Title Page

Title: "Human Limbal MSC express ABCB5 and can grow on Amniotic membrane"

Running title: "limbal MSC express ABCB5"

Bakiah Shaharuddin[1,2] Charles Osei-Bempong[1]Sajjad Ahmad[3,4] Paul Rooney[5] Simi Ali[6]

Rachel Oldershaw[7] Annette Meeson[1]

[1]Institute of Genetic Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle Upon-Tyne, UK

[2]Advanced Medical and Dental Institute, UniversitiSainsMalaysia, Pulau Pinang, Malaysia

[3]St Paul's Eye Unit, Royal Liverpool University Hospital, Prescot Street, Liverpool, UK.

[4]Department of Eye and Vision Sciences, Institute of Ageing and Chronic Disease, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, UK.

[5]Tissue Development Laboratory, NHS Blood and Transplant, Estuary Banks LiverpoolL24 8RB, UK.

[6]Institute of Cellular Medicine, Newcastle University, Newcastle Upon-Tyne, UK.

[7]Department of Musculoskeletal Biology Group I, Institute of Ageing and Chronic Disease, Faculty of Health and Life Sciences, University of Liverpool, Leahurst Campus, Chester High Road, Neston, Cheshire, CH64 7TE

Authors' contribution:

1. Bakiah Shaharuddin: Conception and design, Collection and/or assembly of data, Manuscript writing
2. Charles Osei-Bempong: Support with use of amniotic membrane
3. Sajjad Ahmad: Conception and design
4. Paul Rooney: Provision of amniotic membrane
5. Simi Ali: Conception and design

6. Rachel Oldershaw: Support with MSC differentiation
7. Annette Meeson: Conception and design, Collection and/or assembly of data, Manuscript writing, Final approval of manuscript.

1) Dr Bakiah Shaharuddin

Address 1:
Institute of Genetic Medicine,
NewcastleUniversity,
International Centre for Life,
Newcastle Upon-Tyne,
NE1 3BZ, United Kingdom.
(Email: b.shaharuddin@ncl.ac.uk)

Address 2:

Advanced Medical and Dental Institute,
UniversitiSainsMalaysia,
13200 Pulau Pinang,
Malaysia.

2) Dr Charles Osei-Bempong

Institute of Genetic Medicine,
NewcastleUniversity,
International Centre for Life,
Newcastle Upon-Tyne,
NE1 3BZ, United Kingdom.
(Email: Charles.osei-bempong@ncl.ac.uk)

3) Dr Sajjad Ahmad

Address 1;

St Paul's Eye Unit,
RoyalLiverpoolUniversityHospital,
Prescot Street,
Liverpool,
L7 8XP, United Kingdom.

Address 2:

Department of Eye and Vision Sciences,
Institute of Ageing and Chronic Disease,
University of Liverpool,
4th FloorUCDBuilding,
Daulby Street,

Liverpool,
L69 3GA, United Kingdom.

(Email: Sajjad.Ahmad@liverpool.ac.uk)

Tel: +44 (0) 207 2533411

4) Dr Paul Rooney

Address:
Tissue Development Laboratory, NHS Blood and Transplant, Estuary Banks
LiverpoolL24 8RB, UK.
(Email: Paul.Rooney@nhsbt.nhs.uk)
Tel: 0151 268 7108

5) Professor Simi Ali

Address:
Institute of Cellular Medicine,
William-LeechBuilding,
NewcastleUniversity,
Newcastle upon Tyne
NE24HH, United Kingdom.
(Email: simi.ali@ncl.ac.uk)
Tel: +44 (0) 1912227158

Fax no: +44 (0) 1912418666

6) Dr Rachel Oldershaw

Address:
Department of Musculoskeletal Biology Group I,
Institute of Ageing and Chronic Disease,
University of Liverpool,
Leahurst Campus,
Chester High Road, Neston,
Cheshire, CH64 7TE, United Kingdom.
(Email: Rachel.Oldershaw@liverpool.ac.uk)
Tel: +44 (0) 151 794 6111

7) Dr Annette Meeson

Address:
Institute of Genetic Medicine,
NewcastleUniversity,
International Centre for Life,
Newcastle Upon-Tyne,
NE1 3BZ, United Kingdom.

(Email: annette.meeson@ncl.ac.uk)
Tel: +44 (0) 1912418856

Corresponding author:

Bakiah Shaharuddin
Institute of Genetic Medicine,
NewcastleUniversity,
International Centre for Life,
Newcastle Upon-Tyne,
NE1 3BZ, United Kingdom.
(Email: b.shaharuddin@ncl.ac.uk)

ABSTRACT

Aim: To isolate and characterise limbal mesenchymal stem cells (LMSC) from human corneo-scleral rings. **Materials and Methods:** Cells were isolated from corneo-scleral rings and cultured in a mesenchymal stem cell (MSC)-selective media and examined for differentiation, phenotyping and characterisation. **Results:** LMSC were capable of tri-lineage differentiation, adhered to tissue culture plastic, expressed HLA Class I and cell surface antigens associated with human MSC whilst having no/ low expression of HLA Class II and negative haematopoietic lineage markers. They were capable for CXCL12-mediated cellular migration. LMSC adhered, proliferated on amniotic membrane (AM), and expressed the common putative limbal stem cell markers. **Conclusion**: Limbal-derived MSC exhibited plasticity, could maintain limbal markers expression and demonstrated viable growth on AM.

Keywords*: limbal stem cells, limbal mesenchymal stem cells, limbal stem cell deficiency, amniotic membrane, ABCB5*

Article highlights:

- limbal stem cells
- limbal mesenchymal stem cells
- limbal stem cell deficiency

1.0 INTRODUCTION

The cornea is located at the anterior part of the eye and maintenance of its transparency is vital for clear vision. It consists of three major layers which are derived from different germ layers of which the stroma and endothelium are mesenchymal in origin. Limbal stem cell deficiency (LSCD) occurs due to multiple pathophysiologies that adversely affect the cornea leading to loss of vision. A treatment first introduced by Pellegrini et al [1] using *ex vivo* expanded limbal epithelium on fibrin sheet as a cellular therapy has shown impressive advances for the treatment of LSCD and other ocular surface disorders [1-5]. A successful outcome was considered when there was restoration of corneal epithelium and this was attained in approximately 75% of cases [2, 4, 6]. The success of limbal stem cell transplantation is however restricted to the availability of healthy limbal tissue taken from the contralateral eye in autologous cases or tissues from living related donors or cadaveric eyes for allogeneic transplantation. Cultures with higher frequency of limbal stem cells or progenitor cells in transplanted tissue constructs have higher success rates [4].

MSC also sometimes referred as mesenchymal stromal cells are non-haematopoietic stem cells with regenerative ability and can differentiate into cell lineages of mesenchymal origin i.e. adipocytes, chondrocytes and osteocytes under optimised culture conditions [7]. However, due to variable defining characteristics for MSC, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has defined several minimal requirements [8]: 1) MSC must be plastic adherent, 2) they must express CD105, CD73 and CD90, but lack of expression of CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR surface molecules. Thirdly they should display multi-potentiality to differentiate into adipocytes, osteoblasts and chondroblasts. As MSC secrete many bioactive

molecules e.g. cytokines, trophic and growth factors, they have become of increasing interest to the regenerative medicine and transplantation fields [9-12].

The corneal limbus has been reported to possess stromal cells which have fibroblastic morphology, are adherent to plastic and able to differentiate into multiple lineages, suggestive of an MSC population [13-15]. Human corneal mesenchymal stromal stem cells, unlike bone marrow-derived MSC, develop from neural crest cells [16] and are believed to support the growth of limbal epithelial cells [17, 18].

We aim to improve the current methods of *ex vivo* expanded corneal epithelial transplantation by investigating alternative stem cells sourced from the limbal region and grown in an MSC-selective media and hypoxic condition. The cells were immunophenotyped, examined for mRNA expression of selected human MSC markers and histologically analysed for tri-lineage differentiation. We also grew these cells on amniotic membrane (AM) thawed from frozen, a biological substrate commonly used in limbal stem cell transplantation. In addition, we looked at chemokine receptor CXCR4 expression and further studied CXCL12-mediated chemotaxis in this cell population.

2.0 MATERIALS AND METHODS

This project was conducted according to the ethical principles of the Declaration of Helsinki for human experiments. The use of human limbal tissues and AM was approved by the Ethics Committee, Faculty of Medicine, Newcastle University, and the limbal tissues were obtained from Central Manchester and Manchester Children's University Hospitals (NHS Trust). The human AM were supplied by the National Health Service (NHS) Blood and Tissue bank, Liverpool, United Kingdom. All experiments were performed according to the Control of

Substances Hazardous to Health (COSHH and BIOCOSHH) regulations. All procedures were in compliance with Newcastle University safety policies. Tissue culture was carried out in compliance with regulations for containment of Class II pathogens.

2.1 Isolation, derivation and culture of LMSC

Cells were isolated from research grade human corneo-scleral rings supplied by the Manchester Eye Bank, United Kingdom, as previously described [19]. Briefly, corneo-scleral rings were washed with Dulbecco's Phosphate buffered saline (PBS) and dissected into $1mm^2$ radial pieces. These pieces were serially trypsinised (0.05% trypsin + 0.53mM ethylene diaminetetraacetic EDTA) [Gibco], for 20 minutes with gentle agitation of the suspension at the 10 minute point of a 20 minute cycle. After 20 minutes, the trypsin was inactivated using media containing 10% foetal bovine serum (FBS) [Lonza]. The cell suspension was centrifuged at 220G for 3 minutes. The cell pellet was then re-suspended in human MSC-growth promotion medium (MGPM; alpha-modified Eagle's medium (α-MEM) [Lonza], 10% (vol/vol), FBS [Lonza], 5ng/ml human recombinant fibroblast growth factor-2 (FGF2) [Pepro Tech, UK]. This was repeated for a total of four cycles (we refer to this as serial trypsinisation). Finally the limbal cell suspensions were pooled together and centrifuged to obtain a cell pellet in MGPM and then seeded at 1×10^6cells/$T75cm^2$ flask and incubated at 37°C, 5% CO_2, 5%O_2. This protocol was selective for the growth of MSC; whilst LEC cultures typically require an enriched media and feeder cells [20]. Non-adherent cells were removed the next day and media was changed every three days thereafter. The established cultures of LMSC at 90% confluence were passaged by enzymatic digestion using TrypLE™ Express [Gibco].

i. Chondrogenic differentiation of LMSC

LMSC were seeded into 15ml centrifuge tubes at a cell density of 500,000 cells/ tube in chondrogenic differentiation medium(α-MEM, 10% FBS (vol/vol), 100μM L-ascorbic acid-2-phosphate , 10nM dexamethasone, 400μl ITS^{+1} (10μg/ml insulin, 5.5μg/ml transferrin, 5ng/ml selenium, 0.5mg/ml bovine serum albumin, 4.7μg/ml linoleic acid) [Sigma] and 10ng/ml transforming growth factor (TGF)-β3 [R&D Systems]. Cell suspensions were centrifuged at 220 G for three minutes to form spherical pellets and left to incubate at 37°C, 5% CO_2,5%O_2 for two weeks with medium changes every three days. Histological analysis for chondrogenic differentiation was then performed. Cell pellets were embedded in optimal cutting temperature (OCT) [BD Bioscoences] and sectioned (6μm sections) using a Leica CM3050 cryostat. Chondrogenic sections were stained with Safranin O to determine the presence of sulphated glycosaminoglycan deposition. OCT was dissolved at room temperature by rinsing in 70% (v/v) ethanol followed by PBS washes. Slides were flooded with pre-filtered Safranin O (0.1% (w/ v) Safranin O dissolved in 0.1 % (v/v) acetic acid in deionised water) for 5 minutes and then rinsed with deionised water.

ii. Adipogenic differentiation of LMSC

LMSC were seeded in MGPM into a 12-well cell culture plate at a cell density of 5×10^4 cells/well and cultured until confluent. MGPM was then removed and replaced with adipogenic differentiation medium, STEMPRO® adipogenesis differentiation kit [Gibco]. From this point onwards, cultures were incubated at atmospheric oxygen (22%) 5% CO_2incubator at 37°C for 30 days with medium changes every three days. For histological confirmation of adipogenesis, cell cultures were stained using Oil Red O. A working solution of stain was freshly prepared for each analysis by diluting a stock solution of Oil Red O (0.3% (w/v) in 99% (v/v) isopropanol) by a third in deionised water and filtering through a

0.45μm filter. Cell cultures were covered with 2 ml of Oil Red O working solution and left for 5 minutes at room temperature. The working solution was then removed and rinsed with tap water until clear. A counter-stain of 2ml Haematoxylin [Sigma] was then added to cover the monolayer for 1 minute and rinsed with tap water until clear.

iii. Osteogenic differentiation of LMSC

LMSC were seeded in MGPM in 12-well cell culture plates at a cell density of 2.5×10^4 cells/well and cultured until confluent. The culture medium was replaced with osteogenic differentiation medium (α-MEM, 10% (vol/vol) FBS, 10nM dexamethasone,10mM β-glycerophosphate and 100μM L-ascorbic acid-2-phosphate). All supplementary reagents were purchased from [Sigma Aldrich, Poole, UK]. Cultures were incubated at 37°C, 5% CO_2, 5%$O_2$for four weeks with medium changes every three days. Osteogenic differentiation of LMSC cultures were confirmed by histological staining with Alizarin Red. The cells were incubated in Alizarin Red (4% w/v Alizarin Red in deionised water, pH 4.2-4.4 with 10% (v/v) ammonium hydroxide) for 2 hours at room temperature before being rinsed with PBS.

Similar number of cells (but grown in MGPM only) were cultured as negative controls and then subjected to staining for histological analysis for lineage differentiation.

2.2 Flow cytometry

Changes in LMSC phenotype were investigated by immunophenotyping. Cells were labelled with antibodies specific for cell surface antigens expressed by human MSC; CD44, CD90, CD105, CD146, CD166, antibodies to negative lineage haematopoietic surface antigens CD19, CD45, and a primary control antibody (murine IgG). Cells were also labelled with antibodies expressing HLA Class I and Class II. All cells were from the same cell

preparation. All antibodies were primarily FITC-conjugated except for anti-HLA Class II. All antibodies were purchased from R&D Systems. Cells were detached from tissue culture flasks with TrypLE express and resuspended in flow cytometry buffer solution (0.1% (w/v) bovine serum albumin in PBS. For direct immunofluorescence, 200,000 cells in 100µl were added to each tube and stained with 5µl primary antibody. After incubation for one hour at 4^0C, cells were washed with buffer solution and resuspended in 200µl buffer solution before flow cytometry analysis. For indirect immunofluorescence, 200,000 cells were added to each tube and stained with 5µl primary antibodies in100µl cell suspension. After incubation for one hour in 4^0C, cells were washed. Following that, goat anti-mouse IgG FITC-conjugated secondary antibody [Jackson Immunology Research Laboratory, USA] was added at a 1:25 dilution in 100µl cell suspension; this was incubated again for 30 minutes at 4^0C. At the end of incubation, cells were washed and resuspended in 200µl of flow cytometry buffer solution. Samples were run using FACS Canto II [BD Biosciences] and was analysed using a FACS Diva software [BD Biosciences].

2.3 Immunocyto/histochemistry (ICC and IHC)

For ICC, 40,000 LMSC were seeded in an 8-chamber slide [BD Falcon] and allowed to proliferate until 90% confluentin 5% O2, 5% CO_2 incubator at 37°C. Upon reaching confluence, culture medium was removed and cells washed three times with PBS. Cells were fixed with cold methanol [BDH Laboratory Supplies] at -20°C for 10 minutes and ICC was performed as described previously [21]. Antibodies used in the study included, goat anti-mouse monoclonal antibody to ABCB5 (1:100 dilution) [Abcam], CXCR4 (1:100 dilution) [R&D Systems], ΔNp63 (clone 4A4 1:50 dilution)[Santa Cruz Biotech] and goat anti-rabbit

polyclonal antibody to ABCG2 (1:100 dilution) [Novus Biologicals]. Negative controls were cells incubated without primary, but with secondary antibody.

For IHC, cryosections of AM were embedded in OCT and frozen for cryosectioning. 6µm sections were cut on a cryostat [Leica CM3050] and mounted on silane-adhesion pre-coated slides for optimal histology staining [CellPath]. Slides were air-dried overnight and stored at -20°C before use. Slides were washed with PBS to remove the OCT embedding media and then sections stained as described previously [21]. Additionally, post-staining sections were incubated in Sudan Black [Fisher Scientific] for 10 minutes, washed with PBS and cover slipped.

2.4 Transwell MigrationAnalysis

To elucidate the signalling pathway for LEC migration to the site of injury, we looked for the expression of CXCR4 and its response to specific chemokine CXCL12 ligand by performing chemotaxis assays using transwells migration analysis. Briefly, 100,000 LMSC were resuspended in 300µl of defined keratinocyte serum-free medium supplemented with growth promoting agents (DKSFM) [Gibco] and added to the upper chamber of an 8µm pore diameter 24-well format transwell chamber. The lower chamber was filled with 200µl media without cells but supplemented with CXCL12 at 0ng/ml (internal control) and 300ng/ml, for 5hours incubation (previously pre-optimised) in 5% CO_2 incubator at 37°C. After 5 hours, the migrant cells were stained with haematoxylin and counted using a light microscope under 20X magnification for 5 randomly selected high power fields/ well.

2.5 Microscopy and Imaging

All images were taken using a Nikon Digital Sight–DSFi1 camera and Nikon NIS-Elements D software [Nikon Metrology U.K. Ltd., Derby, UK]. Images were assembled using Adobe Photoshop® CS3 [Adobe Systems]. Fluorescence microscopy was undertaken using an Axioplan F [Zeiss]. Images were then processed using the AxioVision40 version 4.8.2.0 software [Zeiss]. For comparison between 2 populations, images were captured under 20X magnification in five random fields. Cell count was performed using Image J software.

2.6 Semi-quantitative Polymerase chain reaction (sq-PCR)

RNA was extracted using RNEasy Plus Micro Kit [Qiagen, UK] as per manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer [Thermo Scientific].complementary DNA (cDNA) was synthesised using a cDNA Synthesis Kit [Bioline,UK] as per manufacturer's instructions. sq-PCR reactions were assembled with 12.5 µl PCR master mix [Promega, UK], 0.5µl forward primer, 0.5µl reverse primers and 2 – 2.5µl cDNA template and DEPC-treated water to a final volume of 25µl reactions. PCR reactions were amplified for each gene and conditions are summarised in Supplementary Table T1.

2.7 Growth of LMSC on AM

i. Plating of LMSC on AM

Research grade cryopreserved AM obtained from NHS Blood and Transplant was used as a substrate for growth of LMSC. The AM was stretched under tension with two 22mm x 22mm cover slips placed in a 6-well plate (Supplemental Figure F1). 24,000 cells/cm^2 of LMSC from passage 4-5 were plated directly onto the membrane, initially suspended in MGPM. On

the next day the media was partially substituted 50:50 with DKSFM and on the third and subsequent days, with DKSFM only. 100µl DKSFM was supplemented daily directly onto the AM. Cultures were incubated in 5% CO_2 20% oxygen incubator at 37°C for 5 days. At the end of 5 days, the media was drained from the well and the AM was rinsed with PBS and embedded in OCT for cryostat sections.

ii. Haematoxylin and eosin (H&E) staining

Frozen sections (6µm) were cut onto silane-adhesion pre-coated slides [CellPath]. Slides were placed in 95% ethanol for 2 minutes to dissolve OCT, and then fixed with cold methanol for 30 minutes. Sections were then stained with haematoxylin and eosin for brightfield microscopy.

iii. ***Harvesting cells for RNA extraction***

Cells grown on AM were harvested after detachment from membrane using 0.05% trypsin-EDTA for 3 minutes, washed with 10% FBS/PBS and centrifuged to get a cell pellet **(**labelled**L-AM).** LMSC were detached from T75cm^2 flask using TrypLE express and centrifuged to get a pellet and subsequently used for RNA extraction **(**labelled **LMSC).** Under direct microscopic examination, an area of AM devoid of any LMSC growth was marked and harvested as an AM only control. The epithelial cells of the membrane were detached using 0.05% trypsin-EDTA for 3 minutes, washed in 10% FBS/PBS and centrifuged to get a cell pellet. Subsequent steps for RNA extraction followed the manufacturer's instructions using RNEasy Plus Micro Kit [Qiagen].

2.8 Statistical analysis

Descriptive statistics were used to describe means, median, or mode. Quantitative data which were normally distributed were analysed for comparison between 2 groups using independent t-test. Results with *P* values of less than 5% (***P***< 0.05) were considered statistically significant.

3.0 RESULTS

LMSC possess the tri-lineage differentiation potential of MSC

Cells derived from LMSC were assessed for tri-lineage differentiation into chondrocytes, adipocytes and osteoblasts (Figure 1). LMSC showed chondrogenic differentiation as indicated by positive staining with Safranin O indicative of matrix deposition (Figure 1A), adipogenic differentiation as indicated by the presence of lipid vesicles within the cells that stained red with Oil Red O (Figure 1B) and osteogenic differentiation as indicated by Alizarin Red staining for matrix mineralisation (Figure 1C). Cells cultured in MGPM only did not demonstrate any differentiated phenotypes and no characteristic staining with Alizarin Red, Safranin O or Oil Red O (Supplementary Figure F2).

Transcriptional analysis confirmed the tri-linage differentiation potential of LMSC with differentiated cells expressing chondrocyte-specific genes aggrecan and Sox9 but lacking expression of collagen type X (Figure 1D). Adipogenic differentiation as confirmed by expression of adipophilin, adipocyte binding protein-2 and sterol regulatory-element-binding protein-1 (Figure 1E) and osteogenic differentiation as indicated by expression of alkaline phosphatase andbone sialoprotein-1 (Figure 1F).

LMSC fulfil the immunophenotyping criteria for human MSC

Flow cytometry profiles of the immunophenotype of LMSC population are presented by dark grey histograms overlayed onto the representative flow cytometry profiles of the unlabelled cell population (light grey) histograms. Analysis of cell surface antigen expression was carried out by calculating the percentage shift between the labelled and unlabelled LMSC populations. Results of immunophenotyping showing mean percentage of positively stained cells from 3 biological replicates are shown in Supplementary Table T2). Positive shifts were indicated in CD44, CD90, CD105, CD146 and CD166 expression, and cells were in the main negative shift for the haematopoietic cell surface antigens CD19and CD45 expression. LMSC also expressed HLA Class I, but negative expression of HLA Class II (Figure 2).

LMSC express putative limbal stem cell markers

Results of ICC analysis of monolayer LMSC cultures using common limbal markers revealed expression of ΔNp63, ABCG2 and ABCB5 (Figure 3 panel A) compared with negative controls (Figure 3 panel B).

LMSC express CXCR4 and migrate in response to CXCL12

Chemotaxis was observed by LMSC when stimulated with CXCL12. Both cell populations displayed migration, however more migrant cells were observed in the LMSC population on addition of CXCL12 (Figure 4A and 4B) and this was due to the expression of CXCR4 receptor as demonstrated by ICC (Figure 4C). Results of the mean number of migrant cells were calculated and showed statistically significant difference ($P<0.05$) (Figure 4D).

LMSC Grow on AM and express putative limbal stem cell markers

LMSC were adherent on AM and observed to proliferate and achieved confluence in DKSFM. There was a change in cellular morphology from the slender shaped, fibroblastic morphology of LMSC (5A –day 3) to more rounded cell bodies on Day 5 (see high magnification image 5A-day 5). Histological analysis of cryostat sections of LMSC on AM at day 5 (5B) showed the presence of cells on the AM (asterisks) compared with AM only (Figure 5C).

mRNA expression of common limbal markers were investigated in cells grown on AM (L-AM), LMSC alone and epithelial cells of the AM (Figure 5D). L-AM and LMSC showed high expression of the transcription factor p63similar to AM. Whereas weak expression of CK3, a corneal epithelium differentiation marker was detected in LMSC culture, expressed in AM, and showed low expression in L-AM. CK3 was detected in some LMSC culture, but absent in other biological replicates (Supplementary Figure S3). Another corneal differentiation marker CK12 was also not expressed (Supplementary Figure S3). However, LMSC expressed mRNA for ABCB5 (5D).

IHC analysis of LMSC grown on AM showed expression of ABCG2 (6A) and ABCB5 (6C), compared to negative controls,(6B) and (6D). p63 was also shown to be expressed by LMSC on AM (6E), the staining was both nuclear and cytoplasmic. Note the same section of LMSC on AM in (6F) showing the cell nuclei of these cells. AM only stained for p63 expression(6G), note the difference in the pattern of staining.

DISCUSSION

Previous studies have demonstrated the isolation of LMSC from corneal tissue, although protocols varied among the investigators. Polisetty et al described harvesting spindle-shaped outgrowths from primary limbal cultures after growing them on AM[22]. What where termed "limbal niche cells" by Tseng group, where cells isolated from corneo-scleral rings using collagenase, or with dispase and grown on matrigel [15]. The Funderburgh group used dispase and collagenase digestion on minced pieces of stromal tissues and successfully grew these cells in a selective media containing 2% FBS; incubated in humidified atmosphere, at 5% CO_2[14, 23]. These cells have all been shown to express MSC markers [14, 15, 22, 23] and have been described as having angiogenic potential [15].

Our group used serial trypsinisation which we believe effectively cleaved the epithelial layers from the stroma as shown by haematoxylin and eosin staining on cross sections of limbal tissues taken from each cycle (unpublished data). Further use of trypsin allowed separation of the epithelial layer into single cells.

Cells were cultured in a selective MSC media and later in differentiation media under hypoxic conditions, with the exception of those being analysed for adipogenic differentiation which requires normal oxygen levels. Hypoxia or low oxygen tension has been shown to be a critical regulator of stem cell biology, through the activation of hypoxia inducible factor pathways and the maintenance of cellular health [24]. Hypoxia or low oxygen tension is more physiologically relevant to maintenance of stem cell function and differentiation [25] and in addition has been shown to be physiologically effective for greater cell vitality, enhanced tri-lineage differentiation and modulation of the paracrine activity of MSC[26].

Immunophenotyping was used to demonstrate that the cells isolated in this study had the characteristics of human MSC; that is they expressed markers of MSC and had had low/no

expression for the haematopoietic commitment markers, comparable to findings of LMSC immunophenotyping reported in previous studies. It has been noted that there are variations of cell surface antigen presentation by LMSC cultured indifferent culture media or in the presence of additional supplements[23], e.g. CD146+ve expressing cells were present in cultures with serum supplement, but no expression of CD146 was detected in cells cultured in commercially available serum-free media [18]. A CD34+/CD105+ve LMSC population has also been shown to be stem-like and have tri-lineage differentiation capabilities[27]. LMSC showed high expression of HLA Class I, but low/no expression of Class II, similar to previous findings [13, 18], which deems this cell population advantageous for allogeneic transplantation.

Chemokines are important molecules for leukocyte migration and mediate their effect through G-protein coupled 7-transmembrane chemokine receptors [28] on the cell surface of specific and well-defined leucocyte subsets [29]. However, some chemokine receptors are found on non-haematopoietic cells; including neuron and microglia[30],colonic epithelial cells [31], endothelial cells [32] and myocardial cells [33]. In these systems, CXCR4 played an important role in the interaction between the target cells and chemokine signalling through CXCL12 mediation. Our LMSC expressed CXCR4 and were capable of migration and this was mediated by the CXCR4/CXCL12 axis. CXCR4 expression by corneal cells has been reported previously [35]. In addition, Polisetti et al reported that vascular endothelial growth factor (VEGF) was present in LMSC [34].This suggests that cornea wound healing involves new vascular formation and cellular migration, possibly regulated by pro-inflammatory cytokines via the CXCR4 - CXCL12 axis, a similar “homing mechanism” observed to occur in bone marrow-MSC migration for bone regeneration [35] and MSC migration to sites of injury [36].

The current study showed that LMSC fulfil the criteria for MSC tri-lineage differentiation to osteogenic, adipogenic and chondrogenic lineages. Oil Red O staining showed lipid laden cells undergoing adipogenesis, while calcium deposition was indicated by Alizarin Red staining, similar to previous findings in LMSC [14, 22, 27] We also showed our LMSC had chondrogenic potential as shown by cartilage deposition indicated by Safranin O staining.

Further, LMSC showed mRNA expression for genes known to be expressed by these lineage specific cell types. However, our LMSC derived chondrocytes lacked expression of collagen type X. Collagen X is normally expressed by hypertrophic chondrocytes during endochondral ossification[37],which is a distinctive characteristic of bone marrow-MSC where Collagen X appears early in the differentiation. Lack of expression of this in our differentiated cells might be due to lack of maturity in our cells. Tri-lineage commitment of our LMSC shows a similar multipotentiality possessed by human bone marrow-MSC[38].

AM has been widely used for ocular surface diseases in the UK since 1998, the outcome of AM transplantation have given varying results under different conditions [39]. The beneficial effects of AM include its anti-angiogenic, anti-inflammatory and wound healing properties, making it the most common biological substrate used for tissue constructs. Long term storage of AM in cryopreserved media does not appear to alter the sterility, histology or biological properties of the membrane[40].

LMSC grown on AM rapidly became a confluent cell layer and in addition, underwent morphological changes from a fibroblast-like morphology [15, 41] to cells with a more rounded cell body with bipolar protrusions. A series of morphological changes have

previously been observed from outgrowths of limbal epithelial cells grown on intact versus denuded AM by the Tseng group [42] which might be pertinent to the changes observed in our cultures. In their publication, they described the morphological changes of small, cuboidal cells (intact AM) versus large, spindle-shaped cells (denuded AM), stratification (intact AM) versus two-layer cells (denuded AM), etc. These changes were attributed to a process of cellular maturation and differentiation by limbal epithelial cells as they dissolved and then reassembled AM basement membrane as they grew on the AM.

Here we show that LMSC share some expression of putative markers that have been widely used to characterise limbal stem cells at both the level of gene and protein expression. These markers include - transcription factor p63and drug resistance transporter ABCG2[43-45]. p63 is responsible for limbal epithelial proliferation [43] and the isomer ΔNp63α of the transcription factor plays a significant role in corneal regeneration, differentiation and wound healing [46]. Adenosine tri-phosphate binding cassette G2 (ABCG2) is a cell surface transport protein, which is preferentially expressed in a variety of adult stem cells [47-50]. ABCG2 is also responsible for efflux of Hoechst 33342 dye, a characteristic of the limbal side population cell phenotype. Side population analysis has been used to isolate enriched stem cell populations [21, 51-55].

We also studied a new marker, ABCB5 which was recently reported to be an important molecular limbal marker for corneal development. ABCB5 was first identified in skin progenitor cells[56] and malignant melanoma cells [57]. Recently, ABCB5 has been demonstrated to play an important role in limbal stem cell maintenance and corneal wound healing, thus denoting it as an important molecular marker for limbal stem cells[58]. We further characterised LMSC growing on AM. mRNA analysis revealed expression ofp63 and

ABCB5, while protein analysis revealed these cells expressedp63, ABCG2 and ABCB5. In addition, the expression of all three proteins was also maintained by LMSC grown on AM. While expression of p63 is generally considered to be nuclear we observed that LMSC grown in culture and on AM exhibited both punctuate nuclear staining and cytoplasmic staining, this may be indicative of cellular differentiation. Galli et al[59] reported that certain genes like MDM2can mediate translocation of ΔNp63from the cell nucleus to the cytoplasm under appropriate stimuli such as cellular differentiation.

CK3 distinguishes undifferentiated LMSC from epithelial cells, and is a good marker for corneal epithelium and also as a negative marker for limbal stem cells [60]. We found mRNA expression of CK3 was not consistent in all our samples which may suggests variability between donors. Variability in patient-derived MSC has been reported for other MSC populations derived from other tissues [61].

FUTURE PERSPECTIVE

Future studies will be directed towards proliferation and maturation of LMSC on biological and non-biological substrates for ocular surface reconstruction. The immunomodulatory effects of LMSC, e.g. paracrine secretion and functions of trophic and growth factors also warrant further investigation. As these cells can be expanded readily in culture, stored frozen and brought back up in culture, they have potential to be a useful cellular tool in allogeneic cellular therapy for bilateral corneal conditions.

EXECUTIVE SUMMARY

Isolation, identification and characterisation of LMSC

- A simpler way of isolating human LMSC from primary corneo-scleral tissues
- The use of serial trypsinisation method initially harvested a heterogenous population of cells followed by LMSC derivations under optimised culture conditions (a selective MSC media and hypoxic conditions).
- Limbal-derived MSC described in this study have characterised of human MSCs, exhibiting tri-lineage commitment into chondrocytes, adipocytes and osteoblasts as determined by histological analysis, mRNA expression and immunophenotyping.

LMSC cultures benefit from feeder-free culture conditions and shorter duration for cellular differentiation

- LMSC are plastic adherent thus easier to grow without a feeder layer or use of a substrate, and the conditions used here offer shorter duration of cultures (5-7 days) compared to 10-14 days used for outgrowth of limbal explants in enriched media [22] or when using cell suspensions from primary tissues [14, 27].
- LMSC can grow and differentiate towards cells with a more mature corneal epithelial lineage on AM

LMSC migration through the CXCR4-CXCL12 axis isa potentially important signalling pathway for corneal epithelial regeneration

- LMSC express the chemokine receptor CXCR4 (gene and protein expression)
- Cellular migration directed by CXCR4 specific ligand CXCL12 is an important property for a cell population to be useful in clinical transplantation for ocular surface regeneration.

CONCLUSION

- The MSCs derived from limbal tissues satisfied minimum criteria defining them as human MSCs.
- Currently there are no clinical trials using LMSC as a cellular tool for LSCD despite many clinical trials using MSC in other organs such as in the lungs, intestine and liver for many clinical conditions such as degenerative disorders, auto-immune, haematological and neuromuscular disorders [62] warranting further exploration of this cell population.
- LMSC can grow and differentiate on intact cryopreserved AM.
- The preliminary data presented here on LMSC cell population suggest potential for this cell population to be useful in furthering our understanding of LSC biology and as a potential tool in developing better constructs for the treatment of ocular surface disorders.

Acknowledgment:

Supported by grants from the Ministry of Higher Education, Malaysia and partially by Universiti Sains Malaysia, Pulau Pinang, Malaysia. The authors thank Ian Dimmick, Lisa Hodgson, academic and technical staff at the Institute of Genetic Medicine, International Centre for Life, Newcastle Upon-Tyne, United Kingdom, for their assistance throughout this study.

Financial Disclosure:

Authors do not have actual or potential conflict of interest including any financial, personal or other relationships with other people or organisations that could inappropriately influence, or be perceived to influence our work.

FIGURE LEGENDS

Figure 1. Characterisation of limbal MSC, N=3. Histological analysis for LMSC differentiated cells. (A) Safranin O staining of cartilaginous deposition (orange) on a representative section of a pellet generated after 2 weeks culture in chondrogenic differentiation media, photo inset is a magnification of the same image. (B) Oil Red O staining of adipogenic differentiated cells showing lipid vesicles stained bright red, nuclei blue, after 30 days in culture, photo inset is a magnification of the same image. (C) Alizarin Red staining of mineral deposition in osteogenic differentiated cells after 3 weeks in culture, photo inset is a magnification of the same image. Analysis of mRNA expression for genes expressed by LMSC that had undergone tri-lineage differentiation (N=3) for (D) Chondrogenic: Lane 1 Aggrecan (170 bp), lane 2 Sox9 (150 bp), lane 3 no expression to Collagen X (730 bp) (E) Adipogenic : Lane 1 Adipocyte differentiation-related protein (ADRP) or Adipophilin (150 bp), lane 2 Adipocyte binding protein-2 (130 bp), lane 3 Sterol regulatory element-binding protein-1 (230 bp) and (F) Osteogenic: Lane 1 Alkaline phosphatase (187 bp), lane 2 Bone sialoprotein-1 (161 bp). GAPDH was used as a loading control (100 bp). *Figure 1 Panels A, B and C, reproduced with kind permission of reproduction from eCM journal ([www.ecmjournal.org]www.ecmjournal.org)[63]*

Figure 2. Immunophenotyping of LMSC, N=3. Representative histograms showing cell populations labelled for surface antigens expressed by human MSC; CD44, CD90, CD105,

CD146, CD166, haematopoietic cell markers; CD19, CD45, and HLA Class I and Class II. Murine IgG was used as control. Antibody profiles (dark grey) were overlayed onto the unlabelled population (light grey).

Figure 3. Immunocytochemistry of p63, ABCG2 and ABCB5 expression in LMSC cultures on panel A, negative control (secondary IgG) onlyon panel B*[DAPI=blue, FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody=red]*.

Figure 4. CXCL12-directed migration analysis of LMSC. (A) Transwell migration analysis in untreated cells (0ng/ml) showing no migrant cells (B) Cells with addition of 300ng/ml CXCL12, incubated for 5 hours, note more migrant LMSC (arrows). (C) Immuno cytochemistry analysis shows expression of CXCR4 receptor in LMSC [*DAPI=blue, CXCR4-FITC= green]* (D) Mean number of migrant LMSC /high power fields in treated and untreated cells (****P***<0.05, Independent t-test), *N=3*.

Figure 5. Growth of LMSC (white arrows) on AM thawed from frozen (A) from Day 1 until Day 5 in culture. On Day 5, there was distinct LMSC colonies growing on AM, a magnified image shows changes in cellular morphology (white arrows) from spindle-shaped cells to rounded bodies with bipolar projections. (B) Haematoxylin and eosin staining of a cross section of AM with areas of LMSC growth (asterisks)(C) a cross section of AM without LMSC for comparison, photo insets show a magnified view.*[Blue –cell nuclei, scale bar: 20 μm]*. (D) mRNA expression of p63 and CK3 in LMSC growing on AM (L-AM), LMSC only, and AM epithelial cells only(E) mRNA expression of ABCB5 in LMSC. GAPDH was used as a loading control. *Figure 5A reproduced with kind permission of reproduction from eCM journal ([www.ecmjournal.org]www.ecmjournal.org)[63]*

Figure 6. Immunohistochemistry of AM sections with LMSC growth. (6A) LMSC expressing ABCG2, (6C) ABCB5 and (6E) p63 (6B) and (6D) are negative control sections stained with secondary IgG only (6F) the same cross section used for p63 staining with DAPI only (6G) and (6H) are cross sections of AM without LMSC growth; (6G) p63 staining (6H) negative control with secondary IgG only. *[DAPI=blue, FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody=red]*.

(Supplementary) Figure S1. Plating of LMSC on AM thawed from frozen. The membrane was kept stretched and taut using two 22mm x 22mm cover slips. The cells were plated in media into an area confined within a plastic ring.

(Supplementary) Figure S2. Undifferentiated LMSC cultures stained with (A) Oil Red O (B) Safranin O and (C) Alizarin Red as negative controls for LMSC tri-lineage commitment *(N=3).*

(Supplementary) Figure S3. mRNA expression of CK3 and CK12 in LMSC, GAPDH was used as a loading control. *[Abbreviations: LMSC – limbal MSC, LEC - Limbal epithelial cells (+ve control) and NTR - no template reactions* (*–ve control)]*

TABLE LEGENDS

(Supplementary) Table T1. Oligonucleotides primers and amplification conditions. **Taqman probe [Applied Biosystems]*.

(Supplementary) Table T2. Results of FACS analysis for percentage of stained-positive LMSC immunophenotyping for three biological replicates. *(*) N=1.*

REFERENCES and annotations

Papers of special note have been highlighted as:
□*of interest*
■*of considerable interest*

■Ex vivo expansion of LEC for transplantation: methods and outcomes (Ref 1-6)

1. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M: Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *The Lancet* 349(9057), 990-993 (1997).
2. Shortt AJ, Secker GA, Notara MD *et al.*: Transplantation of Ex Vivo Cultured Limbal Epithelial Stem Cells: A Review of Techniques and Clinical Results. *Survey of Ophthalmology* 52(5), 483-502 (2007).
3. Shortt AJ, Secker GA, Rajan MS *et al.*: Ex Vivo Expansion and Transplantation of Limbal Epithelial Stem Cells. *Ophthalmology* 115(11), 1989-1997 (2008).
4. Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G: Limbal Stem-Cell Therapy and Long-Term Corneal Regeneration. *New England Journal of Medicine* 363(2), 147-155 (2010).
5. Tseng SCG: Amniotic Membrane Transplantation for Ocular Surface Reconstruction. *Bioscience Reports* 21(4), 481-489 (2001).
6. Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S: 13 years of cultured limbal epithelial cell therapy: A review of the outcomes. *Journal of Cellular Biochemistry* 112(4), 993-1002 (2011).
7. Baustian C, Hanley S, Ceredig R: Isolation, selection and culture methods to enhance clonogenicity of mouse bone marrow derived mesenchymal stromal cell precursors. *Stem Cell Research & Therapy* 6(1), 151 (2015).
8. Dominici M, Blanc KL, Mueller I *et al.*: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315-317 (2006). ***(□ defining criteria for human human MSC)***
9. Levis HJ, Massie I, Dziasko MA, Kaasi A, Daniels JT: Rapid tissue engineering of biomimetic human corneal limbal crypts with 3D niche architecture. *Biomaterials* 34(35), 8860-8868 (2013).
10. Pellegrini G, Rama P, Matuska S *et al.*: Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regenerative Medicine* 8(5), 553-567 (2013).
11. Langer R, Vacanti J: Tissue engineering. *Science.* 260(5110), 920-926. (1993).
12. Miguel MD, Fuentes-Julián S, Blázquez-Martínez A *et al.*: Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr Mol Med.* 12, 574-591. (2012).
13. Garfias Y, Nieves-Hernandez J, Garcia-Mejia M, Estrada-Reyes C, Jimenez-Martinez MC: Stem cells isolated from the human stromal limbus possess immunosuppressant properties. *Mol Vis.* 18, 2087-2095 (2012).

■ investigations into human limbal stromal MSC(References 14-17, 22, 23,34)

14. Branch MJ, Hashmani K, Dhillon P, Jones DRE, Dua HS, Hopkinson A: Mesenchymal Stem Cells in the Human Corneal Limbal Stroma. *Investigative Ophthalmology & Visual Science* 53(9), 5109-5116 (2012).
15. Li G-G, Zhu Y-T, Xie H-T, Chen S-Y, Tseng SCG: Mesenchymal Stem Cells Derived from Human Limbal Niche Cells. *Investigative Ophthalmology & Visual Science* 53(9), 5686-5697 (2012).
16. Hoar RM: Embryology of the eye. *Environmental Health Perspectives* 44, 31-34 (1982).

17. Pinnamaneni N, Funderburgh JL: Concise Review: Stem Cells in the Corneal Stroma. *STEM CELLS* 30(6), 1059-1063 (2012).
18. Bray LJ, Heazlewood CF, Munster DJ, Hutmacher DW, Atkinson K, Harkin DG: Immunosuppressive properties of mesenchymal stromal cell cultures derived from the limbus of human and rabbit corneas. *Cytotherapy* 16(1), 64-73 (2014).
19. Shaharuddin B, Harvey I, Ahmad S, Ali S, Meeson A: Characterisation of Human Limbal Side Population Cells Isolated Using an Optimised Protocol From an Immortalised Epithelial Cell Line and Primary Limbal Cultures. *Stem Cell Rev and Rep* 10(2), 240-250 (2014).
20. Osei-Bempong C, Henein C, Ahmad S: Culture conditions for primary human limbal epithelial cells. *Regenerative Medicine* 4(3), 461-470 (2009).
21. Shaharuddin B, Harvey I, Ahmad S, Ali S, Meeson A: Characterisation of Human Limbal Side Population Cells Isolated Using an Optimised Protocol From an Immortalised Epithelial Cell Line and Primary Limbal Cultures. *Stem Cell Rev and Rep*, 1-11 (2013).
22. Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemugan GK: Mesenchymal cells from limbal stroma of human eye. *Mol Vis.* 14, 431-442 (2008).
23. Du Y, Funderburgh ML, Mann MM, Sundarraj N, Funderburgh JL: Multipotent Stem Cells in Human Corneal Stroma. *STEM CELLS* 23(9), 1266-1275 (2005).
24. Mohyeldin A, Garzón-Muvdi T, Quiñones-Hinojosa A: Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell.* 7(2), 150-161 (2010). ***(□ hypoxia for MSC cultures)***
25. Bath C: Human Corneal Epithelial Subpopulations: Oxygen Dependent Ex Vivo Expansion and Transcriptional Profiling. *Acta Ophthalmologica* 91(thesis4), 1-34 (2013).
26. Das R, Jahr H, Van Osch GJVM, Farrell E: The Role of Hypoxia in Bone Marrow–Derived Mesenchymal Stem Cells: Considerations for Regenerative Medicine Approaches. *Tissue Engineering Part B: Reviews* 16(2), 159-168 (2009). ***(□ hypoxia for MSC cultures)***
27. Hashmani K, Branch MJ, Sidney LE *et al.*: Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation. *Stem Cell Research & Therapy* 4(75), (2013).
28. Trosko JE, Chang CC, Wilson MR, Upham B, Hayashi T, Wade M: Gap junctions and the regulation of cellular functions of stem cells during development and differentiation. *Methods* 20(2), 245-264 (2000).
29. Groves DT, Jiang Y: Chemokines, a Family of Chemotactic Cytokines. *Critical Reviews in Oral Biology & Medicine* 6(2), 109-118 (1995).
30. Lavi E, Strizki JM, Ulrich AM *et al.*: CXCR-4 (Fusin), a co-receptor for the type 1 human immunodeficiency virus (HIV-1), is expressed in the human brain in a variety of cell types, including microglia and neurons. *The American Journal of Pathology* 151(4), 1035-1042 (1997).

■ ***investigations into CXCR4/CXCL12-directed cellular migration (References 31-33, 36)***

31. Dwinell MB, Eckmann L, Leopard JD, Varki NM, Kagnoff MF: Chemokine receptor expression by human intestinal epithelial cells. *Gastroenterology* 117(2), 359-367 (1999).
32. Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM: Chemokine Receptors in Human Endothelial Cells: functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *Journal of Biological Chemistry* 273(7), 4282-4287 (1998).
33. Nilsson J, Ali S, Harvey I, Kirby JA, Meeson AP: Stem cell therapy: A role for CXCR4 in homing bone marrow side population cells to areas of myocardial damage. *International Journal of Cardiology* 145(3), 554-555 (2010).
34. Polisetti N, Agarwal P, Khan I, Kondaiah P, Sangwan VS, Vemuganti GK: Gene expression profile of epithelial cells and mesenchymal cells derived from limbal explant culture. *Mol Vis* 16, 1227-1240 (2010).

35. Yu L, Tu Q, Han Q *et al.*: Adiponectin Regulates Bone Marrow Mesenchymal Stem Cell Niche Through a Unique Signal Transduction Pathway: An Approach for Treating Bone Disease in Diabetes. *STEM CELLS* 33(1), 240-252 (2015).
36. Marquez-Curtis LA, Janowska-Wieczorek A: Enhancing the Migration Ability of Mesenchymal Stromal Cells by Targeting the SDF-1/CXCR4 Axis. *BioMed Research International* 2013, 561098 (2013).
37. Rucklidge GJ, Milne G, Robins SP: Collagen Type X: A Component of the Surface of Normal Human, Pig, and Rat Articular Cartilage. . *Biochemical and Biophysical Research Communications* 224(2), 297-302 (1996).
38. Pittenger M: *Mesenchymal Stem Cells from Adult Bone Marrow*. In: *Mesenchymal Stem Cells*, Prockop D,Bunnell B,Phinney D (Ed.^(Eds). Humana Press, 27-44 (2008).
39. Saw VPJ, Minassian D, Dart JKG *et al.*: Amniotic membrane transplantation for ocular disease: a review of the first 233 cases from the UK user group. *British Journal of Ophthalmology* 91(8), 1042-1047 (2007).
40. Thomasen H, Pauklin M, Noelle B *et al.*: The Effect of Long-Term Storage on the Biological and Histological Properties of Cryopreserved Amniotic Membrane. *Current Eye Research* 36(3), 247-255 (2011).
41. Barbero A, Ploegert S, Heberer M, Martin I: Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. *Arthritis & Rheumatism* 48(5), 1315-1325 (2003).
42. Li W, He H, Kuo C-L, Gao Y, Kawakita T, Tseng SCG: Basement Membrane Dissolution and Reassembly by Limbal Corneal Epithelial Cells Expanded on Amniotic Membrane. *Investigative Ophthalmology & Visual Science* 47(6), 2381-2389 (2006).

□ ***characterisation of limbal SC by p63, ABCG2and Side Population Ref 43-54,60)***

43. Hsueh YJ, Kuo PC, Chen JK: Transcriptional regulators of the ΔNp63: Their role in limbal epithelial cell proliferation. *Journal of Cellular Physiology* 228(3), 536-546 (2013).
44. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M: Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A* 102(27), 9523-9528 (2005).
45. Kawasaki S, Tanioka H, Yamasaki K, Connon CJ, Kinoshita S: Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. *Experimental Eye Research* 82(2), 293-299 (2006).
46. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M: Isoforms of ΔNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proceedings of the National Academy of Sciences of the United States of America* 102(27), 9523-9528 (2005).
47. Alfakir M, Dawe N, Eyre R *et al.*: The temporal and spatial expression patterns of ABCG2 in the developing human heart. *International Journal of Cardiology* 156(2), 133-138 (2012).
48. Doyle MJ, Zhou S, Tanaka KK *et al.*: Abcg2 labels multiple cell types in skeletal muscle and participates in muscle regeneration. *The Journal of Cell Biology* 195(1), 147-163 (2011).
49. Emmert MY, Emmert LS, Martens A *et al.*: Higher frequencies of BCRP+ cardiac resident cells in ischaemic human myocardium. 34, (2013).
50. Meissner K, Heydrich B, Jedlitschky G *et al.*: The ATP-binding Cassette Transporter ABCG2 (BCRP), a Marker for Side Population Stem Cells, Is Expressed in Human Heart. *Journal of Histochemistry & Cytochemistry* 54(2), 215-221 (2006).
51. Akinci MaM, Turner H, Taveras M, Wolosin JM: Differential Gene Expression in the Pig Limbal Side Population: Implications for Stem Cell Cycling, Replication, and Survival. *Investigative Ophthalmology & Visual Science* 50(12), 5630-5638 (2009).
52. Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MaM, Wolosin JM: Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *Journal of Cell Science* 118, 1715-1724 (2005).

53. Chan RK, Garfein E, Gigante PR *et al.*: Side Population Hematopoietic Stem Cells Promote Wound Healing in Diabetic Mice. *Plastic and Reconstructive Surgery* 120(2), 407-411 410.1097/1001.prs.0000267696.0000298789.0000267666 (2007).
54. De Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li D-Q: ABCG2 Transporter Identifies a Population of Clonogenic Human Limbal Epithelial Cells. *Stem Cells* 23(1), 63-73 (2005).
55. Park K-S, Lim CH, Min B-M *et al.*: The Side Population Cells in the Rabbit Limbus Sensitively Increased in Response to the Central Cornea Wounding. *Investigative Ophthalmology & Visual Science* 47(3), 892-900 (2006).
56. Frank NY, Pendse SS, Lapchak PH *et al.*: Regulation of Progenitor Cell Fusion by ABCB5 P-glycoprotein, a Novel Human ATP-binding Cassette Transporter. *Journal of Biological Chemistry* 278(47), 47156-47165 (2003). ■***ABCB5 as a new stem cell marker***

57. Frank NY, Margaryan A, Huang Y *et al.*: ABCB5-Mediated Doxorubicin Transport and Chemoresistance in Human Malignant Melanoma. *Cancer Research* 65(10), 4320-4333 (2005). ■***ABCB5 as a new stem cell marker***

58. Ksander BR, Kolovou PE, Wilson BJ *et al.*: ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 511(7509), 353-357 (2014). ■***ABCB5 as a new stem cell marker***

59. Galli F, Rossi M, D'alessandra Y *et al.*: MDM2 and Fbw7 cooperate to induce p63 protein degradation following DNA damage and cell differentiation. *Journal of Cell Science* 123(14), 2423-2433 (2010).
60. Harkin DG, Barnard Z, Gillies P, Ainscough SL, Apel AJG: Analysis of p63 and cytokeratin expression in a cultivated limbal autograft used in the treatment of limbal stem cell deficiency. *The British Journal of Ophthalmology* 88(9), 1154-1158 (2004).
61. D'ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA: Age-Related Osteogenic Potential of Mesenchymal Stromal Stem Cells from Human Vertebral Bone Marrow. *Journal of Bone and Mineral Research* 14(7), 1115-1122 (1999).
62. Sharma RR, Pollock K, Hubel A, Mckenna D: Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* 54(5), 1418-1437 (2014).
63. Shaharuddin B, Charles Osei-Bempong SA, Paul Rooney, Simi Ali, Rachel Oldershaw, Meeson A: Limbal mesenchymal stem cells; alternative stem cells for transplantation. *European Cells and Materials* 28(4), 30 (2014).

Figure 1

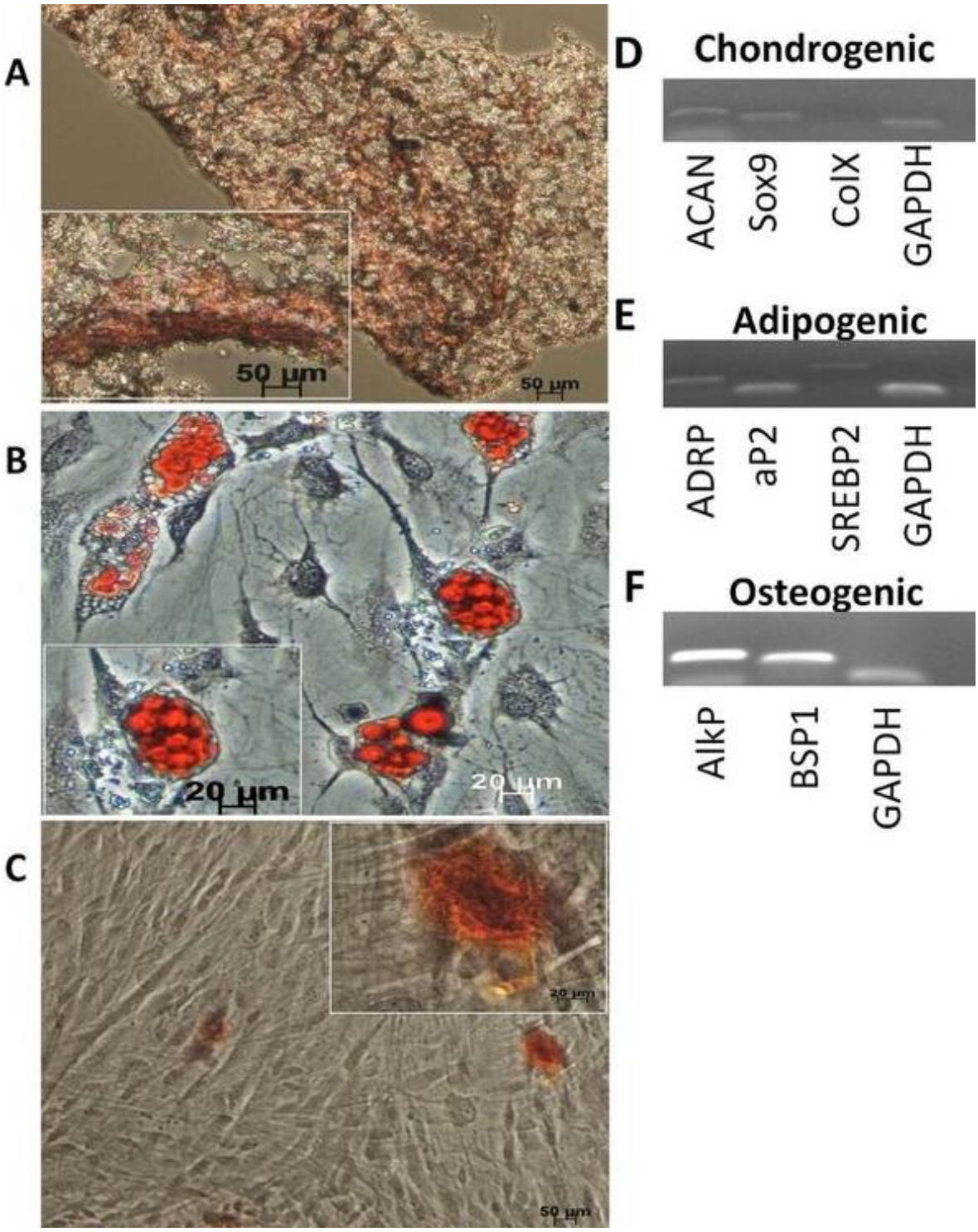

A
50 µm
50 µm
B
20 µm
20 µm
C
50 µm
D
Chondrogenic
ACAN
Sox9
ColX
GAPDH
E
Adipogenic
ADRP
aP2
SREBP2
GAPDH
F
Osteogenic
AlkP
BSP1
GAPDH

Figure 2

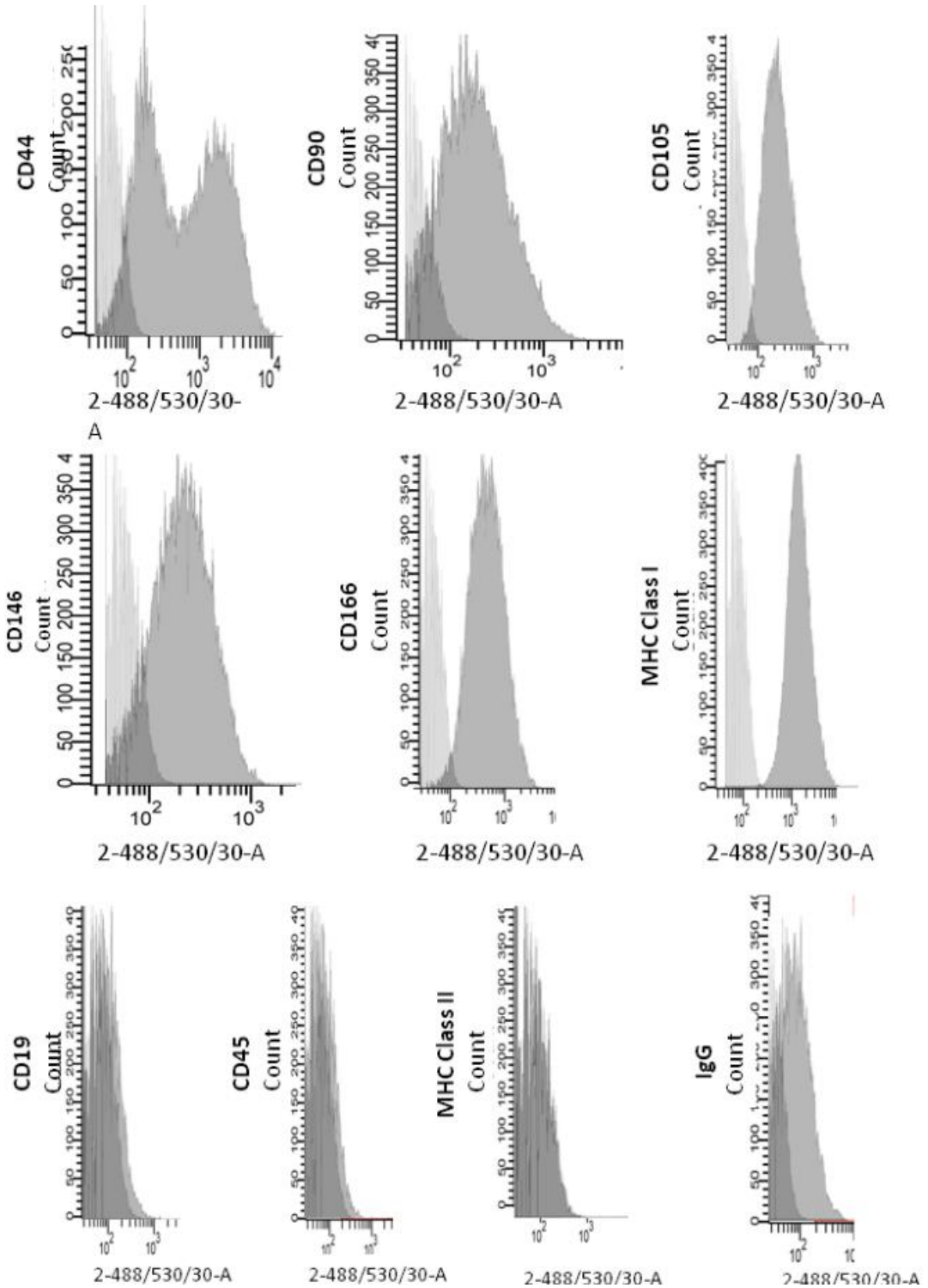

CD44
Count
2-488/530/30-A
CD90
Count
2-488/530/30-A
CD105
Count
2-488/530/30-A
CD146
Count
2-488/530/30-A
CD166
Count
2-488/530/30-A
MHC Class I
Count
2-488/530/30-A
CD19
Count
2-488/530/30-A
CD45
Count
2-488/530/30-A
MHC Class II
Count
2-488/530/30-A
IgG
Count
2-488/530/30-A

Figure 3

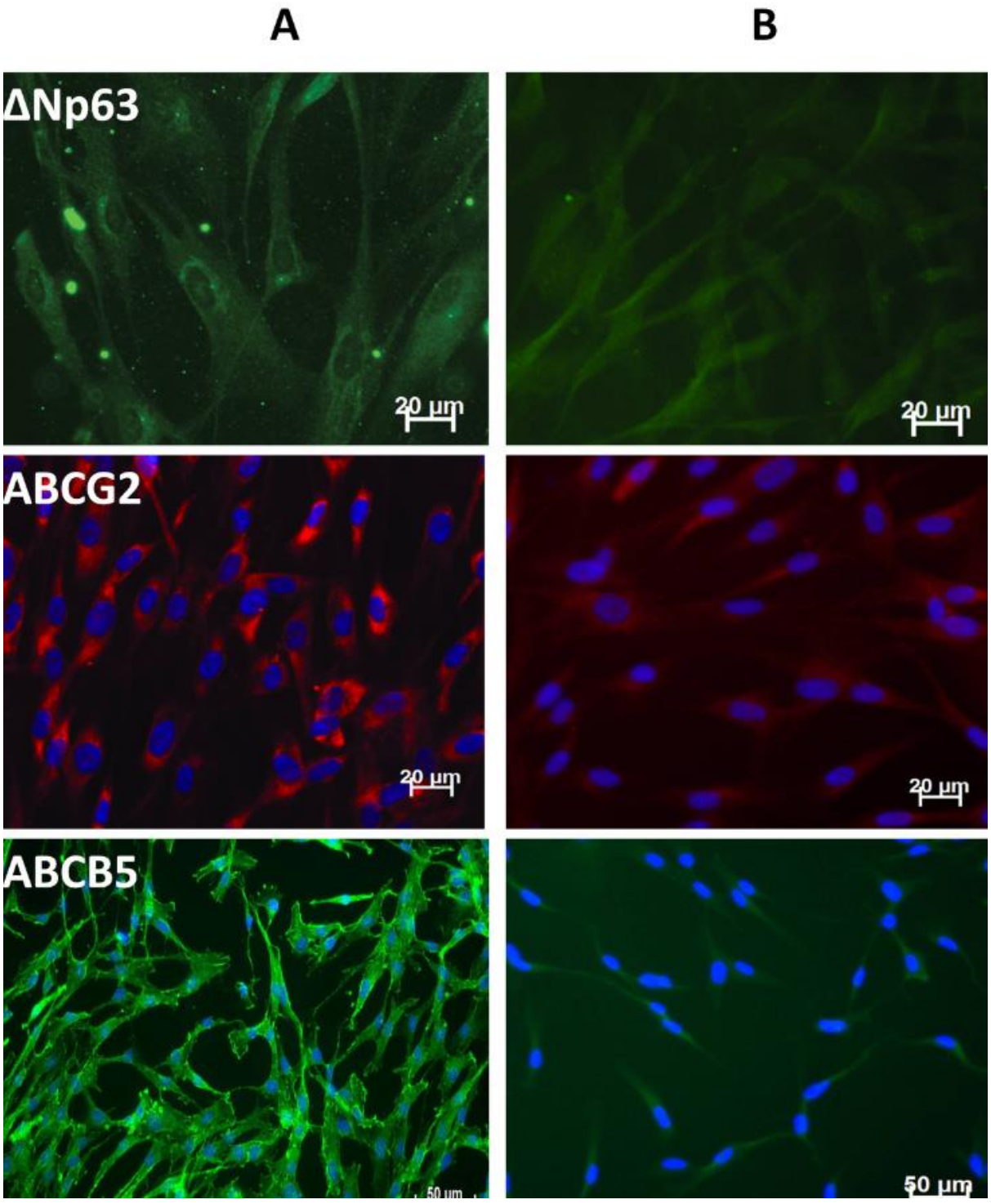


Figure 4

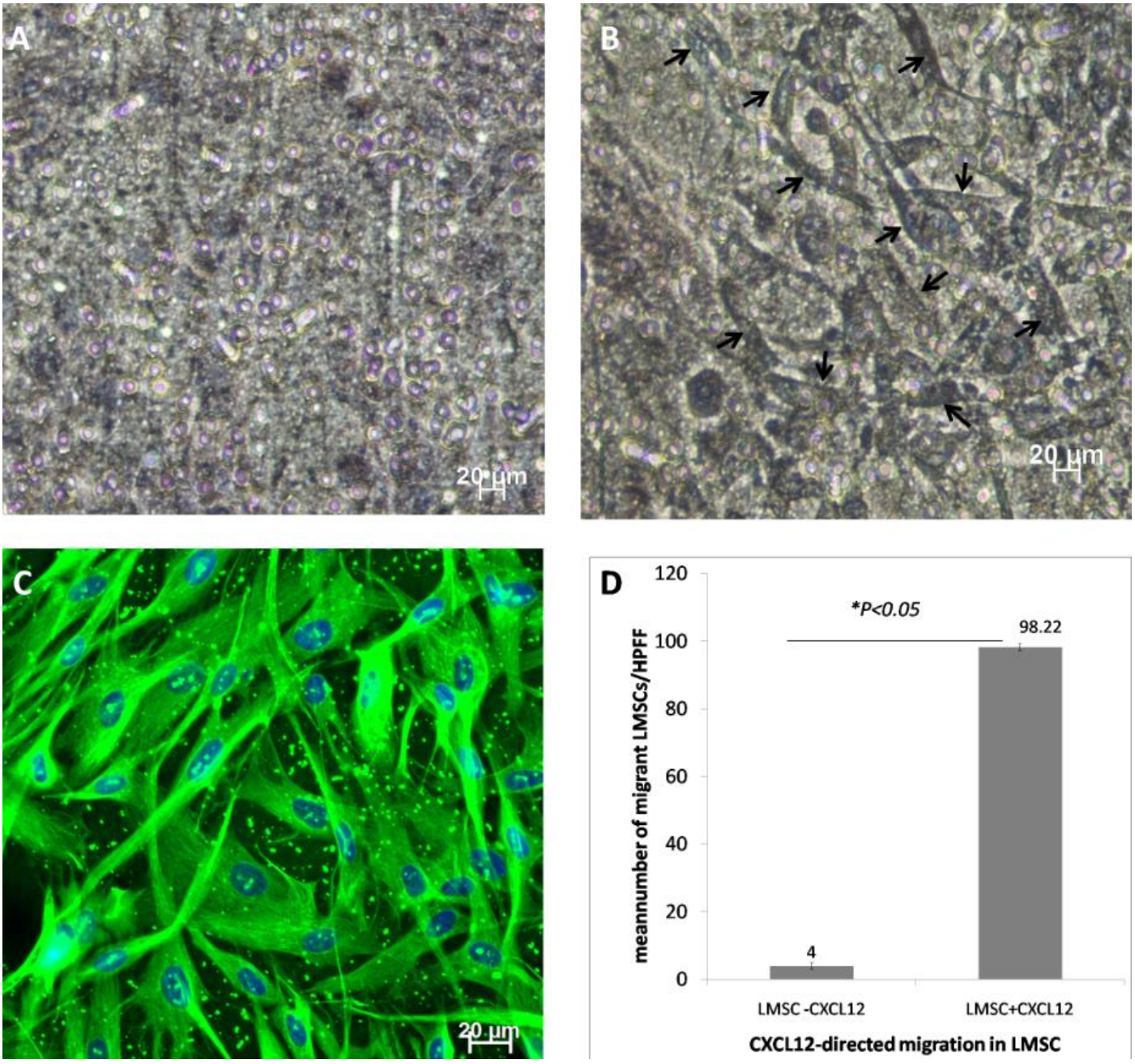

A
20 µm
B
20 µm
C
20 µm
D
*P<0.05
98.22
4
120
100
80
60
40
20
0
meannumber of migrant LMSCs/HPFF
LMSC -CXCL12
LMSC+CXCL12
CXCL12-directed migration in LMSC

Figure 5

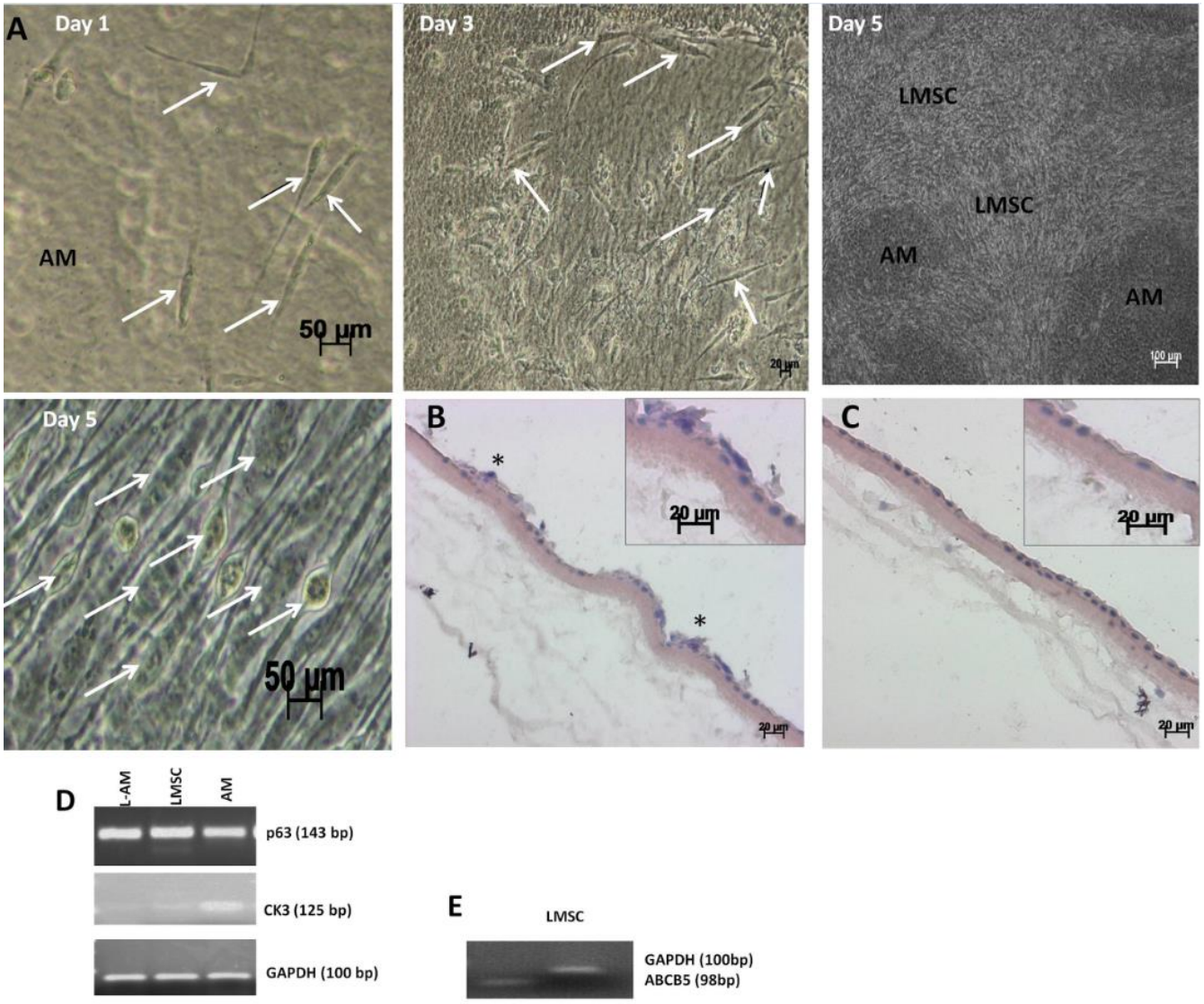
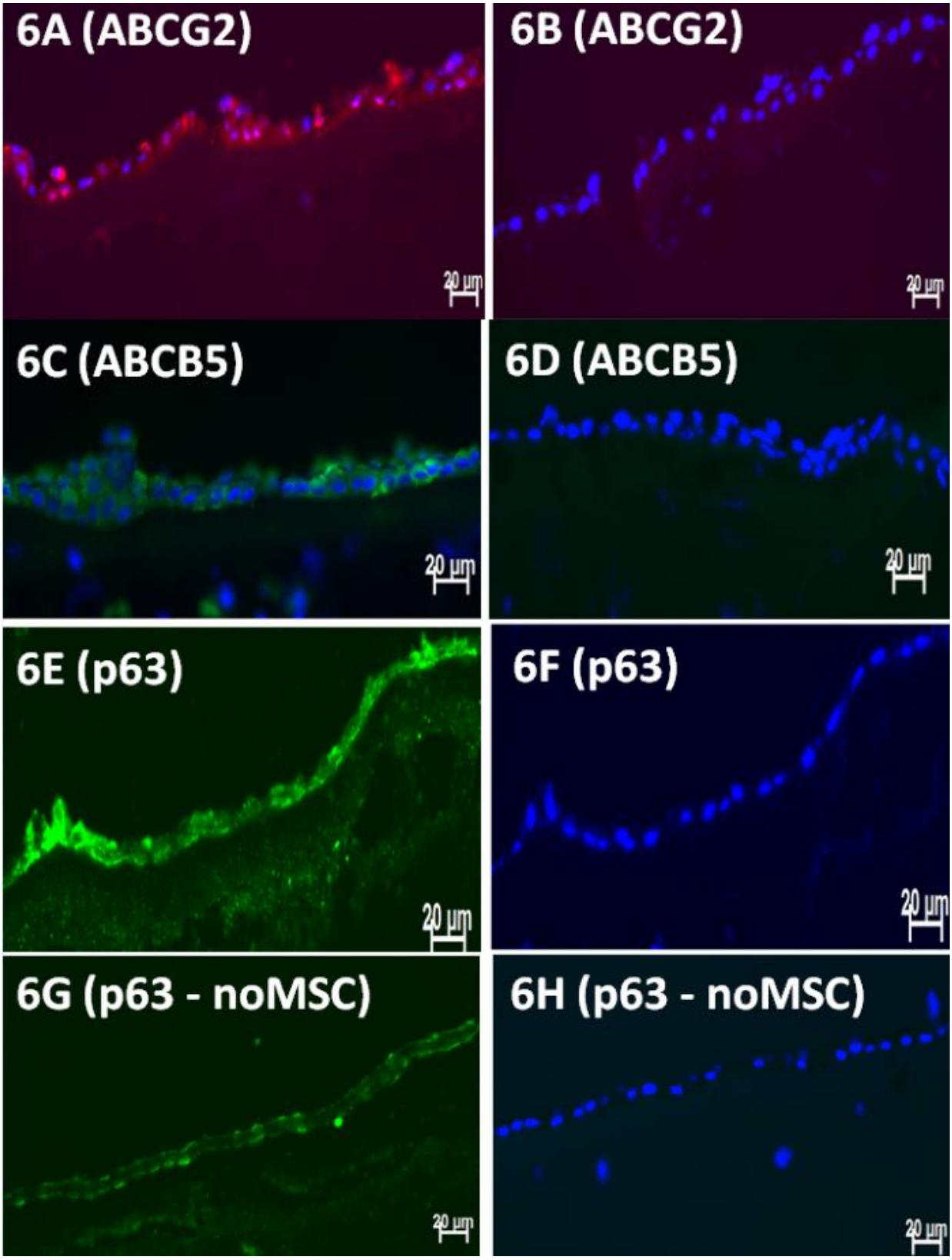
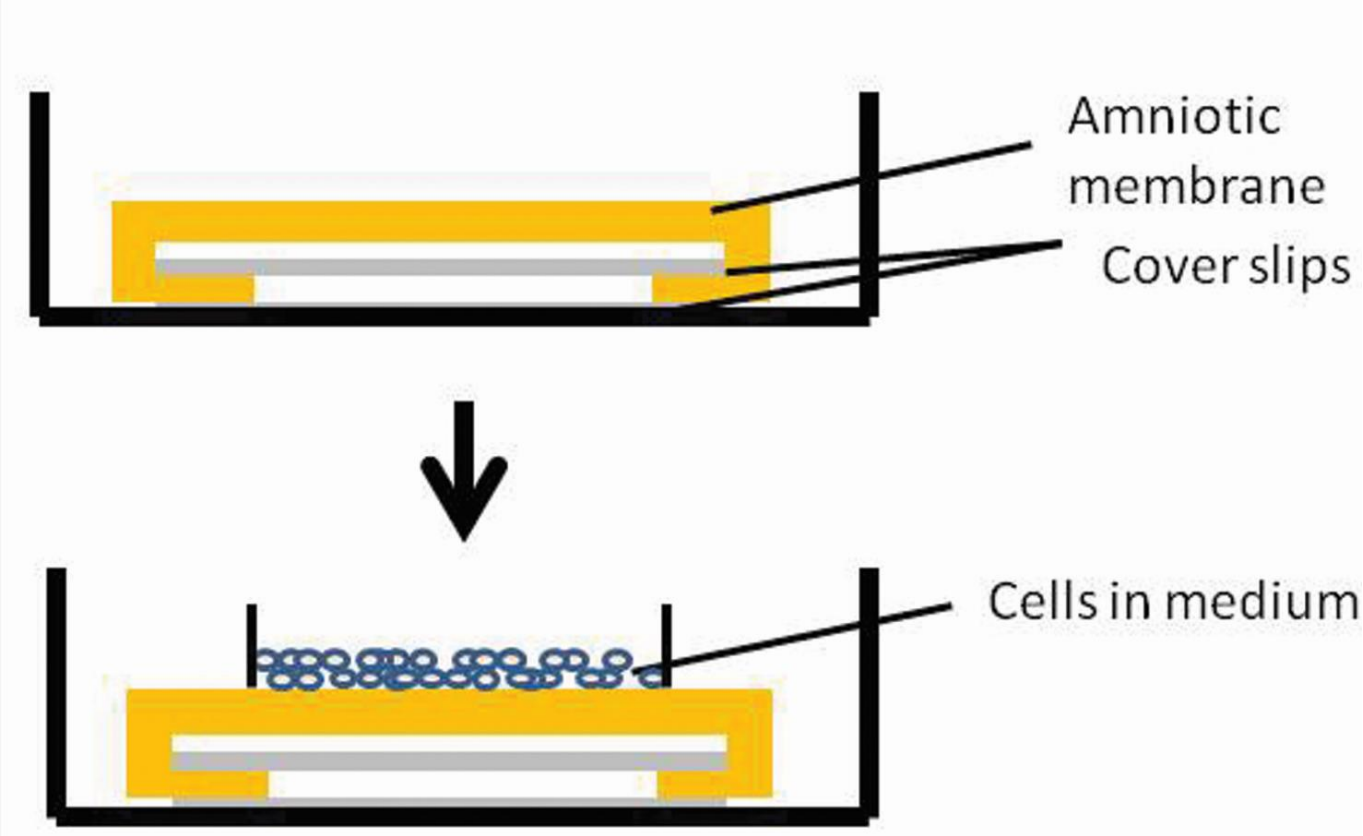

A
Day 1
AM
50 µm
Day 3
Day 5
LMSC
LMSC
AM
AM
Day 5
50 µm
B
20 µm
C
20 µm
D
L-AM
LMSC
AM
p63 (143 bp)
CK3 (125 bp)
GAPDH (100 bp)
E
LMSC
GAPDH (100bp)
ABCB5 (98bp)

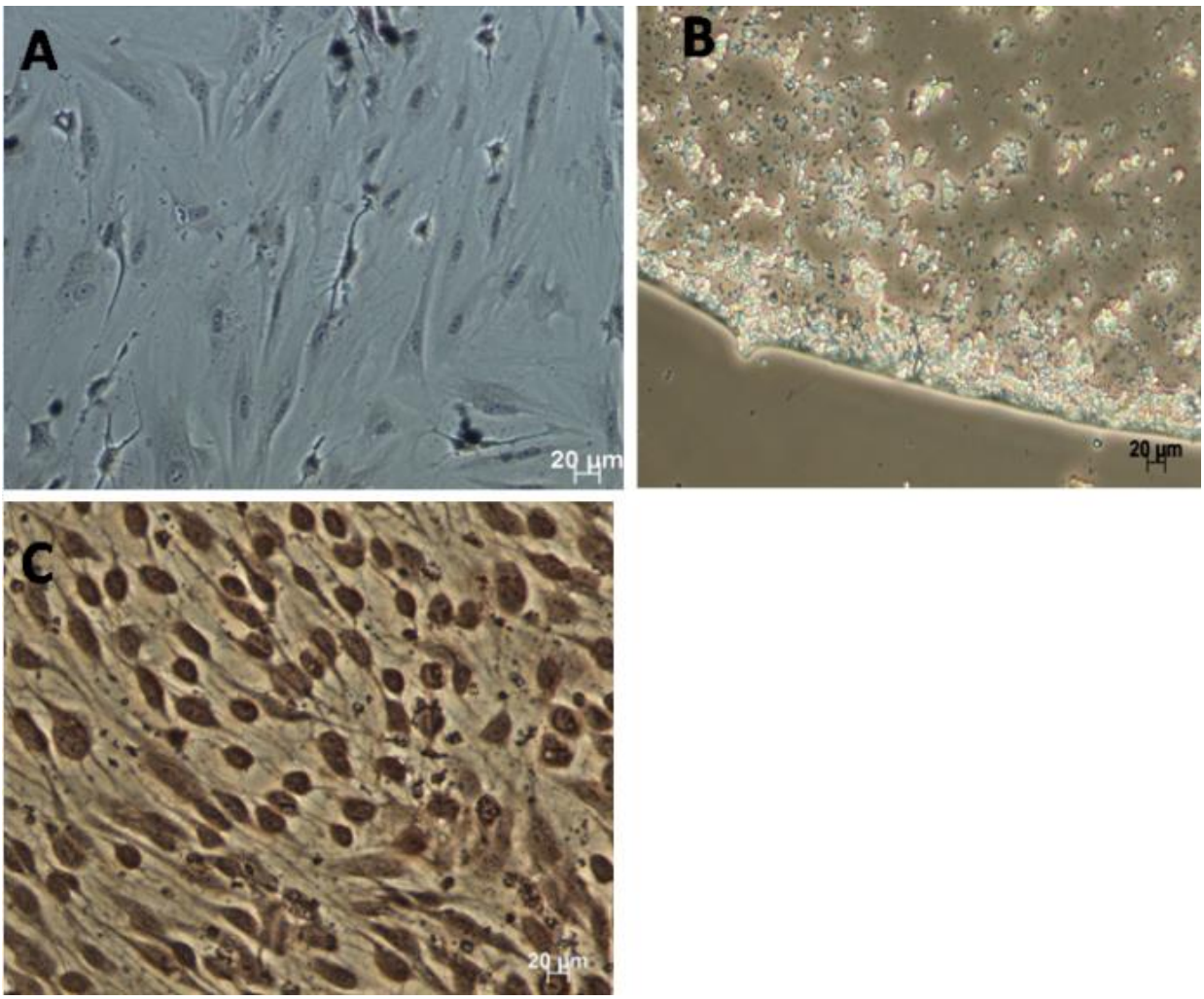
Figure 6


6A (ABCG2)
6B (ABCG2)
6C (ABCB5)
6D (ABCB5)
6E (p63)
6F (p63)
6G (p63 - noMSC)
6H (p63 - noMSC)
20 µm

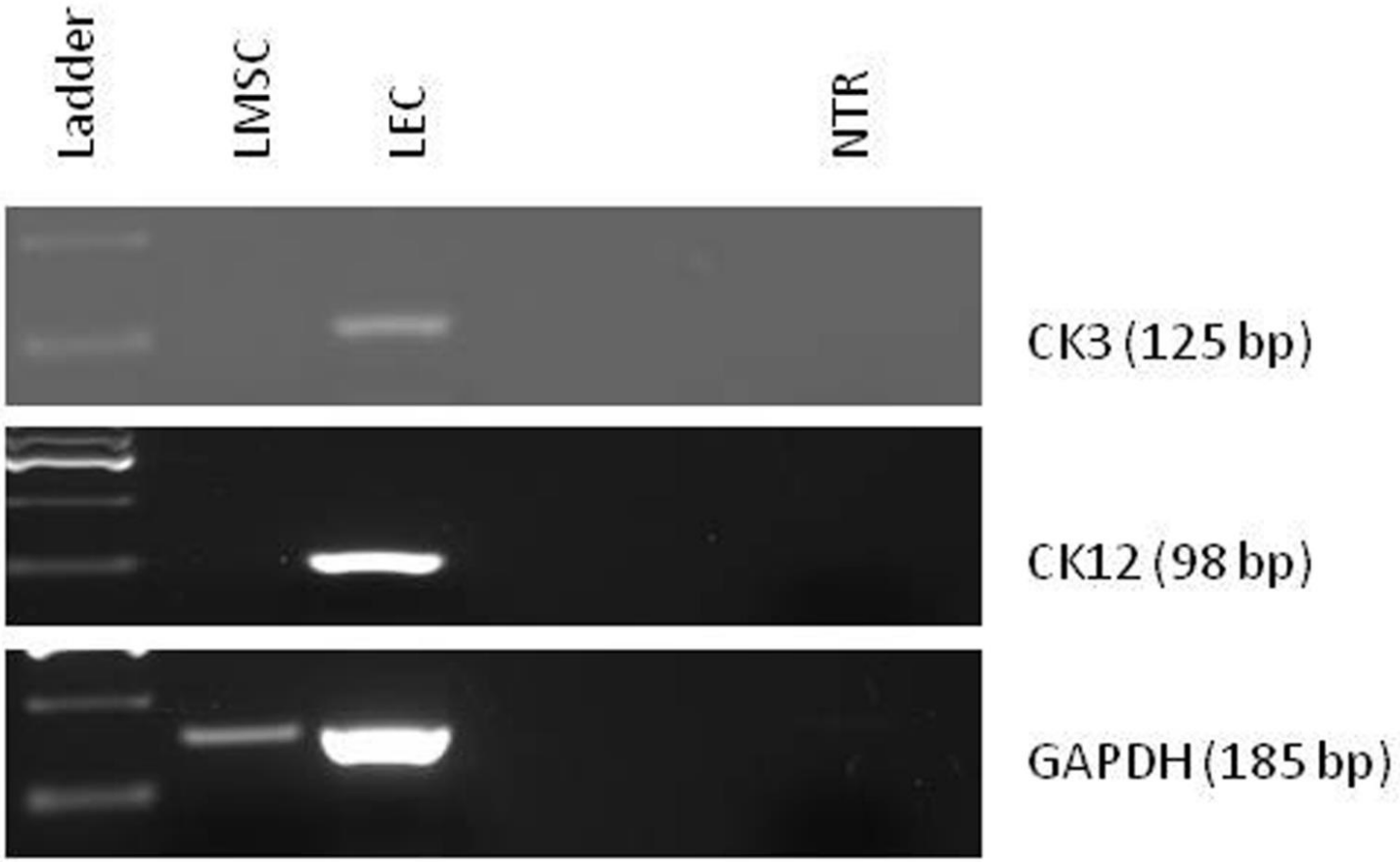
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Table 1

Gene	Primers	Sequences	Product Length (bp)	Annealing temperature (°C)	Amplification Cycles
CHONDROGENIC					
Collagen X			730	55	35
	Forward	CAA GGC ACC ATC TCC AGG AA			
	Reverse	AAA GGG TAT TTG TGG CAG CAT ATT			
Aggrecan			129	55	35
	Forward	TGA GTC CTC AAG CCT CCT GT			
	Reverse	TGG TCT GCA GCA GTT GAT TC			
Sox9			150	55	35
	Forward	GACTTCCGCGACGTGGAC			
	Reverse	GTTGGGCGGCAGGTACTG			
OSTEOGENIC					
Bone sialoprotein-1			161	55	35
	Forward	AAA GTG AGA ACG GGG AAC CT			
	Reverse	GAT GCA AAG CCA GAA TGG AT			
Alkaline phosphatase			187	55	40
	Forward	GGT GAA CCG CAA CTG GTA CT			
	Reverse	CCC ACC TTG GCT GTA GTC AT			
ADIPOGENIC					
SREBP-1			230	55	35
	Forward	GGA GCC ATG GAT TGC ACT TTC			
	Reverse	ATC TTC AAT GGA GTG GGT GCA G			
Adipocyte binding protein-2 (aP2)			130	55	35
	Forward	ATGGGATGGAAAATCAACCA			
	Reverse	GTGGAAGTGACGCCTTTCAT			
Adipophilin			200	55	35
	Forward	CGCTGTCACTGGGGCAAAAGA			
	Reverse	ATCCGACTCCCCAAGACTGTGTTA			
LIMBAL STEM CELL MARKERS					
Δp63α			143	55	35
	Forward	GTGATGATGGTTCACGTTGG			
	Reverse	ACATGACGTCGGGTGTTTTT			
CK3			145	53	35
	Forward	GGATGTGGACAGTGCCTATATG			
	Reverse	AGATAGCTCAGCGTCGTAGAG			
ABCG2			143	55	35
	Forward	GCGTGCTGTGCCCACTCAAA			
	Reverse	AGCATGTGCACGGTGCGTTC			
ABCB5* Hs 02889060_m1		N/A	98	55	35
GAPDH			100	55	35

Forward	ATG GGG AAG GTG AAG GTC G
Reverse	TAA AAG CAG CCC TGG TGA CC

Supplementary Table T1. Oligonucleotides primers and amplification conditions. **Taqman probe [Applied Biosystems]*.

Supplementary Table 2

Markers	Mean	SE
Unlabeled	0.5	0
CD44	78	2.06
CD90	70	6.54
CD105	78	8.9
CD166	74	10.7
CD146	55	10.7
CD19	7.6	2.1
CD45	6.8	2.5
*HLA Class I	100	0
*HLA Class II	0.5	0
IgG	19.4	8.5

Supplementary Table T2. Results of FACS analysis for percentage of stained-positive LMSC immunophenotyping for three biological replicates. *(*) N=1.*