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Citation style: Sikora Bartosz, Skubis-Sikora Aleksandra, Kimsa-Furdzik Małgorzata, Ciszek Wojciech, Kostrzewski Marek, Stojko Jerzy i in. (2019). Adipose-Derived Stem Cells undergo differentiation after co-culture with porcine Limbal Epithelial Stem Cells. "Stem Cell Research" (24 October 2019, Art. Id 101609), doi 10.1016/j.scr.2019.101609

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PII: S1873-5061(19)30239-9
DOI: https://doi.org/10.1016/j.scr.2019.101609
Reference: SCR 101609

To appear in: *Stem Cell Research*

Received date: 13 May 2019
Revised date: 12 September 2019
Accepted date: 2 October 2019

Please cite this article as: Bartosz Sikora , Aleksandra Skubis-Sikora , Ma lgorzata Kimsa-Furdzik , Wojciech Ciszek , Marek Kostrzewski , Jerzy Stojko , Urszula Mazurek , Joanna Gola , Adipose-Derived Stem Cells undergo differentiation after co-culture with porcine Limbal Epithelial Stem Cells, *Stem Cell Research* (2019), doi: https://doi.org/10.1016/j.scr.2019.101609

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Adipose-Derived Stem Cells undergo differentiation after co-culture with porcine Limbal Epithelial Stem Cells

Running title: Transdifferentiation of adipose MSC into corneal epithelium

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Highlights

Average diameter of adipose-derived stem cells (ADSC) is 12.2 µm (N=171)

Average diameter of porcine limbal epithelial stem cells (LESC) is 16.1 µm (N=216)

Porcine limbal stem cells show high proliferation rate after cultivation in DMEM

Human ADSC express *CK3* after co-culture with porcine LESC

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ABSTRACT

Mesenchymal stem cells (MSCs) are objects of interest in regenerative medicine. They are used for various therapies such as for the regeneration of bone, chondrocytes and other tissues. Adipose derived stem cells (ADSCs) *inter alia* are particularly easy to access, they are relatively abundant in fat tissue. ADSCs could be differentiated into many types of cells. To date, it has been proven that ADSCs only differentiate into mesodermal cell lineages. In this study, we present the differentiation of ADSCs into the corneal epithelium. Human ADSCs were placed in a co-culture with porcine limbal epithelial stem cells (LESCs). After 14 days of cultivation, total RNA was extracted for the analysis of the molecular markers (expression of genes of interest). The gene expression was assessed by real-time RT-qPCR. The expression of the surface molecular markers of ADSCs is modulated after co-culturing. We have observed the decrease in *CD73*, *CD90* and *CD105* mRNA expression, while the expression of mRNA coding for *CK3* and *CK12* mRNA was increased in ADSCs co-cultured with porcine limbal epithelial stem cells as compared to the control. We conclude that the co-culture of LESCs and ADSCs changed ADSCs' molecular markers gene expression indicating initiation of differentiation towards limbal cells.

Keywords: adipose derived stem cells; co-culture; cornea; mesenchymal stem cells; transdifferentiation

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INTRODUCTION

Corneal damage can be caused by congenital and inflammatory diseases, chemical injuries, mechanical factors or temperature (Jiang et al., 2010; Joyce et al., 2012; Morrow, 2015). Such injuries, if affect the limbal area, may also result in limbal stem cells deficiency (LSCD). The treatment of LSCD is limited because of the deficit of donor tissues. The corneal limbus contains the limbal epithelial stem cells (LESCs) whose role is to maintain the intact layer of corneal epithelium by replacing the damaged cells (Joyce et al., 2012; Mikhailova et al., 2016; Morrow, 2015).

Adipose tissue is one of the sources of mesenchymal stem cells (MSCs) (Ranera et al., 2011). MSCs have a broad differentiation capacity and a high proliferation rate (Szala et al., 2014). The MSCs express the cell surface markers: CD105, CD73, CD90, CD44 and do not express CD45, CD34 or HLA-DR. MSCs take part in tissue reconstruction (Maleki et al., 2014; Ranera et al., 2011; Suzuki et al., 2015; Szala et al., 2014). Stem cells markers expression varies at some levels of differentiation (Mohsen-Kanson et al., 2013).

MSCs can differentiate into mesodermal cell types. It is known that they can transform into adipocytes, chondrocytes and osteocytes (Skubis et al., 2017). Tremain et al. (2001) revealed the expression of more than two thousand genes in undifferentiated human MSCs including genes that are characteristic for endothelial, epithelial and neuronal cell lineages (Tremain et al., 2001). Providing the appropriate culture conditions should allow MSCs to transdifferentiate into various cells of the three embryonic germ layers (Gu et al., 2009; Tremain et al., 2001).

The induction of stem cells differentiation can be performed by co-culturing (Duan et al., 2013). Indirect method of co-culture is when a barrier, which can be a porous membrane, occurs between cell lines. The membrane should contain pores of which diameter is smaller than the size of cells in co-culture (Duan et al., 2013). We hypothesize that adipose derived

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mesenchymal stem cells (ADSCs) can transdifferentiate into a corneal epithelium in the co-culture with porcine LESCs.

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MATERIALS AND METHODS

Cell lines

Normal human mesenchymal stem cells from adipose tissue (adipose derived stem cells-ADSCs) were standardized cell line obtained from Lonza company (Basel, Switzerland) with catalog number:PT-5006.

LESCs were obtained from 2 mm^2 fragments of the porcine limbus from 6 pigs. The limbus was acquired from the pigs *post mortem*. To obtain the cells, explants were left on six-well culture plates (Nunc, Wiesbaden, Germany) for approximately 15 min under a laminar flow and then flooded with medium.

Medium choice

In spite of MSCs, the culture conditions for porcine LESCs have not been standardized. To achieve the most efficient cells proliferation the WST-1 assay was carried out to determine the best medium for LESCs primary culture and for the experiment. The culture of LESCs was set up in 4 different kind of media: DMEM (Lonza), DMEM: F12 (Lonza), EpiLife™ Medium (Thermo Fisher Scientific) and Epilife+FBS in 96-well plate. The plate was incubated for 96 h at 37°C and 5% CO_2. Next, 10 µl of WST-1 per well was added for 1 hour of incubation and the absorbance was measured (Figure 1 D). Cell proliferation assay were performed in six replicates (N=6).

Additionally, the expression of selected genes (*pABCG2, pTP63*) was examined to assess which medium is appropriate for porcine LESCs in an aspect of phenotype stability (Figure 1 E-F). Experiments were made in six biological and three technical replicates (N=18).

Cell culture conditions

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In relation to the results obtained from viability assay (Figure 1 D) the ADSCs and LESCs were maintained in a DMEM medium (Dulbecco's Modified Eagle Medium, Lonza) that was supplemented with fetal bovine serum (FBS, EuroClone, Italy), amphotericin B (Lonza) and a penicillin-streptomycin (Lonza) at 37˚C in a 5% CO_2 incubator (Direct Heat CO_2; Thermo Fisher Scientific, Waltham, MA). The ADSCs used for the experiment were at passage 4. Identification of ADSCs was done by FACS analysis (Human Mesenchymal Stem Cell Marker Verification Multi-Color Flow Cytometry Kit -R&D Systems, Minneapolis, MN). LESCs' identification was performed with real time RTqPCR by analyzing the expression of *pABCG2*, *pTP63* and *pKRT12*.

Diameter measurement of cells

Both types of cells were measured in order to ensure that they would not migrate through the barrier in the co-culture. Cells were suspended in PBS, 10 µl of suspension was spotted on slide and left to evaporate. Fixed cells were observed (autofluorescence) using a Nikon inverted confocal laser scanning microscope (CLSM) equipped with a 60x objective lens (1.40 NA) and oil immersion. After initial focusing in white light (zoom 600x), the most homogenous regions of cell distribution were selected in the blue light from a PRIOR illumination lamp. A confocal image series were collected after z-axis refocusing. Measurements of cell sizes were carried out after image acquisition by direct line length measurement in three different directions (Figure 1 A).

Co-culture of adipose derived stem cells with limbal epithelial stem cells

The co-culture technique was used to induce the differentiation of the ADSCs. The cells were cultured in 0.4 µm pore size Transwell® system inserts, which ensured cell-cell interactions without any direct contact (Greiner Bio-One, Wemmel, Belgium) at density of 2.2 x 10^4 cells per insert (Figure 1 C). LESCs were seeded into six-well culture plates (Nunc, Wiesbaden,

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Germany). The co-culture was set up one day after the cells had migrated out from the explants (Figure 1 B). The ADSCs and LESCs controls were cultured in monolayers. LESCs' control cells were seeded in plates and ADSCs were culture in inserts separately in order to keep the same conditions. The co-cultures were maintained for 14 days in DMEM supplemented with 10% FBS and 1% of antibiotics.

The experiment was performed on cells in the logarithmic phase of growth under conditions of ≥ 98% viability as assessed by trypan blue exclusion. The cells were assessed using an Olympus IX81 microscope (Olympus, Shinjuku, Tokyo, Japan) and a DP70 camera (Olympus) was used for the photographic documentation. The culture medium was changed at intervals of 3 days.

After 14 days of co-culture, the cells were pelleted and frozen at -70˚C for 24 hours until the extraction of nucleic acids.

Ribonucleic acid extraction from tissue specimens

Total RNA was extracted from the cells using a TRIzol reagent (Invitrogen, Carlsbad, CA). Nucleic acid concentration was determined using a GeneQuant II RNA/DNA spectrophotometer (Pharmacia Biotech, Cambridge, UK).

Quantitative real-time polymerase chain reaction assay

Detection of the expressions of human: *CD73*, *CD90, CD105, CK3, CK12, CK15, CK19, GJA1,* and porcine *pTP63* and *pABCG2,* β-actin (*ACTB*), *GAPDH* mRNAs was carried out using a real-time RT-qPCR with SYBR Green chemistry (SYBR Green Quantitect RT-PCR Kit, Qiagen, Valencia, CA, USA) and an Opticon™ DNA Engine Continuous Fluorescence detector (MJ Research) as previously described (Strzalka et al., 2008). *ACTB* and *GAPDH* were included as the endogenous positive controls of the amplification and the integrity of the extracts. The oligonucleotide primers that are specific for *CD73*, *CD90*, *CD105, pTP63* and

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pABCG2 were designed based on the reference sequences (GenBank accession No. NM_002526.3, NM_006288.3, NM_000118.3, XM_003483293.2 and NM_214010.1, respectively) using Primer ExpressTM Version 2.0 software (PE Applied Biosystems, Foster City, CA) (Table 2). The primers that are specific for *CK3*, *CK12, CK15, CK19* and *GJA1* were obtained commercially (Sigma-Aldrich, Germany) (Table 2).

Statistical analyses

Statistical analyses were performed using Statistica 12.0 software (StatSoft, Tulsa, Oklahoma, USA) and the level of significance was set at $p<0,05$. Values were expressed as the median (Me) with the 25th and 75th quartiles and the minimum and maximum. The Mann-Whitney U test was applied to assess any differences in the expression of genes between two groups and Kruskal Wallis was performed for multiple comparison.

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RESULTS

Identification of cells lines

FACS analysis showed the expression of cell surface CD73, CD90, and CD105-specific markers of MSCs to assess the phenotype of the examined cells.

LESCs were identified by RTqPCR analysis which showed the expression of *pABCG2* (Me=8892 copies/µg RNA), *pTP63* (Me=62447 copies/µg RNA) and *pKRT12* (Me=938693 copies/µg RNA).

Medium choice

The assessment of the influence of media (DMEM, DMEM: F12, Epilife, Epilife + FBS) on LESCs were analyzed based on the viability by the analysis of the mitochondrial activity of cells and analysis of gene expression *pABCG2* and *pTP63* with RTqPCR.

WST-1 viability test has shown that the best medium for LESCs cultivation from all that were tested was DMEM (Kruskal-Wallis test, $p<0.05$) (Figure 1 D). We observed a statistically significant increase in viability of cells after culture in DMEM medium as compared to medium DMEM: F12 ($p=0.024$), Epilife ($p<0.0001$) and also Epilife+FBS ($p=0.033$). Moreover, we proved that cell culture in DMEM: F12 showed higher viability compared to cell culture in Epilife medium (p =0,033. It was noticed also statistically significant lower mitochondrial activity in cell culture in Epilife medium compared to Epilife with FBS ($p=0.024$).

These results were also confirmed by RT-qPCR (Kruskal-Wallis test, $p<0.05$). It was noticed that *pABCG2* were significantly lower in LESCs after culture in Epilife as compared to medium DMEM ($p=0.0016$), DMEM: F12 ($p=0.0203$) and also Epilife + FBS ($p=0.0003$) (Figure 1 B). We noticed that selected media do not make a difference in the induction of s*TP63* expression in LESCs (Figure 1 E).

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Diameter measurement

The both cell lines were analyzed by confocal microscopy to measure their diameter to exclude the possibility of cell migration through the pores of the inserts used for co-culture. The diameter of cells prevents them from migrating through the pores of the 0,4 µm diameter. Group size consisted of total number of measurements, average, median, minimum and maximum values, lower and upper quartiles and standard deviation are presented in Table 1.

The mRNA level of *CD73*, *CD90* and *CD105* in the ADSCs after co-culture with the LESCs

CD73, *CD90* and *CD105* mRNAs were detected in all of the tested samples that were obtained from both the control and the ADSCs after co-culture. The level of *CD73* mRNA copies/µg of total RNA was 1,67-fold lower (Me=159005,7) compared to the control cells (Me=265645) and a statistical significance was found (p=0,0137, Mann-Whitney U test) (Figure 2). In the case of *CD90* mRNA, there was 3,32-fold decrease in the ADSCs after co-culture (Me=70853,125) compared to the control cells (Me=235035,85) and a statistical significance was found (p=0,00001, Mann-Whitney U test) (Figure 2). The mRNA level of *CD105* was 1,92-fold lower in the ADCSs after the co-culture (Me=37575) than in the control samples (Me=72043,015) and this difference was also statistically significant (p=0,002, Mann-Whitney U test). (Figure 2). The above results indicate that co-culture of ADSCs with LESCs may have initiated differentiation.

The mRNA level of *CK3*, *KRT12*, *CK15*, *CK19*, *GJA1* in ADSCs after co-culture with LESCs

Furthermore, there was an almost 23,48-fold statistically significant increase of the *CK3* mRNA copies/µg of total RNA in the ADSCs after the co-culture (Me=188,2) compared to

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the control cells (Me=8,012) (p=0,016, Mann-Whitney U test) (Figure 2). Moreover, there was no significantly different expression of *CK12* mRNA between the ADSCs after the co-culture (Me=2,352) and the control cells (Me=0,217) (p=0,08, Mann-Whitney U test) (Figure 2). The expression of *CK15* was 3-fold decreased but not significantly different after co-culture (Me=0,55) compared to the control cells (Me=1,66) (p=0,108, Mann-Whitney U test) (Figure 2) and the 20,53-fold statistically significant decrease of *CK19* was noticed in ADSCs after the co-culture (Me=1,088) compared to the control cells (Me=22,375) (p=0,00008, Mann-Whitney U test) (Figure 2). The mRNA level of *GJA1* was 1,802-fold lower after co-culture (Me=521,75) than in the control samples (Me=940,2) and the difference was not statistically significant (p=0,148, Mann-Whitney U test) (Figure 2). The above results indicate that co-culture of ADSCs with LESCs have activated in ADSCs the expression of genes typical for LESCs.

The mRNA level of *TP63* and *ABCG2* in LESCs after co-culture with ADSCs

In the case of *TP63* mRNA, there was a significantly different expression between the LESCs after the co-culture (Me=19450) and the control cells (Me=382,05) (p=0,0077, Mann-Whitney U test) (Figure 2). However, number of *ABCG2* copies was 7,7-fold lower in LESCs after co-culture (Me=651,5) compared to the control samples (Me=5015,9) (p=0,0001, Mann-Whitney U test) (Figure 2).

Box and whisker plots in Figure 2 present the medians ±quartiles as well as the extreme values of the copy numbers per 1 μg of total RNA; *p<0,05, Mann-Whitney U test.

DISCUSSION

Human MSCs undergo differentiation through its interaction with the multiple compounds that are present in the extracellular environment that surrounds a cell (Bao et al., 2016; Fong et al., 2016; Rajangam et al., 2016). One of the differentiating methods is based on the

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cultivation of cells from different tissues in order to alter their phenotype (Cieślar-Pobuda et al., 2016; Kubosch et al., 2016; Szaraz et al., 2016). It has been proven that the cultivation of hBMSCs (human Bone-marrow derived Mesenchymal Stem Cells) in a co-culture with the RPE (Retinal Pigment Epithelium) cells induces the transdifferentiation of hBMSCs into the RPE (Mathivanan et al., 2015). Trials of the differentiation of hBMSCs that had been injected into the corneal epithelium of rabbits were successfully completed *in vivo* and *ex vivo* (Gu et al., 2009).

Important in ADSCs study is to determine the expression of the cell surface markers at various steps of its differentiation (Mohsen-Kanson et al., 2013). This help to determine the molecular changes that occur during this process and also provides information about the steps in the maturation of ADSCs. Undifferentiated cells express CD73 and CD105 markers. However, the CD105 expression was inhibited during differentiation (Mohsen-Kanson et al., 2013). Lee et al. (2015) documented that the CD90 and CD105 expression decreased during sequential passages (Lee et al., 2015). A similar effect was observed in porcine amniotic fluid-derived multipotent stem cells. The expression level of CD90 decreased during 16 passages (Chen et al., 2011). On the other hand, it was revealed that the expression of CD73 and CD90 in stromal vascular fraction (SVF) cells were initially low and increased in the next passages. Authors showed that at passage 4, the CD73 and CD90 markers were permanently present in the ADSCs (Mitchell et al., 2006). Culture conditions also have an enormous influence on cell differentiation and the expression of characteristic markers. Ahearne et al. (2013) showed that culture media have an impact on the differentiation pathways (Ahearne et al., 2014). It has been reported that the propagation condition alone, especially the supplementation of media with antibiotics may affect the differentiation pattern of ADSC (Skubis et al., 2017). Therefore, it is necessary to optimize the culture conditions for the proper orientation of the differentiation of ADSCs. Few reports describe differentiation ability

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of MSCs into corneal epithelial cells (Du et al., 2010; Gu et al., 2009; Jiang et al., 2010; Ma et al., 2006; Nieto-Miguel et al., 2013).

Increase in the expression of *CD73*, *CD90* and *CD105* markers in the stem cells from extraocular human adipose tissues in various conditions was observed using the fluorescence intensity technique (Nieto-Miguel et al., 2013). Unfortunately, the results of many studies are ambiguous. Ho et al. (2011) showed that MSCs that differentiated into corneal epithelial cells had a lower expression of CD105 (Ho et al., 2011). Similarly, in our study a statistically significant decrease of CD105 mRNA level was observed.

ABCG2 is a proposed LESCs marker (Chen et al., 2004). ABCG2 protects LESCs from oxidative stress by its ability to transport the small molecule proteins that are associated with proliferation, differentiation and apoptosis (Yoon, 2014). There is an agreement that the *ABCG2* gene is a molecular marker of the stemness (Khan et al., 2016; Kramerov et al., 2015; Rajabi Fomeshi et al., 2016, p. 133; Sándor et al., 2016; Sun et al., 2017). We noticed a downregulation of the *ABCG2* expression in LESCs after the co-culture. Our findings suggest that LESCs began to lose their stemness. We assume that ADSCs may also have an influence on the differentiation of LESCs.

It has been suggested that P63 is responsible for the maintenance of the stem cell population (Pellegrini et al., 2013; Rama et al., 2010). A high expression of this gene has been observed in cells of basal layer (Chen et al., 2004). We noticed its expression upregulated after the co-culture.

Cytokeratins are the proteins that are specially expressed in epithelia. Those putative LESCs markers include cytokeratins encoded by *CK15*, *CK19* which are both considered as positive markers (Mort et al., 2012). The presence of *CK15* and *CK19* was confirmed at limbal basal layer but not at the cornea (Păunescu et al., 2007; Saghizadeh et al., 2011). The expression of these genes should be downregulated in cells that had differentiated into the

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corneal epithelium. Our results confirmed that the expression of *CK19* has been downregulated, which suggests that the process of differentiation has occurred. However, the change of *CK15* expression was not significant. We assume that the elongation of the time of co-culture would have a high impact on the change of its expression and probably would be significant. Furthermore, corneal epithelial cells express *CK3* and *CK12*. The presence of these markers has not been proven in epithelial cells from limbal region (Mikhailova et al., 2015; Sareen et al., 2014; Szabó et al., 2015). Our results showed that a co-culture of porcine LESCs with human ADSCs change the expression of ADSCs markers. Moreover, our study showed that co-culturing ADSCs with porcine LESCs initiated the expression of *CK3*, which is characteristic for corneal epithelial cells.

Connexins play an important role in cell proliferation, differentiation, and regeneration. Connexin 43 is a protein encoded by the *GJA1* gene. Cx43 was found in many epithelial tissues, including cornea (Chen et al., 2006). Its expression was noticed in corneal and limbal suprabasal cells but it was not in basal cells of the limbal epithelium. It was also noticed that Cx43 is not present at small, less-differentiated proliferating cells in limbal primary culture (Chen et al., 2006; Grueterich et al., 2002). It indicates that Cx43 could be a positive marker of corneal epithelial cells but also it could be a negative marker of limbal stem cells (Chen et al., 2006). We have not noticed the significant change in the expression of *GJA1* in the ADSCs after co-culture comparing to the control cells, but the number of *GJA1* mRNA copies was high, both at the examined and the control samples.

CONCLUSION

We conclude that limbal epithelial stem cells act on the gene expression of mesenchymal stem cells and probably might be used as a differentiation factor. We proved that paracrine factors secreted by porcine LESCs are able to impact on the expression of ADSCs markers and have

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an ability to induce the expression of genes characteristic for corneal epithelial cells. This suggest that porcine LESCs are a potential differentiating factor that may be used for targeted ADSCs differentiation. However additional studies are necessary to prove these results at the protein level.

Disclosure Statement

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

Authors kindly acknowledge the support from Medical University of Silesia in Katowice (Upper Silesia, Poland) grant no. KNW-1-062/N/8/I.

Ethical guidelines

The study was conducted on animal tissues obtained from abattoir. The ethical committee approval is not required.

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FIGURE LEGENDS

Figure 1. A – method of cell diameter measurement; **B-C –** morphologic presentation of LESCs, and ADSCs**,** B - primary culture of porcine LESCs, C – human ADSCs, passage 4; **D** – the absorbance of formazan after wst1 reduction, *p<0,05, ***p<0,001 (Kruskal Wallis); **E-F** – Level of *TP63*, *ABCG2* mRNA copies in LESCs after cultivation in examined media, *p<0,05 vs III (Kruskal Wallis); **G** – Level of *CD73, CD90, CD105, CK3, CK12, CK15, CK19, GJA1* mRNA copies/µg of total RNA in ADSCs after 14 days of co-culture compared to the C (control cells)**,** *p<0,05 (Mann-Whitney U test)

Figure 2. Level of TP63, ABCG2 in LESCs after 14 days of co-culture compared to the C (control cells), *p<0,05 (Mann-Whitney U test)

Table 1. Changes in shape induced in ADSC upon co-culture with LESC. Descriptive statistics of cell measurements [µm]

	N	Average diameter	Median	Minimum	Maximum	lower quartile	upper quartile	standard deviation
ADSC	171	12,198	11,670	7,130	23,770	9,860	13,630	3,105
LESC	216	16,123	15,155	8,250	30,900	13,295	18,690	4,325

Diameter measurement of examined cells presented in microns. ADSC - Adipose-tissue Derived Stem Cells, LESC – Limbal Epithelial Stem Cells, N – group size.

Table 2. Characteristics of the primers that were used for real-time RT-qPCR

Gene	species	Sequence of primers	Length of amplicons [bp]	Tm [˚C]
CD73	*human*	Forward: 5’-GGGCACTATCTGGTTCACCGTGTA-3’ Reverse: 5’-ATTGGAAATTTGGCCTCTTTGAGGA-3’	153	82
CD90	*human*	Forward: 5’-CCATTCCCCACCCATCTCCTC-3’ Reverse: 5’-GGACATGAAATCCGTGGCCTG-3’	151	83
CD105	*human*	Forward: 5’-TGCGTCCCAAGACCGGGTC-3’ Reverse: 5’-CCCCGATGAGGAAGGCACCA-3’	153	83
ACTB	*human*	Forward: 5’-TCACCCACACTGTGCCCATCTACGA-3’ Reverse: 5’-CAGCGGAACCGCTCATTGCCAATGG-3’	295	85
CK3	*human*	Forward: 5’-ATTTGTGACTCTGAAGAAGG-3’ Reverse: 5’-TCCTTAAGAAGTCGATCTCATC-3’	101	83
CK12	*human*	Forward: 5’-TCTAAAGACCCAACCAAAAC-3’ Reverse: 5’-CAGCATGTTACTCTCTGAAAGG-3’	168	83
CK15	*human*	Forward: 5’-CCCTCGGTCTTTTATTTCAG-3’ Reverse: 5’-ACACTGTACAAATGAGCTTG-3’	117	82
CK19	*human*	Forward: 5’-AACCATGAGGAGGAAATCAG-3’	129	78

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		Reverse: 5'-CATGACCTCATATTGGCTTC-3'		
GJA1	*human*	Forward: 5'-ATCCTCCAAGGAGTTCAATC-3' Reverse: 5'-AATGAAAAGTACTGACAGCC-3'	163	80
P63	*pig*	Forward: 5'-GGAATGAACCGCCGTCCAA-3' Reverse: 5'-CGAGACTTGCTGCTTCCTGATGC-3'	153	83
ABCG2	*pig*	Forward: 5'-TGGGTCTGGATAAAGTGGCCG-3' Reverse: 5'-GGAGTCTAAGCCAGTCGTGGGC-3'	152	80
GAPDH	*pig*	Forward: 5'-TGTCGCCATCAATGACCCC-3' Reverse: 5'-TGACAAGCTTCCCATTCTC-3'	116	80

Table presents the sequence of forward and reverse primers used to amplify in real-time RT-qPCR. The melting temperature (Tm) and amplified fragments size (bp) are also reported.

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