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Immunological Elimination of Undifferentiated Human Pluripotent Stem Cells Using Pluripotent State-Specific Antigen, Glypican-3

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Thank you for reviewing our manuscript entitled **"Immunological elimination of undifferentiated human pluripotent stem cells using pluripotent state-specific antigen, glypican-3. "** and favorable comments on our manuscript. Based on valuable suggestions, we have revised the manuscript. We would like to ask you to consider our revised manuscript for publication in ***The Journal of Visualized Experiments***.

According to suggestion about introduction, we state clearly the purpose of this method and added the description of the rationale, the advantage, and the information about the application of this method. We also added other detail descriptions according to valuable suggestions.

Please note that this manuscript has been fully revised by a professional native English editor with a PhD in biomedical research, ensuring the quality of the English language of the text. The manuscript contains 2811 words of text, 24 references, 1 page of figure legends, 1 figure and 1 table. This work has not been published previously and is not being considered for publication elsewhere in whole or in part in any language. All the co-authors have read and approved the manuscript for submission. The authors declare that they have no competing financial interests.

We hope that this manuscript will be considered suitable for publication in ***The Journal of Visualized Experiments***. Please contact me if you have any questions concerning the manuscript. Thank you very much for your consideration.

Yours sincerely,

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TITLE:

Immunological Elimination of Undifferentiated Human Pluripotent Stem Cells Using Pluripotent State-Specific Antigen, Glypican-3

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KEYWORDS:

induced pluripotent stem cells, regenerative therapies, cytotoxic T cells, glypican-3, immunotherapy, tumorigenesis

SUMMARY:

Regenerative therapies using human induced pluripotent stem cells (hiPSCs) have recently attracted much attention. In this study, we use anticancer immunotherapy with peptide vaccination to prevent hiPSC-derived tumorigenesis. Our results show that glypican-3 (GPC3) works as a pluripotent state-specific immunogenic antigen in hiPSCs.

ABSTRACT:

Much attention has been focused on human pluripotent stem cells (hPSCs) due to their potential as cell sources in regenerative therapies. Especially in the area of cardiac regenerative medicine, which is challenged by organ shortage, transplantation of human induced pluripotent stem cells (hiPSC)-derived cardiomyocytes has potential to treat many patients with severe heart failure. However, to achieve transplantation of hiPSC-derived cardiomyocytes, removal of contaminated immature cells with high accuracy is essential to eliminate the risk of teratoma formation caused by residual undifferentiated hiPSCs. Peptide vaccination is well-known as an effective anticancer immunotherapy because of selective cellular cytotoxicity. To establish immunological elimination of contaminated immature hiPSCs, we identified glypican-3 (GPC3) as a pluripotent state-specific carcinoembryonic antigen. Immunostaining showed that hiPSCs expressed GPC3, especially in pluripotent states. Undifferentiated hiPSCs were rejected by cytotoxic T cell (CTL) clones sensitized with HLA-class I-restricted GPC3 peptides. These results indicate that GPC3-specific CTLs can prevent hiPSC-derived tumorigenesis, which may occur by contamination by

undifferentiated cells. Our results indicate that GPC3 works as a pluripotent state-specific immunogenic antigen in hiPSCs. These results show the applicability of GPC3-mediated immunotherapy to ensure safety in regenerative medical procedures using hiPSCs.

INTRODUCTION:

Recently, regenerative therapies using human induced pluripotent stem cells (hiPSCs) have attracted much attention as new cell sources for regenerative therapies. Especially in cardiac regenerative medicine, transplantation of hiPSC-derived cardiomyocytes is expected to resolve the challenge of organ shortage[1,2].

Retinal regeneration requires a small number of cells; hence, the possibilities of tumor formation due to residual undifferentiated stem cells are negligible. In contrast, regenerative procedures of the heart and liver, which require a large number of cells, are difficult to perform safely.

Until now, many different methods of eliminating undifferentiated human pluripotent stem cells (hPSCs) from hiPSC derivatives have been reported because of teratoma formation induced by undifferentiated hPSC contamination in hiPSC derivatives[3-7]. However, to achieve transplantation of hiPSC-derived cardiomyocytes, complete removal of residual undifferentiated cells in vivo is important because a massive number of cells is required for transplantation.

Peptide vaccination has been used for cancer patients as an anticancer immunotherapy with selective cellular cytotoxicity[8]. In this study, we aim to prevent hiPSC-derived tumorigenesis with CTLs using peptide vaccination methods.

GPC3 is one of the carcinoembryonic antigens and is widely expressed in human embryos[9-11]. It is also overexpressed in 72–81% of patients with hepatocellular carcinoma (HCC)[12], and expression of GPC3 has been reported in melanoma, Wilms tumor, hepatoblastoma, ovarian clear cell adenocarcinoma, yolk sac tumor, and other carcinomas[11,13-19]. In this study, we report that hiPSCs uniquely express the oncofetal antigen GPC3, and GPC3-specific CTLs can be used in immunotherapy for removal of undifferentiated hiPSCs from hiPSC derivatives for future regenerative medical procedures.

PROTOCOL:

1. Immunofluorescence staining of hiPSCs with GPC3 and OCT4

1.1. Grow cultured hiPSCs in stabilized feeder-free maintenance medium (mTESR1; **Table of Materials**) at a density of 3 x 10^4 cells/cm^2 using 12 well plates. Incubate the dish at 37 °C in a 5% CO_2 incubator overnight.

1.2. Remove the medium of each well and wash with 1 mL of phosphate-buffered saline (PBS) for each well 1x and then remove the PBS.

1.3. Fix cells with 500 µL of 4% paraformaldehyde for 30 min at 4 °C.

1.4. Wash 3x with 1 mL of PBS for each well and then remove PBS.

1.5. Permeabilize with 500 µL of 0.2% triton X-100 in PBS for 15 min at room temperature (RT).

1.6. Remove triton X-100 in PBS, wash cells with 1 mL of PBS 3x for 5 min, and then remove PBS.

1.7. Incubate cells in the anti-GPC3 and anti-OCT4 antibodies diluted 1:200 in 2% fetal bovine serum (FBS) in PBS overnight at 4 °C.

1.8. Decant the solution and wash the cells 3x for 5 min in PBS.

1.9. Incubate the cells with the secondary antibodies (Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 546 anti-rat IgG) diluted 1:200 in 2% FBS in PBS for 1 h at RT in the dark.

1.10. Decant the secondary antibody solution and wash with PBS 1x for 5 min in the dark.

1.11. Stain cells with 4',6-diamidino-2-phenylindole (DAPI; 1 µg/mL final concentration) for 5 min at RT.

1.12. Decant the solution containing DAPI and then wash the cells 2x with PBS for 5 min in the dark. After the wash, observe by fluorescence microscopy.

2. Cell viability assays

2.1. Culture hiPSCs in 96 well plates at a density of 1 x 10^4 cells/cm^2 in mTESR1 with ROCK inhibitor at a final concentration of 10 µM at 37 °C.

2.2. Culture hiPSC-derived cardiomyocytes in 96 well plates at a density of 2 x 10^4 cells per well with alpha minimum essential medium (αMEM) with 5% FBS at 37 °C in static culture.

NOTE: hiPSC-derived cardiomyocytes were generated as previously described[20,21].

2.3. After 24 h, remove the medium in steps 2.1 and 2.2.

2.4. Adjust HLA-A2 restricted-GPC3 specific CTLs to 5 x 10^5 cells in 100 µL of each medium and add to both plates in steps 2.1 and 2.2.

NOTE: HLA-A*02:01-restricted $GPC3_{144–152}$ (FVGEFFTDV)-reactive CTL clones were previously established using peripheral blood mononuclear cells from HCC patients administered a GPC3 vaccination[22].

2.5. After 48 h of coculture with CTLs, stain cells with 1 µM calcein-AM by adding 1 µL of calcein-AM to 100 µL of αMEM (cardiomyocytes) or mTESR1 (hiPSCs) in each well.

2.6. Incubate plates for 15 min at 37 °C.

2.7. Remove each medium with calcein-AM and wash the wells 2x with PBS.

2.8. Observe live cells by fluorescence microscopy.

3. Coculture of hiPSCs and hiPSC-derived cardiomyocytes with GPC3-specific CTLs

3.1. Culture hiPSCs at a density of 1 x 10^5 cells/cm^2 in 6 well plates with mTESR1 for 5−7 days at 37 °C in a 5% CO_2 incubator.

3.2. Culture hiPSC-derived cardiomyocytes at a density of 5 x 10^4 cells/cm^2 in 12 well plates with αMEM for 3 days at 37 °C in a 5% CO_2 incubator.

3.3. Remove the mTESR1 medium from hiPSC culture and collect the cells with 500 µL of dissociation buffer for 5 min at 37 °C. Centrifuge cells in a 10 mL conical tube at 200 x *g* for 5 min at RT.

3.4. Remove the supernatant and fluorescently label hiPSCs with 1 mL of mTESR1 containing 1 µM green 5-chloromethylfluorescein diacetate (CMFDA), then incubate at 37 °C for 1 h.

3.5. After 1 h of incubation, replace the mTESR1 medium containing CMFDA with basic mTESR1 medium.

3.6. Seed labeled hiPSCs from step 3.5 (at a density of 1.5 x 10^3 cells/cm^2) in the 12 well plates where the cardiomyocytes are cultured. Then return the plates to the incubator.

3.7. After 24 h from seeding, adjust HLA-A2 restricted-GPC3 specific CTLs to 3 x 10^6 cells in 100 µL of mTESR1 and coculture cells at 37 °C in a 5% CO_2 incubator in either the presence or absence of GPC3 CTLs.

3.8. After 48 h of coculture with CTLs, wash cells with PBS 3x for 5 min to remove CTLs. Then analyze by flow cytometry or immunofluorescence staining.

3.9. Dissociate with 500 µL of dissociation buffer for 5 min and collect the cells by centrifugation in 10 mL conical tubes at 200 x *g* for 5 min at RT.

3.10. After aspirating the supernatant, suspend the cells in 2% FBS in PBS using a pipette to disaggregate cells.

3.11. Prior to data analysis, vortex each tube to avoid cell aggregates.

3.12. Use flow cytometry to calculate the percentage viability of hiPSCs in cocultured cells

(fluorescently labeled cells are hiPSCs). Collect a minimum of 10,000 events. For analyses, gate the live cell population excluding the dead cells. Determine the percentage of positive cells within the gated population.

3.13. To analyze using immunofluorescence staining (similar to section 1), incubate the cells from step 3.8 with diluted anti-troponin T and anti-OCT4 antibodies (use each at a dilution of 1:200) at 37 °C in a 5% CO_2 incubator. Then wash the cells 2x with PBS for 5 min in the dark. After the wash, observe by fluorescence microscopy.

REPRESENTATIVE RESULTS:

Immunofluorescent staining showed that GPC3 and the typical pluripotent stem cell marker OCT4 were expressed in pluripotent states of hiPSCs (**Figure 1A**). GPC3-specific CTL clones revealed cytotoxic effects against hiPSCs after coculture but not against hiPSC-derived cardiomyocytes (**Figure 1B**).

After 48 h of coculture, using flow cytometry analysis, the viability (%) of hiPSCs in cocultured hiPSCs and hiPSC-derived cardiomyocytes decreased from approximately 25% to 5% (**Figure 1C**). GPC3-specific CTL clones could selectively eliminate undifferentiated hiPSCs from mixed cells of hiPSCs (immunofluorescence stained with OCT4) and hiPSC-derived cardiomyocytes (immunofluorescence stained by TroponinT) using immunofluorescence staining (**Figure 1D**).

FIGURE LEGENDS:

Figure 1: In vitro assay of suppression of pluripotent cells with GPC3 specific CTL. (**A**) Immunofluorescence staining with anti-GPC3 and anti-OCT4 antibodies in hiPSCs (n = 3 independent experiments). Scale bars = 100 µm. (**B**) hiPSCs were cultured with GPC3-specific CTLs for 48 h. After the coculture, live cells were labeled with calcein-AM. Relative intensity was analyzed by fluorescence microscopy (n = 3 independent experiments). Scale bars = 100 µm. (**C**) hiPSCs were mixed with hiPSC-derived cardiomyocytes and cocultured in either the absence (control) or presence (GPC3-CTL) of GPC3-specific CTLs. After 48 h of coculture, using flow cytometry analysis, the viability (%) of hiPSCs in cocultured hiPSCs and hiPSC-derived cardiomyocytes was observed (n = 3 independent experiments). Data are presented as the mean ± SD. *$p < 0.05$. (**D**) Immunofluorescent staining of undifferentiated hiPSCs (stained with anti-OCT4 antibody) and hiPSC-derived cardiomyocytes (stained with anti-Troponin T antibody) cocultured in either the absence or presence of GPC3-specific CTL clones (n = 3 independent experiments). Scale bars = 400 µm.

DISCUSSION:

In this study, we identified glypican-3 (GPC3) as a pluripotent state-specific immunogenic antigen and validated the applicability of GPC3 to remove undifferentiated cells from hPSC derivatives.

To date, many methods for eliminating undifferentiated hPSCs in hiPSC derivatives have been reported, such as use of toxins, small molecules, and pluripotent state-specific antigens[3-7]. Antibody-mediated FACS and transfection of drug-resistant genes have also been used to condense the target differentiated cells in other fields. However, the former is not applicable to

a large number of cells, and the latter requires integration of external genes into the genome, which sometimes causes safety-related issues. We also recently attempted to purify cardiomyocytes and eliminate residual undifferentiated stem cells using mitochondrial fluorescent dye or specific culture conditions based on metabolic differences between cardiomyocytes and the other proliferating cells[21,23,24]. Thus, for safer clinical applications, elimination of residual undifferentiated stem cells from their derivatives represents a barrier that may hinder clinical applications in regenerative medicine. In this study, we aimed to eliminate undifferentiated hiPSCs using an anticancer immunotherapy called peptide vaccination.

Peptide vaccination has been used for anticancer treatment with selective cellular cytotoxicity, especially with CTLs. We found that GPC3 is a carcinoembryonic antigen, which is also expressed in hiPSCs, and is a good target antigen for immunotherapy. As a result, GPC3-specific CTL could selectively eliminate hiPSC in hiPSC-derived cardiomyocytes without cytotoxic effects to the cardiomyocytes. Although we have not determined whether the treated hiPSC-derived cardiomyocytes with CTLs are indeed less teratogenic after transplantation so far, GPC3-specific CTLs are expected to prevent teratoma formation or eliminate existing teratomas in vivo after their administration. We propose that they will serve as an effective therapy for teratoma eradication, for which surgical approaches are difficult.

Moreover, peptide vaccinations have several advantages, as they are already being safely used for clinical applications. However, peptide vaccines have an HLA-associated restriction, and they cannot be applied within the general population. Because their action is not sufficiently strong compared with previous methods, stronger immunotherapies must be established for use in the general population. Our method also offers the possibility of increased accuracy when used in combination with other methods because of our unique approach for removing pluripotent cells.

As a conclusion, HLA-restricted GPC3-derived CTLs have potential as a pluripotent state-specific immunogenic antigen in hiPSCs. Our results indicate that the immunotherapies against GPC3 may prevent teratoma formation.

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DISCLOSURES:

S.T., J.F., and K.F. own equity in Heartseed, Inc. K.F. is CEO of Heartseed, Inc. The remaining authors have no conflicts of interest to disclose.

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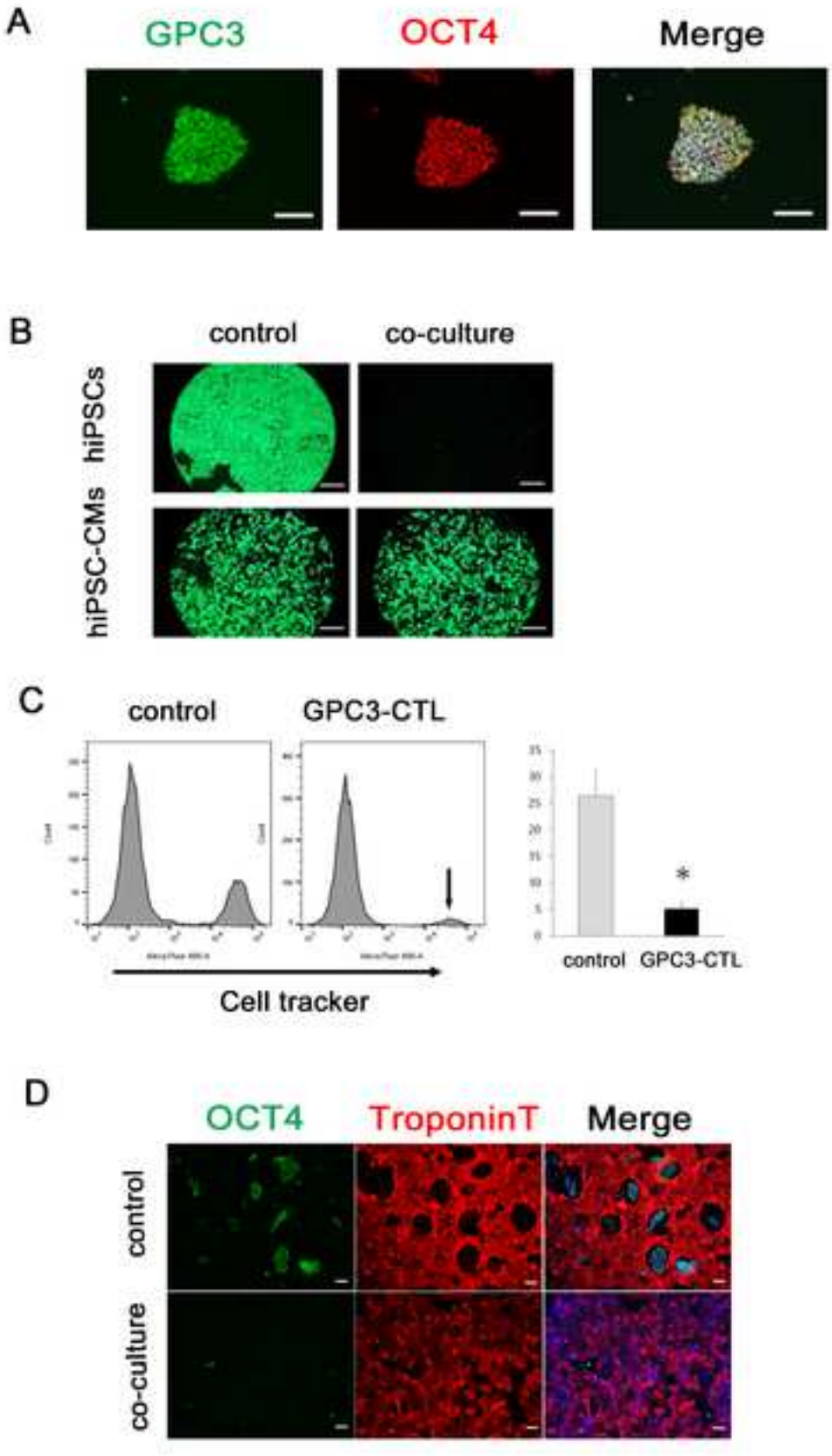
Figure 1



Name of Material/Equipment	Company	Catalog Number	Comments/Description
100-mm tissue culture dish	Falcon	353003	
15ml Centrifuge Tube	Greiner Bio-One	188271	
50ml Centrifuge Tube	Greiner Bio-One	227261	
96-well tissue culture plate	Falcon	353078	
Alexa Fluor 488 anti-mouse IgG	invtrogen	A-21200	
Alexa Fluor 488 anti-rat IgG	invtrogen	A-21470	
Alexa Fluor 546 anti-mouse IgG	invtrogen	A-11003	
Alexa Fluor 546 anti-rabbit IgG	invtrogen	A-11010	
BD Matrigel Matrix Growth Factor Reduced	BD Biosciences	354230	Thaw completely at 4℃ overnight and dilute it 50 times with Dulbecco's Modified Eagle's Medium before coating culture dishes
Calcein-AM	Dojindo	C396	
Cell Tracker Green CMFDA	Thermo Fisher Scientific	C7025	
cTroponin I antibody	abcam	ab52862	
DAPI	Thermo Fisher Scientific	D1306	
D-PBS(–)	Wako	045-29795	
GPC3 antibody	biomosaics	B0025R	
Human/mouse OCT-3/4 antibody	R&D	MAB1759	
mTeSR1 medium kit	STEM CELL	5850	Warm at room temperature before use
Serum, Fetal Bovine	biowest	S1780	
StemProAccutase	Thermo Fisher Scientific	A1110501	dissociation buffer
troponin T antibody	thermo	MA5-12960	
Y-27632	Wako Pure Chemical Industries		

αMEM	Thermo Fisher Scientific	12571048

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