The Constantly Highly Expression of Limbal Stromal Cells Compared to the Bone Marrow Mesenchymal Stromal Cells, Adipose-Derived Mesenchymal Stromal Cells and Foreskin Fibroblasts

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Abstract

Limbal epithelial stem cells (LESC) have great potential in treating the blindness caused by corneal damage. LESC are maintained in stem cell niche called Palisade of Vogt. Limbal stromal (LS) cells are critical component of LESC niche and help in their self renewal. These cells resemble mesenchymal stromal/stem cells with multilineage differentiation potential. However little is known about their gene expression profile compared to MSC derived from various sources.

Keywords: limbal stromal cells; bone marrow mesenchymal stromal cells; adipose mesenchymal stem cells; gene expression profiling; microarray; limbal epithelial stem cells

Introduction

Human cornea on the front surface of eye is very critical for vision. The corneal transparency, continuous regeneration and functionality of corneal epithelium play an important role in refraction of light on to the retina. Corneal epithelium is regenerated by unique population of stem cells called limbal epithelial stem cells (LESC) that are located in the basal region of limbus. LESC differ from the corneal epithelium due to the lack of corneoscleral specific differentiation keratins (K3/K12) expression [1-3], connexin 43-mediated gap junction intercellular communication [4-6], p63 nuclear transcription factor [7,8], cell cycle duration [9], and label retaining property [10]. The limbalstroma provides a unique stem cell niche or microenvironment which is important for the modulation of stemness as it is heavily pigmented, highly innervated and vascularized. Clinically, destruction of LESC or the limbal stromal niche can lead to a pathological stage of LESC deficiency with severe loss of vision [11]. Chronic inflammation in the limbal deficient stroma is sufficient to cause detrimental damage to the conjunctiviallimbalautograft transplanted to patients or experimental rabbits [12]. These findings suggest that the limbal stromal niche is critical in regulating the self-renewal and the fate of LESC. Although the mechanism remains elusive, modulation of epithelial proliferation, differentiation, proliferation and apoptosis by the limbalstroma has been reported to favor stemness [13]. Limbal stromal (LS) cells are very important component of limbal stromal niche that helps in self renewal of LESC. Recently, LS cells were shown to have multilineage differentiation potential [14-17]. In one of the studies, an ABCG2-expressing FACS sorted side population cells from limbalstroma were able to differentiate into chondrocytes and neurons following differentiation induction [14]. In other studies, multipotent cells were also found in corneal stroma [15] and limbalstroma [16-17]. Earlier, we have reported that an ex vivo expanded LS cells possess multipotent differentiation potential towards adipocytes, osteocytes and chondrocytes [18]. Other stromal cells such as mesenchymal stem/stromal cells (MSC) can also be isolated and expanded in vitro for tissue regeneration applications [19-22]. MSC were first identified from bone marrow aspirates [23,24] and subsequently in Wharton’s jelly of human umbilical cords [25], adipose tissue [26], dental tissues [27,28] and skin [29]. Most of the stromal cells derived from various sources expressed the markers of MSCs such as CD44, CD73, CD90, CD105, STRO1 and do not express markers of hematopoietic lineage such as CD14, CD34, CD45 and HLA-DR [30].

In order to find out the specific molecular signature, cellular function and potential biomarkers of the LS cells, we compared the global gene expression profile including long non-coding RNA (lincRNA) of the expanded LS cells with the MSCs derived from bone marrow, adipose tissue and foreskin fibroblasts. In addition, we also evaluated the effects of two different culture conditions on the LS cells gene expression.

Methods

Establishment of limbal stromal cell culture

Corneoscleral rims from three cadaveric donors were obtained from post cornea graft transplantation with informed consent from the donor’s relative. The rims were washed with phosphate buffer saline (PBS; Invitrogen Corporation, Carlsbad, CA) and then trimmed to remove the sclera. The limbal tissues were incubated at 37°C for 2 h with dispase (BD Biosciences, Mississauga, Canada) at a concentration of 5 mg/mL. The
The limbal tissues were then cut into approximately 2 mm explants after washing with PBS. The limbal explants were cultured on matrigel (BD Biosciences, Mississauga, Canada) coated plates with complete medium containing Dulbecco's Modified Eagle's Medium (DMEM)/F12, 10% knockout serum replacement, 10 µg/mL insulin, 5 µg/mL transferrin, 5 µg/mL selenium-X, 100 IU/mL penicillin, 100 µg/mL streptomycin (all from Invitrogen Corporation, Carlsbad, CA, USA), 10 ng/mL leukemia inhibitory factor (LIF) (Sigma-Aldrich Chemie, Steinheim, Germany) and 4 ng/mL basic fibroblast growth factor (bFGF; BD Biosciences, Mississauga, Canada) [17]. The expanded limbal stromal cells were subjected to fluorescence-activated cells sorting (FACS) for the isolation of stage-specific embryonic antigen 4 (SSEA-4+) cells as reported previously [18]. The sorted SSEA-4+ cells were propagated on matrigel coated plate with the medium as mentioned previously. The limbal stromal cells that were maintained in this matrigel system were named as LS-matrigel. On the other hand, some of the sorted cells were maintained on normal plates with Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin (all from Invitrogen Corporation, Carlsbad, CA, USA). These cells were identified as LS-FBS.

**Human bone marrow mesenchymal stromal/stem cells (BM-MSC) culture**

Bone marrow MSC from three different lots (Millipore, Billerica, MA) were propagated and cultured according to manufacturer’s protocol. Briefly, cells at passage 4 were cultured on 0.1% gelatin coated plates with Mesenchymal Stem Cell Expansion Medium (Millipore, Billerica, MA) supplemented with 8 ng/mL fibroblast growth-factor-2 (FGF-2) (Millipore, Billerica, MA). When the cells were approximately 80% confluent, they were dissociated with trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) and passaged or alternatively frozen for later use.

Human adipose-derived mesenchymal stromal/stem cells (AD-MSC) and human foreskin fibroblast cells (HFF) culture.

Cryopreserved AD-MSC and HFF (n=3) at early passage (2-3) were obtained from Stempeutics Research Malaysia and propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin (all from Invitrogen Corporation, Carlsbad, CA). When the cells were approximately 80% confluent, they were dissociated with trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) and passaged or alternatively frozen for later use.

**Total RNA extraction and quality assessment**

LS-FBS (S1-P3, S3-P4 and S6-P3), LS-matrigel (S1-P6, S3-P6 and S6-P5), BM-MSC (BM01, BM05 and BM06), AD-MSC (AD001, AD002 and AD003) and human foreskin fibroblasts (HFF01, HFF02 and HFF03) at early passage (<5) were harvested with 0.25% trypsin-EDTA (Invitrogen Corporation, Carlsbad, CA) upon reaching 80-90% confluency. About 2-3 x 106 cells from each sample were lysed and total RNA was isolated using the RNAeasy kit (Qiagen Hamburg GmbH, Hamburg, Germany) according to the manufacturer’s protocol. The extracted RNA was quantified by reading the absorbance at 260 nm, and its purity was evaluated from the 260/280 ratio of absorbance with NanoDropTM 1000 (Thermo Fisher Scientific Inc.). The total RNA integrity was evaluated using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA).

**Gene expression profiling by microarray experiments**

Genome-wide expression profiles of all the samples were analyzed using Agilent SurePrint 3G 8x60K arrays (Agilent Technologies, Santa Clara, CA) that combined both coding and long intergenic non-coding RNA (lincRNA) for human genome. Prior to Cy3 labeling, 2µL of Agilent One-Color Spike Mix dilution was added to 100ng of total RNA for each sample. The total RNA was converted to cDNA and then to Cy3-labeled cRNA using Agilent One-Color RNA Spike-In Kit as per the manufacturer's protocol. The labeled cRNA was purified and quantitated prior to hybridization in hybridization oven at 65°C for 17 hr.

**Microarray image and data analysis**

Microarray image analysis was done using Feature Extraction version 10.7 and data analysis was done by using GeneSpring 11.5 (both from Agilent Technologies, Santa Clara, CA). The threshold was set to intensity value of 1.0. Normalization was done by 75 percentile shift. Baseline transformation was based on the median of samples. The data were further filtered by probset on flags and expression less than 20. The data has been deposited in Gene Expression Omnibus (GEO) with accession number GSE38947. Unpaired Student’s t test was used for statistical analysis. Genes up or downregulated by two-fold change were selected for further analysis. The false discovery rate (FDR) of 5% was estimated with the Benjamini-Hochberg method.

The gene expression profile of LS-FBS and LS-matrigel was compared. LS-FBS were chosen for the subsequent comparisons to other lineages. Hierarchical clustering was performed for LS-FBS versus BM-MSC, AD-MSC and HFF using Pearson Centered and Average-linkage clustering algorithm. Venn diagrams were drawn for the genes upregulated or downregulated in LS-FBS as compared to other lineages. Gene Ontology (GO) analysis was carried out for the upregulated genes and downregulated genes. Significant pathway analysis was also performed wherever possible. Gene functional classification was further carried out by DAVID software [40].

**Real time RT-PCR**

First strand cDNA was synthesized with Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Nonnenwald, Penzberg, Germany) as per manufacturer’s protocol. Then, quantitative real time polymerase chain reaction (RT-PCR) was performed by using a LightCycler instrument (Roche Diagnostics, Nonnenwald, Penzberg, Germany). Primers for the panel of genes used in this study are listed in Table 1. Products of PCR amplification were detected through intercalation of the SYBR green dye from LightCyclerFastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Nonnenwald, Penzberg, Germany). The amplification cycles were as follows: 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 62°C for 5 s and 72°C for 20 s. The concentration of MgCl2 in all cycling reactions was 2.4 mM. Gene specific products were confirmed by melting curve analysis. Expression of the genes was normalized with the expression of GAPDH and the expression ratio was calculated by REST software [41].

**Table 1 : Human primer sequences used for real time RT-PCR**

| Gene                | Accession | Sense primer         | Antisense primer        | Product size (bp) |
|---------------------|-----------|----------------------|-------------------------|------------------|
| GPNMB               | NM_00100534 | TTCTTGGCTTCTCAAGGAGGACG | GCCTGGTGTTTGACAGACCAT | 75               |
| SFRI1               | NM_0030121  | KOSTGGCGATACAGGAATGTGG | GCCTGGTGTTTGACAGACCAT | 184              |
Results

Cell culture

The LS cells were established from corneoscleral rim tissues and cultured in two different conditions as mentioned in the methods. Cell outgrowths were observed after a few days of plating and the cells reached confluence in about 2-3 weeks. The LS cells appeared to be fibroblastic, elongated and spindle shape growing pattern (Figure 1A). LS-matrigel cells have more elongated feature compared to LS-FBS. The LS-matrigel cells could be cultured up to 10 passages or more. The LS cells derived from the samples using both methods were used in the subsequent experiments. The BM-MSC, AD-MSC and HFF showed spindle and fibroblastic morphology when cultured and expanded (Figures 1B-1D).

Gene expression profiling

A total of 871 entities were found upregulated in LS-matrigel compared to LS-FBS (p<0.05, fold change >=2). The differentially expressed genes (fold change >10) of LS-matrigel versus LS-FBS are depicted in Table 2. Hierarchical cluster analysis was performed to determine the relationship of the four different cell types (LS-FBS, BM-MSC, AD-MSC and HFF). The dendrogram in Figure 2 demonstrates that MSC isolated from the same source were clustered together. A total of 340 significant differentially expressed genes (p <0.05, fold change >=2) were identified between LS-FBS and BM-MSC. Whereas, 399 and 146 differentially expressed genes were identified for AD-MSC and HFF when compared to LS-FBS respectively.

Figure 1: Morphological observations

Figure 2: Hierarchical clustering of all the samples from different sources

Table 2: List of differentially expressed genes

| Gene Symbol | Gene Description | Fold Change | p-value |
|-------------|------------------|-------------|---------|
| ADAMTS8     | ADAM metalloproteinase with thrombospondin type 1 motif 8 | 12.459421 | 12.480957 |
| ADR2A       | Adrenergic, alpha-2A, receptor | NM_000681 | 424.33313 |
| ANGPTL4     | Angiopoietin-like 4 | NM_139314 | 14.670821 |
| ANGPTL7     | Angiopoietin-like 7 | NM_021146 | 14.409806 |
| ANK3        | Ankyrin 3, node of Ranvier (ankyrin G) | NM_020987 | 14.409806 |
| APCDD1      | Adenosinotaxis polyposis coli down-regulated 1 | NM_152000 | 11.659266 |
| APOD        | Apolipoprotein D | NM_001010000 | 12.362466 |
| AQP3        | Aquaporin 3 (Gill blood group) | NM_004925 | 25.748945 |
| ATRX        | Ataxia telangiectasia 1 | NM_01010000 | 11.816478 |
| ASCl2       | Ascl2, achaete-scute complex homolog 2 (Drosophila) | NM_005170 | 11.49502 |
| CALM1       | Calcinophilin | NM_018398 | 12.480957 |
| C6orf1       | Complement factor I | NM_000204 | 12.210217 |
| COMP        | Collagen, type XV, alpha 1 | NM_000090 | 17.54856 |
| COL15A1     | Collagen, type XV, alpha 1 | NM_000093 | 12.480957 |
| COL21A1     | Collagen, type XXI, alpha 1 | NM_000090 | 17.54856 |
| COL3A1      | Collagen, type III, alpha 1 | NM_000090 | 17.54856 |
| COL4A6      | Collagen, type IV, alpha 6 | NM_033641 | 12.49421 |
| COL5A1      | Collagen, type V, alpha 1 | NM_000093 | 10.208904 |
| COMP        | Complement factor I | NM_000095 | 23.414925 |

Figure 3: Venn diagrams showing the numbers of all up- (panel A) or downregulated genes (panel B) of LS-FBS when compared to BM-MSC (bone marrow mesenchymal stem cells), AD-MSC (adipose-derived mesenchymal stem cells) and HFF (human foreskin fibroblasts)
MSCs as shown in Tables 2 and 5 and Appendix 3, 4 and 5. Most of the sharing some common genes that are highly expressed compared to other osteocytes and chondrocytes [42–44]. However, they differ in terms of regulatory properties [45].

**Discussion**

In this study, we compared the gene expression of stromal cells derived from different sources namely limbal stromal cells (LS-FBS and LS-matrigel), bone marrow mesenchymal stem cells (BM-MSC), adipose-derived mesenchymal stem cells (ADMSC) and human foreskin fibroblasts (HFF). Morphologically, these cells resembled the fibroblasts with a slight difference in their size and shape. The MSCs derived from various sources are known for their multipotential differentiation towards adipocytes, osteocytes and chondrocytes [42–44]. However, they differ in terms of growth factor, cytokine secretion and immunomodulatory properties [45].

The LS-FBS and LS-matrigel have different molecular signatures despite sharing some common genes that are highly expressed compared to other MSC as shown in Tables 2 and 5 and Appendix 3, 4 and 5. Most of the
differentially expressed genes in LS-matrigel are involved in the extracellular components such as collagen, type XXI, alpha 1 (COL21A1), matrix metalloproteinase 27 (MMP27), cartilage oligomeric matrix protein (COMP), collagen, type XV, alpha 1 (COL15A1), collagen, type III, alpha 1 (COL3A1), collagen type IV, alpha 6 (COL4A6) and collagen type V, alpha 1 (COL5A1). The results demonstrated that when LS cells were cultured with FBS without matrigel, the expression of these matrix proteins was downregulated. The matrigel provided an efficient culture microenvironment supporting the production of ECM. Our findings concurred with others that culturing method can have influence on the gene expression profile of stem cells [46]. Higher expression of ECM proteins in LS-matrigel as compared to LS-FBS might mimic the stem cell niche environment for LS cells and might be useful in the maintenance of the limbal epithelial stem cells. Different culture conditions have effect on cell characteristic and gene expression. We believe this maybe an adaptive response to stimuli during damage or pathogenesis of limbal epithelial stem cell niche. Due to this adaptive response, LS cells may generate necessary paracrine factors and ECM proteins to help in recovery process. In addition, LIF has been reported to play a role in self renewal and differentiation of human and mouse stem cells [47]. Murine embryonic stem cells for instance depend strictly on LIF for self renewal and maintenance of pluripotency but LIF is not able to maintain human embryonic stem cells. However, our result showed that both LIF and matrigel were not able to induce pluripotency of the SSEA-4+ LS cells.

Although cell culture conditions, growth factors and even FBS affect the gene expression of the cultured cells, there is still no standard culture protocol for MSC derived from various sources. The characteristics of MSC are always confirmed by immunophenotyping and differentiation assay towards adipocytes, osteocytes and chondrocytes [30]. However, the ex vivo expanded MSC are normally heterogeneous. Therefore, a systematic ex vivo global molecular characterization of MSC is needed in the future to define MSC. Thus, gene expression profiling provides an important tool for comparison and characterization of stromal cells from various sources.

In this study, LS-FBS and LS-matrigel were compared to BM-MSC, AD-MSC and HFF cultured in FBS. This study demonstrates a set of novel differentially expressed genes in LS-FBS compared to BM-MSC, AD-MSC and HFF. We also found different set of common genes that were highly expressed by LS-matrigel compared to BM-MSC, AD-MSC and HFF cultured in FBS. This might be due to the culture media components such as LIF, bFGF and matrigel. For LS-FBS, the highest expressed gene, SCIN is a Ca2+-dependent actin severing and capping protein [48] which is presumed to regulate exocytosis by affecting the organization of the microfilament network underneath the plasma membrane. This may play an important role in secretion of various growth factors required for maintenance and self renewal of LESC. It also regulates chondrocytes proliferation and differentiation. The second highly expressed gene, Ras-related GTP binding D is a monomeric guanine nucleotide-binding protein, or G protein. The G proteins act as molecular switches in numerous cell processes and signaling pathways (supplied by OMIM). The intracellular fatty acid binding protein 3 (FABP3) is another highly expressed gene in LS cells. The fatty acid binding proteins (FABP) belong to a multigene family. FABP are thought to participate in the uptake, intracellular metabolism and/or transport of longchain fatty acids. They might be responsible in regulating cell growth and proliferation. One of the FABP genes, FABP4 has been reported to be upregulated during adiogenesis of MSC [31,49].

Conclusion

We report a novel set of genes that are consistently highly expressed in LS cells compared to the bone marrow MSC, adipose-derived MSCs and foreskin fibroblasts. The LS cells have unique molecular signature compared to other MSC lineages. Thus, the highly upregulated genes in LS cells could be used as biomarkers by using real time RT-PCR which is less labourious and quicker as compared to microarray analysis. The knowledge gained can help us to improve our understanding of the cellular signaling pathways involved in LESC self-renewal, survival and differentiation, and may aid in the development of strategies to improve the tissue regeneration potential of these cells.

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