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ORIGINAL ARTICLE

Human Embryonic Stem Cell Derived from Early Stage Fertilized Ovum: Non Immunogenic and Universal, Neuronal and Non-neuronal Cell Lines

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Background: Human embryonic stem cells (hESCs) have the potential to treat various human disorders currently labeled as incurable and/or terminal illness. However, the fear that the patients' immune system would recognize them as non self and lead to an immune rejection has hampered their use. The main cause for immune rejection is usually the incompatibility of both donor and recipient's major histocompatibility complex (MHC).
Methods: We describe a hESC line developed through a patented technology that does not lead to immune reaction upon transplantation. We have transplanted these cells in >1,400 patients with chronic/terminal conditions and did not observe any immune reaction. No immunosuppressant were administered to these patients. We analyzed the expression levels of MHC-I and MHC-II on the surface of these hESCs using microarray technology. The gene targets for miRNA were analyzed using Gene ontology and DAVID database and pathways for these genes were determined using Reactome and Panther databases.
Results: Our results showed that the levels of expression of MHC-I and MHC-II on hESCs is almost negligible and thus the hESCs are less susceptible to an immune rejection.
Conclusions: The hESCs cultured at our facility expresses low levels of MHC-I and do not produce an immune reaction. These can be administered universally and need no cross matching before transplantation.

Keywords: Human embryonic stem cells, Major histocompatibility complex, DAVID, Microarray technology, Reactome, miRNA

Introduction

Human Embryonic stem cells (hESCs) are the potential therapeutic targets for various chronic/terminal conditions (1). hESC lines are excellent candidates in transplantation medicine because they have the capacity to grow indefinitely in culture without losing pluripotency (2). The first FDA approved clinical trial of hESCs therapy was conducted in 2009; a product derived from hESCs was applied for stimulating nerve growth in patients (3). But, the clinical transplantation of hESCs requires immunosuppressive therapy as immune rejection is the bottleneck which hinders the application of hESCs as transplantation therapy (4).

The underline cause of immune rejection are the major histocompatibility antigens (cell surface antigens), essential for the acquired immune system which usually vary between the donor and host as they are perceived as non self by the recipient's immune system (5, 6). Swijnenburg and his co-workers proved that the embryonic stem cells

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(ESC) trigger an accelerated infiltration of immune cells, indicating the immune response towards developing ESCs in allotransplant (genetically non-identical) that increases over time (7). Similarly, Fandrich and his co-workers showed that the rat ESC-like cells express very low levels of major histocompatibility complex (MHC) -I antigens and completely lack MHC-II and co stimulatory molecules. This helps to decrease the chances of immune rejection as the donor MHC is absent that could mismatch with the recipient's MHC (8). Low levels of MHC-I result in escape of hESCs from an immune rejection (9). But, low levels of MHC I antigen increases after differentiation both *in vitro* and *in vivo* and is sufficient for immune rejection (10, 11). The present study describes hESCs of pre-blastomeric origin derived at 2-celled stage and cultured using a patented technology that do not induce any immune rejection. The study also describes the differentially expressed genes profile and their related pathways for immune reactions.

Materials and Methods

Origin of cell line

The study was approved by an independent ethics committee (IEC). Cell lines used in this study were cultured from a spare fertilized ovum obtained during natural *in vitro* fertilization (IVF) process with due consent from the donor. The hESCs were cultured and maintained as per our patented technology (United States Granted Patent No US 8592, 208, 52) in a good manufacturing practice (GMP), good laboratory practice (GLP) and good tissue practice (GTP) compliant laboratory. The cell lines were stable and free from any contamination. The detailed cell culture and differentiation techniques are explained in our previous paper (12).

Cell culture and derivation

The fertilized ovum was suspended in Roswell Park Memorial Institute medium (RPMI) and broken by mechanical means. βhCG and progestin was added and the cells were incubated in a CO_2 water jacketed incubator for 24 hrsin an aerobic condition. The cell suspension was divided into two and one of them was re-incubated in the same incubator after adding Dulbecco's Modified Eagle's Medium (DMEM, Himedia Labs, Mumbai, India) and the other in RPMI in anaerobic condition. The details of the cell culture and derivation are detailed in our previous paper (12).

RNA extraction and RT-PCR

Three samples were selected for the polymerase chain reaction (PCR) analysis and RNA extraction was performed using Qiagen RNeasy micro kit. RNA concentration was estimated using Nanodrop spectrophotometer. RNA purity and integrity were checked by employing an Agilent Bioanalyzer. The cDNA synthesis and primer sequences and annealing temperatures for genes Nestin, Sox 2, HLA-G and β-HCG are mentioned in our previous paper (12). β-actin gene was used as house keeping control gene. The amplified PCR products were analyzed by electrophoresis on 1% agarose gels.

miRNA microarray analysis

Samples were hybridized for microarray experiment. microRNA (miRNA) molecules in total RNA were labeled with Agilent miRNA labeling reagent and hybridization kit (Cat # 5190-0456). Labeling method used ligation of one cyanine 3–pCp molecule to the 3'end of RNA molecule with greater than 90% efficiency that generates fluorescent miRNA. After hybridization, the samples were scanned with Agilent Scanner. Images were analyzed using Agilent's Feature extraction software. Raw data was normalized using GeneSpring GX 12.6 software. Complete miRNA in the array detected on the basis of intensities.

For filtering the high expression miRNA from complete, lobase 2 value$\geq$0.6 was used. The target genes for differentially regulated miRNA's for up-regulation and down-regulation were checked using GeneSpring GX 12.6 software with an integrated target scan database.

Functional annotation analysis

To examine the gene pool of detected miRNA, Database for Annotation, Visualization and Integrated Discovery (DAVID)was used (13). It covers more than 40 annotation categories, including Gene Ontology (GO; www.geneontology.org/) terms, protein–protein interactions, protein functional domains, disease associations and biological pathways. GO terms organize genes into hierarchical categories consisting of three main layers and the first layer included three branches: biological process, cellular component and molecular function.

We analyzed the potential target genes associated pathways as per the Kyoto Encyclopedia of Genes and Genomes, Reactome and Panther pathway database (14-16). A p value of <0.05 was used as the cut-off criterion.

Results

The hESC cell line analyzed was a mixture of the two

cell lines; neuronal and non-neuronal. Thus, the analysis plan is focused on both of them. Mixture batch (M-batch) which is a mixture of both the cell lines was used as a control to compare the analysis of neuronal and non-neuronal cell lines.

Cell line differentiation

Differentiation of the hESC line into neuronal and non-neuronal cells was observed under appropriate culture conditions on DMEM and RPMI media. The detailed protocol is explained in our previous paper. hESCs expressed high levels of nestin (neuronalprogenital cells, NPCs) and NeuN (neuronal marker undifferentiated cells) which indicates the neuronal differentiation nature of these cells (12).

RNA Quality Control (QC) check

All the three samples were found to be suitable for microarray experiments as they showed high purity and concentration of RNA.

Surface markers analyzed by RT-PCR

Markers expression for HLA-G, a major histocompatible factor, 5-methyl cytosine gene activation marker, telomerase maintenance of genomic integrity and pluripotency of stem cells and β- human chorionic gonadotropin (β-hCG) which is an immune modulator was found to be amplified indicating that these genes are present and expressive in hESCs at mRNA level. Expression profile of all the markers was explained in our previous paper (12).

miRNA potential target gene analysis

Hybridized samples predicted the differentially expressed miRNA in the individual test sample. Each miRNA has a unique mirbase accession number and ability to regulate the expression of several hundred target genes. GeneSpring GX provided the gene target and their location on chromosome for each miRNA as shown in Table 1. Hence, GO term and their description for each miRNA target gene was determined for immune reactions.

Functional analysis

Biological processes of the predicted miRNAs gene targets were classified by GO analysis. Genes involved in each pathway are then determined by Reactome and Panther databases. Significant p-values showed the up and down-regulation of genes involved in various pathways of immune rejection (Table 2). After differentiation of the cell lines, MHC-I receptor activity (p=0.0059), MHC-I protein complex (p=0.0057) and total MHC protein complex (p=0.0284) had statistically significant values, implying that their function and pathways are down-regulated and hence the expression of MHC-I on hESCs is low. Antigen processing and presentation of peptide antigen via MHC-I was significant (p=0.0269) indicating a higher potential

Table 1. Protein coding gene targets for miRNA's analyzed by GeneSpring GX

Sr. No	Mirbase accession number	GO term	GO_Process	Full name	Chromosome location	p-value
1	MIMAT0005828	GO:0030183	B-cell Differentiation	ADP-ribosylation factor-like 1	2	0.0582
2	MIMAT0005828	GO:0030183	B-cell Differentiation	B-cell CLL/lymphoma 11A (zinc finger protein)	2	0.0683
3	MIMAT0005828	GO:0030183	B-cell Differentiation	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	10	0.0808
4	MIMAT0005828	GO:0030183	B-cell Differentiation	Histone deacetylase 4	2	0.1070
5	MIMAT0005828	GO:0030183	B-cell Differentiation	Enhancer binding protein (C/EBP), gamma	19	0.1683
6	MIMAT0005828	GO:0030183	T cell receptor signaling pathway	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	5	0.1795
7	MIMAT0005828	GO:0030183	B-cell Differentiation	One cut homeobox 1		0.2662
8	MIMAT0005865	GO:0002520	Immune system development	SMAD family member 3	15	0.6051
9	MIMAT0005865	GO:0042113	B cell activation	Taxilin alpha	1	0.6462
10	MIMAT0005828	GO:0042113	Immune response-activating signal transduction	src kinase associated phosphoprotein 2		0.4767
11	MIMAT0005828, MIMAT0005878	GO:0042113	B cell activation		7	0.3143

Table 2. Transcriptomic Profile of Genes

Sr No	GO term	Description	Count	%	p-value	Genes
1	GO:0030183	B cell differentiation	4	1.005025	0.0678	BCL2, SP3, BAX, CD79A
2	GO:0002520	Immune system development	9	2.261307	0.1764	HOXA3, BCL2, BAX, CD4, CD79A, FGF3, ERCC2
3	GO:0042113	B cell activation	4	1.005025	0.1873	BCL2, SP3, BAX, CD79A
4	GO:0001782	B cell homeostasis	2	0.502513	0.1959	BCL2, BAX
5	GO:0032943	Mononuclear cell proliferation	3	0.753769	0.2136	BCL2, BAX, CD79A
6	GO:0045058	T cell selection	2	0.502513	0.3139	BCL2, CD4
7	GO:0030217	T cell differentiation	3	0.753769	0.3657	BCL2, SP3, CD4
8	GO:0050870	Positive regulation of T cell activation	3	0.753769	0.4413	RARA, CD4, THY1
9	GO:0042110	T cell activation	4	1.005025	0.4509	BCL2, SP3, BAX, CD4
10	GO:0006959	Humoral immune response	3	0.753769	0.4611	CR2, LY86, BCL2
11	GO:0045087	Innate immune response	4	1.005025	0.5109	CYBA, CR2, IRGM, TBKBP1
12	GO:0002429	Immune response-activating cell surface receptor signaling pathway	2	0.502513	0.5388	CD79A, THY1
13	GO:0002253	Activation of immune response	3	0.753769	0.5535	CR2, CD79A, THY1
14	GO:0045580	Regulation of T cell differentiation	2	0.502513	0.6367	CLPTM1, RARA
15	GO:0002757	Immune response-activating signal transduction	2	0.502513	0.6439	CD79A, THY1
16	GO:0002764	Immune response-regulating signal transduction	2	0.502513	0.6711	CD79A, THY1
17	GO:0002684	Positive regulation of immune system process	5	1.256281	0.6905	CR2, RARA, CD4, CD79A, THY1
18	GO:0002252	Immune effector process	3	0.753769	0.7428	CPLX2, CR2, BCL2
19	GO:0050778	Positive regulation of immune response	3	0.753769	0.7811	CR2, CD79A, THY1
20	GO:0002443	Leukocyte mediated immunity	2	0.502513	0.8191	CPLX2, CR2
21	GO:0050776	Regulation of immune response	4	1.005025	0.8240	CR2, RARA, CD79A, THY1
22	GO:0042287	MHC protein binding	1	0.251256	1.0000	CD4
23	GO:0042289	MHC class II protein binding	1	0.251256	1.0000	CD4
24	GO:0032395	MHC class II receptor activity	1	0.251256	1.0000	HLA-DRB1
25	GO:0019815	B cell receptor complex	1	0.251256	1.0000	CD79A
26	GO:0019814	Immunoglobulin complex	1	0.251256	1.0000	CD79A
27	GO:0042613	MHC class II protein complex	1	0.251256	1.0000	HLA-DRB1
28	GO:0042612	MHC class I protein complex	9	0.418994	0.0057	AZGP1, MICA, ULBP1, ULBP2, HLA-A, HFE, HLA-C, HLA-G, HLA-F
29	GO:0032393	MHC class I receptor activity	7	0.325885	0.0059	MICA, ULBP1, ULBP2, HLA-A, HLA-C, HLA-G, HLA-F
30	GO:0002474	Antigen processing and presentation of peptide antigen via MHC class I	1	0.263158	1.0000	HLA-E

Count- Number of genes in the respective term. %- Percentage of involved genes/total genes.

of MHC-I to process the antigen only if they are present on hESCs. MHC -II receptor activity, and for protein binding in MHC-II class protein showed down-regulation (p=1.000) which states that the hESCs did not express MHC-II. Our results clearly prove that B-cell lymphoma-2 (BCL 2) is involved in B cell lineage commitment (p=1.00), B cell differentiation (p=0.0678), B cell activation (p=0.1873), B cell homeostasis (p=0.1959), T cell selection, differentiation and activation (p=0.4509), humoral immune response (p=0.4611) and all these processes are down-regulated indicating that these functional processes are absent in hESCs. Thymocyte differentiation antigen 1 (THY1) is involved in immune system development (p=0.1764), immune surface receptor signaling pathway (p=0.5388), activation of immune response (p=0.5535), positive regulation of immune system process (p=0.6905), regulation of immune response (p=0.8240) and for all these processes GO analysis showed the down-regulation suggesting that the immune response to hESCs is down-regulated by THY1.

Discussion

Since hESCs were first isolated, it has been widely accepted that these cells hold the potential to change the face of medicine as they have the capacity to differentiate in every cell type of the human body (17). But, the immune rejection by the patients' immune system acts as a barrier to the hESC therapy (18).

Previous experiments conducted by Drukker and his co-workers observed that in the mouse strains with different types of immune deficiency, T cell-deficient animals failed to reject hESC-derived graft, whereas the lack of NK cells or B-cells did not interfere with hESC rejection; thus suggesting that T cells play a pivotal role in the rejection of hESCs and their differentiated derivatives (19). Our results showed a down regulation for T cell activation indicating that the hESCs are unable to induce proliferation of T cell population in the host, thus, these hESCs can easily escape immune rejection pathway. It has also been proven that hESCs are able to inhibit T cell proliferation in response to allogeneic antigen presenting dendritic cell (20). Immune system is regulated by several genes but BCL 2 plays a major role. BCL 2 is an anti-apoptotic gene and it regulates cell differentiation processes. Our results showed that BCL 2 down regulates the B-cell and T-cell activation; thus, the functional processes of B-cell and T-cell activation are absent in hESCs.

It was recently suggested that immunological maturity or expression of antigens on the surface of hESCs is a late event during the gestational period of human embryos (21). Our microarray data support this notion. The expression of immune related genes, MHC-I and MHC-II was not up-regulated during *in vitro* differentiation of hESCs. These hESCs were of pre-blastomeric origin and at 2-celled stage; wherein the levels of expression of MHC-I and MHC-II are almost negligible (12). Besides, hESCs might provoke less of an immune response because expression of MHC-I and MHC-II protein was not detected on the surface of either undifferentiated or their differentiated progeny (20) But, hESCs express high levels of MHC-I after differentiation both *in vitro* and *in vivo* hence hESCs can be rejected on transplantation (9). However, our results proved that after pre differentiation, the expression of MHC class I protein complex ($p=0.0057$), and MHC class I receptor activity ($p=0.0059$) showed a down-regulation which means that hESCs grown at our facility showed low levels of MHC-I and MHC-II even after differentiation and hence are capable to escape an immune rejection. We have transplanted these cell lines in over 1,400 patients with terminal conditions where the traditional therapies had not worked and found them to be safe and effective. For all these patients, we did not observe an immune reaction. We have never administered any immunosuppressant to these patients (22-26).

Another key player of immune system activation is THY1 also known as CD90. It is a cell surface antigen which has neuronal expression in nervous system and is found to activate the immune system processes. Since, hESCs have no antigens expressed on their surface and are not involved in any immune reaction; thus, the host cells are not able to identify the injected hESC as foreign and the pathway for immune rejection is down-regulated.

Conclusion

The present study revealed that the hESCs cultured at our facility are non-immunogenic as they express very low levels of MHC-I and MHC-II. These hESCs found to be suitable for transplantation without the use of immunosuppressant and are universal in their applicability.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

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