

Human conjunctival epithelial precursor cells and their progeny in 3D organotypic culture

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ABSTRACT We report on an *in vitro* organ culture method to investigate human conjunctival epithelial basal precursor cells and their progeny within a more natural three-dimensional microenvironment. Conjunctival fragments were cultured on gelatin sponges in medium with 10% FBS. The conjunctival phenotype of the epithelium was confirmed by the expression and distribution of a panel of markers (p63, CK-13/CK-10, CK-19, Ki-67, PAS for goblet cells, CD45 for infiltrating interlamellar leukocytes and nestin for mesenchymal and ocular epithelial precursor cells). After 7 days, the epithelium had exfoliated its superficial layers (mostly CK-19 positive cells and all goblets), maintaining only 1-2 layers of basal/parabasal cells, p63, CK-13/CK-10 and nestin positive cells, firmly attached to the specimen. After 14 days, a new multilayered epithelium was formed, consisting of p63, CK-13/CK-10, nestin positive cells and in the high-zone CK-19 positive cells with new goblets. Additionally, we found interlamellar leukocytes which had probably migrated from capillaries that continued to be well maintained in the subepithelial stroma. Cells dispersed from conjunctival epithelium and co-cultured with feeder post-mitotic NIH3T3 fibroblasts formed mosaics displaying a basal epithelial phenotype. These cells expressed CD133 as revealed by RT-PCR. These organ cultures provide new opportunities to investigate epithelial reconstitution of the conjunctival surface and changes that may have occurred to their stem/precursor cells during adaptation to varying conditions *in vitro*.

KEY WORDS: *epithelial basal precursor, stem cell, niche, epithelium regeneration, interlamellar leukocytes*

The conjunctival epithelium is a stratified, non-keratinized epithelium containing goblet epithelial cells. It can be distinguished by the corneal epithelium by the expression of different cytokeratins, mucins and by the presence of glycocalix (Meller *et al.*, 2002). In the human conjunctiva, goblet cells secreting gel-forming mucins, can occur singly or in greater numbers lining epithelial infoldings or crypts (Shatos *et al.*, 2003). Decrease or loss of mucin/glycocalix production (Paladino *et al.*, 2004; Pflugfelder *et al.*, 1997) generate squamous metaplasia, which may lead to dry eye and ocular surface diseases. In the conjunctiva, putative epithelial stem cells are concentrated mainly in the fornix region, but can be found also along the whole inner epithelium layer (Lavker *et al.*, 1998). Conjunctival epithelial precursor cells are bipotent cells giving rise to either goblet and non-goblet keratinocytes and good candidate to be conjunctival stem/precursor cells (Wei *et al.*, 1997; Pellegrini *et al.*, 1999). Conjunctival epithelial stem/precursor cells can be maintained and expanded *ex vivo*, if cultivated upon appropriate matrix components, on which both goblet and

non-goblet keratinocytes could be expanded (Santos *et al.*, 2005; Connon *et al.*, 2006).

Intact small fragments of conjunctiva were cultivated in an appropriate medium and analysed at different time intervals [prior to culture (T0), after 7 days (T1) and 14 days (T2)] for histology and for the expression of different cellular markers.

3D-organotypic cultures on sponges

Conjunctival fragments on gelatin-supports, revealed good maintenance of 3D-tissue-architecture and cellular heterogeneity throughout the whole period of cultures. At each time in culture, the stromal compartment appeared well maintained, with few capillaries that remained apparently intact and remarkably un-

Abbreviations used in this paper: 3D, three dimensional; RT-PCR, reverse transcriptase- polymerase chain reaction.

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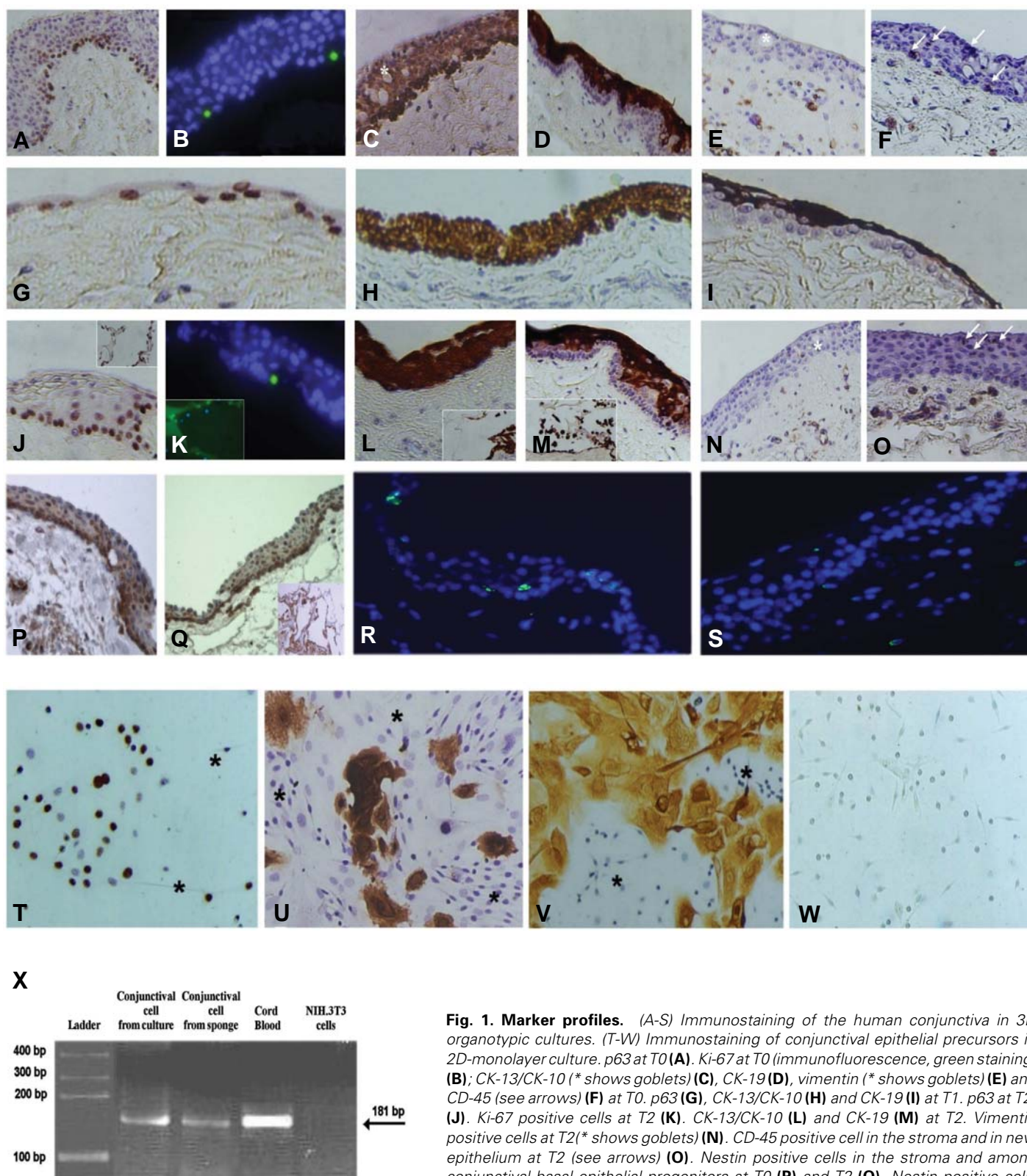


Fig. 1. Marker profiles. (A–F) Immunostaining of the human conjunctiva in 3D organotypic cultures. (T–W) Immunostaining of conjunctival epithelial precursors in 2D-monolayer culture. p63 at T0 (A). Ki-67 at T0 (immunofluorescence, green staining) (B); CK-13/CK-10 (* shows goblets) (C), CK-19 (D), vimentin (* shows goblets) (E) and CD-45 (see arrows) (F) at T0. p63 (G), CK-13/CK-10 (H) and CK-19 (I) at T1. p63 at T2, (J). Ki-67 positive cells at T2 (K). CK-13/CK-10 (L) and CK-19 (M) at T2. Vimentin positive cells at T2 (* shows goblets) (N). CD-45 positive cell in the stroma and in new epithelium at T2 (see arrows) (O). Nestin positive cells in the stroma and among conjunctival basal epithelial progenitors at T0 (P) and T2 (Q). Nestin positive cells migrated into the sponge (insert Q). Apoptotic cells (<1%) were equally detected at

T0 (R) and at T2 (S) in culture. Epithelial cells infiltrating the underlying sponge are shown in the insets of images (J, K, L, M and Q). Epithelial cells isolated from fresh conjunctival fragments after trypsin incubation, were pipetted on a monolayer of mit-c treated murine NIH.3T3 fibroblasts as a source of feeder layer (*). Colonies were positive for p63 (T), CK-13/CK-10 (U), CK-19 (V) but nor for CK-3 (W). (For all figures original magnification, 400X). RT-PCR analysis of CD133 mRNA (X). Positive control: human cord blood; negative control: murine NIH3T3 cells.

changed throughout the experiment.

T0

p63 expression (Fig. 1A) was exclusively confined to the nucleus of cells of the basal layer of the conjunctiva. $5\% \pm 2.2\%$ of basal epithelial cells were cycling, (positive immunostaining for Ki-67, Fig. 1B). CK-13/CK-10 were detected on the whole conjunctival epithelium, highly expressed in the cytoplasm of basal/parabasal cells and gradually less in the upper layers (Fig. 1C). In contrast, CK-19 was never expressed in the basal/parabasal layers (Fig. 1D). CK-3 was never detected in the conjunctival epithelium (not shown). A small number of scattered goblet cells was detected in the more superficial epithelial layers of the epithelium (asterisk, Fig. 1C). Vimentin was expressed in endothelial and dispersed subepithelial stromal cells (Fig. 1E) and CD45 within the stroma in interlamellar monocyte/dendritic-like cells infiltrating the epithelium (Fig. 1F). Nestin was detected in the basal/parabasal layer of the epithelium (Fig. 1P). CD117 was never expressed at any culture time (not shown).

T1

The most superficial epithelial cell layers had detached, while the remaining epithelium was reduced to a mono/bi-layer. Epithelial precursor cells which had remained, selectively expressed p63 and CK-13/CK-10 (Figs. 1G and 1H). Basal cells never expressed CK-19 (Fig. 1I). Cycling cells (Ki-67⁺) were $15\% \pm 3\%$ of these cells in the explants.

T2

Conjunctival epithelium appeared to have reconstituted a non-keratinized squamous epithelium (3-5 layers) lining over the whole external surface of the fragment. Basal/parabasal layers contained a prominence of p63 positive cells (Fig. 1J) with a proportion of $10\% \pm 1.25\%$ cycling cells (Ki-67⁺) (Fig. 1K). The cells of the new epithelium expressed CK-13/CK-10 (Fig. 1L) with only a small proportion of more superficial cells expressing CK-19 (Fig. 1M). A very small number of goblet cells (<1%) was also detected in the more superficial epithelial layers of the new epithelium. This new epithelium showed the tendency of infiltrating into the underlying sponge with cells that were stained for p63 and both CK-13/CK-10 and CK-19 (see inserts of Fig. 1J, Fig. 1L and Fig. 1M). Vimentin and CD45 positive cells were present within small capillary structures and dispersed within the conjunctival stroma. The presence of a small fraction of interlamellar leukocytes phenotypically resembling monocyte/dendritic-like cells was detected infiltrating the new epithelium (Fig. 1N and Fig. 1O).

Nestin-staining remained positive in the basal and parabasal layers of the epithelium.

In the sponge approximately 70% of cells expressed nestin. In addition, both at T0 and at T2, in the stroma several mesenchymal-like cells were positive for nestin immunoreactivity (Fig. 1P and Fig. 1Q).

At T0d, as well as at T1 (not shown) and T2, a limited fraction of apoptotic cells was equally detected (<1%) (Fig. 1R and Fig. 1S respectively), within the epithelium and the stroma, demonstrating that the conjunctival epithelium and stromal elements were well preserved *in vivo* as well as in prolonged 3D-organotypic culture

Isolation and expansion of epithelial basal stem/precursor cells in co-culture with murine NIH.3T3 fibroblasts as a feeder layer

Only a small fraction of epithelial cells isolated directly from the conjunctival explant by enzymatic digestion (< 0.1% of the cells) became adherent and could initiate colonies, forming mosaics of enlarging clusters of tightly adherent polygonal cells. After each passage only a limited proportion of adherent cells (about 1%) formed enlarging colonies with high self-replication efficiency. These cells could be further sub-cultivated by division at sub-confluence for at least 2-4 passages. The presence of post-mitotic murine fibroblasts as a source of feeder cells markedly enhanced the survival and self-renewal of these epithelial cell subsets. In the absence of the feeder layer, once the primary cells had reached saturation density and were divided for further expansion, very few of them became adherent generating in general aborted colonies. These self-replicating epithelial cells showed expression of p63 (>70% positive) (Fig. 1T) and nestin (not shown), CK-13/CK-10 (>60% positive) (Fig. 1U), CK-19 (>90% positive) (Fig. 1V) and were constantly CK-3 negative (Fig. 1W). Ki-67 were rare (< 2.5% positive) and PAS-positive goblet cells were never detected in these late cultures.

In addition, these cells expressed *CD133* mRNA, as detected by RT-PCR analysis (Fig. 1X). Negative controls included murine NIH.3T3 fibroblasts and primary cultured human corneal fibroblasts (the latter not shown).

These findings thus demonstrated a selected expansion of an enriched and rather homogeneous subset of conjunctival epithelial precursor cells with phenotypic characteristics of basal epithelial cells. Contamination of corneal epithelial elements was excluded by no expression of CK-3 by the conjunctival epithelial cells, in line with previous reports (Joseph *et al.*, 2004, Papini *et al.*, 2005). Probably in response to the culture conditions, all samples from different donors revealed that early in culture the most superficial levels sharply detached, including the positive CK-19 and less intensively positive CK13/CK-10 cells and goblet cells which were all released in the medium, while the inner basal and part of the parabasal cell compartment containing p63 and nestin positive cells remained stably anchored to the submucosa. At T2, the new epithelium appeared slightly different from the one observed at T0. It was probably generated from the basal and parabasal keratinocyte precursor cells that remained attached to the fragment at T1. These cells were rapidly adapting to the culture conditions entering into an active proliferative phase (judging by the increasing Ki-67 staining and constantly low apoptotic cells), as reported in general in squamous epithelia maintained in similar cultures (Michellini *et al.*, 2004; Papini *et al.*, 2005). The basal precursor cells of the conjunctival epithelium kept for at least 2 weeks in culture within their natural environment, seem to have a less complex structure with simple patterns that generate a single phenotypic lineage and apparently have more autonomy with less dependence in extra-epithelial influences for their patterning and survival. Consistent with previous observations (O'Connell *et al.*, 2001), we detected no direct relation between p63 expression and cell cycle activity in cultured immature conjunctival basal epithelial cells.

Interestingly, conjunctival epithelial cells were able to migrate and line over the fragment's surface while maintaining their posi-

TABLE 1

ANTIBODIES USED, ANTIGEN DETECTION AND ANTIBODY SPECIFICITY

Antigen	Specificity	Species	Clone	Code	Source	References
CD117	Stem/progenitor cells	Rabbit		A4502	DakoCytomation	Vascotto <i>et al.</i> , 2006
Nestin	Mesenchymal and ocular epithelial precursor cells	Mouse	10c2	SC-23927	Santa Cruz Biotechnology	Borzanic <i>et al.</i> , 2006
p63	Epithelial progenitor cells	Mouse	4A4	SC-8431	Santa Cruz Biotechnology	Signoretti <i>et al.</i> , 2000
MUC5AC	Goblet cells	Mouse	45M1	SC-21701	Santa Cruz Biotechnology	Bara <i>et al.</i> , 1991
Ki-67	Proliferating cells	Mouse	MIB-1	M7240	DakoCytomation	Borre <i>et al.</i> , 1998
CK-13/CK-10	Conjunctival basal cells	Mouse	DE-K13	M 7003	DakoCytomation	Itakura <i>et al.</i> , 1996;
CK-19	Conjunctival epibasal cells	Mouse	RCK108	M-0888	DakoCytomation	Stasiak <i>et al.</i> , 1989
CK-3	Corneal epithelium	Mouse	AE5	CBL 218	Cymbus	Papini <i>et al.</i> , 2005
Vimentin	Endothelial and stromal cells	Mouse	VIM3B4	M-7020	DakoCytomation	Azumi and Battifora 1987
CD45	Leukocytes	Mouse	2B11	M 0701	DakoCytomation	Warne <i>et al.</i> , 1983
Pan-Cytokeratins	All epithelial cells	Mouse	AE1/AE3	M3515	DakoCytomation	Tseng <i>et al.</i> , 1982

tion as a lining sheet of cells that differentiate and maintain tissue structure and function.

The presence of fibroblasts as feeder cells markedly enhanced the prolonged survival and self-renewal capacity of these cells, thus demonstrating the requirement of an exogenous suitable micro-environmental *niche* for epithelial conjunctival stem/precursor cells.

The conjunctiva normally contains *in vivo* all the components of the myeloid and tertiary lymphoid compartment, which are fundamental for immune defense of the ocular surface (Yamagami *et al.*, 2005). In our present study, at T0 blood-derived monocyte/dendritic-like cells (judging from the morphology of CD45⁺ cells) were present close to the basal lamina in the underlying stroma and infiltrating within the multilayered epithelium of the conjunctiva. At T1, when the upper epithelium detached, these cells were almost completely absent in the left epithelium, while their presence was still consistent in the underlying stroma. At T2, monocytes/dendritic-like cells were found again infiltrating the newly reconstituted epithelium, suggesting that their precursors had remained within the capillaries and maintained their capacity to migrate. The method offers important advantages, such as monitoring inducing factors or drugs affecting subpopulations of resident leukocytes within the conjunctiva, or determining how monocyte/dendritic-like cells can be induced to migrate and differentiate into the intra-epithelial compartment.

Experimental Procedures

Human conjunctival tissue

Six human conjunctival explants were obtained from the Corneal Tissue and Cell Bank of Tuscany, Center "P. Perelli", Lucca, Italy. The tissue explants were maintained in EUSOL-C (Graft TEC, Alchimia Transplant s.r.l., Padova, Italy, cat. N° REF-10040) for 2-4 days at 4°C to avoid contamination before being processed.

Cultures

Tissue fragments and dispersed epithelial cells were maintained in EpiLife medium as described in Papini *et al.*, 2005.

3D- organotypic cultures on gelatin sponges

The method was adapted from Chishima *et al.* (1997) with modifications (Papini *et al.*, 2005).

Isolation and expansion of epithelial basal stem/precursor cells in co-culture with feeder NIH.3T3 fibroblasts

Prior to culture conjunctival fragments were treated as described in Papini *et al.*, 2005.

Histology and immunohistochemistry

At each given pre-determined time interval (i.e. at T0, T1 and T2) of culture, tissue fragments were embedded in paraffin and sections (<5 µm of thickness) were stained with Gill's hematoxylin (Bio-optica, Milan, Italy,) and eosin (Eosin Y Sigma-Aldrich). PAS-staining was used for goblet detection (Pellegrini *et al.*, 1999). Preliminary experiments performed using monoclonal antibody MUC5AC (Santa Cruz biotechnology, Santa Cruz, CA, U.S.A.) demonstrated the same targeting as the PAS reaction, as previously reported. Table 1 reports each antibody used, antigens detected, antibody specificity, antibodies's clones for monoclonal antibodies, source and references. Cells grown in monolayer on glass culture-chamber slides were first fixed for 6 min in absolute ethanol and then stained with antibodies. Immunohistochemistry was performed on sections using Dako Envision+TM System HRP (DakoCytomation, Carpinteria, CA, USA). Ki-67 (clone MIB-1) was revealed by polyclonal rabbit IgG conjugated with FITC (DakoCytomation). In order to measure cell apoptosis, tunel technology was performed by using *In Situ* Cell Death Detection Kit (Roche, Penzberg, Germany) and the detection of the DNA strand break was performed by fluorescent microscopy. Percentage of positive cells was evaluated after counting 500 cells in at least 3 different sections. All samples stained by immunofluorescence were counterstained by D.A.P.I. (Sigma-Aldrich).

RT-PCR analysis for CD133 expression

Total RNA was extracted from conjunctival epithelial cells according to the protocol of the manufacturer (QIAamp RNA Mini Kit, Qiagen GmbH, Hilden, Germany). Human cDNA CD133 (GenBank accession n° AF027208) was amplified (forward primers 5'CAGTCTGACCAGCGTGAAAAA3' and reverse primers 5'TTCCATCCGGGTCTGTCTCTAA3'), using Taq Platinum (Invitrogen, Merelbenke, Belgium). RT-PCR was performed following the protocol of the manufacturer (ImProm-II™ Reverse Transcription System, Promega Madison, WI, U.S.A.) obtaining a cDNA sequence of 181 bp.

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References

- AZUMI N. and BATTIFORA H. (1987). The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. A comprehensive immunohistochemical study on formalin- and alcohol-fixed tumors. *Am. J. Clin. Pathol.* 88: 286-296.
- BARA J., GAUTIER R., MOURADIAN P., DECAENS C. and DAHER N. (1991). Oncofetal mucin M1 epitope family: characterization and expression during colonic carcinogenesis. *Int. J. Cancer* 47: 304-310.
- BORRE M., STAUSBOL-GRON B., NERSTROM B. and OVERGAARD J. (1998). Immunohistochemical BCL-2 and Ki-67 expression predict survival in prostate cancer patients followed expectantly. *Prostate Cancer* 5: 268-275.
- BOZANIC D., BOCINA I. and SARAGA-BABIC M. (2006). Involvement of cytoskeletal proteins and growth factor receptors during development of the human eye. *Anat. Embryol.* 211: 367-77.
- CHISHIMA T., YANG M., MIYAGI Y., LI L. and TAN Y. (1997). Baranov E, Shimada H, Moossa AR, Penman S, Hoffman RM. Governing step of metastasis visualized in vitro. *Proc. Natl. Acad. Sci. USA* 94: 11573-11576.
- CONNOR C.J., NAKAMURA T., QUANTOCK A.J. and KINOSHITA S. (2006). The persistence of transplanted amniotic membrane in corneal stroma. *Am. J. Ophthalmol.* 141: 190-192.
- ITAKURA Y., SASANO F., DATE F., KATO K., SEKINE H., MORI S. and NATURA H. (1996). DNA ploidy, P53 expression and cellular proliferation in normal epithelium and squamous dysplasia of non-cancerous and cancerous human oesophagi. *Anticancer. Res.* 16: 201-208.
- JOSEPH A., POWELL-RICHARDS A.O., SHANMUGANATHAN V.A. and DUA H.S. (2004). Epithelial cell characteristics of cultured human limbal explants. *Br. J. Ophthalmol.* 88: 393-398.
- LAVKER R.M., WEI Z.G. and SUN T.T. (1998). Phorbol ester preferentially stimulates mouse fornical conjunctival and limbal epithelial cells to proliferate in vivo. *Invest. Ophthalmol. Vis. Sci.* 39: 301-307.
- MELLER D., PIRES R. T. and TSENG S.C. (2002). Ex vivo preservation and expansion of human limbal epithelial stem cells on amniotic membrane cultures. *Br. J. Ophthalmol.* 86: 463-471.
- MICHELINI M., ROSELLINI A., SIMONCINI T., PAPINI S. and REVOLTELLA R.P. (2004). A three-dimensional culture of the human exocervix for studying epithelial differentiation and migrating leukocytes. *Differentiation* 72: 1-12.
- O'CONNELL J.T., MUTTER G.L., CVIKO A., NUCCI M., QUADE B.J., KOZAKIEWICH H.P., NEFFEN E., SUN D., YANG A., MCKEON F.D. and CRUM C.P. (2001). Identification of a basal/reserve cell immunophenotype in benign and neoplastic endometrium: a study with the p53 homologue p63. *Gynecol Oncol.* 80: 30-36.
- PALADINO G., MARINO C., LA TERRA MULE S., CIVIALE C., RUSCIANO D. and ENEA V. (2004). Cytokeratin expression in primary epithelial cell culture from bovine conjunctiva. *Tissue Cell* 36: 323-332.
- PAPINI S., ROSELLINI A., CAMPANI D., DEMATTEIS A., SELLI C. and REVOLTELLA R.P. (2004). Selective growth of epithelial basal cells from human prostate in a three-dimensional organ culture. *Prostate* 59: 383-392.
- PAPINI S., ROSELLINI A., NARDI M., GIANNARINI C. and REVOLTELLA R.P. (2005). Selective growth and expansion of human corneal epithelial basal stem cells in a three-dimensional-organ culture. *Differentiation* 73: 61-68.
- PELLEGRINI G., GOLISANO O., PATERNA P., LAMBIASE A., BONINI S., RAMA P. and DE LUCA M. (1999). Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J. Cell. Biol.* 145: 769-782.
- PFLUGFELDER S.C., TSENG S.C., YOSHINO K., MONROY D., FELIX C. and REIS B.L. (1997). Correlation of goblet cell density and mucosal epithelial membrane mucin expression with rose bengal staining in patients with ocular irritation. *Ophthalmology* 104: 223-235.
- POTTEN C.S. (1997). Stem cell. *Academy Press, Harcourt Brace & Co, London UK.*
- SANTOS M.S., GOMES J.A., HOFLING-LIMA A.L., RIZZO L.V., ROMANO A.C. and BELFORT R. JR. (2005). Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. *Am. J. Ophthalmol.* 140: 223-230.
- SHATOS M.A., RIOS J.D., HORIKAWA Y., HODGES R.R., CHANG E.L., BERNARDINO C.R., RUBIN P. A.D. and DARTT D.A., (2003) Isolation and characterization of conjunctival goblet cells. *Inv. Ophthalmol. Vis. Sci.* 44: 2477-2486.
- SIGNORETTI S., MONTIRONI R., MANOLA J., ALTIMARI A., TAM C., BUBLEY G., BALK S., THOMAS G., KAPLAN I., HLATKY L., HAHNFELDT P., KANTOFF P. and LODA M. (2000). p63 is a prostate basal cell marker and is required for prostate development. *Am. J. Pathol.* 157: 1769-1775.
- STASIAK P.C., PURKIS P.E., LEIGH I.M. and LANE E.B. (1989). Keratin 19: predicted amino acid sequence and broad tissue distribution suggest it evolved from keratinocyte keratins. *J. Invest. Dermatol.* 92: 707-716.
- TSENG S.C., JARVINEN M.J., NELSON W.G., HUANG J.W., WOODCOCK-MITCHELL J. and SUN T.T. (1982). Correlation of specific keratins with different types of epithelial differentiation: monoclonal antibody studies. *Cell* 30: 361-72.
- VASCOTTO S.G. and GRIFFITH M. (2006). Localization of candidate stem and progenitor cell markers within the human cornea, limbus and bulbar conjunctiva in vivo and in cell culture. *Anat. Rec. A. Discov. Mol. Cell. Evol. Biol.* 288: 921-931.
- WARNKE R.A., GATTER K.C., FALINI B., HILDRETH P., WOOLSTON R.E., PULFORD K., CORDELL J.L., COHEN B., DE WOLF-PEETERS C. and MASON D.Y. (1983). Diagnosis of human lymphoma with monoclonal antileukocyte antibodies. *N. Engl. J. Med.* 309: 1275-1281.
- WEI Z.G., LIN T., SUN T.T. and LAVKER R.M. (1997). Clonal analysis of the in vivo differentiation potential of keratinocytes. *Invest. Ophthalmol. Vis. Sci.* 38: 753-761.
- YAMAGAMI S., YOKOO S., USUI T., YAMAGAMI H., AMANO S. and EBIHARA N. (2005). Distinct populations of dendritic cells in the normal human donor corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 46: 4489-4494.

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