Establishment of a Cell Line From Conjunctival Squamous Cell Carcinoma: PeCa-UkHb-01

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Purpose. Until now, no epithelial cell line from conjunctival squamous cell carcinoma (SCC), to our knowledge, has existed; therefore, the establishment of a model cell line would be a useful tool for further studies. In particular, the phenotypic and molecular characterization in comparison to other SCC cells is of high interest because this would enable the development of new treatment options for clinical application in ophthalmic oncology.

Methods. Epithelial cells were isolated from a bulbar conjunctiva SCC obtained from a 74-year-old male, harvested by stepwise trypsinization and named PeCa-UkHb-01. Cell doubling and the number of passages were determined. Short tandem repeats (STR) and karyotype analyses were performed. Semiquantitative real-time PCR and immunocytochemical fluorescence staining were carried out to detect tumor and epithelial cell markers.

Results. The cells had an epithelial and conjunctival phenotype. They grew above passage number 50 in a doubling time at approximately 34.5 hours. Short tandem repeat analyses confirmed the cell origin, although loss of heterozygosity occurred. Karyotype analyses revealed a heterogeneous composition of the cell culture and the karyogram itself showed aberrations and changes in the chromosome numbers. Real-time PCR and immunocytochemical fluorescence staining revealed the expression of the stem cell markers such as ABCG2, p63, OCT4, c-MYC, and SOX2 as well as the conjunctival cytokeratin K19.

Conclusions. PeCa-UkHb-01 cells fulfill the criteria of a cell line. They display characteristics of ocular carcinoma cells and therefore the presented cell line might serve for further basic research in ophthalmic oncology.

Keywords: squamous cell carcinoma, conjunctiva, cell line

Squamous cell carcinoma (SCC) is a malignant neoplasm that occurs in a variety of epithelial tissues. It is prominent in the skin,[1] but also comes up in other parts of the body, including the esophagus,[2] urinary bladder, or the uterine cervix.[3] In addition, the ocular surface, including cornea and, most prominently, conjunctival epithelium, is among the tissues that are prone to develop SCCs. The conjunctiva consists of nonkeratinized epithelium, both stratified squamous and stratified columnar, with interspersed goblet cells.

Squamous cell carcinomas of the conjunctiva are rather uncommon. For example, Sun et al.[4] reported incidence rates of conjunctival SCC of 0.03 cases per year per 100,000 in the United States. The main risk factors for conjunctival SCC formation are known to be sex, age, and ultraviolet radiation.[5–7] An infection with the human immune deficiency virus (HIV) associated with an immunosuppressed status also elevates the risk of neoplastic tumors in ocular surface epithelia.[8,9] Despite the low incidence, conjunctival SCC is considered the most common malignancy of the ocular surface.[10]

These carcinomas develop from precursor states, the cornea/conjunctival intraepithelial neoplasias (CIN), which are classified in a range of gradings from Grade I to III, with Grade III being the direct precursor of SCC. In conjunctival SCCs the neoplastic cells penetrate the basement membrane infiltrating into the conjunctival stroma, while in CIN the atypical cells remain still within the overlying epithelium without crossing the basement membrane, representing preinvasive disease.[6] If not treated, conjunctival SCCs are able to invade other parts such as the anterior chamber of the eye or the orbital tissue.[11,12] Conjunctival SCC cells may gain access to the blood vessels and lymphatic system, but regional and distant metastases are rather uncommon.[12] Because of the potential invasive and sometimes aggressive properties of conjunctival SCCs, they are a sight-threatening condition. In cases of advanced tumor formation, enucleation or orbital exenteration is often the only treatment option.

The importance of conjunctival SCCs in ophthalmology leads to the necessity of developing appropriate model systems to study these tumors. The character of tumors is prominently analyzed with the help of model cell lines, regarding especially the tumor formation ability, genetic deviations of cancer cells from regular cells[13] and the sensitivity toward different conditions including new therapeutic approaches[14] such as radiation[15] or chemicals.[16]

The establishment of cell lines from SCCs started decades ago, so that today there are a huge variety of cell lines available from different tissues. This includes human and rabbit head and neck SCCs,[17,18] uterine cervix SCCs,[19] and skin SCCs.[20] Despite this, the availability of cell lines derived from conjunctival SCCs is rather limited. To the best of our knowledge, there are currently no SCC cell lines established from conjunctival SCCs.

This report describes the isolation, cultivation, and first characterization of cells derived from a primary untreated SCC

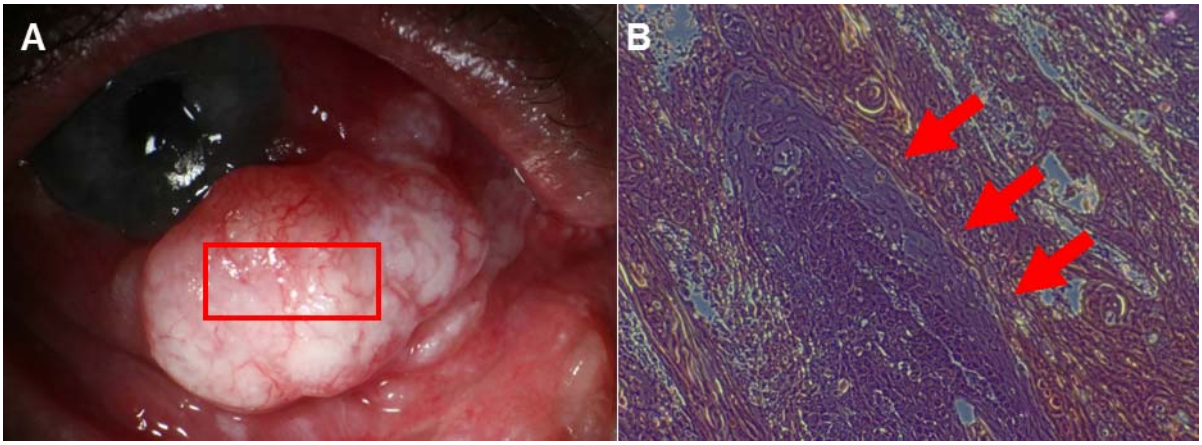

FIGURE 1. The tissue of origin of PeCa-UkHB-01. (**A**) Picture from the tumor, a highly prominent conjunctival squamous cell carcinoma located in the right eye of a 74-year-old donor. *Marked area*: localization of the removed tissue sample for cell culture. (**B**) Histologic section from tumor tissue stained with H&E (magnification: ×100) showing invasive tumor tissue (*arrows*) and surrounding stromal tissue.

of conjunctival localization in order to establish a conjunctival SCC cell line for further studies on this type of cancer. The focus of the initial characterization was laid on ocular surface-, stem cell-, and cancer-related markers in order to evaluate possible similarities of cancer and tissue-specific stem cells because it has been postulated that ocular surface SCCs arise from ocular surface stem cells.[21]

Materials and Methods

Tissue

Samples of a recurrent conjunctival SCC were acquired from the right eye of a tumor patient of the Department of Ophthalmology at the University Hospital Essen who received an orbital exenteration. The examined tissue was obtained after informed consent by the donor, and handled according to the tenets of the Declaration of Helsinki as approved by a vote of the ethics committee of the Faculty of Medicine at the University of Duisburg-Essen.

The male donor was 74 years old at the time the sample was collected. He was a smoker (~six cigarettes a day for the last 50 years); other risk factors contributing to conjunctival SCCs formation such as an immunocompromised state were not known. He was not tested for human immunodeficiency virus (HIV) infection because this procedure is not routinely performed. The donor was first examined in a peripheral eye hospital elsewhere. At this time the primary tumor was a chestnut-sized malignoma located at the right eye, and all macroscopic visible parts of the malignant tissue were removed (R1 resection). The pathological evaluation revealed an invasive squamous cell carcinoma which was staged according to a common international cancer staging notation system (TNM) as pT3, G2, R1. Within 2 months the tumor had resumed to grow almost to its original size. The patient was referred to our hospital and orbital invasion was diagnosed. Orbital exenteration was performed as the only possible treatment option. Samples for cell cultures and histology were collected from the lower nasal part of the large tumor mass (Fig. 1A) during orbital exenteration.

Cell Culture and Separation of Putative Epithelial Cancer Cells

The tissue was digested for 16 hours at 37°C using collagenase A (10103578001; Roche Applied Sciences, Mannheim, Germany) at a concentration of 1 mg/mL in Hanks buffered saline solution. Cells were washed in 5 mL culture medium, Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and centrifuged at 200*g* for 5 minutes. The supernatant was discarded and the pellet was suspended in 5 mL culture medium. Afterward, the resulting cell suspension was equally distributed into the wells of two 24-well plates followed by incubation at 37°C and 5% CO_2. The wells were monitored daily by microscopy for growth and the occurrence of cells with epithelial morphology.

As soon as those cells occurred, they were separated from morphologically distinct cells by means of selective trypsinization. The cell cultures were treated with 0.25% Trypsin/EDTA until fibroblast-like cells detached while the epithelioid cells remained attached. This step was repeated until only cells with epithelial morphology were visible (Fig. 2).

These cells were passaged into T25 flasks and further cultivated. They were labeled PeCa-UkHb-01. Cells were passaged upon reaching 70% to 90% confluence. At different passages, samples were taken for molecular or immunocytochemical characterization.

After establishment of a continuous cell culture, it was routinely screened for mycoplasma contamination based upon a commercially available PCR assay (VenorGeM, Mycoplasma Detection Kit for conventional PCR; Minerva Biolabs, Berlin, Germany). Furthermore, the supernatant from the cultured cell line was analyzed in the department of virology of the University Hospital Essen for HIV, hepatitis C virus (HCV) with a standardized RT-PCR protocol, hepatitis B virus (HBV), as well as human papillomavirus (HPV) with a standardized DNA-based PCR.

Gene expression patterns of PeCa-UkHb-01 cells were compared with the established cell line A431 derived from an epidermoid carcinoma of the vulva of an 85-year-old female patient[22] (kindly provided by Frank Wettey). The A431 cells were cultivated in DMEM supplemented with 15% FBS and passaged at 80% confluence.

Determination of Cell Doubling Time

To determine the cell doubling time, cells from passage number 21 through number 49 were seeded at a defined density in T25 flasks and cultivated until reaching 70% to 90% confluence. At this stage cells were harvested, counted, and passaged. The doubling time was calculated using the following formula: $Td = t^*[\ln(2)/\ln(A/A_O)]$, with Td = doubling time, t = time between plating and passaging, A = number of

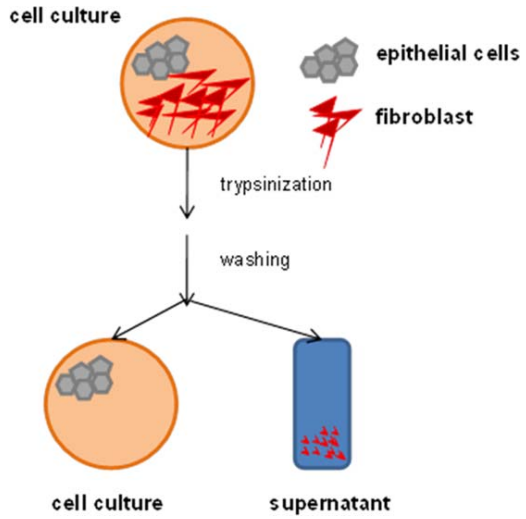

Figure 2. Schematic description of the principle of selective trypsinization. Trypsin was added to a mixed culture of epithelial cells and fibroblasts. After 5 to 10 minutes the fibroblasts started to detach from the culture vessel while the epithelial cells still stayed attached. Afterward, the detached fibroblasts were removed by rinsing carefully in the culture dish. The fibroblasts were collected in the supernatant, while the epithelial cells remained attached to the culture dish.

cells before passaging, and A_O = plated number of cells.[23] A growth curve based on the level of confluency at an examined time point was created and included in the Supplementary Material (Supplementary Fig. S3).

Short Tandem Repeat Analysis

The analysis of STR markers for verification of cell origin was performed independently by MVZ Eberhard & Partner (Dortmund, Germany) and in cooperation with the Institute of Legal Medicine at the University Hospital Essen. Nine microsatellites (D21S11, VWA, D8S1179, D3S1358, D5S818, D7S820, D13S317, D18S379, and FGA) in DNA extracted from patient's blood and cell line samples were investigated at both institutes. At the Institute of Legal Medicine of the University of Duisburg-Essen, we also analyzed the microsatellites Amelogenin, TH01, Penta E, D16S539, CSF1PO, Penta D, D2S1338, D19S433, D1S1656, D2S441, D10S1248, D12S391, D22S1045, SE33, and TPOX. MVZ Eberhard & Partner used a technique published by Sachetti et al.[24]; our analysis was performed with STR multiplex assays Powerplex 16 and Powerplex ESX17 (Promega, Darmstadt, Germany) according to the manufacturers' instructions in a 12.5-µL assay with 0.5 to 1 ng DNA extracted from different samples. Amplification products were evaluated by means of an ABI 3130 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany) with a GeneMapper program (Life Technologies GmbH, Darmstadt, Germany).

Cytogenetic Analysis

Chromosome analysis was performed to screen for chromosomal aberrations in cultivated cells at passage number 20. Samples of blood from the patient served as controls. The analysis was performed by MVZ Eberhard & Partner.

Molecular Biology

The expression of RNA of genes for epithelial cells of the ocular surface (*K3*, *K15*, *K19*, and *EpCAM*) as well as cancer and stem cells (*ABCG2*, *ΔNp63α*, *SOX2*, *c-MYC*, and *OCT4*) was analyzed in cells from passages number 8 to 20 (designated as early) as well as one cell sample from passage 42 (designated as late) by means of semiquantitative real-time PCR. The definition of early and late passages is only for the semiquantitative real-time PCR experiment. Taqman gene expression assays (Applied Biosystems/Life Technologies, Darmstadt, Germany) were used (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as endogenous control gene. Semiquantification was performed with the $2^{-\Delta\Delta ct}$ method (Sequence detection software 2.1; Applied Biosystems). Expression levels of the examined genes were compared with those from the SCC cell line A431.[22] The figures were drawn with GraphPad prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Table 1. Taqman Gene Expression Assays Used to Evaluate the mRNA Expression of Biomarkers in the Cells

N	Target	Refseq	Assay
1	*ABCG2*	NM_004827.2	Hs00184979_m1
2	*ΔNp63α*	NP_001108452.1	Hs00978338_m1
3	*OCT4*	NM_002701.4	Hs01895061_u1
4	*SOX2*	NM_003106.2	Hs00602736_s1
5	*K15*	NM_002275.3	Hs00267035_m1
6	*c-MYC*	NM_002467.4	Hs99999003_m1
7	*K3*	NM_057088.2	Hs00365080_m1
8	*K19*	NM_002276.4	Hs01051611_gH
9	*GAPDH*	NM_002046.3	Hs99999905_m1
10	*EpCAM*	NM_002354.2	Hs00901885_m1

Target, gene of interest; Refseq, ID of the NCBI Reference sequence of the target gene; Assay: Taqman gene expression assay used.

Immunohistochemical and Immunocytochemical Characterization

Tumor tissue embedded in paraffin was sliced, deparaffinized, and stained with hematoxylin & eosin (H&E), K5/6, and TTF1 according to the standardized protocols based on peroxidase stainings of the Institute of Pathology from the University Hospital Essen.

Cells from different early passages (no. 7, 9, 11) and late passages (no. 17, 42) were stained by fluorescence staining for markers of epithelial cells (EpCAM and K19) and for stem cells (ABCG2, p63, OCT4, and SOX2).

Cells were seeded on eight-chamber slides at a density of 10,000 cells per chamber and allowed to attach overnight. After 3 to 5 days of cultivation, cells were fixed in ice-cold methanol at −20°C for at least 10 minutes. Cells were blocked in PBS with 10% FBS (Linaris Biologische Produkte GmbH, Dossenheim, Germany) and 0.1% Triton X-100 (Sigma-Aldrich Chemie GmbH, Munich, Germany) for 45 minutes at room temperature (RT). The slides were then incubated with primary antibodies at appropriate dilutions in PBS (Life Technologies) with 5% FBS and 0.1% Triton X-100 overnight at 4°C. Slides stained without addition of primary antibodies as well as slides with fibroblasts stained according to the same protocols served as negative controls. Alexa Fluor–conjugated secondary antibodies were applied for 45 minutes at RT (Table 2). Cell nuclei were counterstained with Hoechst dye (Sigma-Aldrich Chemie GmbH). Between the main preparation steps the slides were rinsed three times with PBS for 5 minutes each. Finally the slides were covered with mounting medium and stored overnight at RT.

The stained slides were viewed under an inverse fluorescence microscope (Olympus IX-51, Olympus Deutschland

Table 2. Antibodies for Fluorescence Staining in Immunocytochemistry

Antibody	Host	Dilution for ICC	Source
Primary antibodies			
ABCG2	Mouse	1:200	Abcam ab3380 (Abcam plc, Cambridge, UK)
p63	Mouse	1:200	Santa Cruz sc-8431 (Santa Cruz Biotechnology, Heidelberg, Germany)
OCT4	Rabbit	1:200	Abcam ab19857
SOX2	Mouse	1:400	Abcam ab79351
K19	Mouse	1:400	Santa Cruz sc-6278
EpCAM	Mouse	1:400	Abcam ab71916
Secondary antibodies			
Alexa Fluor anti-mouse	Goat	1:400	Invitrogen A11017 (Invitrogen, Life Technologies GmbH, Darmstadt, Germany)
Alexa Fluor anti-rabbit	Donkey	1:400	Invitrogen A21206

ICC, immunocytochemistry; Source, manufacturer and catalog number of the antibody.

GmbH, Hamburg, Germany) and documented with cell^F software (Olympus Deutschland GmbH).

Results

Cell Culture and Doubling Time

Adherent cells mostly displaying fibroblast-like, spindle-shaped morphology were found in 25 of 48 wells at day 1 after seeding.

Epithelial-like cells were noticed after 23 days of cultivation in one well. After removing fibroblasts by stepwise trypsinization, pure epithelial cells were cultivated and labeled PeCa-UkHb-01. During proliferation the cells formed colonies of tightly adhered cells, which subsequently formed a confluent monolayer (Fig. 3).

Mycoplasma screening demonstrated that the cell line was not contaminated. HIV, HBV, HCV, and HPV could not be detected in the supernatant.

The calculated doubling time of the analyzed passages of these cells was 34.7 ± 3.8 hours. A growth curve for the cultivation within one passage is displayed in the Supplementary Material (Supplementary Fig. S3). The cells were continuously cultivated for 60 passages to evaluate their potential for long-term proliferation.

Table 3. Results of the STR Analysis

Microsatellites (Chromosome)	Passage No. 10, 18, and 25	Blood
Amelogenin (X Y)	X(Y)*	X**Y**†
D3S1358 (3)	14	14
TH01 (11)	6	6,**9**
D21S11 (21)	27,29,30	27,__,30
D18S51 / D18S379 (18)	13	13
Penta E (15)	10,15	10,15
D5S818 (5)	13	**11**,13
D13S317 (13)	12	**9**,12
D7S820 (7)	8	8,**11**
D16S539 (16)	11	11
CSF1PO (5)	12	12
Penta D (21)	10,13	10,13
VWA (12)	16,17	16,17
D8S1179 (8)	13,14	13,14
TPOX (2)	8,11	8,11
FGA (4)	22,23	22,23

Cells from PeCa-UkHb-01 at different passages were compared to a blood sample of the donor. The numbers in the two left columns represent the number of repeats for each microsatellite according to the accepted nomenclature.

* Only minimal traces of the microsatellite from the Y chromosome were detected in the 10th passage.

† Deviations are displayed in bold.

STR Analysis

The results from the nine microsatellites analyzed with both methods were identical. Both concluded that the cell line most likely originates from the patient with a probability of error of P less than 4.96×10^{-9}, despite the fact that in several STRs loss of heterozygosity could be seen. For confirmation of this statement, we analyzed another 14 STRs. The investigation of samples from three different passages of the cell line (passages 10, 18, and 25) led to the conclusion that for the time frame of these passages the cell line is stable regarding the analyzed DNA regions. However, we noticed variation between the results of the blood and of the cell line; they are marked in bold in Table 3. The microsatellites of the Y chromosome were not detectable in later passages; only in passage 10 a very small amount could be measured, indicating a loss of the chromosome.

Chromosome Analysis

In the peripheral blood, 20 chromosomes in metaphase could be analyzed, visualized, and arranged in a karyogram (Fig. 4 shows, respectively, 1 karyogram). It is cytogenetically one of a

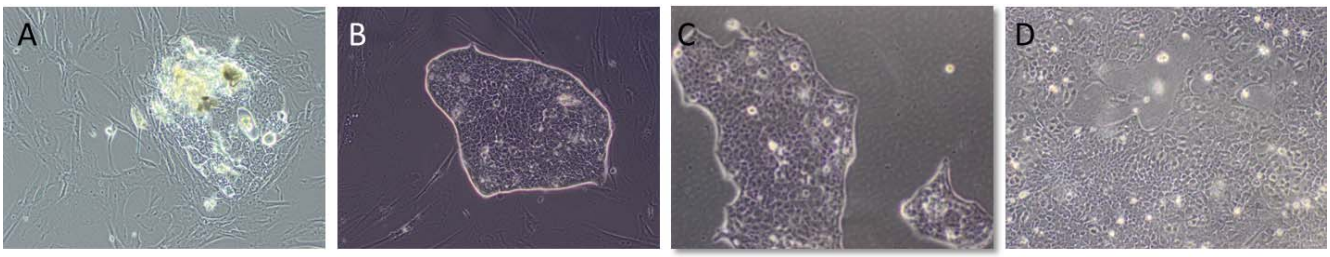

Figure 3. Representative morphology of PeCa-UkHb-01 cells in different stages of purification and selective enrichment of cells with epithelial morphology by trypsin treatment over several passages. The second passage still contained an almost equal mixture of epithelial and fibroblast-like cells, (**A**) while the passage no. 3 (**B**) was composed mainly of epithelial cells. The passage no. 9 (**C**) finally was completely void of fibroblast-like cells. Passage no. 54 (**D**) displays morphology comparable to the earlier pure epithelial cell passages.

Investigative Ophthalmology & Visual Science

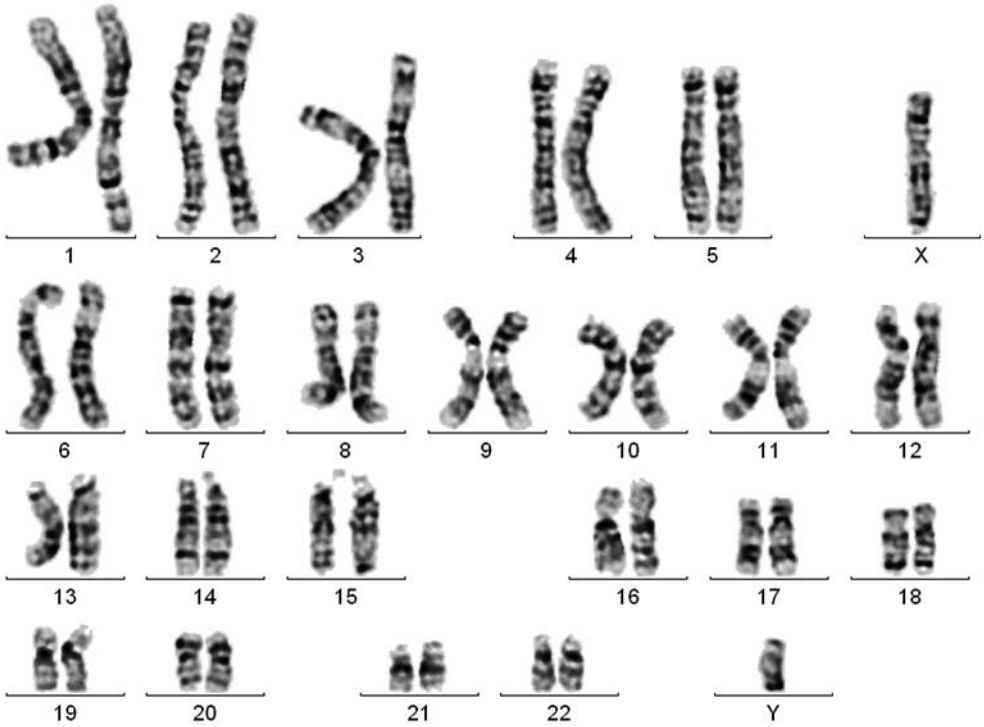

FIGURE 4. Karyogram derived from peripheral blood of the donor. It displays a regular chromosomal arrangement with a complete set of intact chromosomes. The numbers indicate the corresponding pair of homologue autosomal chromosomes. X and Y chromosomes are shown separately.

normal male with 46 chromosomes, including the X- and Y-chromosome.

In Figures 5A and 5B, 2 out of 20 karyotypes from the cell line are shown. The total analysis showed that the cell line is very heterogeneous due to a high variety in the analyzed metaphases regarding chromosome number and aberration locations. In three additional metaphases up to 197 chromosomes appeared. In the metaphases, which could be karyotyped, chromosome number varied between 56 and 61 (i.e., between hyperdiploidy and hypotetradiploidy).

It was not possible to assign all found chromosomal parts to their original location. Therefore, only the allocated chromosomes could be analyzed. The most frequent appearance of the chromosome is mentioned below in the brackets behind the particular chromosome. Chromosomes 18 (19) and Y (19), and also half of chromosomes 4 (19), 5 (18), and 21 (14), were lost completely. On the other hand, chromosomes 9 (14), 11 (15), 12 (15), 16 (12), and 20 (16) became trisomeric; chromosome 15 was found nine times as either trisomeric or normal. Additional (+i) or a change to an isochromosome (i) could be presumably observed by chromosome 8 (+i; 12), 10 (i; 12), 13 (1; 10) and 14 (+i; 10). Chromosome 2 (18), 6 (16), 7 (19), 17 (18), 19 (15), 22 (18), and X (20) appeared as expected. Chromosome 3 (19) received a third chromosome with a deletion of q-arm. A rearranged chromosome 1 occurred 13 times as shown in both Figures 5A and 5B. A specific rearranged chromosome occurred between one and nine times.

Characterization by Immunohistochemistry and Immunocytochemistry

The H&E staining of the tumor tissue showed regular SCC morphology (Fig. 1B). It was positive for the general SCC marker K5/6 and negative for lung-specific SCC marker TTF1 (Supplementary Fig. S1).

The fluorescence staining of the cultured cells was positive for the epithelial marker EpCAM and the conjunctival epithelial marker K19 (Fig. 6), as well as the evaluated cancer- and stem-cell markers ABCG2, p63, OCT4, and SOX2 (Figs. 7, 8), indicating the epithelial and conjunctival origin. Positive staining was detected in early and late passages. The staining of p63 in late passages seemed less prominent than in earlier passages.

Molecular Biological Characterization

PeCa-UkHb-01 cells expressed mRNA of the ocular surface cytokeratins K3, K15, and K19, as well as the epithelial marker EpCAM. Expression of the markers ABCG2, ΔNp63α, SOX2, c-MYC, and OCT4 were also detected in the cell line.

A comparison between the expression values of PeCa-UkHb-01 with those in the established A431 SCC cell line showed similarities and differences. While expression of K3 and EpCAM were comparable, the expression of K19 showed a 1000-fold increase in the PeCa-UkHb-01 cells compared with the A431 cells. Cytokeratin K15 expression was lower in PeCa-UkHb-01 as in A431 (Fig. 9).

The markers OCT4 and SOX2 revealed a 4- and 20-fold higher expression in the PeCa-UkHb-01 cells compared with A431 cells. The other examined markers ABCG2, ΔNp63α, and c-MYC were comparable in their expression pattern (Fig. 10). Comparison analyses between early and late passages from PeCa-UkHb-01 did not show any changes outside the SD except for c-MYC.

DISCUSSION

We described the establishment of the conjunctival SCC cell line PeCa-UkHb-01. The aim of this work was to develop a model for further in vitro studies on conjunctival SCCs. Although nonmalignant conjunctival cell lines have been

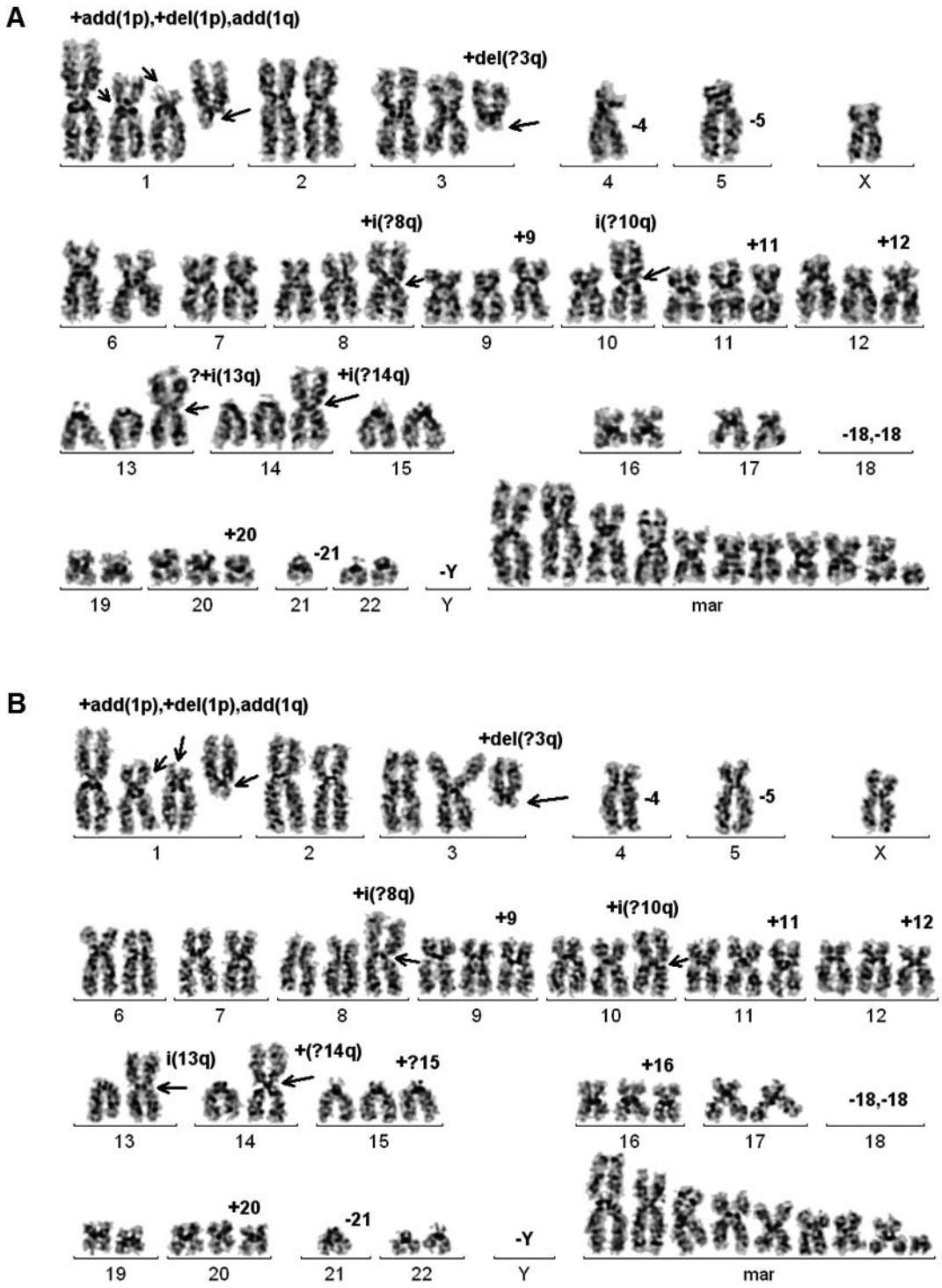

Figure 5. Representative karyograms (**A**, **B**) derived from the PeCa-UkHb-01 cells displaying a range of chromosomal aberrations. The numbers below the chromosomes indicate the corresponding pair of chromosomes. Small numbers above the chromosomes show chromosomal aberrations. Several chromosomes, like chromosome 1, 3, 11, or 12 are present in more than the normal diploid appearance. The Y-chromosome and the whole chromosome 18 are missing. Several chromosomes and chromosomal fragments could not be assigned; they are summed up and named mar. mar, marker chromosome.

generated,[25,26] establishment of cell lines from this carcinoma, to our knowledge, has not been reported so far.

To isolate the cells, we used a tissue sample from a conjunctival tumor histologically confirmed to be a conjunctival SCC. The protocol applied to generate the cell line corresponded to protocols reported for the establishment of cell lines from other SCCs.[17] The pure epithelial cell line obtained by selective trypsinization was subjected to further characterizations.

PeCa-UkHb-01 cells were maintained in culture for at least 60 passages, thus demonstrating that they are capable of long-term culture as compared with regular conjunctival cells.

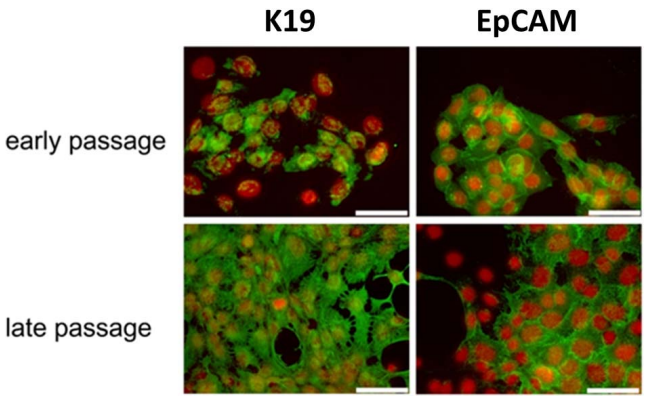

FIGURE 6. Immunocytochemical staining of the cultivated cells for the epithelial markers K19 and EpCAM. *Above*: early passage cells (passage no. 10 for K19 and 12 for EpCAM); *below*: late passage cells (passage no. 42 for both markers). Expression of the markers was detected in the cytoplasmatic cell compartments. *Scale bars*: 50 μm.

Primary cultures of conjunctival cells were reported to be able to remain in culture for up to 1 month without passaging and, depending on the culture conditions, capable of being passaged up to five times.[26,27] There are established conjunctival epithelial cell lines from nonmalignant tissue which are able to be cultivated over extended periods of time. One of them, IOBA-NHC, spontaneously immortalized in culture; others were immortalized by transfection[28] or contained chromosomes of cancer cells.[25] However, PeCa-UkHb-01 cells were not manipulated to gain the ability of unlimited growth.

The calculated doubling time of 34.7 hours was in line with respective times from head and neck SCCs, which had doubling times between 17 and 240 hours with a median of 26.5 hours.[29]

Recently, cell lines from other tissues which have been used for extensive molecular genetic or immunohistological analyses were proven to be derived not from the original tumor, but were contaminated by other cell lines or even cells of handling technicians.[30] Therefore, STR analyses were performed to prove the origin of PeCa-UkHb-01 from the donor. Despite minor differences between blood DNA and cell line DNA, especially loss of heterozygosity in the cell line, the data seemed to confirm the origin of the cells. For instance, in

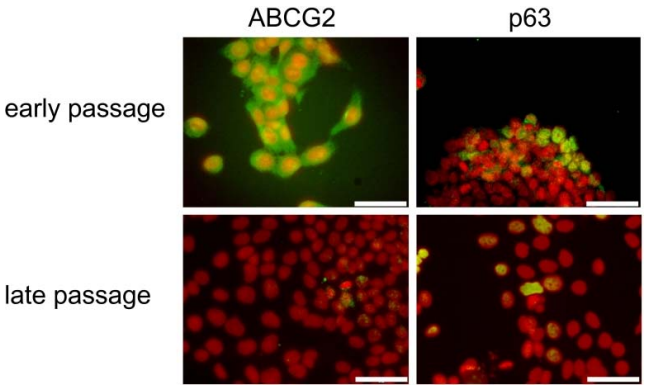

FIGURE 7. Immunocytochemical staining of the cultivated cells for the cancer markers ABCG2 and p63; *Above*: Early passage cells (passage no. 9 for ABCG2 and no. 3 for p63); *Below*: Late passage cells (passage no. 42 for ABCG2 and for p63 passage no. 17*). *Scale bars*: 50 μm. Because passage 3 is in early passage for this marker the picture from passage 17 was considered late passage

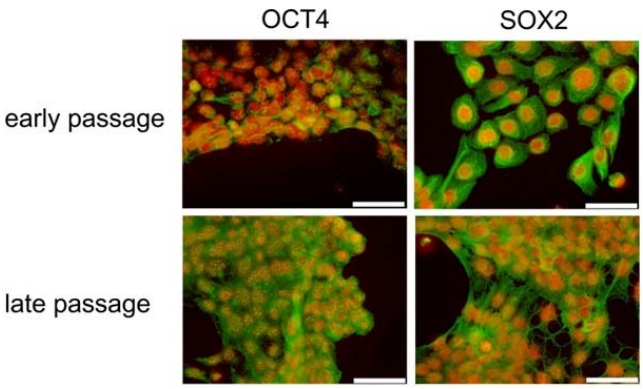

FIGURE 8. Immunocytochemical staining of the cultivated cells for the stem cell/pluripotency markers OCT4 and SOX2. *Above*: Early passage cells (passage no. 10 for OCT4 and passage no. 12 for SOX2); *below*: Late passage cells (passage no. 42 for OCT4 and SOX2). *Scale bars*: 50 μm.

passages numbers 18 and 25 no STRs from the Y chromosome were detected. The differences between the STR results of the blood and cell line samples are probably explained by mutations or cross-chromosomal rearrangements. Due to the lack of tumor tissue DNA, it is not possible to say if the mutations have already occurred during tumor development or in cell culture. In the second case, it could be assumed that further mutations happened between ongoing passages, but given the almost identical STR results of passage numbers 10 and 25, this seems unlikely. To solve this open question, further experiments have to be performed in the future.

The karyotype analysis of PeCa-UkHb-01 detected abnormal chromosomal arrangements and heterogeneity between the donor's blood cells and different cells of the cell line, as well as among the cells of a particular passage (Figs. 4, 5). A comparison of cell culture–, blood-, and tumor-samples was not possible, because the procedure to generate karyograms is not applicable on tissue samples. For karyogram generation it is necessary to perform a metaphasis arrest on proliferating cells.

In PeCa-UkHb-01 cells, mainly deletions or additions of one chromosome and the addition of an isochromosom to a newly attached chromosome were observed. These cytogenetic features are not surprising because chromosomal aberrations caused by an inherent genomic instability[31] are a common

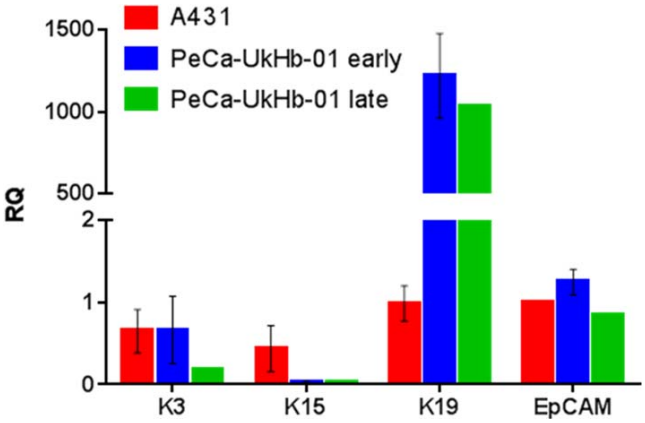

FIGURE 9. Semiquantitative real-time PCR analysis of ocular surface epithelial cytokeratins. The expression of K3, K15, K19, and EpCAM was compared between A431 (*red*) cells ($n = 12$), PeCa-UkHb-01 cells early passage (*blue*; $n = 8$) and PeCa-UkHb-01 cells late passage (*green*; $n = 1$). Data are normalized to the expression value found in A431. RQ, relative quantification.

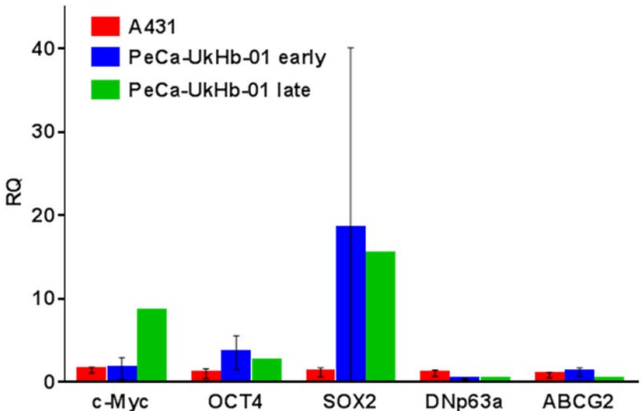

Figure 10. Semiquantitative real-time PCR analysis of the expression of pluripotency marker in PeCa-UkHb-01 and A431. The expression of c-MYC, OCT4, SOX2, ΔNp63α, and ABCG2 were compared between A431 (*red*; $n = 12$) and PeCa-UkHb-01 cells early passage (*blue*; $n = 8$) and PeCa-UkHb-01 cells late passage (*green*; $n = 1$). Data are normalized to the expression value found in A431.

feature in many cancer cells, including SCC cell lines.[17,32–34] The detected loss of the Y chromosome in PeCa-UkHb-01 is a process that also occurs frequently in SCC cell lines. Hunter et al.[35] have published that 8 of 13 (62%) SSC cell lines from esophagus of male donors have lost their Y chromosome. Loss of the Y chromosome was also observed in regular cells from elderly male donors.[36,37] In general, the results of STR analysis and karyotype determination are in line. The heterogeneity of PeCa-UkHb-01 might limit the ability to use them in in vitro studies emphasizing the need to generate PeCa-UkHb-01 cultures with a homogenous chromosomal arrangement. A lot of different SCC cell lines available are heterogeneous in their chromosomal arrangement.[32] They originated from a tumor, but for a really homogenous cell line a single cell must be used. As far as we know, only the cell lines USC-HN1 and USC-HN2 fulfill these criteria.[17,34] Recently, a study by Asnaghi et al.[38] examined DNA copy number alterations in a series of conjunctival SCCs. They detected chromosomal alterations, which are potentially important in tumor formation and growth, especially the frequent amplification of the p-arm of chromosome 8. This kind of alteration could not definitely be identified in the cell line. While chromosome 8 was found in triplicates, with possible addition of an isochromosom at the q-arm, there was no clear hint of an amplification of the p-arm. Nevertheless, because there were a number of chromosomes and fragments which could not be assigned, it cannot be ruled out that p-arm amplification took place. Further studies on the cells are needed to validate this.

The characterization of PeCa-UkHb-01 by means of immunologic staining and gene expression analysis by real-time PCR was performed to determine the phenotype of the new cell line. The cell line A431 used as comparison in real-time PCR experiments is a model cell line for cancer research.[22] The results of the comparison displayed some similarities and differences. The epithelial markers K3, K15, K19, and EpCAM were detected by immunocytochemistry and PCR analysis (Figs. 6, 9). The expression of EpCAM was initially detected as a dominant surface antigen in human colon carcinoma tissue and is highly expressed in various human carcinomas.[39] The cytokeratin K3 is a characteristic epithelial marker of the corneal surface, while K15 is a popular marker of corneal epithelial progenitor cells.[40,41] Cytokeratin K19 is a marker of conjunctival epithelium and corneal epithelial progenitor cells.[41,42] The almost 1000-fold increased expression of K19 in our cell line compared with the epidermoid derived A431 cells is likely due to the conjunctival origin of the cells. The expression of K19 was confirmed in immunocytochemistry. The staining and expression pattern for K19 and EpCAM was comparable in early and late passages with no differences found, indicating no relevant phenotypic changes in the course of the cultivation.

ABCG2 is expressed in cancer cells and associated with resistance of the tumor against chemotherapeutics, making it a potential target for anticancer therapies.[43] The marker ΔNp63α is an isoform of the p63 protein, which is involved in cancer cell metabolism.[44] Ye et al.,[45] for example, demonstrated the value of ΔNp63α for cellular proliferation in esophageal squamous cell carcinoma cell lines by silencing its expression. Both markers were detected by PCR as well as immunocytochemistry. It should be noted that, unlike the real-time PCR assay, the monoclonal antibody used to detect p63 was not isoform specific for ΔNp63α. The staining for p63 revealed a less intense staining of this marker in late passages compared with early passages. It remains unclear if this observation is caused merely by coincidence or by a real decrease in expression, which might occur after extended cell culture. With the isoform ΔNp63α used in the semiquantitative real-time PCR no expression differences were noted. Nevertheless, it is noteworthy that all examined passages showed expression of ABCG2 and p63. Polymerase chain reaction data showed a comparable expression of the markers in PeCa-UkHb-01 and A431. It was lower for DNp63a in PeCa-UkHb-01 early and late, and higher for ABCG2 in PeCa-UkHb-01 early, but lower for PeCa-UkHb-01 late; the differences did not seem to be significant.

Real-time PCR further revealed the expression of OCT4, SOX2, and c-MYC in PeCa-UkHb-01. These proteins have been found in neoplastic cancers and ocular surface epithelial progenitor cells.[41,46] In cancer cell lines, an artificially increased OCT4 expression demonstrated the ability of cells to initiate tumors.[47] SOX2, a key gene that controls transcriptional networks required for pluripotency, has a prognostic value for the progression and clinical outcome in other SCC types.[46,48,49] Increased amplification of SOX2 was reported to be a common incidence in SCCs.[46] The biomarker cMYC was demonstrated to be amplified more frequently in advanced stages of esophageal SCC.[50] The expression analysis revealed that c-MYC expression in A431 was comparable with PeCa-UkHb-01 in early passages. However, the one sample of the late passage of PeCa-UkHb-01 displayed a higher expression. OCT4 and SOX2 were expressed at higher levels. Immunocytochemical analysis of cell culture confirmed the expression of OCT4 and SOX2 in PeCa-UkHb-01 cells. The staining for SOX2 revealed a strong cytoplasmic staining for the protein (Fig. 8). As a transcription factor, SOX2 was reported to be predominantly localized in the nucleus, but in case of cultivated cells depending on the used antibodies or staining conditions, a positive-staining signal could also occur in the cytoplasm.[51]

The noted discrepancies in the biomarker expression patterns of early and late passages of PeCa-UkHb-01 can be indicative for modifications gained during cultivation or the result of the chromosomal heterogeneity. The significance of these findings in PeCa-UkHb-01 has yet to be examined.

The results of the characterization demonstrate that PeCa-UkHb-01 cells display functional features of SCC cells. They are able to be maintained in culture for long periods of time and display chromosomal aberrations and heterogeneity common for tumor cells.

Finally, the expression of tumor associated biomarkers by our cells, which are also present in the model SCC cell line A431 as well as in other SCCs, underline that cellular mechanisms working in SCCs may be also active in PeCa-UkHb-01. In the future, the PeCa-UkHb-01 cell line might be a

useful experimental tool to develop new therapeutic modalities in ophthalmic oncology.

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