Comparative global gene expression profile of human limbal stromal cells, bone marrow mesenchymal stromal cells, adipose-derived mesenchymal stromal cells and foreskin fibroblasts

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Abstract

Background: Limbal epithelial stem cells (LESC) have great potential in treating the blindness caused by corneal damage. LESCs are maintained in stem cell niche called Palisade of Vogt. Limbal stromal (LS) cells are critical component of LESCs 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.

Methods: In this study, we compared the gene expression profile of LS cells expanded in two different culture conditions: basal media with bFGF, LIF and matrigel (LS-matrigel), as well as basal media with 10% FBS (LS-FBS). In addition, gene expression profile of LS-FBS cells were compared to bone marrow, adipose-derived MSC and foreskin fibroblasts. Total RNA was extracted from various cell types upon achieving confluency and subjected to microarray experiments (Agilent platform) using Human GE 8x60k gene chips. Data analysis was done with GeneSpring software.

Results: LS cells cultured in matrigel system showed upregulation of 871 genes as compared to LS-FBS and 58 genes were consistently differentially expressed in LS-FBS as compared to other cell types. Despite many long intergenic non-coding RNA and function unknown genes, differentially expressed genes represent gene ontology for cell signaling molecules including various growth factors, cell metabolism and extracellular matrix components for various biological processes. Samples from the same source were closely clustered by hierarchical clustering analysis.

Conclusions: The two culture conditions used in the study affected the gene expression profile of LS cells significantly. The LS cells showed distinct molecular signature as compared to MSC from various other sources. This comparative study will help in understanding the limbal stem cell niche biology and the novel set of genes could be used as biomarkers for LS cells.

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. LESCs differ from the corneal epithelium due to the lack of corneo-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 limbal stroma 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 LESCs or the limbal stromal niche can lead to a pathological stage of LESCs deficiency with severe loss of vision [11]. Chronic inflammation in the limbal deficient stroma is sufficient to cause detrimental damage to the conjunctival limbal autograft 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 LESCs. Although the mechanism remains elusive, modulation of epithelial proliferation, differentiation, proliferation and apoptosis by the limbal stroma 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 limbal stroma 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 limbal stroma [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].

The advent of microarray technology has enabled the monitoring of individual and global gene expression patterns across multiple cell populations. Numerous studies have now examined the global gene expression profile of MSC derived from different tissues that exhibit varying levels of proliferation, secretome and differentiation potentials. These studies showed that stromal cells isolated from different sources have unique molecular signatures despite sharing some common genes belonging to MSC when compared to mature fibroblasts [31-33]. It was also shown that gene expression profile of MSC remained stable during ex vivo expansion and subculture and was proven more sensitive to define MSC [34]. Other studies have reported gene expression profile of LESC [35-38], but comparative gene expression analysis of LS cells that are critical component of limbal stem cell niche and help in maintaining self renewal of LESC has not been well studied. As far as genome-wide differential gene expression profiling of LS cells is concerned, there is only one report where analysis were performed on mesenchymal cells derived from limbal explants culture [39]. Gene expression profile of LS cells also has not been compared with other stromal cells such as adipose stromal cells and foreskin fibroblast.

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

The research protocol was approved by the Medical Research

and Ethics Committee, Ministry of Health and the Medical Research Secretariat, UKM (University Kebangsaan Malaysia).

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 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 Chem, 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 2-3x10⁶ cells from each sample were lysed and total RNA was isolated using the RNeasy 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 NanoDrop™ 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 G3 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 probeset 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 LightCycler FastStart 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

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

Gene	Accession No.	Sense primer	Antisense primer	Product Size (bp)
<i>GAPDH</i>	NM_002046	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498
<i>SCIN</i>	NM_033128	ATGGCTTCGGGAAAGTTTATGT	CATCCACCATATGTGCTGGG	117
<i>RRAGD</i>	NM_021244	CTAGCGGACTACGGAGACG	ATGAGCAGGATTCTCGGCTTC	122
<i>FABP3</i>	NM_004102	ATGGGGACATTCTCACCTAAA	GCTGTTGTCTCATCGAACTCCA	91
<i>TFAP2B</i>	NM_003221	TTCCTCCCAAATCGGTGACTT	CGCCGGTGTGACAGACAT	75
<i>GPNMB</i>	NM_001005340	CTTCTGCTTACATGAGGGAGC	GGCTGGTGAGTCACTGGTC	164
<i>SFRP1</i>	NM_003012	ACGTGGGCTACAAGAAGATGG	CAGCGACACGGGTAGATGG	184

The table details the primers sequences, their accession number and expected product size after RT-PCR.

the expression ratio was calculated by REST software [41].

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 (as shown in Supplement figure S1). 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).

Figure 1. Morphological observations. Phase contrast microscopic image of a confluent culture of (A) Limbal stromal cells, (B) Bone marrow stromal cells, (C) Adipose derived stromal cells and (D) Human foreskin fibroblast. The stromal cells derived from the different sources appeared to be fibroblastic, elongated and spindle shape. Magnification 40x.

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. Venn diagram (Figure 3) shows that among the differentially expressed genes, 23 entities were upregulated (including one lincRNA) in LS-FBS (Table 3) whereas 35 entities (including of 4 lincRNA) were downregulated in LS cells versus the other three cell types (Table 4). Among these were several genes contributing to the cell signaling receptors such as Frizzled family receptor 5 (*FZD5*) involved in Wnt signaling, extra cellular matrix proteins (*SPON2*), transmembrane glycoprotein (*GPMNB*) involved in G protein coupled receptor activity and genes related to cell signaling and metabolism such as phosphatidylserine binding (*SCIN* and *SCARB1*) or lipid binding proteins (*SCIN*, *SCARB1*, *NPC2*, *FABP3*, and *HS1BP3*). In addition, transcription factor involved in sequence specific DNA binding including transcription factor AP-2 beta (*TFAP2B*) was also upregulated.

However, we found different set of upregulated or downregulated genes when LS-matrigel was compared to the BM-MSC, AD-MSC and HFF. The common upregulated and downregulated genes in LS-matrigel are listed in Appendix 1 and 2. Venn diagrams of the comparison are shown in Supplement figures S2 and S3. In addition, genes that are differentially expressed when LS-matrigel was compared to BM-MSC, AD-MSC and HFF separately are shown in Appendix 3, 4 and 5 respectively. Some of the common genes that upregulated in LS-FBS and LS-matrigel when compared to BM-MSC were insulin-like growth factor binding 5 (*IGFBP5*), fibrillin 2 (*FBN2*), proprotein convertase subtilisin/kexin type 9 (*PCSK9*) and adrenomedullin (*ADM*). On the other hand, common genes that upregulated in LS-matrigel and LS-FBS when compared to HFF were Xg blood group (*Xg*), insulin-like growth factor binding protein 2 (*IGFBP2*) and laminin alpha 3 (*LAMA3*). However, common upregulated genes could not be found in LS-FBS and LS-matrigel when compared to AD-MSC.

Further functional classification of genes that were highly expressed (more than 10-15 folds) in LS-FBS was done by using DAVID software. The differentially expressed genes as shown in Table 5 were classified as secreted proteins, signaling protein, extra cellular matrix, cell differentiation, basement membrane and cell adhesion protein. Some of the secreted proteins that were highly expressed when compared to BM-MSC and AD-MSC were EGF-like-domain, multiple 6 (*EGFL6*), angiopoietin-like 7 (*ANGPTL7*), insulin-like growth factor binding protein 2 (*IGFBP2*) and insulin-like growth factor binding 5 (*IGFBP5*). These proteins might play important role in proliferation and self renewal of LESC in paracrine fashion in the stem cell niche. Most of the Hox gene family members were downregulated as they are involved in differentiation. This also shows that LS-cells maintain multipotent phenotype by suppressing Hox genes.

For LS-FBS versus BM-MSC, significant pathway analysis ($p < 0.05$) showed that the genes involved in several pathways such as IL-2 (*STAT5A*, *BCL2*, *PLCB4*) and IL-7 (*IL-7R* and *STAT5A*), tnfr 2 signaling (*MAP3K1*), angiotensin II mediated activation of JNK pathway via pyk2 dependent signaling (*MAP3K1*, *AGT*),

Table 2. Differentially expressed genes in limbal stromal cells cultured in matrigel system versus non-matrigel system supplemented with fetal bovine serum.

Gene Symbol	Gene Name/Description	Acc. No.	Fold Change
<i>ADAMTS8</i>	ADAM metallopeptidase with thrombospondin type 1 motif, 8	NM_007037	14.913507
<i>ADRA2A</i>	adrenergic, alpha-2A-, receptor	NM_000681	47.013718
<i>ANGPTL4</i>	angiopoietin-like 4	NM_139314	424.33313
<i>ANGPTL7</i>	angiopoietin-like 7	NM_021146	14.670821
<i>ANK3</i>	ankyrin 3, node of Ranvier (ankyrin G)	NM_020987	14.409806
<i>APCDD1</i>	adenomatosis polyposis coli down-regulated 1	NM_153000	11.659266
<i>APOD</i>	apolipoprotein D	NM_001647	12.362466
<i>AQP3</i>	aquaporin 3 (Gill blood group)	NM_004925	25.748945
<i>ARHGAP28</i>	Rho GTPase activating protein 28	NM_001010000	11.816478
<i>ASCL2</i>	achaete-scute complex homolog 2 (Drosophila)	NM_005170	11.49502
<i>CACNA2D3</i>	calcium channel, voltage-dependent, alpha 2/delta subunit 3	NM_018398	12.480957
<i>CFI</i>	complement factor I	NM_000204	12.210217
<i>CILP</i>	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	NM_003613	12.177848
<i>CLCA2</i>	chloride channel accessory 2	NM_006536	26.598017
<i>COL15A1</i>	collagen, type XV, alpha 1	NM_001855	22.379642
<i>COL21A1</i>	collagen, type XXI, alpha 1	NM_030820	48.873936
<i>COL3A1</i>	collagen, type III, alpha 1	NM_000090	17.54856
<i>COL4A6</i>	collagen, type IV, alpha 6	NM_033641	12.459421
<i>COL5A1</i>	collagen, type V, alpha 1	NM_000093	10.208904
<i>COMP</i>	cartilage oligomeric matrix protein	NM_000095	23.414925
<i>CPZ</i>	carboxypeptidase Z	NM_001014448	21.976519
<i>EGFL6</i>	EGF-like-domain, multiple 6	NM_001167890	73.905075
<i>FAM65B</i>	family with sequence similarity 65, member B	NM_014722	16.607714
<i>FGF18</i>	fibroblast growth factor 18	NM_003862	15.78104
<i>FOSB</i>	FBJ murine osteosarcoma viral oncogene homolog B	NM_006732	13.191734
<i>GABRE</i>	gamma-aminobutyric acid (GABA) A receptor, epsilon	NM_004961	11.908827
<i>GADL1</i>	glutamate decarboxylase-like 1	NM_207359	38.765053
<i>GAP43</i>	growth associated protein 43	NM_002045	27.63465
<i>H19</i>	H19, imprinted maternally expressed transcript (non-protein coding)	NR_002196	42.17927
<i>HGF</i>	hepatocyte growth factor (hepapoietin A; scatter factor)	NM_001010931	13.644942
<i>HTRA3</i>	HtrA serine peptidase 3	NM_053044	11.428729
<i>IGSF10</i>	immunoglobulin superfamily, member 10	NM_178822	27.802753
<i>IRF4</i>	interferon regulatory factor 4	NM_002460	15.386851
<i>LOC100506700</i>	hypothetical LOC100506700	XR_110229	24.45396
<i>LRRC17</i>	leucine rich repeat containing 17	NM_001031692	26.013319

Continuation of Table 2.

Gene Symbol	Gene Name/Description	Acc. No.	Fold Change
<i>LRRC17</i>	leucine rich repeat containing 17	NM_005824	23.40266
<i>LSP1</i>	lymphocyte-specific protein 1	NM_001013254	80.217
<i>MFAP4</i>	microfibrillar-associated protein 4	NM_002404	10.018822
<i>MGP</i>	matrix Gla protein	NM_000900	33.745953
<i>MIAT</i>	myocardial infarction associated transcript (non-protein coding)	NR_003491	23.861937
<i>MMP27</i>	matrix metalloproteinase 27	NM_022122	35.801075
<i>N/A</i>	lincRNA:chr22:27053483-27072438 forward strand	N/A	31.898119
<i>N/A</i>	lincRNA:chr17:67547498-67549996 forward strand	N/A	23.818346
<i>N/A</i>	lincRNA:chr12:46826133-46974783 forward strand	N/A	23.46723
<i>N/A</i>	lincRNA:chr22:27065125-27066565 forward strand	N/A	13.99885
<i>N/A</i>	lincRNA:chr2:74193717-74210392 reverse strand	N/A	11.909513
<i>N/A</i>	lincRNA:chr2:179803305-179829380 reverse strand	N/A	10.683858
<i>N/A</i>	lincRNA:chr22:27066072-27067126 forward strand	N/A	10.115315
<i>NDNF</i>	neuron-derived neurotrophic factor	NM_024574	10.847972
<i>OGN</i>	osteoglycin	NM_033014	158.96016
<i>OMD</i>	osteomodulin	NM_005014	20.503307
<i>OSR2</i>	odd-skipped related 2 (Drosophila)	NM_053001	10.601532
<i>PCOLCE2</i>	procollagen C-endopeptidase enhancer 2	NM_013363	12.823184
<i>PDGFD</i>	platelet derived growth factor D	NM_025208	41.041542
<i>PKD4</i>	pyruvate dehydrogenase kinase, isozyme 4	NM_002612	13.998885
<i>PGA3</i>	pepsinogen 3, group I (pepsinogen A)	NM_001079807	34.137875
<i>RARRES1</i>	retinoic acid receptor responder (tazarotene induced) 1	NM_002888	19.60683
<i>RASSF2</i>	Ras association (RalGDS/AF-6) domain family member 2	NM_014737	12.719816
<i>SORCS2</i>	sortilin-related VPS10 domain containing receptor 2	NM_020777	16.55429
<i>STRA6</i>	stimulated by retinoic acid gene 6 homolog (mouse)	NM_001199042	13.068887
<i>TDRD6</i>	tudor domain containing 6	NM_001010870	12.645858
<i>THBS4</i>	thrombospondin 4	NM_003248	42.90762
<i>TMEM26</i>	transmembrane protein 26	NM_178505	22.539011
<i>TRIL</i>	TLR4 interactor with leucine-rich repeats	NM_014817	29.37157
<i>TXNIP</i>	thioredoxin interacting protein	NM_006472	17.168047
<i>WNT2</i>	wingless-type MMTV integration site family member 2	NM_003391	49.876865
<i>N/A</i>	PREDICTED: Homo sapiens hypothetical LOC729420 (LOC729420), miscRNA [XR_110129]	XR_110129	10.492821
<i>N/A</i>	MGC13nov.3.1.L1.1.G04.F.1 NIH_MGC_331 Homo sapiens cDNA clone MGC13nov.3.1.L1.1.G04, mRNA sequence [EG328730]	EG328730	10.060941
<i>N/A</i>	PREDICTED: Homo sapiens FLJ46836 protein (FLJ46836), miscRNA [XR_108962]	XR_108962	10.016423

Fold change >10.

Figure 2. Hierarchical clustering of all the samples from different sources. Dendrogram showing samples isolated from the same sources (limbal stromal cells cultured in fetal bovine serum, LS-FBS; bone marrow mesenchymal stromal/stem cells, BM-MSC; adipose-derived mesenchymal stromal/stem cells, AD-MSC and human foreskin fibroblasts, HFF) were clustered together by upregulated genes in limbal stromal cells. LS-FBS have different molecular signature when compared to other MSC as shown by the hierarchical tree (left panel). The left panel shows the whole hierarchical tree and the right panel shows zoom in on a set of significantly upregulated genes.

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). All gene expressions are ≥ 2 fold change, $p < 0.05$. The common up- or downregulated differentially expressed genes are listed in (Tables 3 and 4).

Table 3. List of common upregulated genes in LS-FBS versus BM-MSC, AD-MSC and HFF.

Gene Symbol	Gene Name/Description	Acc. No.	BM-MSC (f.c.)	AD-MSC (f.c.)	HFF (f.c.)
<i>SCIN</i>	scinderin	NM_033128	330.80643	416.52576	369.1616
<i>RRAGD</i>	Ras-related GTP binding D	NM_021244	218.22987	18.668407	178.51025
<i>FABP3</i>	fatty acid binding protein 3, muscle and heart	NM_004102	123.3219	155.64526	76.11089
<i>TFAP2B</i>	transcription factor AP-2 beta	NM_003221	43.46775	31.220913	35.57746
<i>GPNUMB</i>	glycoprotein (transmembrane) numb	NM_001005340	26.490465	12.710969	11.586283
<i>NAP1L2</i>	nucleosome assembly protein 1-like 2	NM_021963	9.873706	18.588165	9.587671
<i>OGDHL</i>	oxoglutarate dehydrogenase-like	NM_018245	10.780043	14.660217	9.35376
<i>GPNUMB</i>	glycoprotein (transmembrane) numb	BC011595	7.3409696	8.595104	6.69056
<i>FZD5</i>	frizzled family receptor 5	NM_003468	8.075183	6.3447533	6.686808
<i>RENBP</i>	renin binding protein	NM_002910	4.8013253	4.9874763	6.3223376
<i>DNAH5</i>	dynein, axonemal, heavy chain 5	NM_001369	6.636916	6.3912625	5.8422527
<i>SPON2</i>	spondin 2, extracellular matrix protein	NM_012445	59.94457	12.877473	5.7284737
<i>UAP1L1</i>	UDP-N-acetylglucosamine pyrophosphorylase 1-like 1	NM_207309	4.6691117	7.7450266	4.8722553
<i>C6orf155</i>	chromosome 6 open reading frame 155	NR_026807	5.532519	5.84893	4.606592
<i>TSPAN10</i>	tetraspanin 10	NM_031945	18.728783	14.794658	4.4555798
<i>SH3RF2</i>	SH3 domain containing ring finger 2	NM_152550	2.2531955	4.0488048	3.829434
<i>NPC2</i>	Niemann-Pick disease, type C2	NM_006432	2.3174524	4.9840603	3.48242
<i>ARSE</i>	arylsulfatase E (chondrodysplasia punctata 1)	AK223183	15.381536	16.5452	3.47192
<i>SCARB1</i>	scavenger receptor class B, member 1	NM_005505	3.3584666	7.5342646	2.8759322
<i>C17orf55</i>	chromosome 17 open reading frame 55	NR_038080	2.2982838	2.7519205	2.544845
N/A	lincRNA:chr3:142845360-142851335 reverse strand	N/A	2.506281	2.3448408	2.4984725
<i>HS1BP3</i>	HCLS1 binding protein 3	NM_022460	2.2680733	4.639097	2.460958
<i>FLVCR1</i>	feline leukemia virus subgroup C cellular receptor 1	NM_014053	2.2281523	5.496619	2.2000937

f.c=fold change; N/A=not available.

activation of PKC through G-protein coupled receptors (*PLCB4*) and FAS signaling pathway (*BCL2*, *MAP3K1*) were over expressed.

Confirmation of differential expression by RT-PCR analysis

Differential expression of six regulated genes and a house-keeping gene, *GAPDH* was determined by RT-PCR as presented in **Figure 4**. RT-PCR confirmed the differential expression as observed in four cell the types. Specific amplification was confirmed by melting curve analysis. The tendency of differential expression in LS-FBS versus the rest was consistent between microarray data and real-time RT-PCR data in all the genes tested.

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 (AD-MSC) 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 sign-

Figure 4. Validation of the expression of genes by relative real time PCR. The bar charts show the mean expression (n=3, p<0.05) of the selected up- or downregulated genes for each group (A: LS-FBS versus BM-MSC; B: LS-FBS versus AD-MSC; C: LS-FBS versus HFF) are consistent when analyzed by microarray and relative real time PCR. For relative real time PCR, expression of genes was normalized with GAPDH.

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 heterogenous. 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

atures 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

Table 4. List of common downregulated genes in LS-FBS versus BM-MSC, AD-MSC and HFF.

Gene Symbol	Gene Name/Description	Acc. No.	BM-MSC (f.c.)	AD-MSC (f.c.)	HFF (f.c.)
<i>HOXA11AS</i>	HOXA11 antisense RNA (non-protein coding)	NR_002795	-104.227234	-166.47116	-673.34393
<i>HOXA10</i>	homeobox A10	NM_018951	-123.810524	-181.91698	-324.92493
<i>HOXA11</i>	homeobox A11	NM_005523	-36.106262	-44.978073	-264.6142
<i>HOXA9</i>	homeobox A9	NM_152739	-643.6674	-382.13437	-190.41304
<i>lincRNA</i>	lincRNA:chr12:54356229-54357908	n.a	-13.816334	-23.520226	-106.52508
<i>LOC442249</i>	similar to keratin 18	XR_019231	-39.17349	-40.84192	-47.219604
<i>HOXA7</i>	homeobox A7	NM_006896	-473.85187	-266.04224	-45.93801
<i>HOXC10</i>	homeobox C10	NM_017409	-41.060524	-73.95265	-41.661804
<i>SFRP1</i>	secreted frizzled-related protein 1	NM_003012	-90.09236	-22.70849	-31.701826
<i>lincRNA</i>	lincRNA:chr12:54356229-54357908 reverse strand	n.a	-4.4482675	-6.152943	-26.052305
<i>EBF3</i>	early B-cell factor 3	NM_001005463	-111.54111	-54.667686	-25.794918
<i>MEIS1</i>	Meis homeobox 1	NM_002398	-9.061138	-7.985613	-23.654848
<i>HOXD8</i>	homeobox D8	NM_019558	-53.95993	-83.73045	-18.824736
<i>HCLS1</i>	hematopoietic cell-specific Lyn substrate 1	NM_005335	-5.432739	-9.804886	-16.318146
<i>HAND2</i>	heart and neural crest derivatives expressed 2	NM_021973	-21.603893	-57.825478	-14.021899
<i>IL17B</i>	interleukin 17B	NM_014443	-15.500265	-9.498544	-10.416743
<i>TMEM90B</i>	transmembrane protein 90B	NM_024893	-195.35493	-142.35834	-7.5122113
<i>FRMPD4</i>	FERM and PDZ domain containing 4	NM_014728	-9.06647	-13.105078	-6.804531
<i>CDH2</i>	cadherin 2, type 1, N-cadherin (neuronal)	NM_001792	-13.568187	-16.845877	-5.71267
<i>lincRNA</i>	lincRNA:chr2:177494316-177502754 reverse strand	n.a	-11.785658	-9.501191	-5.4580956
<i>lincRNA</i>	lincRNA:chr2:177494316-177502302 reverse strand	n.a	-11.574628	-9.275996	-5.1087427
<i>MYCBP</i>	c-myc binding protein	NM_012333	-4.902564	-3.403298	-4.6458
<i>GATA6</i>	GATA binding protein 6	NM_005257	-14.644289	-8.480716	-4.317858
<i>SLC35E2</i>	solute carrier family 35, member E2	NM_182838	-2.3155887	-3.433437	-4.1792254
<i>SEMA7A</i>	semaphorin 7A, GPI membrane anchor	NM_003612	-10.770672	-4.537565	-3.8937697
<i>NCKAP5</i>	NCK-associated protein 5	NM_207363	-7.6027536	-2.4458163	-3.5850215
<i>NR2F2</i>	nuclear receptor subfamily 2, group F, member 2	NM_021005	-2.2446127	-2.1425328	-3.4852633
<i>TSHZ3</i>	teashirt zinc finger homeobox 3	NM_020856	-3.4907699	-2.9466493	-3.434508
<i>MLXIP</i>	MLX interacting protein	NM_014938	-2.431865	-3.559918	-2.3397596

f.c = fold change; n.a = not available.

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 long-chain 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 adipogenesis of MSC [31,49]. Fourthly, the transcription factor AP-2 beta (*TFAP2B*) is involved in the regulation of cell proliferation and

differentiation during embryonic development. This protein functions both as transcriptional activator and repressor by binding to different promoters. Mutation in *TFAP2B* gene has been reported in autosomal dominant Char syndrome indicating its function in the differentiation of neural crest cell derivatives. Since cornea is one of the derivative of neural crest during embryo development, the over expression of this gene is significant. In addition, angiopoietin-like 7 (*ANGPTL7*) was highly expressed in LS cells. The protein acts as a negative regulator of angiogenesis besides playing a role as morphogen in inducing a corneal phenotype *in vivo* [50]. Another highly expressed gene related to the eye was tetraspanin 10 (*TSPAN10*) [51] and the function of this protein

Table 5. Differential biological process associated gene expression in LS-FBS as compared to BM-MSC, AD-MSC and HFF.

Genes Over expressed in LS-FBS vs BM-MSC (> 15 fold change)	Genes Over expressed in LS-FBS vs AD-MSC (> 10 fold change)	Genes Over expressed in LS-FBS vs HFF (> 10 fold change)
Secreted Protein EGF-like-domain, multiple 6 [EGFL6] MAM domain containing 2 [MAMDC2] Adrenomedullin [ADM] Angiopietin-like 7 [ANGPTL7] Angiotensinogen (serpin peptidase inhibitor) [AGT] Chromosome 10 open reading frame 58 [C10orf58] Fibrillin 2 [FBN2] Gremlin 2 [GREM2] Insulin-like growth factor binding protein 5 [IGFBP5] Noggin [NOG] Podocan [PODN] Proprotein convertase subtilisin/kexin type 9 [PCSK9] Retinoic acid receptor responder (tazarotene induced) 2 [RARRES2]	Signal Protein 24-dehydrocholesterol reductase [DHCR24] Acid phosphatase 5, tartrate resistant [ACP5] Angiopietin-like 7 [ANGPTL7] Arylsulfatase E (chondrodysplasia punctata 1) [ARSE] Bone morphogenetic protein 4 [BMP4] Carboxypeptidase A4 [CPA4] Glycoprotein (transmembrane) nmb [GPNMB] Heparanase [HPSE] Laminin, alpha 3 [LAMA3] Sema domain, immunoglobulin domain (Ig), Short basic domain, secreted, (semaphorin) 3A [SEMA3A] Similar to Mast/stem cell growth factor receptor precursor (SCFR) (Proto-oncogene tyrosine-protein kinase Kit) (c-kit) [CD117] Sodium channel, voltage-gated, type I, beta [SCN1B] Spondin 2, ECM [SPON2]	Signal Protein EGF-like-domain, multiple 6 [EGFL6] Xg blood group [XG] Deleted in bladder cancer 1 [DBC1] Glycoprotein (transmembrane) nmb [GPNMB] Insulin-like growth factor binding protein 2, 36kDa [IGFBP2] Integrin, alpha 8 [ITGA8] Laminin, alpha 3 [LAMA3] Podoplanin [PDPN] Retinoic acid receptor responder (tazarotene induced) 2 [RARRES2]
Extra Cellular Matrix EGF-like-domain, multiple 6 [EGFL6] MAM domain containing 2 [MAMDC2] Fibrillin 2 [FBN2] Podocan [PODN]	Transmembrane 24-dehydrocholesterol reductase [DHCR24] GTPase, IMAP family member 2 [GIMAP2] N-acetyltransferase 8-like (GCN5-related, putative) [NAT8L] NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa; UDP-N-acetyl-alpha-D-galactosamine: polypeptide N acetylgalactosaminyl transferase 14 [GalNAc-T14] Adrenergic, beta-2-, receptor surface [ADRB2] Epoxide hydrolase 4 [EPHX4] Glycoprotein (transmembrane) nmb [GPNMB] Potassium inwardly-rectifying channel, subfamily J, member 2 [KCNJ2] Purinergic receptor P2X, ligand-gated ion channel, 7 [P2RX7] Receptor accessory protein 2 [REEP2] Sodium channel, voltage-gated, type I, beta; solute carrier family 4, sodium bicarbonate cotransporter, member 8 [SLC4A8] Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 [SLC7A2] Tetraspanin 10 [TSPAN10] Transmembrane and tetratricopeptide repeat containing 1 [TMTC1]	Basement Membrane EGF-like-domain, multiple 6 [EGFL6] Laminin, alpha 3 [LAMA3]
Cell Differentiation EGF-like-domain, multiple 6 [EGFL6] Forkhead box L2 [FOXL2] Glycoprotein M6B [GPM6B] Integrin, alpha 8 [ITGA8] Noggin [NOG]		Cell Adhesion Protein EGF-like-domain, multiple 6 [EGFL6] Integrin, alpha 8 [ITGA8] Laminin, alpha 3 [LAMA3]
Unknown function lincRNA:chrX:115401722-115413847 reverse strand		Differentiation EGF-like-domain, multiple 6 [EGFL6] Glycoprotein M6B [GPM6B] Integrin, alpha 8 [ITGA8]
		Unknown function lincRNA:chr10:134136360-134144210 forward strand

is unknown. The transmembrane glycoprotein (*GPNMB*) was also reported to be expressed in the lowly metastatic human melanoma cell lines and xenografts and it could add in melanogenesis as the limbus of the human eyes is rich in melanin. Spondin 2 (*SPON2*) which was also highly expressed in LS cells is a cell adhesion protein that promotes adhesion

and outgrowth of hippocampal embryonic neurons while inhibit angiogenesis [52]. The antiangiogenesis properties of this protein might help in maintaining transparency of cornea by inhibiting neoangiogenesis.

Polisetti et al., recently reported a different set of differentially expressed genes in LS cells related to extracellular

components, cell adhesion molecules (microfibrillar associated protein 5, syndecan 2, matrix-remodelling associated 5, chondroitin sulfate proteoglycan 4 and collagen 8 alpha 1) as compared to BM-MSC [36]. However, by using different approaches such as single color versus dual color hybridization and isolation methods, our results revealed novel genes involved in the biological processes and molecular functions that have not been reported for ex vivo cultured LS cells. Besides extracellular related genes (*LAMA3*, *IGFBP2*, *IGFBP5*) and cell adhesion molecules (*SPON2*, *JAM2*, *EGFL6*, *ITGA8*), most of the genes significantly overexpressed belonged to the various biological process and molecular functions. In addition, many of the downregulated genes encode the class of transcription factors called homeobox genes that are involved in differentiation (*HOXD8*, *HOXA9*, *HOXA7*, *HOXC10*, *HOXA10*, and *HOXA11*). This shows that the LS cells are multipotent and suppressing the expression of *HOX* gene family members. These DNA-binding transcription factors may regulate gene expression, morphogenesis, and differentiation. The *HOX* genes have been reported to play a role in both the early stem cell function as well as in the later stages of hematopoietic differentiation, and perturbation of *HOX* genes expression can be leukomogenic [53].

The Wnt pathway has been implicated in the regulation of self-renewal and cell fate determination in embryogenesis and stem cells [54]. Optimal level of Wnt/ β catenin signalling increased the proliferation and colony-forming efficiency of primary LESC while maintaining the stem cell phenotype of these cells [55]. This was also shown in *Dkk2* knockout mice where they displayed epidermal differentiation on the ocular surface [56]. *PAX6* expression was also lost in the corneal epithelial cells of these mice, suggesting it is downstream of *Dkk2* [56]. Deficiencies in *PAX6* leads to aniridia resulting in impaired corneal epithelial function and eventual LESC failure, which may be due to altered niche development. Interestingly, we found high expression of frizzled family receptor 5 (*FZD5*) in LS-FBS compared to the other stromal cells. High level of Wnt signaling pathway in LS-FBS and LS-matrigel cells (as shown in Table 3 and Appendix 3) may help to modulate the proliferation, self-renewal and differentiation of LESC. This demonstrates the importance of limbal niche control over LESC fate during development.

In this study, we also reported a set of unknown genes and long intergenic non-coding RNAs (lincRNAs) which are highly expressed in LS cells (Tables 2 and 5). LincRNAs have been shown to act in the circuitry controlling self renewal and differentiation of stem cells by epigenetic modifications [57,58]. Further studies would be needed to elucidate the functions of these lincRNAs.

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.

Additional files

Appendix 1
Appendix 2
Appendix 3
Appendix 4
Appendix 5
Supplement figure S1
Supplement figure S2
Supplement figure S3

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	ML	NH	AO	TU	GS	PB	RJ	ZZ
Research concept and design	✓	✓	✓	✓	--	--	✓	--
Collection and/or assembly of data	✓	✓	--	✓	--	✓	--	--
Data analysis and interpretation	✓	✓	--	✓	✓	--	--	--
Writing the article	✓	✓	✓	--	✓	--	--	--
Critical revision of the article	✓	✓	--	--	✓	✓	✓	✓
Final approval of article	✓	--	--	--	--	--	--	✓
Statistical analysis	✓	--	--	--	--	--	--	--

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