# Epithelial-Mesenchymal Transdifferentiation in Pediatric Lens Epithelial Cells

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**Purpose.** Posterior capsule opacification (PCO) is a complication after cataract surgery, particularly in children. Epithelial-mesenchymal transition (EMT) of lens epithelial cells, mediated by transforming growth factor beta (TGFβ), contributes to PCO. However, its pathogenesis in children is poorly understood. We correlated cell growth in culture with patient characteristics, studied gene expression of pediatric lens epithelial cells (pLEC), and examined the effects of TGFβ-2 on these cells in vitro.

**Methods.** Clinical characteristics of children with cataracts correlated with growth behavior of pLEC in vitro. mRNA expression of epithelial (αB-crystallin, connexin-43) and mesenchymal ($\alpha_V$-integrin, α-smooth muscle actin, collagen-Iα2, fibronectin-1) markers was quantified in pLEC and in cell line HLE-B3 in the presence and absence of TGFβ-2.

**Results.** Fifty-four anterior lens capsules from 40 children aged 1 to 180 months were obtained. Cell outgrowth occurred in 44% of the capsules from patients ≤ 12 months and in 33% of capsules from children aged 13 to 60 months, but in only 6% of capsules from children over 60 months. TGFβ-2 significantly upregulated expression of αB-crystallin (HLE-B3), $\alpha_V$-integrin (HLE-B3), collagen-Iα2, and fibronectin-1 (in pLEC and HLE-B3 cells).

**Conclusions.** Patient characteristics correlated with growth behavior of pLEC in vitro, paralleling a higher clinical incidence of PCO in younger children. Gene expression profiles of pLEC and HLE-B3 suggest that upregulation of $\alpha_V$-integrin, collagen-Iα2, and fibronectin-1 are involved in EMT.

Keywords: cataract, children, posterior capsule opacification, epithelial-mesenchymal transformation, lens epithelial cells, transforming growth factor beta-2

Opacification of the lens (cataract) can significantly impair vision. Although more common in older adults, cataracts occur in childhood (congenital and juvenile cataracts). Congenital cataracts are responsible for 5% to 20% of cases of treatable blindness in children.[1] Childhood cataracts may be isolated or in association with other entities, including trauma, infection, inflammation, and metabolic and syndromic disorders.[1,2] A significant proportion of bilateral childhood cataracts is hereditary.[3,4] Visually significant congenital cataracts are usually operated within the first few weeks of life to prevent refractory amblyopia.[5]

Posterior capsule opacification (PCO), an obscuration of the lens capsule after cataract surgery, is especially common in young children. If extending centrally, visual axis opacification (VAO) may result and reduce vision. This postoperative complication has been reported in up to 44% of children within the first year after cataract surgery, and it occurs more frequently in younger children.[6,7] Although surgical techniques such as a primary posterior capsulotomy and anterior vitrectomy reduce the risk for VAO in children, these surgical steps are associated with a higher risk of postoperative complications such as retinal detachment, cystoid macular edema, glaucoma, endophthalmitis, and inflammation.[5,8–12]

The knowledge of the pathogenesis of PCO in children and its prevention remains limited. As it is virtually impossible to remove all lens epithelial cells during cataract surgery, remaining cells can undergo epithelial-mesenchymal transition (EMT), defined as a loss of polarity, cell-to-cell contact, and epithelial characteristics.[13] Transformed cells may proliferate and migrate to the visual axis, where the resulting opacification can necessitate surgical intervention.[14]

Transforming growth factor beta (TGFβ) appears to play a key role in the pathology of PCO in adults.[15,16] In the aqueous humor of the anterior chamber, TGFβ is secreted as an inactive complex.[17] In the eye, the isoform TGFβ-2 dominates and appears to be the most potent isoform to be associated with the development of PCO.[15,17,18] It is thought that TGFβ is upregulated and activated by postoperative inflammation and that surgical complications may further increase its levels in the anterior chamber.[15]

The signaling pathways of TGFβ in adult PCO are well described. In lens epithelial cells, activated TGFβ increases the expression of mesenchymal markers such as fibronectin-1 (*FN1*), α-smooth muscle actin (*aSMA*), and collagen-Iα2 (*COL1A2*).[15,16,19] TGFβ also decreases the expression of epithelial markers such as αB-crystallin (*CRYAB*) and connexin-43 (*Cx43*) in cells undergoing EMT.[15,16] Furthermore, PCO is

associated with upregulated levels of α-integrin subunits (*ITGAV*), which are involved in TGFβ activation and signaling.[20,21] The latent TGFβ complex is marked by a noncovalent association between propeptides and TGFβ[22] Recent reports suggest that $\alpha_V\beta6$-integrin[21] and other alpha integrins have the ability to disrupt this association, activating TGFβ and allowing it to interact with its receptor.[20] Nevertheless, these findings are based only on the induction of EMT by TGFβ in animal cells,[23,24] adult human lens epithelial cells,[25] and human lens cell lines.[26,27] Experimental studies that describe the molecular basics of pediatric lens epithelial cells (pLEC) are lacking. Since regrowth of the lens from pLEC was presented as a new therapeutic approach for pediatric cataract patients recently,[28] a better understanding of the molecular pathomechanisms involved in the formation of PCO in children becomes more important. Due to the rarity of childhood cataracts, the pathogenesis and signaling cascades remain largely unexplored.

In order to study the cellular characteristics of pLEC in culture, we established a protocol for cell culture. We then correlated patient characteristics with growth behavior of pLEC in vitro, and generated a gene expression profile to define the differentiation status of these cells. Confirmatory experiments were performed on the lens cell line HLE-B3. We then investigated the effect of TGFβ-2 on the gene expression profiles to study the molecular basis of EMT in pLEC in children.

## Methods

### Patient Characteristics

Forty children (age 1 to 180 months) with uni- or bilateral cataracts presenting to the Department of Ophthalmology, Charité–Universitätsmedizin Berlin, participated in this study. The parents provided informed consent to participate in the study. The study protocol was approved by the ethics committee (institutional review board of Charité–Universitätsmedizin Berlin). All procedures involving human material were performed in accordance with current ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

The following clinical parameters were collected: age at cataract surgery, sex, laterality of cataract, type of cataract, etiology (e.g., family history of cataracts, presence of disorders related to cataract), and other ocular comorbidities such as strabismus and nystagmus.

### Cell Culture

**Cultivation of Pediatric Lens Epithelial Cells.** Fifty-four anterior lens capsules of 40 children were harvested during cataract surgery by means of manual curvilinear capsulorhexis (Fig. 1A). Modifying the method described by Ibaraki,[29] we established a protocol to cultivate pLEC as follows:

1. Anterior lens capsules of approximately 5-mm diameter with attached pLEC, obtained during cataract surgery in children, were immediately transported to the laboratory in balanced salt solution and were transferred to 35-mm culture wells (Eppendorf, Hamburg, Germany) within 30 minutes of collection.
2. After allowing them to rest for 5 minutes, the lens capsules were covered with 60 µL Dulbecco's modified Eagle's medium/F-12 (Sigma-Aldrich Corp., Schnelldorf, Germany) supplemented with 20% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) and 1% penicillin-streptomycin (Biochrom AG, Berlin, Germany) and secured at the bottom of the well with 12-mm glass coverslips. This concentration of serum has been described as optimal for culturing human lens epithelial cells.[29–32]
3. Lens capsules were cultured at 37°C in a 5% $CO_2$ atmosphere. Based on other studies, all cells were cultured at atmospheric oxygen levels.[30,33,34] After 24 hours, 1 mL culture medium was carefully added to the culture plates.
4. The medium was changed every 3 days. At this stage, 16 capsules showed cell outgrowth and pLEC from six capsules were immediately used for analysis of mRNA expression. The remaining 10 capsules were subcultured on day 13 using Accutase (PAA) and transferred to a 12-well plate, while empty lens capsules were removed.
5. After reaching confluence in 4 to 6 days, pLEC were subcultured again and seeded on two culture plates.
6. Upon reaching a confluence of 70%, cells of six capsules were serum starved for 24 hours followed by stimulation with 10 ng/mL TGFβ-2 (Sigma-Aldrich Corp.) for 48 hours in serum-free medium to study its effect on the cells. Serum-free cells cultivated without TGFβ-2 served as the control. For comparison, each experiment and control was performed on samples of the same lens capsule (see step 5 above).

Cells were monitored daily by phase contrast microscopy, and capsules with cell outgrowth were correlated with patient characteristics. Anterior lens capsules without proliferation tendency were classified as proliferation negative. Other authors performed experiments using lens epithelial cells in passages 1–5.[30,33,35] To ensure comparability, we used early-passage cells (passage 2) to study the effect of the growth factor TGFβ-2. To determine the properties of primary isolated pLEC, cells in passage 0 (p0) were used.

**Cultivation of HLE-B3 Cells.** To validate the results obtained on pLEC, cells of the commercially available human lens epithelial cell line HLE-B3 (ATCC, Manassas, VA, USA) were cultured at 37°C in a 5% $CO_2$ atmosphere in Eagle's Minimum Essential Medium (EMEM; ATCC) containing 20% fetal calf serum (PAA) and 1% penicillin-streptomycin (Biochrom AG). As in pLEC, gene expression profiles of serum-free HLE-B3 cells in the absence and presence of TGFβ-2 were studied. A short tandem repeat analysis certificate was provided by the manufacturer.

### RNA Isolation and Reverse Transcription

Due to the small number of pLEC obtained from the donor capsules, RNA of pLEC was isolated with a reagent (TRI-Reagent; Sigma-Aldrich Corp.) and a visible dye-labeled carrier (Pellet Paint; Novagen, San Diego, CA, USA). RNA was extracted from HLE-B3 cells in passage 5 to 9 using a kit (RNeasy Micro Kit; Qiagen, Hilden, Germany).

In pLEC and HLE-B3 cells, a reverse transcription kit (QuantiTect; Qiagen) was used to synthesize cDNA. To eliminate genomic DNA contamination, 2 µL gDNA wipeout buffer was added to the samples.

### Quantitative Real-Time PCR and Gel Electrophoresis

Gene expression of pLEC and HLE-B3 cells was assessed by quantitative real-time PCR (qRT-PCR) using Rotor-Gene SYBR Green PCR Mix (Qiagen) on a real-time PCR cycler (Rotor-Gene Q; Qiagen). For analysis of each candidate gene, triplicates were prepared. Primers were designed using Primer-BLAST

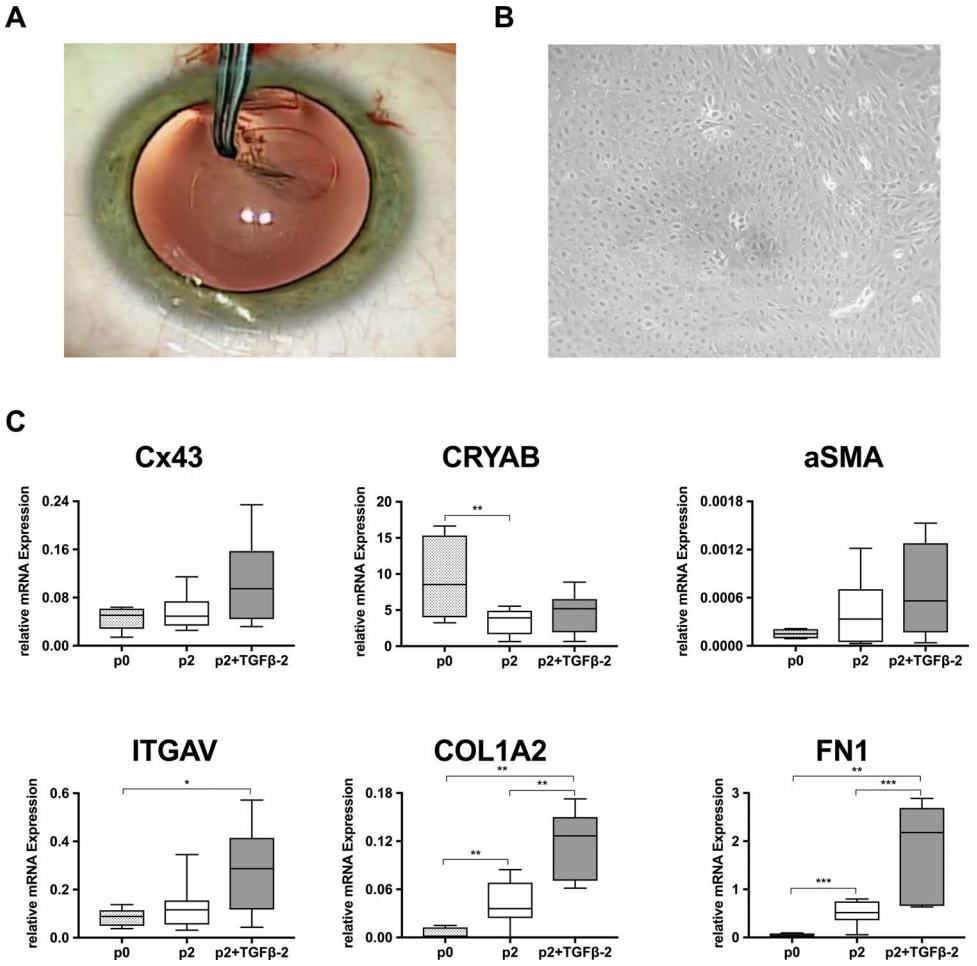


FIGURE 1. Gene expression profile of pediatric lens cells. (**A**) Manual capsulorhexis during cataract surgery on a child. The anterior lens capsule with the attached primary lens epithelial cells is harvested. (**B**) Cultivated pLEC were monitored by phase contrast microscopy on day 6 (objective ×20). Cells near the capsule were predominantly cuboid, whereas remote cells had an elongated character. (**C**) Quantitative RT-PCR was used to analyze mRNA expression for *Cx43*, *CRYAB*, *aSMA*, *ITGAV*, *COL1A2*, and *FN1*. TGFβ-2 (10 ng/mL for 48 hours) increased gene expression of *COL1A2* and *FN1* significantly ($P < 0.05$) in pLEC. *GAPDH* was selected as housekeeping gene. p0, passage 0; p2, passage 2.

(https://www.ncbi.nlm.nih.gov/tools/primer-blast; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). *GAPDH* was selected as internal control gene for relative quantification. Primers were obtained from Eurofins MWG Operon (Eurofins Genomics, Ebersberg, Germany). Primer sequences and expected product sizes are listed in Table 1.

Data were analyzed with software (Rotor-Gene; Qiagen) and relative mRNA expression was calculated by employing the comparative $C_T$ method.[36]

Gel electrophoresis was used to test for specific amplification and primer dimers.

Molecular imager Chem Doc XRS (Biorad, Munich, Germany) allowed identification of PCR amplicons by their product size (see Supplementary Fig. S1).

## Immunocytochemistry (ICC)

At passage p0, pLEC, as well as stimulated and unstimulated HLE-B3 cells on glass coverslips, were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After incubation in a blocking solution containing 5% BSA in Tris-buffered saline for 30 minutes, the following primary antibodies were applied overnight at 4°C: anti-actin, aSMA-Cy3 (1:500, mouse monoclonal; Sigma-Aldrich Corp.), and anti-fibronectin (1:250, mouse monoclonal; Sigma-Aldrich Corp.). For visualization of fibronectin, another incubation step at room temperature for 1 hour was added using the secondary antibody goat anti-mouse (AF546, 1:5000; Thermo Fisher, Darmstadt, Germany). The negative control was performed with HLE-B3 cells stained only with secondary antibody without primary antibody incubation (see Supplementary Fig. S2). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Corp.). Subsequently, coverslips were mounted onto glass slides and subjected to an Axio Imager 2 and Zen lite 2012 Software (Zeiss, Jena, Germany). Fluorescence signal analysis was performed by measuring color pixels per square micrometer of 13 cells per group. To determine the mean corrected total cell fluorescence (CTCF) the following formula was used[37]:

$$CTCF = Integrated\ Density - (Area\ of\ selected\ cell \times Mean\ fluorescence\ of\ background\ readings).$$

Pixel analysis was done with ImageJ 2.0 software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health).[38]

**Table 1.** Primer Sequences Used for mRNA Quantification by qRT-PCR

| Gene | Forward | Backward | Product Size, bp |
|---|---|---|---|
| *Cx43* | CAAAATCGAATGGGGCAGGC | GCTGGTCCACAATGGCTAGT | 136 |
| *CRYAB* | ACTCTCAGAGATGCGCCTGG | GCTGGGATCCGGTATTTC | 186 |
| *aSMA* | AGCTTTCAGCTTCCCTGAACA | CACCATCACCCCCTGATGTCT | 223 |
| *ITGAV* | AGGCTGGAACTCAACTCTTAGC | GCTCCCAGTTTGGAATCGGA | 222 |
| *COL1A2* | GGAGGTTTCGGCTAAGTTGGA | CGGCTGGGCCCTTTCTTACA | 228 |
| *FN1* | GAGCTGAGTGAGGAGGGAGA | GCATCAGGCGCTGTTGTTT | 176 |
| *GAPDH* | TCAACGACCACTTTGTCAAGCTCA | GCTGGTGGTCCAGGGGTCTTACT | 119 |
| *TGFβ-2* | GTGCTTTGGATGCGGCCTA | GGCATGCTCCAGCACAGAA | 147 |

*GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

### Statistical Analysis

All experiments were performed at least three times. Statistical analysis was done with statistical software (GraphPad Prism 7; GraphPad Software, Inc., La Jolla, CA, USA). First, the ROUT method was employed to detect outliers. Normality was assessed using the Shapiro-Wilk test.

To explore statistical significance, Fisher exact test, $\chi^2$ test, Student's *t*-test or Mann-Whitney *U* test were performed, with a *P* value $< 0.05$ ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) considered as statistically significant. Box plot whiskers represent the range, and mean ± standard error of the mean was reported.

## Results

### Patient Characteristics

Of 40 children with cataracts included in this study, 38% were female and 68% were diagnosed with bilateral cataracts (Table 2). In 25% of cases, family history was positive for childhood cataracts. Associated diagnoses such as chromosomal anomalies, craniofacial syndromes, dermatologic disorders, aniridia, or uveitis were noted in 23% of patients. In 53% of children, the etiology of the cataracts remained unclear (Table 2).

### Patient Characteristics and Cell Outgrowth

Of the 54 harvested anterior capsules, cell outgrowth was observed in 16 (30%). Gender did not affect the likelihood of cell outgrowth. While 44% of capsules from children ≤12 months of age at the time of surgery showed cell outgrowth, this was the case in 33% of capsules from children aged 13 to 60 months and in only 1 of 17 capsules (6%) from a child older than 60 months (Fig. 2A; Table 3). There was a significant association between age at cataract surgery and cell outgrowth in vitro ($\chi^2 = 7.153$; $df = 2$; $P = 0.03$).

Outgrowth of pLEC was observed in 43% of the capsules obtained from patients with a positive family history for childhood cataract, 36% of capsules from children with cataracts associated with other entities, and 21% of capsules from patients with idiopathic cataracts (Fig. 2B, Table 3).

As for the type of cataract, cell outgrowth was observed in 8/10 capsules from lenses with total cataracts, in 1/2 lenses with posterior polar cataracts, and in 2/3 lenses with persistent fetal vasculature (PFV). Two of 13 capsules with zonular cataract, 1/9 with complex cataract, and 2/9 capsules without specified cataract morphology showed cell outgrowth. Capsules from lenses with other types of cataracts did not show cell outgrowth (Fig. 2C; Table 3). There was a significant association between cataract type and cell growth in vitro ($\chi^2 = 20.89$; $df = 7$; $P = 0.004$).

**Table 2.** Characteristics of 40 Child Study Participants With Cataracts

| | Number of Patients | Percentage |
|---|---|---|
| Sex | | |
| Female | 15 | 37.5 |
| Male | 25 | 62.5 |
| Age at cataract surgery | | |
| Average in mo | 41.4 | |
| ≤12 mo | 19 | 47.5 |
| 13–60 mo | 8 | 20.0 |
| >60 mo | 13 | 32.5 |
| Laterality | | |
| Unilateral | 13 | 32.5 |
| Bilateral | 27 | 67.5 |
| Etiology | | |
| Family history of childhood cataract | 10 | 25.0 |
| Cataract associated with other disorders | 9 | 22.5 |
| Idiopathic, isolated | 21 | 52.5 |

### mRNA Expression of Primary pLEC Versus pLEC in Passage 2

ICC and qRT-PCR were performed to investigate the molecular properties of the primary isolated pLEC (p0). In order to determine possible changes caused by the cell culturing and passaging, mRNA expressions were compared with pLEC in passage 2.

Subculturing did not have significant effects on mRNA expression of *Cx43*, *aSMA*, and *ITGAV*. However, there was a significant increase in mRNA expression of *COL1A2* ($P = 0.005$, $n_{p0} = 6$, $n_{p2} = 9$) and *FN1* ($P = 0.0009$, $n_{p0} = 6$, $n_{p2} = 10$) of cells in passage 2 compared to primary isolated pLEC (Fig. 1C). mRNA expression levels of *CRYAB* showed a significant reduction during subculturing ($P = 0.009$, $n_{p0} = 6$, $n_{p2} = 9$). Immunocytochemistry confirmed expression of mesenchymal markers aSMA and FN1 as proteins in primary isolated pLEC (see Supplementary Fig. S3).

### Effect of TGFβ-2 on mRNA Expression in pLEC

The effect of TGFβ-2 on pLEC was examined at passage 2 (Fig. 1B). There was a significant increase in mRNA expression of *COL1A2* ($P = 0.003$, $n_{control} = 9$, $n_{TGFβ\text{-}2} = 5$) and *FN1* ($P = 0.0009$, $n_{control} = 10$, $n_{TGFβ2} = 6$) in response to TGFβ-2 (Fig. 1C). However, TGFβ-2 stimulation did not have a significant effect on mRNA expression of *ITGAV*, *Cx43*, and *aSMA*. Although mRNA expression of *CRYAB* in pLEC was the highest of all investigated genes, no significant change after stimulation with TGFβ-2 was observed (Fig. 1C).

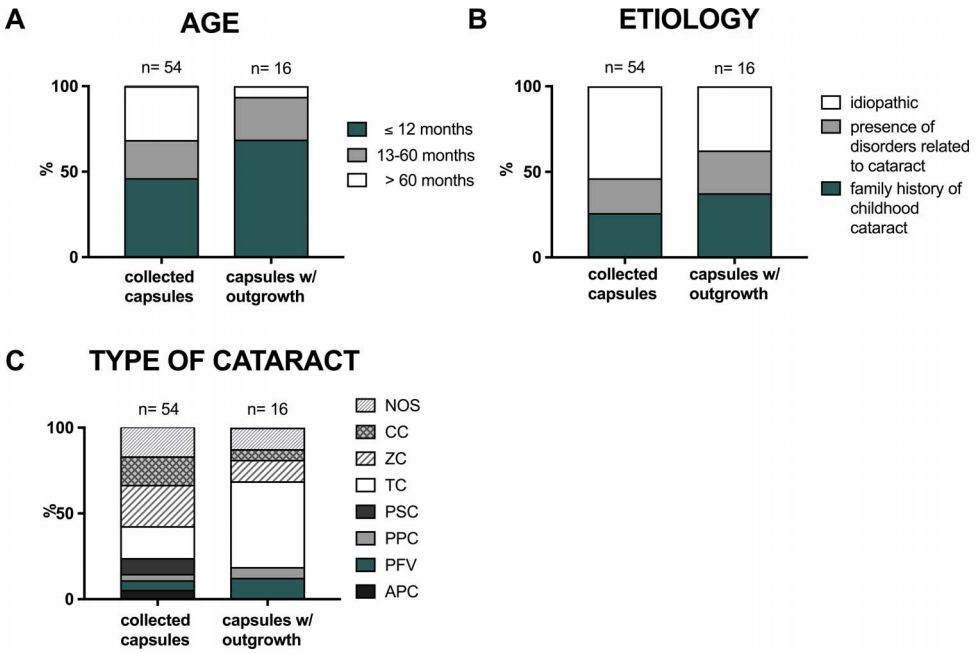


**Figure 2.** Frequency distribution of properties of all collected capsules versus capsules with outgrowth of pLEC. (**A**) Effect of age at time of surgery on outgrowth of pLEC in culture. Of capsules showing cell growth, 69% came from children 12 months or younger, 25% of children aged 13–60 months, and the remaining 6% from children older than 60 months. (**B**) Effect of cataract etiology on outgrowth of pLEC in culture. (**C**) Effect of clinical type of cataract on outgrowth of pLEC in culture. APC, anterior polar cataract; CC, complex cataract; NOS, not otherwise specified; PFV, persistent fetal vasculature; PPC, posterior polar cataract; PSC, posterior subcapsular cataract; TC, total cataract; ZC, zonular cataract.

## Effect of TGFβ-2 on mRNA Expression and Immunocytochemistry in HLE-B3

Stimulation of HLE-B3 cells (Fig. 3A) with TGFβ-2 significantly increased mRNA expression of *CRYAB* ($P = 0.0003$, $n = 9$), *ITGAV* ($P = 0.01$, $n = 9$), *COL1A2* ($P = 0.0002$, $n = 9$), and *FN1* ($P < 0.0001$, $n = 9$) (Fig. 3B). As in pLEC, TGFβ-2 did not significantly affect expression of *Cx43* and *aSMA* (Fig. 3B). Stimulated and unstimulated cells showed comparable levels fluorescence signals from staining of αSMA protein (Fig. 4A, C), while the protein expression of FN appeared significantly increased in the immunofluorescence staining in response to TGFβ-2 ($P = 0.009$, $n = 13$) (Fig. 4B, C).

**Table 3.** Properties of the Capsules Included in the Study Regarding Patients' Features

| | Capsules | Capsules With Cell Outgrowth (%) |
|---|---|---|
| Cases | 54 | 16 (29.6) |
| Sex | | |
| Female | 22 | 7 (31.8) |
| Male | 32 | 9 (28.1) |
| Age at cataract surgery | | |
| ≤12 mo | 25 | 11 (44.0) |
| 13–60 mo | 12 | 4 (33.3) |
| >60 mo | 17 | 1 (5.9) |
| Etiology | | |
| Family history of childhood cataract | 14 | 6 (42.9) |
| Cataract associated with other disorders | 11 | 4 (36.4) |
| Idiopathic, isolated | 29 | 6 (20.7) |
| Type of cataract | | |
| Anterior polar | 3 | 0 (0) |
| Persistent fetal vasculature | 3 | 2 (66.7) |
| Posterior polar | 2 | 1 (50.0) |
| Posterior subcapsular | 5 | 0 (0) |
| Total cataract | 10 | 8 (80.0) |
| Zonular cataract | 13 | 2 (15.4) |
| Complex cataract | 9 | 1 (11.1) |
| Not otherwise specified | 9 | 2 (22.2) |

## Comparison of mRNA Expression in Primary pLEC and HLE-B3

In unstimulated primary pLEC (p0), total mRNA levels were higher than in unstimulated HLE-B3 cells for most investigated genes. Particularly, mRNA expressions of *COL1A2* (10-fold), *CRYAB* (194-fold), and *aSMA* (15-fold) were higher in pLEC than in HLE-B3 cells; mRNA expressions of *Cx43* (4-fold) and *ITGAV* (3-fold) were moderately higher (Fig. 5A). The higher expression rates of these genes were associated with significantly higher mRNA levels of *TGFβ-2* in primary pLEC compared to HLE-B3 cells ($P = 0.0005$, $n_{p0} = 5$, $n_{HLE\text{-}B3} = 6$) (Fig. 5B). Primary isolated pLEC showed lower mRNA levels of *FN1* (0.3-fold) compared to unstimulated HLE-B3 cells.

## Discussion

Pediatric cataracts are rare,[2,4,39] and research concerning the pathogenesis of both childhood cataract pathogenesis and PCO is limited, making therapeutic strategies difficult to develop. We provide insight into the pathophysiology of after-cataracts in children, which could serve as basis for future research to better understand the molecular mechanisms of this process and its prophylaxis.

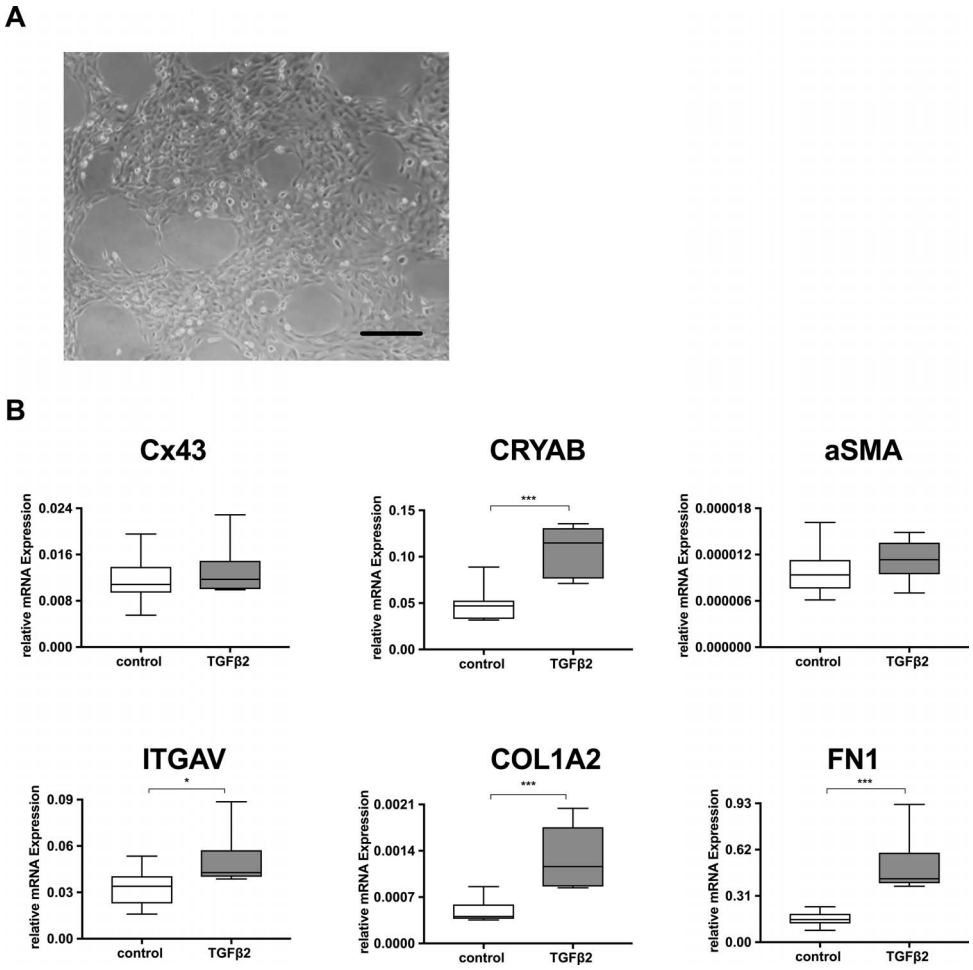


FIGURE 3. Effects of TGFβ-2 on HLE-B3 cells. (**A**) Phase contrast microscopy of cell line HLE-B3 in passage 5 (objective ×20). *Scale bar*: 1 mm. (**B**) Relative mRNA expression of all investigated genes in absence and presence of 10 ng/mL TGFβ-2. *CRYAB*, *ITGAV*, *COL1A2*, and *FN1* were significantly increased after stimulation with TGFβ-2 for 48 hours ($P < 0.05$).

## Patient Characteristics Affect Growth Behavior of Cells in Vitro

The purpose of this study was to establish a standardized protocol for cell culture of pediatric pLEC and to correlate cell outgrowth with clinical characteristics. Using the established cell-culturing protocol, 16 of the 54 capsules showed outgrowth of cells. We found that pLEC from lens capsules from younger children more often showed cell outgrowth in culture than did those from older children. This in vitro finding agrees with clinical experience that young age is a risk factor for PCO after pediatric cataract surgery.[7]

Cataract type also affected pLEC outgrowth. While cell outgrowth in vitro happened in most capsules from lenses with a total cataract, PFV, or a posterior polar cataract, it occurred less frequently in capsules from zonular and complex cataracts. Capsules from anterior polar cataracts and posterior subcapsular cataract showed no cell outgrowth.

Of note, cataract type is often related to patient age at surgery. For example, it is likely that denser (total) cataracts are diagnosed and operated on earlier than for subtler ones. A larger number of cases would be desirable, and future research may further study age and type of cataract as factors affecting behavior of pLEC in cell culture.

A recent report suggested that regrowth of the lens from pLEC remaining in the lens capsule at the time of surgery may be possible.[28] While this approach has the potential to revolutionize current concepts on optical rehabilitation after cataract surgery in children, the validity and reproducibility of this strategy remains to be confirmed. Our results would suggest that cell outgrowth may be more frequent in younger children with total cataracts, but whether a regenerated lens is sufficiently clear needs to be confirmed in clinical studies. However, understanding the cellular mechanisms underlying proliferation of pLEC in children is a prerequisite for the evaluation and implementation of novel therapeutic approaches for pediatric cataracts.

## Effect of TGFβ on pLEC

EMT is thought to be involved in the development of after-cataracts in adults.[16] Our data support the hypothesis that the same mechanism applies for pLEC. Prior research described the induction of EMT by TGFβ in rat lens cells,[23,24] adult human lens epithelial cells,[25] and human lens cell lines[26,27] as a characteristic feature of after-cataract formation.[40] In after-cataract formation, TGFβ is known to act via the well-studied SMAD cascade and various SMAD independent signaling pathways.[19,40–43] EMT is characterized by an increased expression of α-smooth muscle actin, collagen-I, integrins, fibronectin, and other proteins considered to be mesenchymal markers. Conversely, epithelial markers are downregulated in the presence of TGFβ.[23,24,44–46] It can be assumed that this growth factor has comparable effects on pediatric lens cells.

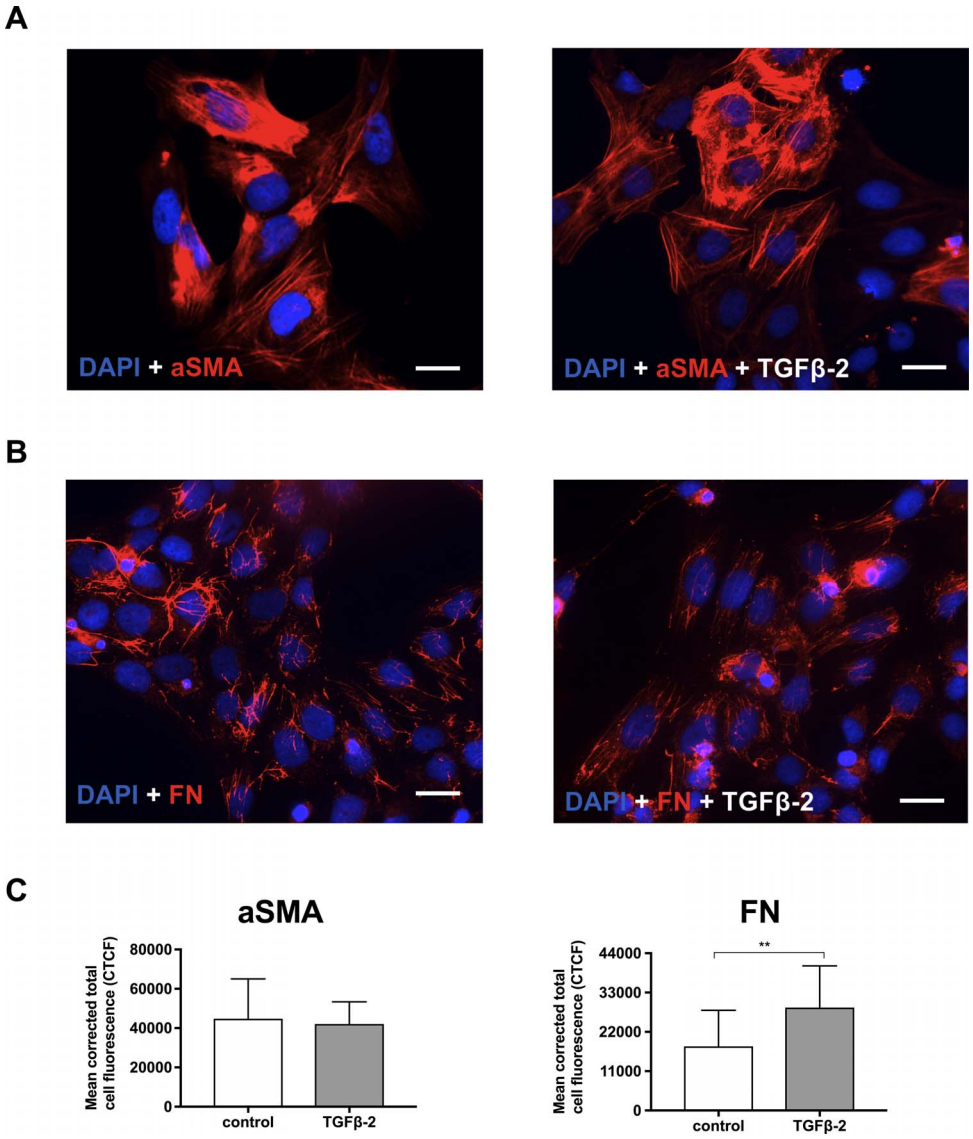


FIGURE 4. Immunocytochemistry of HLE-B3 cells. Immunocytochemistry of HLE-B3 cells stained with antibodies against αSMA (**A**) and FN (**B**) in absence and presence of 10 ng/mL TGFβ-2. Nuclei were stained with DAPI. Mean corrected cell fluorescence of FN was significantly increased by TGFβ-2 (**C**), $P < 0.05$. *Scale bar*: 50 μm.

However, to our knowledge, no studies have qualified the influence of TGFβ-2 on these cells. Building on these studies, the present work describes the response of pediatric lens cells to TGFβ-2 that, however, might differ in its mechanism from that of adult EMT.

We found an increased mRNA expression of extracellular matrix genes in presence of TGFβ-2 in pLEC. Stimulation with TGFβ-2 promoted an intense mRNA upregulation of *FN1* and *COL1A2* as features of a myofibroblastic transformation in pLEC.

FN, an extracellular matrix protein, is reported to be involved in EMT.[16,47] It was found to accumulate in experimental PCO, specifically in anterior subcapsular plaques in vitro[16] and appears to be essential for integrin-promoted activation of TGFβ[47] Moreover, FN supports migration of rabbit lens epithelial cells in vitro and therefore might promote migration of lens epithelial cells to the posterior lens capsule.[48] Consistent with these findings, pLEC showed significantly upregulated mRNA levels of *FN1* in response to TGFβ-2. It appears likely that this pathway is involved in the formation of PCO, and it may be particularly responsible for fibrotic types of PCO in children.

We demonstrated that TGFβ-2-mediated upregulation of mRNA for *COL1A2* also occurs in pLEC. This substantiates previous immunohistologic studies identifying collagen-I-rich cells in congenital cataracts.[49] In general, collagen-I is involved in TGFβ-related diseases such as idiopathic lung fibrosis, systemic sclerosis, and certain types of cancer.[50,51] Overexpression of *COL1A2* in pediatric lens cells with its fibrotic and migrating patterns may contribute to high incidence of PCO and VAO in children.

TGFβ-induced upregulation of *aSMA* has been reported as a key factor in EMT.[16,46] However, we found that TGFβ-2 had no significant effect on *aSMA* expression in pLEC or in HLE-B3 cells. The most likely explanation is that TGFβ signaling in juvenile cataract differs from that in adult cataract. TGFβ-mediated stimulation of *aSMA* expression appears to be age dependent.[52] A lower proportion of advanced glycation end products in lens epithelial cells of younger donors may be associated with a weaker TGFβ-dependent effect on *aSMA*

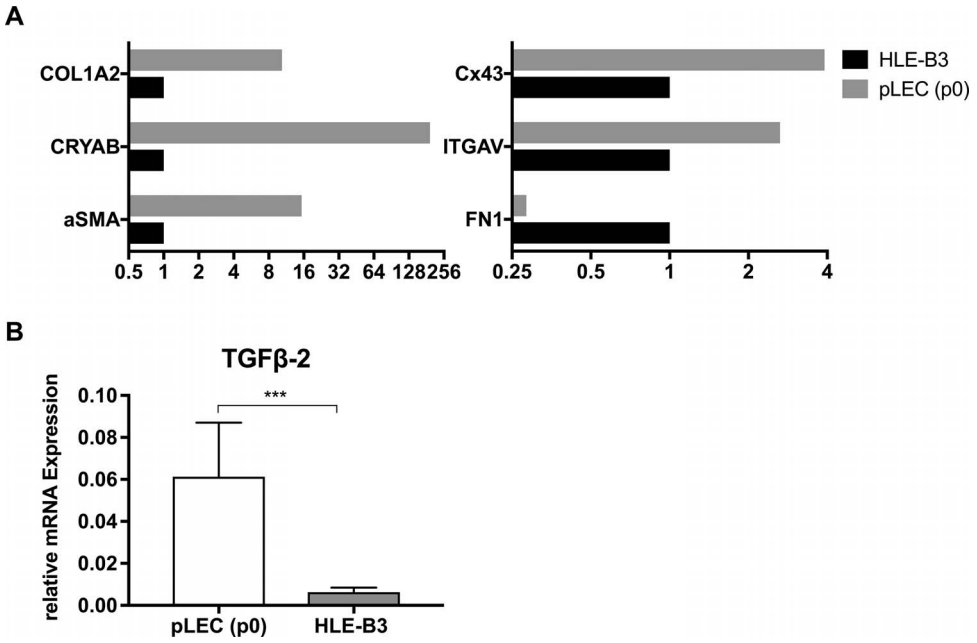


**Figure 5.** Fold expression of unstimulated primary pLEC and unstimulated HLE-B3 cells. (**A**) Fold expression of *COL1A2*, *CRYAB*, and *aSMA* are up to 194-fold higher in primary pLEC (p0) compared to HLE-B3. mRNA expression of *Cx43* and *ITGAV* are up to 4-fold higher in primary pLEC compared to HLE-B3, while expression of *FN1* was lower in primary pLEC (p0). mRNA expression of HLE-B3 was normalized to 1. (**B**) Relative mRNA expression of *TGFβ-2* was significant higher in primary isolated pLEC (p0) compared to HLE-B3 cells ($P < 0.05$).

expression.[52] The exact underlying mechanism of this TGFβ-mediated *aSMA* expression has not yet been identified. Although TGFβ may induce the expression of *aSMA* directly, indirect pathways and interactions with other TGFβ-induced proteins should be considered.[53] In our study, a lack of TGFβ-2 effect on mRNA expression of *aSMA* might result from already elevated mRNA levels of *aSMA* in pLEC so that TGFβ-2 may not further upregulate this gene.[54] Finally, it is important to note that variations in experimental methodology may affect stimulation results. Further research into delineating the underlying mechanisms is necessary.

Detectable basal gene expression rates in primary pLEC for mesenchymal markers in the absence of TGFβ-2 suggest that these cells may have lost their epithelial features to some extent. These changes could be caused by an overexpression of TGFβ in congenital cataracts[55,56] or an increased level of TGFβ following cataract surgery.[57] Indeed, our study demonstrated higher basal expression rates of *TGFβ-2* in pLEC compared to HLE-B3 cells. Stimulation of pLEC with TGFβ-2 further led to a significant increase in mRNA expression of mesenchymal genes, *COL1A2* and *FN1*, implying transdifferentiation toward myofibroblastic characteristics. Interestingly, there was no significant concomitant reduction of epithelial markers in response to TGFβ-2. This is in line with the clinical observation that lens epithelial cells continue to produce lens material while proliferating and migrating on the remaining lens capsule. It remains to be demonstrated if success of recent therapeutic approaches such as regeneration of the lens from pLEC is limited by the transdifferentiation status of these cells. *FN1* and *COL1A2* seem to be characteristic genes in EMT in pLEC, so they can be recommended as potential targets to reduce PCO in children.

As a limitation, one should consider that qRT-PCR measures gene expression but does not reflect changes on the protein level. The main obstacle to quantitative protein levels by Western blotting was the limited number of pLEC and the low amount of RNA per harvested lens capsule. Moreover, mRNA expression was investigated 48 hours after stimulation with TGFβ-2. One should consider that gene expression may vary at different times. Nevertheless, qRT-PCR is a highly reproducible and sensitive method, allowing quantification of specific DNA sequences.[58,59]

## Similar Effect of TGFβ-2 in HLE-B3 and pLEC

Consistent with findings in pLEC, we found TGFβ-2-induced gene expression profiles compatible with EMT in HLE-B3 cells. These cells served as a control for the experimental setup used to analyze pediatric lens cells. TGFβ-2 promoted a significant upregulation of *ITGAV*, *FN1*, and *COL1A2* gene expression in HLE-B3 compared to untreated cells. Contrary to the well-studied effects of TGFβ-2 on mesenchymal markers,[16,21,23,24,44] the present study also detected a significant upregulation of *CRYAB* in HLE-B3 cells after stimulation with TGFβ-2. With its chaperone-like and heat-shock protein activity, α-crystallin suppresses stress-induced cell death and lenticular protein aggregation and therefore preserves transparency of the lens.[60,61] Overexpression of *CRYAB* under conditions of stress contributes to increased stress resistance of cells.[62] Moreover, mutations in the α-crystallin gene cause congenital cataracts.[63] Intriguingly, *CRYAB* also appears to be involved in EMT.[62] One study found that depletion of *CRYAB* by siRNA has reduced TGFβ-induced mesenchymal changes in lens cells.[62] It is possible that TGFβ-2 increases the expression of *CRYAB*, which subsequently results in an increased expression of EMT genes,[62] and that increased *CRYAB* levels lead to an increased nuclear localization of Smad4, which induces mesenchymal genes and increased proliferative and migratory capacity.[62,64] We detected high basal expression levels of *CRYAB* in pLEC and a TGFβ-2-promoted mRNA elevation of *CRYAB* in cell line HLE-B3. Whether this was due to an abortive protective mechanism of α-crystallin in lens cells undergoing EMT or whether high *CRYAB* levels contribute to the pathogenesis of PCO due to their antiapoptotic properties remains unclear.[65]

We show that pLEC and HLE-B3 cells respond similarly to stimulation with TGFβ-2 with respect to mesenchymal markers

*FN1* and *COL1A2*, but that total expression rates of most examined genes were considerably higher in pLEC. This may reflect the greater vitality and metabolic activity of pLEC in children compared with the cell line. Changes in molecular expression cell lines can occur due to subculturing and growth medium composition. HLE-B3 cells are, although lens cells, not native cells and may not accurately reflect the behavior of pLEC.

We found that pLEC from younger children were more likely to grow in cell cultures. These cells exhibit a gene expression profile that indicates a phenotypic shift from epithelial to mesenchymal properties (EMT). In the light of recent approaches for pediatric cataracts, our findings may help design research to better understand the molecular mechanisms involved in the formation of PCO.

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