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Pigment epithelial cells isolated from human peripheral iridectomies have limited properties of retinal stem cells

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ABSTRACT.

Purpose: **The identification of cells with properties of retinal progenitor cells (RPCs) in the adult human ciliary margin (CM) prompted a number of studies of their proliferative and differentiation potential. One of the remaining challenges is to find a feasible method of isolating RPCs from the patient's eye. In the human CM, only the iris pigment epithelium (IPE) is easily obtained by a minimally invasive procedure. In the light of recent studies questioning the existence of RPCs in the adult mammalian CM, we wanted to assess the potential of the adult human IPE as source of RPCs.**

Methods: **The IPE were isolated from peripheral iridectomies during glaucoma surgery, and IPE and ciliary body (CB) epithelium were also isolated from post-mortem tissue. Cells were cultivated in sphere-promoting conditions or as monolayers. Whole-tissue samples, undifferentiated and differentiated cells were studied by immunocytochemistry, RT-PCR and transmission electron microscopy.**

Results: **The adult human IPE, like the CB, expressed markers of RPCs such as Pax6, Sox2 and Nestin *in vivo*. Both sphere-promoting and monolayer cultures preserved this phenotype. However, both IPE/CB cultures expressed markers of differentiated epithelial cells such as Claudin, microphtalmia-associated transcription factor (MITF) and Cytokeratin-19. Ultrastructurally, IPE spheres displayed epithelial-like junctions and contained mature melanosomes. After induced differentiation, IPE-derived cells showed only partial neuronal differentiation expressing β-III-tubulin, Map-2 and Rhodopsin, whereas no mature glial markers were found.**

Conclusion: **Proliferative cells with some properties of RPCs can be isolated from the adult human IPE by peripheral iridectomies. Yet, many cells retain properties of differentiated epithelial cells and lack central properties of somatic stem cells.**

Key words: adult human – ciliary body – iris pigment epithelium – peripheral iridectomy – progenitor cell – retinal stem cell

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Introduction

In adults, retina is considered to have limited regenerative potential, and severe injury can lead to permanent damage (Klassen et al. 2004). Even though retinal degenerative diseases may be slowed or halted using gene therapy or medical treatment, there are no therapeutic options today that can regenerate damaged retinal neurons. During the development of the human retina, neuroepithelial stem/progenitor cells (NSCs) give rise to both the distinct cell types of the neuroretina and the retinal pigment epithelium (RPE) (Livesey & Cepko 2001). While the proximal retina becomes an integrated part of the central nervous system (CNS), the distal ciliary margin (CM) gives rise to two non-neural structures: the double-layered epithelium of the ciliary body (CB) and the iris pigmented epithelium (IPE) (Perron & Harris 2000). In cold-blooded vertebrates such as fish and amphibians, NSCs located in the CM can regenerate new retinal neurons throughout life (Perron & Harris 2000). Indications of an analogous stem cell-like population in the CM of the adult human retina (Tropepe et al.

2000; Coles et al. 2004; Xu et al. 2007a) and in the retina itself (Klassen et al. 2004; Mayer et al. 2005; Das et al. 2006; Carter et al. 2007; Lawrence et al. 2007) have prompted a number of investigations into their proliferative and differentiation potential *in vitro* and *in vivo* (Bhatia et al. 2009).

It has previously been demonstrated that transplantation of photoreceptor precursors can improve visual function in animal models of retinal degeneration (MacLaren et al. 2006). If NSCs exists in the adult human eye, they could be utilized as an autologous source for retinal regenerative or disease- modifying therapy, thereby avoiding ethical concerns and reducing the risk of immunological reactions and tumour formation that are associated with the use of pluripotent stem cells (Klassen et al. 2004). One of the major remaining challenges is to find a clinically and practically feasible method of obtaining NSCs from the patient's eye. Of the different cell types with neuroepithelial origin, only the IPE is easily obtained by a minimally invasive procedure, peripheral iridectomy (PI). Studies performed in chicken (Sun et al. 2006) and non-human mammals (Haruta et al. 2001; Akagi et al. 2005; Asami et al. 2007; MacNeil et al. 2007) indicate that NSCs are present in the IPE, while one study of human tissue shows that post-mortem (PM) IPE cells have less proliferative capacity compared with CB cells (Coles et al. 2004). There are also some prior clinical experiences with transplantation of IPE for RPE substitution in age-related macular degeneration (Abe et al. 2007), indicating that these cells are suited for cell-based therapy. However, little is known about the neuroepithelial potential of the adult human IPE and whether cells with the desired stem cell-like properties may be successfully cultured from patient biopsies.

A somatic stem cell is commonly defined as a cell with the ability to self-renew and give rise to all the functional cell types of the organ from which they originate (Gage 2000; Reh 2002). *In vitro*, NSCs have the ability to form spheres in suspension culture, a process where individual cells proliferate to form free-floating structures with a uniform well-defined spherical contour (Reynolds & Weiss 1992). Sphere formation has been documented to give an indication of the proliferative capacity of stem/progenitor cells (Gage 2000; Tropepe et al. 2000; Coles et al. 2004; Moe et al. 2005). Whether the sphere-forming cells of the adult mammalian CM represent a population of true NSCs, retinal progenitor cells (RPCs) with a restricted lineage potential or simply differentiated epithelial cells with the ability to proliferate and ectopically express certain stem cell markers is a matter of debate (Moe et al. 2009; Cicero et al. 2009; Gualdoni et al. 2010).

In the light of these recent studies questioning adult mammalian CB as a source of retinal stem cells, we wanted to test whether this also applies for adult human IPE, because the IPE is the only region of the human CM that is readily accessible for cell isolation in a future scenario RPC autotransplantation of patients with retinal degenerative diseases.

Materials and Methods

Dissection procedure

All experiments were conducted in accordance with the Declaration of Helsinki, and all tissue harvesting was approved by the Local Committees for Medical Research Ethics. Eyes were enucleated from human cadavers (mean age 64 years, max/min 82/32 years, $n = 15$) as previously described (Slettedal et al. 2007). Peripheral iridectomies were also obtained from trabeculectomies performed in patients with medically intractable glaucoma (mean age 54 years, max/min 85/14 years, $n = 16$) after informed written consent.

Post-mortem iris biopsies were carefully dissected free, separated and rinsed in Leibowitz-15 medium (L15; Invitrogen, Carlsbad, CA, USA). Peripheral iridectomies obtained during glaucoma surgery (Fig. 1A,B) were immediately transferred to L15. The pigmented anterior and posterior IPE were strongly attached. Both layers

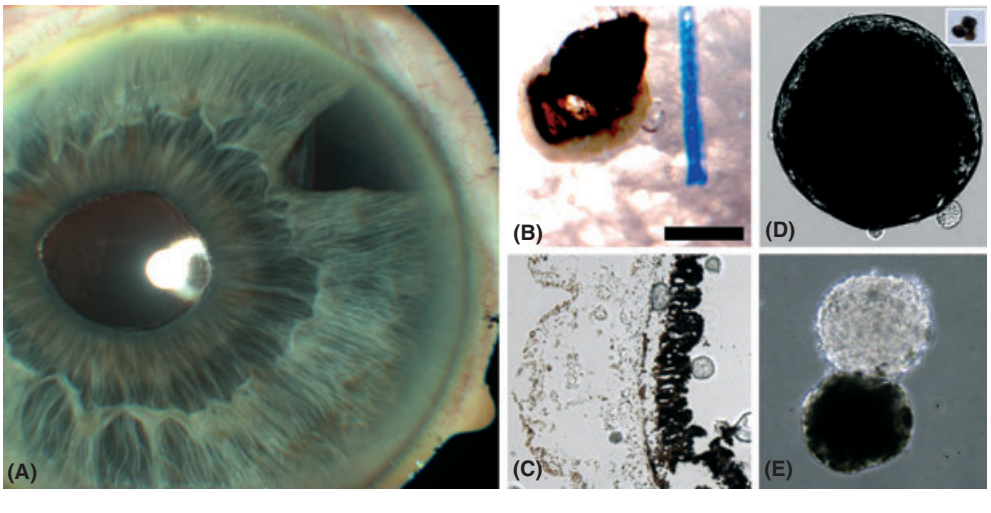

Fig. 1. Iris pigment epithelial cells (IPE) were isolated from peripheral iridectomies (picture for illustrational purposes – the iridectomies are usually much smaller and covered by the superior margo) (A). The isolated peripheral iridectomy with the posterior IPE in black (B). The anterior and the posterior IPE were strongly attached, and both layers were easily separated from the underlying stroma (C). Under sphere-promoting conditions, single pigmented cells started to proliferate (D, inset) to form typical spheres with a smooth outer surface (D). At later passages, some spheres lost most of their pigmentation, while others still showed intense pigmentation (E). Scale bar: B, 1 mm.

were easily separated from the underlying stroma (Fig. 1C) using a cell scraper and incubated with trypsin–ethylenediaminetetraacetic acid (EDTA) (0.05%, Invitrogen) for 5 min followed by careful trituation. The CB epithelial cells were accordingly isolated as previously described (Moe et al. 2009).

In vitro cultivation

The cell suspension was passed through a 70-μm strainer (BD Biosciences, San Diego, CA, USA) before cells were plated in DMEM/F12 containing Hepes buffer (1 M, 0.8%, Sigma-Aldrich, St. Louis, MO, USA), B27 supplement (2%, Invitrogen), epidermal growth factor (EGF) (20 ng/ml; R&D Systems, Minneapolis, MN, USA), basic fibroblast growth factor (bFGF) (10 ng/ml, R&D Systems), Heparin (2.5 μg/ml; LEO Pharma, Ballerup, Denmark) and Penicillin/Streptomycin (100 U/ml; Sigma-Aldrich) in non-adherent culture flasks at 37 °C in 6% CO_2 and 20% O_2. Spheres were supplemented with bFGF and EGF twice a week and passaged every two weeks by incubating the tissue at 37 °C in collagenase (78U) and hyaluronidase (38U) for 40 min, followed by trypsin–EDTA (0.05%, Invitrogen) for 2×4 min. Spheres were normally studied at passage 2–3, but some were kept in culture for up to six passages.

For adherent monolayer cultivation, cells were plated in tissue culture plates with medium containing 1% fetal calf serum (FCS; Sigma-Aldrich). Monolayer cultures were split when reaching confluency using trypsin–EDTA (0.05%, Invitrogen). To induce differentiation, single-cell suspensions were plated on glass coverslips coated with poly-L-ornithine (10 μg/ml) and cultured in the absence of growth factors and addition of 3% FCS, B27 with vitamin A (2%, Invitrogen) and laminin (100 ng/ml; BD Biosciences) for 3 weeks.

Immunocytochemistry

Spheres

A mixture of human plasma and thrombin (Sigma-Aldrich) was used to clot spheres before fixation in 4% paraformaldehyde and embedment in paraffin. Three-micrometre sections were cut and stained using LabVision Autostainer360 (Lab Vision Corporation, Fremont, CA, USA).

Monolayer and differentiated cells

Adherent cultivated cells and differentiated cells were fixed with 4% formaldehyde, and staining was performed as previously described (Olstorn et al. 2007).

Antibodies

The following primary antibodies, dilutions and positive controls were used (rb: rabbit, ms: mouse, gp: guinea pig, gt: goat, shp: sheep, pc: positive control): Sox-2 [rb, 1:500; Chemicon, Temecula, CA, USA, pc: neural progenitor cells (Moe et al. 2005)], Pigment Epithelium-Derived Factor (PEDF, ms, 1:200; Chemicon), Vimentin (rb, 1:200; NeoMarkers, Fremont, CA, USA, pc: cornea), Glutamine Synthetase (GS, 1:2000, Chemicon, pc: retina), E-cadherin (ms, 1:50; Dako, Glostrup, Denmark, pc: skin epithelium), N-cadherin (ms, 1:50; Dako, pc: kidney), Claudin-1 (Rb, 1:200; LabVision, Fremont, CA, USA, pc: breast carcinoma), Glial Fibrillary Acidic Protein (GFAP) (ms, 1:1000; Dako, pc: neural progenitor cells), Cytokeratin 19 (ms, 1:100; Dako, pc: skin epithelium), Nestin (rb, 1: 200; Sigma-Aldrich, pc: neural progenitor cells), Nestin (ms, 1:80; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Map-2 (rb; 1:1000; Chemicon, brain tissue), β-III-tubulin [ms, 1:1000; Sigma-Aldrich, pc: differentiated neural progenitor cells (Moe et al. 2005)], Rhodopsin (ms, 1:1500; Sigma-Aldrich, pc: retina), Ki-67 (rb, 1:200; Neo Markers, pc: appendix), Connexin-43 (ms, 1:500; Sigma-Aldrich, pc: fetal tissue), Chx10 (shp, 1:200; Chemicon), Nerurofilament M (NFM, ms, 1:000; Sigma-Aldrich), Syntaxin1 (HPC-1) (ms, 1:1000; Sigma-Aldrich) and Doublecortin (DCX, gt, 1:100; Santa Cruz Biotechnology, pc: differentiated neural progenitor cells). The secondary antibodies had the fluorescent markers Cy3 (1:1000; Sigma-Aldrich) and Alexa Fluor 488 (1:500; Invitrogen). Hoechst (1:500; Invitrogen) was used for nuclear staining. For negative controls, the primary antibody was omitted. The sections were analysed using an Olympus BV 61 FluoView confocal microscope (Olympus, Hamburg, Germany) and a ZEISS Axio Observer.Z1 fluorescence microscope (ZEISS, Oberkochen, Germany). The expression pattern was evaluated by two independent investigators.

Transmission electron microscopy

The spheres were fixed for 30–60 min at room temperature by immersion in freshly prepared mixed aldehyde fixation containing 0.1 M sodium cacodylate buffer, 2% glutaraldehyde, 2% paraformaldehyde and 0.025% $CaCl_2$, pH 7.4. Fixation was continued overnight at 4 °C, postfixed in 1% osmium tetroxide and dehydrated through a graded series of ethanol up to 100%. The spheres were then immersed in propylene oxide for 20 min and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, USA). Ultra-thin sections (60–70 nm thick) were cut on a Leica Ultracut Ultramicrotome UCT (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate and examined using a Tecnai12 transmission electron microscope (Phillips, Amsterdam, the Netherlands).

RT-PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). Potential DNA was degraded using Dnase1 (Ambion, Austin, TX, USA). RNA concentration and purity was quantified by spectrophotometry (Nanodrop, Wilmington, DE, USA), and quality and integrity were confirmed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The cDNA was synthesized from 200 ng total RNA using Multiscribe Reverse Transcriptase (Applied Biosystems, Abingdon, UK). RT-PCR analyses were performed using StepOnePlus Real-Time PCR System (Applied Biosystems) and TaqMan Gene Expression assays (Applied Biosystems). The analyses were performed in 96-multiwell plates with a total reaction volume of 15 μl, containing $1 \times$ TaqMan Gene Expression Master Mix, $1 \times$ fwd./rev. primer and probe mix and 0.5 μl cDNA (Table 1). The PCR conditions included an initial holding stage, 50 °C for 2 min and 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 seconds and 60 °C for 1 min. The PCR was carried out in duplicates for each

Table 1. Primers used in RT-PCR.

Gene name	Gene symbol	Assay ID	Amplicon length (bp)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Hs99999905_m1	122
Claudin-1	CLDN1	Hs00221623_m1	82
Keratin 19	CK-19	Hs00761767_s1	116
N-Cadherin	CDH2	Hs00983062	78
E-cadherin	CDH1	Hs01023894_m1	61
Serpin peptidase inhibitor, clade F, member 1	PEDF/SERPINF1	Hs01106937_m1	84
Doublecortin	DCX	Hs00167057_m1	77
Glial fibrillary acidic protein	GFAP	Hs00157674_m1	75
Nestin	NES	Hs00707120_s1	81
Microphtalmia-associated transcription factor	MITF	Hs01117294_m1	81
Cone-rod homeobox	CRX	Hs00230899m1	73
Visual system homeobox 2	VSX2	Hs01584046_m1	116
Sonic hedgehog homologue	Shh	Hs00179843_m1	70
Nanog homeobox	Nanog	Hs02387400_g1	109
SRY(sex-determining region Y)-box 2	Sox2	Hs01053049_s1	91
POU class 5 homeobox 1	Oct4/POU5F1	Hs03005111_g1	64
Paired box 6	Pax6	Hs01088112	55

sample, at least three samples in each condition, and a template-free (H_2O-only) reaction was included for each primer pair as a negative control. PCR was also carried out with RNA as template, to ensure no genomic DNA amplification. PCR products were visualized using MCE-202 MultiNA Microchip Electrophoresis System for DNA/RNA analysis (Shimadzu Corporation, Kyoto, Japan) according to the manufacturer's instruction.

Statistics

The results are presented as mean ± SD or median (range). Differences between groups were tested with two-tailed independent sample *t*-tests. Data that were not normally distributed were normalized by log transformation. The significance level was set to $p < 0.05$, and data were analysed using SPSS version 14.0.

Results

Isolation, proliferation and sphere-forming capacity

After removal of the PI from the operation theatre, we could only identify the IPE (Fig. 1B–C) in seven out of 16 specimens, while the other IPE samples were lost from iris stroma during removal from the eye or from the field of operation. In all cultures from PM tissue ($n = 15$) and from peripheral iridectomies with identified IPE ($n = 7$), pigmented cells proliferated and formed heavily pigmented spheres after 4–5 days in culture (Fig. 1D). After repetitive passages, many spheres lost their light-microscopic appearance of pigment, while some spheres continued to be heavily pigmented (Fig. 1E). The measured growth rate of individual spheres ten days after the first passage (P1 + 10D) showed an average diameter of 134 ± 64 μm for PM spheres and ±42 μm for PI spheres ($n = 14$). There were no statistically significant differences in sphere size between the groups ($p = 0.15$).

Expression of epithelial and neural markers

We have recently shown that adult human CB spheres contain a population of proliferating epithelial-like cells with decreased expression of neural stem cell markers compared with CNS neurospheres (Moe et al. 2009). To elucidate whether the IPE, which, unlike the CB, can be isolated by a minor surgical procedure, also contain a mixed epithelial and neural phenotype, we compared whole tissue (WT), primary spheres (P0) and tertiary spheres formed after two passages (P3) from IPE and CB samples.

We first characterized the composition of intermediate filaments (IFs) that are expressed in distinct patterns during epithelial and neuroepithelial differentiation (Schlotzer-Schrehardt & Kruse 2005; Moe et al. 2009). We found CK-19 transcripts of both PM IPE and CB spheres especially at P3 (Fig. 2J), while only weak bands of GFAP were detected in WT and at P3 of IPE samples and at P3 in CB spheres (Fig. 2J). A distinct population of cells positive for Nestin, a marker normally expressed in neural precursors (Lendahl et al. 1990), was found in both PM IPE/CB spheres and PI IPE spheres (Fig. 2A–C). This population displayed intense staining, in contrast to the surrounding cells, which were negative. Even though RT-PCR analysis revealed Nestin in all WT samples and at P3 of both IPE and CB, Nestin expression was much weaker at P0 (Fig. 2J). At P3, 38 (29–42)% of cells in the CB spheres and 35 (29–42)% ($n = 3$) of the cells in the IPE spheres were Nestin positive.

In an attempt to distinguish epithelial-like cells from neural progenitor cells in the spheres, we used Claudin to identify cells with epithelial tight-like junctions (Furuse et al. 1998), as such junctions are not found in CNS-derived neurospheres (Kohno et al. 2006; Moe et al. 2009). Claudin-1 was clearly expressed in both IPE and CB spheres at PO and P3 (Fig. 2J), but significant double staining for Claudin-1 and Nestin was not observed (Fig. 2A–C). In PM IPE spheres at P2, 17 (6–25) % of the cells were Claudin-1 positive ($n = 4$). Both Nestin-positive and Nestin-negative cells were mitotically active, seen as positive Ki-67 staining in both PM IPE/CB spheres and PI spheres (Fig. 2D–F). The percentage of cells positive for Ki-67 was 22 (20–41)% and 25 (15–38)% for CB and IPE spheres, respectively ($n = 3$) (Fig. 4B). These data indicate that the sphere-forming cells from the IPE, like the CB, contain both neural-like and non-neural cells with a proliferative potential.

The IPE spheres also expressed PEDF (Figs 2J and 4E), which is known to be a potent endogenous inhibitor of retinal angiogenesis and a neuroprotective agent normally secreted by the RPE and the CM epithelium (Dawson et al. 1999; Aymerich et al. 2001; Bilak et al. 2002).

Next, we compared the expression of transcription factors known to regulate RPCs (Belecky-Adams et al. 1997; Rowan & Cepko 2004; Lamba et al. 2010). Of the selected immature stem cell markers, Sox2 and Nanog

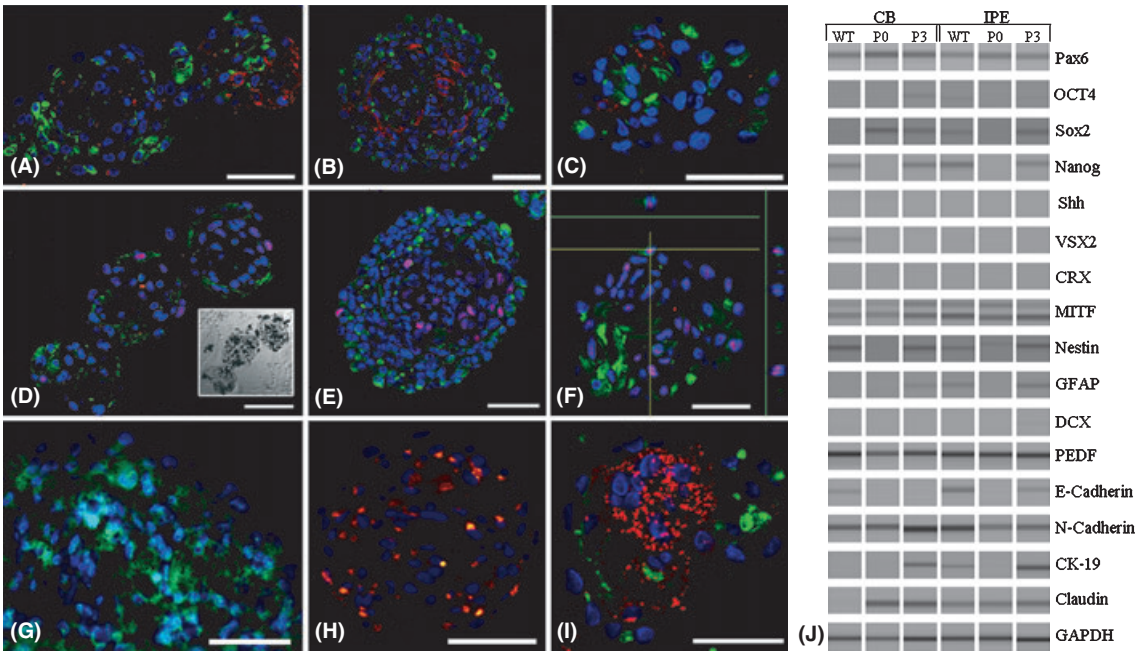

Fig. 2. Neuroepithelial stem/progenitor markers in adult human iris pigment epithelium (IPE) compared with the adult human ciliary body (CB) epithelium. Immunocytochemical analysis of secondary spheres derived from post-mortem (PM) CB (A + D), PM iris (B + E) and peripheral iridectomies obtained during glaucoma surgery (C + F). Co-staining of Nestin (green) and Claudin-1 (red) (A–C). Co-staining of Ki-67 (nuclear stain, red) and Nestin (green) (D–F). Orthogonal projections showing Ki-67 staining within the Nestin-positive cell (F). N-cadherin staining (green) in secondary IPE sphere (G). Chx10 staining (red) in secondary IPE sphere (H). Connexin 43 (red) and Nestin (green) staining in secondary IPE sphere (I). Nuclear staining with Hoechst (blue). Scale bars: A-I 50 μm. Right panel: RT-PCR analyses comparing the expression of stemness markers (Oct4, Sox2, Nanog, Shh, p63), early eye-field markers (Pax 6, VSX2, Crx, MITF), neural markers (Nestin, GFAP, DCX, N-cadherin) and epithelial markers (PEDF, E-cadherin, CK-19, Claudin-1) in whole-tissue (WT) samples and spheres at passage 0 (P0) and passage 3 (P3), isolated from the PM CB and IPE (J).

were present in WT IPE samples, while Sox2 was not found in WT of the CB samples. Both markers were found at P3 of IPE and CB spheres (Fig. 2J). Only weak transcripts of the immature stem cell marker Oct4 were detected. Of the presumptive eye-field transcription factors, Chx10/VSX2, which plays a critical role in RPC proliferation and bipolar cell determination (Liu et al. 1994; Rowan & Cepko 2004), has previously been detected in rodent CB spheres (Xu et al. 2007a), but not in chick IPE progenitors (Sun et al. 2006; Asami et al. 2007). We were not able to detect VSX2 transcripts in any of the IPE samples (Fig. 2J). Still, careful immunocytochemical analysis showed weak but clearly evident perinuclear Chx10 staining of the IPE spheres at early passages (Fig. 2H). In addition, we found significant mRNA levels of the retinal progenitor marker Pax6 (Marquardt et al. 2001) in both WT, P0 and P3 of both IPE and CB samples. Microphtalmia-associated transcription factor transcripts were also found in IPE and CB samples, while the pan-photoreceptor marker Crx was not (Fig. 2J). Even though MITF is also expressed in the developing RPE (Nakayama et al. 1998), the co-expression of several immature eye-field transcription factors indicates that the adult human IPE, comparable with the CB, display some of the gene expression profile found in retinal stem/progenitor cells *in vivo* and that this profile is preserved during *in vitro* cultivation.

Ultrastructural characterization

Next, we wanted to explore whether adult human IPE spheres similar to CB spheres (Moe et al. 2009) contain ultrastructural characteristics of epithelial cells, in addition to structures found in CNS neurospheres (Kohno et al. 2006; Moe et al. 2009). Transmission electron microscopy revealed polymorphic cells with free IFs and cytoplasmic processes at the centre of the IPE spheres, resulting in a compact architecture with areas displaying considerable membrane convolution (Fig. 3A). The cells on the sphere surface were flattened and some had microvilli on their apical side (Fig. 3A). We could only detect scattered mature stage IV melanosomes (Schraermeyer & Heimann 1999; Gualdoni et al. 2010), in which melanin deposition is complete and the organelle striations are not visible (Fig. 3A), indicating no active melanin synthesis. Vesicles, indicating active exocytosis/endocytocis, were also seen (Fig. 3C inset). Similar to cells of both adult human CB and CNS neurospheres (Moe et al. 2009), cells of the IPE spheres appeared to be connected by immature adherence-like structures (Fig. 3C), also indicated by N- and E-cadherin expression (Fig. 2G,J). Of the PM IPE spheres at P2, 40 (31–48)% of the cells were N-cadherin positive and 23 (8–57)% were E-cadherin positive (n = 3). We also found indications of gap junctions in the IPE spheres evident by Connexin-43 staining (Fig. 2I) that have previously been identified as a marker of the mammalian CM epithelium (Gualdoni et al. 2010). In addition, epithelial junctions not found in CNS neurospheres, such as desmosomal-like adhesion complexes in various stages of maturation (Fig. 3B) and tight

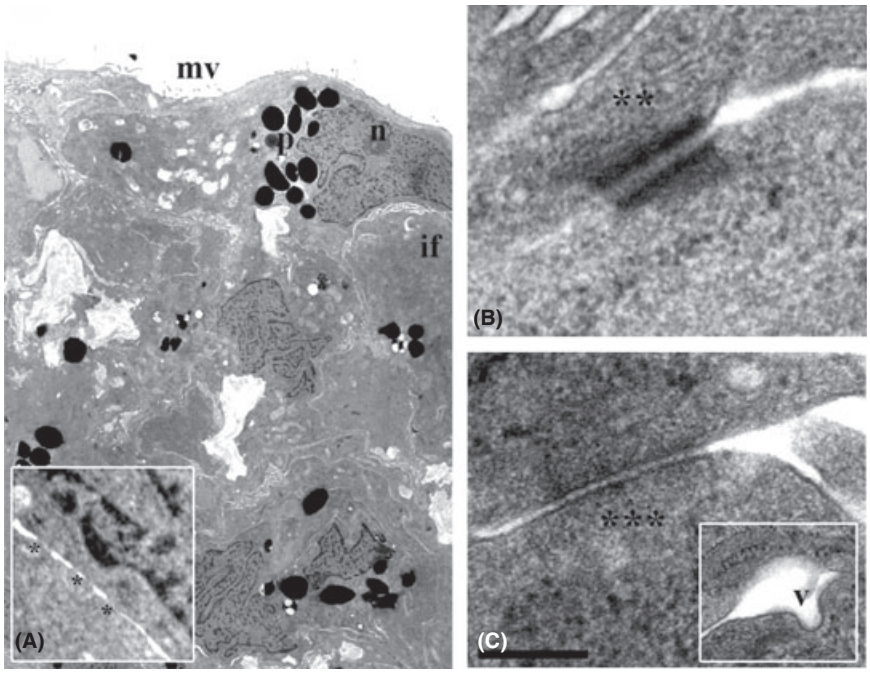

Fig. 3. Transmission electron micrographs of adult human iris pigment epithelial cells grown as spheres. Overview showing pigmented cells with small villi on surface (A) and tight junction-like structures (A inset). Peripheral cell with desmosomal-like junction (B). Central cell with adherent-like junction (C) and vesicle (C inset). N, nucleolus; p, pigmented melanosomes; mv, microvilli; if, intermediate filaments; v, vesicles; *, tight-like junctions; **, desmosomal-like junction; ***, immature adherence-like junctions. Scale bar C: 200 nm.

junction-like structures with the appearance of punctuate fusions of the two plasma membranes (Fig. 3A inset), and corresponding Claudin-1 staining (Fig. 2B,C) were also found in the IPE spheres.

Minimal serum adherent cultivation

To utilize autologous IPE progenitors for transplantation, a large number of cells may be needed. Because of the limited proliferative potential of adult human CM progenitors in sphere-forming cultures (Coles et al. 2004; Xu et al. 2007a; Moe et al. 2009), we tested the use of adherent monolayer culture (Coles et al. 2004) for expanding adult human IPE cells isolated from peripheral iridectomies (Fig. 4A). Because high serum concentration can negatively select for slow-cycling stem cells (Mannello & Tonti 2007), we utilized a minimal serum (1% FCS) culture media. Compared with sphere cultures, monolayering increased the number of cells; while monolayer cultured PI IPE cells at P1 doubled in 10 days (20 000–38 000 cells), sphere cultured cells were halved [20 000–8000 cells (n = 154 spheres)]. Importantly, the adherent cells ceased cell division upon confluency and required regular growth factor supplementation and passaging to maintain proliferation (not shown), indicating that the culture conditions did not immortalize the cells. We then investigated whether minimal serum adherently cultivated IPE cells retain some of the neuroepithelial progenitor markers found in the IPE spheres. As for IPE spheres, the monolayer IPE cells from both PM and PI stained for Nestin (Fig. 4C,D,F), cytoplasmic Sox-2 (Fig. 4D), PEDF (Fig. 4E) and Vimentin (Fig. 4F). Only weak perinuclear GFAP staining was observed (Fig. 4E). At P3, the number of Nestin-

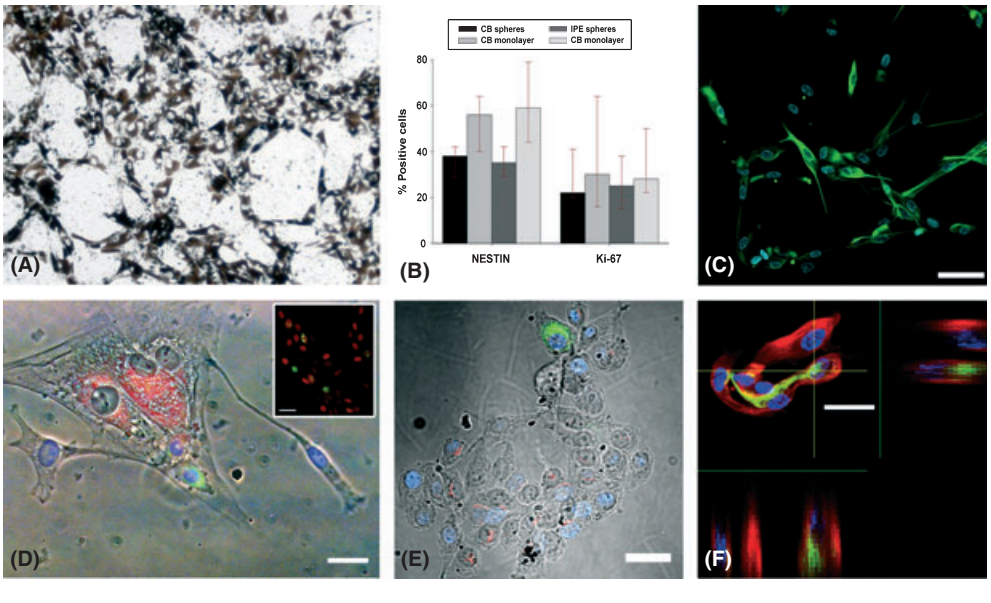

Fig. 4. Adherent cultivation of adult human iris pigment epithelial cells (IPE) in minimal (1%) serum media. Light-microscopic appearance of IPE monolayer cells from peripheral iridectomies (A). Comparison of IPE cells with ciliary body (CB) epithelial cells for Nestin and Ki-67 expression in sphere-promoting and monolayer cultures, presented as median (range) (n = 3) (B). Immunocytochemical staining of monolayer IPE cells from peripheral iridectomies with Nestin (green) (C), co-staining of Sox-2 (red) and Nestin (green) (D), HuN (red) and Ki-67 (green) (D inset), glial fibrillary acidic protein (red) and pigment epithelium-derived factor (green) (E) and Vimentin (red) and Nestin (green) with orthogonal projections (F). Nuclear staining with Hoechst (blue). Scale bars: C, 50 μm; D–E, 20 μm; F, 10 μm.

expressing cells in the PM IPE monolayer was 59 (44–79)% and the number of actively dividing cells, evident by Ki-67 staining, was 28 (22–50)% (n = 3)(Fig. 4B). Approximately one-fourth of the Nestin-positive cells at P1 contained light-microscopic-visible pigment. Together, these findings indicate that adult human IPE can be readily expanded using minimal serum adherent cultivation without losing their limited properties of neuroepithelial progenitors.

Differentiation potential

Finally, to determine the potential for retinal differentiation of adult human IPE cells in culture, a differentiation protocol known to promote neuronal differentiation of NSCs (Moe et al. 2005) was utilized. After induced differentiation, cells migrated out of the spheres (Fig. 5A) to form a sheet of cells with sparse pigmentation and mixed morphology (Fig. 5B). A few cells developed a mature neuronal-like morphology with multiple branching extensions (Fig. 5C). Cells grown and passaged in minimal serum monolayer cultures could also be induced to differentiate in a similar manner (not shown). Immunocytochemical analysis revealed only weak perinuclear staining of GFAP (Fig. 5E) and no staining of GS, while strong Vimentin staining was still found in most cells (Fig. 5F). This indicates that the adult human IPE cells were not able to differentiate into mature glial cell types, like RPCs do during eye development (Fischer et al. 2010). However, many (>80%) of the differentiated cells strongly expressed the neuronal marker β-III-tubulin (Fig. 5D), and some also displayed Map-2 (not shown). The β-III-tubulin-positive population included both cells with ganglion cell-like morphology with a polarized appearance and long dendrites (Fig. 5D right cell), as well as cells with round cell soma and short extensions (Fig. 5D left cells), resembling immature neuroblasts or photoreceptor precursors in culture (Inoue et al. 2010). Although we did not find any significant number of cells displaying markers of retinal neurons (HPC-1, DCX, NFM, Rhodopsin), less than 5% of the rounded cells with short extensions showed co-staining of β-III-tubulin and Rhodopsin (Fig. 5G). These results suggest that isolated and expanded adult human IPE cells can be induced to partly differentiate into a neuronal direction. Still, the present *in vitro* protocol does not produce any fully differentiated neurons or glia found in the retina.

Discussion

Peripheral iridectomy is routinely performed during trabeculectomies for medically intractable glaucoma, with few surgical complications, and can therefore be utilized for future harvest of cells for autotransplantation. In the

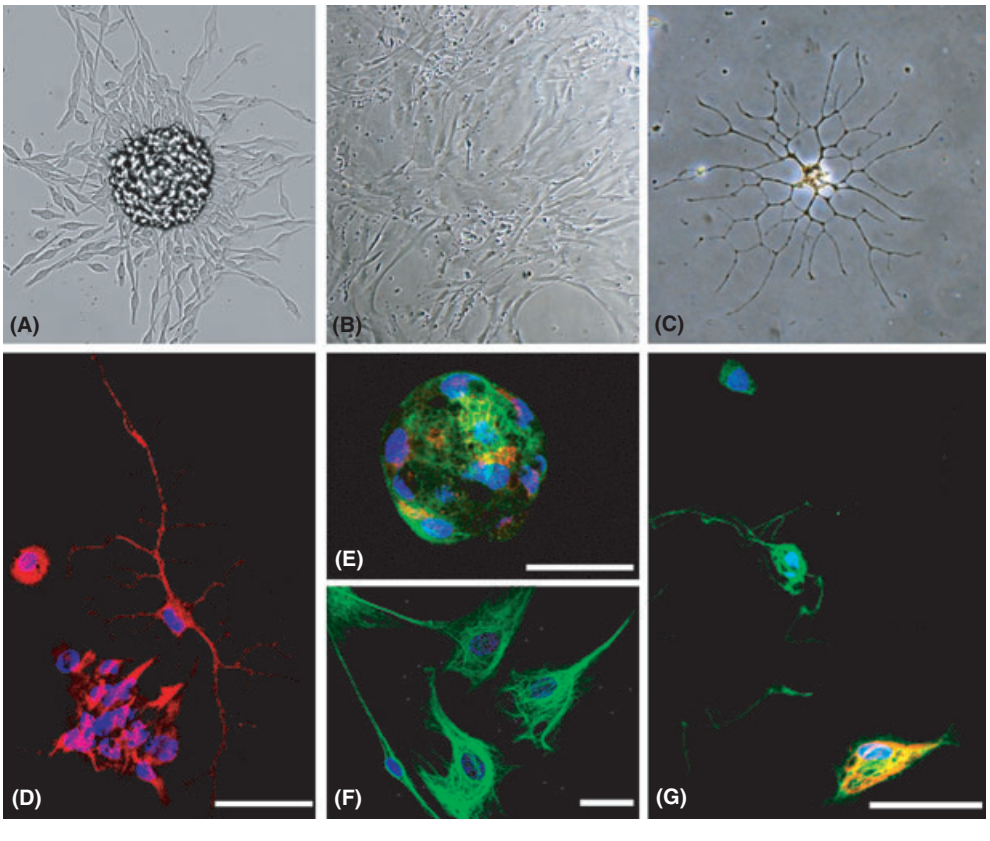

Fig. 5. Differentiation of adult human iris pigment epithelial cells (IPE) cultivated as spheres. After enzyme digestion, small spheres were still present, and cells migrated out of the spheres after induced differentiation (A) to form a sheet of cells with sparse pigmentation and mixed morphology (B). Some cells acquired a distinct neuronal morphology including long, polarized outgrowths (C). Robust β-III-tubulin staining (red), showing cells with photoreceptor-like and ganglion cell-like morphologies (D). Cells that did not migrate out of the small spheres also stained for β-III-tubulin (green), while only weak, perinuclear staining of GFAP (red) was detected (E). Strong staining of Vimentin was present also after differentiation (F). Double staining with β-III-tubulin (green) and Rhodopsin (red) (G). Nuclear staining with Hoechst (blue). Scale bars: D,E,G, 50 μm; F, 10 μm.

present paper, we have shown that a population of proliferative cells with some properties of RPCs can be isolated from peripheral iridectomies. Yet, these cells retain properties of differentiated epithelial cells and lack central properties of somatic stem cells such as targeted multipotent differentiation. This is an important finding, as almost all previous research on IPE as a source of retinal stem cells has been performed on non-human cell types and needs to be confirmed in humans.

Recently, Cicero et al. have reinterpreted previous findings supporting the existence of retinal stem cells in the CB epithelium of adult mammals, suggesting instead that these cells have the ability to proliferate and ectopically express RPC markers and some mature retinal markers, while they still retain their pigmented ciliary epithelial phenotype (Cicero et al. 2009). Our findings support this conclusion to a certain degree. We have previously compared spheres from the adult human CB and brain, finding that CB spheres contain progenitor cells with more epithelial properties and limited expression of NSC markers compared with brain neurospheres (Moe et al. 2009). In the present paper, we show that proliferating cells of the adult human IPE also have properties of differentiated epithelial cells; they contain junctional complexes such as gap-like, adherence-like and desmosomal-like junctions, contain mature melanosomes and express markers found in the ciliary epithelium such as PEDF, MITF and CK-19. This is not surprising, given that the IPE and CB have the same developmental origin.

However, our data indicate that IPE, like the CB, does not contain a homogenous population of epithelial cells without properties of NSCs or RPCs. Firstly, we found expression of transcription factors prominent in NSCs such as Sox-2, Nestin and Nanog, as well as the RPC-marker Pax6, both *in vivo* and after repetitive passages *in vitro*. This is also partly supported by a recent study (Bhatia et al. 2009), showing that the human CB *in situ* express Vimentin, Sox2 and Chx10, while Nestin, in contrast to our study, was not evident. Secondly, immunocytochemistry revealed two distinct populations of cells: one Claudin-1-positive and one Nestin-positive population. It thus seems plausible that the cultured cells do not diffusely upregulate Nestin, but rather that a sphere consists of a subpopulation of Nestin-expressing cells with neighbouring epithelial-like cells positive for Claudin-1. Thirdly, we only found mature melanin granules and a gradual loss of pigmentation with time in culture, which is in coherence with other studies of adult mammalian CB/IPE cultures (Sun et al. 2006; Gualdoni et al. 2010; Yamamoto et al. 2010). Thus, our data support that repeated passaging may positively select for neuroepithelial properties in the sphere-forming IPE/CB cells (Kohno et al. 2006; Asami et al. 2007). Cicero et al. studied spheres mostly at P0 after 7 days in culture, and it is possible that the cells at this time display a more homogeneous epithelial phenotype than if they are kept in culture for a longer period of time. A recent study performed on mice iris tissue (Yamamoto et al. 2010) also strengthens the hypothesis of a mixed population of neural progenitor cells and epithelial cells in the adult mammalian IPE. They showed that within a neurosphere, cells positive for the neurotrophin receptor $p75^{NTR}$ are more Nestin positive, have less or no melanin and have a greater ability to differentiate into neuronal-like cells than the $p75^{NTR}$ -negative population. Still, as we did not differentiate between the anterior and the posterior IPE during cultivation, there might be differences in the cellular composition originated from these two layers. In addition, the IPE were only isolated from patients with glaucoma or PM biopsies which may also have an effect on the composition of the cultured cells.

Even though cells with some properties of NSCs may reside in CM of adult humans, their differentiation into functional retinal cell types remains a challenge. Although some previous studies have shown the presence of retinal markers (Coles et al. 2004; Sun et al. 2006; Yamamoto et al. 2010) and immature neuronal activity (Das et al. 2005) in differentiated progenitors from the mammalian CB/IPE, most studies have failed to produce convincing data of functional retinal cell types (MacNeil et al. 2007; Cicero et al. 2009; Gualdoni et al. 2010). These studies are in agreement with our differentiation experiments using adult human IPE cells, where only partial differentiation into a retinal direction was observed. Furthermore, we did not find proper glial differentiation, as previously described for adult human CB epithelial cells in culture (Coles et al. 2004). As one of the key features of somatic stem cell is their ability to differentiate into all cell types of the organ from which they originate (Gage 2000; Reh 2002), the lack of multipotent retinal differentiation indicates that the adult human IPE does not contain *bona fide* retinal stem cells. It can be argued that *in vitro* differentiation is a non-physiological event that does not necessarily impact on the ability of IPE cells to integrate and differentiate into a recipient retina. On the other hand, MacLaren et al. have shown that the optimal donor cell for retinal transplantation is a cell that has recently committed to the photoreceptor fate (MacLaren et al. 2006). Thus, it seems necessary to use a source of stem cells with the ability to effectively generate such committed precursor cells *in vitro* to succeed in clinical transplantations for retinal degenerative diseases.

Given the neuroepithelial origin of the IPE, they may be ontogenetically closer to RPCs than embryonic stem cells or induced pluripotent stem cells (iPS) and should therefore be suited for novel reprogramming strategies utilizing gene transfer to express key transcription factors (Asami et al. 2007). Pax6 is one of the earliest eye-field factors and is considered a master control gene for retinal development. Conversely, loss of Pax6 function results in aniridia and Peter's anomaly (Xu et al. 2007b; Davis et al. 2009). It has been suggested that the lack of retinal differentiation of mammalian CM progenitors may be due to the loss of Pax6 during repetitive passaging (MacNeil et al. 2007). Yet, in our study, Pax6 expression was found both in P0 and P3 spheres of both CB and IPE spheres. However, Crx (Haruta et al. 2001), another key transcription factor for photoreceptor development, was not detected in our experiments. Studies have been successful in generating cells with a photoreceptor-like phenotype from mammalian IPE (Haruta et al. 2001)

(Akagi et al. 2005) and human CB (Inoue et al. 2010; Jomary et al. 2010) when Crx gene transfer was performed alone or in combination with other transcription factors. Further studies are thus needed to find out whether similar techniques may be utilized to guide neuroretinal differentiation of adult human IPE cells. In addition, mapping the epigenetic status of such key regulating genes in the IPE cells could give valuable information about the ability of the human IPE to act as RPCs in adulthood.

In summary, we have shown that a population of proliferative IPE cells with some properties of retinal precursor cells can be isolated with a minimal surgical procedure from the human eye. However, these cells retain properties of differentiated epithelial cells and lack central properties of somatic stem cells.

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