Human Oral Mucosal Fibroblasts from Limbal Stem Cell Deficient Patients as an Autologous Feeder Layer for Epithelial Cell Culture

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Abstract

Purpose: To investigate if human oral mucosal fibroblasts (HOMF) from patients with limbal stem cell deficiency (LSCD) can be used as an autologous feeder layer to support the culture of epithelial cells for potential clinical use.

Methods: HOMF were isolated from oral mucosal biopsies obtained from the following groups of patients with LSCD: Aniridia, Mucous Membrane Pemphigoid, Stevens Johnson Syndrome, and Ectodermal Dysplasia. The ability of these cells to support the culture of human limbal epithelial cells (HLE) was compared to that of HOMF from non-LSCD donors and 3T3s commonly used to culture epithelial cells for use in the clinic to treat LSCD.

Results: HOMF were successfully obtained by explant culture for 3/3 Aniridia patients, 3/3 Mucous Membrane Pemphigoid patients, 1/3 Stevens Johnson Syndrome patients, and 1/1 Ectodermal Dysplasia patients. All HOMF cultured from these LSCD groups supported the expansion of HLE with epithelial culture times and total colony forming efficiency comparable to those achieved on HOMF isolated from donors without LSCD. PCR showed that all HLE cultured on LSCD donor HOMF expressed p63α, CK15, PAX6, CK12, and MUC16 as did HLE cultured on the control non-LSCD donor HOMF and 3T3s. Western blotting detected CK15 and MUC16 protein expression in all groups.

Conclusion: HOMF from patients with LSCD can be successfully used to support the expansion of epithelial cells. These cells may therefore be useful as autologous feeder fibroblasts for the expansion of epithelial cells for use in the clinic to treat LSCD.

Keywords

oral mucosal fibroblasts, limbal epithelial stem cell deficiency, cornea, limbal epithelial cells

Introduction

Limbal stem cell deficiency (LSCD) occurs when the population of stem cells responsible for ensuring a healthy corneal epithelium (limbal epithelial stem cells, LESC) are lost, depleted, or unable to properly function due to injury or disease. As a result of LSCD the cornea loses its transparency as it becomes epithelialised with opaque conjunctival epithelium. This affects vision and can lead to blindness. In addition to conjunctivalisation of the cornea, other characteristics of LSCD include vascularisation, inflammation, discomfort and pain [1]. Injury such as chemical burn, hereditary disease such as Aniridia, and acquired diseases such as Stevens Johnson Syndrome and Mucous Membrane Pemphigoid can result in LSCD.

Cultured epithelial stem cell therapies utilising human limbal epithelial cells (HLE) or human oral mucosal epithelial cells (HOME) have been used to successfully treat LSCD. These epithelial cells are typically expanded on a murine 3T3 feeder layer prior to transplantation. Although this culture method is still in use, the search for a 3T3-free culture method (either feeder-free or with human-derived feeders) is currently underway to ensure the safest possible therapy for the patient. Ideally cell therapies should be produced without the use of any animal-derived products in order to meet regulatory requirements. Although few if any 3T3s are likely to be transferred to the patient with the cultured epithelial cells, and no harmful effect for the patient has been shown with the clinical use of 3T3s during the culture process [2], the only way to eliminate any possible risk associated with these cells is to remove them from the culture process.

A number of human-derived cell types have been investigated as 3T3 alternatives to support the expansion of HLE including dermal fibroblasts [3, 4], oral mucosal fibroblasts [5-7], limbal fibroblasts [5], limbal stromal fibroblasts [3], the MRC-5 cell line [3, 8], the human fibroblast cell lines Hs68 and CCD1112Sk [9], amniotic epithelial cells [10], mesenchymal stem cells [4, 11], limbal mesenchymal cells [12, 13], human processed lipoaspirate cells [14], human Tenon's fibroblasts [15] and umbilical cord derived human unrestricted somatic stem cells [16]. Other human-derived cell types which have also been investigated for their ability to support HLE culture include limbal niche cells [17], limbal stromal cells [17] and corneal stromal stem cells [18]. Although several of these have shown promise as being suitable 3T3 alternatives for the culture of autologous or allogeneic HLE, their use clinically in the production of limbal epithelial cells for the the treatment of LSCD has not been reported. Some of these human-derived feeder cells (such as oral mucosal fibroblasts investigated here) have the potential to

be autologous which offers an advantage to the patient that immunosuppression is not required to prevent rejection when used to support autologous epithelial cell expansion. HOMF can be easily isolated from a small biopsy taken from inside the patient's mouth from the buccal oral mucosa. This is an easily accessible location, and heals quickly with minimal scarring following biopsy excision. We have previously demonstrated that HOMF from non-LSCD donors can be used as a feeder layer to sucessfully expand HLE and HOME [5]. HOMF were shown to be comparable to 3T3s for epithelial expansion for a number of parameters including the number of epithelial population doublings achieved in culture [5]. HOMF may therefore have potential as an alternative and autologous source of feeder cells thus eliminating murine 3T3s from the culture system when expanding epithelial cells for cultured limbal epithelial transplantation (CLET) or cultivated oral mucosal epithelial sheet transplantation (COMET) to treat LSCD. For LSCD caused as a result of injury to the eye such as acid or alkali burn, patients will have normal HOMF. However, since certain ocular surface diseases are known to affect other mucous membranes e.g. Stevens Johnson Syndrome and Mucous Membrane Pemphigoid it is important to assess whether HOMF from patients with LSCD as a result of disease are suitable for use as feeder layers for epithelial cell expansion. Here we isolated HOMF from the following LSCD patient groups: Aniridia, Mucous Membrane Pemphigoid, Stevens Johnson Syndrome, and Ectodermal Dysplasia and compared their ability to support HLE expansion with that of HOMF from non-LSCD donors and 3T3s commonly used for the culture of HLE/HOME for clinical application. HOMF isolated from donors with LSCD will be referred to as "LSCD HOMF", and those isolated from donors without LSCD referred to as "normal HOMF".

Materials and Methods

Isolation and culture of epithelial cells

HLE and HOME were isolated and cultured as previously described [5]. For primary expansion HLE/HOME were cultured in Corneal Epithelial Culture Medium (CECM) on Mitomycin C (MMC) growth arrested 3T3s (one T25 flask per donor). Following primary expansion HLE/HOME were cryopreserved in 70% CECM (without EGF) plus 20% fetal bovine serum (FBS) and 10% DMSO until required for experiments involving further expansion on growth arrested feeder layers.

HLE were isolated from cadaveric limbal rims (obtained from the Moorfields Lions Eye Bank (London, UK)) using Dispase II. Each rim was incubated in 1.2 U/ml Dispase II (Roche Diagnostics, Basel, Switzerland) for either 1–2 hours at 37°C or overnight at 4°C. Following removal from Dispase II, the epithelial cells were scraped from the limbal region of the tissue. Following initial expansion, HLE were split equally onto the following MMC growth arrrested feeder fibroblasts: 3T3, Aniridia HOMF, Stevens Johnson Syndrome HOMF, Mucous Membrane Pemphigoid HOMF, Ectodermal Dysplasia HOMF, and normal HOMF (one T25 flask for each condition). Six experiments were set up each with HLE from a different donor. Where possible, different donor fibroblasts were used for each experiment i.e Aniridia and Mucous Membrane Pemphigoid. Since only one set of Ectodermal Dysplasia and Stevens Johnson Syndrome HOMF were obtained, these fibroblasts were used for all the HLE experiments. HLE were harvested prior to confluency at passage 1, RNA (n=3) or protein (n=3) extracted, and colony forming efficiency (CFE) assays set up (n=3).

Oral mucosal biopsies were taken from healthy volunteers and patients with LSCD as previously described [5]. All tissue had appropriate research consent and ethics approval. Oral

mucosal biopsies were obtained from three patients with Aniridia, three patients with Stevens Johnson Syndrome, three patients with Mucous Membrane Pemphigoid, one patient with Ectodermal Dysplasia, and three healthy volunteers with no ocular surface disease. HOME were isolated from the oral mucosal biopsies using Dispase II and trypsin:EDTA. Following incubation in 1.2U/ml Dispase II for either 1 hour at 37˚C or overnight at 4˚C, biopsies were incubated in 0.25% trypsin:EDTA for 30 mins at room temperature Trypsin was quenched with CECM and the tissue gently scraped using forceps to remove the epithelial cells. For passage 1, LSCD HOME were seeded at $1.5x10^4$ cells/cm^2 onto MMC growth arrested 3T3s. Protein and RNA were isolated from sub-confluent passage 1 cells and CFE assays also set up using these cells.

Fibroblast culture and growth arrest

Following epithelial cell isolation from the oral mucosal tissue using Dispase II and trypsin:EDTA, the remaining stroma was used for explant culture of HOMF. HOMF were growth arrested at passage number 4 for use as feeder fibroblasts for the culture of HLE. HOMF and 3T3 J2s were cultured and MMC growth arrested as previously described [5]. In brief, for growth arrest HOMF were treated with 10μg/ml MMC in HOMF culture media for 3 hours, and 3T3s treated with 4μg/ml MMC in 3T3 culture media for 2 hours at 37˚C. For use as a feeder layer, MMC growth arrested 3T3s were seeded at $2.7x10^4$ cells/cm^2, and MMC treated HOMF at $1.2x10^4$ cells/cm^2.

Differential trypsinisation

Differential trypsinisation was used to separately remove fibroblasts and epithelial cells from co-cultures as previously described [19]. In brief, feeder fibroblasts (HOMF or 3T3) were firstly

removed using 1x trypsin:EDTA followed by removal of the epithelial cells with 10x trypsin:EDTA. Prior to epithelial harvest, cultures were observed under a microscope to ensure fibroblast removal.

Colony forming efficiency (CFE)

To assess the CFE of HLE which had been expanded on LSCD HOMF (as well as normal HOMF and 3T3 controls), 100-500 HLE (passage 1) were seeded onto MMC growth arrested 3T3s in a 6 well plate ($2x10^5$ 3T3s/well) and cultured for 10-11 days in CECM. Ice-cold methanol was used to fix the cells which were then stained with 2% rhodamine B (Sigma-Aldrich). Image J software was used to count the total number of colonies per well, and the total CFE calculated as follows: CFE(%)=(number of colonies/number of epithelial cells seeded)x100. For HOME the CFE assay was performed as above using passage 1 cells, and fixed after 8-12 days in culture.

PCR

RNA was isolated from epithelial cells using the RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) and quantified using an Eppendorf Biophotometer. From each sample, 1µg was used to synthesise cDNA using the Quantitect Reverse Transcriptase kit (Qiagen). PCRs were performed on equal amounts of cDNA using the primers and methods previously described [5] with the following modifications to the number of cycles in the thermocycling program: 32 cycles for CK12; 25 cycles for CK15, GAPDH and PAX6; 30 cycles for MUC16. Primer sequences were taken from published references (CK12[20], CK15[21], ΔNp63α[22], GAPDH [20], MUC16[23] and PAX6[20]) and checked using Primer-BLAST (National

Centre for Biotechnology Information), in addition sequencing of primer products was also performed. No reverse transcriptase and no sample controls were also performed.

Western blotting

Protein extraction, quantification, and western blotting were performed as previously described [5]. Briefly, 40µg of each HLE sample (or 20µg for HOME) was used for western blotting experiments using the following primary antibodies: CK15 (AB52816, Abcam, 1:10,000), p63α (4892, Cell Signaling Technology, 1:800), PAX6 (PRB-278P, Covance, 1:500), MUC16 (AB134093, Abcam, 1:10,000) and GAPDH (MAB374, Millipore, 1:1500). The secondary antibodies used were goat anti-mouse HRP (P0447 Dako, 1:10,000) and goat anti-rabbit HRP (P0448, Dako; 1:5000). For CK15 two bands were observed, an upper band of approximately 50kDa and a lower band of approximately 45kDa. For MUC16 a range of bands was detected starting at approximately 80kDa. For p63α and PAX6, bands were observed at approximately 75kDa and 50kDa respectively. Densitometric analysis was performed using Image J software, and results normalised to GAPDH.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. One way ANOVA was performed followed by Dunnett's multiple comparison test with $P<0.05$ considered to be statistically significant.

Results

HOMF were successfully explant cultured from all the patients with Aniridia, Mucous Membrane Pemphigoid, Ectodermal Dysplasia and the healthy volunteers (Table 1 and Figure 1). However only one out of the three Stevens Johnson Syndrome biopsies yielded HOMF despite HOME being successfully isolated and cultured from the same biopsies (supplementary Figure 1). For the other two Stevens Johnson Syndrome biopsies, no HOMF had migrated out of the tissue after 50 days in culture. HOME displayed typical epithelial cell morphology in culture (supplementary Figure 1), successfully formed epithelial colonies when the CFE assay was performed (supplementary Figure 2), and expressed CK15 and p63α (detected by PCR and western blotting, supplementary Figure 2).

HLE were successfully cultured on all the LSCD HOMF obtained as well as normal HOMF and 3T3 controls (Figure 2). HLE were harvested upon reaching at least 75% confluency. The time to this confluency for HLE co-cultured with the different LSCD HOMF was comparable to that of HLE co-cultured with normal HOMF (Figure 3). The number of epithelial cells obtained per flask in this time was also comparable for LSCD HOMF and normal HOMF supported cells.

A significantly longer time to confluency was observed for HLE cultured on 3T3s compared to any HOMF group (normal and LSCD) ($P<0.05$, one way ANOVA). However this was accompanied by a higher epithelial cell number obtained in this time (Figure 3).
The total CFE for HLE cultured on all the different feeder fibroblast types was not significantly different for any of the groups studied (Figure 4). PCR showed that the putative stem cell markers p63α and CK15 were detected in HLE cultured on all the different feeder layers for all 3 sets of donor HLE (Figure 5). The differentiation marker CK12, as well as MUC16 and PAX6 which are expressed in the cornea were also detected by PCR (Figure 5).

Western blots showed MUC16 and CK15 protein expression in HLE co-cultured on all feeder fibroblast types (Figure 6). As previously described [5] two protein bands were observed for CK15 in HLE, an upper band at approximately 50kDa and a lower band at approximately 45kDa. The lower band was not observed in HOME (supplementary data figure 2). PAX6 and p63α proteins were not detected in all samples (Figure 6) and samples with higher expression of PAX6 protein also had higher p63α protein levels. Interestingly, (by eye, Figure 6) PAX6 and p63α protein expression seemed to correlate with CK15 upper band expression. Where CK15 was predominantly in the upper band higher expression of p63α and PAX6 proteins was observed and when predominantly in the lower band, lower levels of p63α and PAX6 proteins were detected (figure 6).

Table 1 here.

Figures 1, 2, 3, 4, 5 and 6 here.

Discussion

Here we have shown that HOMF can be successfully isolated and cultured from the following LSCD patient groups: Aniridia, Mucous Membrane Pemphigoid, Stevens Johnson Syndrome, and Ectodermal Dysplasia. Furthermore, we have demonstrated that these LSCD HOMF can support the expansion of HLE. LSCD HOMF in this study were from patients with LSCD caused as a result of disease. Normal HOMF were from healthy volunteers with no LSCD. Patients with LSCD caused as a result of injury such as acid or alkali burn will also have normal HOMF. To our knowledge isolation of HOMF from patients with LSCD has not previously been reported. In previous work HOMF (from donors without LSCD) were found to be comparable to 3T3 as feeder fibroblasts for the expansion of HLE over multiple

passages [5]. Here we assessed HLE expansion on LSCD HOMF, as well as on normal HOMF and 3T3 controls.

Although we have shown that it is possible to isolate and culture HOMF from buccal oral mucosal biopsies taken from patients with Stevens Johnson Syndrome, the success rate was only 33% (n=3) compared to 100% success rate for Aniridia (n=3) and MMP (n=3) patients. Stevens Johnson Syndrome is a hypersensitivity disorder which affects the skin and mucous membranes. All the Stevens Johnson Syndrome patients in this study had very advanced disease, none of them smoked, and none were on any systemic medication at the time each biopsy was taken (see supplementary Table 1 for further information). Although only 3 Stevens Johnson Syndrome biopsies were included in this study our results suggest that it may be difficult to isolate and culture HOMF from patients with Stevens Johnson Syndrome for use in cell therapies (at least using the explant method described here). However the Stevens Johnson Syndrome HOMF that were successfully isolated did support the expansion of HLE. Epithelial cells were successfully isolated and cultured from 3/3 Stevens Johnson Syndrome buccal oral mucosal biopsies (the same biopsies that were used for fibroblast explant culture), suggesting that there was no processing issue with these samples.

Further repeats would be required to assess whether HOMF can be isolated from other patients with Stevens Johnson Syndrome and if so, whether these cells can also support HLE expansion. Further experiments may need to be performed to optimise HOMF isolation and culture for patients with Stevens Johnson Syndrome. Interestingly the initial migratory potential of HOME from Stevens Johnson Syndrome patients has been shown to be reduced compared to HOME from non-LSCD donors [24]. If oral fibroblasts in Stevens Johnson Syndrome are similarly affected for some patients (since there was no problem obtaining

HOMF by explant culture for one patient with SJS, this is clearly not the case for all patients) it is possible that isolation methods that do not require the oral fibroblasts to migrate out of the tissue such as collagenase digestion may be more successful than explant culture for these patients.

Although ED HOMF were successfully isolated in this study (n=1), further repeats would be required to give a better indication of whether HOMF can be consistently isolated from patients with ED and used to successfully support the culture of HLE.

Although HLE took longer to reach confluency on 3T3s compared to on HOMF, this correlates with a greater cell number achieved in this time. Possible explanations for these differences include the following: the difference in feeder size and seeding density between 3T3s and HOMF, HLE were either smaller or more tightly packed in the 3T3 condition, HLE were adapting to the new feeder support in the HOMF conditions (having been previously expanded on 3T3s for the primary culture). However, HLE cultured on LSCD HOMF were comparable to those cultured on normal HOMF in terms of HLE time to confluency, HLE cell number achieved in culture, and total CFE. Total CFE was comparable for HLE co-cultured with 3T3s or HOMF (both normal and LSCD), and both the putative stem cell marker CK15 and the corneal marker MUC16 were detected by PCR and western blotting in all cultures.

More repeats would be required to properly compare protein expression of p63α and PAX6 in all groups since detectable levels of these proteins were not observed in all the controls (HLE cultured on either 3T3 or normal HOMF). HLE co-cultured with Aniridia HOMF all had relatively high p63α and PAX6 protein expression, it would be interesting to see if this was a

consistent trend in further repeats. We have previously observed PAX6 protein expression in HLE co-cultured with 3T3 and HOMF feeder fibroblasts [5]. EGF has been shown to inhibit PAX6 expression in rabbit corneal epithelial cells [25]. Since cultures were not all harvested at the same time due to differing times to confluency it is possible that variations in media change time (and therefore EGF addition) prior to cell harvest may have affected PAX6 expression. This data suggests that further culture optimisation may be required in order to maintain expression of the putative stem cell marker p63α and the corneal epithelial marker PAX6 but importantly we have shown that HLE can be expanded on LSCD HOMF. Future immunostaining would be useful to detect p63α and PAX6 expression, and also determine the percentage of p63 positive cells in the epithelial cell cultures since HLE cultures containing more than 3% p63-bright cells are associated with a more successful clinical outcome for the treatment of LSCD [26].

HOMF from LSCD patients may be useful as autologous feeder fibroblasts for the expansion of epithelial cells for use in the clinic to treat LSCD. Future work could investigate this in a long term study to determine the success of using HLE produced using this method for transplantation to treat LSCD compared to currently used methods. Using a patient's own cells as feeder fibroblasts would remove the need to use murine 3T3s which are commonly used to support the expansion of epithelial cells for use in the clinic. This would create a safer cell-product for clinical use by removing any potential risks associated with the use of these murine feeder cells. In the case of unilateral LSCD where a patient's own HLE could be used from a healthy eye, and expanded using the same patient's HOMF, an autologous cell graft could be produced. Using only autologous cells in the production of a cell therapy has the advantage that immunosuppression is not required to prevent graft rejection. Long term

immunosuppression carries a high risk of serious eye and systemic complications [27] and autologous grafts are therefore preferable if this is possible.

Although the ability of LSCD HOMF to support HLE culture was studied here, these cells may also be suitable for supporting the expansion of other epithelial cell types such as HOME for use in the treatment of LCSD. We have previously shown that normal HOMF can support the expansion of HOME [5]. For cases of bilateral LSCD (where both eyes are affected by the condition) autologous HLE are not available and autologous HOME can be used as a cultured cell therapy for LSCD treatment (COMET). If autologous HOMF were used for the expansion of autologous HOME, a graft could be produced for the treatment of bilateral LSCD in which all cells are autologous.

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Declarations of interest

The authors report no conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author, AROC, upon reasonable request.

Ethics statement

This study was carried out in accordance with the principles outlined in the Declaration of Helsinki and was approved by the following ethics committee: Moorfields Biobank, reference: 10/H0106/57-2011ETR10. HLE were isolated from research-grade limbal rims obtained from the Moorfields Lions Eye Bank (London, UK) and oral mucosal biopsies were taken from healthy volunteers and patients with LSCD. All tissue had appropriate research consent.

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	Aniridia	Stevens Johnson Syndrome	Mucous Membrane Pemphigoid	Ectodermal Dysplasia
HOMF obtained	3/3	1/3	3/3	1/1
HOME obtained	3/3	3/3	3/3	1/1

Table 1. Summary of HOME and HOMF culture success from buccal oral mucosal biopsies from patients with LSCD. HOME were successfully cultured from all biopsies. HOMF were successfully explant cultured from all Aniridia, Mucous Membrane Pemphigoid and Ectodermal Dysplasia patients. However, only one out of three Stevens Johnson Syndrome biopsies yielded HOMF.

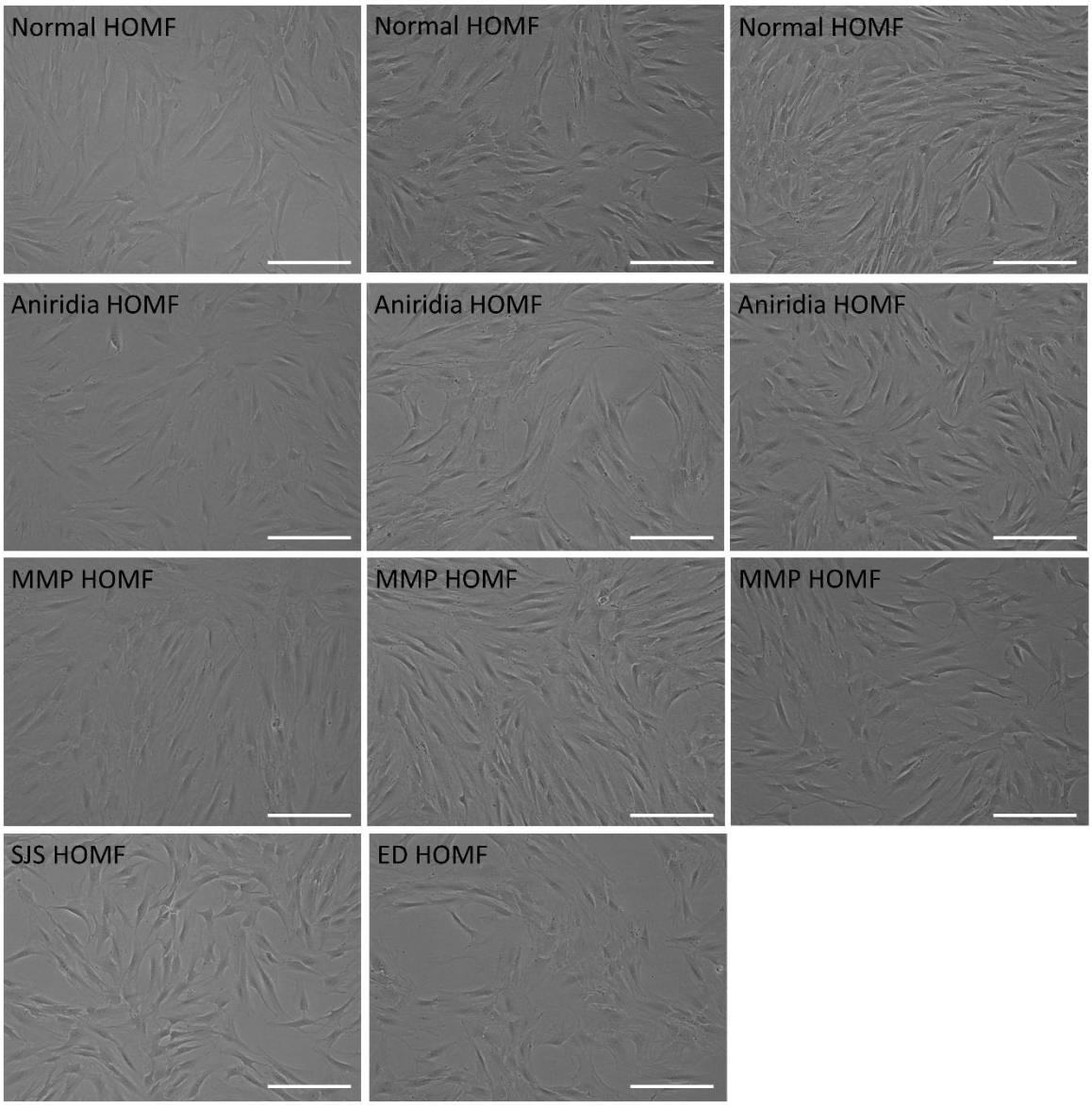


Figure 1. HOMF were successfully isolated from LSCD patients. Photos of passage 4 HOMF are shown from the following disease groups: Aniridia, Mucous Membrane Pemphigoid (MMP), Stevens Johnson Syndrome (SJS), and Ectodermal Dysplasia (ED) as well as normal HOMF controls. Scale bars are 200μm. Each image is of cells from a different donor.

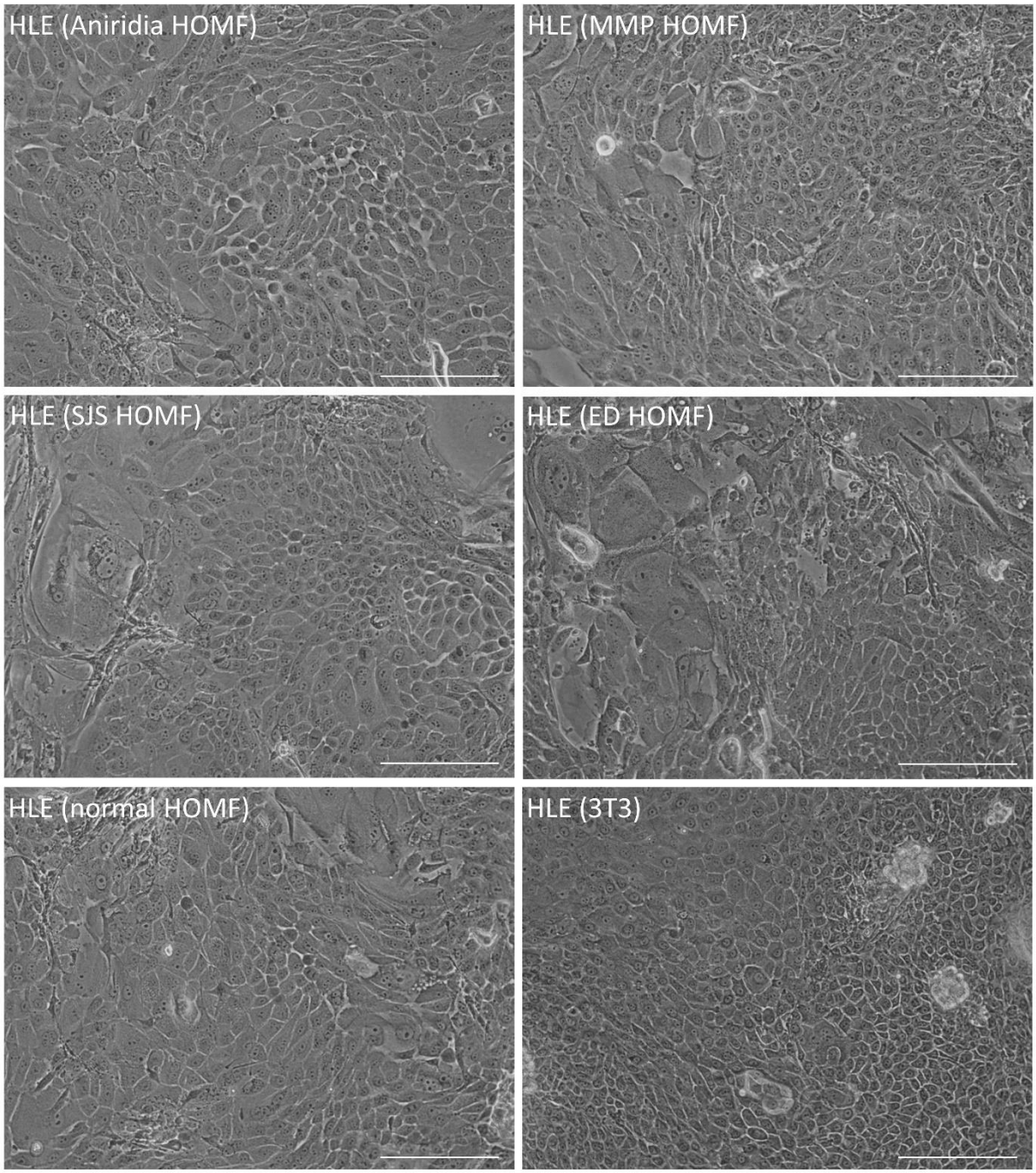


Figure 2. HLE were successfully expanded on HOMF from patients with LSCD. Cells with typical epithelial morphology were observed in all conditions. Photos shown were taken at passage 1 after 7 days in culture for HLE co-cultured with normal, Aniridia, Mucous Membrane Pemphigoid (MMP) and Stevens Johnson Syndrome (SJS) HOMF. The Ectodermal Dysplasia (ED) photo was taken at day 9, and 3T3 photo at day 10. All images shown are of HLE from the same donor. Scale bars are 200µm.

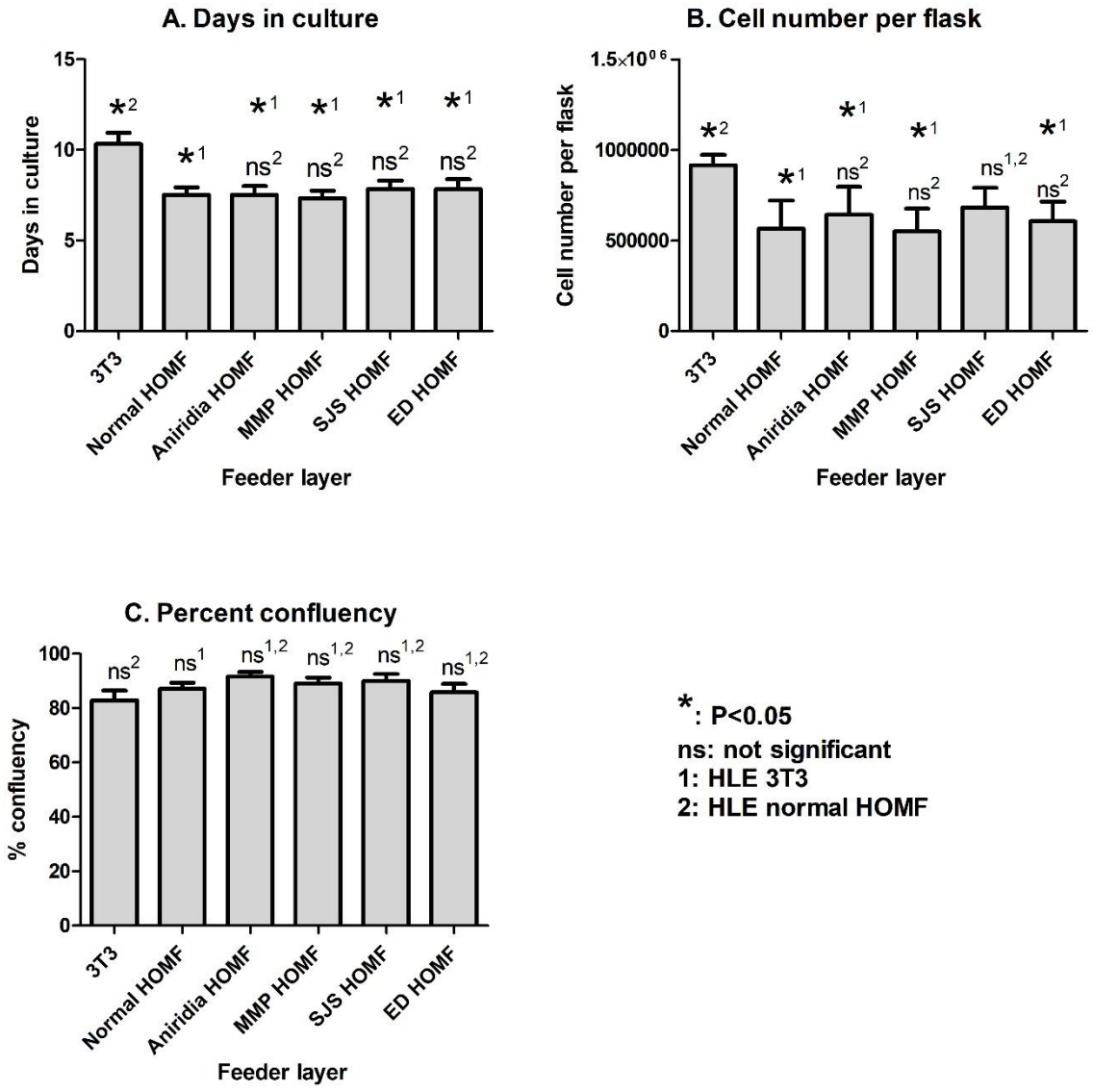


Figure 3. Epithelial cell number, percentage confluency and time in culture for HLE expanded on LSCD HOMF and the following controls: normal HOMF and 3T3s. The number of days in culture for HLE to reach at least 75% confluency as well as the epithelial cell numbers obtained in this time were comparable for all HLE cultured on HOMF from LSCD patients, and normal HOMF controls. HLE took longer to reach confluency on 3T3s compared to on HOMF (normal or LSCD), with more epithelial cells obtained in this time. For days in culture and percent confluency, n=6. For cell number, n=3. *P<0.05 compared to: HLE cultured on 3T3 ($*^1$), HLE cultured on normal HOMF ($*^2$), or both ($*^{1,2}$) controls (one way ANOVA). Non-significant (ns) results are indicated as: ns^1 compared to HLE cultured on 3T3, ns^2 compared to HLE cultured on normal HOMF, or $ns^{1,2}$ compared to both controls.

Results shown are for passage 1 HLE. MMP: Mucous Membrane Pemphigoid, SJS: Stevens Johnson Syndrome, ED: Ectodermal Dysplasia.

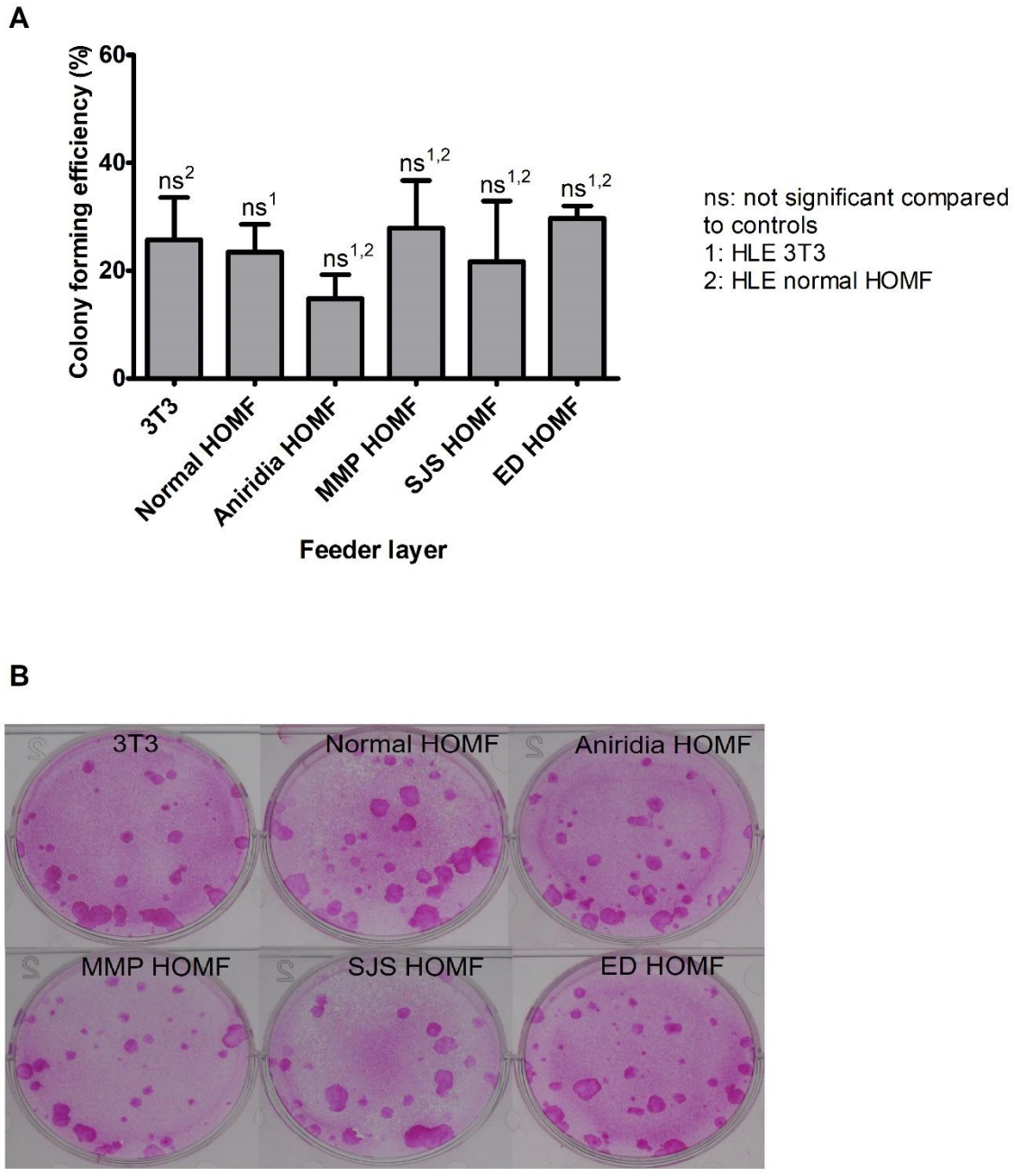


Figure 4. Colony forming efficiency (CFE) of HLE cultured on HOMF from patients with LSCD and the following controls: normal HOMF and 3T3s. Colonies of epithelial cells were observed for HLE cultured on HOMF from all LSCD groups: Aniridia, Mucous Membrane Pemphigoid (MMP), Stevens Johnson Syndrome (SJS), and Ectodermal Dysplasia (ED) as well as on normal HOMF and 3T3 controls. A. No significant difference in total CFE was observed between any of the conditions and the controls ($P>0.05$, one way

ANOVA). N=3 (ie. 3 donor HLE) with 3 replicates per donor. HLE obtained at passage 1 were used for the CFE assay. B. Colonies observed when 250 HLE (from the same donor) previously cultured on the feeder layers indicated (passage 1) were seeded onto 3T3s for the CFE assay and cultured for 11 days. Wells shown are from a 6 well plate, approximate diameter 35mm. Non-significant (ns) results are indicated as: ns[1] compared to HLE cultured on 3T3, ns[2] compared to HLE cultured on normal HOMF, or ns[1,2] compared to both controls.

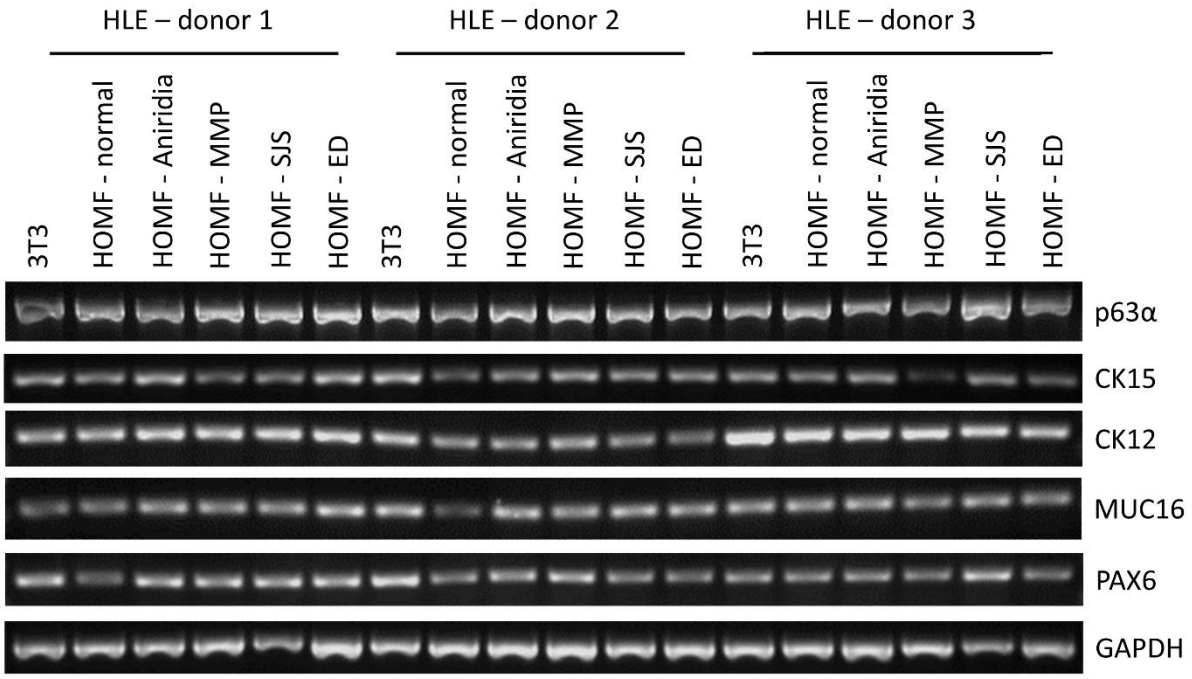


Figure 5. Marker expression in HLE co-cultured with LSCD HOMF, normal HOMF and 3T3s. PCR showed that the putative stem cell markers p63α and CK15 were detected in passage 1 HLE cultured on all the different feeder layers for 3 sets of donor HLE. The differentiation marker CK12, as well as MUC16 and PAX6 which are expressed in the cornea were also detected by PCR in all HLE cultures. MMP: Mucous Membrane Pemphigoid, SJS: Stevens Johnson Syndrome, ED: Ectodermal Dysplasia.

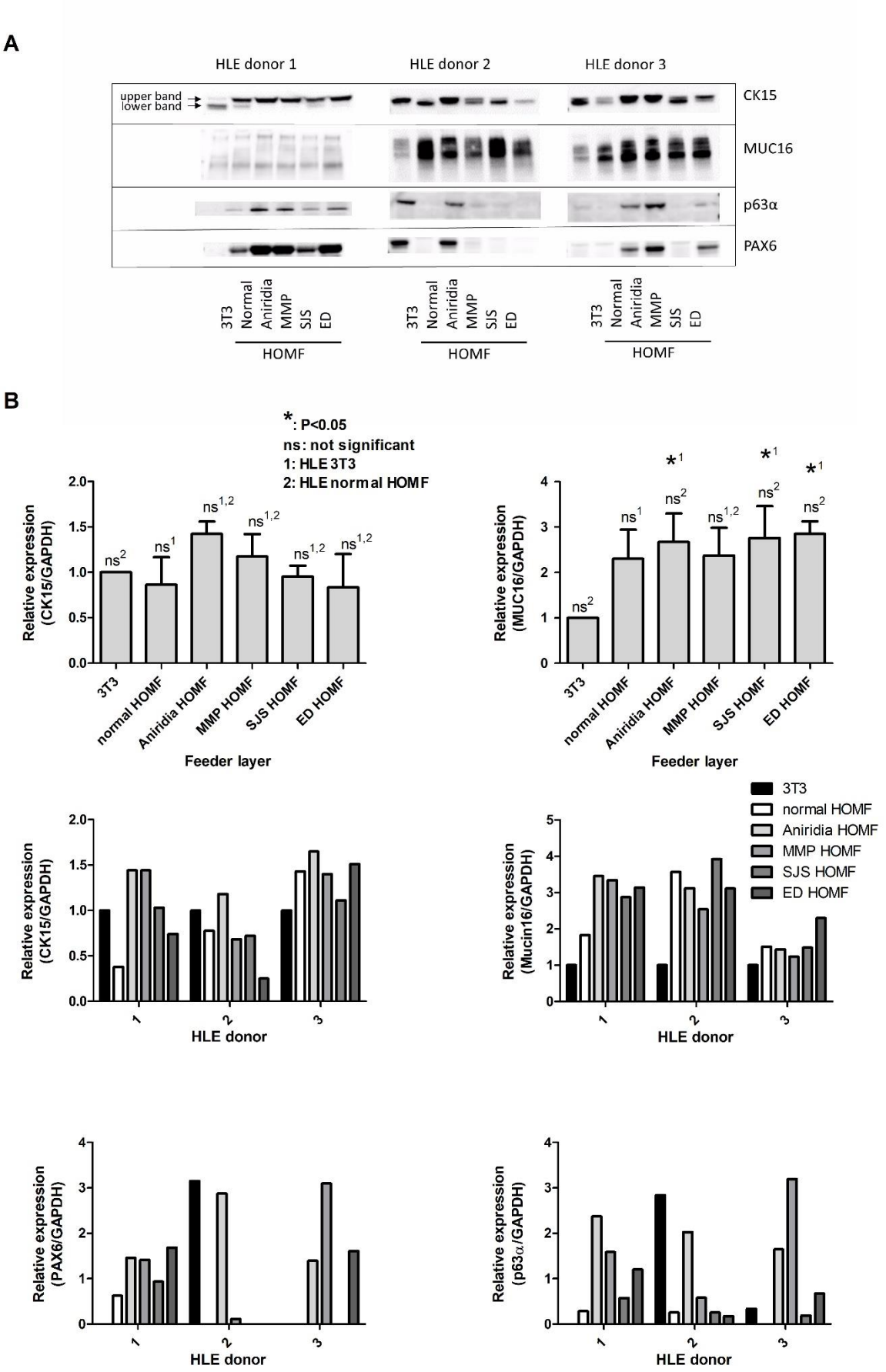
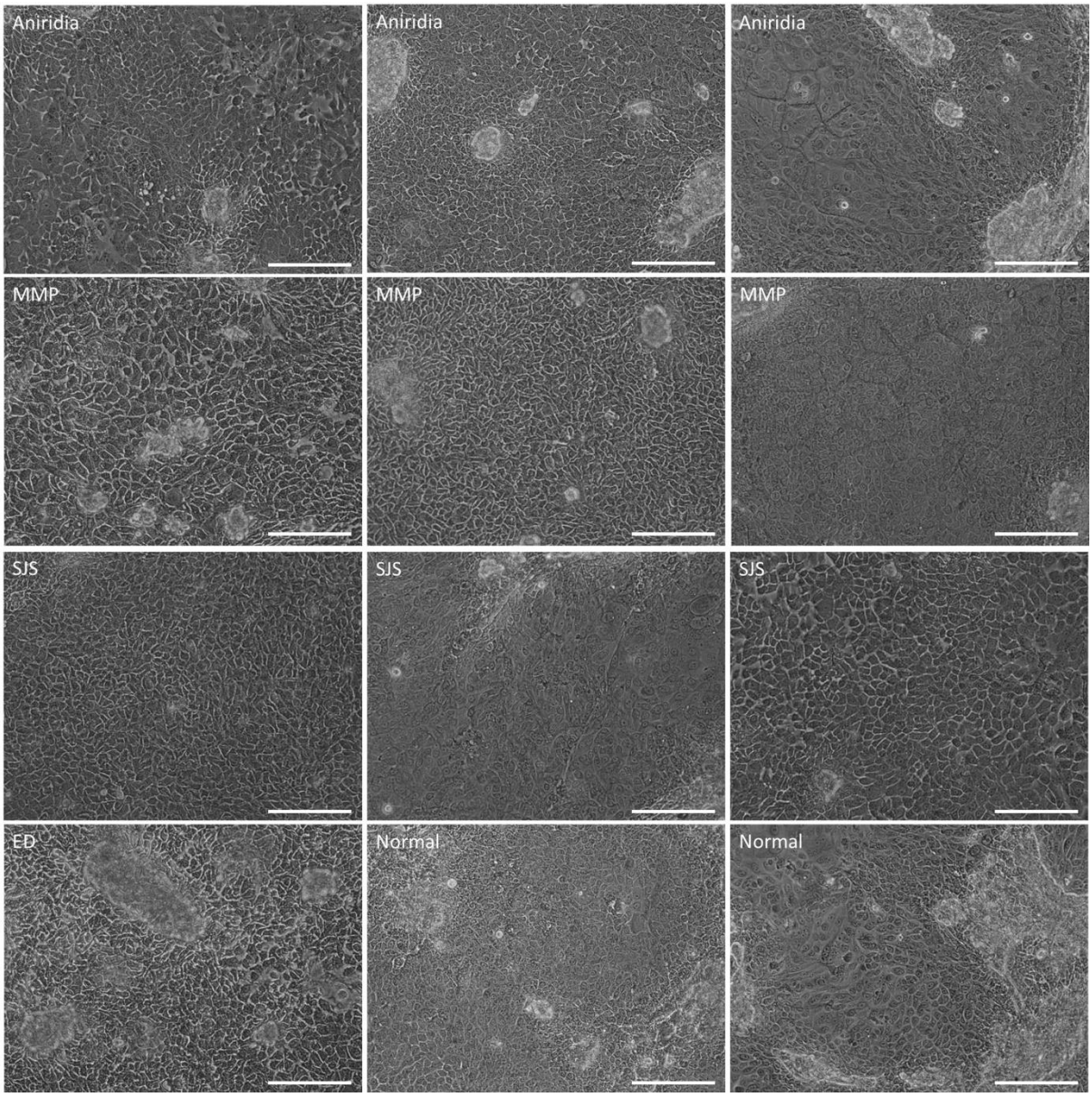

A
HLE donor 1
HLE donor 2
HLE donor 3
upper band
lower band
CK15
MUC16
p63α
PAX6
3T3
Normal
Aniridia
MMP
SJS
ED
HOMF
B
*: P<0.05
ns: not significant
1: HLE 3T3
2: HLE normal HOMF
Relative expression (CK15/GAPDH)
Relative expression (MUC16/GAPDH)
Feeder layer
3T3
normal HOMF
Aniridia HOMF
MMP HOMF
SJS HOMF
ED HOMF
Relative expression (CK15/GAPDH)
Relative expression (Mucin16/GAPDH)
HLE donor
Relative expression (PAX6/GAPDH)
Relative expression (p63α/GAPDH)

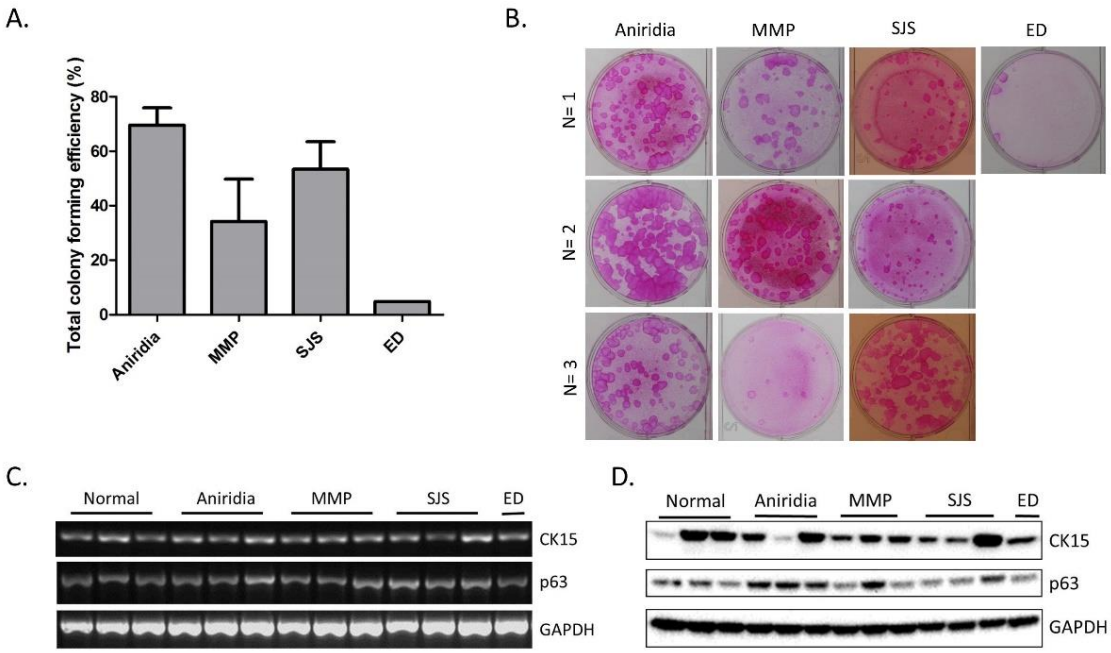
Figure 6. Protein expression detected by western blotting for HLE co-cultured with LSCD HOMF, normal HOMF and 3T3s. MUC16 and CK15 protein expression was detected in HLE co-cultured on all feeder fibroblast types. The proteins p63α and PAX6 were not detected in all samples. A. Protein bands obtained by western blotting. B. Quantification of protein bands. For CK15 and MUC16, protein expression was normalised to GAPDH and relative to 3T3 controls. Average protein expression for n=3 HLE donors is shown**.** For CK15 no significant difference between LSCD HOMF and controls (HLE cultured on 3T3 or normal HOMF) was observed. For MUC16 there was no significant difference between normal HOMF and any LSCD HOMF groups, however when compared to 3T3 the following showed significantly higher expression of MUC16: Aniridia HOMF, SJS HOMF, and ED HOMF (*P<0.05, one way ANOVA, Dunnett's multiple comparison test). Since controls (HLE cultured on 3T3 or normal HOMF) did not all show detectable levels of PAX6 and p63α, average protein expression for n=3 is not shown for these markers. MMP: Mucous Membrane Pemphigoid, SJS: Stevens Johnson Syndrome, ED: Ectodermal Dysplasia. Significant differences are shown compared to the following controls: HLE cultured on 3T3 ($*^1$), HLE cultured on normal HOMF ($*^2$), or both ($*^{1,2}$). Non-significant (ns) results are indicated as: ns^1 compared to HLE 3T3, ns^2 compared to HLE normal HOMF, or $ns^{1,2}$ compared to both controls.

Donor	HOMF isolated?	Medicines
1	Yes	Not on any systemic treatment at time of biopsy but had previously been on treatment with Azathioptine, systemic steroid (oral prednisolone) and oral Mycophenolate.
2	No	No systemic medicines related to eye condition. Had had a bone marrow transplant and also had graft versus host disease. Previous oral surgery.
3	No	No systemic medicines of note.

Supplementary data Table 1. Stevens Johnson syndrome patients extra data.



Supplementary data Figure 1. Images of primary HOME from the following LSCD groups in culture: Aniridia, Mucous Membrane Pemphigoid (MMP), Stevens Johnson Syndrome (SJS), and Ectodermal Dysplasia (ED) as well as cells from non-LSCD patients. Epithelial cells were successfully cultured from all the HOME biopsies obtained. Each photo is of cells from a different donor. Scale bars are 200µm.



Supplementary data Figure 2. Colony forming efficiency (CFE) and marker expression of HOME from patients with LSCD. Epithelial colonies were successfully obtained from HOME from the following LSCD patient groups: Aniridia (n=3), Mucous Membrane Pemphigoid (MMP, n=3), Stevens Johnson Syndrome (SJS, n=3), Ectodermal Dysplasia (ED, n=1). A. Mean total forming efficiency (SEM error bars). B. Images of the epithelial colonies obtained when 250 cells (from passage 1 cultures) were seeded per well of a 6 well plate (300 cells/well for Aniridia donor 1). Each image is of HOME colonies from a different donor. C. CK15 and p63α were detected by PCR in all HOME samples. D. CK15 and p63α proteins were detected by Western blotting in HOME obtained from all LSCD patient groups as well as normal controls. The CK15 band observed was approximately 50kDa.