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Research Article

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 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 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 conjunctivallimbalautograft 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 Chemic, Steinheim, Germany) and 4 ng/mL basic fibroblast growth factor (bFGF; BD Biosciences, Mississauga, Canada) [17]. The expanded limbal stromal cells were subjected to fluorescenceactivated 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 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 , 2uL 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 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].

Gene	Accession	Sense primer	Antisense primer	Product Size (bp)
GAPDH	NM_002046	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498
SCIN	NM_033128	ATGGCTTCGGGAAAGTTTATG	CATCCACCATATTGTGCTGGG	117
RRAGD	NM_021244	CTAGCGGACTACGGAGACG	ATGAGCAGGATTCTCGGCTTC	122
FABP3	NM_004102	ATGGGGACATTCTCACCCTAAA	GCTGTTGTCTCATCGAACTCCA	91
TFAP2B	NM_003221	TTCCTCCCAAATCGGTGACTT	CGCCGGTGTTGACAGACAT	75
GPNMB	NM_001005340	CTTCTGCTTACATGAGGGAGC	GGCTGGTGAGTCACTGGTC	164
SFRP1	NM_003012	ACGTGGGCTACAAGAAGATGG	CAGCGACACGGGTAGATGG	184

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

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).

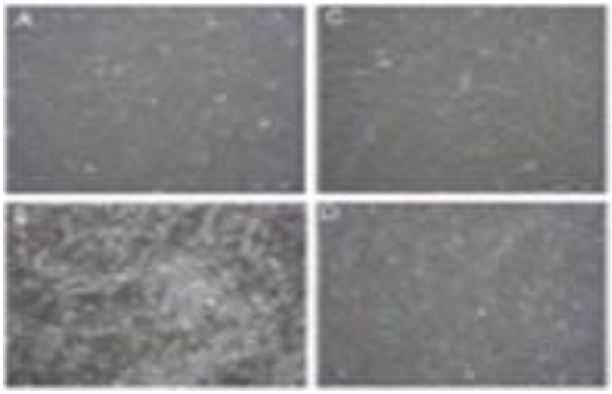

Figure . 1 : Morphological observations

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 LSFBS 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.

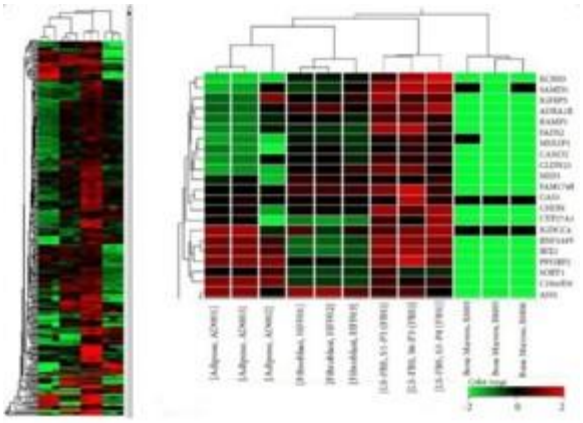

Figure . 2 : Hierarchical clustering of all the samples from different sources

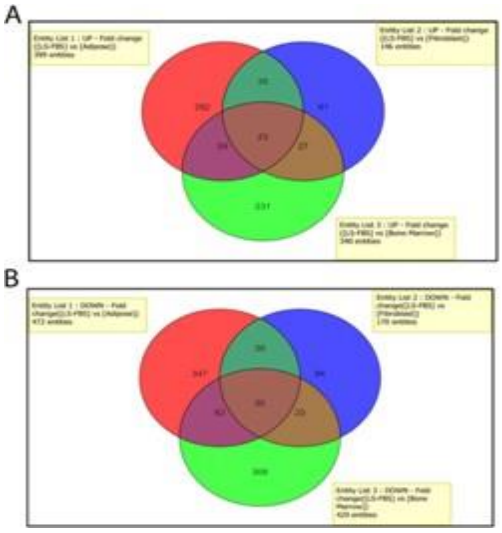

Figure . 3 : Venn diagrams showing the numbers of all up- (panel A) or downregulated genes (panel B) of LS-FBS when compared to BM-MSC (bone marrow mesenchymal stem cells), AD-MSC (adipose-derived mesenchymal stem cells) and HFF (human foreskin fibroblasts)

ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8		
ADRA2A	adrenergic, alpha-2A-, receptor	NM_000681 NM_007037	47.013718 14.913507
ANGPTL4	angiopoietin-like 4	NM_139314	424.33313
ANGPTL7	angiopoietin-like 7	NM_021146	14.670821
ANK3	ankyrin 3, node of Ranvier (ankyrin G)	NM_020987	14.409806
APCDD1	adenomatosis polyposis coli down-regulated 1	NM_153000	11.659266
APOD	apolipoprotein D	NM_001647	12.362466
AQP3	aquaporin 3 (Gill blood group)	NM_004925	25.748945
ARHGAP28	Rho GTPase activating protein 28	NM_001010000	11.816478
ASCL2	achaete-scute complex homolog 2 (Drosophila)	NM_005170	11.49502
CACNA2D3	calcium channel, voltage-dependent, alpha 2/delta subunit 3	NM_018398	12.480957
CFI	complement factor I	NM_000204	12.210217
CILP	cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	NM_003613	12.177848
CLCA2	chloride channel accessory 2	NM_006536	26.598017
COL15A1	collagen, type XV, alpha 1	NM_001855	22.379642
COL21A1	collagen, type XXI, alpha 1	NM_030820	48.873936
COL3A1	collagen, type III, alpha 1	NM_000090	17.54856
COL4A6	collagen, type IV, alpha 6	NM_033641	12.459421
COL5A1	collagen, type V, alpha 1	NM_000093	10.208904
COMP	cartilage oligomeric matrix protein	NM_000095	23.414925

CPZ	carboxypeptidase Z	NM_001014448	21.976519
EGFL6	EGF-like-domain, multiple 6	NM_001167890	73.905075
FAM65B	family with sequence similarity 65, member B	NM_014722	16.607714
FGF18	fibroblast growth factor 18	NM_003862	15.78104
FOSB	FBJ murine osteosarcoma viral oncogene homolog B	NM_006732	13.191734
GABRE	gamma-aminobutyric acid (GABA) A receptor, epsilon	NM_004961	11.908827
GADL1	glutamate decarboxylase-like 1	NM_207359	38.765053
GAP43	growth associated protein 43	NM_002045	27.63465
H19	H19, imprinted maternally expressed transcript (non-protein coding)	NR_002196	42.17927
HGF	hepatocyte growth factor (hepapoietin A; scatter factor)	NM_001010931	13.644942
HTRA3	HtrA serine peptidase 3	NM_053044	11.428729
IGSF10	immunoglobulin superfamily, member 10	NM_178822	27.802753
IRF4	interferon regulatory factor 4	NM_002460	15.386851
LOC100506700	hypothetical LOC100506700	XR_110229	24.45396
LRRC17	leucine rich repeat containing 17	NM_001031692	26.013319
LRRC17	leucine rich repeat containing 17	NM_005824	23.40266
LSP1	lymphocyte-specific protein 1	NM_001013254	80.217
MFAP4	microfibrillar-associated protein 4	NM_002404	10.018822
MGP	matrix Gla protein	NM_000900	33.745953
MIAT	myocardial infarction associated transcript (non-protein coding)	NR_003491	23.861937
MMP27	matrix metallopeptidase 27	NM_022122	35.801075
N/A	lincRNA:chr22:27053483-27072438 forward strand	N/A	31.898119
N/A	lincRNA:chr17:67547498-67549996 forward strand	N/A	23.818346
N/A	lincRNA:chr12:46826133-46974783 forward strand	N/A	23.46723
N/A	lincRNA:chr22:27065125-27066565 forward strand	N/A	13.99885
N/A	lincRNA:chr2:74193717-74210392 reverse strand	N/A	11.909513
N/A	lincRNA:chr2:179803305-179829380 reverse strand	N/A	10.683858
N/A	lincRNA:chr22:27066072-27067126 forward strand	N/A	10.115315
NDNF	neuron-derived neurotrophic factor	NM_024574	10.847972
OGN	osteoglycin	NM_033014	158.96016
OMD	osteomodulin	NM_005014	20.503307
OSR2	odd-skipped related 2 (Drosophila)	NM_053001	10.601532
PCOLCE2	procollagen C-endopeptidase enhancer 2	NM_013363	12.823184
PDGFD	platelet derived growth factor D	NM_025208	41.041542
PDK4	pyruvate dehydrogenase kinase, isozyme 4	NM_002612	13.998885
PGA3	pepsinogen 3, group I (pepsinogen A)	NM_001079807	34.137875
RARRES1	retinoic acid receptor responder (tazarotene induced) 1	NM_002888	19.60683
RASSF2	Ras association (RalGDS/AF-6) domain family member 2	NM_014737	12.719816
SORCS2	sortilin-related VPS10 domain containing receptor 2	NM_020777	16.55429
STRA6	stimulated by retinoic acid gene 6 homolog (mouse)	NM_001199042	13.068887
TDRD6	tudor domain containing 6	NM_001010870	12.645858
THBS4	thrombospondin 4	NM_003248	42.90762
TMEM26	transmembrane protein 26	NM_178505	22.539011
TRIL	TLR4 interactor with leucine-rich repeats	NM_014817	29.37157
TXNIP	thioredoxin interacting protein	NM_006472	17.168047
WNT2	wingless-type MMTV integration site family member 2	NM_003391	49.876865
N/A	PREDICTED: Homo sapiens hypothetical LOC729420 (LOC729420), miscRNA [XR_110129]	XR_110129	10.492821
N/A	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
N/A	PREDICTED: Homo sapiens FLJ46836 protein (FLJ46836), miscRNA [XR_108962]	XR_108962	10.016423

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

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 metallopeptidase 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 BMMSC, 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 adipogenesis 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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