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...
(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 CO₂ water jacketed incubator for 24 hrs in 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, lobe 2 value ≥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**

Differentialiation 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 (neuronal progenitor 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

| 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 | 2 | 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 | 7 | 0.4767 |
| 11     | MIMAT0005828, MIMAT0005878 | GO:0042113 | B cell activation | | | 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:002943 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:003017 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, CD79A |
| 10    | GO:006959 Humoral immune response | 3     | 0.753769 | 0.4611 | CR2, LY86, BCL2 |
| 11    | GO:0045087 Innate immune response | 4     | 1.005025 | 0.5109 | CYB, 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:002757 Immune response-activating signal transduction | 2     | 0.502513 | 0.6439 | CD79A, THY1 |
| 16    | GO:002764 Immune response-regulating signal transduction | 2     | 0.502513 | 0.6711 | CD79A, THY1 |
| 17    | GO:002684 Positive regulation of immune system process | 5     | 1.256281 | 0.6905 | CR2, RARA, CD4, CD79A, THY1 |
| 18    | GO:002252 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:002443 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 | CD79A |
| 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 | CD79A |
| 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:002474 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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