UNIVERSITY OF CALIFORNIA, SAN DIEGO

MHC Class I & II-Deficient Human Embryonic Stem Cell Line as a Promising Tool to Abrogate Allogenic Immune Rejection in Cell-Based Therapy

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by

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The thesis of Zheng Qian is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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ABSTRACT OF THE THESIS

MHC ClassI & II - Deficient Human Embryonic Stem Cell Line as a Promising Tool to Abrogate Allogenic Immune Rejection in Cell-Based Therapy

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Professor Xu, Chair
Professor Lu, Co-Chair

Human embryonic stem cells (hESCs) hold great promise for regenerative medicine as an unlimited source of cells for replacement of the tissues destroyed by injuries or diseases. One of the major impediments of fully realizing its poetical is the immune rejection from allogenic recipients. To overcome the histocompatibility, we established hESCs that lack the expression of Major Histocompatibility Complex (MHC)

class I or II, which are the primary mediators for allograft rejection. [31] Briefly, CRIPR-Cas9n was used to delete exon 1 of beta-2 micro globulin (B2M), leading to a complete deficiency of MHC I on the cell surface of hESCs. In the same manner, exon 2 and 3 of Class II transactivator (CIITA) were deleted, aiming to abrogate any potential of inducible MHC II expression in hESCs. Despite of this genetic manipulation, MHC I-deficient hESCs maintained its self renewal capacity, expressed pluripotent genes and were pluripotent as indicated by their ability to form teratoma in NSG mice. Moreover, we demonstrated that the teratoma derived from MHC I - deficient hESCs were immune protected in Hu-mouse reconstituted with allogenic human immune system, while the teratoma from the parental hESCs showed apparent tissue necrosis and a mount of T cell infiltration, an indication of immune rejection. All these findings indicate that our MHC I-deficient hESCs are hypoimmunogenic, and might be employed as an appealing source of cells for tissue replacement in cell-based therapy without requiring the long-term systemic immune suppression.

INTRODUCTION

A. Human Embryonic Stem Cell Therapy

Human embryonic stem cells (hESCs) are cells isolated from the inner cell mass of blastocyst-stage embryos, and are capable of propagating themselves continuously while maintaining their pluripotency to differentiate into all cell types. [1,2] hESCs and its derivatives hold great promise in cell-based therapy to replace the tissues destroyed by injuries or diseases such as cardiomyocytes [3], pancreatic B cells [5], pneumocytes [6], neurons [4], etc. Since 1998 when the first hESCs line was established in Thomas lab, hESCs-based therapy has made significant progress in regenerative medicine. [1] In 2010, Dr. Wang's lab showed the therapeutical potential of alveolar type II epithelial cells derived from hESCs (hES-ATIICs) to treat acute lung injury in a immune-compromised SCID mouse. [6] In his study, with the treatment of hES-ATIICs, all mice subjected to acute lung injury showed improvement on symptoms such as restored respiration and survived, while all control mice were reported to suffer severe respiratory failure and deceased from lung injury. These findings suggested great therapeutic potential of hESCs derivatives to replace the damaged tissue.

While hESCs-based therapy is promising, one key bottleneck to its clinical application is the immune rejection of hESC-derived cells by allogenic recipients. [11] Many initial studies suggested that hESCs derivatives are highly immunogenic, and will

elicit robust allograft rejections during the transplantation. [7, 11, 28] To overcome the immune rejection, long-term immunosuppression medicine is often administrated to recipients. However, it is reported that immunosuppressed organ allograft recipients have an increased risk of developing cancers.[9] Therefore, we proposed to generate hypoimmunogenic hESCs as an alternative approach to overcome the allograft rejection of hESCs-derivatives without treating the recipients with immunosuppressive regime.

B. Hypoimmunogenic hESCs generation by knocking out MHC Class I and II expression.

MHC proteins are displayed on cell surface of hESCs, and are considered as a primary mediator to initiate and maintain an alloreactive immune response through presentation of antigen to T cells. [11, 31] It can be divided into 2 classes. Class I is found in all of the nucleated cells, while class II is restricted to antigen-presenting cells (APCs) such as B cells, macrophage, and dendritic cells. Both Grinnemo's and Drukker's labs reported that hESCs express a low level of MHC class I molecules on cell surface which is sufficient to elicit rapid cytolytic T cell killing response in allogenic or xenogeneic recipient. [7,8] Thus, to address the immunogenicity of hESCs, we first aimed to generate a hESCs line deficient of MHC I expression on its cell surface. To do so, we proposed to functionally disrupt the expression beta-2 microglobulin (B2M), a monomorphic light chain of MHC I, which is necessary for MHC I's presentation on cell membrane of hESCs to function. [10]

Some earlier studies showed that hESCs don't express MHC II before or after differentiation [7,8], yet there is accumulating evidence suggesting that MHC II expression can be induced in cell types that normally don't express MHC class II, such as epithelial cells and schwann cells. [15, 16] Besides a low level of mRNA encoding MHC class II molecules were detected in hESC according to Grinner's lab, indicating the potential of MHC II expression in hESCs. [7] Thus, in order to generate hypoimmungenic hESCs, MHC II proteins must be depleted from the cell surface of hESCs as well. To do so, we proposed to disrupt Class II MHC Transactivator (CIITA) gene whose expression is both necessary and sufficient for MHC class II expression. [17]

C. Gene Editing in hESCs using CRISPR/Cas9 Nickase

An effective gene-targeting technology to disrupt, over-express, or repair genes is critical in basic biology research and regenerative medicine. For a long time, Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been considered as two most efficient site-specific genome editing tools. [22] These two chimeric nucleases are composed of a programmable and sequence-specific DNA-binding domain and a nonspecific DNA cleavage domain [22,23], which can bring the nuclease to a specific genome site to introduce a DNA double-strand break (DSB) and to stimulate cellular DNA repair mechanisms homology-directed repair (HDR) or non homologous end joining (NHEJ) [24]. Despite of their ability to customize the DNA-binding module to recognize mostly any sequence, ZFNs and TALENs have several

limitations such as high risk of off-targeting mutations and time-consuming experimental design. [21]

Recently, a new gene-targeting technology, denoted the clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) system, has emerged as an efficient and user-friendly alternative to ZFNs and TALENs. [18] Distinct from these two site-specific nucleases, CRISPR utilizes DNA-RNA recognition to introduce a DSB instead of DNA-protein recognition. Since 2013 when it was first introduced by Zhang's lab, CRISPR has been successfully applied in gene editing of ESCs. [19, 26] While promising, Cas system is still facing concerns such as the off-target mutation effect. [25] To reduce the off-target, a D10A mutant nickase version of Cas9 (Cas9n) has been generated. Compared to wild type Cas9, Cas9n offers a higher ratio of HDR to NHEJ, resulting lower off-target mutations. [25] To achieve precise gene targeting via HDR without random integration, Cas9n system was thus employed in our present studies to disrupt B2M and CIITA gene in hESCs.

D. Humanized Mice Model

While immune system has been extensively studied in mice model, human immune response to allografts is still poorly understood due to the significant difference between mouse and human immunity. Immune system is species specific, and data drawn from mice model is often found conflicting to clinical data and thus can't be applied in developing therapeutics for human. [29] Therefore, it's crucial to use a physiologically

relevant model to better understand human immune response to allogenic transplanted hESCs.

To address this difficulty, we used humanized mice (Hu-mice) reconstituted with functional human immune system by transplanting human fetal thymus fragments and CD34+ liver cells into immunodeficient NOD/SCID/IL-2y -/- (NSG) mice as described in Rong's paper [28]. (figure 2) This Hu-mice model is well constituted with human T and B cells to mount robust allo-immune responses, and has been successful employed to study human immune response to allogenic hESC grafts. [27, 28, 34]

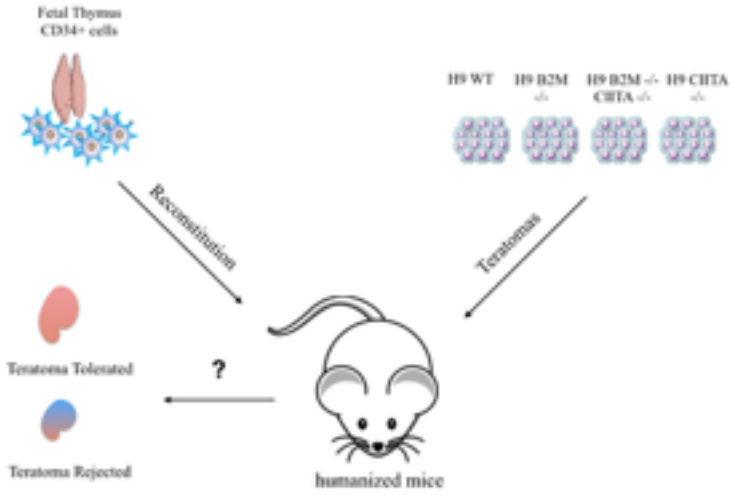

Figure 1: **Strategy to study the immunogenicity of MHC- Null hESCs in humanized mice model.** Human fetal thymus and autologous CD34+ fetal liver cells were transplanted into NSG mice to generate Hu-mice. H9 WT, H9 B2M -/-, H9 B2M-/- CIITA -/-, and H9 CIITA -/- are injected into four hind legs separately in one Hu-mice to form teratoma.

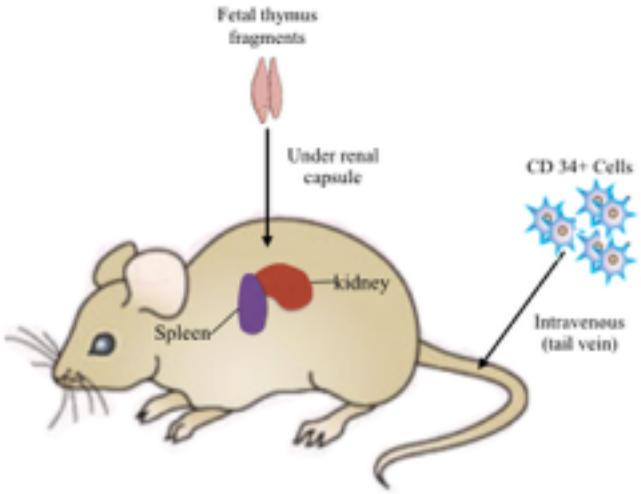

Figure 2: **Approaches for the engraftment of human immune system in immunodeficient mice.** To develop Hu-mice, fetal thymus fragments was implanted under the renal capsule in irradiated adult NSG mice, and CD34+ liver cells are injected intravenously through the tail vein.

MATERIALS AND METHODS

Construction of B2M-Targeting Vector and CIITA-Targeting Vector

The B2M-targeting vector and the CIITA-targeting vector were both constructed by reengineering the CRISPR/Cas9 nickase vector with a IRES-Puro-pA fragment and the F5 flanked selection cassette as described in Rong's paper, 2012. (19) Briefly, the CRISPR/Cas9 nicks vector was digested with BaeI overnight. The guideRNA fragments targeting B2M and CIITA were ligated into the BaeI site of CRISPR/Cas9 nickase vector by using gibson assembling. The guideRNA fragments targeting B2M are 'cctgtcatgtttaacgtcct' denoted as g1 and 'tagctgtgctcgcgctactc' denoted as g2. The guideRNA fragments targeting CIITA are 'catgtgcctggcgtatagg' denoted as g1 and 'ggtgcttcctcaccgatat' denoted as g2.

Cell Culture for hESCs

The hESC line, HuES 9, was cultured on CF-1 mouse embryonic fibroblast feeder (CF-1) layer in DMEM F12 (1;1) supplemented with 1% penStrep, 2mM Glutamate, 0.1 mM nonessential amino acids, 10 ng/ml basic fibroblast growth factor, and 55 uM beta-mercaptoethanol. hESCs were dissociated with Collegenase IV and passaged on CF-1 feeder with 1:9- 1:13 dilution. All tissue culture reagents were purchased from life technologies unless indicted elsewhere. All human ESC work has been approved by UCSD institutional ESCRO and IRB.

Generation of B2M-knockout hESCs

To target B2M gene, approximately 3.2 million of hESCs were resuspended in 500 µl phosphate-buffered saline (PBS), mixed with 6 µg of CRISPR-Cas9n B2M targeting vector, and then electroplated at 320 V and 200 uF. The transfected cells were evenly placed on 3 CF1- coated 6-cm plates in MEF-conditioned hESC medium. After 48 hours, the transfected cells were selected with 0.5 µg/ml puromycin (puro) for 48 hours. The stably transfected hESC colonies that survived puromycin selection were picked and screened by PCR analysis to identify double B2M allele-targeted hESC clones (B2M-/- hESCs). To generate B2M & CIITA- null hESCs, the selected B2M -/- hESCs # 22 cell line was prepared and transfected with CIITA targeting vector as described above.

PCR Analysis of B2M-Targeted and CIITA-targeted hESC Clones

Genomic DNA was isolated from hESC clones. To identify B2M-/- hESC clone, the following primers were used: forward primer (F1): ggactccaccaccacgaaat; forward primer 2 (F2): ccagtctagtgcatgccttct; reverse primer (R): gacgcttatcgacgccctaa. The B2M wild type PCR fragment is 575 bp and 1530 bp, while the B2M knockout fragment is 427 bp. To screen for CIITA-/- hESC clones, the following primers were used: forward primer (F): aagcagtagagcattgtggt; reverse primer (R): tcttccctggagtctccgtt. The CIITA wild type PCR fragment is 1195 bp and the CIITA knockout fragment is 349 bp.

Reverse Transcription-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction.

Total RNA was isolated from wild type hESCs, B2M-/- hESCs # 22 with PureLink RNA Mini Kit (12183025, Life Technologies). Total RNA (4 μg) was reversely transcribed into cDNA and analyzed by quantitative real time PCR with FastStart Universal SYBR Green Master (ROX) (Roche). To examine the B2M mRNA expression levels, the following B2M specific primers located in exon 2 was used: qB2M -F: 5' GGC TAT CCA GCG TAC TCC AA3' qB2M- R: 5' ACG GCA GGC ATA CTC ATC TT 3'. To examine the pluripotency of B2M-null hESCs clone #22, their relative mRNA levels of the hESC-specific pluripotent genes and those of wide type hESCs and H460 were determined. The following pluripotent genes were tested: NANOG, OCT3/4. SOX2, LIN28, REX1, TDGF-1, GABRB3, DNMT3B. For all the real-time quantitative PCR assays, the average threshold (Ct) was determined for each gene and normalized to GAPDH mRNA levels.

Flow Cytometry Analysis (FACs)

To detect MHC I surface expression in B2M -/- hESCs, hESCs were stained with 1:50 diluted mouse anti-human major histocompatibility complex I (BD Bioscience), which recognizes HLA-A, HLA-B, HLA-C. To analyze T cell infiltration of teratomas, the digested single cells from teratomas were stained with PE-conjugated anti-hCD3 antibody (12-0038, eBioScience), APC-conjugated anti-hCD19 antibody (555485, BD Pharmingen). All of the samples were measured on a BD LSR-II, and the analysis was performed using FACS Diva software (Becton Dickinson).

Differentiation of B2M-Null hESCs into Cardiomyocytes

A small molecule-driven cardiomyocyte differentiation protocol was used. [32] B2M-/- hESCs were cultured on Matrigel-coated plates in differentiation medium (DM) which contains 80% knockout0DMEM, 20% fetal bovine serum (FBS), and 1% nonessential amino acid, and 1 mM L-glutamine. When confluence was reached, cells were plated with GSK3 inhibitor CHIR99021 in RPMI/B27-insulin medium for 24 hours, and subsequently changed to RPMI/B27-insulin. From day 7, B2M-/- hESCs were cultured in RPMI/B27 medium.

Teratoma Formation and Immunohistochemistry Analysis

To examine the pluripotency and immunogenicity of MHC I - deficient hESCs, B2M -/- hESCs and parental hESCs were resuspended at 10 million in 100 μl of hESC medium and injected subcutaneously into the right and left hind legs of the isoflurane-anesthetized NSG mice or immunocompetent Hu-mice respectively. Two month after implatation, tumors were measured and surgically dissected from the euthanized mice. Teratomas were fixed in 10% (V/V) buffered formalin, embedded in paraffin and sectioned as described in Zhao's paper in 2011. [33] The sections were stained with hematoxylin and eosin for histological analysis. For frozen samples, teratomas were embedded in optimal cutting temperature compound (OCT) and sectioned. Sections were fixed with 4% (V/V) paraformaldehyde in PBS for 15 mins at room temperature (RT). After being washed three times with PBS, cells were incubated in 0.3% Triron-X-100 in

TBS for 10 mins at RT. Coverslips were mounted on the glass slides using Vectashieled mounting medium for fluorescence. The stained sections were examined and the images were captured using an Olympus confocal microscope.

CHAPTER 1: Generation of MHC Class I & II deficient hESCs.

Introduction

Histoincompatibility is a major obstacle of hESCs-based therapy. [7] To address the histoincompatibility issue, we proposed that an abolition of surface-expressed MHC I and MHC II on hESC-derived cells could mitigate their immunogenicity to T cells from allogenic recipients during stem cell transplantation. To test this possibility, we generated a hESC line whose expression of MHC class I or MHC class II was completely ablated. To do so, we sought to disrupt B2M gene encoding the MHC class I light chain [10] and CIITA gene whose expression is found to be sufficient to induce MHC class II expression. [17] To introduce precise gene targeting via HR, CRISPR/Cas9 nickase was used. [19] As shown in figure 3, exon 1 of B2M gene on chromosome 15, exon 2 and 3 of CIITA gene on chromosome 16 were deleted.

A. PCR Analysis son the disruption of B2M and CIITA gene in hESCs.

To first generate B2M -/- hESCs, parental hESCs were transfected with the B2M -targeting vector, and then plated onto three CF-1 coated 6-cm plates with puro selection for 48 hours. Of 3.2 million transfected hESCs, there were 27 colonies survived after puro selection. From PCR analysis, 4 of the 27 selected colonies were found to contain

both WT fragment (1530 bp, 575 bp) and KO fragment (427bp), indicating that the CRISPR-Cas9n vector has correctly targeted one of the B2M alleles, and 1 of the 27 selected colonies, B2M -/- hESCs 22, was found to contain only 427bp KO fragment, indicating that both of the B2M alleles has been correctly disrupted.

Similarly, to generate CIITA -/- hESCs, 3.2 million parental hESCs 9 were transfected with the CIITA targeting vector. From PCR analysis, 8 of the 31 selected colonies were found to contain both WT fragment (1195 bp)and KO fragment (349 bp), and only 1 out of the 31 selected colonies, CIITA -/- hESCs 27 was found to contain only KO fragment (349 bp), indicating that both of the CIITA alleles have been disrupted.

In order to generate MHC I & II - deficient hESCs, the established B2M-/- hESCs was subsequently transfected with the CIITA - targeting vector. Of 4 million transfected B2M-/- hESCs, 30 colones were picked and screened for successful abolition of both alleles of CIITA. According to the PCR analysis, 7 out of 30 clones contained both WT fragment (1195 bp) and KO fragment (349 bp), and 3 clones, B2M-/- CIITA -/- #1,#7, #9, contained only KO fragment (349 bp).

B. CRISPR-Cas9n vector introduced precise gene editing, yet low targeting efficiency.

To examine the off-target mutations that might be introduced by CRISPR Cas9n, the KO fragments of all identified B2M-/- hESCs, CIITA -/- hESCs, and B2M-/- CIITA-/- hESCs were sent for DNA sequencing test. According to the sequencing data, CRISPR

Cas9n introduced precise deletion of exon 1 of B2M gene and exon 2 and 3 of CIITA gene with no random insertion of the targeting vector sequence. (figure 5)

While CRISPR-Cas9n offered a lower rate of off-target mutation, as anticipated it has a relatively low targeting efficiency as compared to wild type Cas9. The targeting efficiency of CRISPR-Cas9n to disrupt B2M or CIITA genes was summarized in table 1. In agreement with Rong's findings, the efficiency of Cas9n to correctly target both alleles is around 5%.

Based on PCR analysis screening as shown in figure 4 a & b , MHC-Class I deficient hESCs (H9 B2M -/- 22), MHC-Class II deficient hESCs (H9 CIITA -/- 27) and MHC Class I &II deficient hESCs (H9 B2M-/- CIITA-/- 7 were generated and was further investigated for their immunogenicity and pluripotency.

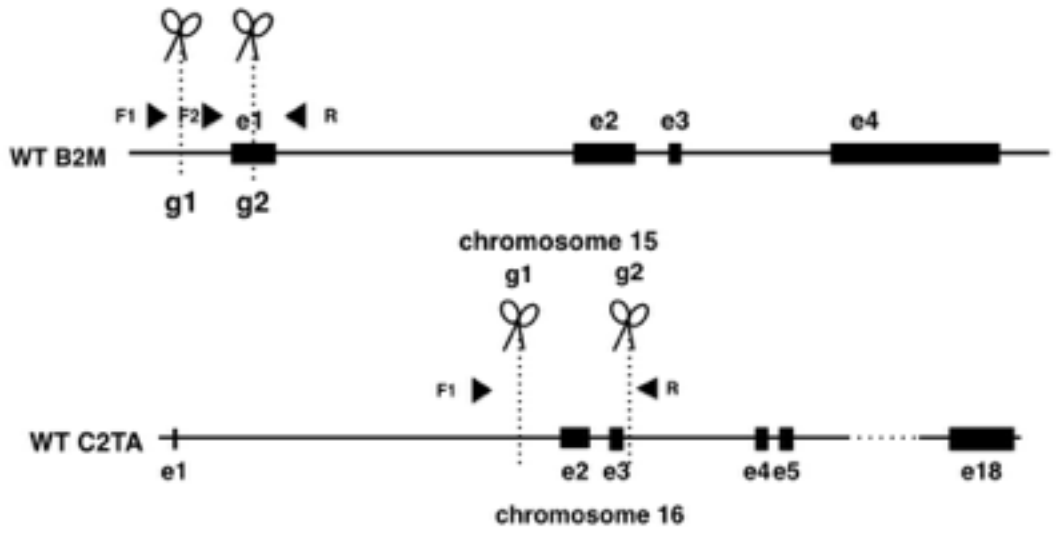

Figure 3 : **CRISPR-Cas 9 targeting strategy of B2M and CIITA knock-out in hESCs.** Filled boxes indicate the coding sequence in exons of B2M on chromosome 15 and those of CIITA on chromosome 16. The guide RNA cutting sites and the binding site of PCR primers used to identify knockout clones were also indicated.

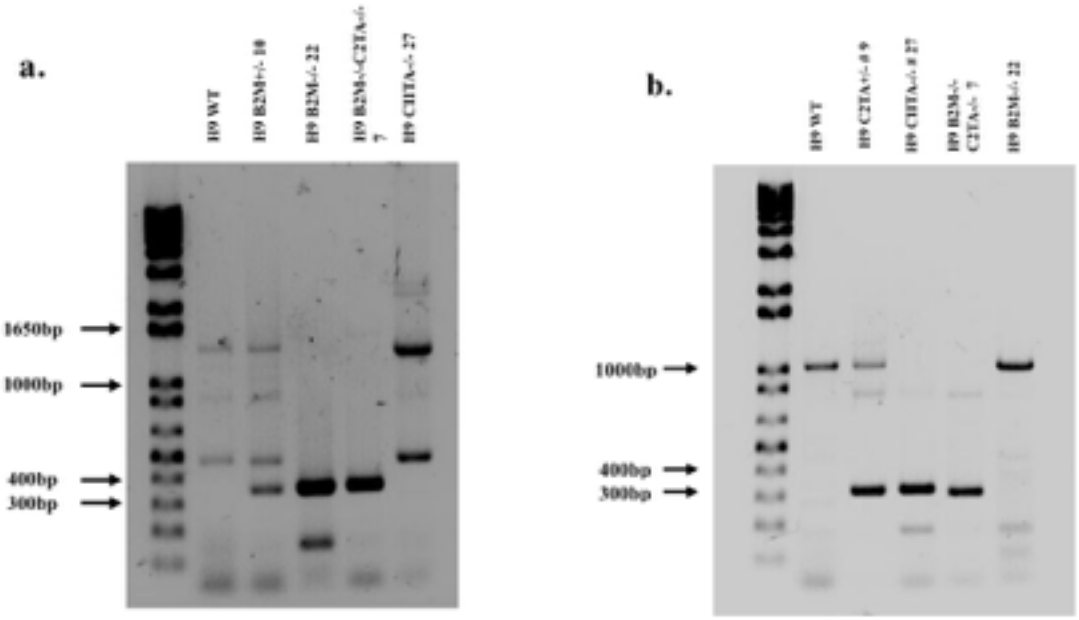

Figure 4: **PCR analysis on B2M-CIITA- Targeted hESCs.** (a) PCR analysis of hESCs clones from the B2M targeting vector transfection showed 575bp, 1530 bp WT and 427bp KO B2M fragments. (b) PCR analysis of CIITA-targeted hESCs clones showed 1195 bp WT and 349 bp KO CIITA fragments.

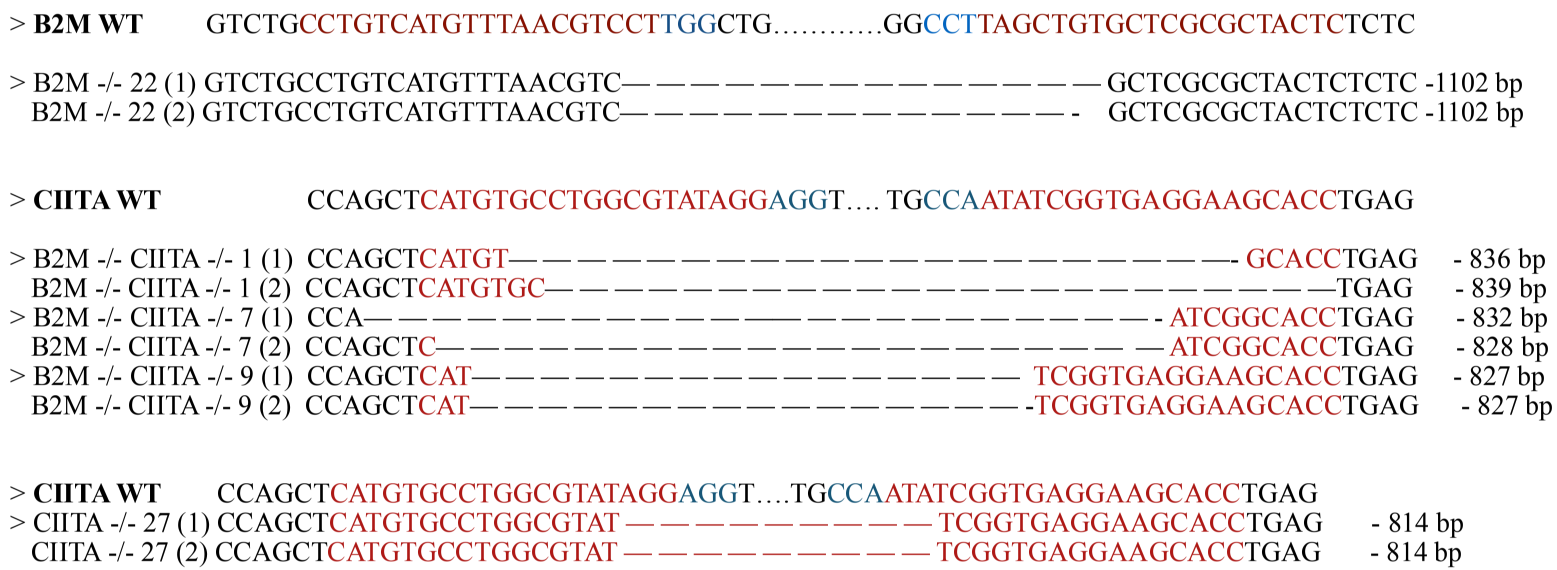

Figure 5: **Alignment of the genomic sequences of mutants and wild types at CRISPR cutting sites.** The wild type is shown at the top with the PAM sequence highlighted in blue, and the guideRNA targeting sequence in red. The dot line in B2M WT sequence represents 1081 basepairs not shown, and that in CIITA WT sequence represents 821 basepairs not shown. The dash line in KO sequence represents the deleted bases. The net change in length is noted to the right side of each sequence.

Table 1: **Efficiency of generating B2M- null hESCs and CIITA-Null hESCs using CRISPR/Cas9 nickase.**

Gene Targeted	No. of clones analyzed	Heterozygous	Homozygous	Targeting Efficiency
B2M	27	4 (14.8%)	1 (3.7 %)	18.5%
B2M & CIITA	30	7 (23.3%)	3 (10.0%)	33.3%
CIITA	31	8 (25.8%)	1(3.2%)	29.0%

CHAPTER 2: Investigation of MHC I expression in B2M -/- hESCs.

Introduction

hESCs have been reported to exhibit immunogenicity when transplanted into allogenic recipients. [7,8,11] Largely, its immunogenicity is due to the amount of MHC I proteins presented on cell surface of hESCs, which will slightly increase upon differentiation and will dramatically increase when treated with IFN-y., a typical inflammation factor released in the course of immune response. [7,8] Recognition of MHC I molecules by cytotoxic T cells can induce vigorous killing of hESCs and its derivatives, leading to robust allogenic immune rejections.

Previously, qPCR result suggested that our B2M -/- hESCs expressed no stable B2M mRNA, and thereby should have eliminated the chances that MHC I protein could be synthesized and displayed on the surface of this B2M- null hESCs line and its derivatives. (figure 6) To test this possibility, the surface expression of MHC I in B2M-/- hESCs was examined using FACs to ensure a complete depletion of MHC I on cell surface.

In contrast, the expression of MHC II molecules was not observed in hESCs regardless of differentiation or cytokine treatment. [7,8] Therefore, in our studies, the

surface expression of MHC II was not investigated in MHC II-deficient hESCs that we have established.

A. RT-PCR analysis on the mRNA expression level of B2M and CIITA in hESCs.

To evaluate B2M mRNA expression in homologous (B2M -/- 22) hESCs, real-time PCR was performed on both B2M-/- hESCs, parental hESCs and h460 which is a human lung cancer cell line. B2M-specific primers located at exon 2 which is an undisrupted exon was used as described in Materials and Methods. B2M mRNA expression was clearly detectable in wild type H9 and was lower compared to h460 by approximately 6 folds. This low level of B2M mRNA expression in wild type H9 compared to that in h460 agrees with the notion that without stimulation of IFNy, undifferentiated hESCs are to some degree immune-privileged as they express a low level of MHC I molecules on surface which decreases the chance of being rejected. [8] As anticipated, no B2M mRNA expression was detected in the B2M -/- hESCs (figure 6). This lack of B2M mRNA expression in B2M -/- hESCs indicated that the disruption of B2M gene at exon 1 has completely abrogated B2M mRNA transcription in hESCs.

B. FACs analysis showed that surface MHC I expression lacking on B2M -/- hESCs.

B2M molecule is covalently associated with the alpha-chain of MHC I, and has been reported necessary for surface MHC I presentation. [10] Therefore, it is anticipated that the disruption of B2M gene will result a complete loss of MHC I proteins expressed on the cell surface of hESCs. To test this possibility, FACs was performed to analyze

surface expression of MHC I protein on B2M -/- hESCs and its parental hESCs using an anti-human MHC-I antibody. Flow cytometry analysis demonstrated that MHC I protein expression level on B2M-/- hESCs was approximately 5 fold lower compared to its parental hESCs, which is in agreement of the result from other paper (12). These data suggested that the functional knockout of the B2M gene had led to surface MHC-I protein deficiency.

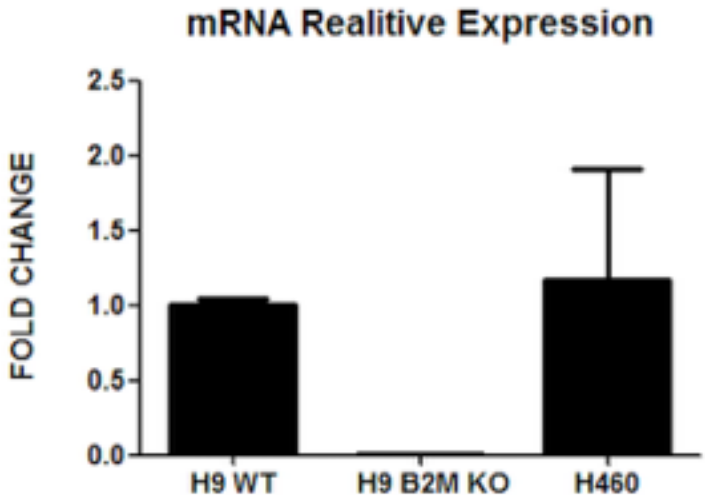

Figure 6: **RT- PCR analysis on mRNA expression level of B2M in MHC I -deficient hESCs.** B2M-specific quantitative reverse transcriptase-PCR was performed using total RNA isolated from both H9 wild type and H9 B2M -/- 22. Bar graph depicts RNA expression level of B2M in the cultures.

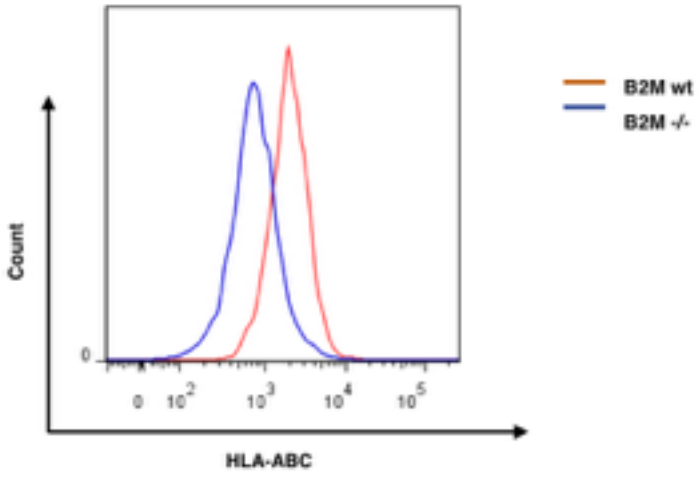

Figure 7: **The lack of surface expression of HLA class I molecules in B2M-null hESCs was confirmed by FACs.**

CHAPTER 3 : Self-renewal capacity, and pluripotency of MHC I deficient hESCs.

Introduction

Isolated from the inner cell mass of blastocyst-stage embryos, hESCs can undergo unlimited self-renewal and retain its pluripotency to differentiate into all cell types in our body. [1,2] hESCs thus hold a great promise in regenerative medicine as a renewable cell source for replacement of tissues injured or diseased. In order to develop hESCs into a safe and effective human therapeutic, extensive effort has been devoted to reduce the immunogenicity of hESCs. Current advances in gene editing, such as CRISPR and TALEN, make such genetic manipulation possible in hESCs. In our studies, we used CRISPR-Cas9n to disrupt B2M gene in hESCs by introducing a deletion at exon 1 on both B2M alleles. (figure 3) While CRISPR Cas9n offers a low off-target mutations [19], the existing possibility of introducing random off-targets and integration of transgene still poses risk of impairing of self-renewal capacity and pluripotency of hESCs, rendering the genetic modified hESCs lose its advantage as an unlimited cell source. Thus, teratoma formation and *in vitro* assay was performed to examine the self renewal ability and pluripotency of MHC I deficient hESCs.

A. *In vitro* self-renewal capacity and pluripotency of MHC I - deficient hESCs.

To test its long-term self-renewal capacity *in vitro*, B2M -/- hESCs were continuously cultured in hESC- supporting conditions as described in Material and Methods. The cell line has been propagating while maintaining its defined morphology as the wild type hESCs did for the entire experimental duration, from January 2017 to now, indicating its self-renewal capacity *in vitro* was not affected.

To examine the pluripotency of B2M-/- hESCs in vitro, RT-PCR was performed to evaluate the mRNA of pluripotent genes, such as Oct 4, Nanog, Sox 2, Dnmt3b, Lin 28, Gabrb3, Tdgf1, in undifferentiated wild type hESCs, B2M-/- hESCs, and h460. As shown in figure 8, the mRNA expression of all of the pluripotent genes was clearly detectable in both undifferentiated wild type and B2M-/- hESCs at approximately the same level, indicating that the knockout of B2M gene in hESCs did not affect mRNA expression of these pluripotent gene. In contrast, as anticipated, none of the pluripotent mRNA expression was detected in H460.

In addition, the pluripotency of MHC I deficient hESCs in vitro was further confirmed when it has been successfully differentiated into cardiomyocytes *in vitro*. (data not shown).

B. *In vivo* self-renewal capacity and pluripotency of MHC I - deficient hESCs.

To test the pluripotency and self-renewal capacity of MHC I - deficient hESCs *in vivo*, B2M-/- hESCs and parental hESCs were implanted intramuscularly into right and left hind legs of NSG mice respectively. Teratomas were harvested and examined two months after injection as described in Materials and Methods.

The size of tumor (1.1 x 0.6 x 0.8 cm) derived from B2M -/- hESCs was similar to that (0.8 x 0.8 x 0.9 cm) derived from parental hESCs, suggesting that B2M -/- hESCs had similar proliferation rate as its parental hESCs and has maintained its self-renewal capacity *in vivo* despite of the disruption of its B2M gene. (figure 9.A.)

Moreover, H&E histological analysis was performed on its teratoma formed in NSG mice to test the pluripotency of B2M -/- hESCs. A large variety of cell types from all three germ layers were observed after hematoxylin-eosin (HE) staining such as cartilage, gut-like epithelium and rosette. (figure 8.B.) These data suggested that MHC I deficient hESCs maintained its capacity to differentiate into all cell lineages *in vivo*.

Taken together, all these findings suggested that despite of the deletion of exon 1 on both B2M alleles, B2M -/- hESCs still maintained its unlimited self-renewal capacity, expressed pluripotent genes, were pluripotent *in vivo* as indicated by their ability to form teratoma in NSG mice.

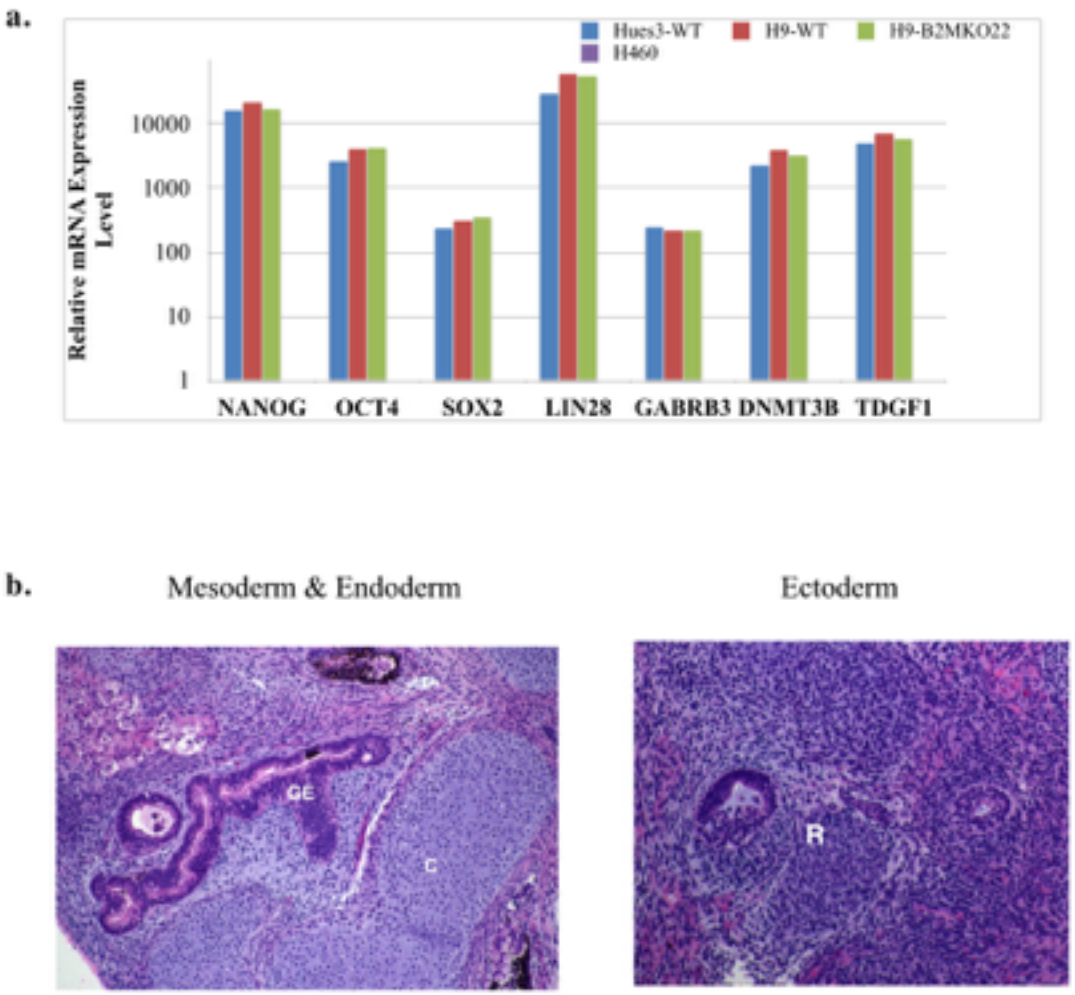

Figure 8: **The pluripotency of MHC class I-deficient hESCs *in vitro*.** a) The relative mRNA levels of hESC-specific genes in B2M-/- hESCs, parental hESCs, and negative control H460 cancer cells were determined by RT-qPCR. the mRNA levels in h460 are arbitrarily set to 1. b) H&E staining of teratomas removed from B2M-/- hESCs and parental hESCs in NSG mice. Three germ layers were identified. C, cartilage; GE, gut-like epithelium; R- rosette.

CHAPTER 4: Immunogenicity of MHC I- deficient hESCs *in vivo*.

Introduction

The mismatch of MHC molecules between hESCs-derived cells and the recipient gives a rise to the allograft rejection, which has been considered as a major impediment to the clinical development of hESCs-based therapy. MHC molecules are responsible for activating both CD4+ T cells and CD8+ cytotoxic T cells, eliciting vigorous immune response to allogenic tissues. There is accumulating evidence that the adaptive immune system, especially the T lymphocytes, is central to allograft response during the transplantation, and the highly polymorphic MHC molecules are a major mediator of the immune rejections in allogenic recipients. [31, 33] Thus, we hypothesized that our established MHC I deficient hESCs line would shield the histoincompatibility of hESCs, avoid being recognized CD8+ cytotoxic T cells, and thus mitigate its immunogenicity even upon differentiation or stimulation of IFNy. To test this possibility, we took advantage of hESCs's capacity to form teratoma in Hu-mice to examine the immunogenicity of various cell types derived from B2M -/- hESCs *in vivo*.

A. Teratoma formed by MHC I-deficient hESCs were protected from allogenic immune rejection.

MHC I -deficient hESCs were injected into the right hind leg region of Hu-mouse. As an internal control, the parental hESCs were injected into the left hind leg region of the same Hu-mouse. While both MHC I -deficient hESCs and parental hESCs formed teratomas in Hu-mouse two months after implantation, apparent tissue damage were observed in the teratoma from parental hESCs. (figure 9.a) Although the size of teratoma derived from hESCs (1 x 1.1 x 1 cm) is similar to that from B2M -/- hESCs (1.1 x 1.2 x 0.8 cm), the teratoma from hESCs was primarily consisted of liquid cyst and few cells. The fact that both MHC I -deficient hESCs and parental hESCs formed teratomas of similar size and composition in NSG mice showed that MHC I -deficient hESCs maintain intact pluripotency and self renewal capacity as the parental hESCs. (figure 9.A.) Therefore, the regression of parental hESCs teratoma was due to immune rejection.

The teratomas were further analyzed histologically using H&E staining and FACs to detect the rejection. The teratoma from parental hESCs showed extensive tissue necrosis with an elevated level of human T cell infiltration, which is a hallmark of immune rejection. (figure 9.a.b.c.) In contrast, many tissues were detected within parts of MHC I-deficient hESCs-derived teratoma (figure 9.c), and the number of human T cell infiltrated into B2M -/- hESCs- derived teratoma is only 28% of that in parental hESCs-derived teratoma, (figure 9.b) indicating that teratomas from hESCs was protected from immune rejection.

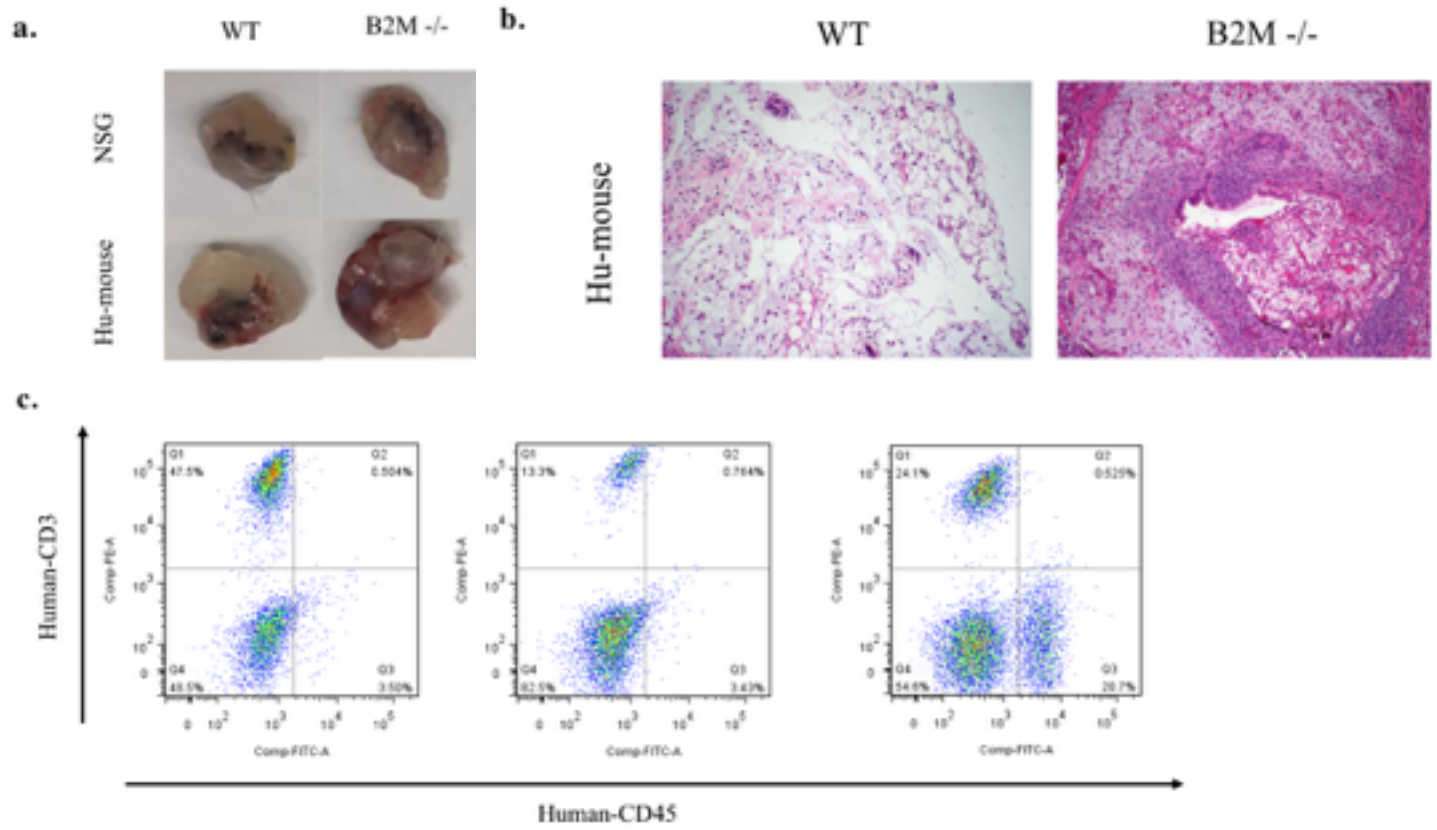

Figure 9. **MHC I-deficient hESCs are tolerated from allogenic immune rejection in Hu-mouse. a)** Images of teratomas derived from parental hESCs and B2M -/- hESCs formed in NSG mice and Hu-mice. WT hESCs and B2M-/- hESCs were injected subcutaneously into the left and right hind leg region of the mice. Two month after the implantation, teratomas were surgically removed and examined as shown above. b) H&E staining of teratomas removed from B2M-/- hESCs and parental hESCs in Hu-mouse. B2M -/- hESCs form teramotas in Hu-mouse which contains a large variety of cells derived from the three germ layers. Apparent tissue necrosis was detected within parts of teratoma formed by parental hESCs. **c)** Flow cytometry analysis showed less T cell infiltration in teratoma formed by B2M-/- hESCs than those formed by wild type hESCs.

DISCUSSION

The immune rejection of hESCs-derived cells in allogeneic recipients remains a key bottleneck for its clinical application. MHC-I expression, known as a primary mediator of allograft rejection, was analyzed in initial studies as an indicator of immunogenicity of hESCs. [8] hESCs was reported to have a reduced expression of MHC I as compared to somatic cell lines, suggesting that hESCs might be immune privileged and thus less likely to be rejected by allogeneic immune system. [8] In agreement with these studies, our results also showed that parental hESCs in its undifferentiated stage expressed a significantly lower level B2M mRNA, and a diminished amount of MHC I proteins on cell surface as compared to H460. (figure 6, 7.) All these findings demonstrated that parental hESCs exhibit reduced immunogenicity. However, as it undergoes differentiation into adult somatic cells, parental hESCs slowly takes on characteristics of an adult cells including an increased expression level of non-self marker, MHC I, and MHC I expression can be dramatically elevated upon the stimulation of inflammatory cytokine IFN y released in the course of immune response, further promoting immune rejection. [12, 14] Therefore, hESCs-derived cells are highly immunogenic, which was also indicated in our *in vivo* studies when apparent regression and extensive tissue necrosis were observed in the teratoma derived from parental hESCs. Therefore, to realize hESCs' potential as an unlimited cell source in hESCs-based therapy, its immunogenicity in allogenic recipients must be addressed.

To overcome the histoincompatibility of hESCs derivatives, we established a stable MHC I- deficient hESCs line whose surface expression of MHC I has been completely ablated. To do so, we deleted the exon 1 of B2M gene in parental hESCs using CRISPR Cas9n. We employed Hu-mice model with a functional human immune system to evaluate the immunogenicity of MHC I -deficient hESCs and its derivatives. According to our pilot *in vivo* studies, this genetically modified hESCs can be protected from the allogenic immune response, giving arise a great promise to be developed into a 'universal donor hESCs' in regenerative medicine.

While our present study showed MHC I -deficient hESCs are effective in evading the immune rejections mediated by alloreactive T cells, the lack of MHC I proteins expressed on its cell surface may render MHC I -deficient hESCs susceptible to natural killer (NK) cell-mediated killing. NK cells express inhibitory receptors, which recognize MHC I molecules expressed on all nucleated cells, and activating receptors which recognize specific NK activating ligands such as NKP46, MICA. The the balance of activating and inhibitory signals determines whether NK cells will initiate lysis of targeted cells. Previous studies have shown that parental hESCs expresses a low but detectable amount of MHC I, and a low amount of activating ligands namely NKp46, NKG2D, MICA, MICB, suggesting that hESCs has the potential to be targets for allogeneic NK cells. [8, 38, 37] Many studies done in mice model have demonstrated the susceptibility of hESCs to NK cells as they can delay the teratoma growth after transplantation, and impair the growth of hESCs in vitro. [37] Moreover, Wang in 2014

showed that an elevated level of NK cells- mediated killing was observed to B2M-null hESCs line when transplanted into immunocompetent mice, indicating that the lack of MHC I molecules expressed on cell surface of our MHC I deficient hESCs can activate NK cells and trigger its cytotoxic activity, which is called 'missing self' phenomenon. [28] However, none of the studies is done in a human physiologically relevant model, and the clear difference between murine and human immune system may give a conflicting data regarding to the NK cells' cytolytic activity against hESCs during allogeneic transplantation. To investigate the immunogenicity of our MHC I deficient hESCs, we employed Hu-mice reconstituted with a function human immune system which bridged the gap between murine and human immunity. In our pilot *in vivo* studies, we demonstrated that MHC I deficient hESCs were tolerated by allogeneic immune system as indicated by well-formed teratoma in Hu-mice and no evident regression that might be due to NK cells killing was detected which is conflicting to previous studies done in mice model. [28, 37] Therefore, the role of NK cells in allogeneic immune rejection of hESCs is worth of investing in the future.

Besides of the NK cells killing, cancer risk is another concern raised in our established MHC I - deficient hESCs. Without MHC I molecules to activate alloreactive T cells, this genetically modified hESCs line are able to evade the immune surveillance, growing rapidly without any suppression even if it's infected or mutated. To make MHC I deficient hESCs a safe cell source for hESCs-based therapy, a strategy to eliminate the immune tolerated hESCs derivatives after transplantation must be achieved. One possible

approach is to incorporate a ‘suicide gene’ in the genome of genetically modified hESCs, which encodes an enzyme that leads to cell death only when activated by a specific compound. One example of such suicide gene is the herpes simplex virus thymidine kinase (HSVTK) gene which has been tested in numerous experimental and clinical settings as a biosafety mechanism to eliminate a selective group of cells. [36] HSVTK converts non-toxic ganciclovir (GCV) into GCV-triphosphate which can be incorporated into the DNA of replicating cells, terminating DNA synthesis and resulting in cell death. It has been reported that HSVTK knock-in hESCs can be efficiently eliminated by GCV administration, solving the safety issue of immune evasive hESCs after transplantation. [34] Therefore, to address the tumorigenesis issue, we might further incorporate a HSVTK gene as a safety check point for our MHC I -deficient hESCs.

CONCLUSION

We have generated stable B2M-/- hESCs, CIITA-/- hESCs, and B2M-/- CIITA -/- hESCs. All these hESCs lines contain no off-target integration nor random mutation. The established B2M-/- hESCs line expresses no stable B2M mRNA, presents no MHC I proteins on its cell surface and exhibits intact self-renewal capacity and pluripotency both *in vitro* and *in vivo*. Our pilot in vivo study demonstrated that the teratoma derived from B2M-/-hESCs were immune protected in allogenic humanized mouse, while the teratoma from parental hESCs were subjected to robust immune rejection. These findings indicate that B2M-/- hESCs-derived cells exhibited much reduced immunogenicity compared to parental hESCs in allogenic recipients, and thus hold great therapeutic potential as a 'universal donor' hESCs in regenerative medicine.

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