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EDITORIAL

Shaping the eye from embryonic stem cells: Biological and medical implications

Gabriele Colozza, Morgane Locker, Muriel Perron

Gabriele Colozza, Morgane Locker, Muriel Perron, Laboratory of Neurobiology and Development, UPR CNRS 3294, University Paris-Sud, 91405 ORSAY Cedex, France
Gabriele Colozza, Howard Hughes Medical Institute University of California, Los Angeles, 5-748 MacDonald Research Laboratories (MRL), 675 Charles E. Young Drive, South Los Angeles, CA 90095-1662, United States
Author contributions: Colozza G wrote the first draft of the manuscript; Locker M and Perron M revised the manuscript.
Correspondence to: Muriel Perron, PhD, Laboratory of Neurobiology and Development, CNRS UPR 3294, Institute of Neurobiology A. Fessard, Bât. 445, University Paris-Sud, 91405 ORSAY Cedex, France. muriel.perron@u-psud.fr
Telephone: +33-1-69157225 Fax: +33-1-69156802
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Abstract

Organogenesis is regulated by a complex network of intrinsic cues, diffusible signals and cell/cell or cell/matrix interactions that drive the cells of a prospective organ to differentiate and collectively organize in three dimensions. Generating organs *in vitro* from embryonic stem (ES) cells may provide a simplified system to decipher how these processes are orchestrated in time and space within particular and between neighboring tissues. Recently, this field of stem cell research has also gained considerable interest for its potential applications in regenerative medicine. Among human pathologies for which stem cell-based therapy is foreseen as a promising therapeutic strategy are many retinal degenerative diseases, like retinitis pigmentosa and age-related macular degeneration. Over the last decade, progress has been made in producing ES-derived retinal cells *in vitro*, but engineering entire synthetic retinas was considered beyond reach. Recently however, major breakthroughs have been achieved with pioneer works describing the extraordinary self-organization of murine and human ES cells into a three dimensional structure highly resembling a retina. ES-derived retinal cells indeed assemble to form a cohesive neuroepithelial sheet that is endowed with the intrinsic capacity to recapitulate, outside an embryonic environment, the main steps of retinal morphogenesis as observed *in vivo*. This represents a tremendous advance that should help resolving fundamental questions related to retinogenesis. Here, we will discuss these studies, and the potential applications of such stem cell-based systems for regenerative medicine.

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Key words: Retina; Optic cup; Embryonic stem cells; Retinal pigment epithelium; Three dimensional culture

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OPTIC CUP MORPHOGENESIS *IN VIVO*

Harmonious formation and patterning of tissues and organs during development relies on complex cellular interactions that are tightly regulated in both time and space. Among well known processes is embryonic induction, in which a group of cells causes changes in adjacent cell morphology, proliferative capacities or destiny. Eye development is often taken as a paradigm for successive and often reciprocal inductions that

progressively shape an organ. By the end of neurulation, the optic vesicle (OV) evaginates from the anterior neural tube, comes in close contact with the head surface or "pre-lens" ectoderm, and instructs it to become a lens placode. As a consequence, this ectodermal region thickens and eventually invaginates to form the lens vesicle. Concomitant to this process, complex structural changes take place in the OV, which in turn invaginates into an optic cup (OC) (Figure 1A). Although still a matter of debate (see below), the pre-lens ectoderm seems to be required for OC morphogenesis[1]. Then, the OC inner wall forms the sensory neural retina while the outer one forms the retinal pigmented epithelium (RPE). The ectoderm was shown to specify the neural retina, while diffusible signals emanating from the periocular mesenchyme determine the RPE. Besides, opposing influences from the neural tube or optic stalk on one hand and from the dorsal OC region on the other hand contribute to dorso-ventral patterning of the OC. This extensive series of interactions ensures the coordinated development of different parts which, together, will constitute a functional eye[2].

FROM MURINE EMBRYONIC STEM CELLS TO OC MORPHOGENESIS *IN VITRO*

In the past few years, major progress has been made in setting up efficient procedures allowing the *in vitro* differentiation of RPE or neural retinal cells from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells[3-9] (Figure 2). Members of Yoshiki Sasai's group are among great contributors. A first milestone was the generation of neural precursors from ES cells cultured under low growth factor conditions (serum-free floating culture of embryoid-body-like aggregates with quick reaggregation, or SFEBq)[10,11]. Then, these neuralized ES cells were shown to acquire a retinal-restricted identity when exposed to biological compounds inhibiting Wnt and BMP/Nodal pathways (a known requirement for eye field formation *in vivo*) in the presence of Activin and serum[12]. However, in these procedures, cells do not self organize into neuroepithelia. Researchers from Sasai's group then turned to a three dimensional (3D) culture system by adding basement-membrane matrix components (matrigel) to the SFEBq culture medium[13-15]. In these conditions, ES cells were not only efficiently "retinalized", but also organized within aggregates into polarized neuroepithelia. A striking phenomenon then started to occur after 6-7 d of culture with the budding of vesicles, followed 2 d later by an invagination process, giving rise to OC-like structures (Figure 1B). These were shown, as *in vivo*, to be composed of a distal portion exhibiting features of neural retina, and a proximal one expressing characteristic markers of RPE. The entire process of this spontaneous OC morphogenesis, monitored in 3D live imaging with multi-photon optics, could be subdivided in 4 main steps, resembling the developmental phases occurring *in vivo*. The first one is the evagination of hemispherical vesicles from neuralized aggregates. Phase 2 is characterized by the flattening of the distal portion of the vesicles. During phase 3 the angles at the junctions (hinge points) between prospective neural retina and RPE become acute. Finally, phase 4 marks the invagination event that shapes the OC. Noteworthy, this whole dynamic process occurs in the absence of a full embryonic environment and in particular without lens or any surface ectoderm. Whether OC formation *in vivo* is indissolubly linked to lens development and *vice versa* has been a large matter of debate[1,16,17]. The findings reported by Eiraku *et al*[13] show that, at least in this simplified *in vitro* context, lens is dispensable for OC invagination.

CELL DIFFERENTIATION WITHIN MURINE ES-DERIVED RETINA

In the developing embryonic eye, newly generated OCs extensively grow as a result of cell proliferation. During this step, apico-basal interkinetic nuclear migration (INM) accompanies cell cycle progression of retinal progenitor cells. Such INM was also observed in ES-derived OC neuroepithelia, highlighting again the great similarity with the *in vivo* situation. Besides, when phase 4 OC were excised and maintained in long-term culture, they progressively acquired a typical lamination, reminiscent of the early postnatal retina. Accordingly, expression of differentiation markers of all retinal neurons and glia could be detected in a correct apico-basal pattern (Figure 1B). Additionally, the typical temporal histogenic sequence was conserved as well, with the birth of ganglion cells first, followed by the progressive genesis of amacrine, cone, horizontal, rod and, finally, bipolar cells. Thus, these synthetic retinas not only undergo spontaneous morphogenesis, but also self-regulate the spatial and temporal order of neuronal differentiation. However, it remains to be determined whether connectivity establishes between the different cell layers of the ES-derived OC and whether it leads to any signs of functionality.

MECHANISMS UNDERLYING MURINE OC MORPHOGENESIS

Certainly, *in vitro* generated OCs represent a powerful system for future studies on several aspects of retinal development. Eiraku *et al*[13] started to address some questions and notably investigated the cellular and molecular mechanisms sustaining murine OC morphogenesis *in vitro*. They found that OC invagination requires the activation of the Rho-associated protein kinase (ROCK), a key regulator of actomyosin contraction in epithelial morphogenesis[18]. During phase 1, ROCK activity is uniformly distributed in the proximal and distal portions of the OV. It then progressively decreases from phase 2 to phase 4 in the neural retina and gets restricted to the prospective RPE and hinge points (Figure 1B). This likely confers them a higher

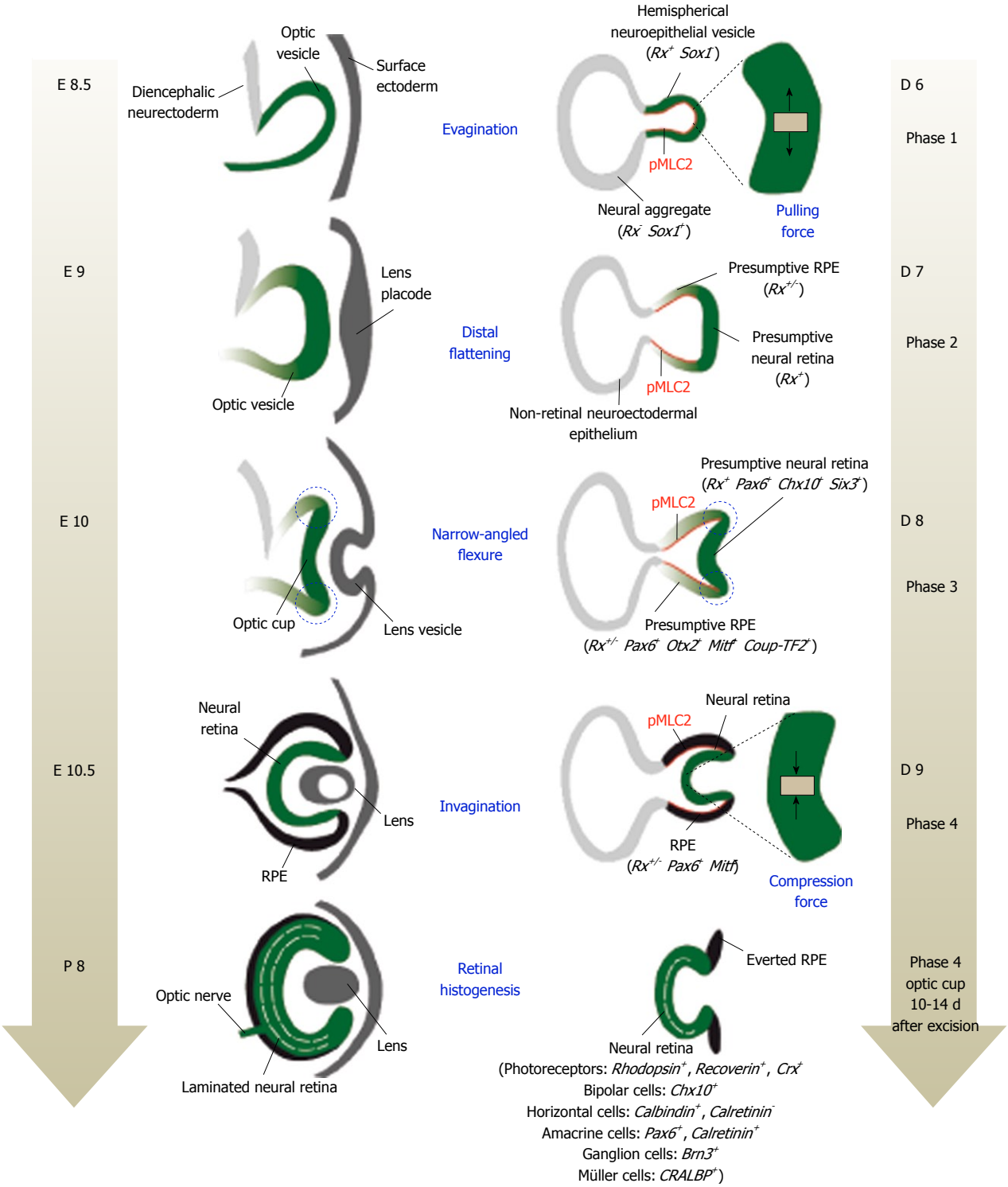

Figure 1 Schematic representation of *in vivo* (A) and *in vitro* (B) retinal development. A: During mouse embryonic development, the *Rx*-expressing optic vesicle (green) evaginates from the diencephalon at embryonic day 8 (E8). After contacting the surface ectoderm, the outer wall of the optic vesicle invaginates to form the optic cup. Its proximal layer forms the retinal pigmented epithelium (RPE), while the distal layer constitutes the neural retina. Retinal histogenesis and consecutive lamination then occurs. The process is completed postnatally (P0-P11); B: Retinal morphogenesis from mouse embryonic stem cells mimics this whole *in vivo* process and can be divided into 4 main phases. In phase 1, the *Rx*-expressing neuroepithelium (green) evaginates from the neural aggregate. In phase 2, its distal portion flattens. During phase 3, the angles at the hinge between the presumptive RPE and the neural retina become acute (dotted blue circles). Finally, a two-walled optic cup is formed during phase 4, following invagination of the retinal neuroepithelium. Pulling and compression forces that contribute to these morphogenetic changes are illustrated with black arrows in the neural retina magnifications. Progressive acquisition of neural retina- and RPE-specific identities is highlighted by the indication of selectively expressed set of genes. From phase 2, both tissues can also be distinguished in terms of mechanical properties, with the RPE (and hinge points) being more rigid than the NR. This correlates with higher levels of Rho-associated protein kinase activity, visualized by the expression of phosphorylated Myosin Light Chain 2 (pMLC2; red outline). Finally, following excision and further culture, phase 4 optic cup recapitulates retinal histogenesis, as observed *in vivo*. As a minor difference compared to the *in vivo* situation, synthetic retinas exhibit scattered ganglion cells and fewer cone photoreceptors. Note also that optic cup excision leads to RPE

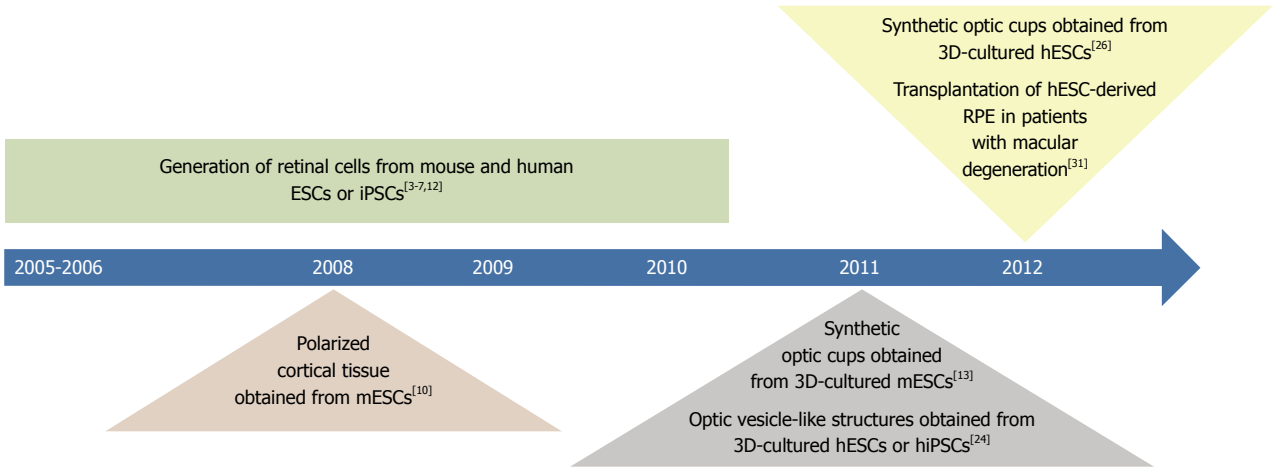

Figure 2 Timeline indicating the major advancements over the past 7 years in derivating retinal cells or three dimensional retinal-like structures from embryonic stem or induced pluripotent stem cells *in vitro*. (m/h)ESCs: Mouse/human embryonic stem cells; (m/h)iPSCs: Mouse/human induced pluripotent stem cells; 3D: Three dimensional.

rigidity compared to that of the neural retina, as inferred by elasticity measures with an atomic force microscope. Such an acquisition of regionally distinct mechanical properties within the OC likely constitutes a major requirement for the invagination process to occur in phase 3. Cell proliferation-dependent tissue expansion in phase 4 then provides the pushing force that contributes to the bending of the neural retina under the more rigid RPE. To support these analyses, the authors performed elegant cell ablation experiments using a 3D-targeted multiphoton laser beam and observed the subsequent changes of the created gap area. This revealed that pulling tensions occur in phase 1 and 2 whereas compression forces are at work during phase 4 (Figure 1B). Based on these observations, a "relaxation-expansion" model for self-driven OC morphogenesis was proposed and validated *in silico* through computer simulation[19]. It stipulates the need of three sequential rules: (1) mechanical relaxation of the neural retina, in contrast to the RPE which remains rigid; (2) apical constriction of hinge cells, that likely directs the orientation of the neural retina epithelium bending; and (3) tangential expansion of the neural retina that promotes its complete invagination.

The authors next asked whether neural retina and RPE fates were irreversibly determined at the OV stage. Phase 1 vesicles excised from the aggregates and cultured without non-retinal tissue retained a neural retina identity, but both RPE differentiation and OC invagination failed to occur. Of note, both processes were restored when the isolated epithelia were exposed to biological or chemical activators of canonical Wnt signaling, consistent with the known requirement of this pathway in RPE specification *in vivo*[20,21]. Conversely, when whole aggregates were cultured in the presence of Wnt inhibitors, RPE differentiation was reduced and OC invagination was hampered. Thus, while the neural retina can autonomously differentiate at this stage, the presumptive RPE still requires inductive signals from the neighboring neural (non retinal) tissues to be determined. Furthermore, these results also highlight again that the invagination process is RPE-dependent.

3D RETINAS FROM HUMAN ES CELLS

Several groups attempted to reconstitute retinal tissue *in vitro* using human cells. For this purpose, different 3D systems, like rotary cultures or cultures in matrix on top of RPE cells[22,23], have been developed. Although the resulting cell aggregates or sheets differentiated towards retinal cell types, no self-organization of OV- or OC-like structures were reported.

In 2011, an important breakthrough has been obtained towards the generation of optic structures using human ES (hES) and iPS cells[24]. Meyer and colleagues took advantage of a stepwise procedure capable of inducing efficient expression of eye field markers such as *Rx* and *Pax6*[25]. Basically, pluripotent cells were cultured as free-floating aggregates, exposed to a neural induction medium and then prompted to form adherent neural clusters on laminin-coated dishes. After 16 d of differentiation, neural rosettes were isolated and grown in suspension. Strikingly, by day 20, most of the resulting aggregates remained spherical but a subset (around 20%) adopted a vesicle-like structure appearance. Importantly, immunofluorescence and comparative gene microarray analyses showed that these OV-like aggregates harbored molecular features of retinal progenitor cells, while the non-vesicular ones expressed forebrain markers[24]. Of note, longitudinal analysis of OV-like structures revealed progressive expression of several differentiation markers, in a temporal order resembling that observed *in vivo*. In addition, spatial cellular segregation reminiscent of retinal lamination could be observed in these aggregates. Finally, both morphological and electrophysiological criteria suggested that photoreceptors derived from such OV-like structures were capable of phototransduction. This work

thus undoubtedly demonstrated that homogeneous 3D populations of neural retinal cells can be obtained and isolated from both human ES and iPS cells. However, despite the high degree of neuroretinal differentiation, RPE was rarely observed in OV-like structures, unless they were treated with Activin A. Furthermore, they never underwent morphological changes to form OCs and even lost with time their vesicular shape. A possible explanation is the lack of both RPE and non-retinal tissue within aggregates.

It is only very recently that formation of complete hES-derived OCs was achieved, again by Sasai and coworkers[26]. Among differences with the protocol applied for murine SFEBq/matrigel cultures are (1) an improvement of the re-aggregation step, using V-bottomed instead of U-bottomed wells; (2) the addition of a Wnt inhibitor (IWR1e) to favor rostral neural cell fates at the expense of caudal ones; and (3) the use of a ROCK inhibitor (Y-27632) to prevent dissociation-induced apoptosis of hES cells[27,28]. Following a 12 d-culture in these conditions, supplementation of aggregates with fetal bovine serum and with the Hedgehog agonist SAG, was found to induce a high degree of "retinalization".

However, although most of the neuroepithelium generated in this way was positive for the neural retina markers Chx10 and Pax6, no sign of RPE differentiation could be detected. As a consequence, self-formation of OC did not occur. This could be circumvented by adding the Wnt agonist CHIR99021 in a specific time window (between days 15-18). Under these conditions, as seen with murine ES cells, the neural retina epithelium first evaginated into an OV-like structure and then spontaneously invaginated to form a double walled OC. Importantly, hES-derived retinas, like their murine counterparts, were also able to acquire a typical layered structure, following proper spatio-temporal differentiation of retinal cell types. In particular, photoreceptors displayed characteristics of differentiated cells, like the presence of inner segments and connecting cilia, although outer segments were not clearly observed.

DIFFERENCES BETWEEN MOUSE AND HUMAN *IN VITRO* GENERATED OCs

Despite the aforementioned similarities, several specific features of human compared to mouse artificial OCs were observed by the authors[26]. In particular, the former took longer to develop (about 24 d *vs* 9 d for mouse) and were bigger (550 μm *vs* 250 μm in diameter) and thicker (approximately 120 μm *vs* 60 μm). This obviously reflects the differences observed *in vivo* during fetal development of the two species, and therefore suggests that both retinal size and developmental timing are intrinsically controlled.

Another intrinsic property harboured by hES-derived retinal neuroepithelium is its ability to spontaneously evert from an apically concave (OV) to an apically convex (OC) structure. While murine synthetic retinas require RPE and hinge points for this process, human ones are able to bend in an apically convex manner independently. Notably, this property was found to be associated with a preferential localization of cell bodies to the apical side of the neuroepithelium and to be integrin-dependent, two features that are specific to human retinas.

Finally, differences were also observed concerning photoreceptor development: much more cones were generated in hES-derived OCs and the overall differentiation process of photoreceptor cells was consistently slower (as observed again during fetal development). Importantly, photoreceptor genesis could be accelerated by counteracting Notch activity through the addition of the γ-secretase inhibitor DAPT.

PERSPECTIVES IN REGENERATIVE MEDICINE

The findings reported in this review not only represent major advances for basic research but also bear profound medical implications, above all in the field of regenerative medicine. One obvious conclusion is that such artificial retinas constitute potential homogeneous sources of stage-selected retinal cells for transplantation in patients affected by neurodegenerative diseases like retinitis pigmentosa or age-related macular degeneration[29]. Of note, photoreceptor transplantation has been shown to be a valid therapeutic strategy, able to restore vision in a mouse model of rod degeneration[30], paving the way for similar treatments in humans. Such a possibility is well illustrated by a recent clinical trial, where hES cell-derived RPE was transplanted in patients affected by different forms of macular degeneration[31]. This study shows for the first time successful attachment and survival of the graft, associated with significant improvement of visual acuity and no tumorigenicity nor transplant rejection after 4 mo. Although further follow-up of the patients is required, this represents an encouraging and promising milestone. It should, however, be noted that such a strategy may not be sufficient to treat retinopathies that primarily result from alterations of the vascular microenvironment, like that occurring in diabetic retinopathy. In these cases, therapeutic revascularization[32] must be considered prior to retinal cell replacement.

As far as genetic retinal disorders are concerned, a fascinating application would be to produce patient's iPS cells, correct the disease-linked genetic mutation, and promote their differentiation into a fully functional retina[33]. *In vitro* generated organs from iPS cells also constitute powerful systems to model human diseases and perform large-scale drug screening. The study by Meyer and collaborators gives an interesting proof of concept of such potential uses through the description of OV-like structure formation from iPS cells derived from a patient affected by gyrate atrophy[24]. This pathology is an autosomal recessive disorder primarily affecting RPE.

Importantly, RPE dysfunction was observable *in vitro* and could be corrected through both pharmacological and gene therapy approaches[24]. This study thus validates iPS-derived 3D retinal models as versatile systems for personalized drug testing and gene rescue. However, in a concomitant work, the authors described a substantial mutational load in the iPS cell line occurring at the time of derivation[34]. Some evidence points to the reprogramming process as a possible critical mutagenic step and many mutations are found enriched in genes known to have causative effects in cancers[35,36]. Thus, extensive genetic characterization of the iPS cell clones to be used will be critical before considering them as potential sources for transplantation and regenerative medicine.

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