Original Paper

Enhanced Immunological Tolerance by HLA-G1 from Neural Progenitor Cells (NPCs) Derived from Human Embryonic Stem Cells (hESCs)

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Key Words

Embryonic stem cells • Transplantation • HLA-G1 • Neural progenitor cells • Mixed lymphocyte reaction • HESCs

Abstract

Background: Despite the great potential of utilizing human embryonic stem cells (hESCs)-derived cells as cell source for transplantation, these cells were often rejected during engraftment by the immune system due to adaptive immune response. **Methods:** We first evaluated HLA-G expression level in both hESCs and differentiated progenitor cells. After that, we generated modified hESC lines that over-express *HLA-G1* using lentiviral infection with the construct contains both HLA-G1 and GFP tag. The lentivirus was first produced by co-transfecting HLA-G1 expressing lentiviral vector together with packaging vectors into packaging cell line 293T. Then the produced virus was used for the infection of selected hESC lines. We characterized the generated cell lines phenotype, including pluripotency and self-renewal abilities, as well as immune tolerance ability by mixed lymphocyte reaction (MLR) and cytotoxicity assays. **Results:** Although the hESCs do not express high levels of HLA-G1, over-expression of HLA-G1 in hESCs still retains their stem cell characteristics as determined by retaining the expression levels of OCT4 and SOX2, two critical transcriptional factors for stem cell function. Furthermore, the HLA-G1 overexpressing hESCs retain the self-renewal and pluripotency characteristics of stem cells, which can differentiate into different types of cells, including pigment cells, smooth muscle cells, epithelia-like cells, and NPCs. After differentiation, the differentiated cells including NPCs retain the high levels of HLA-G1 protein. In comparison with conventional NPCs, these HLA-G1 positive NPCs have enhanced

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immune tolerance ability. **Conclusions:** Ectopic expression of HLA-G1, a non-classical major histocompatibility complex class I (MHC I) antigen that was originally discovered involving in engraftment tolerance during pregnancy, can enhance the immunological tolerance in differentiated neural progenitor cells (NPCs). Our study shows that stably overexpressing HLA-G1 in hESCs might be a feasible strategy for enhancing the engraftment of NPCs during transplantation.

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Introduction

Stem cells and progenitor cells transplantation is an attractive application in the clinic. Pluripotent stem cells, including embryonic stem cells and induced pluripotent stem cells (iPS), are derived from totipotent cells that are capable of self-renew and differentiate into other types of cells [1]. Because of this character, stem cells are ideal source to provide unlimited cell source for transplantation [2-9]. However, there are several challenges for the current available strategy. These challenges include the lack of suitable donor hESCs available that can satisfy the current clinical needs, and the rejection during transplantation [10-12]. HLA-G1 is one of the candidates that potentially can overcome the engraftment rejection during transplantation. It is originally discovered during pregnancy as a non-classical major histocompatibility complex class Ib (MHC Ib) antigen responsible for the maternal immune tolerance to the fetus [13-15]. Unlike highly polymorphic HLA-A and HLA-B genes, HLA-G has few alleles and is produced by the placenta cells that directly interface with maternal blood and tissues. The soluble isoform of HLA-G produced by placental cells after migrating into maternal uterus will interact with inhibitory receptors on leukocytes ILT2 and ILT4, which in turn suppresses cytotoxic CD8⁺ T lymphocytes and natural killer (NK) cells [16-18]. We are utilizing this naturally occurring MHC I protein to overcome the rejection during engraftment. Here we show by introducing HLA-G1 into hES stem cells, they still maintain the pluripotency ability of stem cells. When these HLA-G1 high cells are differentiated into NPC cells, they have enhanced immune tolerance through high level of HLA-G1 expression. Our study provides us a novel strategy to improve the application of utilizing hESCs and progenitor cells for transplantation with increased engraftment ability.

Materials and Methods

Cell culture

hESCs were maintained in ES medium (DMEM with serum replacement, 8 ng/ml bFGF, 2 mM L-glutamine, 1X NEAA, 0.1mM 2-ME, 100 u/ml penicillin, 100u/ml streptomycin). NPC cells were maintained in DMEM/F12 medium supplied with 1X B-27 supplement, 20ng/ml bFGF and 20ng/ml EGF. 293T and MEF cells were cultured in DMEM+10% FBS media.

MEF cells were isolated from 13-15 dpc embryos of mice (ICR background). After removal of head, visceral tissues and tail, the remaining bodies were washed, minced and dissociated with 0.25% trypsin. The dissociated cells were cultured in DMEM +10% FBS in 10-cm dish pre-coated with 0.1% gelatin. Floating cells were washed with 1X PBS the next day. For coculturing with hESCs as feeder cell, MEF cells were pre-treated with mitomycin c for 2-3 hrs prior to coculture.

HLA-G1 expressing lentivirus production and infection of host cells

A human HLA-G1 template was cloned from placenta obtained after cesarean section. After reverse transcribe total RNA (RNeasy plus mini kit, Qiagen) into cDNA using MMLV reverse transcriptase, HLA-G1 was cloned using the primers: Forward, 5'- ATCGTCTAGAATGGTGGTCATGG-3' and reverse, 5'- ATCGTGTACATCACTTGTTCATCGTCGTCCTTGTAGTCATCTGAGCTCT -3'. The amplified cDNA was cloned in pGEM-T easy construct using TA-clone and further subcloned into iDuet101a-IRES-eGFP lentiviral vector. The lentivirus was produced by transfecting HLA-G1-iDuet101a-IRES-eGFP plasmid with packaging vectors CMVΔ8.92 and pMDG into 293T cells using lipofectamine 2000 (ThermoFisher Scientific). The supernatant

was collected 24 hrs and 48 hrs after transfection and filtered with 0.45µm filter and concentrated at 4000x g for 20 min at 4 °C. The hESCs were infected with packaged lentivirus in the presence of 3-5 µg/ml polybrene (sigma). After infection, the hESCs were cultured together with MEF cells in ES medium. The infection efficiency was determined by the presence of GFP under fluorescent microscope and Western blot for the expression level of HLA-G1.

Western Blot

The cells were lysed by RIPA lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40) with 0.5% sodium deoxycholate. The extracted protein samples were loaded on 10% SDS-PAGE, transferred into nitrocellulose membrane and immunoblotted with antibodies against Oct-4 (Santa Cruz), HLA-G (clone 4H84, Abcam), Sox2 (Chemicon). Fluorescent labeled secondary antibodies FITC-goat anti-mouse IgG1, TRITC-goat anti-mouse IgG1, FITC-goat anti-rabbit IgG and TRITC goat anti-rabbit IgG were used for detection, and membranes were detected on Odyssey (LI-COR).

Immunofluorescent (IF) staining

The cultured cells or O.C.T. (International equipment) embeded sections were permeabilized with 0.5% PBST 5 min for three times, washed with PBS and stained with antibodies against Oct-4 (Santa Cruz), HLA-G (Abcam), Sox2 (Chemicon), Nestin and Pax6. For fluorescence detection, secondary antibodies were used (FITC or TRITC labeled) and counterstained with DAPI for nuclei. Slides were mounted and examined using fluorescent microscope Olympus BX51.

Alkaline phosphatase (ALP) activity assay

The activity of alkaline phosphatase was detected by NBT (nitro-blue tetrazolium chloride) /BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) substrate. Briefly, cells were washed 3 times using TSM1 buffer (100mM Tris-HCl, pH8.0, 100mM NaCl, 10mM MgCl₂) for 3 min at RT, followed 3 times washes with TSM2 buffer (100mM Tris-HCl, pH9.5, 100mM NaCl, 50mM MgCl₂) for 3 min each. After wash, NBT/BCIP substrate was added for 10~15 min to detect ALP activity. When it yielded black-purple precipitate, the reaction was terminated by adding distilled water, and the cells were examined under the microscope.

Teratoma formation assay

The hESCs were harvested by digesting with 0.25% trypsin for 3-5 min. The digested cells were separated into single cells and centrifuged at 1000x rpm for 5 min and the cell pellets were resuspended in 1x PBS. The cells were injected via intramuscular injection into the rear leg of SCID mice at 5x10⁵/mouse. The growth of the teratomas was monitored until they reach to the desired size. Tumors were isolated and fixed and embeded in paraffin block for H&E staining and histological analysis.

Neural progenitor cells (NPCs) differentiation from hESCs

hESCs were digested with 0.1% collagenase IV for 10-20 min at 37 °C and cultured in NPC culture medium for 1 wk. The cells were then cultured in albumin coated culture dishes and continued culturing in NPC medium as described above. After another week, the cells were digested with acutase and examined using IF staining and Western blot.

Mixed lymphocyte reaction (MLR)

HLA-G1 expressing neural progenitor cells (NPCs) derived from hESCs were irradiated at 20 Gy for 1 hr to inhibit the cell growth. After irradiation, freshly isolated PBMC from healthy donor were co-cultured with irradiated hESCs at different ratios in the presence of RPMI media supplemented with IL-2 (100ng/ml). The proliferation of the cells was determined using Cell Counting Kit-8 (Dojindo), and the relative cell number was measured using plate reader at 450nm.

Cytotoxicity assay

NPC cells that derived from HLA-G1 expression hESCs were coated in 96-plate for overnight in culture media. The next day, NK-92MI cells were co-cultured with NPC cells at different ratios for 4 hrs at 37 °C. The cytotoxicity was determined by using CytoTox 96 kit (Promega) to determine the levels of cytosolic enzyme lactate dehydrogenase (LDH) release upon co-culture according to the manuals.

Results

Evaluation of HLA-G expression in both hESCs and differentiated progenitor cells

In order to determine the endogenous expression levels of HLA-G in both stem cells and progenitor cells, we chose three different hESCs and induced them to randomly differentiate into different types of progenitor cells. It is well characterized that introducing Oct4, Sox2, c-Myc and Klf4 is sufficient to induce pluripotent stem cells from fibroblast [3, 19]. We chose OCT4, one of the characteristic markers to determine the differentiation status of the stem cells that being examined. All three undifferentiated hESCs have high levels expression of OCT4, and when they differentiate into progenitor cells or somatic cells, the levels of OCT4 are significantly reduced, as determined by Western blot (Fig. 1a). On the other hand, HLA-G was not detectable either in the undifferentiated hESCs, or in the cells after differentiation (Fig. 1a). The antibody we used can detect both HLA-G1 and HLA-G5, two different isoforms of HLA-G family [20]. We continued to monitor the expression levels of targeting proteins at different time points after hESCs differentiation, HLA-G is not detectable from week 1 (Fig. 1a, top panel) till week 4 (Fig. 1a, lower panel). K562 is a human leukemia cell line serves as negative control for both HLA-G and stem cell marker OCT4. JEG-3 is a human placenta choriocarcinoma cell line that has been reported previously to have high levels of HLA-G [21, 22]. We used it as the positive control for HLA-G and negative control for OCT4. Consistent with previous reports, we detected high expression level of HLA-G in JEG-3 cells (Fig. 1a).

We further confirmed our result by immunofluorescent (IF) staining, since this would allow us to examine the target proteins expression in each single cell. Consistent with what

Fig. 1. Endogenous levels of HLA-G in both hESCs and differentiated progenitor cells. (a) Western blot showing OCT4 and HLA-G in both parental hESCs and differentiated cells at 1 wk (upper panel) or 4 wks (lower panel) after differentiation induction. K562 and JEG-3 are used as negative and positive controls for HLA-G, respectively. (b) Immunofluorescent staining from three different hESC cells H-1 hESCs (A), Peng hESCs (B) and Li hESCs (C) for the expression of proteins OCT4 (green) and HLA-G (red). JEG-3 is used for positive control for HLA-G (D). Cell nucleic are counter stained in blue.

Fig. 2. Generation and examination of HLA-G1 over-expression hESCs. (a) hESCs were infected by HLA-G1 overexpression lentivirus and examined by bright-field microscope (A) and IF microscope for the presence of GFP (B). The protein levels of HLA-G1 and OCT4 of infected cells as well as packaging cells 293T were examined by SDS-PAGE (right panel). Scale bar: 50 μ m (b) flow cytometry analysis of the GFP positive cells. Left, GFP intensity from control vector infected hESCs. Middle, GFP percentage from HLA-G1 lentivirus infected hESCs. The expression levels of GFP are shown as log scale fluorescent intensity (x axis) of each cell (y axis). Light gray: negative control of hESCs without infection. Thick black, mock (left) or HLA-G1 (middle) transfected samples. Right, quantification of the percentage of GFP positive HLA-G1 expression cells among total hESCs. (c) IF staining of OCT4 (green) /HLA-G1 (red) (A) or SOX2 (green)/HLA-G1 (red) (B). The stem cell property is also examined by alkaline phosphatase activity (C). Right panels, karyotype of HLA-G1 overexpression hESCs. Scale bar: 75 μ m.

we discovered using Western blot (Fig. 1a), all three hESCs including H-1 hESCs (Fig. 1b, panel A), Peng hESCs (Fig. 1b, panel B) and Li hESCs (Fig. 1b, panel C), express high levels of OCT4. Furthermore, OCT4 is mainly present in the nucleic area, and this indicates that OCT4 is in active form as a transcriptional factor to maintain the status of stem cells. Consistent with our Western blot results (Fig. 1a), all three types of hESCs examined have no detectable HLA-G expression. In contrast, JEG-3 showed detectable expression of HLA-G on the cell membrane (Fig. 1b, panel D), the major location for the expressed MHC molecules. The expression of OCT4 was not detectable in JEG-3 cells (Fig. 1b, panel D). Our results indicate that the expression of HLA-G is highly controlled in a spatial and temporal fashion that it can only be detected in the placenta cells during pregnancy in the natural condition.

Generation of modified hESC lines that over-express HLA-G1

Next, we generate lentiviral construct that allows us to stably over-express HLA-G1 in the hESC cells to study the role of HLA-G1 expression in hESCs and differentiated progenitor cells. This construct also contains GFP tag under the control of IRES site, which allows the simultaneous expression of HLA-G1 and GFP in the same cell. We were able to detect about 60~70% GFP positive cells from the hESCs 6 weeks after lentiviral infection and selection (Fig. 2a, left panel), indicating HLA-G1 expressing lentivirus has already integrated the target genes into the chromosome of the infected cell, and the integrated genes can be stably inherited by the daughter cells during hESCs self-renewal. This result was further confirmed by Western blot showing that not only GFP was present in the selected hESCs as a tracking

Fig. 3. Pluripotency ability of HLA-G1 positive hESC cells. (a) teratoma assay of HLA-G1 positive cells. Upper panels, H&E staining showing differentiated pigment cells (left), smooth muscle cells (middle) and epithelia-like cells (right). Lower panels, IHC staining showing expression levels of S100 (left), desmin (middle) and cytokeratin (right). (b) Bright-field microscopy showing morphology changes of differentiated NPCs from HLA-G1+ hESCs after induction at day 7 (A), 10 (B) and 14 (C). HLA-G1+ hESCs differentiated NPCs were also examined by IF for the expression of NES-TIN (D-F), PAX6 (G-I) and HLA-G (J-L). Control NPCs differentiated from mock lentivirus infected hESCs were also evaluated for HLA-G expression (M-O).

marker, but HLA-G1 protein was detectable from the generated hESC stable cell line (Fig. 2a, right panel). More importantly, after HLA-G1 integrated and expressed into the host hESCs, these cells still retained high levels of OCT4 expression (Fig. 2a, right panel). This indicates although hESCs do not express HLA-G1, ectopic expression of HLA-G1 does not change the characteristics of the host stem cell phenotype. We further quantified the percentage of GFP/HLA-G1 positive hESCs after infection using flow cytometry analysis, there were 50.59% cells that were HLA-G1 positive after the selection (Fig. 2b)

To confirm over-expression of HLA-G1 does not change the stem cell property of infected cells, we further examine the infected cells for different properties that are unique to stem cells. We found that after lentivirus infection and HLA-G1 overexpression, these cells retained expression levels of stem cell markers OCT4 and SOX2 (Fig. 2c, panel A, B). Moreover, we also detected high levels of alkaline phosphatase activity (Fig. 2c, panel C), which was only present in the hESCs but not progenitor cells [1]. These evidences indicate that those HLA-G1 still maintains stem cell property. Furthermore, karyotype analysis showed normal chromosomal characteristics as 46XY (Fig. 2c, right panel).

Fig. 4. Elevated immune tolerance of differentiated NPCs by HLA-G1 (a) Mixed lymphocyte reaction (MLR) assay by co-culturing CD8⁺ T cells from PBMC with irradiated HLA-G1⁺ NPCs at indicated ratios. The proliferation of CD8⁺ T cells was determined using cell counting kit-8. (b) Cytotoxicity assay using co-culturing NK cell line NK-92MI with HLA-G1⁺ NPCs at indicated ratios. The cytotoxicity of the NK cells were determined by CytoTox 96 kit 4 hrs after co-culturing.

HLA-G1 over-expression does not interfere with the pluripotency ability of the host hESCs

We performed the teratoma assay to further evaluate the pluripotency ability of HLA-G1 positive hESCs *in vivo*. Our results showed that in teratoma from HLA-G1⁺ hESCs, there are cell types coming from all three layers, including pigment cells (ectoderm) (Fig. 3a, upper left), smooth muscle cells (mesoderm) (Fig. 3a, upper middle) and epithelia-like cells (endoderm) (Fig. 3a, upper right), as determined by H&E staining for pathological assessment. Furthermore, we also performed the IHC staining to evaluate the markers for those differentiate cell types, and they are all positive for the markers of the cell types they belong to: neural ectoderms are positive for S100 (Fig. 3a, lower left), smooth muscle cells are positive for desmin (Fig. 3a, lower middle), and epithelia-like cells are positive for cytokeratin (Fig. 3a, lower right). When we extracted the tissue samples from those formed teratomas, there is still detectable HLA-G1 protein (data not shown).

We also differentiated the HLA-G1⁺ hESCs into neural progenitor cells and monitored the process during differentiation. The cells show typical NPC cell morphology 7 days after differentiation induction (Fig. 3b, panel A). After differentiation, we put them in 2D culture for 3 days (Fig. 3b, panel B), and monitored the cells at day 7 since differentiation (Fig. 3b, panel C). We further assessed the expression of markers for NPC cells using IF staining. And we found that after differentiation, almost all the differentiated cells are NESTIN positive (Fig. 3b, panel D-F), some of the cells have PAX6 expression (Fig. 3b, panel G-I). This indicates all the cells successfully differentiated into NPC cells. More importantly, after differentiation, cells still retained HLA-G1 protein expression (Fig. 3b, panel J-L). In contrast, those differentiated NPC cells from mock lentivirus infected hESCs were negative for HLA-G1 expression (Fig. 3b, panel M-O). This indicates that it is possible to differentiate HLA-G1⁺ hESCs into other types of cells including NPCs, which can be potentially used for transplantation. However not all the cells express HLA-G1, which is due to the infection efficiency of the HLA-G1⁺ expression virus.

HLA-G1 enhances immune tolerance ability of differentiated NPC cells

After obtaining HLA-G1 positive NPCs that are differentiated from hESCs, we want to further evaluate whether HLA-G1 can enhance the immune tolerance ability. Since HLA-G1 is found to interact with inhibitory receptors as an MHC Ib antigen, we want to determine whether HLA-G1 expression on NPCs can facilitate cytotoxic CD8⁺ T cells proliferation inhibition, and whether it can reduce cytotoxicity of NK cells *in vitro*. In order to do that, we co-cultured HLA-G1+ NPCs with human T cells that were derived from PBMC cells at different ratios (Fig. 4a). We discovered that at the ratios of 5:1 and 2.5:1 (T cells vs NPC), HLA-G1 expression significantly suppressed the proliferation of the T cells in response to IL-2 stimulation and NPC co-culturing (Fig. 4a). However, since we were using total PBMC and specific stimulate T cell proliferation by adding IL-2. We might see better response by adding pure human T cells for the co-culture assay.

In order to study the effect of HLA-G1+ NPCs on NK cells cytotoxicity ability, we used NK-92MI cell line, which has NK cells characteristics, to perform cytotoxicity assay. NK cells were co-cultured with HLA-G1+ NPCs at different ratios as indicated (Fig. 4b). At ratio of higher than 2.5:1 (NKs: NPCs), HLA-G1+ NPCs significantly suppressed the cytotoxicity of NK cells for about 25%. At ratios of 5:1 and 10:1 (NKs: NPCs), there were statistical significant differences between HLA-G1+ NPCs and control HLA-G1- NPCs for their ability to suppress NK cell cytotoxicity.

Discussion

Pluripotent stem cells, including embryonic stem cells and induced pluripotent stem cells, are capable of self-renewing and differentiating into other types of cells [1, 3, 19]. They are very attractive cell sources for the regenerative medicine, disease model generation and drug screening platform build-up [10, 23-26]. However, there are also shortcomings and obstacles during the application of stem cell [27, 28]. In this study, we sought to evaluate the possibility of overcoming transplantation rejection of progenitor cells derived from ES cells, and whether this would be a feasible strategy for the application for transplantation of differentiated cells derived from ES cells.

HLA-G family protein has been well characterized responsible for the fetus tolerance during pregnancy [20]. It is also shown responsible for transplantation tolerance and immune evasion during cancer progression [29, 30]. The idea of utilizing HLA-G to help overcoming transplantation rejection for the stem cells has been discussed by several groups previously [10, 31]. However, currently there is no systemic study to evaluate the feasibility of this application.

Our study shows for the first time that by stably over-expression HLA-G1 in the hESCs using lentivirus system, we are able to generate HLA-G1+ hESCs that still function as normal stem cells. They still retain the expression of OCT4 and SOX2, two markers important for stem cells function. Furthermore, these HLA-G1+ hESCs also have the capability of differentiating into different types of cells *in vivo*, including those derived from ectoderm, mesoderm and endoderm. However, the percentage of HLA-G1+ hESCs after virus infection and selection is about 50%. It would be meaningful to further purify those infected cells, such as picking single-cell colony or using fluorescence activated cell sorting for those GFP positive infected cells. This would help us further address whether all those HLA-G1 positive hESCs retain HLA-G1 protein expression during propagation, and whether this can further improve the immune tolerance ability of those differentiated progenitor cells. It is known that lentiviral infection might induce the development of malignant tumor cells, due to the random incorporation of target cDNA to host chromosome, which has potential risk of interfering the function of several oncogenes. Other alternative methods could potentially be used for avoiding this type of risk [32]. Besides utilizing hESC cells, other patient-derived stem cell preparations as well as iPSCs could also potentially used with HLA-G1 expression to increase the immune tolerance for clinical applications to avoid bioethics concerns. However, it should be noted

that not like hESCs having extremely low level or no expression of HLA class I and HLA class II, other types of stem cell and progenitor cells might have higher levels of HLA molecules [33], which will increase the risk of rejection during transplantation. Our pilot study will help us to further explore and expand utilizing stem cells as a valuable source for transplantation and engraftment with improved immune tolerance.

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Disclosure Statement

No.

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