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Generating Hypoimmunogenic Human Embryonic Stem Cells by the Disruption of Beta 2-Microglobulin

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Abstract Immune rejection hinders the application of human embryonic stem cells (hESCs) in transplantation therapy. Human leukocyte antigens (HLAs) on the cell surface are the major cause of graft rejection. In this study, we generated HLA class I-deficient hESCs via disruption of beta 2-microglobulin (β2m), the light chain of HLA Class I. We found that HLA class I proteins were not present on the cell surface of β2m-null hESCs. These cells showed the same pluripotency as wildtype hESCs and demonstrated hypoimmunogenicity. Thus, HLA class I-deficient hESCs might serve as an unlimited cell source for the generation of universally compatible "off-the-shelf" cell grafts, tissues or organs in the future.

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Introduction

Since the first human embryonic stem cells (hESCs) were established in 1998 [1], hESC-based replacement therapy has provided great promise in future transplantation medicine because these cells could theoretically serve as a potentially inexhaustible source of all cell types in the body. Many protocols have described the generation of directed differentiated cells [2–4], and three-dimensional structural tissues from hESCs have been successfully established in vitro [5, 6].

However, immune rejection often occurs between genetically unrelated individuals during cell, tissue or organ transplantation, and this is a bottleneck that hinders the clinical applications of hESCs [7]. Although autologously induced pluripotent stem cell (iPSC) technology [8, 9] provides a potential approach to avoid immune rejection, reprogramming-associated mutations and genetic instabilities have raised safety concerns regarding this technique [10–12]. Moreover, the generation of individualized iPSCs for each patient is impractical, and the cost of establishing and maintaining iPSC [13] or hESC banks [14] that could fulfill at least partial HLA matching would also be high. Furthermore, the different characterizations among these iPSC or hESC lines in the banks also hinder the development of standard differentiation protocols. Thus, generating hypoimmunogenic and universally compatible hESCs might be a feasible approach to promote hESC-based therapy and realize hESC-related large-scale industrialized production.

HLA is a cell surface molecule that is encoded by a large gene family and can be divided into class I and class II molecules. HLA class I molecules are found on the surface of every nucleated cell; however, class II molecules are found only on antigen-presenting cells (APCs) and some

lymphocytes [15]. HLA mismatch between donor cells and the recipient's immune cells during transplantation often results in immune rejection [7]. Classical HLA class I complexes structurally consist of a polymorphic heavy chain consisting of HLA class I peptides (including HLA-A, HLA-B and HLA-C) and a light chain β2m. In the absence of β2m, class I HLAs cannot be properly assembled and are not presented on the cell membrane where they function [16, 17]. In this study, we generated hypoimmunogenic hESC lines by the disruption of β2m expression and depletion of HLA from the cell surface in hESCs.

Materials and Methods

Cell Culture

Irradiated CF1 feeder cells were seeded onto Matrigel-coated culture flasks at a density of 30,000 cells/cm^2 for 2 days prior to hESC passage. The culture medium for the feeder cells consisted of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10 % fetal bovine serum (FBS; Hyclone). The culture medium was then changed into hESC medium just prior to hESC passaging. The hESCs were maintained in hESC medium, which contained DMEM/F12 (Invitrogen) supplemented with 20 % knockout serum replacement (Invitrogen), 4 ng/mL basic fibroblast growth factor (bFGF; Invitrogen), 2 mmol/L L-glutamine (Invitrogen), 1 % nonessential amino acids (Invitrogen) and 0.1 mmol/L β-mercaptoethanol (Sigma-Aldrich). The hESCs were passaged approximately once a week. Collagenase IV was used to dissociate the cells from the feeders as cell clumps. The hESCs were then washed twice with hESC medium until the clumps were dissociated to an appropriate size before being passaged onto newly prepared feeder cells. The cryopreservation media for the hESCs consisted of 20 % qualified embryonic stem cell FBS (Gibco), 70 % hESC medium and 10 % dimethyl sulfoxide (DMSO). 293 T cells were maintained in DMEM (Invitrogen) supplemented with 10 % FBS (Hyclone).

TALEN Efficiency Detection

The TALENs were constructed with FastTALE TALEN Assembly Kit (Sidansai). The constructed TALEN constructs were transfected into 293 T cells. The genomic DNA of these 293 T cells was harvested 3 days after transfection. Next, PCR and sequencing were performed to examine the efficiency of the TALENs.

Derivation of HLA Class I-Deficient hESC Lines

H1 or X1 was used to establish the HLA class I-deficient hESC lines. First, the most efficient TALENs were transfected into hESCs using the FuGENE HD transfection reagent (Promega) in feeder-free culture. The transfected colonies were dissociated into single cells using TrypLE (Invitrogen) 5 days post-transfection and seeded onto CF1-coated plates at a density of 500 cells/cm^2. Two weeks after passaging, the colonies derived from the single cells were transferred into fresh CF1-coated wells, and in parallel, a direct cell PCR kit (F140, Life Technologies) was used to identify the mutants.

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

Total RNA was prepared using an RNeasy kit (Qiagen), which was then used as a template for real-time polymerase chain reaction (RT-PCR). RT-PCR was performed in an Eppendorf Mastercycler® ep realplex real-time PCR system using SYBR Green-based PCR Master mix (TOYOBO). Standard curves were acquired for both the gene of interest and internal control (G3PD). The primers used are listed in Supplementary Table 1.

Western Blotting

Cells were harvested, and the following antibodies were used: anti-beta 2-microglobulin (1:1000, Santa Cruz Biotechnology), anti-HLA-A/B/C (LY5.1, 1:1000, Santa Cruz Biotechnology) and anti-GAPDH (1:5000, Abcam).

FACS

hESCs were dissociated using TrypLE, and 5×10^5 cells were stained for 30 min at 37 °C in 100 μL of 0.5 % FBS in PBS containing an appropriate dilution of phycoerythrin (PE) or PE-CY7-conjugated antibody. Primary antibodies included human beta 2-microglobulin (BD Biosciences) and HLA -A, -B and -C (BD Biosciences). The sample measurement was performed on a FACSCalibur flow cytometer system, and the analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Immunostaining and Cell Transplantation

The primary antibodies used were anti-Oct4 (1:100, Santa Cruz Biotechnology), anti-Sox2 (1:1000, Millipore), anti-Nanog (1:150, Santa Cruz Biotechnology), anti-Tra-1-81 (1:150, Chemicon), anti-Tra-1-60 (1:150, Chemicon), anti-CDH1 (1:100, BD), anti-SSEA1 (Ascites, 1:500, Developmental Studies Hybridoma Bank), anti-SSEA3 (Ascites, 1:400, Developmental Studies Hybridoma Bank) and anti-SSEA4 (Ascites, 1:400, Developmental Studies Hybridoma Bank) for hESCs staining and anti-Sox17 (1:100, R&D), anti-Myoblast (1:100, Developmental Studies Hybridoma Bank) and anti-Tuj1 (1:100, Santa Cruz Biotechnology) for

embryoid bodies (EB) staining. 1×10^6 hESCs were harvested and then resuspended in 100-μL culture medium. These cells were injected into the tibialis anterior muscles of 6–8-week-old Balb/c mice. The muscles were harvested 48 h post-transplantation, fixed in 4 % paraformaldehyde and dehydrated in 20 % and then 30 % sucrose at 4 °C overnight. The entire muscle was sectioned serially every 20 μm using a cryostat, and sections were selected for staining every 10 serial sections. The primary antibodies used for immunostaining included rabbit antibodies against mouse CD3 (1: 400, Abcam) and mouse KLRA1 (1:200, Abcam).

For the analysis of the immune response in xeno-transplantation, three to four transplanted muscles were used for each donor hESC line. The CD3- or KLRA1-positive cells were quantified on each section selected for immunostaining.

Teratoma Formation

Cells were injected intramuscularly into non-obese diabetic/severe combined immune-deficient (NOD/SCID) mice (approximately 5×10^6 cells per site). After approximately 2 months, the tumors were processed for hematoxylin-eosin staining. All of the animal experiments were conducted in accordance with the Guide for the Care and Use of Animals for Research Purposes and approved by the Zhejiang University Animal Care Committee.

Elispot Assay

For the allogeneic immune response in vitro, human peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood drawn from healthy blood volunteers. Elispot assays using 5×10^5 mitomycin C-treated hESCs as stimulator cells and 5×10^5 human PBMCs as responder cells were performed according to the manufacturer's instructions (Mabtech) using IFN-gamma coated plates. The spots were automatically quantified using an Elispot plate reader (CTL) for scanning and analyzing.

Statistical Analysis

All of the data are represented as the mean ± SEM. Statistical significance was calculated using an unpaired Student's *t*-test, and differences were considered significant at $p<0.05$.

Results

Disruption of Beta 2-Microglobulin in hESCs by TALENs

The β2m gene was disrupted using transcription activator-like effector nucleases (TALENs) [18–20] targeting exon 2 (Fig. 1a). Sixteen pairs of TALENs (Fig. S1A) were constructed, and their activities were confirmed in 293 T cells (Fig. S1B). The most efficient TALEN pairs (L86&R102) were selected to target β2m in H1 [1] and X1 [21] hESCs (Fig. 1a). Both heterozygous ($\beta2m^{+/-}$) and homozygous ($\beta2m^{-/-}$) hESCs were obtained in one targeting round (Fig. 1b). In the absence of any drug selection, 44 %–87 % of the clones were targeted in one or both alleles (Fig. 1c).

Fig. 1 Disruption of beta 2-microglobulin in hESCs by TALENs. **a** Target sequences on exon 2 of human β2m targeted for cleavage by TALENs. **b** Alignment of the genomic sequences of mutants and wildtypes at the TALEN target site. The number of deleted (*dashes*) or inserted (letters in *red*) nucleotides compared with the wildtype sequences (wt) is indicated on the right of each sequence. **c** Efficiency of generating β2m-deficient hESCs using TALENs

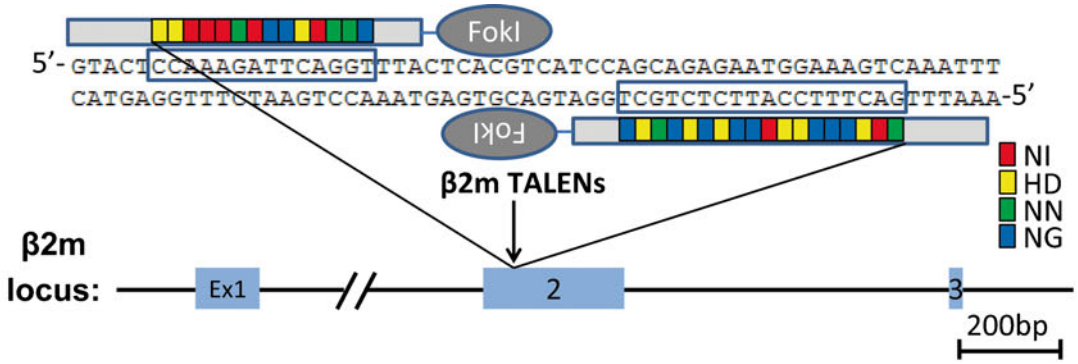
Investigation of HLA Class I Expression in β2m Null hESCs

To investigate β2m and HLA class I expression in heterozygous ($\beta2m^{+/-}$) and homozygous ($\beta2m^{-/-}$) hESCs, RT-PCR, western blot and FACS analysis were performed. β2m mRNA could be detected, although it was decreased in both heterozygous ($\beta2m^{+/-}$) and homozygous ($\beta2m^{-/-}$) hESCs compared with wildtype hESCs (Fig. 2a), suggesting that β2m expression might be regulated by a positive feedback loop. Interferon gamma (IFN-γ), a typical inflammatory factor, has been reported to induce the expression of β2m and HLA-A, -B and -C [22]. Moreover, β2m mRNA increased after IFN-γ treatment in wildtype, $\beta2m^{+/-}$ and $\beta2m^{-/-}$ hESCs. However, no β2m protein (Fig. 2e) or cell surface β2m molecules (Fig. 2f) in $\beta2m^{-/-}$ hESCs were detected, even after IFN-γ treatment, while the expression of β2m protein in both $\beta2m^{+/-}$ and wildtype hESCs was largely increased after treatment (Fig. 2e). There was also no difference between the wildtype, $\beta2m^{+/-}$ and $\beta2m^{-/-}$ cells at the HLA-A, -B and -C mRNA (Fig. 2b, c and d) or protein expression levels (Fig. 2e), with or without IFN-γ treatment. However, we could not detect any HLA class I molecules on the surface of $\beta2m^{-/-}$ hESCs (Fig. 2g), even after IFN-γ treatment. Heterozygous hESCs expressed less HLA class I on the cell surface compared with wildtype hESCs. In addition, HLA-A, -B and -C molecules on the cell surface of both wildtype and $\beta2m^{+/-}$ hESCs increased after treatment with IFN-γ (Fig. 2g). Thus, we successfully established HLA class I-deficient hESCs by disrupting the β2m gene.

The Pluripotency of HLA Class I-Deficient hESCs

Next, we characterized the undifferentiated state and pluripotency of established HLA class I-deficient hESCs. $\beta2m^{-/-}$ hESCs expressed pluripotent genes, such as Oct4, Nanog, Sox2, Dppa2, Dppa4, Nodal, Dnmt3b and Rex1, similar to wildtype hESCs (Fig. 3a). Furthermore, immunostaining

a



b

>β2m wt	TCCAAAGATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCA	
>H1-1	TCCAAAGAT----------CCAGCAGAGAATGGAAAGTCA	-19 bp
>H1-2	TCCAAAGATTCA----------TCCAGCAGAGAATGGAAAGTCA	-15 bp
>H1-5	TCCAAAGATTCAGGTTTA---------TCCAGCAGAGAATGGAAAGTCA	-9 bp
>H1-6	TCCAAAGATTCAGGTTTACT--------CCAGCAGAGAATGGAAAGTCA	-8 bp
>H1-14(1)	TCCAAAGATT----------GAATGGAAAGTCA	-26 bp
H1-14(2)	TCCAAAGATT----------CAGCAGAGAATGGAAAGTCA	-19 bp
>H1-16	TCCAAAGATTCAGGTTTACTCACCACGTCATCCAGCAGAGAATGGAAAGTCA	+3 bp
>H1-17	TCCAAAGAT----------TCATCCAGCAGAGAATGGAAAGTCA	-15 bp
>H1-20(1)	TCCAAAGATTCAGGTTTACTC-----ATCCAGCAGAGAATGGAAAGTCA	-5 bp
H1-20(2)	TCCAAAGATTC----------ACGTCATCCAGCAGAGAATGGAAAGTCA	-10 bp
>H1-24(1)	----------ATCCAGCAGAGAATGGAAAGTCA	-49 bp
H1-24(2)	----------ATCCAGCAGAGAATGGAAAGTCA	-49 bp
>H1-25(1)	TCCAAAGATTCAGGTT----------TGGAAAGTCA	-23 bp
H1-25(2)	TCCAAAGAT----------CCAGCAGAGAATGGAAAGTCA	-19 bp
>H1-28(1)	TCCAA----------TGGAAAGTCA	-34 bp
H1-28(2)	TCCAAAGATTCAGGTTTACTC-----ATCCAGCAGAGAATGGAAAGTCA	-5 bp
>H1-29(1)	TCC----------AGCAGAGAATGGAAAGTCA	-49 bp
H1-29(2)	TCCAAAGATTCAGGTTTACTCA-----TCCAGCAGAGAATGGAAAGTCA	-5 bp
>H1-30(1)	TCCAAAGATTCAGGTTTACTCAC----------	-675 bp
H1-30(2)	TCCAAAGATTCAGGTTTACTCAC----------	-675 bp
>H1-32(1)	TCCAAAGATTCAGGTTTACTCACG----------AAAGTCA	-18 bp
H1-32(2)	TCCAAAGATT----------CCATCCAGCAGAGAATGGAAAGTCA	-14 bp
>H1-38	TCCAAAGATTC----------AGGAAAGTCA	-28 bp
>X1-1(1)	TCCAAAGATTCAGGTTTACT--------CCAGCAGAGAATGGAAAGTCA	-8 bp
X1-1(2)	TCCAAAGATTCAGGTTTACTCAC----------	-178 bp
>X1-2(1)	TCCAAAGATTCAGGTT----------TGGAAAGTCA	-23 bp
X1-2(2)	TCCAAAGATTCAGGTT----------TGGAAAGTCA	-23 bp
>X1-4	TCCA----------GCAGAGAATGGAAAGTCA	-27 bp
>X1-5	----------GAGAATGGAAAGTCA	-213 bp
>X1-6	TCCAAAGATTC----------CAGCAGAGAATGGAAAGTCA	-18 bp
>X1-11(1)	TCCAAAGATTCAGGTTTACTCA-----TCCAGCAGAGAATGGAAAGTCA	-5 bp
X1-11(2)	TCCAAAGATTCAGGTTTACTCA-----TCCAGCAGAGAATGGAAAGTCA	-5 bp
>X1-14	TCCAAAGATTCAGGTTTACTCACGCACGTCATCCAGCAGAGAATGGAAAGTCA	+4 bp

c

Cell line targeted	No. of clones analyzed	Heterozygous	Homozygous	Targeting efficiency
H1	38	9(23.7%)	8(21.0%)	44.7%
X1	8	4(50.0%)	3(37.5%)	87.5%

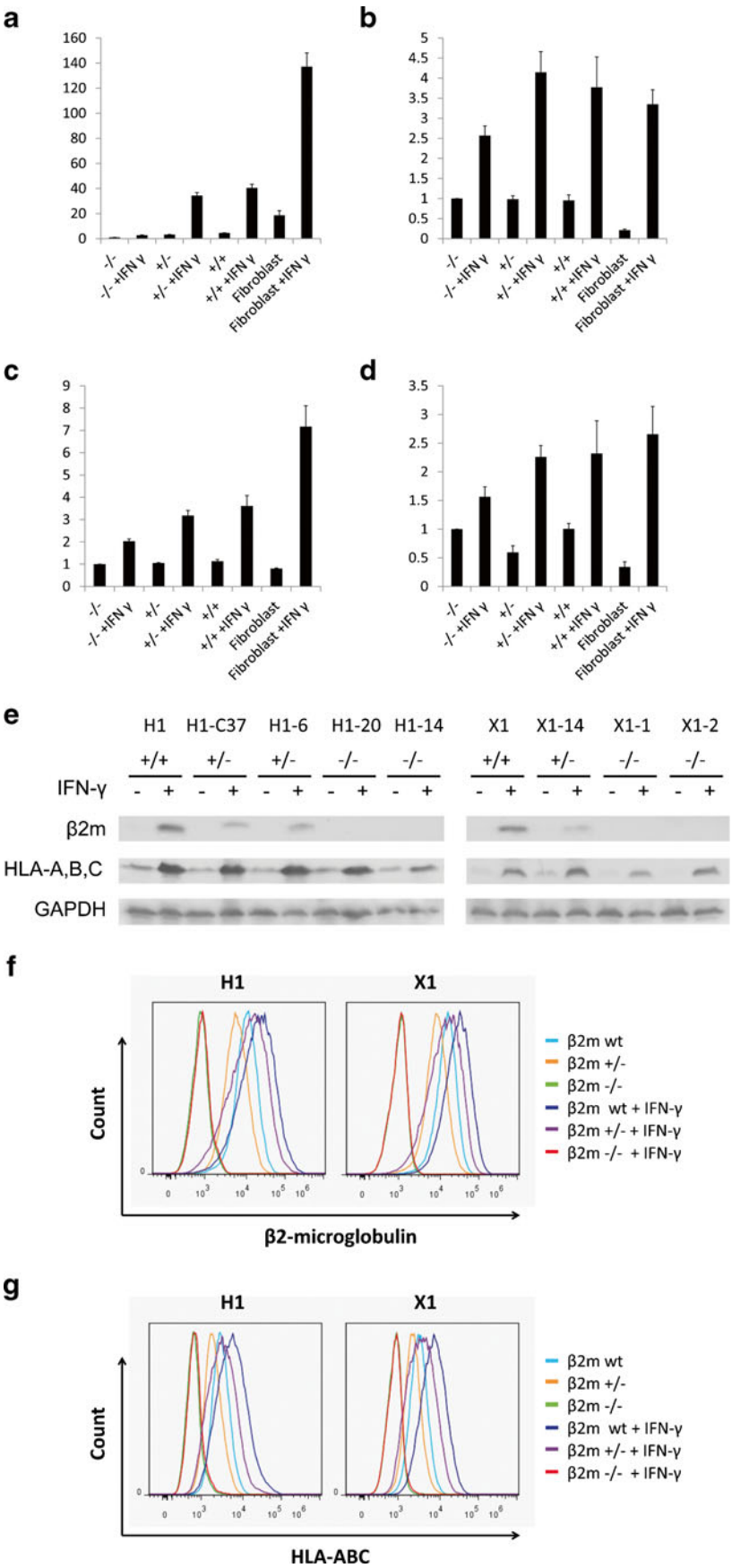


Fig. 2 Investigation of HLA class I expression in β2m null hESCs. **a**–**d** Relative mRNA levels of the β2m gene (**a**) and HLA-A (**b**), -B (**c**) and -C (**d**). **e** Western blotting analysis of β2m and HLA-A, -B and -C protein expression in β2m-deficient hESCs after treatment with IFN-γ (500 U/ml) or control treatment for 24 h. **f**–**g** FACS analysis of β2m (**f**) and HLA-A, -B and -C (**g**) protein expression on the cell surface in β2m-deficient hESCs after IFN-γ (500 U/ml) or control treatment for 24 h

showed that the β2m$^{-/-}$ hESCs were positive for Oct4, Sox2, Nanog, Tra-1-60, Tra-1-81, CDH1, SSEA3 and SSEA4 but not SSEA1 (Fig. 3b). We also examined the differentiated potential of β2m$^{-/-}$ hESCs in vitro and in vivo. The embryoid bodies of β2m$^{-/-}$ hESCs contained all three germ layers (Fig. 3c and d). When β2m$^{-/-}$ hESCs were injected into NOD/SCID mice, teratomas formed after 2 months, and all three germ layers were observed after hematoxylin-eosin (HE) staining (Fig. 3e). We found that the karyotypes were normal in both heterozygous (Fig. 3f) and homozygous (Fig. 3g) hESCs.

The Immunogenicity of HLA Class I-Deficient hESCs

To test the alloimmunogenicity of the β2m-deficient hESCs, an enzyme-linked immunospot (ELISPOT) assay was performed to detect the IFN-γ secretion of human peripheral blood mononuclear cells (PBMCs) when co-cultured with

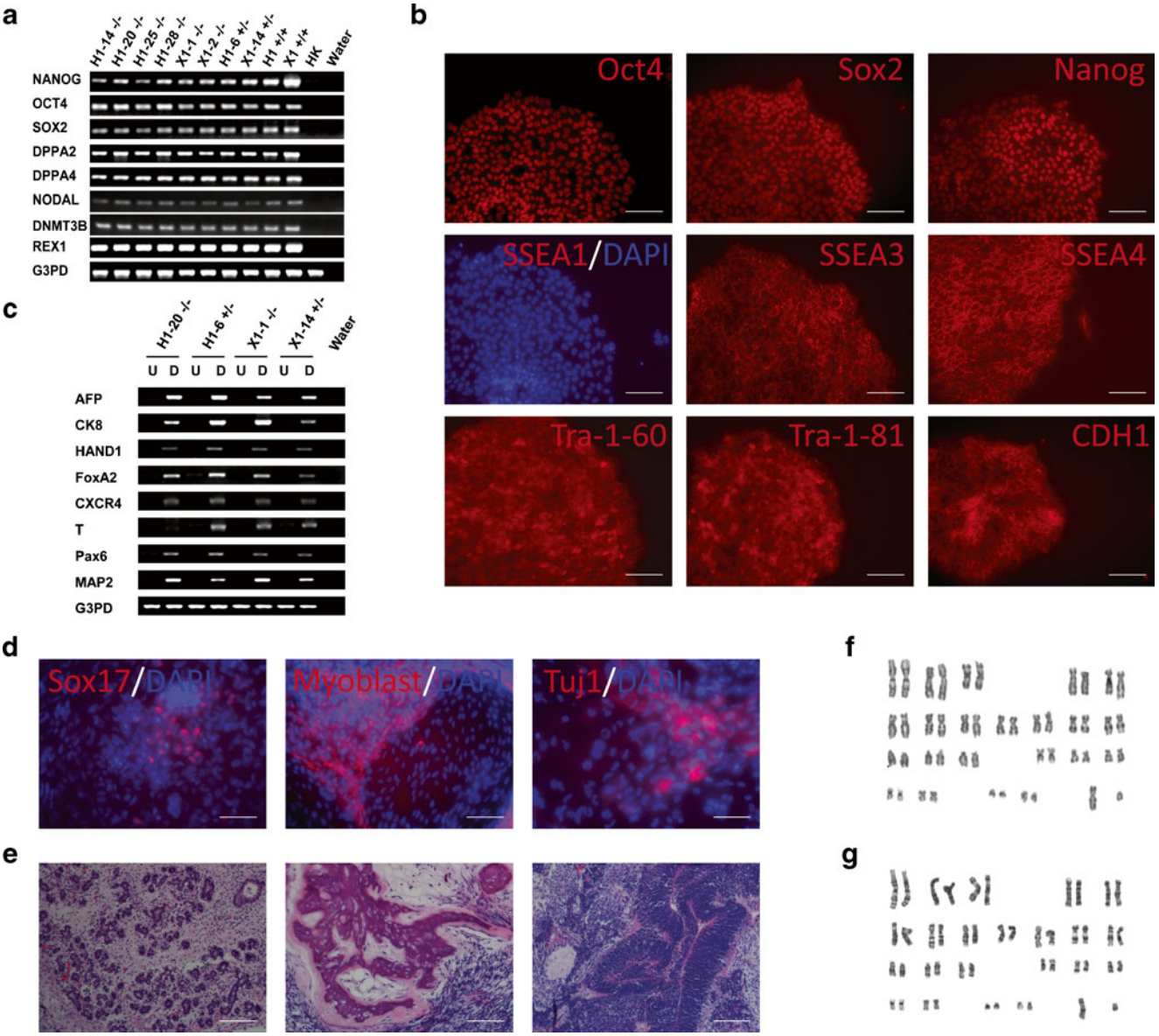


Fig. 3 The pluripotency of HLA class I-deficient hESCs. **a** RT-PCR analysis of pluripotent marker expression in β2m-deficient hESCs. **b** Immunostaining of pluripotent markers in β2m$^{-/-}$ hESCs. Scale bar, 100 μm. **c** RT-PCR analysis of differentiated marker expression in β2m-deficient hESC-derived embryoid bodies. U, undifferentiated; D, differentiated. **d** Immunostaining of markers for all three germ layers in β2m-deficient hESC-derived embryoid bodies. Scale bar, 100 μm. **e** HE staining identified three germ layers (endoderm (*left*), mesoderm (*middle*) and ectoderm (*right*)) in teratomas formed from the β2m$^{-/-}$ hESCs. **f**–**g** Karyotype analysis of β2m heterozygous (**f**) and homozygous (**g**) hESCs

wildtype or β2m-deficient hESCs in vitro. Wildtype hESCs dramatically stimulated PBMCs to secrete IFN-γ, while $\beta2m^{-/-}$ hESCs significantly attenuated the secretion of IFN-γ (Fig. 4a). These results indicated that homozygous ($\beta2m^{-/-}$) hESCs demonstrated hypo-alloimmunogenicity in vitro.

To investigate the immunogenicity of β2m-deficient hESCs in vivo, wildtype, $\beta2m^{+/-}$ and $\beta2m^{-/-}$ hESCs were transplanted into the tibialis anterior muscles of Balb/c mice. Tibialis anterior muscles were harvested 2 days post-transplantation. Immunohistochemistry was performed, and the results showed that both T (Fig. 4b) and natural killer (NK) (Fig. 4c) lymphocytes were decreased in the injected sites in both the heterozygous ($\beta2m^{+/-}$) and homozygous ($\beta2m^{-/-}$) hESCs compared with wildtype hESCs. The β2m-deficient hESCs showed hypo-immunogenicity in vivo, despite the lack of teratoma formation when injected into Balb/c mice.

Discussion

These results have some important implications. First, HLA class I-deficient hESCs may serve as a universal donor cell source for transplantation therapy. Universal donor cells can facilitate the production of commercial "off-the-shelf" hESC medicine.

Second, there are some advantages of HLA class I-deficient hESCs compared with iPSCs. There is little evidence showing that off-target events occur when using TALENs to modify the genome [23, 24], and many unpredictable mutations have occurred during the reprogramming process [11, 12]. Thus, HLA class I-deficient hESCs might raise less safety concerns compared with iPSCs. In HLA class I-deficient hESCs, pluripotency is ensured, and the differentiation potential is consistent with wildtype hESCs. However, it has been reported that partially reprogrammed iPSCs exhibit pluripotency defects, tendentious differentiation and occasionally elicit immune rejections [25, 26]. Additionally, the cost of establishing iPSCs for every patient or even an iPSC or hESC bank would be high, and the diversity in these banks may impede the clinical application of human stem cells. In this study, we generated universal hESC donor cells, which are much more economical and convenient for these applications.

Third, if HLA Class I-deficient hESC-derived cells were applied in future clinical medicine, the use of immunosuppressants on patients will decrease, and the side effects of these drugs may be diminished or even avoided. Importantly, using immunosuppressant made cells of the whole body of patients out of immune system monitoring, which may cause the danger of mutagenesis or tumorigenesis. While our method might only enable these hESC-derived-cell grafts to escape from immune system monitoring, decreasing the risk of carcinogenesis.

Fourth, the recognition of HLA class I molecules by cytotoxic T lymphocytes (CTL) could stimulate the killing of HLA class I presenting cells [27]. Although HLA class I molecules can also serve as ligands for NK cells, in contrast to CTL, the recognition of HLA class I by NK cells can inhibit NK-mediated cytotoxicity [28]. These HLA class I-deficient hESCs should be able to escape the attack of CTL and activate the cytotoxicity of NK cells. Unlike the role of T cells [29], it has been reported that NK cell-mediated resistance to alloengraftment is relatively weak [30, 31]. This is consistent with our results, which showed that a decreased total output of immune rejection. Thus, β2m-null hESCs exhibit hypoimmunogenicity. One recent report also showed similar results [32]. In order to allow an effective clinical

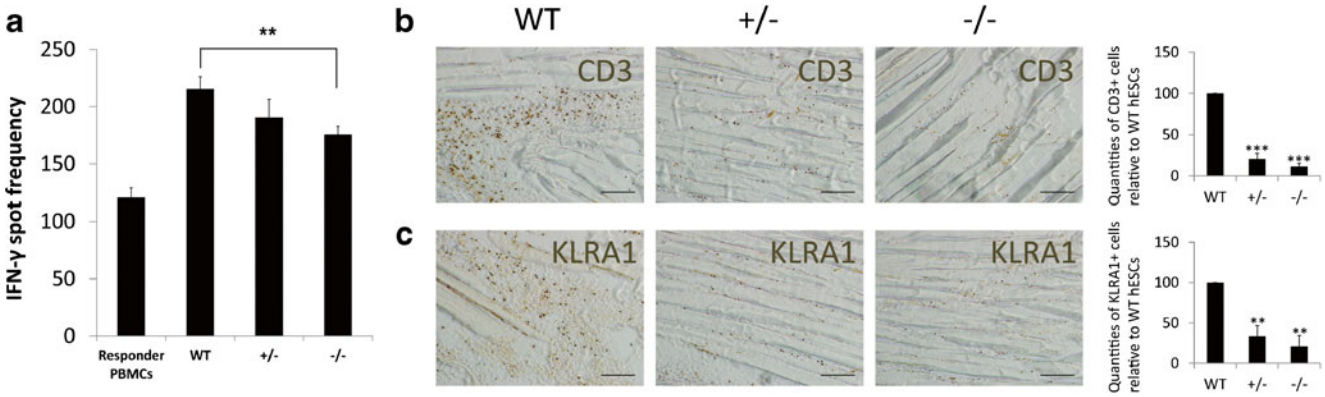


Fig. 4 The immunogenicity of HLA class I-deficient hESCs. **a** IFN-γ secretion of human peripheral blood mononuclear cells (PBMCs) when cocultured with β2m-deficient and wildtype hESCs. $N>20$. ** $p<0.01$. The data are expressed as the mean ± SEM. **b**–**c** Both the β2m-deficient and wildtype hESCs were injected into the tibialis anterior muscles of Balb/c mice. Two days post-transplantation, the tibialis anterior muscles were harvested, and immunostaining using the T lymphocyte marker CD3 (**b**) and NK lymphocyte marker KLRA1 (**c**) was performed. Quantification of the relative cell number is also shown. Scale bar, 100 μm. ** $p<0.01$, *** $p<0.001$. The data are expressed as the mean ± SEM

translation, further experiments should be done to clarify whether the β2m-null hESCs activate the attack of NK cells or not. Moreover, HLA class I-deficient hESCs might also be considered as new material for the study of human immunology.

Finally, if these hypoimmunogenic hESCs could serve as universal donor cells, the destruction of human embryos in the establishment of hESCs banks can be avoided.

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