Expression of ΔNp63 in Response to Phorbol Ester in Human Limbal Epithelial Cells Expanded on Intact Human Amniotic Membrane

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Purpose. To evaluate the effect of phorbol 12-myristate 13-acetate (PMA) on the expression of ΔNp63 in human limbal epithelial cells (HLECs) during ex vivo expansion on amniotic membrane (AM).

Methods. Primary HLECs were cultured either on AM or plastic surfaces and were treated with 1 μg/mL PMA for 24 hours. Expression of ΔNp63 and the differentiation-associated gap junctional protein connexin 43 (Cx43) were studied by laser scanning microscopy.

Results. The labeling index (LI) of ΔNp63 was higher in HLECs cultured on AM than in HLECs grown on plastic (81.4% ± 12.2% and 66.6% ± 16.5%, respectively; $P < 0.001$). After PMA treatment, ΔNp63 expression in HLECs on plastic dramatically decreased to 20.4% ± 11.4%. However, HLECs cultured on AM showed only a moderate decrease in ΔNp63 expression (56.4% ± 10.9%, $P < 0.001$) after PMA treatment. It was also observed that 72.8% ± 17.5% of the ΔNp63-positive cells in untreated HLECs cultured on plastic coexpressed Cx43, in contrast to only 21.9% ± 3.7% of the ΔNp63-positive cells in HLECs cultured on AM ($P < 0.001$). The latter indicates that growth over AM preserves limbal phenotype, whereas growth over plastic surface induces or allows transition toward corneal peripheral phenotype.

Conclusions. ΔNp63 protein is typically detected in human corneal epithelial cells with high proliferative capacity including, limbal epithelial stem cells (SCs) and probably also transient amplifying cells (TACs). AM supports ΔNp63 protein expression in HLECs and maintains a higher resistance against phorbol ester–induced differentiation, indicating that characteristic signs of limbal epithelial progenitor cells may be preserved during ex vivo expansion on AM. (*Invest Ophthalmol Vis Sci.* 2003;44:2959–2965) DOI:10.1167/iovs.02-0776

The protein p63, which has been recently identified as a very close homologue of the prototypical tumor suppressor p53[1,2] has been shown to be transcribed into at least six different protein isotypes with various functions. In contrast to p53, p63 exhibits a rather tissue-specific distribution.[1,2] These transcripts are divided into two groups that are distinguished by the presence or absence of an acidic NH_2-terminal transactivation domain. Protein isotypes containing an acidic NH_2-terminal transactivation domain (TAp63) are capable of inducing apoptosis,[3,4] whereas those without the NH_2-terminal transactivation domain may function as dominant–negative p63 isotypes (ΔNp63). In addition, alternative splicing of the p63 gene leads to three different C-terminal forms, α, β, and γ, for both TAp63 and ΔNp63.[1,2] ΔNp63α has been reported to be strongly expressed in the epidermis[5–7] and is selectively expressed in the basal cell compartment of a variety of epithelial tissues (e.g., cervix, urogenital tract, prostate, breast,[5–10] and cornea).[11] Therefore, it has been suggested that ΔNp63α may be essential in the maintenance of a stem cell (SC) population in various epithelial tissues. Furthermore, ΔNp63 has been also implicated in the control of epithelial cell proliferation and migration during wound healing.[7,9]

SCs maintain tissue integrity by continuous generation of cells destined to self-renewal of the target tissue. SCs are long-living, relatively undifferentiated cells that exhibit a high potential for cell division (i.e., they are clonogenic cells). SCs exhibit a very low mitotic activity and thus are slow cycling. Replication of SCs gives rise to, on average, one SC that remains in the SC environment and one transit amplifying cell (TAC) that replicates relatively rapidly but has only a limited proliferative potential and therefore has a short life span.[12–14] Recent cumulative evidence indicates that SCs of the corneal epithelium are localized in the limbus, the narrow transitional zone between the cornea and the bulbar conjunctiva.[12,15–20] Three subpopulations of limbal epithelial cells have been distinguished in vitro[21,22]: holoclones, which correspond to SCs; paraclones, which contain most of the TACs; and meroclones, which are considered to represent early, young TACs with a greater proliferative capacity than TACs found in paraclones.[21,22] Pellegrini et al.[11] observed that the ΔNp63 transcription factor is expressed strongly in holoclones (SC) and weakly in meroclones (early TACs), but is absent in paraclones (TACs), indicating that ΔNp63 may be essential in the maintenance of corneal epithelial SC population. Therefore, the level of ΔNp63, but not the presence, can serve as an indicator to cell stemness.

Previous studies[23,24] have shown that cultured epidermal cells have divergent responses toward tumor promoter phorbol esters characterized by proliferation of less differentiated cells, whereas more mature cells are induced to accelerated terminal differentiation. Moreover, in vitro studies[25,26] have identified that limbal epithelial SCs are also more resistant to tumor promoter phorbol esters than TACs. Furthermore, phorbol esters induce corneal epithelial cells to proliferate and thereby exhaust the proliferative capacity of TACs that finally undergo terminal differentiation. In contrast, SCs preserve their proliferation capacity after withdrawal of phorbol ester.[25,26] This effect is commonly used to select SCs in vitro by means of reduction or elimination of the TAC subpopulation.

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Various ocular surface disorders are characterized by limbal SC deficiency (for review see also Refs. 17,19,27) and the renewal of the limbal epithelial SC population is required for restoration of vision and normal corneal surface (for reviews see also Refs. 28–30). Recent advances in tissue engineering techniques have facilitated the ex vivo expansion of HLECs using different culture substrates or adjuncts such as amniotic membrane (AM), fibrin, or 3T3 feeder layer systems.[31–41] Ex vivo expansion of autologous and allogenic HLECs on AM has been considered an attractive technology for its potency in a variety of clinical applications.[32,33,35,36] Moreover, experimental studies have shown that outgrowth rate, cell-cycle kinetics, and cell phenotype characteristic of limbal and conjunctival epithelial progenitor cells are preserved during ex vivo expansion on AM,[38,39,42,43] supporting the assumption that this tissue engineering technique might be useful to renew SC populations of the entire ocular surface.

In the present study we sought to study whether characteristic features of the limbal epithelial progenitor cells are preserved using this tissue engineering technique. To this end, we determined the expression of ΔNp63 protein in HLECs during ex vivo expansion on preserved human AM and the effect of phorbol 12-myristate 13-acetate (PMA) treatment on ΔNp63 expression. The data indicates that AM supports ΔNp63 protein expression in HLECs and maintains a higher resistance against phorbol ester-induced differentiation indicating that characteristic signs of limbal epithelial progenitor cells may be preserved during ex vivo expansion on AM.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), Ham's F-12 nutrient medium, HEPES-buffer solution, amphotericin B, Hank's balanced salt solution (HBSS), and fetal bovine serum (FBS) were purchased from Invitrogen GmbH (Karlsruhe, Germany). Dimethyl sulfoxide (DMSO), cholera toxin (subunit A), hydrocortisone, epidermal growth factor (EGF), Dispase II, gentamicin, insulin-transferrin-sodium selenite media supplement, poly-L-lysine, bovine serum albumin (BSA), RNase A, glycerol, saline sodium citrate (SSC) buffer, PMA, and embedding medium (Moviol) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). The nylon transfer membrane (Hybond-N+, pore size: 0.45 μm) was obtained from Amersham Biosciences (Freiburg, Germany). The tissue culture plastic plates (six-well) and plastic culture dishes were from Falcon (BD Biosciences, Bedford, MA). Culture plate inserts used to construct the culture system were from Millipore (Eschborn, Germany). Mouse monoclonal antibody against ΔNp63 protein,[8,9] clone 4A4, was purchased from BD Biosciences-PharMingen (Heidelberg, Germany) and an affinity purified rabbit polyclonal antibody to Cx43 was obtained from Alpha Diagnostic International (San Antonio, TX). Fluorescence-conjugated (Alexa Fluor 546) goat anti-mouse exhibiting a orange fluorescence, fluorescence-conjugated (Alexa Fluor 633) goat anti-rabbit (red fluorescence), and green nucleic acid stain (SYTO 16) were obtained from Molecular Probes, Leiden, The Netherlands.

Tissue Procurements

Human tissue was handled according to the tenets of the Declaration of Helsinki. Corneoscleral tissue from human donor eyes, aged between 30 to 50 years, was obtained from the Cornea Bank of Essen (Germany). After obtaining proper informed consent, AM was processed and preserved according to Tseng et al.[44] Briefly, the human placenta was collected shortly after elective cesarean delivery. Human immunodeficiency virus, human hepatitis virus types B and C, and syphilis were excluded by serologic tests. In addition, hepatitis virus type C was excluded by PCR. Under a lamellar-flow hood, the placenta was cleaned of blood clots with sterile phosphate-buffered saline (PBS) solution (pH 7.4) containing 50 μg/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL neomycin, and 2.5 μg/mL amphotericin B (Invitrogen GmbH). The amnion was separated from the chorion by blunt dissection and flattened onto a positively charged nylon transfer membrane (Hybond-N+; Amersham Biosciences) with the epithelium-basement membrane surface up. The nylon membrane with the adherent AM was stored at −80°C in a sterile vial containing DMEM (Invitrogen) and sterile glycerol (Sigma-Aldrich Chemie GmbH) at the ratio of 1:1 (vol/vol).

Three-Dimensional Cultures on AM

After careful removal of excessive sclera, iris, corneal endothelium, conjunctiva, and Tenon's capsule, the remaining tissue was placed in a culture dish, and limbus explants of approximately 1 × 1.5 × 2.5 mm were cut with a scalpel. Preserved human intact AM was fastened onto a culture insert as recently reported.[38] On the center of the AM a limbal explant was placed and cultured in supplemental hormonal epithelial medium (SHEM) consisting of an equal volume of HEPES-buffered DMEM containing bicarbonate and Ham's F12. The medium was supplemented with 5% FBS, 0.5% DMSO, 2 ng/mL mouse EGF, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin, 5% FBS, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B.[38,43,45] Cultures were incubated at 37°C, 5% CO_2, and 95% humidity for 4 to 5 weeks. A set of cultures was treated with medium containing 1 μg/mL PMA for 24 hours and afterward switched to PMA-free medium for 3 days. All cell cultures were terminated and fixed in 4% paraformaldehyde PBS for 30 minutes. Four cultures per group were included in the present study. Air lifting of cell cultures, as described by several groups,[34,45] was avoided to minimize the promotion of differentiation processes in vitro.

HLECs cultured on plastic dishes were used as the control group. Although the interactions of HLECs with the substrate by adhesion proteins and the effect of these on development, growth, and differentiation of epithelial cells are important elements to consider, conventional cultures on plastic were chosen to demonstrate the suitability of AM as a substrate and carrier for transplantation in ocular surface tissue engineering techniques.

Immunofluorescence Studies and Confocal Laser Scanning Microscopy

Ten-micrometer-thick sections of cornea were cut at −20°C with a cryotome (Kryostat 1720; Leica, Heidelberg, Germany), collected on poly-L-lysine–coated glass slides and fixed in 4% paraformaldehyde in PBS for 30 minutes. Corneal sections and cell cultures were pretreated with 1% Triton X-100 for 10 minutes. In addition, permeabilization was performed with 10% methanol for 10 minutes. BSA (3%) was applied for 1 hour at room temperature to block nonspecific binding sites. Afterward, the specimens were incubated overnight with mouse monoclonal antibody against ΔNp63 at 1:100 in PBS. After extensive washing in PBS, the specimens were incubated for 1 hour with a secondary fluorescence-conjugated goat anti-mouse antibody (1:400; Alexa Fluor 546; Molecular Probes) that exhibits an orange to red fluorescence. Thereafter, specimens were treated with RNase A in 2× SSC buffer for 30 minutes at 37°C. Green fluorescent nucleic acid stain (SYTO; Molecular Probes) was used as a nuclear counterstain. Finally, sections were mounted with in embedding medium (Moviol; Sigma-Aldrich Chemie, GmbH) and images were obtained by means of a confocal laser scanning microscope (LSM 510; Carl Zeiss Meditech, Oberkochen, Germany). The ΔNp63 expression was calculated by counting positive nuclei in at least 10 200 × 200-μm^2 areas of each HLEC culture ($n = 4$), and the labeling index (LI) of ΔNp63 was displayed as the number of ΔNp63 positive cells/total number of cells × 100%.

Coexpression of ΔNp63 and Cx43 was shown by incubating HLEC cultures with mouse anti-p63 and a rabbit affinity purified polyclonal antibody against Cx43 (both at 1:100 in PBS, overnight). Afterward, specimen were incubated with fluorescence-conjugated goat anti-

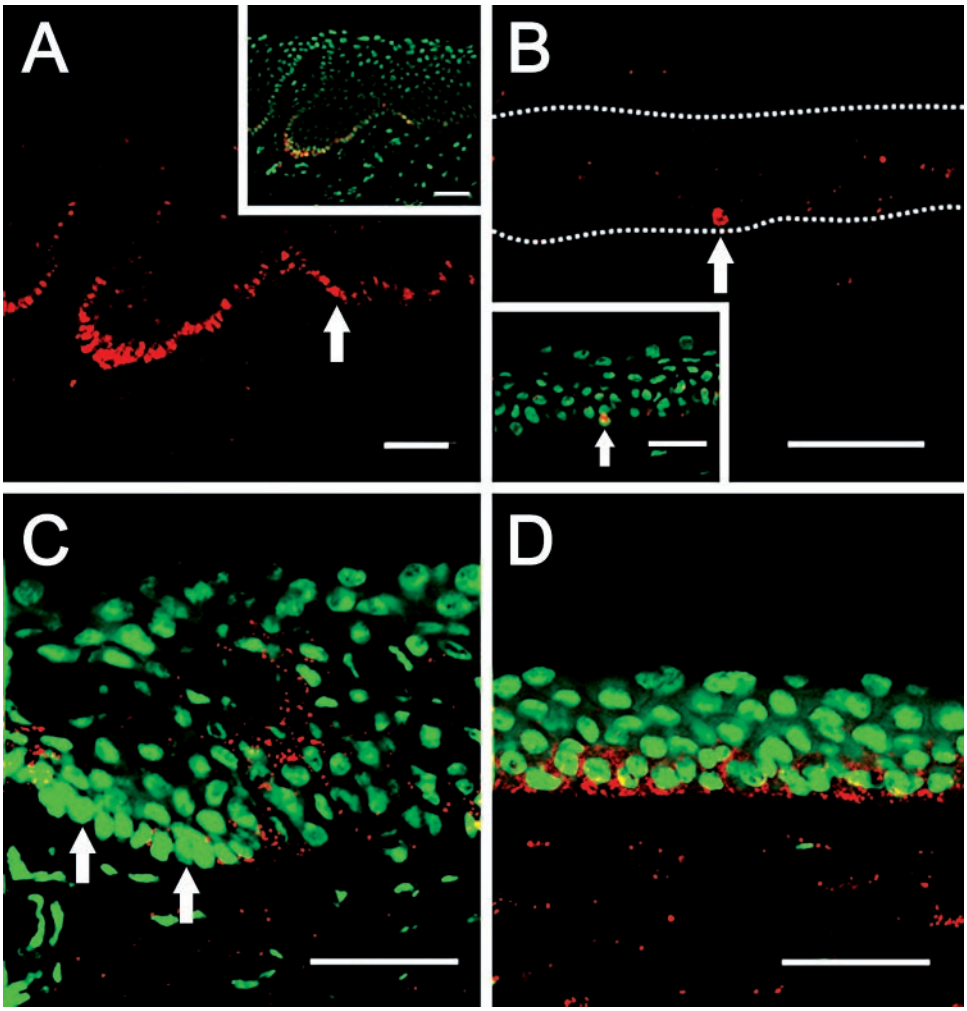

FIGURE 1. Expression of ΔNp63 protein (**A**, **B**) and Cx43 (**C**, **D**) in human limbal and corneal epithelial cells. (**A**) In vivo, nuclear ΔNp63 staining was strongly detected in the basal layer of limbal epithelium and weakly in basal epithelial cells of the peripheral cornea. *Arrow*: border between limbus (*left*) and cornea (*right*). *Inset*: A counterstain of the presented area. (**B**) ΔNp63 was weakly detected in basal epithelial cells of peripheral cornea. A counterstain of the presented area is illustrated in the *inset*. (**C**, **D**) Subpopulations (*arrows*) of the basal limbal epithelium did not express Cx43; however, strong punctate labeling of Cx43 (*red*) was observed in basal and suprabasal epithelial cells of the peripheral cornea (**D**). Cell nuclei were counterstained with SYTO-16 (*green*). Scale bar, 50 μm.

mouse (1:400; Alexa Fluor 546; Molecular Probes) for 1 hour, followed by an incubation with fluorescence-conjugated goat anti-rabbit (Alexa Fluor 633; Molecular Probes; 1:400 for 1 hour; dark red fluorescence). Quantitative analysis of ΔNp63 and Cx43 expression between the described experimental groups was performed using the same recording parameters (objective, stack size, pinhole, gain amplitude, and contrast) on the laser scanning microscope. LI of Cx43 was displayed as number of Cx43-positive cells/total number of cells × 100%. In addition, the number of ΔNp63-positive cells coexpressing Cx43 (Cx43 and ΔNp63 positive cells/total number of ΔNp63-positive cells × 100) was calculated.

Statistical Methods

The statistical analysis was performed by an ANOVA (analysis of variance) with an additional post hoc analysis (the Scheffé test).

RESULTS

Distribution of ΔNp63 and Cx43 in Human Limbus and Cornea

Analysis of the ΔNp63 expression pattern revealed a strong nuclear labeling in basal epithelial cells of the limbus (Fig. 1A), whereas ΔNp63 was almost absent in suprabasally located limbal epithelial cells. Labeling was occasionally detected in basal epithelial cells of the peripheral cornea (Figs. 1A, 1B) and absent in the central cornea.

Subpopulations of basal limbal epithelial cells were devoid of Cx43, with isolated and faint Cx43-positive labeling of suprabasal epithelial cells (Fig. 1C). However, in the cornea, a strong and punctate membrane-bounded pattern for Cx43 was predominantly observed in the basal epithelial cell layer and to lesser extent in the suprabasal epithelial cells (Fig. 1D).

Expression of ΔNp63 Protein in Ex Vivo Expanded HLECs before and after PMA Treatment

Next, we investigated the influence of PMA-induced differentiation on ΔNp63 expression. The data showed a high and homogeneous expression of ΔNp63 protein in HLECs expanded on AM prior and after PMA exposure (Table 1). The LI for ΔNp63 in the untreated group (Fig. 2A, 2B; PMA−) was higher in HLECs cultured on AM than in HLECs cultured on plastic (81.4% ± 12.1% and 66.6% ± 16.5%, respectively, $P < 0.001$). After a 24-hour treatment with PMA, decreased ΔNp63 expression was evidenced in both groups (Figs. 2C, 2D, Table 1). HLECs cultured on AM showed a higher resistance to phorbol ester than HLECs grown on plastic, which was reflected by a significantly higher LI for ΔNp63 in HLECs cultured on AM (56.4% ± 10.9%) compared with HLECs grown on plastic (20.42% ± 11.4%, $P < 0.001$, Table 1).

We also noted some topographic differences of the LI for ΔNp63 in the different cell populations. ΔNp63 expression in HLECs on AM was homogenous, whereas ΔNp63 labeling of HLECs cultured on plastic was reduced from the center of the

TABLE 1. The Effect of PMA on the LI of ΔNp63 in HLECs

	Total	Central Area	Peripheral Area
HLEC/AM PMA−	81.4 ± 12.2	80.6 ± 10.4	82.3 ± 13.8
HLEC/PL PMA−	66.6 ± 16.5*	72.8 ± 18.1	60.4 ± 12.2†
HLEC/AM PMA+	56.4 ± 10.9*	58.8 ± 11.6	54.0 ± 9.9
HLEC/PL PMA+	20.4 ± 11.4*	26.9 ± 8.6	13.9 ± 10.0†

LI data are expressed in percent ± SD. HLEC/AM = Human limbal epithelial cells cultured on AM; HLEC/PL, human limbal epithelial cells cultured on plastic; PMA+, treatment with PMA (1 μg/mL for 24 hours); PMA−, no treatment with PMA.

* $P < 0.001$ compared with HLEC/AM PMA−.

† $P < 0.05$ compared with central area of corresponding HLEC culture.

organ culture to the periphery of the culture (Table 1). LI of ΔNp63 in the central and peripheral areas of untreated HLEC cultures grown on plastic was 72.8% ± 18.1% and 60.4% ± 12.2%, respectively ($P < 0.05$) indicating increased differentiation processes of HLECs toward peripheral culture compartments. After PMA treatment, this difference remained consistent (LI = 26.9% ± 8.6% for central and 13.9% ± 10% for peripheral areas; $P < 0.05$, Table 1).

Colocalization Studies of ΔNp63 and Cx43 in HLECs

The observed response of HLECs to PMA suggested the existence of a subpopulation of ΔNp63-positive cells that are sensitive for PMA. Therefore, these cells might represent early TAC, which also express ΔNp63 as previously suggested by Pellegrini et al.[11] To identify this subpopulation of cells in culture, we examined coexpression of ΔNp63 with the differentiation-associated marker Cx43 (Table 2). In the untreated groups, a higher expression of Cx43 was observed in HLECs cultured on plastic (63.1% ± 21.5%) than in HLECs on AM (20.0% ± 2.9%, $P < 0.001$, Table 1) confirming our previous data.[42] Moreover, Cx43 appeared as small, immunoreactive dots in HLECs cultured on AM that rarely encircled cells, whereas HLECs cultured on plastic exhibited Cx43-immunoreactive signals that had an elongated appearance and clearly encircled the cells. Of note, 72.8% ± 17.5% of ΔNp63-positive cells in untreated HLECs cultured on plastic were labeled by Cx43, in contrast to only 21.9% ± 3.7% in HLECs grown on AM (Figs. 3A, 3B; $P < 0.001$). This indicates that growth over AM preserves limbal phenotype, whereas growth over plastic sur-

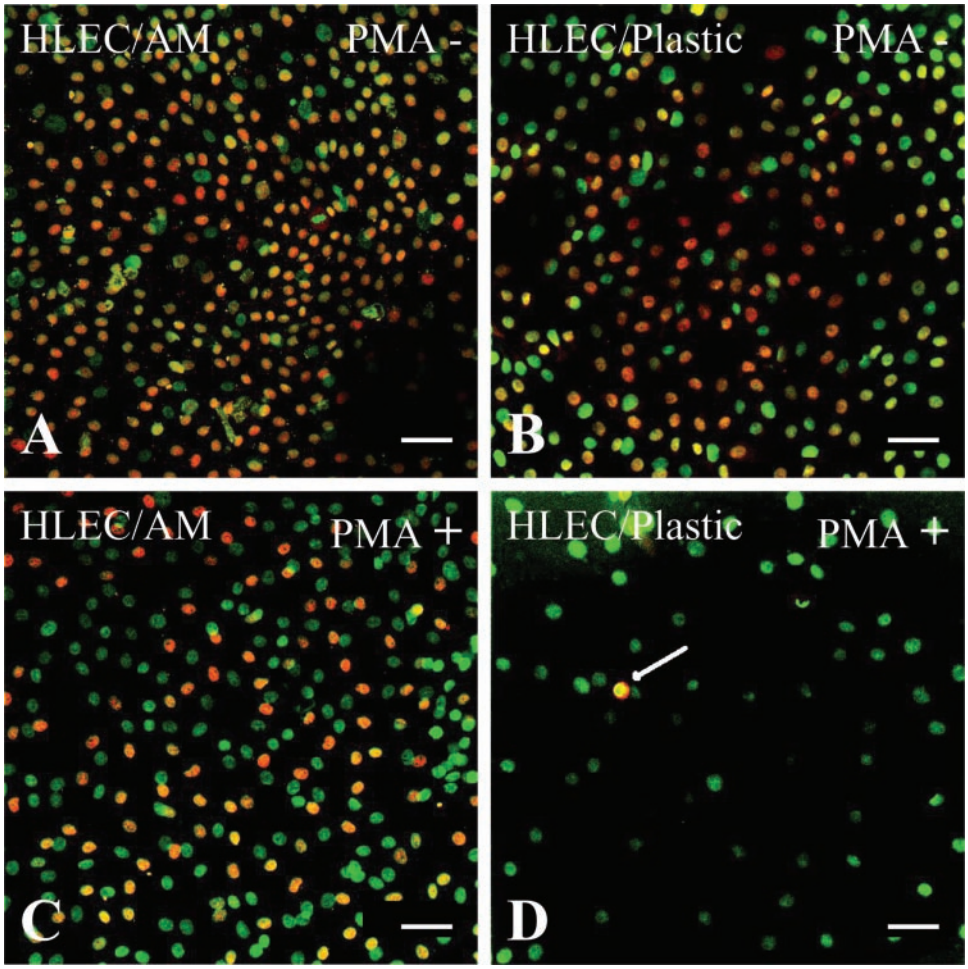

FIGURE 2. Confocal laser scanning microscopy of ΔNp63 expression in HLECs on AM or plastic. Immunoreactivity of ΔNp63 protein (*orange-red*) is higher in HLECs cultured on AM (**A**) compared with HLECs cultured on plastic (**B**). HLECs on AM (**C**) showed a higher resistance against the phorbol ester PMA compared with HLECs on plastic (**D**). Cell nuclei were counterstained with nucleic acid stain (*green*). Scale bar, 50 μm.

TABLE 2. Colocalization LI of ΔNp63 and Cx43

	Cx43+ and ΔNp63+ LI*	Cx43 LI†
HLEC/AM	21.9 ± 3.7	20.0 ± 2.9
HLEC/PL	72.8 ± 17.5‡	63.1 ± 21.5‡

LI data are expressed as percent ± SD. Abbreviations are as defined in Table 1.

* Cx43 expression in ΔNp63 positive cells: Cx43- and ΔNp63-positive cells/total number of ΔNp63-positive cells × 100.

† Number of Cx43 positive cells/total number of cells × 100.

‡ $P < 0.001$ compared with HLEC/AM.

face induces or allows transition toward corneal peripheral phenotype.

DISCUSSION

The p63 gene encodes at least six different protein isotypes resulting from alternative splicing and the use of an alternative promoter. The ΔNp63 isotypes that lack the NH_2-terminal transactivation domain block some of the activities of the corresponding full-length proteins.[1] For instance, ΔNp63 isotypes have been shown to exhibit antiapoptotic properties in UV-induced epidermal damage.[4] Expression of p63 appears to vary at different stages of growth and differentiation in a variety of epithelial tissues. Moreover, cell differentiation typically triggers an increase of TAp63 levels and a concomitant decrease of dominant-negative ΔNp63.[1,7,46] In accordance, ΔNp63 has been reported to be strongly expressed in basal epithelial cells of different tissues, such as cervix, urogenital tract, prostate, breast,[5–9] and cornea.[11] Therefore, it has been suggested that ΔNp63α may play an important role in the maintenance of SC populations in various epithelial tissues.

In the present study, we evaluated the expression pattern of ΔNp63 in HLECs ex vivo, cultured on different substrates and the effect of PMA on ΔNp63 expression. Furthermore, colocalization of ΔNp63 and the differentiation-associated antigen Cx43 was examined. The herein presented data on ΔNp63 expression in human cornea and limbus confirm previously published observations.[11] No ΔNp63 expression was observed in the human central cornea, but it was occasionally observed in basal cells of the peripheral cornea. These cells may represent young, or "early", TACs that have been reported also to express ΔNp63.[11] However, ΔNp63 labeling of basal and suprabasal limbal epithelial cells was compared with adjacent cells gradually more dense as recently reported,[11] which may be attributed to the higher sensitivity of the microscopy system used in this study.

Based on the notion that ΔNp63 does not selectively label SCs but also labels young TACs,[11] we used PMA to eliminate ΔNp63-positive young TACs. This is reflected by the dramatic decrease of LI for ΔNp63 in HLECs cultured on plastic compared with HLECs cultured on AM , which indicates that HLECs cultured on AM are more resistant to phorbol ester, a characteristic feature of limbal epithelial progenitor cells.[25,26]

A simple classification of the progeny of limbal epithelial cells based on the discrimination between strong or weak labeling for ΔNp63 is not sufficient for characterization of these cells. We therefore performed double labeling with ΔNp63 and the well-established differentiation associated antigen Cx43. Cx43 is known to be absent in limbal epithelial SCs (Fig. 1C) whereas it is predominantly expressed in TACs localized in limbal suprabasal epithelium and/or corneal basal epithelium.[47,48]

These studies indicate three subpopulations of HLECs in our cell cultures: cells positive for ΔNp63 only, cells positive for Cx43 only, and cells with a simultaneous expression of ΔNp63 and Cx43. According to recent publications,[11,47,48] HLECs with an exclusive ΔNp63 labeling may represent SCs, whereas HLECs labeled with Cx43 only may be mature TACs. The simultaneous labeling for both antigens may indicate young TACs, which still retain a high but limited proliferative capacity and, in contrast to SCs, have already entered into the process of cellular differentiation as indicated by the expression of the differentiation-associated marker Cx43. These observations imply that colocalization of different antigens, as demonstrated by double-labeling and laser confocal microscopy, facilitates the determination of certain cell phenotypes of the spectrum of epithelial cell progeny.

Furthermore, the most ΔNp63-positive HLECs cultured on plastic compared with HLECs cultured on AM were also Cx43 positive, indicating that the substrate specifically regulates the state of differentiation. It is important to point out that the ΔNp63-positive subpopulation of HLECs cultured on AM was predominantly free of Cx43 expression and therefore presumably contained SCs, in contrast to the apparent predominance of TACs in HLECs on plastic. However, further studies are needed to establish a more reliable method that may specifically identify SCs.

Our data also suggest that conventional plastic cell culture dishes provide a relatively unfavorable environment for the growth of epithelial cells. This dramatic contrast suggests the AM culture model as a superior carrier for transplantation of ex

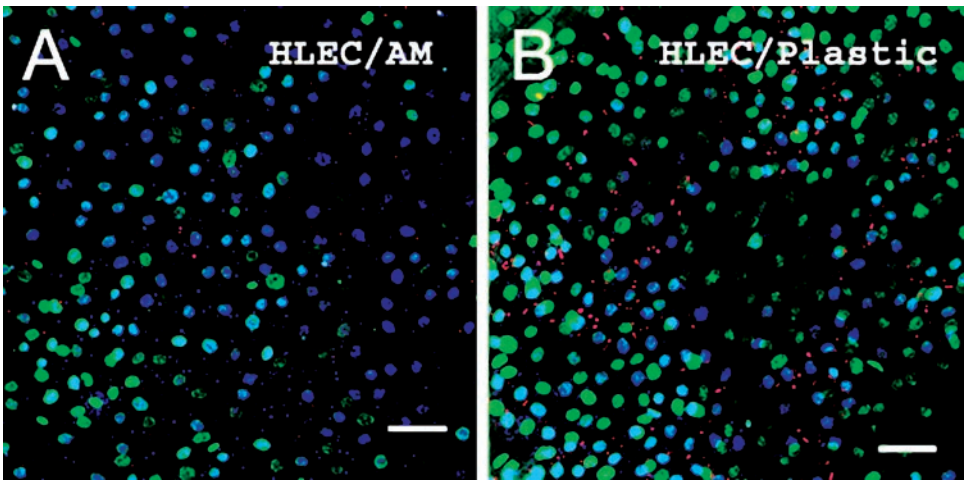

FIGURE 3. Coexpression of ΔNp63 and Cx43 in HLECs on AM or plastic. (**A**) The coexpression of Cx43 (*red*) and ΔNp63 (*blue*, pseudocolor for fluorescent stain [Alexa Fluor 546; Molecular Probes, Leiden, The Netherlands]) was significantly higher in HLECs cultured on plastic (**B**) than on AM (**A**). Cell nuclei were counterstained with nucleic acid stain (*green*). Scale bar, 50 μm.

vivo–expanded HLECs. However, other culture substrates, such as the 3T3 fibroblast feeder layer[31,33,49,50] and fibrin,[37] should be evaluated to compare benefits and disadvantages between these substrates and AM.

In conclusion, in comparison with cell culture on plastic, culture on AM supports ΔNp63 protein expression of HLECs and maintains a higher resistance against phorbol ester. This indicates that characteristic features of limbal epithelial progenitor cells may be preserved during ex vivo expansion on AM, whereas they are suppressed on plastic surfaces. These data support the use of AM as substrate for tissue engineering and an alternative therapeutic strategy for corneal surface reconstruction in distinct ocular surface diseases.

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