion includes a EGFP gene. The term "deletion" refers to a loss or removal of one or more nucleotides in a DNA sequence or a loss or removal of the function of a gene. In some cases, a deletion can include, for example, a loss of a few nucleotides, an exon, an intron, a gene segment, or the entire sequence of a gene. In some cases, deletion of a gene refers to the elimination or reduction of the function or expression of a gene or its gene product. This can result from not only a deletion of sequences within or near the gene, but also other events (e.g., insertion, nonsense mutation) that disrupt the expression of the gene. In various embodiments a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1. In various other embodiments a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second

allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1.

[0028] In various embodiments the human pluripotent stem cells are human embryonic stem cells (hESC). hESCs are a good option in terms of safety, but the derivation of hESCs is always ethically controversial and the applications of hESC derivatives are limited.

[0029] In various embodiments the human pluripotent stem cells are induced pluripotent stem cells (iPSC). Methods of generating iPSC from various sources are known. Currently, most iPSCs are generated by reprogramming adult somatic cells. The choice of starting somatic cells will affect not only the efficiency and kinetic of reprogramming, but also the practicality of generating GMP-grade iPSCs. Although fibroblasts are the most commonly used somatic cells, they are not very GMP-compliant. Skin sample collection through punch biopsy is invasive and growing fibroblasts from skin biopsy sample is time-consuming (up to 3 weeks). The derivation of fibroblasts under GMP itself is already a daunting task.

[0030] In various embodiments the human pluripotent stem cells are induced from peripheral blood cells (PBC (iPSC)). Using iPSC has the advantage that they can be generated from a reliable, unlimited and standardizable starting cell source such as peripheral blood mononuclear cells (PBMC) isolated from donors or patients allowing iPSCs derivatives to be used in both autologous and allogeneic applications and it further doesn't have any of the ethical concerns of using hESC. Using PBCs to generate iPSCs (hP-iPS) is a practical option since peripheral blood collection is convenient and isolation of mononuclear cells from peripheral blood sample only takes 15 minutes. The easiness of implementing GMP in sample collection, transportation and processing renders PBCs an attractive starting material for iPSC derivation. Advantageously, human mesenchymal stromal cells can be directly induced from B2M knockout hP-iPS cells in large scale showing negative for B2M and HLA class I expression. These B2M knockout hP-iPS cells could be a permanent cell source for generating functional iPS-derived MSCs with reduced or low immunogenicity.

[0031] In various embodiments the methods and compositions described herein can further comprise introducing one or more of each of Oct-4, Sox2, Nanog, c-MYC and Klf4 or other reprogramming compositions known in the art for reprogramming. As noted above, the exact method used for reprogramming is not necessarily critical to the methods and compositions described herein. However, where cells differentiated from the reprogrammed cells are to be used in, e.g., human therapy, in one aspect the reprogramming is not effected

by a method that alters the genome. Thus, in such embodiments, reprogramming can be achieved, e.g., without the use of viral or plasmid vectors.

[0032] In various embodiments the method further comprises determining that the mutation is present in both alleles of the B2M gene. In various embodiments determining that the mutation is present in both alleles comprises sequencing both alleles of the B2M gene to detect any insertions or deletions in the B2M gene. In various embodiments the sequencing comprises using primers of SEQ ID NOS. 5 and 6. In various embodiments the sequencing comprises using primers of SEQ ID NOS. 7 and 8. In various embodiments the sequencing comprises using primers selected from the group of SEQ ID NOS. 5-14. In various embodiments the sequencing comprises using any one of the primers selected from the group of SEQ ID NOS. 5-14. In various embodiments determining that the mutation is present in both alleles of the B2M gene comprises determining whether the mutation is present in exon 1 of the B2M gene. In various embodiments determining that the mutation is present in both alleles comprises determining whether a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and determining whether a second allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1. In various other embodiments determining that the mutation is present in both alleles comprises determining whether a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and determining whether a second allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1. In various embodiments determining that the mutation is present in both alleles comprises detection methods such as southern blotting. In various embodiments determining that the mutation is present in both alleles comprises detection methods such as cell sorting for the presence or absence of HLA class I molecules wherein the absence of expression of HLA class I molecules on a mutated cell in comparison to a wild type cell indicates that the mutation is present in both alleles. In various embodiments determining that the mutation is present in both alleles comprises detection methods such as cell sorting for the presence or absence of any one of B2M, HLA-A, HLA-B or HLA-C or a combination thereof wherein the absence of expression of any one of B2M, HLA-A, HLA-B or HLA-C or a combination thereof on a mutated cell in comparison to a wild type cell indicates that the mutation is present in both alleles.

[0033] In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene express at least one of CD29, CD44,

CD73, CD90, CD105, CD166, or a combination thereof and comprise low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45, B2M, HLA-A, HLA-B or HLA-C or a combination thereof. In various embodiments the derived human mesenchymal stromal cell are stained with antibodies of at least one of CD29, CD44, CD73, CD90, CD105, CD166, CD14, CD34, HLA-DR, CD25, CD45 B2M, HLA-A, HLA-B or HLA-C or a combination thereof and sorted in a flow cytometer to determine which cells are expressing at least one of CD29, CD44, CD73, CD90, CD105, CD166, CD14, CD34, HLA-DR, CD25, CD45 B2M, HLA-A, HLA-B or HLA-C or a combination thereof. In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene express any 2, any 3, any 4, any 5, or all of CD29, CD44, CD73, CD90, CD105, CD166. In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene comprise low or no expression of any 2, any 3, any 4, any 5, any 6, any 7, any 8, or all of CD14, CD34, HLA-DR, CD25, CD45, B2M, HLA-A, HLA-B and HLA-C.

[0034] In various embodiments the alleles are mutated with one or more plasmid comprising a guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and an endonuclease.

[0035] In various embodiments the endonuclease may comprise CRISPR-associated endonucleases, such as Cas9, Cpf1 and the like, to permanently edit within or near the genomic locus of the B2M gene or other DNA sequences that encode regulatory elements of the B2M gene. In this way, examples set forth in the present disclosure can help to reduce or eliminate the expression of the B2M gene. In various embodiments, the endonuclease is guided by a guide nucleic acid sequence. In various embodiments, the Cas9 endonuclease or Cpf 1 endonuclease is selected from *Streptococcus pyogenes* Cas9, *S. aureus* Cas9, *Neisseria meningitidis* Cas9, *S. thermophilus* CRISPR1 Cas9, *S. thermophilus* CRISPR 3 Cas9, *Treponema denticola* Cas9, *Lachnospiraceae bacterium* ND2006 Cpf1 and Acidaminococcus sp. BV3L6 Cpf1 or any others known in the art.

[0036] In various embodiments, the guide nucleic acid sequence is a single-guide RNA (sgRNA) that contains a targeting sequence (crRNA sequence) and a RNA-guided nuclease-recruiting sequence (tracrRNA)). In various embodiments the plasmid comprises a px260 (Addgene, Cambridge, MA, USA) containing a CRISPR/cas9 system targeted to a portion of the beta-2-microglobulin gene comprising SEQ ID NO. 3. In various embodiments, the guide nucleic acid sequence comprises a crRNA-tracrRNA-Cas9 complex that can guide the complex to the target nucleic acid to which the crRNA can hybridize. Hybridization of the

crRNA to the target nucleic acid can activate Cas9 for targeted nucleic acid cleavage. The target nucleic acid in this CRISPR system is referred to as a protospacer adjacent motif (PAM). In nature, the PAM is essential to facilitate binding of a site-directed polypeptide (e.g., Cas9) to the target nucleic acid. In various embodiments the plasmid comprises a px459 (Addgene, Cambridge, MA, USA) containing a CRISPR/cas9 system targeted to a portion of the beta-2-microglobulin gene comprising SEQ ID NO. 4.

[0037] In various other embodiments the guide nucleic acid sequence is a single RNA-guided endonuclease that, in contrast to the above described system, lacks tracrRNA. In fact, CpfI-associated CRISPR arrays can be processed into mature crRNAs without the requirement of an additional trans-activating tracrRNA. This Type of CRISPR array can be processed into short mature crRNAs of 42-44 nucleotides in length, with each mature crRNA beginning with 19 nucleotides of direct repeat followed by 23-25 nucleotides of spacer sequence.

[0038] In various embodiments methods of mutating genes described herein include methods of using site-directed nucleases to cut deoxyribonucleic acid (DNA) at precise target locations in the B2M gene, thereby creating single-strand or double-strand DNA breaks at particular locations within the gene. Such breaks can be and are regularly repaired by natural, endogenous cellular processes, such as homology-directed repair (HDR) and non-homologous end joining (NHEJ). These two main DNA repair processes consist of a family of alternative pathways. NHEJ directly joins the DNA ends resulting from a double-strand break, sometimes with the loss or addition of nucleotide sequence, which may disrupt or enhance gene expression. HDR utilizes a homologous sequence, or donor sequence, as a template for inserting a defined DNA sequence at the break point. The homologous sequence can be in the endogenous genome, such as a sister chromatid. Alternatively, the donor can be an exogenous nucleic acid, such as a plasmid, a single-strand oligonucleotide, a double-stranded oligonucleotide, a duplex oligonucleotide or a virus, that has regions of high homology with the nuclease-cleaved locus, but which can also contain additional sequence or sequence changes including deletions that can be incorporated into the cleaved target locus. A third repair mechanism can be micro-homology-mediated end joining (MMEJ), also referred to as "Alternative NHEJ," in which the genetic outcome is similar to NHEJ in that small deletions and insertions can occur at the cleavage site. MMEJ can make use of homologous sequences of a few base pairs flanking the DNA break site to drive a more favoured DNA end joining repair outcome.

[0039] In various embodiments, nickase variants of RNA-guided endonucleases, for example Cas9, can be used to increase the specificity of CRISPR-mediated genome editing. Wild type Cas9 is typically guided by a single guide RNA designed to hybridize with a specified -20 nucleotide sequence in the target sequence (such as an endogenous genomic locus). However, several mismatches can be tolerated between the guide RNA and the target locus, effectively reducing the length of required homology in the target site to, for example, as little as 13 nt of homology (about 65%), and thereby resulting in elevated potential for binding and double-strand nucleic acid cleavage by a CRISPR/Cas9 complex elsewhere in the target genome - also known as off-target cleavage. Because nickase variants of Cas9 each only cut one strand, in order to create a double-strand break it is necessary for a pair of nickases to bind in close proximity and on opposite strands of the target nucleic acid, thereby creating a pair of nicks, which is the equivalent of a double-strand break. This requires that two separate guide RNAs - one for each nickase - must bind in close proximity and on opposite strands of the target nucleic acid. This requirement essentially doubles the minimum length of homology needed for the double-strand break to occur, thereby reducing the likelihood that a double-strand cleavage event will occur elsewhere in the genome, where the two guide RNA sites - if they exist - are unlikely to be sufficiently close to each other to enable the double-strand break to form. As known in the art, nickases can also be used to promote HDR versus NHEJ. HDR can be used to introduce selected changes into target sites in the genome through the use of specific donor sequences that effectively mediate the desired changes.

[0040] In various embodiments any of these genome editing mechanisms can be used to create desired mutations in both B2M alleles. A step in the mutating process can be to create one or two DNA breaks, the latter as double-strand breaks or as two single-stranded breaks, in the target locus as near the site of intended mutation.

[0041] A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) genomic locus can be found in the genomes of many prokaryotes (e.g., bacteria and archaea). In prokaryotes, the CRISPR locus encodes products that function as a type of immune system to help defend the prokaryotes against foreign invaders, such as virus and phage. There are three stages of CRISPR locus function: integration of new sequences into the CRISPR locus, expression of CRISPR RNA (crRNA), and silencing of foreign invader nucleic acid. Five types of CRISPR systems (e.g., Type I, Type II, Type III, Type U, and Type V) have been identified.

[0042] A CRISPR locus includes a number of short repeating sequences referred to as "repeats." When expressed, the repeats can form secondary structures (e.g., hairpins) and/or comprise unstructured single-stranded sequences. The repeats usually occur in clusters and frequently diverge between species. The repeats are regularly interspaced with unique intervening sequences referred to as "spacers," resulting in a repeat-spacer-repeat locus architecture. The spacers are identical to or have high homology with the target sequences. A spacer-repeat unit encodes a crisprRNA (crRNA), which is processed into a mature form of the spacer-repeat unit. A crRNA comprises a "seed" or spacer sequence that is involved in targeting a target nucleic acid. A spacer sequence is located at the 5' or 3' end of the crRNA.

[0043] A CRISPR locus also comprises polynucleotide sequences encoding CRISPR Associated (Cas) genes. Cas genes encode endonucleases involved in the biogenesis and the interference stages of crRNA function in prokaryotes. Some Cas genes comprise homologous secondary and/or tertiary structures.

[0044] In various embodiments the endonuclease comprises a Clustered Regulatory Interspaced Short Palindromic Repeat (CRISPR) associated nucleic sequence Cas9.

[0045] In various embodiments the guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene comprises a sequence selected from SEQ ID NO. 3 or SEQ ID NO. 4. In various embodiments the portion of the beta-2-microglobulin gene comprises exon 1 of the B2M gene.

[0046] In various embodiments the human pluripotent stem cell is mutated by: (a) Transfecting the human pluripotent stem cell with a first plasmid to mutate a first allele of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and at least one selection marker; (b) selecting the cell with a mutation in the first allele of a beta-2-microglobulin gene via the at least one selection marker; (c) transfecting the cell with the mutation in the first allele of a beta-2-microglobulin gene with a second plasmid to mutate a second allele of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and at least one further selection marker; and (d) selecting the cell with the mutation in the first allele of a beta-2-microglobulin gene and a mutation in the second allele of a beta-2-microglobulin gene via the at least one further selection marker.

[0047] In various embodiments any selection marker known in the art to differentiate between a cell that has been mutated and a cell that has not been mutated by selection or screening. In various embodiments the selection marker comprises a nucleic acid sequence expressing antibiotic resistance whereby mutated cells are selected by being the only cells that survive when the antibiotic is applied. In various embodiments the selection marker comprises a nucleic acid sequence expressing neomycin resistance whereby mutated cells are selected by being the only cells that survive when neomycin is applied. In various embodiments the selection marker is a nucleic acid sequence expressing a green fluorescence protein (GFP) whereby mutated cells are selected via fluorescence activated cell sorting. In various embodiments the selection marker comprises both a nucleic acid sequence expressing neomycin resistance gene and a nucleic acid sequence expressing a green fluorescence protein (GFP) whereby mutated cells are first selected by being the only cells that survive when neomycin is applied and thereafter via fluorescence activated cell sorting. In various embodiments the selection marker comprises a nucleic acid sequence expressing hygromycin resistance whereby mutated cells are selected by being the only cells that survive when hygromycin is applied. In various embodiments the selection marker is a nucleic acid sequence expressing mcherry whereby mutated cells are selected via fluorescence activated cell sorting. In various embodiments the selection marker comprises both nucleic acid expressing a hygromycin resistance and a nucleic acid sequence expressing mcherry whereby mutated cells are first selected by being the only cells that survive when hygromycin is applied and thereafter via fluorescence activated cell sorting. In various embodiments the selection marker comprises a nucleic acid sequence expressing puromycin resistance whereby mutated cells are selected by being the only cells that survive when puromycin is applied. In various embodiments the selection marker in the first or second plasmid is at least one of a nucleic acid expressing neomycin resistance; a nucleic acid expressing hygromycin resistance; a nucleic acid sequence expressing puromycin resistance; a nucleic acid sequence expressing a green fluorescence protein (GFP); a nucleic acid sequence expressing mcherry whereby the selection marker or markers in the first plasmid differ from the selection markers in the second plasmid to allow a different selection of a mutation in both alleles.

[0048] In various embodiments the human pluripotent stem cell is transfected with a plasmid to mutate both alleles of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and a selection marker; and selecting the cell with a mutation in both alleles of a beta-2-microglobulin gene via the selection marker.

[0049] In various embodiments the selection marker comprises a nucleic acid sequence expressing antibiotic resistance whereby mutated cells are selected by being the only cells that survive when the antibiotic is applied. In various embodiments the selection marker comprises a nucleic acid sequence expressing puromycin resistance whereby mutated cells are selected by being the only cells that survive when puromycin is applied. In various embodiments the selection marker is a transient marker. In various embodiments the selection marker is a lack of expression of HLA class I molecules selected by cell sorting. In various embodiments the selection marker comprises both a nucleic acid sequence expressing antibiotic resistance and the lack of expression of HLA class I molecules whereby mutated cells are first selected by being the only cells that survive when the antibiotic is applied and thereafter selected by cell sorting. This has the advantage of screening for cells with a mutation in both alleles without having to prepare two separate mutations and selection for each mutation. In various embodiments the antibiotic may be puromycin. In various embodiments the selection marker does not include a nucleic acid sequence expressing fluorescence molecule. This has the advantage of not interfering with subsequent fluorescence staining or imaging of the cells.

[0050] Another aspect of the invention includes genetically modified human mesenchymal stromal cells comprising cells expressing no beta-2-microglobulin and expressing reduced or no HLA class I molecules on the cell surface.

[0051] As used herein the term “genetically modified human mesenchymal stromal cells” refers to human mesenchymal stromal cells that have been differentiated from human pluripotent stem cells whereby the B2M gene has been artificially modified such that it does not express beta-2-microglobulin and HLA class I molecules. The resulting human mesenchymal stromal cells also do not express beta-2-microglobulin and HLA class I molecules. As described herein the human pluripotent stem cells can be induced pluripotent stem cells (iPSCs). An advantage of using iPSCs is that the cells can be derived from the same subject to which the mesenchymal stromal cells are to be used or administered. That is, a somatic cell can be obtained from a subject, reprogrammed to an induced pluripotent stem cell, and then re-differentiated into a mesenchymal stromal cells to be used for treatment or administered to the subject (e.g., autologous cells). Because the beta-2-microglobulin and HLA class I molecules are not expressed, the risk of engraftment rejection or allergic response can be reduced compared to the use of other human mesenchymal stromal cells. In addition, the use of iPSCs negates the need for cells obtained from an embryonic source. In various embodiments the pluripotent stem cells used in the disclosed

methods are not embryonic stem cells. Although differentiation is generally irreversible under physiological contexts, several methods have been developed to reprogram somatic cells to iPSCs. Exemplary methods are known to those of skill in the art.

[0052] In various embodiments the HLA class I molecules comprises any one of B2M, HLA-A, HLA-B or HLA-C or a combination thereof. In various embodiments the genetically modified human mesenchymal stromal cells are absent the expression of any one of B2M, HLA-A, HLA-B or HLA-C or a combination thereof. In various embodiments the genetically modified human mesenchymal stromal cells are absent the expression of all of B2M, HLA-A, HLA-B or HLA-C. Since B2M is the key component in HLA class I complex, knockout of B2M can disrupt the HLA class I expression on the cells. HLA class I complex plays a role of presenting cytosolic peptides as self-antigens for histocompatibility recognition. The HLA class I negative phenotype resulting from B2M knockout could significantly reduce the immunogenicity of such human mesenchymal stromal cells with little antigen presenting. Hence, risk of allogenic rejection could also be reduced since hosts' alloreactive lymphocytes could not be activated by recognizing allo-antigens presented on cell surface of any grafts with these cells.

[0053] In various embodiments the genetically modified human mesenchymal stromal cells express at least one of CD29, CD44, CD73, CD90, CD105, CD166, or a combination thereof and with low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45 B2M, HLA-A, HLA-B or HLA-C or a combination thereof.

[0054] In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene express at least one of CD29, CD44, CD73, CD90, CD105, CD166, or a combination thereof and comprise low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45, B2M, HLA-A, HLA-B or HLA-C or a combination thereof. In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene express any 2, any 3, any 4, any 5, or all of CD29, CD44, CD73, CD90, CD105, CD166. In various embodiments the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene comprise low or no expression of any 2, any 3, any 4, any 5, any 6, any 7, any 8, or all of CD14, CD34, HLA-DR, CD25, CD45, B2M, HLA-A, HLA-B and HLA-C. Such cells have the advantage of having features of a human mesenchymal stromal cell such as expression of any one of CD29, CD44, CD73, CD90, CD105, CD166; low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45; and the ability to be contribute to adipogenesis, osteogenesis and chondrogenesis but have the advantage that they do not

express HLA class I molecules. Even after differentiation to adipocytes, osteocytes and chondrocytes HLA class I expression remains low.

[0055] In various embodiments a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1.

[0056] In various embodiments a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second allele of the beta-2-microglobulin gene is mutated to delete base pair 46 of SEQ ID NO. 1.

[0057] In various embodiments the first allele comprises a mutation in exon 1 comprising the nucleic acid sequence set out in SEQ ID NO. 57 (CCGAGATGTCTCGCTCCGTGGTTAGCTGTGCTCGCGCTACTCTCT). In various embodiments the second allele comprises a mutation in exon 1 comprising the nucleic acid sequence set out in SEQ ID NO. 58 (CCGAGATGTCTCGCTCCGTGGCCCTTAGCTGTGCTCGCGCTACTCTCT). In various embodiments the second allele comprises a mutation in exon 1 comprising the nucleic acid sequence set out in SEQ ID NO. 59 (CCGAGATGTCTCGCTCCGTGCCTTAGCTGTGCTCGCGCTACTCTCT).

[0058] In various embodiments the cells as described herein are suitable for use in a treatment. As the HLA class I expression was blocked in B2M knockout human mesenchymal stromal cells, there was little antigen presenting on B2M knockout iMSCs. In this case, B2M knockout iMSCs would not provoke host-versus-graft rejection as an allogeneic graft. Besides, they could escape from the lysis of alloreactive T cells and prolong their survival after infusion. These B2M knockout hP-iPSC-derived MSCs could be used as allogeneic biomaterials in an "off-the-shelf" scenario for therapeutic purposes such as use in regenerative medicine. These B2M knockout iPS cell-derived MSCs are effective as primary adult mesenchymal stromal cells in treating numerous diseases from tissue damage to immune disorders.

[0059] Another aspect of the invention includes a kit for generating human mesenchymal stromal cells with reduced immunogenicity, the kit comprising:

(a) one or more plasmid capable of mutating both alleles of a beta-2-microglobulin gene in a human pluripotent stem cell;

(b) a mesenchymal stromal cell medium for differentiating human pluripotent stem cells into human mesenchymal stromal cells.

[0060] In various embodiments the kit, further comprises two or more primers sequences selected from SEQ ID NOS. 5-28. In various embodiments the primer pairs comprise a forward and reverse primer selected from any one of SEQ ID NOS. 5 and 6; SEQ ID NOS. 7 and 8; SEQ ID NOS. 9 and 10; SEQ ID NOS. 11 and 12; SEQ ID NOS. 13 and 14; SEQ ID NOS. 15 and 16 wherein one or more additional primer pairs may be selected from SEQ ID NOS. 17 and 18; SEQ ID NOS. 19 and 20; SEQ ID NOS. 21 and 22; SEQ ID NOS. 23 and 24; SEQ ID NOS. 25 and 26; SEQ ID NOS. 27 and 28; SEQ ID NOS. 29 and 30; SEQ ID NOS. 31 and 32; SEQ ID NOS. 33 and 34; SEQ ID NOS. 35 and 36; SEQ ID NOS. 37 and 38; SEQ ID NOS. 39 and 40; SEQ ID NOS. 41 and 42; SEQ ID NOS. 43 and 44; SEQ ID NOS. 45 and 46; SEQ ID NOS. 47 and 48; SEQ ID NOS. 49 and 50; SEQ ID NOS. 51 and 52; 43 and 54; and SEQ ID NOS. 55 and 56.

[0061] In various embodiments the one or more plasmid comprises a guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and an endonuclease.

[0062] In various embodiments the guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene is as described herein above. In various embodiments the endonuclease is as described herein above.

[0063] In various embodiments the targeted portion of the beta-2-microglobulin gene is exon 1 of the B2M. In various embodiments the one or more plasmid comprises a CRISPR/cas9 system targeted to a portion of the beta-2-microglobulin gene. In various embodiments the guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene comprises a sequence selected from SEQ ID NO. 3 or SEQ ID NO. 4.

[0064] In various embodiments the plasmid comprises a px260 (Addgene, Cambridge, MA, USA) containing a CRISPR/cas9 system targeted to a portion of the beta-2-microglobulin gene comprising SEQ ID NO. 3. In various embodiments the plasmid comprises a px459 (Addgene, Cambridge, MA, USA) containing a CRISPR/cas9 system targeted to a portion of the beta-2-microglobulin gene comprising SEQ ID NO. 4.

[0065] In various embodiments the endonuclease comprises a Clustered Regulatory Interspaced Short Palindromic Repeat (CRISPR) associated nucleic sequence (Cas9).

[0066] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by a skilled person to which the subject matter herein belongs. As used herein, the following definitions are supplied in order to facilitate the understanding of the present invention.

[0067] Throughout this document, unless otherwise indicated to the contrary, the terms “comprising”, “consisting of”, “having” and the like, are to be construed as non-exhaustive, or in other words, as meaning “including, but not limited to”.

[0068] Furthermore, throughout the specification, unless the context requires otherwise, the word “include” or variations such as “includes” or “including” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0069] As used in the specification and the appended claims, the singular form “a”, and “the” include plural references unless the context clearly dictates otherwise.

Examples

[0070] Described is various embodiments to generate HLA class I negative MSCs. Human PBMC-derived iPSC cells (hP-iPSCs) were first generated and genetically modified by knocking out the B2M gene in hP-iPSCs with specifically designed CRISPR/Cas9 systems. The B2M knockout hP-iPSC single cell clones were screened to select the clones negative for HLA class I complex expression while maintaining the pluripotency and genetic normality. The selected clones were then differentiated into B2M knockout iPSC-derived MSCs (iMSCs) that express relevant MSC markers without HLA class I expression and display multipotency to differentiate into osteoblasts, chondrocytes, and adipocytes. Importantly, iMSCs display lower immunogenicity to allogenic immune cells as compared to wild type iMSCs. Hence, disclosed is a technology that uses the B2M knockout hP-iPSCs as the “off-the-shelf” cell resources to generate MSCs with low immunogenicity. The iMSCs generated have a great potential in regenerative medicine.

Cell culture of iPSCs from peripheral blood cells

[0071] Human PBMC-derived induced pluripotent stem cells were cultured in mTeSR™1 medium (STEMCELL Technologies, Vancouver, Canada) on Matrigel™ hESC-qualified Matrix (BD Biosciences, Franklin Lakes, NJ, USA) coated plates. Culture medium was refreshed everyday while hP-iPSCs were sub-cultured every week. To form embryoid

bodies from hP-iPSCs, confluent iPSC colonies were dissociated with 1mg/mL Dispase (Thermo Fisher Scientific, Waltham, MA, USA) as large cell aggregates. Those cell aggregates were cultured in low attachment plate with embryoid body (EB) medium as Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco™, Thermo Fisher Scientific) supplemented with 20% KnockOut serum replacement (Gibco™), 2mM L-glutamine (Lonza, Basel, Switzerland), 0.1mM Non-Essential Amino Acid (NEAA, Gibco™) and 0.1mM β -Mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). The EB medium was refreshed every two to three days.

Western blotting and flow cytometry analysis

[0072] For Western blot analysis, sample proteins were extracted by lysing cells with Radio-immunoprecipitation assay (RIPA) buffer (Nacalai Tesque, Kyoto, Japan), analyzed in SDS-PAGE gel under reducing condition and then electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). Rabbit Anti-B2M antibody clone EP2978Y (1:5000 dilution, Abcam, Cambridge, UK) and mouse Anti- β -actin antibody clone GT5512 (1:1000 dilution, Abcam) were used as primary antibodies. Goat anti-rabbit IgG-HRP (1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat anti-mouse IgG-HRP (1:2000 dilution, Santa Cruz Biotechnology) were used as secondary antibodies. The membrane was developed and visualized for chemiluminescence by MYECL™ Imager (Thermo Fisher Scientific). For flow cytometry analysis, iPS and MSC cells were stained with antibodies in autoMACS® Running Buffer (Miltenyi Biotec) and analyzed by BD Accuri™ C6 Flow Cytometer (BD Biosciences). Data were analyzed by CFlow® Sampler software (BD Biosciences).

Southern blotting

[0073] Southern blot was performed whereby for each sample, 15 μ g genomic DNA was digested with 50U HindIII-HF (New England Biolabs, Ipswich, MA, USA) overnight. Digested DNA was loaded on a 1% agarose gel and gel electrophoresis was performed at 40V for 5 hours. DNA was then transferred to a positively-charged nylon membrane by using iBlot® Dry Blotting System (Invitrogen™, Thermo Fisher Scientific). The membrane was washed with 1.5M NaCl/0.5M NaOH denaturing solution and then air-dried. Ultraviolet cross-linking was performed at 130mJ/cm². The membrane was first pre-hybridized in DIG Easy Hyb (Roche Diagnostics) buffer for one hour and then hybridized with DIG-labeled probe overnight. Afterwards, the membrane was first washed twice with 2× saline-sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 40°C and then twice with 0.1× SSC/0.1% SDS

at 50°C. Then, membrane was blocked and washed with DIG Wash and Block Buffer Set (Roche Diagnostics) and incubated with an anti-digoxigenin-AP (Roche Diagnostics). Finally, the membrane was detected with CDP-Star (Roche Diagnostics) as a substrate for chemiluminescence by using MYECL™ Imager (Thermo Fisher Scientific). The probes were synthesized by using the PCR DIG Probe Synthesis Kit (Roche Diagnostics) with donor plasmid for B2M gene as a template. Primers for probe synthesis are listed in Table 1.

[0074] Table 1: List of oligos and primers

Application	SEQ ID NO.	Direction (Fwd/Rev)	Sequence	Amplicon size
B2M Target1	5	Fwd	AAACggccgagatgtctcgctccgGT	NA
	6	Rev	TAAAAcCggagcgagacatctcggc c	
B2M Target2	7	Fwd	CACCcgcgagcacagctaaggcca	NA
	8	Rev	AAACtggccttagctgtgctcgcg	
B2M EX1 WT	9	Fwd	TCTCGAATGAAAAATGCA GGTCCG	780 bp
	10	Rev	TGACGCTTATCGACGCCCT AAACTT	
B2M EGFP HDR	11	Fwd	CCACAACGTCTATATCATG GCCGAC	1630 bp
	12	Rev	GTTTGCTCTGGAGAATCTC ACGCAG	
B2M mCherry HDR	13	Fwd	TCTAGAGCCTGCAGTCTCG ACAAGC	1420 bp
	14	Rev	GTTTGCTCTGGAGAATCTC ACGCAG	
Probe EGFP	15	Fwd	CACCTACGGCAAGCTGAC CCTGAAGTTCAT	517 bp
	16	Rev	ATGTGATCGCGCTTCTCGT TGGGGTCTTTG	
B2M Off-target1	17	Fwd	CCCCAACCAGATTGCCCCA T	619 bp
	18	Rev	TCCCTGCAGCGGATCCTCA A	
B2M Off-target2	19	Fwd	TCCAGCTGCAAAAGGGAG AAGAGA	572 bp
	20	Rev	AGGGAAGAAGCCAAAGCG AA	
B2M Off-target3	21	Fwd	TGCCTGAGGTCCAAGGAA GAGCT	605 bp
	22	Rev	TGAGCCCAAATCCAGCCA CA	
B2M Off-target5	23	Fwd	AGCTGTATGACAGCCCCTC TGTGC	589 bp

	24	Rev	TAGACTCACGAGGCCCGGC GA	
B2M Off- target8	25	Fwd	CGGTGACTGTGGCTGAGA CTGAGAC	474 bp
	26	Rev	CGCCGCTACCCCGAAGAA AA	
B2M Off- target16	27	Fwd	AGGAACTGGGGCTTGAAT GGGG	621 bp
	28	Rev	GGCTGTTGCCCAGTCCTGG AAA	
Oct4	29	Fwd	GAGCAAAACCCGGAGGAG T	310 bp
	30	Rev	TTCTCTTTCGGGCCTGCAC	
Sox2	31	Fwd	GGGAAATGGGAGGGGTGC AAAA	151 bp
	32	Rev	TTGCGTGAGTGTGGATGGG ATT	
Nanog	33	Fwd	GCTTGCCTTGCTTTGAAGC A	256 bp
	34	Rev	TTCTTGACCGGGACCTTGT C	
β -actin	35	Fwd	TGGCACCCAGCACAAATGA AG	149 bp
	36	Rev	GATGGAGGGGCGGACTC	
Pax6	37	Fwd	CAATAATGTTGACGGTGAC TATC	179 bp
	38	Rev	AGAAGGAAGCGACACTCT G	
MHC- α	39	Fwd	GTCATTGCTGAAACCGAG AATG	413 bp
	40	Rev	GCAAAGTACTGGATGACA CGCT	
AFP	41	Fwd	CCCGAACTTTCCAAGCCAT A	101 bp
	42	Rev	TACATGGGCCACATCCAG G	
LPL	43	Fwd	TCAACTGGATGGAGGAG	169 bp
	44	Rev	GGGGCTTCTGCATACTCAA A	
PPAR- γ	45	Fwd	CTCCTATTGACCCAGAAAG C	350 bp
	46	Rev	GTAGAGCTGAGTCTTCTCA G	
ALP	47	Fwd	TGGAGCTTCAGAAGCTCA ACACCA	454 bp
	48	Rev	ATCTCGTTGTCTGAGTACC AGTCC	

OCN	49	Fwd	GGCAGCGAGGTAGTGAAG AG	195 bp
	50	Rev	CAGCAGAGCGACACCCTA GAC	
COL2a	51	Fwd	ATGATTTCGCCTCGGGGCTC C	390 bp
	52	Rev	CATTACTGGGAAGTGGGC GC	
ACAN	53	Fwd	CACTGTTACCGCCACTTCC C	190 bp
	54	Rev	ACCAGCGGAAGTCCCCTTC G	
GAPDH	55	Fwd	CCCCTTCATTGACCTCAAC TACA	342 bp
	56	Rev	TTGCTGATGATCTTGAGGC TGT	

RT-PCR

[0075] Total RNA was isolated from each cell sample by TRIzol Reagent (Thermo Fisher Scientific). The reverse transcription was performed by SuperScript III First-Strand Synthesis System (Invitrogen™). PCR amplification was performed by KAPA Taq ReadyMix PCR Kit (Kapa Biosystems, Roche Diagnostics) with according annealing temperatures for 35 cycles. The PCR products were resolved by 1.5% agarose gel for analysis. Primers and their relevant annealing temperatures were listed in Table 1.

Generation of B2M knockout hP-iPSC clone

[0076] The B2M knockout hP-iPSCs were generated by two methods. The first method was a double colour selection and the second method was a one-shot puromycin selection.

Example 1 using CRISPR/Cas9 with double colour selection

[0077] Plasmid construction

[0078] The pX260 plasmid (Addgene, Cambridge, MA, USA) containing a CRISPR/Cas9 system, which was described previously (L. Cong et al., Science 339, 819 (Feb 15, 2013)), were used in this study. Target sequences of CRISPR/Cas9 were designed in exon 1 by CHOPCHOP (<http://chopchop.cbu.uib.no/>). The B2M Target1 (SEQ ID NO. 3: GGCCGAGATGTCTCGCTCCG) was subcloned into pX260. Two donor plasmids for B2M Exon 1 homology-direct integration were constructed. One was designed with an EF1 α (eukaryotic translation elongation factor 1 α) promoter driving the expression of an EGFP

gene and a PGK (Mouse phosphoglycerate kinase 1) promoter driving the expression of the Neo gene (the neomycin resistant gene) while the other was designed with the EF1 α promoter driving the expression of an mCherry gene and an SV40 (Simian virus 40) promoter driving the expression of the Hygro gene (the hygromycin resistant gene). Both donor sequences were flanked by homologous DNA sequences from B2M Exon 1 locus (chromosome 15: nucleotides 44,710,501–44,711,401 and nucleotides 44,711,615–44,712,485, GRCh38.p2 Primary Assembly).

[0079] For the double colour selection, hP-iPS cells were dissociated by Accutase™ (Merck Millipore), washed by phosphate-buffered saline (PBS, Lonza) and resuspended in Opti-MEM® I Reduced Serum Medium (Gibco™) as single cell suspension. 1×10^6 cells were transfected with 2.5 μ g pX260 plasmid and 2.5 μ g donor plasmid with GFP by electroporator (Nepa Gene, Chiba, Japan). Transfected single cells were recovered in NutriStem® hPSC XF Medium (Biological Industries, Beit-Haemek, Israel) and seeded on Matrigel™ coated plates. Four days after electroporation, culture medium was changed back to mTeSR™1 medium and cells were selected by 25 μ g/mL Geneticin® (G418 Sulfate, Gibco™) for two weeks. Selected cells were subjected to fluorescence-activated cell sorting (FACS) for single cell seeding, which was performed by BD FACSAria™ I Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Selected hP-iPS cells were dissociated by Accutase™ as single cells and the GFP positive population was seeded as one cell per well on Matrigel™ coated 96 well-plates in NutriStem® medium. Single cell clones were expanded, genotyped by PCR and sequencing. A mono-allele knockout single cell clone was confirmed and preceded to a second round of knockout on the other allele. The mono-allele knockout single cell clone was transfected with 2.5 μ g pX260 plasmid and 2.5 μ g donor plasmid with mCherry by electroporation. Transfected cells were selected by 10 μ g/mL Hygromycin B (Gibco™) for two weeks and subjected to single cell seeding as well. The double colour single cell clones were collected, expanded and confirmed by genotyping.

[0080] To disrupt the B2M gene in human PBMC-derived iPS cells, B2M was first targeted by CRISPR/Cas9 with the help of colour selection. hP-iPSCs were co-transfected with a CRISPR/Cas9 plasmid pX260 to target B2M gene and a donor plasmid containing EGFP and an Neomycin resistant gene flanked by B2M homologous sequences (Figure 1A). Transfected cells were selected by geneticin for two weeks and sorted by GFP positive expression as single cells. Total eighteen single cell clones were expanded for analysis. All the 18 single clones were confirmed with the integration of EGFP selection marker into the B2M site (Figure 2A, 2B) but they still bore normal B2M expression. Sequencing analysis

showed that they each had an intact wild type allele of B2M (Figure 2C), which indicated that these 18 clones were only monoallelic knockout.

[0081] To disrupt the remaining wild type B2M allele, one of the monoallelic knockout clone #18 was transfected with B2M targeting CRISPR/Cas9 plasmid again but accompanied with a donor containing mCherry and Hygromycin selection marker (Figure 1A). Similarly, transfected cells underwent Hygromycin B selection for two weeks and cell sorting which was based on EGFP and mCherry double colors. Total six single cell clones were collected and expanded showing positive for both selection markers (Figure 1B). Besides, all the six clones were confirmed with site specific integration of both makers in B2M site and no wild type B2M allele could be detected by PCR (Figure 1C). This suggests that both alleles of B2M were knocked out in the six double colour hP-iPSC clones. As expected, B2M expression could not be detected on those biallelic knockout clones by Western blotting, even when cells were treated by IFN- γ for 48 hours (Figure 1D). Monoallelic knockout clone, however, maintained potent B2M expression when compared to wild type clone. This finding was also supported by flow cytometry assay showing the surface expression of B2M while only biallelic knockout ones were negative for B2M expression (Figure 1E, 3A). In addition, biallelic knockout clones were also negative for HLA class I (Figure 1E). As B2M is the key component of HLA class I complex, disruption of B2M has blocked HLA class I expression and substantially reduced the immunogenicity. Here, the B2M knockout hP-iPS cells with double color selection were achieved.

[0082] Maintenance of pluripotency on B2M knockout hP-iPSCs

[0083] The pluripotency was examined in both the mono and bi-allelic of B2M knockout iPS cells. RT-PCR results showed equivalent expression of Oct4, Sox2 and Nanog in mono- and bi- allelic B2M knockout iPS cells comparing to wild type cells (Figure 3B). hP-iPS cells were also induced to form embryoid bodies (EBs). All three germ layer differentiation markers were detected in B2M knockout iPS-derived EBs (Figure 3C, 3D), which suggested intact differentiation capacity of B2M knockout iPS cells.

Example 2 One step generation of B2M knockout hP-iPSC clone by CRISPR/Cas9 without colour selection

[0084] Plasmid construction

[0085] The pX459 plasmid (Addgene, Cambridge, MA, USA) containing a CRISPR/Cas9 system, which was described previously (F. A. Ran et al., Nature protocols

8, 2281 (Nov, 2013)), was used in this study. Target sequences of CRISPR/Cas9 were designed again in exon 1 by CHOPCHOP (<http://chopchop.cbu.uib.no/>) The B2M Target2 SEQ ID NO. 4 (CGCGAGCACAGCTAAGGCCA) was subcloned into pX459.

[0086] For the one-shot puromycin selection, 1×10^6 hP-iPS cells were transfected with 5 μ g pX459 plasmid with the B2M target2 (Figure 4A) by electroporation and recovered in NutriStem® medium on Matrigel™ coated plates overnight. Afterwards, transfected cells were selected by 1 μ g/mL puromycin (Thermo Fisher Scientific) in NutriStem® medium for 24 hours. Survival single cells were culture in fresh Nutristem® medium for three to four days before changed back to mTeSR™1 culture. Single cell clones were isolated and subjected to genotyping and phenotyping. B2M negative clones were confirmed from those single cell clones.

[0087] Although the B2M negative hP-iPS cells were well established with the help of colour selection, the entire process was still time-consuming and not efficient. Further, the cells with fluorescence could potentially interfere with subsequent assays using relevant fluorescent reagents. Potential integration of selection marker into genomic region besides B2M was also an arising concern as evidence was shown by southern blotting (Figure 3A). To overcome these issues, the method to knock out B2M on hP-iPS cells was modified without colour selection.

[0088] It was observed that, after transfection of CRISPR/Cas9 plasmid, some cells were already B2M negative but they simply could not be selected. Therefore, the plasmid pX459 with the B2M target2 (Figure 4A) was introduced as the transient expression of Cas9 which could be selected by puromycin. The hP-iPS cells were transfected with pX459 plasmids targeting B2M by electroporation. When transfected cells were settled down, they were subsequently selected by puromycin for only 24 hours. Within the surviving cells, large amount of B2M negative cells could be observed by flow cytometry assay (Figure 4B). These survival cells were seeded as single cells and twelve single cell clones were successfully expanded. Among the 12 clones, five of them were tested as B2M negative by flow cytometry assay. B2M knockout (B2MKO) clone #3 and #8 were randomly selected for subsequent analysis. Both of the B2M knockout clones were negative for B2M and HLA class I molecules (Figure 5A). By genotyping, either deletion or insertion in the B2M targeting site was found in those B2M knockout clones (Figure 5B). Such specific mutation, which was induced by CRISPR/Cas9 mediated cleavage, caused a frame shift of B2M translational reading frame and brought an early stop codon to terminate the B2M expression.

[0089] Off-target analysis and karyotyping

[0090] The off-target sites of B2M CRISPR/Cas9 target was predicted by benchling (<https://benchling.com/>). Primers for the top three high ranking off-target sites in total prediction and in coding sequences were designed accordingly. Those primer sequences were included in Table 1. Genomic DNA of B2M knockout hP-iPSCs clones was isolated by DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Sequences of off-target sites were amplified by PCR respectively, purified and analyzed by Sanger Sequencing (AITbiotech, Singapore). For karyotyping, confluent hP-iPSCs colonies were sent to NUH referral laboratory (NRL) for chromosome analysis.

[0091] The accuracy of this CRISPR/Cas9 mediated B2M knockout was also examined by sequencing high risk off-target sites. Only one off-target was spotted in clone #3, which did not affect the reading frame. No observable off-target was found in clone #8, which ensured the precision of this gene editing (Table 2). In addition, karyotyping also showed the normal status of chromosome in the B2M knockout clone #8 (Figure 5C). Here, B2M gene on hP-iPS cells was knocked out precisely without introducing any selection marker or random genomic damage.

[0092] Table 2: Sequencing results for potential off target sites.

No.	Sequence	PAM	Score	Gene	Chromosome	Strand	Position	Mismatches	Off-target on #3	Off-target on #8
0	CGCGAGCACAGCTAAGGCCA	CGG	100	B2M	chr15	-1	44711563	0		
1	CGAGAGCACTGCTAAGGCCG	GGG	1.603603		chr19	-1	2269919	3	No	No
2	CCCAAGCACAGTTAAGGCCA	TGG	1.52		chr12	-1	12716484	3	No	No
3	CAATGCACAGCTAAGGCCA	GAG	1.325519		chr1	1	4351231	4	No	No
5	CAGGAGCACAGCCAAGGCCA	GGG	1.226744	FGFR4	chr5	-1	1.77E+08	3	6bp deletion	No
8	CAAGAGTCAGCTAAGGCCA	AAG	0.995487	HIST1H1D	chr6	-1	26234357	4	No	No
16	GGACAGCATAGCTAAGGCCA	TGG	0.913282	SERPINA6	chr14	1	94314432	4	No	No

[0093] Maintenance of pluripotency on B2M knockout hP-iPSCs

[0094] The pluripotency of B2M knockout iPS cells was examined. RT-PCR results showed equivalent expression of Oct4, Sox2 and Nanog in mono- and bi- allelic B2M knockout iPS cells comparing to wild type cells (Figure 6A). hP-iPS cells were also induced to form embryoid bodies (EBs). All three germ layer differentiation markers were detected in B2M knockout iPS-derived EBs (Figure 6B, 6C), which suggested intact differentiation capacity of B2M knockout iPS cells.

[0095] Generation of mesenchymal stromal cells from B2M knockout

[0096] Many studies have unveiled the feasibility of deriving mesenchymal stromal cells (MSCs) from human embryonic stem cells (hESCs) or iPS cells (Y. Du et al., Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 43, 611 (2017)). Mesenchymal stromal cells (MSCs) were differentiated from hP-iPSCs directly as described previously (Y. Du et al., Cellular physiology and biochemistry: international journal of experimental cellular physiology, biochemistry, and pharmacology 43, 611 (2017)). When hP-iPSCs reached confluence, culture medium was replaced by Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Gibco™), 10% fetal bovine serum (FBS, Hyclone™ GE Healthcare, Little Chalfont, UK) and 2mM L-glutamine (Lonza). The culture medium was first refreshed every day for four days and then refreshed every two days for 6 to 12 more days. Afterwards, cells were dissociated by Trypsin (Hyclone™ GE Healthcare) and seeded on Matrigel coated 6-well plates at a density of 1×10^6 cells/well. When cells reached confluence, they were dissociated by Trypsin and seeded on 0.1% Gelatin water (Merck Millipore, Billerica, MA, USA) coated 6-well plates at a density of 2×10^5 cells/well. The morphology of attached cells was gradually turned like fibroblast cells. These differentiated cells were sub-cultured every three to four days when they reached 80% confluence as mesenchymal stromal cells for subsequent analysis.

[0097] The mesenchymal stromal cells were successfully derived from B2M knockout iPS cells as well as wild type cells, showing the phenotype of fibroblast like cells (Figure 7A). Flow cytometry assay confirmed negative for B2M and HLA class I on MSCs from B2M knockout iPS cells (Figure 7B). Further phenotyping supported that those B2M knockout cells maintained the characteristics of MSCs as they were negative for CD14, CD24, CD34, CD45 and HLA-DR but positive for CD29, CD44, CD73, CD90, CD105 and CD166 (Figure 7C).

[0098] In vitro differentiation of hP-iPSC-derived MSCs

[0099] The multipotency of B2MKO iPS-derived MSCs were analyzed by inducing further differentiation of those MSCs into adipocytes, osteocytes and chondrocytes. Adipogenesis was confirmed by positive staining of lipid content in differentiated cells with Oil Red O (Figure 8A). Robust osteogenesis was verified by positive red staining of calcific deposition with Alizarin Red S (Figure 8B). In addition, chondrogenesis was achieved with formation of chondrocytes aggregates and blue staining of acidic polysaccharides (Figure

8C). RT-PCR also showed up-regulation of relevant markers in differentiated cells supporting the differentiation capacity of B2MKO iPS-derived MSCs. Thus, functional mesenchymal stromal cells could be generated from B2M knockout hP-iPS cells.

[00100] In this case, the methods for generating MSCs from B2M knockout hP-iPS cells directly were adapted. For adipogenesis, iPSC-derived MSCs were seeded at a density of 10,000 cells/cm² for two to four days to reach confluence. Then culture medium was changed to differentiation medium from StemPro™ Adipogenesis Differentiation Kit (Gibco™) and refreshed every three to four days for more than two weeks. Cells were then fixed with 4% formaldehyde (Sigma-Aldrich) and histologically stained by Oil Red O (Sigma-Aldrich) for lipid content.

[00101] For osteogenesis, iPSC-derived MSC cells were seeded at a density of 5,000 cells/cm² for two to four days. Then culture medium was changed to differentiation medium from StemPro™ Osteogenesis Differentiation Kit (Gibco™) and refreshed every three to four days for more than three weeks. Cells were then fixed with 4% formaldehyde (Sigma-Aldrich) and histologically stained by Alizarin Red S solution (Merck Millipore) for calcification.

[00102] For chondrogenesis, iPSC-derived MSC cells were concentrated to 1.6×10⁷ cells/mL. Cells were loaded onto plate by 5μL droplets and cultured as pellets for two hours to form cell clusters. Then culture medium was changed to differentiation medium from StemPro™ Chondrogenesis Differentiation Kit (Gibco™) and refreshed every three to four days for more than three weeks. Cells were then fixed with 4% formaldehyde (Sigma-Aldrich) and histologically stained by Alcian blue 8GX (Sigma-Aldrich) for acidic polysaccharides.

[00103] Hypoimmunogenicity of B2MKO hP-iPSC-derived MSCs

[00104] To examine the immunogenicity of hP-iPSC-derived MSCs, allogenic human peripheral blood mononuclear cells (PBMCs) were first primed with wild type hP-iPSC-derived MSCs (iMSCs). Fresh peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor's buffy coat with Ficoll®-Paque PREMIUM 1.084 (GE Healthcare) by density gradient centrifugation. PBMCs were primed with MSCs by co-culture in AIM V® Medium (Gibco™) with 5% human AB serum (Valley Biomedical, Winchester, VA, USA) and 300 IU/mL IL-2 (PeproTech, Rocky Hill, NJ, USA) for T cell activation.

Activated CD3+ T cells were re-stimulated with MSCs at a ratio of 5:1 every seven days to achieve the population of MSC-specific cytotoxic T lymphocytes (CTLs).

[00105] Human PBMCs were co-cultured with wild type iMSCs directly and re-stimulated by fresh iMSCs every week. Primed PBMCs were phenotyped by flow cytometer to delve into the specific population. Most primed donors would end up with a major CD3+CD56- T cell population with CD8+ T dominant (Figure 9A). These primed PBMCs population indicated that alloreactive cytotoxic T lymphocytes had been stimulated by iMSCs, although mesenchymal stromal cells or iMSCs were reported with immunosuppressive characteristics (M. Giuliani et al., Blood 118, 3254 (Sep 22, 2011)).

[00106] Cell cytotoxicity assay

[00107] Cell cytotoxicity was assessed in a standard two-hour Europium-release assay with DELFIA® EuTDA Cytotoxicity Reagents (PerkinElmer, Waltham, MA, USA). Assay was performed according to the manufacturer's instructions. Target cells were first labeled with BATDA Reagent at 37°C for 5 to 15 minutes and then washed three times with PBS. Effector cells and labeled target cells were mixed in triplicate at different effector to target (E:T) ratios in AIM V® Medium with 5% human AB serum. Mixed cells were incubated at 37°C in humid incubator for two hours. Spontaneous and maximum releases were determined by incubating target cells without effector cells and with lysis buffer respectively. After incubation, supernatants were transferred to mix with the Europium solution and analyzed by VICTOR™ Time-resolved fluorometer (PerkinElmer). The percentage of specific lysis was calculated as:

[00108]

$$\% \text{ Lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

[00109] When both wild type and B2MKO iMSCs were challenged with primed PBMCs in cell cytotoxicity assay, those primed PBMCs would preferentially kill wild type iMSCs but ignore the B2MKO iMSCs (Figure 9A). The B2MKO iMSCs could escape from the alloreactive cytotoxicity due to the loss of HLA class I antigen presenting while wild type iMSCs would be eliminated by alloreactivity.

[00110] Similarly, when both wild type and B2MKO iMSCs were challenged with primed PBMCs in the presence of OKT3 compared to PBMCs in the presence of OKT3 the B2M knockout cells demonstrated the lowest percentage of proliferation (Figure 10). This was a statistically significant reduction. Human peripheral

blood-derived mononuclear cells (hPBMCs) were stained by CFES/Far red staining. Following this 1×10^5 were isolated by gradient centrifugation, added to each well, and stimulated with an OKT-3 to stimulate proliferation. OKT-3-activated hPBMCs were then cultured on both wild type and B2MKO iMSCs for 3–4 days. Proliferation levels were assessed after 18 h. The proliferation of hPBMCs was suppressed when they were co-cultured with naïve MSCs; proliferation was further suppressed in B2M knockout derived iMSCs, as shown in Figure 10.

[00111] For certain donors, substantial amount of CD3-CD56+ Natural Killer (NK) cells could remain accompanied with alloreactive T cells after priming (Figure 9B). When this type of cells was used as effectors in cell cytotoxicity assay, B2MKO iMSCs could also be killed dose-dependently but their lysis was dramatically lower than the wild type counterpart (Figure 9B). To evaluate iMSCs' susceptibility to NK cells, both wild type and B2MKO iMSCs were challenged with primary NK cells.

[00112] Primary Natural Killer (NK) cells were expanded from fresh PBMCs with inactivated modified K562 cells as feeder cells. The expanded NK cells were collected seven to ten days after co-culture with K562 cells as a purity of more than 90% CD3-CD56+ cells for cell cytotoxicity assay.

[00113] Slightly higher lysis of B2MKO iMSCs was observed comparing with lysis of wild type iMSCs (Figure 9C), which is consistent with the "missing-self" theory (M. G. Morvan, L. L. Lanier, Nature reviews. Cancer 16, 7 (Jan, 2016)). Hence, B2MKO hP-IPSC-derived MSCs showed lower immunogenicity but higher susceptibility to NK lysis.

[00114] Statistical analysis

[00115] Data were collected as described above and summarized by Prism Version 7 software (GraphPad). Data were presented as mean ($\pm$ SD) and analyzed by Two-way Anova, Tukey's multiple comparisons test and Independent Samples Student's t-test. Representative histograms and graphs were chosen from independent repetition on the basis of the average values.

[00116] It should be further appreciated by the person skilled in the art that variations and combinations of features described above, not being alternatives or substitutes, may be combined to form yet further embodiments falling within the intended scope of the invention.

Claims:

1. A method of generating human mesenchymal stromal cells with reduced immunogenicity, the method comprising:
 - (a) mutating a human pluripotent stem cell at both alleles of a beta-2-microglobulin gene;
 - (b) differentiating the mutated human pluripotent stem cell into a derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene;wherein the derived human mesenchymal stromal cell expresses no beta-2-microglobulin and express reduced or no HLA class I molecules on the cell surface.
2. The method according to claim 1, wherein the human pluripotent stem cells are human embryonic stem cells.
3. The method according to claim 1, wherein the human pluripotent stem cells are induced pluripotent stem cells (iPSC).
4. The method according to claim 3, wherein the iPSC are induced from peripheral blood cells.
5. The method according to any one of claims 1 to 4, wherein the method further comprises determining that the mutation is present in both alleles of the Beta-2-microglobulin gene.
6. The method according to any one of claims 1 to 5, wherein the derived human mesenchymal stromal cell having a bi-allelic mutation in the beta-2-microglobulin gene express at least one of CD29, CD44, CD73, CD90, CD105, CD166, or a combination thereof and comprise low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45 B2M, HLA-A, HLA-B, HLA-C, or a combination thereof.
7. The method according to any one of claims 1 to 6, wherein the alleles are mutated with one or more plasmid comprises a guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and an endonuclease.
8. The method according to claim 7, wherein the endonuclease comprises a clustered regulatory interspaced short palindromic repeat associated nucleic sequence Cas9.

9. The method according to claim 7 or 8, wherein the guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene comprises a sequence selected from SEQ ID NO. 3 or SEQ ID NO. 4.
10. The method according to any one of claims 1 to 9, wherein the human pluripotent stem cell is mutated by:
- i. Transfecting the human pluripotent stem cell with a first plasmid to mutate a first allele of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and at least one selection marker;
 - ii. selecting the cell with a mutation in the first allele of a beta-2-microglobulin gene via the at least one selection marker;
 - iii. transfecting the cell with the mutation in the first allele of a beta-2-microglobulin gene with a second plasmid to mutate a second allele of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and at least one further selection marker; and
 - iv. selecting the cell with the mutation in the first allele of a beta-2-microglobulin gene and a mutation in the second allele of a beta-2-microglobulin gene via the at least one further selection marker.
11. The method according to any one of claims 1 to 9, wherein the human pluripotent stem cell is transfected with a plasmid to mutate both alleles of a beta-2-microglobulin gene comprising an endonuclease, guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and a selection marker; and selecting the cell with a mutation in both alleles of a beta-2-microglobulin gene via the selection marker.
12. Genetically modified human mesenchymal stromal cells comprising cells expressing no beta-2-microglobulin and expressing reduced or no HLA class I molecules on the cell surface.

13. The cells according to claim 12, expressing at least one of CD29, CD44, CD73, CD90, CD105, CD166, or a combination thereof and with low or no expression of any one of CD14, CD34, HLA-DR, CD25, CD45 B2M, HLA-A, HLA-B, HLA-C, or a combination thereof.

14. The cells according to claim 12 or 13 wherein a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second allele of the beta-2-microglobulin gene is mutated to include an inserted cytosine between base pairs 46 and 47 of SEQ ID NO. 1.

15. The cells according to claim 12 or 13 wherein a first allele of the beta-2-microglobulin gene is mutated to delete base pairs 47 and 48 of SEQ ID NO. 1 and a second allele of the beta-2-microglobulin gene is mutated to delete base pair 46 of SEQ ID NO. 1.

16. The cells according to claim 12 to 15, for use in a treatment.

17. A kit for generating human mesenchymal stromal cells with reduced immunogenicity, the kit comprising:

(a) one or more plasmid capable of mutating both alleles of a beta-2-microglobulin gene in a human pluripotent stem cell;

(b) a mesenchymal stromal cell medium for differentiating human pluripotent stem cells into human mesenchymal stromal cells.

18. The kit according to claim 17, further comprising two or more primers sequences selected from SEQ ID NOS. 5-28.

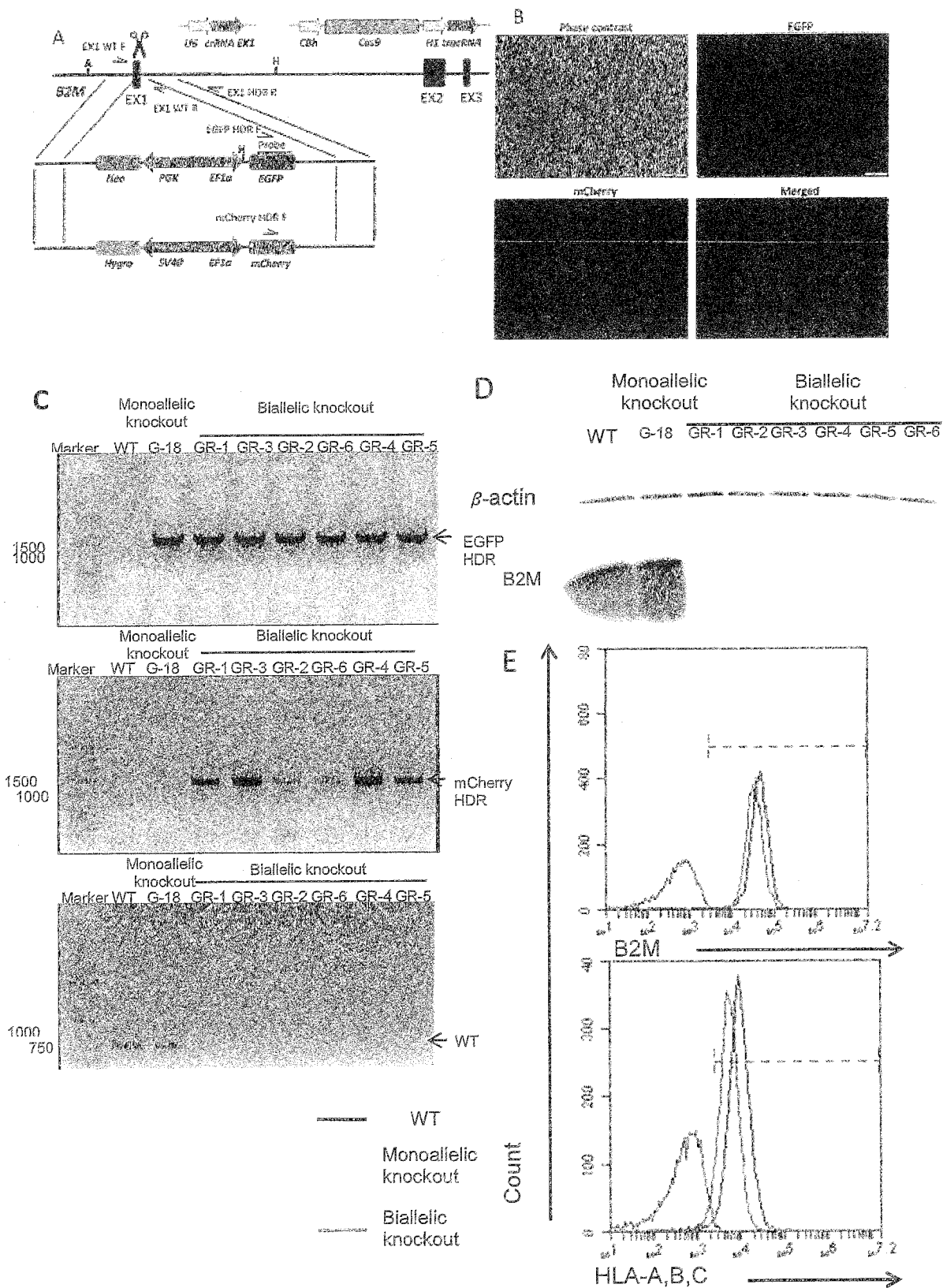
19. The kit according to claim 17 or 18, wherein the one or more plasmid comprises a guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene and an endonuclease.

20. The kit according to claim 19, wherein the endonuclease comprises a clustered regulatory interspaced short palindromic repeat associated nucleic sequence Cas9.

21. The kit according to claim 19 or 20, wherein the guide nucleic acid sequence targeted to a portion of the beta-2-microglobulin gene comprises a sequence selected from SEQ ID NO. 3 or SEQ ID NO. 4.

1/11

Figure 1



2/11

Figure 2

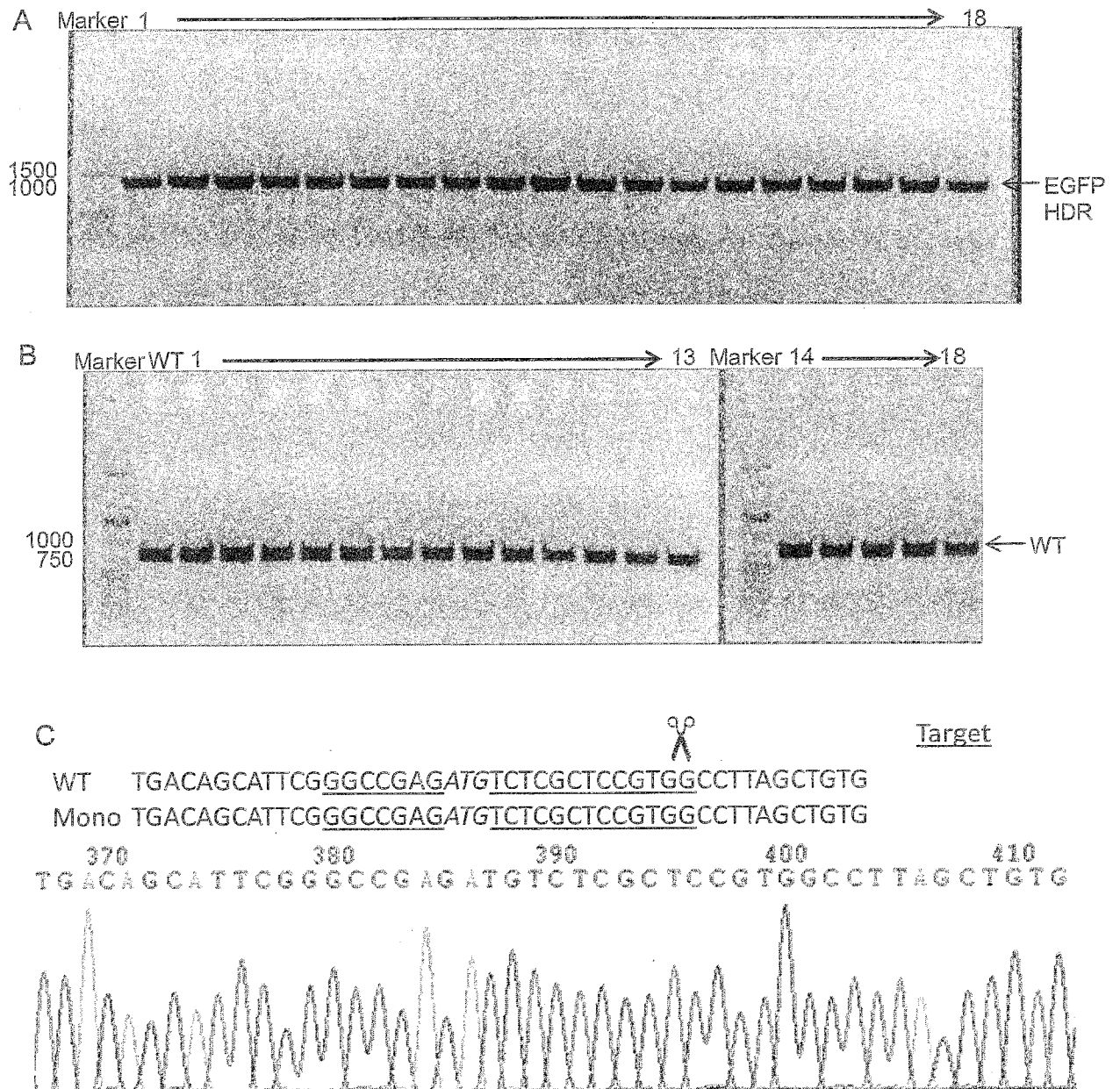


Figure 3

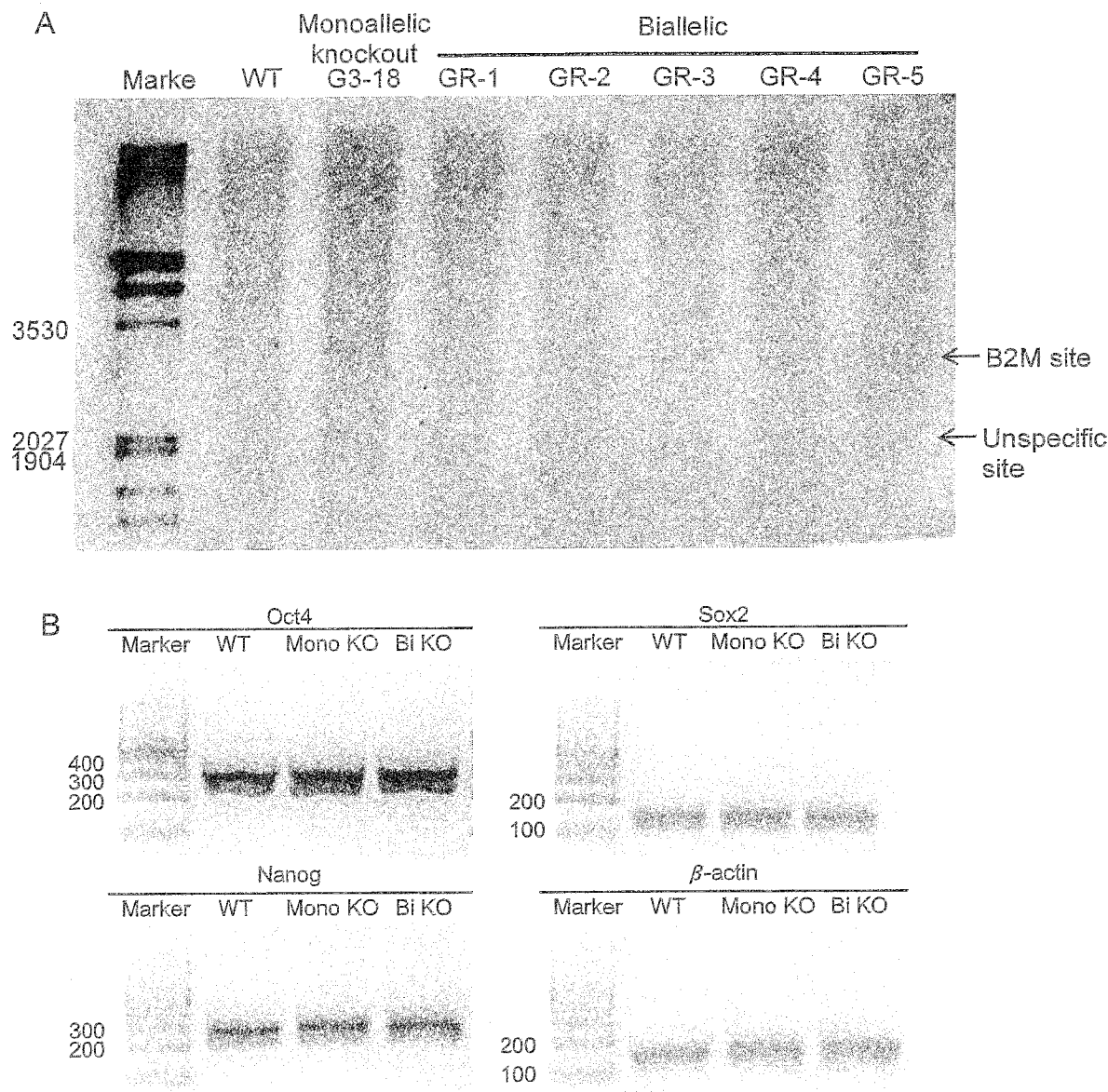
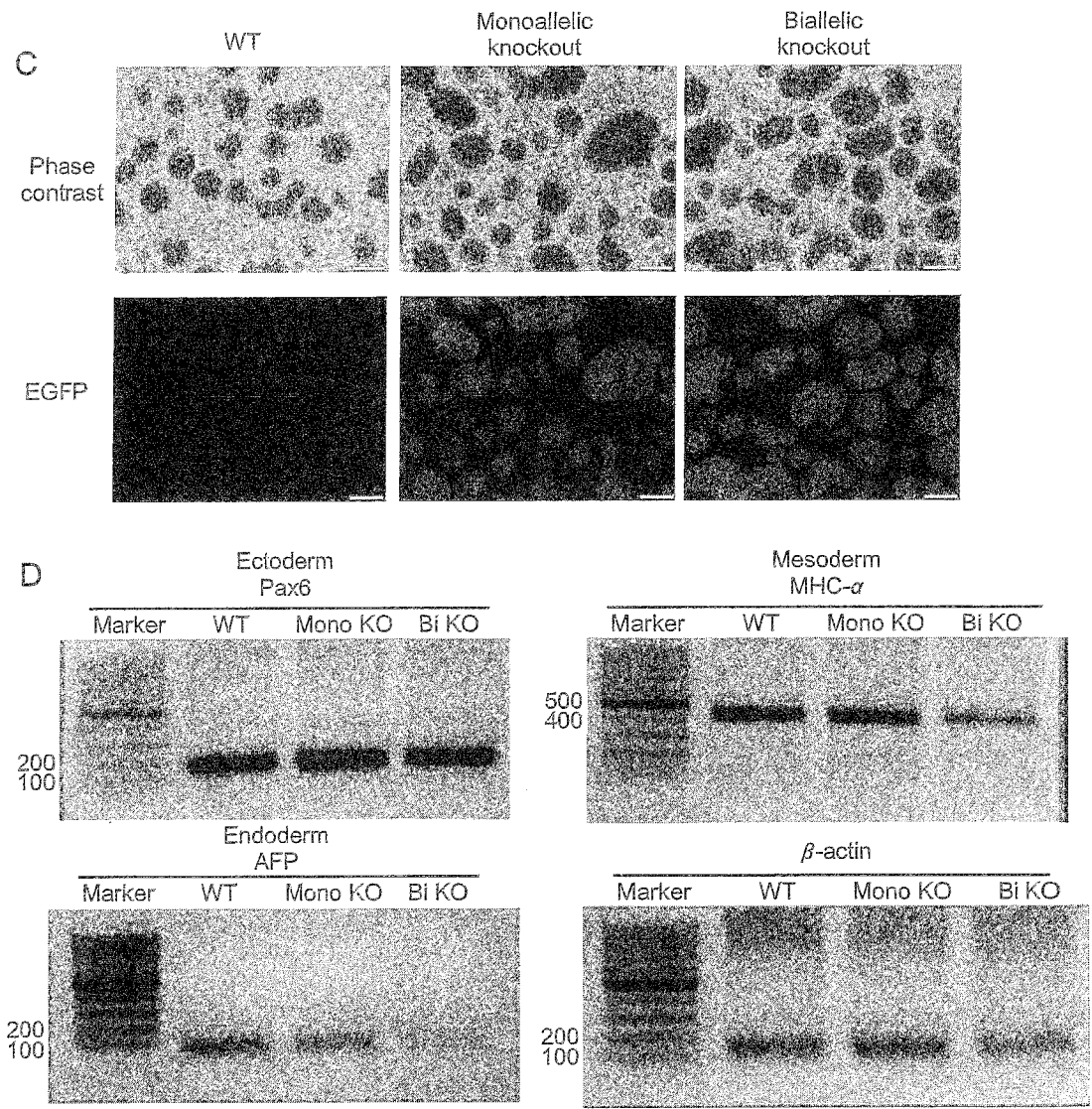


Figure 3 continued



5/11

Figure 4

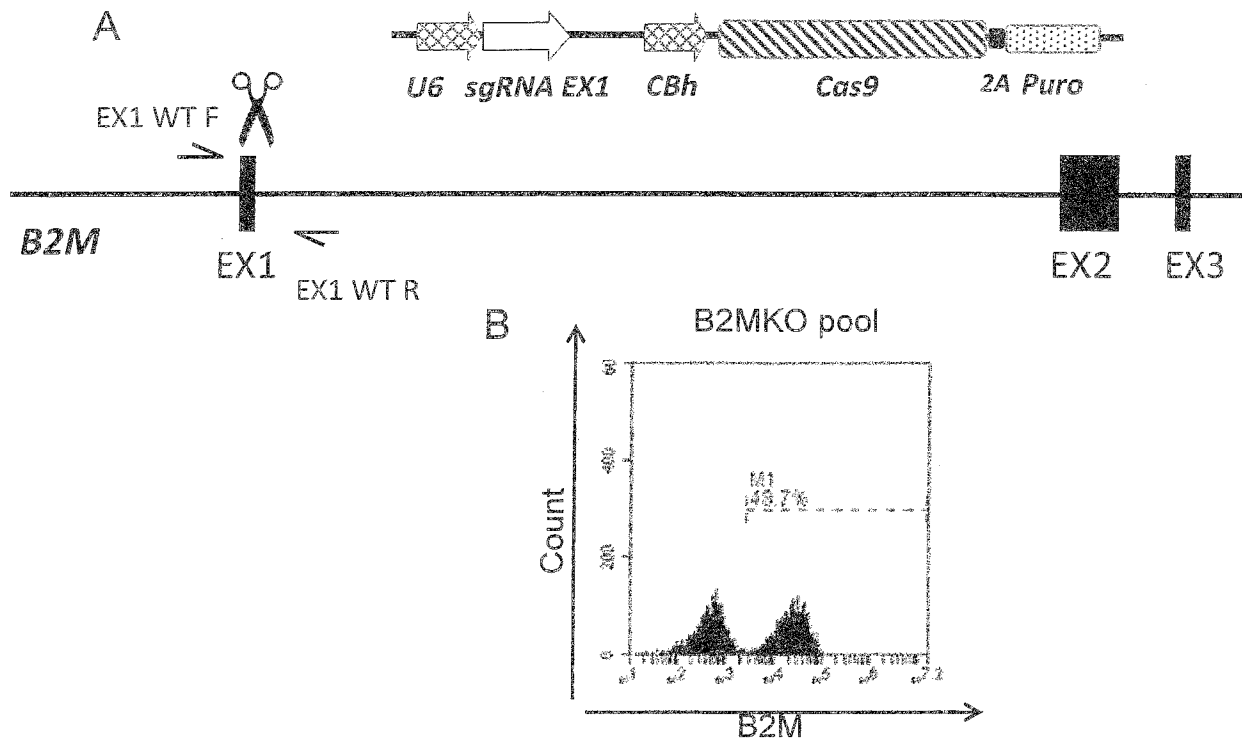
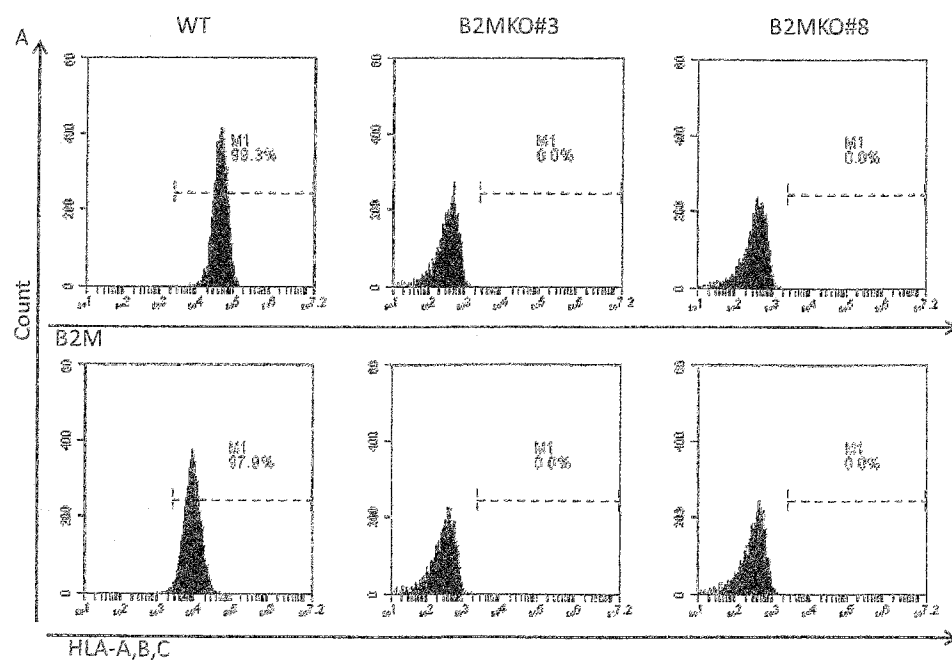


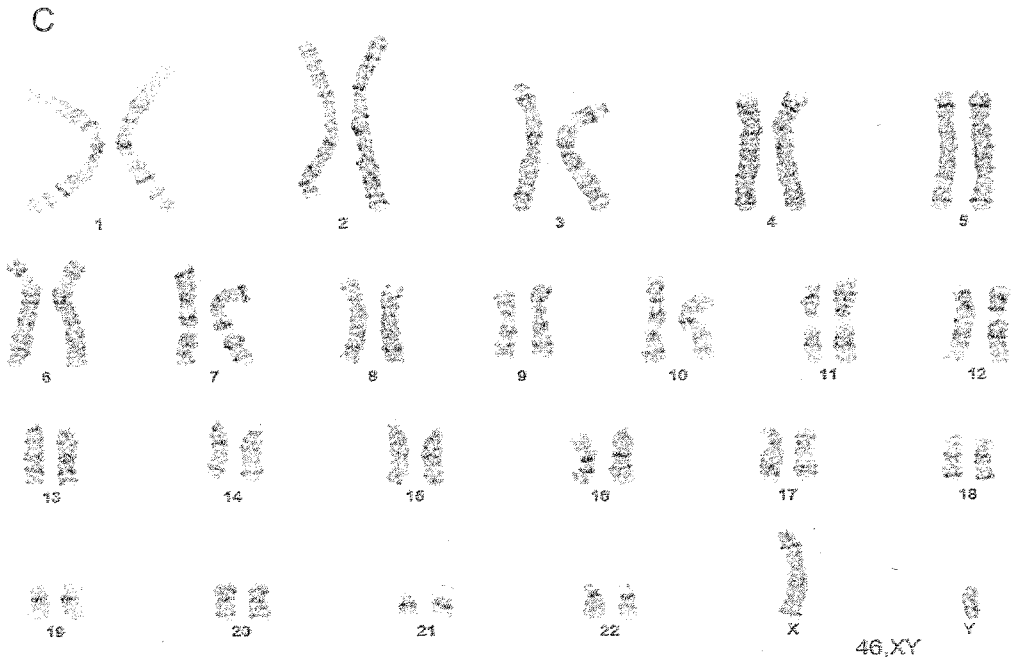
Figure 5



6/11

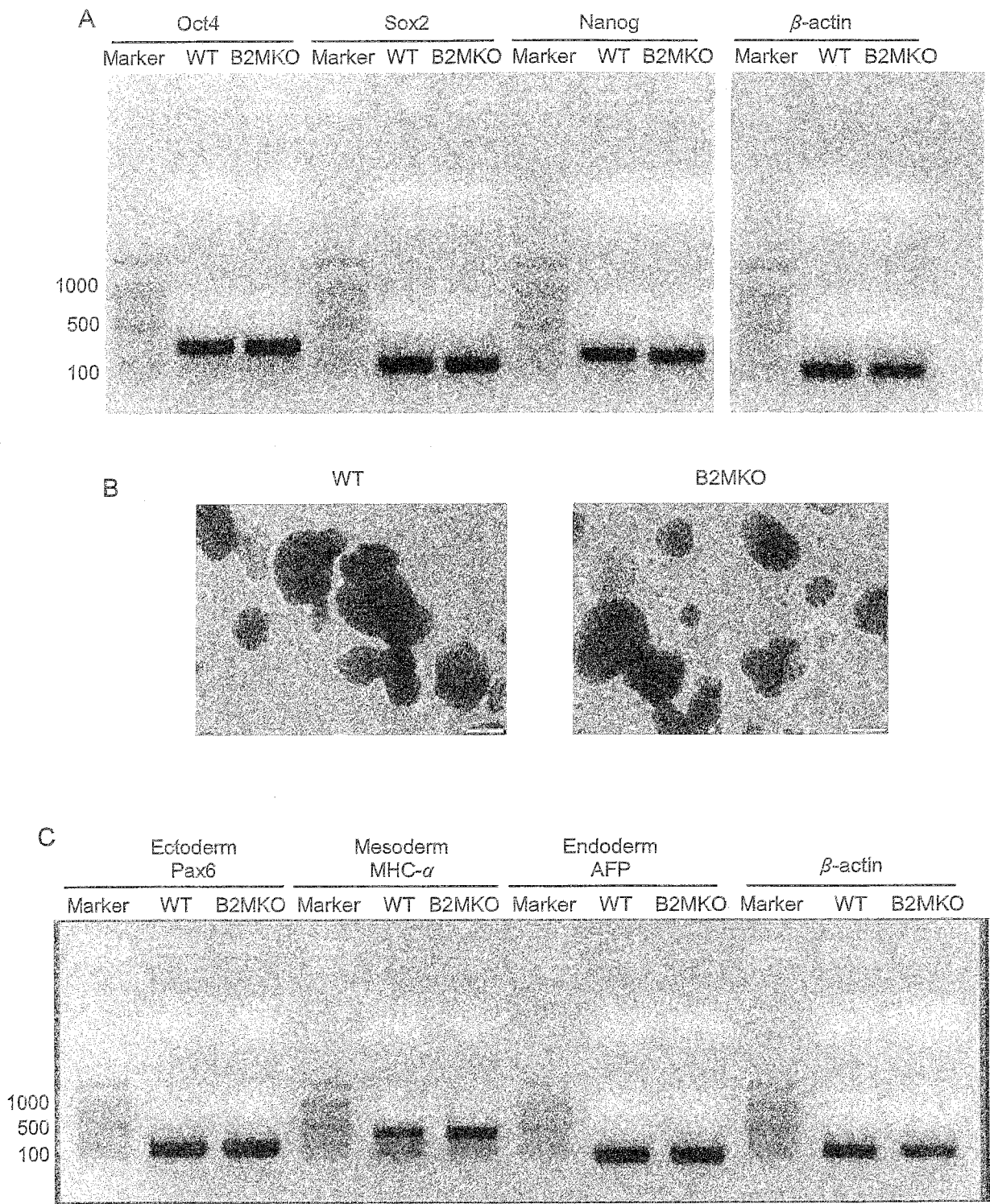
Figure 5 continued

B	WT	5'-CCGAGATGTCTCGCTCCGTGGCCCTTAGCTGTGCTCGCGCTACTCTCT-3' 3'-GGCACCAGGAATCGACACGAGCGC-5'	Target region Target sequence Start codon
B2MKO #3	TA-1	5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2
B2MKO #3	TA-2	5'-CCGAGATGTCTCGCTCCGTGGCCCTTAGCTGTGCTCGCGCTACTCTCT-3'	+1
B2MKO #3	TA-3	5'-CCGAGATGTCTCGCTCCGTGGCCCTTAGCTGTGCTCGCGCTACTCTCT-3'	+1
B2MKO #3	TA-4	5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2
B2MKO #8	TA-1	5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2
B2MKO #8	TA-2	5'-CCGAGATGTCTCGCTCCGTG--CCTTAGCTGTGCTCGCGCTACTCTCT-3'	-1
B2MKO #8	TA-3	5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2
B2MKO #8	TA-4	5'-CCGAGATGTCTCGCTCCGTG--CCTTAGCTGTGCTCGCGCTACTCTCT-3'	-1
B2MKO #3			
		5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2 allele 1
		5'-CCGAGATGTCTCGCTCCGTGGCCCTTAGCTGTGCTCGCGCTACTCTCT-3'	+1 allele 2
B2MKO #8			
		5'-CCGAGATGTCTCGCTCCGTGG---TTAGCTGTGCTCGCGCTACTCTCT-3'	-2 allele 1
		5'-CCGAGATGTCTCGCTCCGTG--CCTTAGCTGTGCTCGCGCTACTCTCT-3'	-1 allele 2



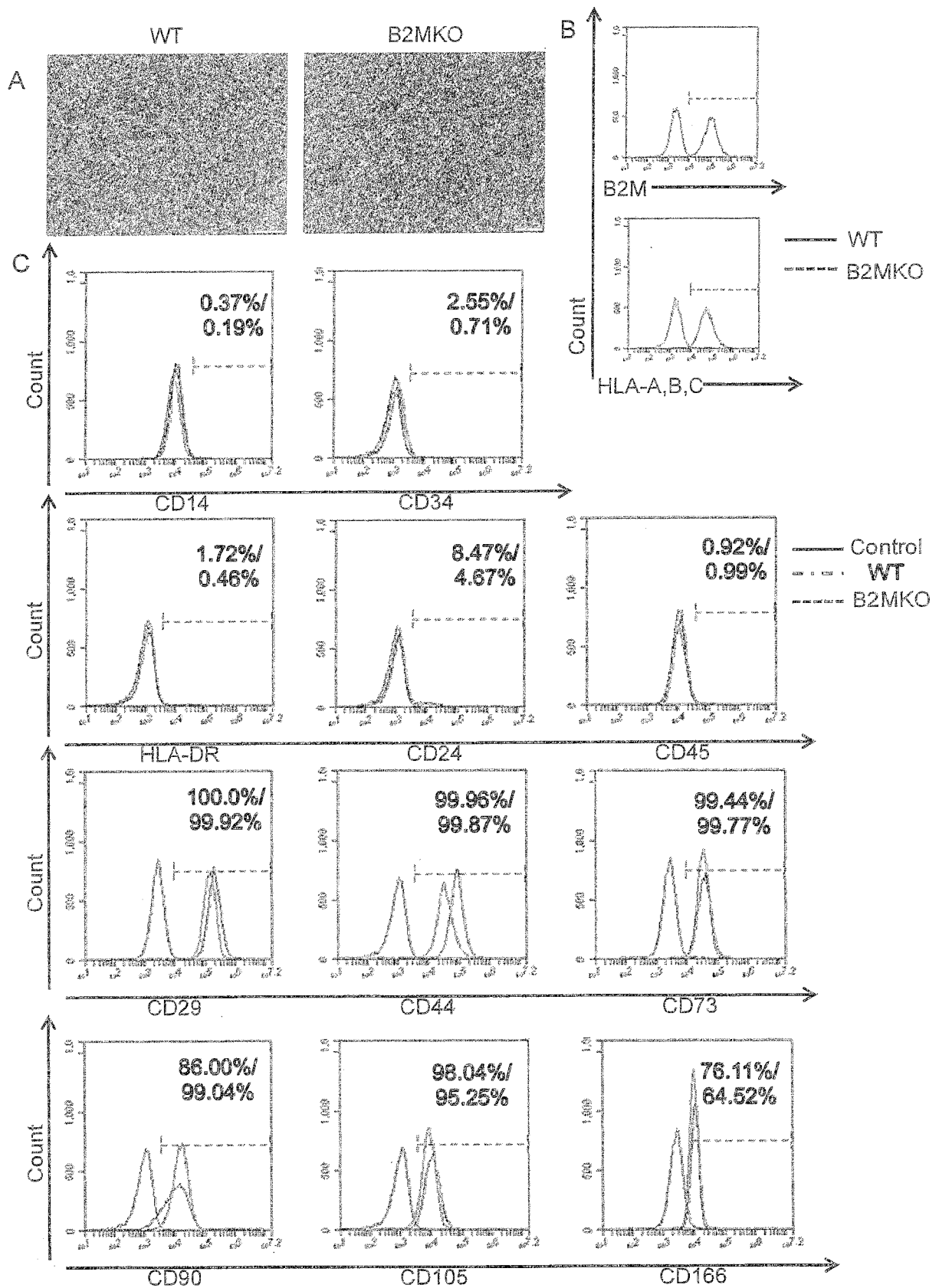
7/11

Figure 6



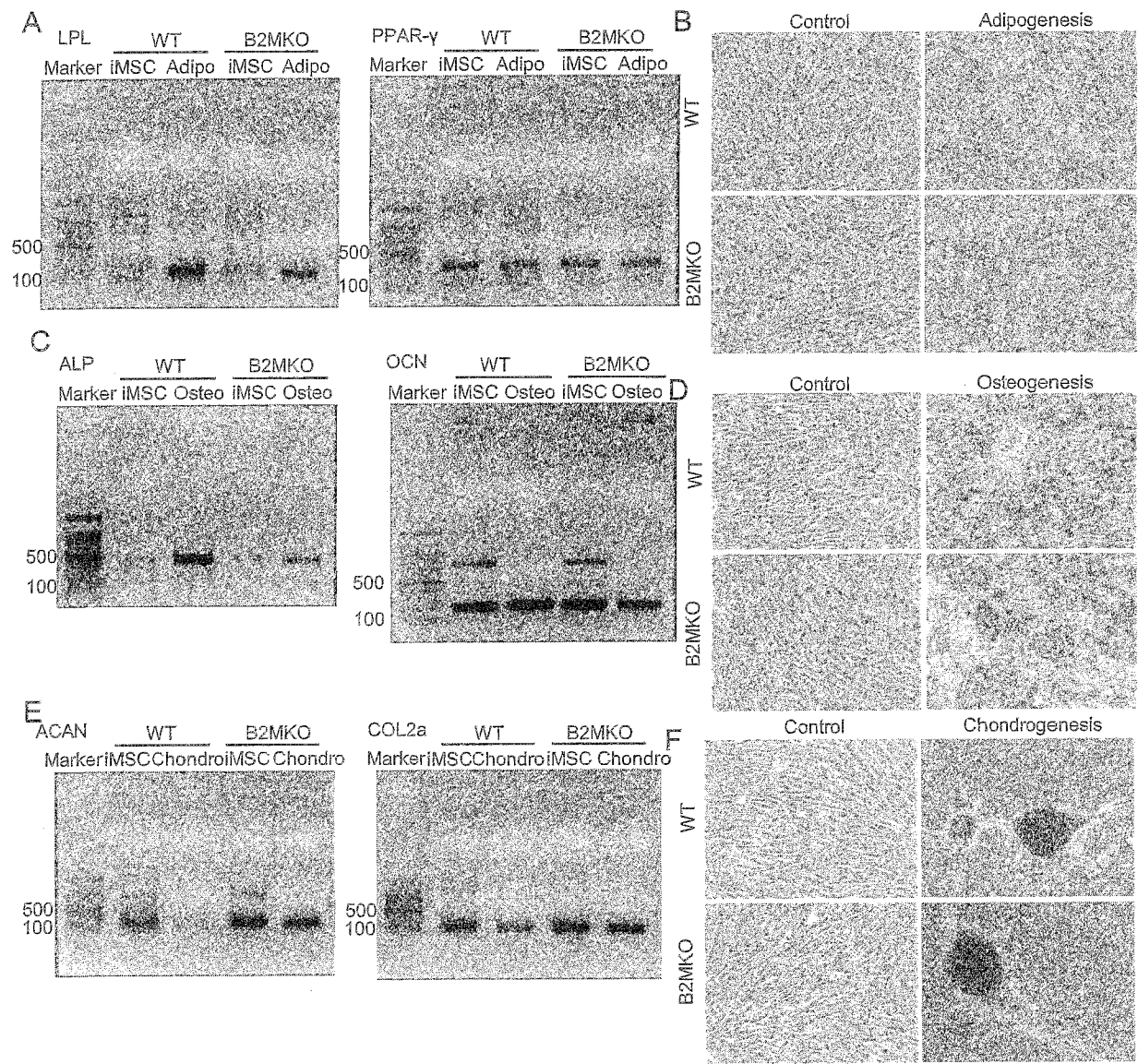
8/11

Figure 7



9/11

Figure 8



10/11

Figure 9

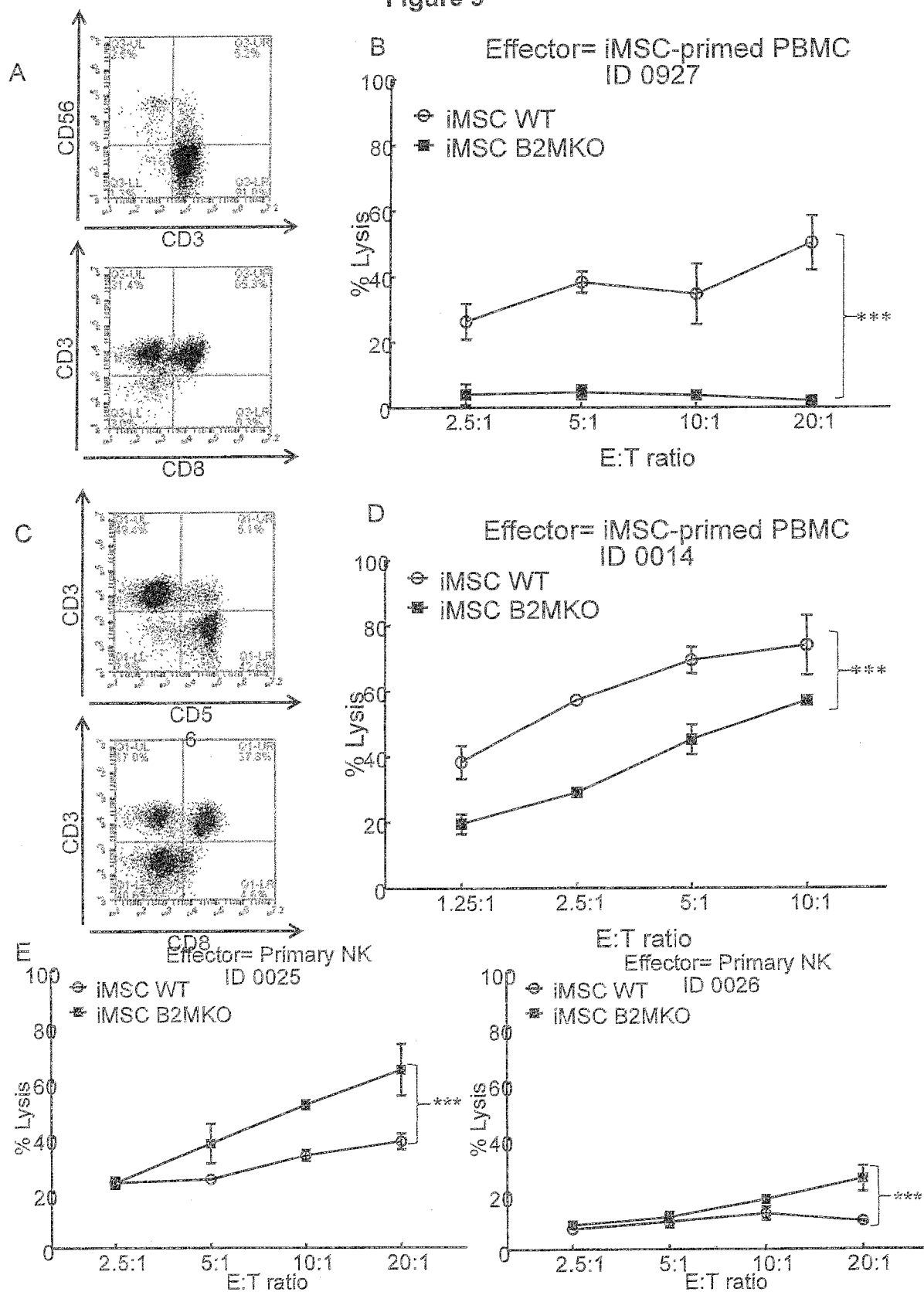
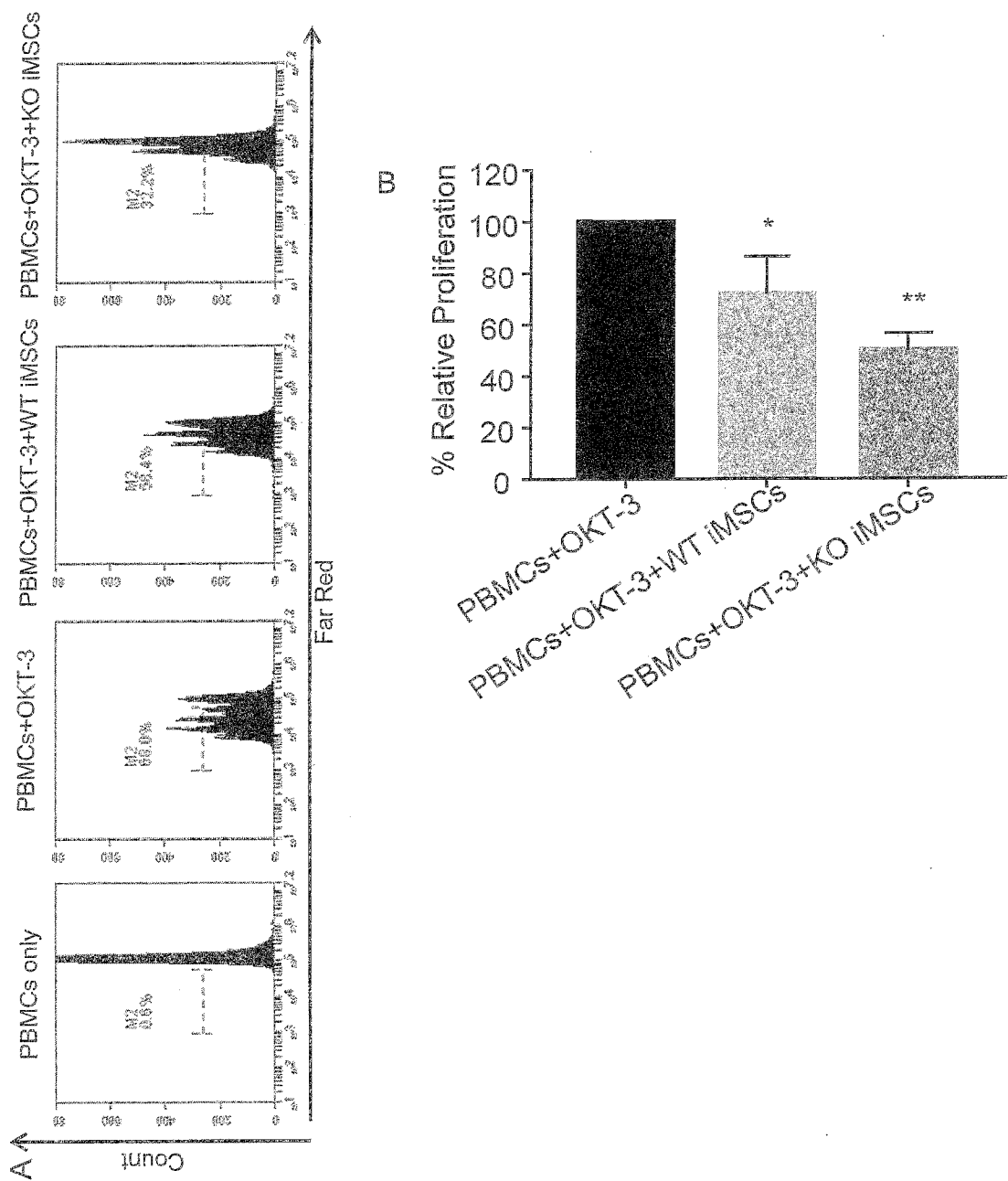


Figure 10



INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050454

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/0775 (2010.01) A61K 35/28 (2015.01)

According to International Patent Classification (IPC)

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

FAMPAT/CAPLUS/BIOSIS/MEDLINE/EMBASE: mesenchymal stromal cells, mesenchymal stem cells, immunogenicity, pluripotent stem cell, beta-2-microglobulin, bi-allelic mutation, and related terms.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/145384 A1 (UNIVERSITY OF WASHINGTON THROUGH ITS CENTER FOR COMMERCIALIZATION) 26 October 2012 Figure 4, Example 1, Para [00010], [00041], [00060]-[00063], [00074], [00092], Claims	1-21
X	XU X. ET AL., Efficient homology-directed gene editing by CRISPR/Cas9 in human stem and primary cells using tube electroporation. <i>Sci Rep.</i> , 3 August 2018, Vol. 8, No. 1, pages 11649:1-11 [Retrieved on 2019-10-30] <DOI: 10.1038/S41598-018-30227-W> Abstract, Page 5-6: Ablation of clinically relevant genes in primary human cells, Figures 4A, 5, 6B	12-15
X	WO 2014/139443 A1 (ZHEJIANG UNIVERSITY) 18 September 2014 Whole document, in particular Examples of the original non-English language document (a machine translation is enclosed only for your reference)	12-16

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

*Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30/10/2019

(day/month/year)

Date of mailing of the international search report

18/11/2019

(day/month/year)

Name and mailing address of the ISA/SG

Intellectual Property Office of Singapore
1 Paya Lebar Link, #11-03
PLQ 1, Paya Lebar Quarter
Singapore 408533

Email: pct@ipos.gov.sg

Authorized officer

Cheng Chang (Dr)

IPOS Customer Service Tel. No.: (+65) 6339 8616

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SG2019/050454

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2018/132783 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 19 July 2018 Whole document, in particular Paragraph [0075], Figure 7	1-21
A	FITZSIMMONS R. E. B. ET AL., Mesenchymal Stromal/Stem Cells in Regenerative Medicine and Tissue Engineering. <i>Stem Cells Int.</i> , 19 August 2018, Vol. 2018, pages 8031718: 1-16 [Retrieved on 2019-10-30] <DOI: 10.1155/2018/8031718> Pages 8-9, point 11	-
A	MANDAL P. K. ET AL., Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. <i>Cell Stem Cell.</i> , 6 November 2014, Vol. 15, No. 5, pages 643-652 [Retrieved on 2019-10-30] <DOI: 10.1016/J.STEM.2014.10.004> Whole document	-
A	WANG D. ET AL., Targeted Disruption of the β_2 -Microglobulin Gene Minimizes the Immunogenicity of Human Embryonic Stem Cells. <i>Stem Cells Transl Med.</i> , 18 August 2015, Vol. 4, No. 10, pages 1234-1245 [Retrieved on 2019-10-30] <DOI: 10.5966/SCTM.2015-0049> Whole document	-

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/SG2019/050454

Note: This Annex lists known patent family members relating to the patent documents cited in this International Search Report. This Authority is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012/145384 A1	26/10/2012	DK 2699593 T3 CA 2833173 A1 JP 2018174946 A US 2014/0134195 A1 JP 2014513948 A EP 2699593 A1	13/11/2017 26/10/2012 15/11/2018 15/05/2014 19/06/2014 26/02/2014
WO 2014/139443 A1	18/09/2014	CN 104046593 A	17/09/2014
WO 2018/132783 A1	19/07/2018	KR 20190103373 A CA 3049766 A1 CN 110177869 A AU 2018207649 A1	04/09/2019 19/07/2018 27/08/2019 11/07/2019