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Organoid technology for retinal repair

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ABSTRACT

A major cause for vision impairment and blindness in industrialized countries is the loss of the light-sensing retinal tissue in the eye. Photoreceptor damage is one of the main characteristics found in retinal degeneration diseases, such as Retinitis Pigmentosa or age-related macular degeneration. The lack of effective therapies to stop photoreceptor loss together with the absence of significant intrinsic regeneration in the human retina converts such degenerative diseases into permanent conditions that are currently irreversible. Cell replacement by means of photoreceptor transplantation has been proposed as a potential approach to tackle cell loss in the retina. Since the first attempt of photoreceptor transplantation in humans, about twenty years ago, several research groups have focused in the development and improvement of technologies necessary to bring cell transplantation for retinal degeneration diseases to reality. Progress in recent years in the generation of human tissue derived from pluripotent stem cells (PSCs) has significantly improved our tools to study human development and disease in the dish. Particularly the availability of 3D culture systems for the generation of PSC-derived organoids, including the human retina, has dramatically increased access to human material for basic and medical research. In this review, we focus on important milestones towards the generation of transplantable photoreceptor precursors from PSC-derived retinal organoids and discuss recent pre-clinical transplantation studies using organoid-derived photoreceptors in context to related *in vivo* work using primary photoreceptors as donor material. Additionally, we summarize remaining challenges for developing photoreceptor transplantation towards clinical application.

1. Introduction

Degenerative diseases of the retina affecting the light sensing photoreceptor cells represent one of the main causes for disability in industrialized societies. As the mammalian retina, including human, does not show significant regenerative capacity the loss of photoreceptors in conditions like Retinitis pigmentosa (RP) or age-related macular degeneration (AMD) remains permanent, leading to vision impairment and eventually blindness. Though diverse therapeutic concepts are currently investigated, no definite cure has been established up to date.

Cell transplantation approaches for the replacement of lost photoreceptors have been investigated over the last three decades in pre-clinical animal models of retinal degeneration (reviewed by Santos-Ferreira et al. (2017)). Starting with primary retinal cells isolated during development as donor material, several studies provided evidence for successful survival and differentiation after grafting. Particularly, young post-mitotic photoreceptors termed photoreceptor precursors, showed the best transplantation success, including restoration of some functionality as observed by vision-based tests (Barber et al., 2013; Pearson et al., 2012; Santos-Ferreira et al., 2015; Singh et al., 2013). The described improvement of visual activity in retinal degeneration mouse models after photoreceptor precursor transplantation might be obtained by two separate mechanisms: either by supporting remaining dysfunctional photoreceptors of the host *via* cytoplasmic material transfer from the healthy donor cells (Ortin-Martinez et al., 2016; Pearson et al., 2016; Santos-Ferreira et al., 2016a; Singh et al., 2016) or by replacement of lost photoreceptors in late stage retinal degeneration models (Singh et al., 2013).

The majority of these preclinical studies were performed using primary photoreceptors isolated from the young, postnatal mouse retina. Aiming to use a similar donor cell population in the clinic would cause major challenges associated with donor material propagation and logistics besides ethical concerns, as the corresponding time period in humans would require the isolation of donor retina material from foetuses within the second trimester of pregnancy. Furthermore, as mice are nocturnal animals their retinas are dominated by rod

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photoreceptors and only contain small amounts of cones (*i.e.* 3% of all photoreceptors), the photoreceptor sub-type providing high acuity daylight vision in humans, making pre-clinical transplantation studies for improving day-light vision challenging due to shortness of donor material (Santos-Ferreira et al., 2015). Therefore, an *in vitro* expandable cell source for the production of high numbers of transplantable rod or cone photoreceptor precursors represents an essential prerequisite on the path towards clinical application.

With the generation of human embryonic (hESC; Thomson et al., 1998) and induced pluripotent (hiPSC; Takahashi et al., 2007) stem cells a major breakthrough for the virtually unlimited propagation of donor cells was made, as pluripotent stem cells (PSCs) can massively be expanded *in vitro* and have the potential to differentiate, in principle, into any distinct cell-type of the body. Furthermore, the identification of culture systems for the production of 3D tissue organoids from PSCs, thereby reflecting a developmental environment more closely related to the *in vivo* situation, now allows the generation of specific cell-types in high precision and amounts as it was demonstrated in self-organizing organoids from the gut, liver, kidney or brain (reviewed by Ader and Tanaka (2014) and Lancaster and Knoblich (2014)). Besides gaining insights to early stages of human development, organoid technology also offers the possibility to perform high throughput drug screening and, combined with patient derived iPSCs and gene editing technology, it allows human disease modeling and repair (Fig. 1).

The ability to generate retinal cells from hPSCs has been a great advance towards the generation of clinically relevant cell populations, specifically for cell replacement therapies in the eye. The last 10+ years have witnessed great progress in the field, with several studies reporting the ability of hESCs and hiPSCs to follow a stepwise differentiation process that results in the generation of anterior neural tissue, eye field, retinal (progenitor) cells and, finally, differentiated cell types (Lowe et al., 2016; Meyer et al., 2009; Nakano et al., 2012; Zhong et al., 2014). Particularly seminal work from the Sasai lab that showed the generation of 3D retinal organoids that closely follow *in vivo* retinogenesis (Eiraku et al., 2011; Nakano et al., 2012) has been of tremendous importance for the production and availability of *in vitro* generated photoreceptors. Work performed in recent years has led to an accumulation of knowledge about factors that are required for and/or enhance retinal specification. This body of evidence has been incorporated into protocols developed by several groups, thereby further optimizing efficiency and robustness of retinal organoid production. In this review, we will summarize current protocols used for the generation of retinal organoids from mouse and human PSCs with the aim to produce transplantable photoreceptors for cell-based therapeutic approaches and discuss remaining challenges and roadblocks towards clinical application.

2. Generation of retinal organoids from pluripotent stem cells (PSC)

2.1. Mouse ESC-derived retinal organoids

Most of the currently used protocols for the production of self-organizing 3D retinal organoids from pluripotent stem cells are based on the seminal work of Yoshiki Sasai and his team (Eiraku et al., 2011, Eiraku and Sasai, 2011). Starting with mouse ESCs the protocol allows to mimic retinal development *in vitro* and has set the basis for multiple studies with different purposes: from optimization of the protocol for the derivation of transplantable rod photoreceptors and retinal sheets to developmental studies and disease modeling (see Fig. 2 for detailed comparison of the different protocols of mESC-derived retinal organoids).

Retinal organoid formation in Sasai's protocol is initiated by quick reaggregation of a defined number of dissociated mESCs (*i.e.* 3000cells/well) in 96 well plates under serum-free floating conditions that results in the formation of embryoid-body-like aggregates (Fig. 2). Addition of extracellular matrix (ECM) components (Matrigel) leads to the formation of a rigid continuous neuroepithelia that evaginate and express eyefield transcription factors like Rx and Pax6 within a week of culture, thus representing optic vesicle-like structures. Interestingly, within the next three days of differentiation around 60% of the optic vesicle-like structures undergo a dynamic

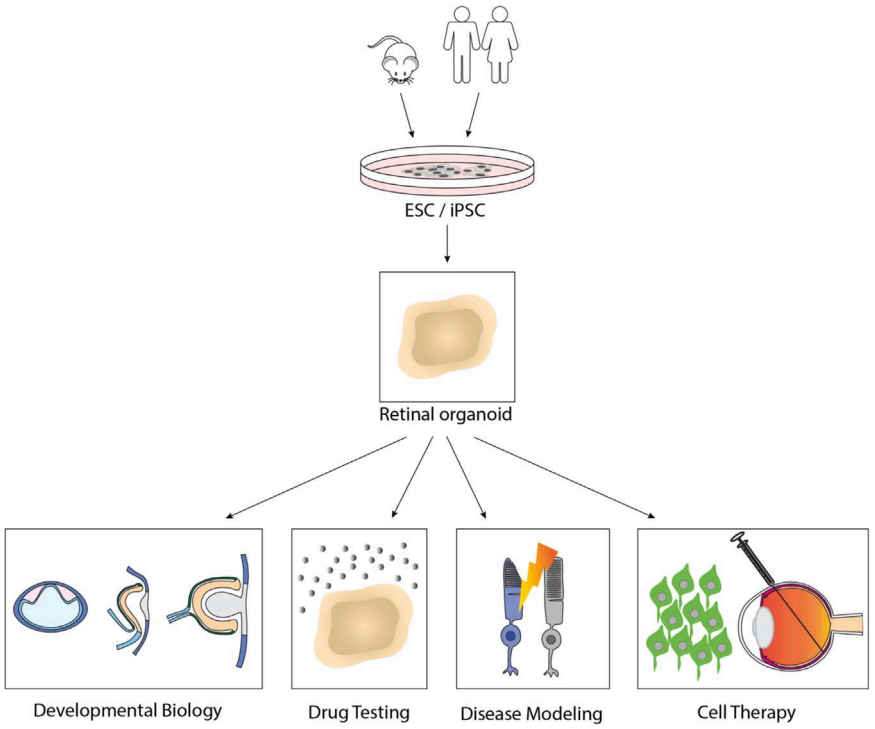


Fig. 1. Summary of potential applications of retinal organoids. Pluripotent stem cells can be used to generate retinal organoids for several applications: from analysis of required factors during retinal development and retinogenesis, to high throughput compound screening, and disease modeling *in vitro*. Another major application is the generation of photoreceptors for cell therapies, the focus of this review.

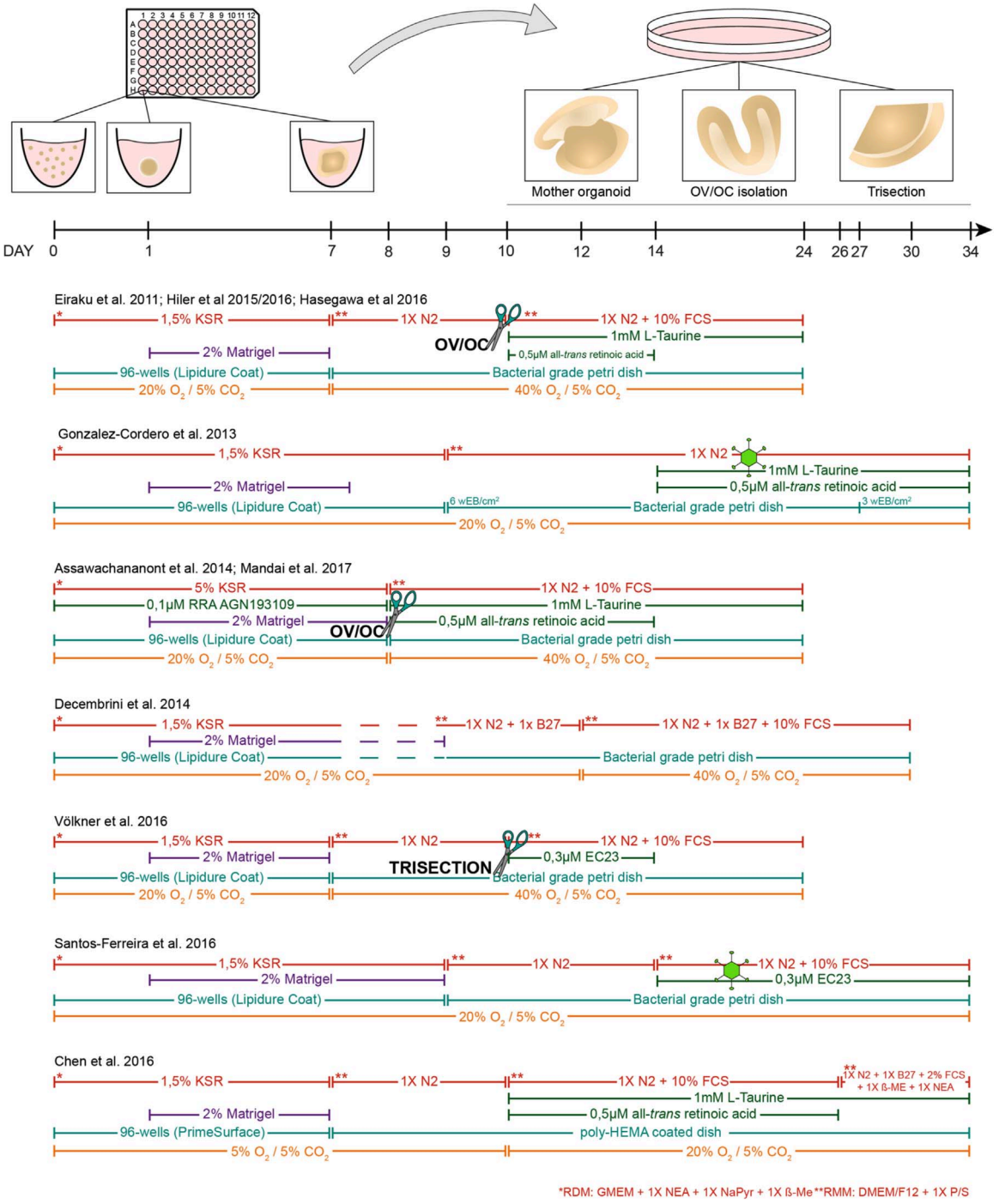


Fig. 2. mESC-derived retinal organoids – protocol comparison. Schematic representation of the key steps for retinal organoid generation. mESC are first dissociated and plated in 96-well plates at D0. Matrigel is added to the media at D1 and organoids are kept in these conditions until D7-9. Then, they are transferred to a bacterial-grade petri dish until the end of the culture. Differences in terms of media components, O_2 concentration, plates used, *etc.* among the protocols used by different research groups are reflected in the specific schematic summaries of the protocol described in each publication. RDM: retinal differentiation media; RMM: retinal maturation media; KSR: knock-out serum replacement; FCS: fetal calf serum; RRA: retinoic acid receptor antagonist.

shape change known as invagination, leading to the formation of optic cup-like structures with comparable size (200–400 µm) to optic cups at embryonic day (E) 10.5 (~ 300 µm) *in vivo*. These optic cup-like structures are then separated from the aggregates for developing individually into stratified neural retina containing an outer nuclear layer (ONL) with photoreceptors (rhodopsin$^+$, recoverin$^+$), an inner nuclear layer (INL) in which horizontal (calbindin$^+$, calretinin$^-$), bipolar (Chx10$^+$, Pax6$^-$) and amacrine (Pax6$^+$, calretinin$^+$) cells as well as Müller glia cells (CRALBP$^+$) are localized, and a ganglion cell layer (GCL) with retinal ganglion cells (Brn3$^+$, Pax6$^+$, calretinin$^+$). Interestingly, the mESC-derived retinal tissue presents an apical-basal polarity, with a convex apical side, which resembles the *in vivo* retina orientation. Furthermore, as retinal organoids spontaneously acquire the dorso-ventral (D-V) polarity comparable to the developing retina *in vivo*, Sasai's team was able to study the underlying mechanisms of crosstalk between BMP and canonical Wnt-signaling in an *in vitro* system (Hasegawa et al., 2016). Actually, the pattern of the D24 retinal epithelia is very similar to that seen in P4 retinas (Eiraku et al., 2011), indicating that the timing of retinogenesis is conserved in mESC-derived retinal organoids.

This impressive recapitulation of the main events in retina formation is mainly based on the self-organization of aggregated mESCs in the presence of ECM components. Interestingly, the formation of invaginated optic cups *in vivo* is orchestrated by a highly controlled signaling system that includes neighboring tissue other than the retina. *In vivo,* evagination of the mouse diencephalon at E8.5 results in the formation of the optic vesicle, that contains cells expressing signaling factors such as BMP4, FGF8 and Delta that induce cells of the surface ectoderm to become the lens placode (Fuhrmann, 2010). During development FGFs secreted by the newly formed lens placode then induce expression of Vsx2 in the optic vesicle, essential for neural retina cell specification. In parallel, the distal part of the optic vesicle in contact with the lens placode show a coordinated invagination that results in the formation of the two-walled optic cup, of which the inner part will eventually develop into neural retina and the outer part into retinal pigment epithelium (RPE) (Fuhrmann, 2010). Interestingly, the series of morphological changes and neural retina specification, that *in vivo* is tightly orchestrated by this reciprocal embryonic induction between the optic vesicle and the surface ectoderm, are also observed in *in vitro* generated retinal organoids despite the absence of surface ectoderm. This observation might be explained by developmental biology studies in which the role of the FGF signaling pathway in optic cup formation and retinal specification (Zhao et al., 2001) and the consequence of ablation of the surface ectoderm was investigated (Hyer et al., 2003). These studies showed that in the absence of surface ectoderm but with an exogenous source of FGF, invagination of the optic vesicle is impaired, but the neural retina is specified, indicating that both processes might be independent from each other (Fuhrmann, 2010). Indeed, retinal organoids are cultured in the presence of FCS from D10 onwards, which, among many other components, also contains several growth factors including FGFs. Furthermore, although the efficiency of optic cup formation *in vitro* is highly variable, *i.e.* it ranges from 0% to 80% (see Suppl. Table 1), the presence of stratified retina at the end of the culture (D24) is induced with high efficiency (at least 70%, see Suppl. Table 1), thus supporting the idea of independence between the invagination process and neural retina specification.

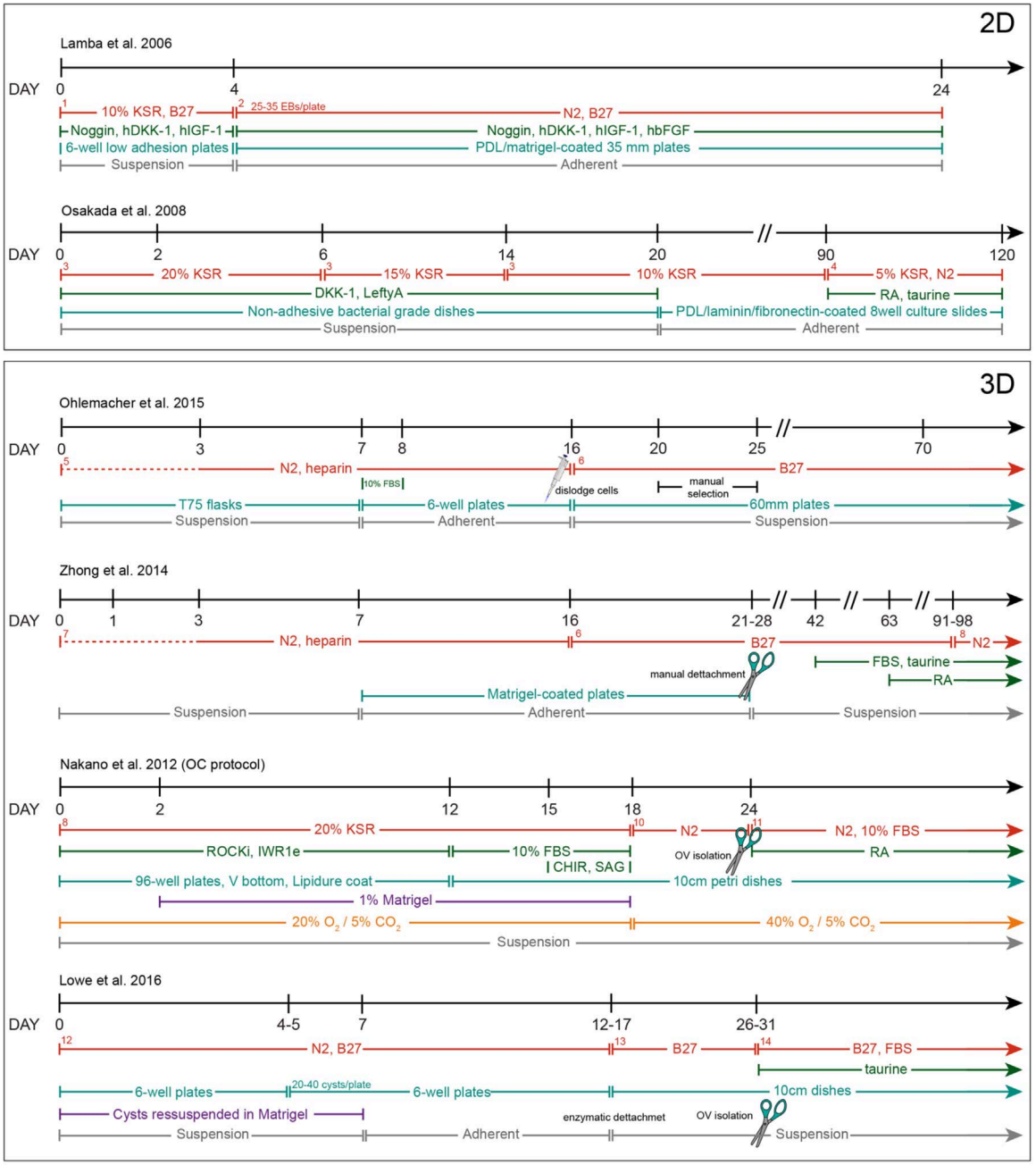
The introduction of the retinal organoid technology represents a corner stone in the field of retinal research as it provides the first culture system for the generation of high numbers of retinal neurons *in vitro*, particularly photoreceptors, which was not accomplished before *via* 2D culture techniques (Lamba et al., 2006; Osakada et al., 2008). However, several laboratories realized that the 3D retinal organoid protocol provided by Sasai's group showed high heterogeneity when used with different pluripotent stem cell lines, particularly in terms of cell composition and areas that were properly patterned in retinal nuclear layers. Indeed, the lab of Michael A. Dyer developed a standardized method to quantify retinogenesis within organoids (STEM-RET) and showed significant changes in differentiation outcomes when comparing the performance of murine ESC and fibroblast/rod-derived iPSC lines due to epigenetic differences in the source cell lines (Hiler et al., 2015). Furthermore, invaginated optic cups, as described by Eiraku et al. (2011), were only rarely observed by other groups (Decembrini et al., 2014; Gonzalez-Cordero et al., 2013; Santos-Ferreira et al., 2016b, personal communication), further impairing the laborious manual separation of optic cups from 3D aggregates. To circumvent the observed variability in the differentiation process *in vitro*, modifications for the production of retinal organoids were introduced to enable robust culturing outcomes.

Robin Ali's team was the first to adapt, optimize and scale up the 3D retinal organoid protocol with the objective of increasing the amount of transplantable photoreceptors (Gonzalez-Cordero et al., 2013; Kruczek et al., 2017). By prolonging the time of the organoids in extracellular matrix and growing the retinal tissue within the mother organoid, *i.e.* without dissection of parts of the organoid, they were able to obtain transplantable rod photoreceptors in high amounts, but at the expense of generating properly stratified retinal tissue. A similar approach was followed by Lakowski et al. (2015) and Santos-Ferreira et al. (2016b) who additionally introduced cell surface markers for the separation of rod photoreceptors from retinal organoids previous to transplantation into degeneration mouse models. By in depth quantification of the retinal organoid differentiation process, Völkner et al. (2016) discovered that in fact the neuroepithelia found in D10 organoids, despite not forming optic vesicle/cup-like structures, are able to develop towards a stratified neural retina, and therefore, the isolation of optic cups used in the original protocol might lead to a loss of potential retinal tissue. The solution offered by the authors consists in trisecting the organoids at D10 and culturing all of the created fragments, instead of processing only isolated optic cups. With this, the authors were able to achieve more than 180% retinal organoids per starting mother aggregate and reduced the previous high variability in organoid composition allowing a more robust and reproducible organoid production pipeline (Fig. 2; Suppl. Table 1). Additionally, by inhibiting notch signaling by *N*-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester (DAPT) at different developmental stages, *i.e.* between D12-14 or D16-18, they showed increased generation of cones or rods, respectively. Other modifications for improved retinal organoid generation included changes in the media composition by adding retinoic acid receptor antagonists (Assawachananont et al., 2014; Mandai et al., 2017a) at early stages of differentiation or neural and/or photoreceptor differentiation supporting supplements like B27 (Decembrini et al., 2014) at later stages. Additionally, several groups have observed an improvement in photoreceptor generation and/or survival by adapting O_2 concentrations at different stages of organoid development (Chen et al., 2016; Decembrini et al., 2014; Mandai et al., 2017a: see Fig. 2 and Suppl. Table 1).

In summary, the introduction of retinal organoid technology, first provided using murine systems, offers an *ex vivo* platform for studying retinal development and cell-based treatment approaches and paved the way for the use of human ESC- and iPSC-derived retinal tissue in vision research.

2.2. hESC- and hiPSC-derived retinal organoids

Since the introduction of human ESCs (Thomson et al., 1998) and iPSCs (Takahashi et al., 2007) several approaches have been investigated for the generation of retinal cells *in vitro*. Mammalian eyes are generated as evaginations from the developing diencephalon, the region that gives rise to posterior forebrain structures. *In vivo* studies performed with rodents have shown that forebrain development requires the concerted inhibition of specific signaling pathways including Wnt/BMP, as well as the activation of others like IGF (Anderson et al., 2002; Bachiller et al., 2000; Mukhopadhyay et al., 2001). Furthermore, microinjection of insulin-like growth factor (IGF) mRNAs in Xenopus embryos caused ectopic formation of eyes (Pera et al., 2001). Based on these findings, pioneer work by Tom Reh's laboratory exposed plated hESCs-derived embryoid bodies to the Wnt/BMP inhibitors DKK-1 and noggin besides IGF-1, resulting in cells committed to a retinal fate, *i.e.* approximately 70% Pax6/Chx10 double positive cells (Lamba et al., 2006). However, the majority of differentiated neurons had an inner retina identity, *i.e.* mainly RGC and amacrine, with around 12% of cells expressing Crx, an early photoreceptor marker, and very few cells expressing mature photoreceptor markers like opsins (Lamba et al., 2006). Culturing cells for an extended period of time and addition of retinoic acid and taurine (see Fig. 3), medium supplements described to have impact on primary photoreceptor development *in vitro* (Altshuler et al., 1993; Kelley et al., 1994), led to an increased number of opsin-expressing photoreceptors at D200 in another 2D culture approach (up to 10% for any opsin, Osakada et al., 2008). However, despite a significant improvement in the number of photoreceptors, there was still a considerable gap in these differentiation systems using plated embryoid bodies to the *in vivo* situation, where photoreceptors constitute approximately 70% of all retinal cells.



1 in DMEM/F12, 10% KSR, B27, 1ng/mL noggin, 1ng/mL Dkk-1, 5ng/mL IGF-1; **2** in DMEM/F12, B27, N2, 1ng/mL noggin, 1ng/mL Dkk-1, 5ng/mL IGF-1; **3** in DMEM/F12, 0.1mM B-Me, 0.1mM NEAA, 2mM L-glutamine, 20, 15 or 10% KSR; **4** in GMEM, 5% KSR, 0.1mM B-Me, 0.1mM NEAA, 1mM pyruvat, 1uM RA, 100uM taurine, N2, Pen/Strep; **5** gradual replacement of mTeSR1 with NIM = DMEM/F12, 1%N2, 1X NEAA, 2mg/mL heparin; **6** in RDM = DMEM/F12 3:1, 2% B27, 1X NEAA, 1% Pen/Strep, 1% antimycotic; **7** gradual replacement of mTeSR1 + blebbistatin with NIM = DMEM/F12, 1% N2, 1X NEAA, 2ug/mL heparin; **8** in RDM = DMEM/F12 3:1, N2, 1X NEAA, 1% Pen/Strep, 1% antimycotic; **9** in retinal diff medium = GMEM, 20% KSR, 0.1mM B-Me, 0.1mM NEAA, 1mM pyruvat, Pen/Strep; **10** in maturation medium 1 = DMEM/F12 Glutamax, 1X N2, Pen/Strep, Fungizone; **11** in maturation medium 2 = DMEM/F12 Glutamax, 1X N2, 10% FBS, 0.5uM RA, Pen/Strep, Fungizone; **12** in N2B27 medium = DMEM/F12+Glutamax and neurobasal medium 1:1, 0.5X B27, 0.5X N2, 0.1mM B-Me, 2mM Glutamax; **13** in medium 2 = DMEM/F12 3:1, 2% B27, 1X NEAA; **14** in medium 5 = DMEM/F12 3:1, 2% B27, 10% FBS, 100uM taurine, 1X NEAA, 2mM Glutamax.

Fig. 3. hPSC-derived retinal organoids – protocol comparison. Schematic representation of key protocols published in recent years for the generation of retinal organoids from human PSCs. Major steps and procedures, as well as key factors used are highlighted for a better comparison between protocols. KSR: Knock-Out Serum Replacement; hDKK1: human Dickkopf-related protein 1; hIGF-1: human Insulin-like Growth Factor 1; hbFGF: human basic Fibroblast Growth Factor; PDL: poli-ɒ-lysine; FBS: Fetal Bovine Serum; RA: retinoic acid; ROCKi: ROCK inhibitor; IWR1: Wnt inhibitor IWR1-endo; CHIR: CHIR99021, GSK3b inhibitor; SAG: Hedgehog agonist smoothened agonist.

Based on the quick aggregation and retinal organoid system developed with mouse ESCs, Sasai's group provided in 2012 a protocol for the generation of 3D retinal tissue from human ESCs, that allowed the formation of typical three layered retinal structures that harbored high numbers of photoreceptors beside several other retinal neurons and Müller glia cells (Nakano et al., 2012). Starting with defined numbers of quickly aggregated hESCs (9000 cells/well) and adding ECM components (Matrigel), the formation of optic cups containing both neuroretina and retinal pigment epithelium (RPE) was achieved by supplementation of the medium with 10% FBS besides the timely addition of Hedgehog agonist smoothened agonist (SAG) and a Wnt agonist (CHIR99021, a GSK3ß inhibitor) (for details see Fig. 3). Even though Wnt inhibition is important for establishment of anterior identity in the neural plate, it has also been shown that the Wnt/ß-catenin signaling pathway is important for the regulation of RPE genes, including MitF and Otx2 (Capowski et al., 2016; Westenskow et al.,

2009). This was the first and only study reporting the formation of a double-layered optic cup structure, albeit in less than 25% of generated retinal organoids. Induction of eye field was highly efficient with approx. 70% of cells expressing Rx at D18; at a later time point (D126), Crx+ photoreceptor cells constituted 12–18% of total cells and showed expression of further photoreceptor markers including opsins, recoverin, the rod-specific marker Nrl and an early cone marker, Rxr-gamma (Nakano et al., 2012). Interestingly, the generation of photoreceptors could be accelerated by Notch inhibition at D29 for 12–14 days resulting in up to 78% recoverin$^+$ photoreceptors. Several other laboratories used this protocol with minor modifications for the generation of human retinal tissue containing photoreceptor-specific fluorescent reporters or for disease modeling using patient derived iPSCs (Aparicio et al., 2017; Arno et al., 2016; Gao et al., 2016; Kaewkhaw et al., 2015; Parfitt et al., 2016; Tanaka et al., 2015). Interestingly, cryopreservation based on a vitrification method allowed en-block storage of hESC-derived stratified retinal tissue, thus facilitating faster availability of donor cells in potential clinical applications. Using stored intermediates of retinal development might significantly reduce remaining culture times for the production of transplantable photoreceptors in this otherwise highly time-consuming culture system (Nakano et al., 2012).

An alternative approach for receiving human retinal tissue was introduced by the Gamm lab, that developed a retina induction protocol for hESCs based on a switch from initial 2D adherent to 3D free-floating culture conditions (Meyer et al., 2009; Ohlemacher et al., 2015, see Fig. 3). The authors reported a stepwise protocol by which cells were first directed towards a neural fate by treatment with N2-containing neural induction medium, while in floating conditions. After attachment, a neuroepithelium (NE) with columnar cell organization developed. By D6 major eye field transcripts were detectable, namely Rx, Six3, Six6, Lhx2, Otx2 and Pax6, and by D10 more than 90% of cells were Pax6/Rx double positive. Interestingly, during eye field specification in this system, both DKK-1 and noggin were up-regulated (Meyer et al., 2009). These adherent eye-field rosettes were mechanically lifted and induced for further retinal differentiation by addition of B27 supplement. By D20-25 two populations of free-floating neurospheres were distinguishable: ca. 20% were optic vesicle-like retinal neurospheres, characterized by a bright, thick NE, whereas the majority constituted darker forebrain neurospheres (Meyer et al., 2011). Manual separation of retinal neurospheres has to be performed around this time point, since after D25 the retinal morphology becomes unstable (Meyer et al., 2011; Ohlemacher et al., 2015). Despite being a time consuming step in an already cumbersome and long protocol, it led to an enrichment of Crx$^+$ cells at D80 (~ 60%, Meyer et al., 2011, *versus* ~ 12%, Meyer et al., 2009). In fact, without isolation of retinal neurospheres, the majority of the Pax6/Rx double positive population did not form an optic vesicle, revealing that those cells remained at a more primitive stage of human eye field induction (Meyer et al., 2009). Interestingly, a minority (~ 17%) of optic vesicles generated from blood-derived hiPSCs by the same protocol showed some degree of lamination, with RGC-like cells in an inner most layer and recoverin$^+$ photoreceptor-like cells in an outer layer (Phillips et al., 2012). The introduction of some modifications to this protocol by Zhong and colleagues, including attachment of cysts to matrigel-coated plates and addition of FBS, RA and taurine to promote photoreceptor differentiation (see Fig. 3) allowed an increase in Chx10$^+$ retinal progenitor cells (RPCs) in the neuroretina domains collected between D21-28 to up to 70%, and a cell-line dependent efficiency of 50–70% in retinal cup formation when cultured in suspension (Zhong et al., 2014). Here, retinogenesis started around week 5 (W5) with the generation of Brn3+ RGCs and progressed until around W22, with the appearance of Chx10$^+$ bipolar cells. Supplementation of culture medium with RA promoted photoreceptor development and maturation, generating more rho$^+$, S-opsin$^+$ and L/M-opsin$^+$ photoreceptors, that also expressed other proteins of the phototransduction cascade. Furthermore, EM analysis at W27 showed the presence of extracellular membrane discs reminiscent of outer segments (Zhong et al., 2014).

The introduction of 3D organoid technology dramatically improved the availability of human retinal tissue and human photoreceptors. However, current protocols are still impeded by high variability in differentiation outcomes that combined with the longevity of the differentiation process (up to 300 days) impairs robust data collection. One reason for the observed heterogeneity might be the undirected aggregation of PSCs into embryoid body-like structures at the start of the described protocols. To circumvent the use of embryoid bodies some laboratories started the differentiation of hPSCs in so-called cyst cultures in which small clumps of PSCs are placed in Matrigel resulting in the formation of neuroepithelial cysts containing a single lumen and apical-basal patterning within five days of culture. Robust induction of retinal phenotypes were observed using this protocol, allowing en-block generation of RPE without the need for selection and isolation of pigmented colonies (Zhu et al., 2013) and efficient formation of retinal organoids containing stratified retinal tissue harboring all major retinal cell types including photoreceptors with outer segment–like structures (Lowe et al., 2016). However, in depth quantification of retinal induction and retinal organoid formation using human PSCs besides direct comparison of different protocols to identify inter-line and inter-experiment variability are still limited and further modifications to improve the robustness of the process will be needed to make full use of the system (Reichman et al., 2017; Wiley et al., 2016a; Zhong et al., 2014).

Furthermore, none of the published studies so far has systematically analyzed generation of inner nuclear neurons, even though markers for bipolars, horizontals, amacines and Müller glia, like PKCa, PROX1, AP2a, Pax6, Otx2, have been detected (Kaewkhaw et al., 2015; Lowe et al., 2016; Zhong et al., 2014). Currently, retinal organoid technology still seems to be unable to produce correct proportions of all retinal cell-types, particularly those localized in the INL. Another, yet correlated, challenge that the field still faces is related to retinal structure, since long term cultures often miss the characteristic retinal organization in three clearly separated nuclear layers. This might not only be related to the altered proportions of inner nuclear neurons, but also to the failure in establishing correct synaptic connections, even though some synaptic proteins have been reported to be expressed (Wahlin et al., 2017; Zhong et al., 2014). Furthermore, it has been shown in the mouse retina *in vivo* that if RPE is abolished relatively late during development (E11.5–12.5) the retina loses its typical lamination, and often forms rosettes (Raymond and Jackson, 1995). Even though some protocols reported also the generation of RPE cells (Kaewkhaw et al., 2015; Lowe et al., 2016; Reichman et al., 2014, 2017; Wahlin et al., 2017; Wiley et al., 2016a; Zhong et al., 2014), such cells were rarely shown to be organized as a monolayer facing the photoreceptors, the only exception being a sub-fraction of retinal organoids generated by Sasai's protocol (Nakano et al., 2012). Thus, absence of RPE cells at the apical side of retinal organoids might be in part accountable for impaired generation of proper lamination.

The emergence of hPSC-based protocols for retinal differentiation that recapitulate major molecular and cellular events of human retinogenesis *in vitro* has opened up immense research possibilities, including developmental studies and disease modeling besides generation of clinically relevant cells (Gill et al., 2016; Ohlemacher et al., 2016). One remarkable advantage is the ability to study early human retinal cell fate decisions and to decipher the role of key transcription factors during early retinal development. Also relevant is the possibility to use hPSC-derived retinal organoids as a tool to model and follow retinal disease progression *in vitro* (Arno et al., 2016; Capowski et al., 2014; Ohlemacher et al., 2016; Parfitt et al., 2016; Tucker et al., 2013; Wiley et al., 2016b). However, it still has to be evaluated in detail to what extend *in vitro* generated retinal organoids recapitulate *in vivo* development and whether retinal diseases can be fully modeled *in vitro* as *in vivo* such conditions often consolidate in adulthood. Another

advantage of the 3D culture system is the generation of high numbers of clinically relevant retinal cell populations for transplantation or high-throughput screening approaches. Furthermore, 3D cultures allow the maintenance of retinal tissue in culture for longer periods than current 2D systems, most likely because of the higher degree of structural organization achieved, thus increasing the likelihood of maturation *in vitro* (Reichman et al., 2017; Wahlin et al., 2017; Zhong et al., 2014). Another reported advantage is the reduction or even absence of undifferentiated cells within retinal organoids (Wiley et al., 2016a), circumventing the need of multiple rounds of cell sorting, that is associated with a considerable drop in cell number and viability, to reduce the likelihood of teratoma formation following transplantation (Tucker et al., 2011). Adaptation of organoid production using bioreactors might allow scaling up the generation of stem cell-derived photoreceptors, a demand that will have to be met in the near future given the requirement of high cell numbers for cell-based therapies or drug-screening approaches.

2.3. Mouse versus human PSC-derived retinal organoids

The generation of retinal progenitor cells from mouse and human PSCs requires different conditions (Viczian, 2013), reflecting the fact that the source material is derived at different stages of embryonic development: mouse ESCs are derived from the inner cell mass of the blastula, whereas human ESCs are from a slightly later stage, the epiblast (Rossant, 2008). For example, whereas mESCs were reported to efficiently undergo retinal differentiation with low concentrations of knockout serum replacement (KSR) (Eiraku et al., 2011), hESCs required the addition of significantly higher concentrations (10–20%) (Nakano et al., 2012). Unlike mESCs, reaggregation of hESCs was slow and often incomplete when cells were seeded in conventional U-bottomed wells (Eiraku et al., 2011), leading to heterogeneity in aggregate size and, consequently, to varying differentiation efficiencies. For this reason, hESCs were seeded in conical shaped wells (Nakano et al., 2012). Alternative approaches include the addition of commercially available medium that promotes aggregation (Aparicio et al., 2017), or the use of blebbistatin, a specific inhibitor of non-muscle myosin II that has been shown to inhibit cell migration and invasiveness (Zhong et al., 2014).

Structural and morphogenetic differences between mouse and human PSCs were also observed during retinal differentiation *in vitro*, including size (250–300 μm *versus* 550 μm) and thickness (60–80 μm *versus* 120–150 μm) of ESC-derived NR at the early optic cup stage (Nakano et al., 2012). Mouse and human PSC-derived retinal organoids differ markedly in retinogenesis timing, reflecting the much longer developmental time in human (Fig. 4). While expression of early eye field markers (e.g. Rx) does not differ much between mouse (~ D7) and human (between D5-D18, depending on the protocol), genesis of early born neurons (e.g. $Brn3^+$ RGCs) is delayed in human (between D24-D54, depending on the protocol) in comparison to mouse (around D16) organoids. Species-specific differences are even more evident when comparing later stages of retinal differentiation and maturation: cone and rod opsins are first detected at D16 and D20 in mouse *versus* D120-150 and D180 in humans, respectively (Eiraku et al., 2011; Lowe et al., 2016; Tucker et al., 2013).

Human *in vivo* retinogenesis is initiated at the site of the future fovea, a rod-free area densely packed with cones that does not exist in the mouse retina, and then spreads across the retina reaching the retinal edges many weeks or months later, so that central regions are well developed at week 20 while in peripheral regions retinal progenitor cells may be just exiting cell cycle (Hendrickson et al., 2008). An exception to this central-to-peripheral rule is the late maturation of foveal cones. It is currently unknown whether human retinal organoids are able to recapitulate fovea formation *in vitro*, however, a significantly higher proportion of cones was reported in human organoids (12.4% to 17.8% , Kaewkhaw et al., 2015 and Gonzalez-Cordero et al 2017) in comparison to the mouse system (< 0.1%, Eiraku et al., 2011). Actually, due to the importance of cones for human vision attempts for increasing cone generation also in mouse organoids have been started and first studies showed, that inhibiting Notch signaling at distinct developmental stages increase the number of cones (Kruczek et al., 2017; Völkner et al., 2016).

In conclusion, though retinal organoid generation shows the same principles, several differences are observed in human and mouse PSC-derived retinal tissue that might allow to identify and investigate species-specific variability during retinal development. Additionally, due to the significantly shorter culturing time mouse retinal organoids represent still a valuable tool in basic research to study mammalian cell fate determination, tissue pattering and neuronal maturation, whereas human retinal organoids open the door to evaluate human retinal development, disease modeling besides providing cells for clinical application.

3. Transplantation of PSC-derived photoreceptors

Photoreceptor transplantation as a therapeutic approach for retinal degenerative diseases is based on the premises of an efficient cell source, survival for long periods after transplantation, *in vivo* maturation, connectivity with the existing retinal circuitry and restoration of lost visual function. Though major developments within the last decade brought therapeutic photoreceptor transplantation closer to clinical application, retinal organoid technology, now representing the major tool in these efforts, has not yet met all of these premises (Jayakody et al., 2015; Reh, 2016; Santos-Ferreira et al., 2017).

Studies conducted with primary mouse rods pioneered photoreceptor transplantation approaches by showing that the most successful grafting outcomes were achieved when donor cells were isolated from the retina around P4, at the peak of rod genesis (Bartsch et al., 2008; MacLaren et al., 2006). The impact of donor cell age and differentiation stage has also been observed in first studies using mESC-derived cells, confirming that a committed, less mature photoreceptor precursor allows improved transplantation success (Decembrini et al., 2014; Gonzalez-Cordero et al., 2013). In line with these observations, also younger grafts were more prone to form organized structures following transplantation of whole PSC-derived retinal sheets (Assawachananont et al., 2014; Mandai et al., 2017a). Enrichment of donor cells based on the expression of photoreceptor-specific fluorescent reporters or cell surface markers by flow cytometry or magnetic activated cell sorting (MACS) has been shown to increase transplantation success using primary photoreceptors (Barber et al., 2013; Eberle et al., 2011; Lakowski et al., 2011; Pearson et al., 2012). Similarly, as current 3D protocols for photoreceptor production also result in a heterogeneous cell population, with several other retinal cell-types present, a purification step before transplantation is required in order to reduce contaminating cells and minimize potential tumor formation from remaining undifferentiated cells (Lakowski et al., 2015; Tucker et al., 2011). Indeed, established protocols for primary photoreceptors using flow cytometry or MACS have been successfully adapted to mESC-derived photoreceptors prior transplantation (Decembrini et al., 2014; Gonzalez-Cordero et al., 2013; Kruczek et al., 2017; Lakowski et al., 2015; Santos-Ferreira et al., 2016b).

Transplantation success has been previously measured by fluorescent reporter expression in photoreceptors located within the host tissue, interpreted as structural photoreceptor 'integration' into the ONL, besides acquisition of polarized morphology as well as development of OSs. However, recent publications have caused a shift in this paradigm (Decembrini and Matrin, 2017; Ortin-Martinez et al., 2016; Pearson et al., 2016; Santos-Ferreira et al., 2016b; Singh et al., 2016), as these studies have elegantly shown that the most common event following transplantation of photoreceptors into retinas still containing endogenous photoreceptors is the exchange of cytoplasmic material including fluorescent reporter proteins between grafted and host

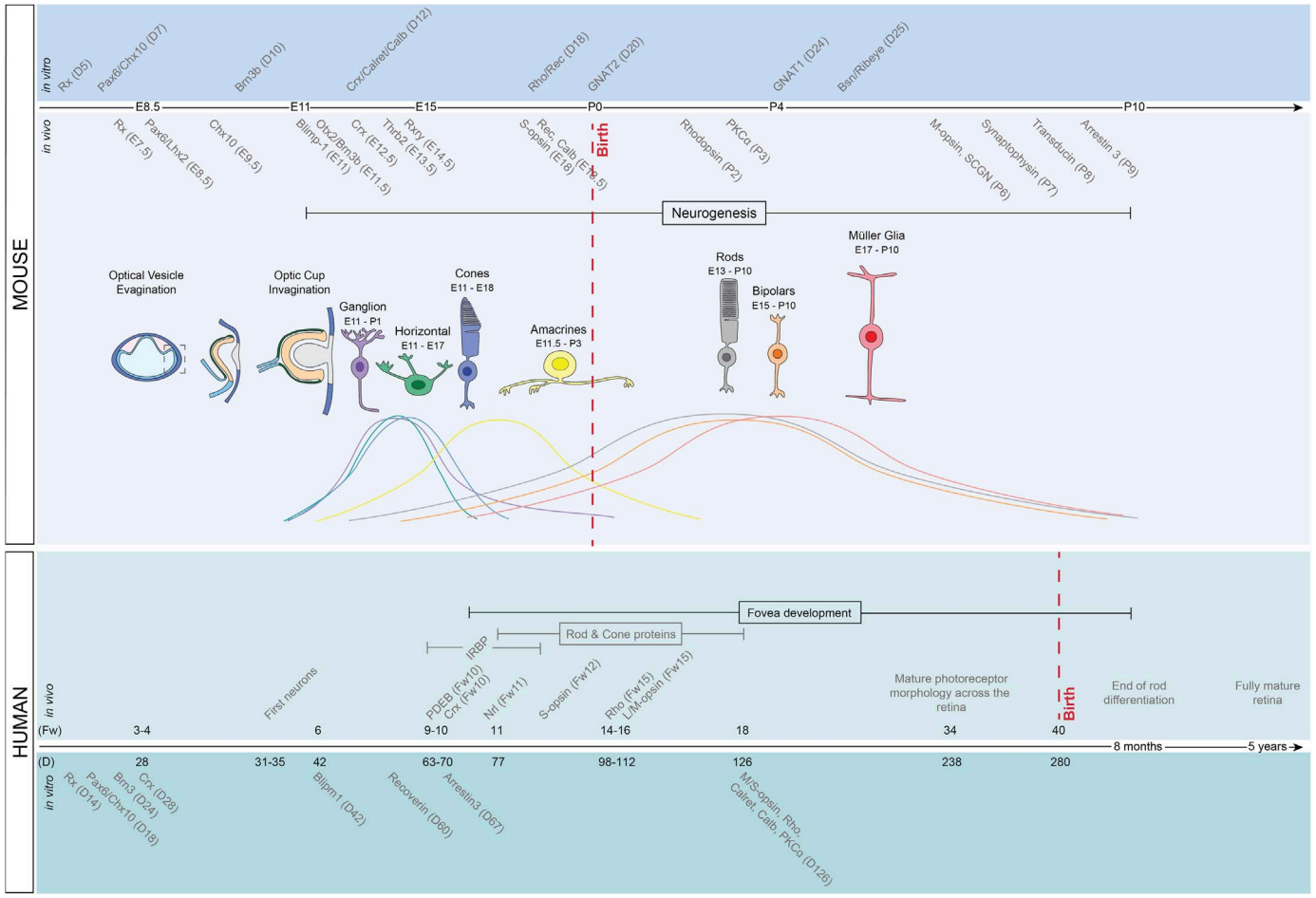


Fig. 4. Comparison of mouse/human *in vivo vs in vitro* retinal development. Schematic representation of events in mouse (upper half) and human (lower half) retina development observed *in vivo* or in retinal organoids *in vitro* based on marker expression (mainly data obtained by immunohistochemistry) characteristic for different developmental stages. In the mouse, retinal development is well studied; therefore, many markers can be used as a reference when analyzing the resemblance of retinal organoid development to the *in vivo* situation. In the case of human, due to lower sample availability, developmental *in vivo* data is less precise. Rho: rhodopsin; Rec: recoverin; Calb: calbindin; GNAT: guanine-nucleotide binding protein subunit alpha; PKC: protein kinase C; Bsn: bassoon; SCGN: segretagogin; PDEB: phosphodiesterase B; IRBP: interphotoreceptor retinoid-binding protein. The information has been collected from Akimoto et al. (2006), Brzezinski and Reh (2015), Brzezinski et al. (2010), Chang et al. (2002), Decembrini et al. (2014), Eiraku et al. (2011), Gonzalez-Cordero et al. (2013), Gordon et al. (2013), Hendrickson et al. (2008), Heyningen and Williamson (2002), Jean et al. (1999), Kaewkhaw et al. (2015), Lagutin et al. (2001), Mathers et al. (1997), de Melo et al. (2016), Nakano et al. (2012), Ng et al. (2001), Puthussery et al. (2010), Roberts (2005), Sharma et al. (2003), Swaroop et al. (2010), Walther and Gruss (1991), Zagozewski et al. (2014) and Zhu et al. (2002).

photoreceptors. This finding has tremendous impact on photoreceptor transplantation approaches, calling for a reevaluation of previous work. Therefore, photoreceptor transplantation as a therapeutic strategy might rely either on cell support in diseases characterized by dysfunction of photoreceptors, or on cell replacement in cases when the majority of photoreceptors have already been degenerated. Indeed, also after transplantation of mouse and human PSC-derived photoreceptors into mouse retinas containing endogenous photoreceptors, fluorophore-labeled cells within the ONL showed an adult-like morphology, correct polarity and expression of mature proteins (Decembrini et al., 2014; Gonzalez-Cordero et al., 2013; Homma et al., 2013; Lakowski et al., 2015; Lamba et al., 2009, 2010; Santos-Ferreira et al., 2016b). Thus, it may be that also PSC-derived photoreceptors engage in material transfer (Kruczek et al., 2017; Pearson et al., 2016).

Evaluation of OS formation, protein expression and connectivity to the retinal circuitry might therefore be investigated in severely degenerated animals that are depleted of endogenous photoreceptors. Transplantation into tg(Cpfl1;Rho$^{-/-}$) and rd1 mice, whose retinas lack endogenous photoreceptors at the time of intervention, has shown that mESC-derived photoreceptors remain as clusters in the subretinal space, and do not acquire a polarized morphology nor show extended OS formation (Barnea-Cramer et al., 2016; Santos-Ferreira et al., 2016b) However, ultrastructural analysis using appropriate reporter cells, as it was performed with primary photoreceptors (Eberle et al., 2011), will be necessary to determine OS formation of retinal organoid-derived photoreceptors in more detail. Furthermore, Kruczek et al. (2017) transplanted mES-derived cone precursor cells into severely degenerated Aipl1-deficient mice and showed peripherin2 positive distal processes in few donor cells. Interestingly, when mESC- and miPSC-derived retinal sheets were used for grafting and had direct contacts with host RPE cells, well-aligned membranous discs in OS-like structures were observed (Assawachananont et al., 2014). Transplantation of hESC-derived retinal sheets into primate models of injury-induced focal retinal degeneration also led to the survival of donor cells, with photoreceptors organized in clusters expressing rod and cone proteins, as well as some synaptic markers (Shirai et al., 2015), however, a detailed analysis of OS formation was not performed in this study.

Another essential feature for a functional replacement by cell transplantation is the establishment of synaptic contacts between donor photoreceptors and host inner nuclear neurons. This has been investigated by the detection of synaptic proteins (Assawachananont et al., 2014; Decembrini et al., 2014; Lamba et al., 2009; Santos-Ferreira et al., 2016b) in pre- and postsynaptic terminals of donor photoreceptors and host second order neurons (Mandai et al., 2017a), and functionally through micro electrode arrays and vision guided

behavioral tests (Mandai et al., 2017a). Here, light response signals could be recorded at the RGC level in grafted areas and some transplanted animals showed light-responsive behaviors in a visually guided shuttle-avoidance test. Other methods for functional readout have also been employed, but less successfully. Electroretinograms (ERGs) have shown no to minimal improvement (Lamba et al., 2009; Santos-Ferreira et al., 2016b; Shirai et al., 2015; Tucker et al., 2011; Zhu et al., 2017), similar to studies using optokinetic tracking (OKT) or light-dark box measurements (Barnea-Cramer et al., 2016; Mandai et al., 2017a). However, miPSC-derived Nrl-eGFP positive cells showed a membrane current similar to that of developing rods, and Ca^{2+} responses to high K^+ stimulation (Homma et al., 2013). However, more reliable data will be necessary to estimate the therapeutic potential of PSC-derived photoreceptors, as current studies that include in detail evaluation of functionality, maturation and proper synaptic connectivity of PSC-derived photoreceptors *in vivo* are severely limited. Actually, in regard to assessing functionality of human photoreceptors in preclinical animal models of retinal degeneration, experimental systems that demonstrate proper synaptic connections and communication between human photoreceptors and mouse second order neurons still have to be established.

Though the retina has an immune-privileged status (Streilein, 2003), inflammation might have important influence on transplant survival and maturation, particularly when using human photoreceptors in preclinical animal models. First studies have dealt with the issue of immune responses by providing immunosuppressant agents during the experiment (Barnea-Cramer et al., 2016; Lamba et al., 2009, 2010; Mandai et al., 2017a; Shirai et al., 2015) or by transplanting into immune-compromised recipients (Decembrini et al., 2014; Zhu et al., 2017). Transplantation of hESC-derived retinal cells into IL2rg-deficient mice provided evidence for higher survival rates and improved donor photoreceptor integration (Zhu et al., 2017); however, IL2rg-deficient mice did not display retinal degeneration and the potential transfer of cytoplasmic material between donor and host photoreceptors might have to be analyzed in further detail (MacLaren, 2017). Additional systematic studies will be necessary to fully understand the role of the immune system for photoreceptor transplantation as this might have important implications for translation towards clinical application.

4. Towards clinical use of PSC-derived retinal organoids

Several therapeutic approaches tailored for retinal degenerative diseases are currently under investigation in clinical trials. An overview of such approaches, including pharmacotherapy, neuroprotection, gene replacement, optogenetic therapy, retinal prosthesis and stem cell therapy has been recently published by Scholl and colleagues (Scholl et al., 2016). For the purpose of this review, we will focus on stem cell-based therapies involving photoreceptor transplantation.

The first attempt of human photoreceptor replacement was performed in 1997 (Kaplan et al., 1997) when two non-sighted patients with advanced RP received a gelatin-encased sheet of photoreceptor cells obtained from adult human cadaveric eyes. The main purpose of the study was to determine the feasibility and safety of the procedure before performing a pilot clinical study. Twelve months after transplantation no visual improvement was observed. However, despite the patients were not given systemic immunosuppression, no evidence of transplant rejection was reported. First indications of visual improvement after retinal sheet transplantations in RP patients were reported in 1999 (Radtke et al., 1999). Since then, different studies reported survival and absence of rejection after transplantation of fetal retinal sheets (Berger et al., 2003; Radtke et al., 2002, 2004). However, due to the severely limited availability of fetal tissue such studies only contained small cohort groups and therefore systematic larger studies were not performed.

The discovery of hESCs in 1998 (Thomson et al., 1998) and hiPSCs in 2007 (Takahashi et al., 2007), together with the development of protocols to generate hESC-derived retinal cells. *i.e.* retinal pigment epithelium (RPE) and photoreceptors (Nakano et al., 2012; Ramsden et al., 2013), offered the possibility to overcome the limited amount of donor retinal tissue as well as the ethical issues associated with the use of fetal human material. Indeed, due to the availability of protocols for the efficient generation of RPE from hPSCs first clinical trials have been initiated in patients suffering from Stargardt disease or AMD using hESC- or hiPSC-RPE. The MA09-hRPE cell line was the first to reach clinical trials (Schwartz et al., 2012, 2015, NCT01345006), however, since hESCs were expanded on mitomycin C-treated fibroblasts, the derived human RPE is considered a xenotransplant product. This first clinical trial has been followed by several others in which hESC/hiPSC-derived RPE transplantation either as cell suspensions or as sheets have been performed (reviewed in Kimbrel and Lanza (2015), Mandai et al. (2017b), Mead et al. (2015), Nazari et al. (2015), Trounson and DeWitt (2016) and Wu et al. (2016)). The published results of these pioneering cell therapy trials for the treatment of retinal degenerative diseases showed the safety of PSC-derived RPE donor cells transplanted into the eye, as no significant side effects have been reported, and the procedure might have protected treated patients from further vision loss (Schwartz et al., 2015). Furthermore, two ongoing clinical trials use *in vitro* expanded human fetal tissue-derived retinal progenitor cells (hRPC) injected in the vitreous cavity (jCyte, NCT02320812, NCT03073733) (Klassen, 2016) or sub-retinally (ReNeuron, NCT02464436; based on a preclinical study, Luo et al., 2014) as an approach to support photoreceptors of patients with Retinitis Pigmentosa.

Based on these first stem cell-based clinical trials for the treatment of retinal degenerative diseases there is significant interest in bringing retinal organoid-derived photoreceptors towards clinical application. An essential prerequisite to use hESC/hiPSC–derived retinal tissue for therapeutic purposes is to modify and optimize xeno-free and feeder-free protocols compliant with Good Manufacturing Procedures (GMP). Tannenbaum et al. (2012) derived, for the first time, xeno-free and GMP-grade hESC lines. Moreover, several groups reported the generation of hiPSCs in xeno-free conditions (Lu et al., 2014; Rodríguez-Pizà et al., 2010; Ross et al., 2009; Wang et al., 2015) and several attempts for setting up cell banks containing GMP grade hiPSCs have been started (Baghbaderani et al., 2016).

Whether hESCs or hiPSCs are favorable to generate transplantable donor cells for clinical use will depend on several stipulations including biological-medical as well as regulatory requirements. Patient-specific iPSCs would be ideal to avoid immune rejection of transplanted cells, however, they might need to be genetically modified using gene editing tools like CRISPR/Cas9 to remove disease causing mutations, so that the disease does not appear again after treatment. Moreover, due to individual reprogramming and genetic modification, each patient-specific iPSC line would need to be validated, increasing the overall costs and length of the procedure. On the other side, hESCs can be validated, expanded and differentiated in large amounts, therefore one cell line could be used for multiple patients. However, ethical concerns arousing from using human embryonic tissue besides regulatory restrictions in some countries that prohibit the generation of hESC lines, and immunological challenges due to HLA-mismatch need to be addressed in this case. A potential way around might be the establishment of cell/tissue banks that contain diverse GMP-approved ESC or iPSC lines from healthy donors with different immunological profiles. Depending on the grade of heterogeneity of the population of a country/region the availability of a few hundred PSC lines might be sufficient to serve the majority of patients (Solomon et al., 2015; Taylor et al., 2012).

First steps for developing PSC-derived photoreceptors towards clinical applications have been taken, as recent studies describe protocols in which photoreceptors derived from human iPSCs were produced in xeno-free/feeder-free cGMP conditions (Reichman et al., 2017; Wiley et al., 2016a). Challenges still remaining include the

extended time lines associated with the generation of photoreceptor-containing 3D retinal organoids, laborious manual working steps (e.g. neural retina/optic cup isolation), besides high heterogeneity and variability between cell lines and differentiation rounds (see above). Furthermore, it will be of utmost importance to develop protocols for the enrichment of human cone and rod photoreceptor precursors from the heterogeneous retinal organoids previous to transplantation by GMP applicable technologies for improving transplantation success and increasing the safety of the procedure to rule out teratoma formation in treated patients. Therefore, more robust, automated, scalable and GMP compliant procedures would be of great advantage to drive photoreceptor transplantation towards a general treatment option for retinal degenerative diseases.

5. Conclusions

The use of culture systems based on 3D organoids have significantly improved the availability of *in vitro* models for studying differentiation and maturation of specific target tissues and cells. Particularly, by taking advantage of human ESCs or iPSCs, new opportunities have arisen to investigate human related development and disease, besides providing sufficient amounts of human cells for clinical applications. With the introduction of PSC-derived retinal organoids an important tool for generating retinal tissue *in vitro* has been established and is now widely used in diverse laboratories world-wide. Indeed, retinal organoids represent currently the most improved *in vitro* system to generate high amounts of photoreceptors that can be further developed towards potential cell-based therapies. High heterogeneity between cell lines and experimental outcomes, extended culturing times and manual and laborious procedures beside missing selection techniques to purify specific photoreceptor sub-types currently limits the use of human retinal organoids for clinical applications. However, by tackling these challenges such 3D retina culture systems will be of utmost importance for regenerative approaches to treat currently incurable retina degenerative diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.09.028.

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