

Cells previously identified as retinal stem cells are pigmented ciliary epithelial cells

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It was previously reported that the ciliary epithelium (CE) of the mammalian eye contains a rare population of cells that could produce clonogenic self-renewing pigmented spheres in culture. Based on their ability to up-regulate genes found in retinal neurons, it was concluded that these sphere-forming cells were retinal stem cells. This conclusion raised the possibility that CE-derived retinal stem cells could help to restore vision in the millions of people worldwide who suffer from blindness associated with retinal degeneration. We report here that human and mouse CE-derived spheres are made up of proliferating pigmented ciliary epithelial cells rather than retinal stem cells. All of the cells in the CE-derived spheres, including the proliferating cells, had molecular, cellular, and morphological features of differentiated pigmented CE cells. These differentiated cells ectopically expressed nestin when exposed to growth factors and low levels of pan-neuronal markers such as beta-III-tubulin. Although the cells aberrantly expressed neuronal markers, they retained their pigmented CE cell morphology and failed to differentiate into retinal neurons *in vitro* or *in vivo*. Our results provide an example of a differentiated cell type that can form clonogenic spheres in culture, self-renew, express progenitor cell markers, and initiate neuronal differentiation that is not a stem or progenitor cell. More importantly, our findings highlight the importance of shifting the focus away from studies on CE-derived spheres for cell-based therapies to restore vision in the degenerating retina and improving techniques for using ES cells or retinal precursor cells.

transdifferentiation | transplantation | neurospheres | differentiation

Over 40 million people worldwide are blind. Macular degeneration accounts for ≈ 8 million cases of blindness. Although the cause of macular degeneration is not known, 1 potential treatment is cell therapy. Stem cells hold great promise for regenerative medicine, and recently many efforts have been devoted to finding suitable candidates for retinal transplants. These candidates include photoreceptor progenitors (1) and embryonic stem cells (2, 3). Others have looked to the ciliary epithelium (CE) as a potential source of retinal stem cells (4, 5).

In 2000, Tropepe and colleagues revealed that the CE of the mouse eye can be dissociated, maintained in culture at clonal density and stimulated to form clonogenic spheres (5). The CE-derived spheres were pigmented, expressed nestin, and could be dissociated and replated to form spheres up to 8 times. When transferred to differentiation conditions in culture, cells from the CE-derived spheres up-regulated genes found in rod photoreceptors, bipolar neurons, and Müller glia (5). These findings were later extended to CE isolated from postmortem human eyes (4). Based on these and other data, it was suggested that the sphere-forming cells in the mammalian CE are retinal stem cells (RSCs) and, thus, hold promise for therapeutic replacement of retinal neurons lost to disease or degeneration.

Given the potential impact of cell replacement therapy to treat retinal degeneration, we sought to characterize the molecular and cellular mechanisms underlying the expansion and differentiation of human and murine RSCs. We present evidence that the clono-

genic spheres derived from the mouse and human CE originate from differentiated pigmented CE cells rather than a rare stem cell population harbored in the ciliary epithelium. These cells proliferate in culture while retaining all of their features of differentiated pigmented CE cells. When transferred to culture conditions that promote differentiation, the pigmented CE cells ectopically up-regulate pan-neuronal markers. However, they do not form bona fide retinal neurons or glia, *in vitro* or *in vivo*. Therefore, the mouse and human CE does not contain retinal stem cells, but instead it contains a population of differentiated pigmented CE cells that can proliferate, clonally expand, and self-renew in stem cell medium and express some neuronal markers while retaining features of pigmented epithelial cells.

Results

The CE of the mammalian eye (Fig. 1*A*) consists of a layer of pigmented epithelial cells adjacent to a layer of nonpigmented epithelial cells (Fig. 1*B*). Both types of CE cells express cytokeratin, which is not expressed in the retina (Fig. 1*C* and *D*). Transmission electron microscopy (TEM) was used to visualize the melanosomes, basal and lateral membrane interdigitations, and epithelial cell–cell junctions in the CE (Fig. 1*E* and *F*). Similar analyses of retinal progenitor cells and neural stem cells [supporting information (SI) Fig. S1] demonstrated that these properties are unique to pigmented CE cells. Real-time RT-PCR confirmed that CE cells have unique molecular profiles (Fig. 1*G–I*).

To determine whether murine CE-derived spheres contain RSCs and retinal progenitor cells as proposed (5), we performed molecular, cellular, and morphologic analyses of CE-derived spheres and compared those data to normal CE, retinal progenitor cells, retinal progenitor cell derived spheres, neural stem cells, and neural stem cell derived spheres. Dissociated CE cell preparations (Fig. 2*A* and *B*) were maintained at clonal density (20 cells per μL) in stem cell medium for 7 days (4–6). Pigmented spheres (Fig. 2*C*) were identified at a frequency indistinguishable from previous results (5). TEM analysis on CE-derived spheres after 7 days in culture revealed that all of the cells in the CE-derived spheres appeared pigmented (Fig. 2*D* and *E*) and had morphologic characteristics of normal pigmented CE cells (Fig. 2*F–H*). Moreover, the cells did not have characteristics that were found in spheres derived from retinal progenitor cells or neural stem cells (Fig. S2, Fig. S3, and Table S1).

To validate this finding, we analyzed gene expression in CE-derived spheres by real-time RT-PCR using TaqMan probes. Consistent with the TEM analysis, the CE-derived spheres expressed genes found in the primary CE (Fig. 2*I*) and those required

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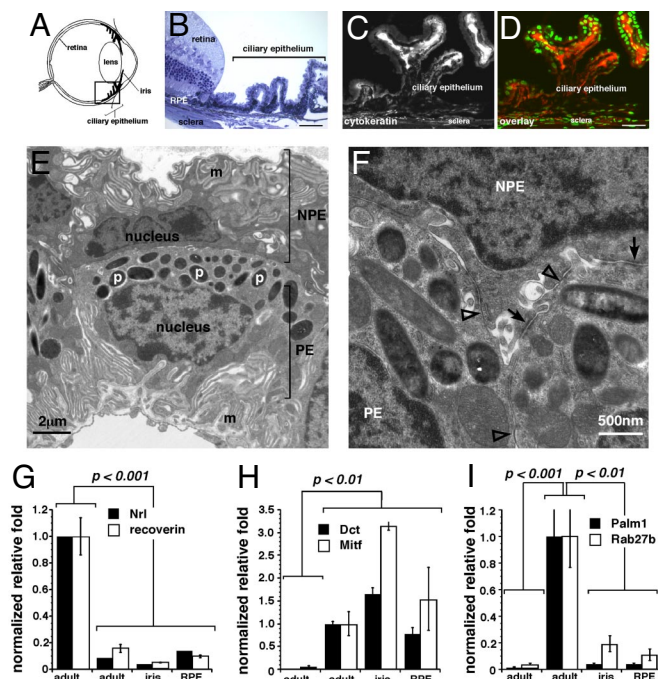


Fig. 1. Isolation and characterization of the mouse ciliary epithelium. (A) Diagram of the location of the ciliary epithelium (CE) in the mammalian eye. (B) Toluidine blue stained 1- μ m thick plastic section of the adult C57BL/6 mouse CE. (C and D) Cytokeratin immunofluorescence in the adult mouse CE (red fluorescence) alone (C) and overlaid with nuclear Sytox green stain (D). (E and F) Transmission electron microscopy of the mouse CE showing membrane interdigitations (m), pigment containing melanosomes (p), and epithelial junctions found in the CE including tight junctions (open arrowheads) and gap junctions (arrows). (G–I) Real time RT-PCR analysis of mouse CE, retina, retinal pigment epithelium, and iris using probes for retinal specific genes (*Nrl*, *recoverin*), pigmented cells (*Dct*, *Mitf*), and CE (*Palm1*, *Rab27b*). Each bar represents the mean and standard deviation from duplicate experiments for at least 3 independent samples for each piece of tissue. Abbreviations: RPE, retinal pigment epithelium; CE, ciliary epithelium; m, membrane interdigitations; p, pigmented melanosomes; NPE, nonpigmented ciliary epithelium; PE, pigmented epithelium. (Scale bars in B–D, 10 μ m.)

for proliferation (Fig. 2J). They did not express genes found in retinal progenitor cells (Fig. S2) or neural stem cells (Fig. S3). Affymetrix gene expression microarray analyses of primary mouse CE, CE-derived spheres, and retinal samples confirmed the high level of similarity between the gene expression profiles of pigmented CE and the CE-derived spheres (Table S2).

We predicted that if pigmented CE cells are the cells of origin for CE-derived spheres, then tissue samples from mutant mice that aberrantly produce more pigmented CE cells will show increased sphere production. Previous studies have shown that in the *Chx10^{orf/orf}* microphthalmic mouse strains, CE-sphere formation is increased (7). To determine whether this finding was the result of an increased number of pigmented CE cells, we analyzed the CE from *Chx10^{orf/orf}* eyes (Fig. S4 A–D). *Chx10^{orf/orf}* eyes gave rise to more spheres per eye (Fig. S4 F–H), and they had more pigmented CE cells per eye than did their wild-type littermates (Fig. S4 E–G). TEM analysis of serial sections of the spheres from the *Chx10^{orf/orf}* eye revealed that each cell was a pigmented CE cell (Fig. S4I). These data suggest that the increase in CE cells in the *Chx10^{orf/orf}* eye is sufficient to account for the increased efficiency of sphere production from these animals.

On the basis of the data presented above, we propose that the murine CE-derived spheres are made up of proliferating pigmented CE cells rather than RSCs or progenitors (Fig. 3 A and B). To test this hypothesis, we identified and characterized the proliferating cells in the CE-derived spheres. We labeled S-phase cells by incubating the spheres with [³H]-thymidine for 1 h on Day 3. The spheres were then immediately harvested, and a 1- μ m section was collected, stained with toluidine blue, and overlaid with autoradiographic emulsion to detect the [³H]-thymidine⁺ cells (Fig. 3C). Adjacent to the 1- μ m section, ten 50-nm serial sections were collected and processed for TEM imaging. By aligning the 1- μ m and 50-nm sections, we were able to characterize the morphologic features of the S-phase cells (Fig. 3 D and E). Every cell analyzed had all of the morphologic features of pigmented CE cells including pigment, membrane interdigitations, and epithelial junctions (Table S3 and Fig. 3E). TEM analysis of M-phase cells revealed that they also had the same features as pigmented CE cells (Fig. 3 F–H).

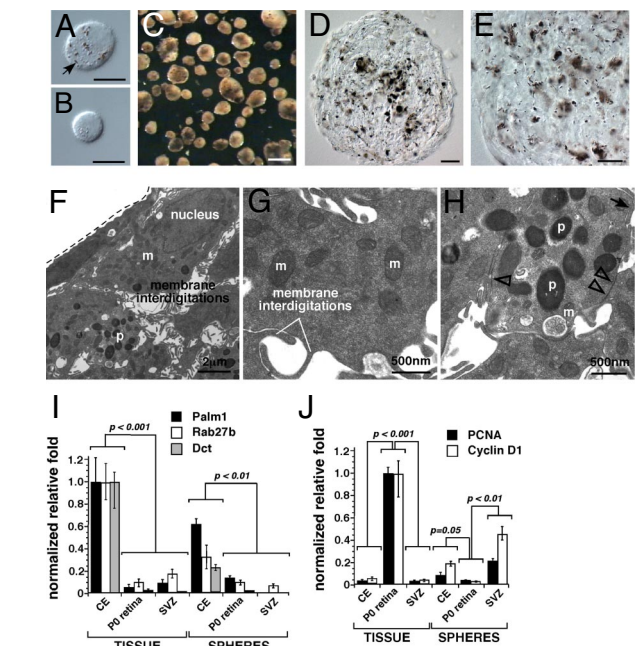


Fig. 2. CE-derived spheres are pigmented. (A and B) Representative DIC images of pigmented and nonpigmented dissociated mouse CE cells. (C) Brightfield image of CE-derived spheres after 7 days in culture from the mouse. (D and E) Cryosections of the mouse CE-derived spheres imaged using DIC optics. (F–H) TEM analysis of mouse CE-derived spheres emphasizing membrane interdigitations (F and G) and epithelial junctions (H) found in the normal CE (tight junctions, open arrowheads; gap junctions, arrow). (I) Real time RT-PCR analysis of CE-derived spheres using probes for genes found in the normal CE (*Palm1*, *Rab27b*, *Dct*). The source of retinal progenitor cells was PO mouse retina, and the source of neural stem cells was adult SVZ. (J) Genes normally expressed in proliferating cells (*PCNA*, *Cyclin D1*) were also analyzed in these samples using real time RT-PCR. Each bar represents the mean and standard deviation from duplicate experiments for at least 3 independent samples for each piece of tissue. Abbreviations: m, mitochondria; p, pigment; CE, ciliary epithelium; SVZ, subventricular zone. (Scale bars in A and B, 5 μ m; C, 100 μ m; D and E, 10 μ m.)

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Although these data indicated that the proliferating cells in CE-derived spheres are pigmented CE cells, they did not exclude the possibility of a rare stem cell population. To analyze every cell in the CE-derived spheres, we performed serial electron microscopic analysis of entire spheres using dual-beam focused ion beam technology (Fig. 4 A and B, and Movies S1–S4). This imaging technique allowed us to serially query the entire sphere in 70- to 150-nm slices with resolution similar to that of standard TEM (Fig. 4 C–E).

After all images were collected, we performed morphometric analysis on 5 datasets per sphere from 3 independent spheres to provide a random sampling of cells at all eccentricities of the sphere. Each dataset consisted of 21 sequential sections selected randomly and scored by 2 independent investigators. Every cell in each

factors (Fig. 5D and Table S5). Exposure to stem cell medium led to the rapid up-regulation of *Nestin* expression before the cells had time to clonally proliferate. Thus, dissociation and exposure to the stem cell medium was sufficient to cause these changes in gene expression rather than selective growth of an epithelial or retinal stem cell population.

To further investigate the morphologic features of the *Nestin*-expressing cells in CE-derived spheres and confirm these findings, we examined a transgenic mouse strain that expresses the human placental alkaline phosphatase gene (*AP*) under the control of the *nestin* promoter and enhancer in the second intron, which regulates *nestin* expression in neural stem/progenitor cells (8) (*Nes-Cre^{ERT2}-IRES-hPLAP*). We grew spheres from dissociated CE of these mice and analyzed them with AP TEM by using lead citrate staining as described in ref. 9. We found that the *Nestin*-expressing (*AP*⁺) cells contained pigment, membrane interdigitations, and epithelial junctions similar to differentiated pigmented CE cells (Fig. 5E–I). Therefore, pigmented CE cells ectopically induced the expression of the stem/progenitor cell gene *Nestin*, but this profile did not mark a stem cell population in the CE or in the CE-derived spheres based on morphological or molecular criteria.

When CE-derived spheres are transferred to medium containing serum and plated on laminin, they were reported to produce cells expressing genes found in rods, bipolar cells, and Müller glia (5). Thus, we propose that these cells can initiate transdifferentiation or reprogramming from pigmented CE cells rather than differentiate from RSCs or progenitors. This model is consistent with previous studies showing robust transdifferentiation between CE, retina, and retinal pigment epithelium in various species (10–14). To test this hypothesis, we maintained spheres on laminin-coated coverslips for 21 days, as described in refs. 4 and 5. The spheres adhered to the coverslip, and pigmented cells migrated from them (Fig. 6A–D). Even under differentiation conditions, every cell retained pigment (Fig. 6A–D). TEM analysis revealed that after 21 days, the cells retained all morphologic features of CE cells (Fig. 6E–G) and none of retinal neurons (e.g., processes, synaptic densities, synaptic vesicles, or synaptic ribbons).

To characterize the changes in gene expression over the course of the differentiation culture, we performed real-time RT-PCR on samples harvested every 2 days for 21 days. Genes that are normally expressed in rods (*Nrl*, *Rhodopsin*, *Gnat1*), bipolar cells (*PKC α*) and Müller glia (*Cralbp*) were not induced compared with levels seen in the normal retina (Fig. 6H). However, genes expressed in the differentiated CE (*Palmdelphin*, *Rab27b*) increased in these cultures (Fig. 6I).

If retinal differentiation is a very rare event in these cultures, it may be difficult to detect using real time RT-PCR. Therefore, we tested this possibility by immunostaining the cells with antibodies to markers of various retinal cells. The vast majority of cells were cytokeratin⁺ (97/100, 93/100; 95 ± 2.8%) (Fig. S5A and B) consistent with their differentiation as pigmented CE cells. As mentioned above, a subset of the cells were *nestin*⁺ (15/100, 19/100; 17 ± 2.8%) (Fig. S5C and D, compared with SVZ controls in Fig. S5E and F), but were pigmented and had the features of differentiated pigmented CE cells. The pan-neuronal marker β -III tubulin (Tuj1) labeled \approx 20% of the cells (22/100, 19/100; 20 ± 2.1%) in the CE-sphere differentiation cultures; those cells showed morphologic features of pigmented adherent epithelial cells. Occasionally (1–2 cells per well), an epithelial cell elongated and resembled a neuron (Fig. S9G); however, those cells also contained pigment (Fig. S5H). Dissociated cortex was used as a control for the Tuj1 immunostaining (Fig. S9J) (16/100, 11/100; 13 ± 3.5%). No cells in the CE differentiation cultures expressed rhodopsin (Fig. S5K–M), but occasionally, nonspecific immunofluorescence was observed along the distorted edge of the pigmented epithelial cells that resembled a rod photoreceptor (Fig. S5M and N). These “false rods” did not contain nuclei and appeared to be the curled-up membrane of pigmented epithelial cells that had trapped the

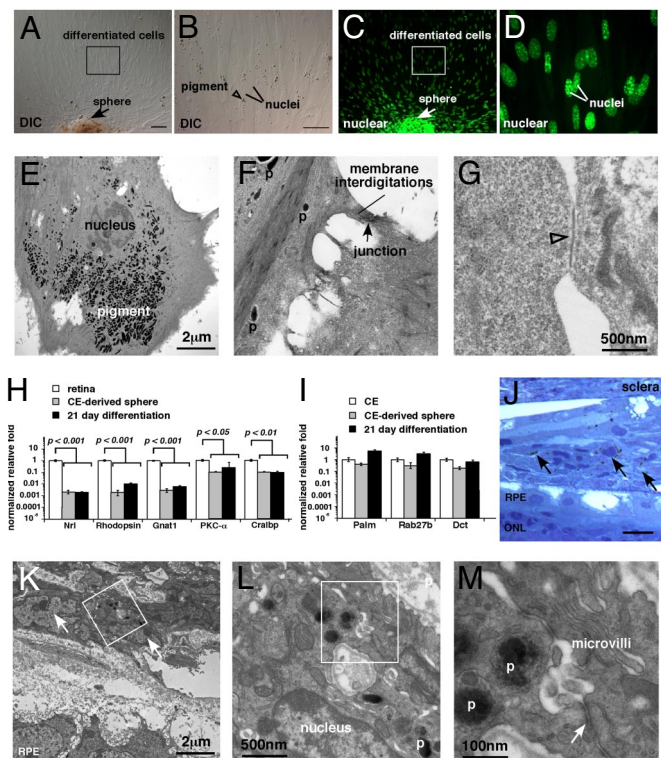


Fig. 6. Differentiated mouse CE-derived spheres resemble pigmented CE cells. (A–D) CE-derived spheres were plated on laminin coated coverglass and grown in differentiation medium for 21 days. The spheres adhered to the coverglass and spread out from there. All of the cells appeared to be pigmented in DIC images (A and B) when compared with the pattern of nuclear staining (C and D). (E–G) TEM analysis of CE-derived sphere differentiation cultures. (H and I) Real time RT-PCR analysis of differentiated CE cells for genes found in retinal neurons or glia (H) and pigmented CE cells (I). (J–M) CE-derived spheres were injected into the subretinal space of newborn rats to study their transdifferentiation into retinal neurons and glia. Tissue was analyzed 21 days after injection. (J) In and around the site of injection, pigmented cells were readily detected in histological sections (arrows). (K–M) TEM analysis of these sections revealed that the cells remained differentiated as pigmented CE cells. The injected cells retained pigment, membrane interdigitations (microvilli), and epithelial junctions (arrow in M). Abbreviations: DIC, differential interference contrast microscopy; p, pigment; RPE, retinal pigment epithelium; ONL, outer nuclear layer. (Scale bars in A, B, and J, 10 μ m.)

primary antibody. Rhodopsin⁺ cells from adult retinæ controls always contained a nucleus (Fig. S5O and P). PKC α ⁺ cells (bipolar cells) were readily detected in dissociated adult retinæ but were absent from CE-derived sphere differentiation cultures. Together, these data suggest that at least 17% of cells become *nestin* positive, but fail to maintain a program to become retinal neurons. There is an ectopic up-regulation of immature neuron markers in the pigmented cells, such as Tuj1; however, very few cells express markers of bipolar neurons or photoreceptors. Without exception, all labeled cells retained the pigment and morphology of CE cells.

To determine whether transplantation of CE-derived spheres into the developing retina promotes their transdifferentiation, we injected pigmented CE-derived spheres (10 spheres/1 μ L vehicle) into the subretinal space of 7 newborn albino rat pups. Twenty-one days later, we identified the pigmented cells in and around the injection site. There was no evidence of their integration into the developing retina (Fig. 6J). TEM analysis of the injected eyes confirmed these results (Fig. 6K–M). Our analysis shows that the injected pigmented cells grouped together and formed a basal lamina, thus isolating the transplanted cells from the surrounding tissue. In all cases, the injected cells failed to integrate within the

production of replacement neurons through reprogramming or transdifferentiation of pigmented CE, because neither mouse nor human CE contains true stem cells. Recent work from the Svendsen lab has indicated that factors secreted by the RPE may promote the expression of retinal progenitor cell genes in human retinal neurospheres, ultimately leading to the expression of genes found in retinal neurons (17). Transdifferentiation of glia and retinal pigment epithelium has been described and perhaps represents a more attractive candidate than the CE for the production of functional retinal neurons. Other promising alternatives include the use of photoreceptor precursor cells from fetal retinae (18–20) or the use of ES cells (21, 22). Of particular interest is the recent report that retinal neurons can be induced to reenter the cell cycle and make more retinal neurons without the need for stem cells or progenitor cells (23). If these studies can be extended to photoreceptors, then we might be able to generate new neurons in situ in a degenerating retina (23).

Materials and Methods

Animals. Timed, pregnant Sprague–Dawley rats were obtained from Charles River Laboratories. All retinal samples and CE cultures were from C57BL/6 male mice 6–10 weeks of age. *Chx10^{crj}* and *Mitf^{pmi}* mice were obtained from Jackson Labs. The *Nes-Cre^{ERT2}-IRES-hPLAP* mice were obtained from Dr. S. Baker (St. Jude Children's Research Hospital). The St. Jude Children's Research Hospital Institutional Animal Care and Use Committee approved all of the animal experiments.

Human Tissue. Adult postmortem eyes were obtained from the Mid-South Eyebank, and fetal eyes were obtained from ABR, Inc. All experiments involving human tissue were approved by the St. Jude Children's Research Hospital IRB.

Histology and Immunostaining. We immunolabeled retinal sections with attached CE and SVZ sections cut on a vibratome and dissociated retinae and SVZ as

described in refs. 24 and 25). The list of antibodies used and their dilutions is provided (*SI Materials and Methods*).

Real Time RT-PCR. Real time RT-PCR experiments were performed as described in ref. 23. Individual probe and primer sequences can be found in the *SI Materials and Methods*, Table S5.

[³H]-thymidine Labeling and Detection. To label S-phase retinal progenitor cells, we incubated freshly dissected retinae in 1 mL explant culture medium containing [³H]thy (5 μ Ci/mL; 89 μ Ci/mmol) for 1 h at 37°C. Autoradiography was carried out as described in refs. 24 and 25.

Microscopy. Bright-field and single-cell fluorescent images were obtained using a Zeiss AxioPlan-2 fluorescent microscope with the Zeiss AxioCam digital camera. Fluorescent images of sections were obtained by using a Leica TCSNT confocal microscope. Electron microscopy was carried out as described in refs. 9 and 26. Detailed protocols and procedures are presented in *SI Text*.

CE, SVZ, and Retinal Dissociation and Culture. Cultures of CE, SVZ, and Retinae to generate clonogenic spheres was carried out as described in refs. 4, 5, 27–30. Detailed protocols are available in *SI Materials and Methods*.

In Vivo Sphere Injections. Spheres were harvested by gravity and resuspended at a density of 10 spheres/ μ L. Injections of 1 μ L of spheres was performed as described in ref. 31.

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