INTRODUCTION

Loss of sight is one of the most feared disabilities. The retina is often called the window to the brain, and dysfunction of retinal neurons in complex multifactorial diseases such as age-related macular degeneration (AMD), glaucoma, or diabetic retinopathy is a major cause of incurable blindness worldwide.\(^1,2\) Mutations in over 200 genes can lead to inherited retinal and macular diseases (IRDs), and genetic variations at numerous loci have been associated with susceptibility to AMD (RetNet, https://sph.uth.edu/retnet/).\(^3-5\) Identification of causative genes has enabled generation of animal models, resulting in elucidation of physiological functions of underlying gene products and development of gene therapies, in particular using adeno-associated virus (AAV) vector platform.\(^6\) In addition, fundamental advances in understanding of retinal development has resulted in rapid preclinical translation of potential cell replacement therapies,\(^7\) especially transplantation of photoreceptors,\(^8,9\) and retinal pigment epithelium (RPE).\(^10\) Although overall morphology, physiology, and molecular profile of the retina are highly conserved, many human clinical phenotypes are not faithfully recapitulated in animal models. Therefore, human stem cell-based in vitro models that complement the studies in mice and in vivo systems are highly desirable, with renewable sources of human cells needed for developing cell-based treatments. Breakthroughs in differentiating retinal tissues from patient-specific induced pluripotent stem cells (iPSCs) offer promising personalized models of human disease as well as provide a resource for evaluating therapeutic strategies. In this article, we will review current status of research on stem cell-derived retinal organoids, including methods for differentiation, and discuss their utility for identifying potential therapies.
2 | DIFFERENTIATION METHODS AND STAGING OF RETINAL ORGANOIDS

Groundbreaking discoveries in reprogramming of somatic cells into iPSCs and their differentiation into retinal lineages have greatly facilitated the study and treatment design of retinopathies. Early differentiation protocols used two-dimensional (2D) adherent cultures to derive retinal cells from pluripotent stem cells (PSCs).\textsuperscript{11,12} Sasai laboratory pioneered the methods for 3D optic cup morphogenesis in vitro from mouse embryonic stem cells,\textsuperscript{13} followed by adaptation of the protocol to produce human retinal organoids.\textsuperscript{14} Subsequently, two major approaches emerged for deriving retinal organoids (Figure 1). In the first approach, PSCs are suspended as single cells, followed by quick aggregation into embryoid bodies in a multiwell format, and then retinal neuroepithelium differentiation is stimulated by addition of basal lamina components present in Matrigel.\textsuperscript{14,15} As Matrigel composition can vary greatly, low concentration of BMP4 can be used in a chemically defined medium instead.\textsuperscript{16} Although this approach is suitable for automation and potential use in high-throughput screens, laminated retinal morphology appears to be preserved for a limited period.\textsuperscript{16} The second set of protocols combine 2-D and 3-D approaches with initial formation of embryoid bodies from detached human PSC (hPSC) colonies and neural induction in suspension, then plating and eye field formation in adherent conditions, followed by isolation of retinal domains and suspension culture as retinal organoids.\textsuperscript{17-19} The initial step of embryoid body formation can be omitted with growth of hPSCs to confluence and neural induction in adherent cultures.\textsuperscript{17,20} Another alternative is mixing hPSC clumps with Matrigel to form epithelialized cysts, which can be then plated, later followed by gentle lifting with dispase to form well-laminated organoids.\textsuperscript{21,22} Replacement of widely used all-trans retinoic acid with 9-cis retinoid acid accelerates differentiation of rod photoreceptors in retinal organoids,\textsuperscript{23} whereas signaling by thyroid hormone facilitates the specification of L/M cone subtypes,\textsuperscript{24} consistent with S-cones being the default fate of photoreceptors.\textsuperscript{25,26} Interestingly, scraping of the adherent culture and selection of subsequently formed optic vesicles improves the yield and efficiency of generating retinal organoids.\textsuperscript{27} Typically, the combination of 2-D and 3-D approaches achieves higher organization and maturity levels in late-stage organoids.\textsuperscript{15,19,23,24} The use of bioreactors significantly improves the culture of brain organoids with photosensitive cells\textsuperscript{28} and has shown promise in culture of retinal organoids.\textsuperscript{29,30} However, customized and efficient bioreactors are required for their wider acceptance in the field for producing organoids.\textsuperscript{31}

The array of methods to derive retinal organoids across independent laboratories has created challenges in comparing distinct studies and assuring faithful recapitulation of native developmental processes. Initially, limited sets of markers of retinal cell types for immunohistochemistry or gene profiling were used to characterize organoid differentiation and correlate it to human eye development.\textsuperscript{18,20,32,33} However, different methods often led to variable timing or robustness of marker expression. In order to facilitate cross-comparison across studies, a light microscopy-based staging system was developed.\textsuperscript{34} In this classification (Figure 2), stage 1 corresponds to phase-bright early retinal neuroepithelium developing into an opaque intermediate stage 2 and followed by stage 3 characterized by emergence of brush-like surface protrusions that correspond to developing photoreceptor outer segments.\textsuperscript{34} In another study, transcriptomes of developing retinal organoids were compared to developmental epochs in human fetal retina.\textsuperscript{35} Expression levels of retinal genes, however, varied considerably among retinal organoids from different iPSC lines.\textsuperscript{23,36} Transcriptional profiling alone\textsuperscript{21,23,32,36} or in combination with open chromatin analysis\textsuperscript{37} revealed similarities to human eye development with stage 1 showing early neurogenesis and retinal progenitor gene signatures, followed by interneuron and synaptogenesis-related gene expression in stage 2 and, finally, photoreceptor differentiation and maturation prominent in stage 3. These studies highlighted broad similarities of organoids to developing native retina tissue, despite protocol differences, based on light microscopy and/or transcriptome-based staging.

3 | RETINAL ORGANOIDS AS DISEASE MODELS

Technologies to convert somatic cells harvested in a patient biopsy into iPSCs have opened new avenues for interrogating disease mechanisms associated with retinopathies. Early studies using iPSC lines from retinitis pigmentosa patients differentiated in adherent conditions identified endoplasmic reticulum stress as a common disease signature.\textsuperscript{38,39} Subsequently progress in 3-D cultures allowed examining disease mechanisms in organoids with more native spatial cell arrangement. Defects in early retinogenesis were modeled in cells carrying R200Q mutation in the transcription factor visual system homeobox 2 (VSX2).\textsuperscript{40} Consistent with the clinical phenotype of microphthalmia, optic vesicles revealed slower growth and loss of retinal bipolar cells that express VSX2 at later stages. During retinal development, a distinct profile of alternative splicing is established, and retinal organoids have been used for disease modeling of patients with mutations in splicing factor PRPF31 (RP11).\textsuperscript{41} Many disease-causing mutations in IRDs are specifically located in genes related to sensory cilia and can be modeled in organoids.\textsuperscript{52} Two of the published studies examined effects of mutations in a centrosome-cilia protein CEP290.\textsuperscript{43,44} Cilia formation was shown to be impaired in fibroblasts...
derived from CEP290 patients with a more severe syndromic disease Joubert syndrome, but not in those diagnosed with Leber congenital amaurosis (LCA), a blinding retinal disease. However, retinal organoids from CEP290-LCA patients exhibited abnormal ciliogenesis demonstrating tissue-specific impact of pathogenic mutations and correlating broader defects with increased disease severity. Retinal organoids from patients with an intronic CEP290 mutation common in LCA patients revealed a more frequent cryptic exon inclusion compared to fibroblasts, iPSCs or RPE cells from the same patient. Reduced CEP290 protein levels in CEP290-LCA organoids led to impaired cilia development. RPGR is another cilia protein commonly mutated in retinitis pigmentosa (RP). Retinal organoids from patients harboring RPGR mutations displayed impairment of cilia biogenesis and abnormal photoreceptor morphology as well as decreased expression of

FIGURE 1 Retinal organoid differentiation methods. A. Two main approaches for generation of retinal organoids: 3D suspension culture throughout, starting from embryoid bodies, which subsequently differentiate into retinal tissues or a mix of 2D/3D culture with isolation of optic regions from adherent cultures. B. Comparison of main steps between the two approaches and key reagents used at each step.
many phototransduction-related genes. However, the molecular basis of how a defective ciliary protein leads to photoreceptor cell death still remains elusive. In addition to genes encoding ciliary proteins, mutations in two genes related to structural integrity of the retina have been studied in organoid cultures. Organoids derived from iPSCs of a X-linked retinoschisis patient with RS1 mutation recapitulated the retinal phenotype. In organoids derived from patient with mutations in the CRB1 gene, the outer limiting membrane was disrupted with ectopic photoreceptor cell bodies detected beyond the apical limit of the neural retina. The aforementioned studies have focused on examining monogenic diseases. With respect to multifactorial traits, such as AMD, glaucoma, or diabetic retinopathy, combining several tissues by incorporation of a vascular network or combining organoids with RPE into a retina-on-a-chip format might be necessary to recreate more physiological interactions. We note that iPSC-derived RPE from AMD patients with common ARMS2/HTRA1 susceptibility variants has demonstrated upregulation of several disease biomarkers, including complement and pro-inflammatory factors. These studies clearly demonstrate the utility of patient stem cell-derived retinal organoids in a dish for modeling endophenotypes of human disease in complement with in vivo animal systems.

### 4 | EVALUATION OF THERAPEUTIC STRATEGIES IN HUMAN RETINAL ORGANOIDS

Most studies to date have used gene editing in iPSCs to rescue disease phenotypes. However, such a strategy is not yet applicable for patient treatment. Most translational research so far has focused on gene therapy using AAV vectors and replacement of RPE or photoreceptors; nonetheless, retinal organoid protocols are now being adapted to search for small molecule therapeutic compounds using high-throughput screening strategies.

#### 4.1 | Gene-based therapies

AAV vector platform has become a gene delivery method of choice for retinal diseases (Figure 3). Transduction of mouse and human retinal organoids as well as RPE tissues has been examined by a panel of commonly used AAV capsid serotypes. Interestingly, ShH10 capsid serotype, which was developed through directed evolution approach of screening AAV mutant libraries, was highly efficient in...
transducing all stem cell-derived tissues examined. In mouse retinal organoids, AAV2 vector rescued the CEP290-associated disease phenotype. For human photoreceptor transduction AAV2 capsid serotype also appears to be highly potent, whereas AAV5 is most effective for RPE cells of both mouse and human origin. Additionally, AAV5 transduced both human photoreceptors as well as Müller glia, albeit at a lower frequency, in a separate study. Interestingly, human cone cells seem to be more effectively transduced by AAV8 vector, suggesting that even cell subtype differences in tropism might exist. Other treatment paradigms are also being explored in organoid models; these include antisense oligonucleotides (ASOs), which are short, chemically modified RNA molecules that can interfere with splicing or translation. ASO treatment is shown to rescue ciliation defects in retinal organoids and RPE cells derived from a patient with a common intronic mutation in the CEP290 gene.

4.2 | Cell therapies

Stem cells and organoids have been widely used as a source for evaluating cell replacement therapy approaches. The degenerative process in retinopathies primarily affects two retinal cell types: the light-sensitive photoreceptors and RPE. As a simple monolayer easily distinguishable by pigmentation and cuboidal morphology, RPE has been one of the first cell types to be efficiently differentiated from PSC sources and the first hESC-derived cell type to be transplanted into humans. Implanting intact RPE sheets alone or grown on a bioscaffold is likely to result in improved outcomes.

With respect to photoreceptor cells, pioneering studies on neural retina transplantation used primary cells derived from either donor mice or human fetal tissue. Similar to RPE, both suspension as well as whole tissue approaches have been studied; however, unlike RPE, much of the studies so far have focused on animal models. Transplantation of donor-derived photoreceptor precursor suspensions rescued some level of visual function in a model of IRD with intact but dysfunctional photoreceptors (Gnat1−/− mice); however, unfortunately rather than true functional replacement, this process involved fusion events and cytoplasmic transfer of reporter and other proteins to the diseased photoreceptors from the graft. Notably, transplantation of stem cell-derived photoreceptors have been performed in both control and models of advanced retinal degeneration where majority of host photoreceptors are lost and assessment of transplanted cells is not confounded by cell fusion. In such instances, survival of a cell mass in subretinal space, expression of phototransduction-related proteins, small structures resembling early outer segments (parts of photoreceptors where light is detected and phototransduction initiated) and presence of synapse-related proteins in the graft have been reported.
and indicate that the transplanted cells can mature in vivo.\textsuperscript{20,71,72} Recently, rods directly derived from fibroblasts using a combination of five small molecules are shown to exhibit similar characteristics when transplanted into rd1 model of end-stage retinal degeneration and partially restored some aspects of visual function.\textsuperscript{9} These observations suggest that, provided they are capable of connecting to the remaining host visual circuit, these transplanted cells might confer some light sensitivity to severely degenerated retina. Whether such a cell replacement strategy would indeed be applicable for treatment remains to be established. Given that donor-host species mismatch in human cell transplantation into animal models might affect cell-to-cell interactions, an interesting, as yet unexplored, avenue would be to use the organoids as in vitro recipient tissue for dissociated human photoreceptors.

Another approach to treat vision loss in advanced retinal degeneration is transplantation of laminated retinal sheets.\textsuperscript{60,61,65} Here, 3D retinal tissue is dissected out from organoids and transplanted whole into the subretinal space to overlay the degenerated retina. The major setbacks associated with this method include difficulty in maintaining correct morphology and polarity while surgically placing the graft into the recipient eye and presence of interneurons in the graft, which may hinder connectivity with remaining host inner retina. In contrast to dissociated cells, retinal sheets transplanted into rat\textsuperscript{60} or nonhuman primate recipients\textsuperscript{61} showed clear elaboration of outer segment structures suggesting advanced photoreceptor maturation following grafting. The presence of synaptic connectivity was also detected between the graft and host. However, obtaining a clear evidence for graft-driven retinal function remains challenging. Experiments performed on explanted retinas from recipient animals using microelectroretinography and micro-electrode arrays have shown signals suggestive of light-evoked responses;\textsuperscript{73} but unequivocal evidence for functionality of the graft itself is difficult to obtain in the presence of activity of the host retina. Stronger and more direct evidence are required for both cell suspension as well as retinal sheet transplantation to demonstrate efficacy of photoreceptor replacement therapy.

4.3 | Small molecule screening

Retinal neurons and organoids derived from human cells are valuable model systems to discover candidate target drugs for various diseases. Such an approach has been successfully applied to identify inhibitors of Zika virus brain infection\textsuperscript{74} and assess efficacy of chemotherapy agents for glioblastoma-like neoplasms.\textsuperscript{75} A 3-D retinoblastoma organoid model has been used to assess cellular responses to currently used chemotherapy drugs.\textsuperscript{76} Challenges of variability and scalability of retinal organoid cultures have so far limited their use in high-throughput compound screens. Organoids grown in a multiwell format were used to determine impact of seeding density, nutrients added to culture media and targets of ophthalmic toxicity of the antibiotic moxifloxacin.\textsuperscript{54} Recent changes in methodology\textsuperscript{23,27} and use of fluorescent reporter-based screening platforms\textsuperscript{53,77} should expedite the use of in vitro-generated organoids for high content screening of small molecules for treatment of retinal diseases.

5 | CURRENT LIMITATIONS OF RETINAL ORGANOID TECHNOLOGY

At present, successful use of retinal organoids in modeling and treatment of retinal diseases is limited by several issues. A hallmark of retinal disease is impairment of visual function, and retinal organoids from even healthy control cell lines typically show poor, if any, response to light.\textsuperscript{19,54} This is likely related to limited development of outer segment discs, where visual pigments and associated phototransduction proteins are concentrated, because of the absence of direct interaction with RPE. In addition, projection neurons in the retina (the retinal ganglion cells) degenerate in organoids probably due to the lack of connection to brain targets.\textsuperscript{19,23,78} Further precluding assessment of functional retinal circuits formation. Other challenges for using the organoid system include a great heterogeneity of cell types,\textsuperscript{21,28,79} their variability in maturation states,\textsuperscript{23} and a lack of consistent laminated structure. The diversity and developmental variability pose a challenge to comparative analysis and assessment of therapeutic effects, yet the recent staging methods may alleviate some of these problems.\textsuperscript{23,34,54}

6 | EMERGING TECHNOLOGIES FOR THE ANALYSIS OF RETINAL ORGANOIDs

Much of the characterization of retinal organoids, at least thus far, has been largely focused on histological and bulk transcriptome examination\textsuperscript{23,34,79} and suggested differentiation of major retinal cell types in organoids similar to the human fetal retina.\textsuperscript{21} However, faithfulness of subtype diversification, molecular marker expression within subtypes and relative proportions of various types are still largely missing. Several recent studies took advantage of the advances in single-cell transcriptional profiling to fill this gap.\textsuperscript{21,79-82} First studies with a relatively small number of cells demonstrated robust specification into photoreceptors and Müller glia.\textsuperscript{21,79} Analysis of cone photoreceptor profiles highlighted their similarity to native human cones.\textsuperscript{21,24,82} Consistent with in vivo mouse retina data,\textsuperscript{83,84} neurogenic retinal progenitors have been identified in the organoids characterized by ASCL1 expression. More comprehensive single-cell resolution analyses of developing human retinal organoids performed in comparison with human fetal retina samples at equivalent developmental stages have identified transitional cell populations, similar to the neurogenic retinal progenitors in vivo.\textsuperscript{80} The single-cell transcriptomic studies lay foundation for future use of this new technology in describing pathological changes in stem cell-derived disease models and more precisely determining the developmental stage of cells for transplantation in cell therapies.

Although transcriptome profiling provides powerful insights into gene expression patterns in retinal organoids, little is known as to how epigenetic regulation of chromatin features contributes to establishment of these transcriptional profiles. In brain organoids derived from human and nonhuman primate stem cells, combining single-cell profiling with open chromatin analysis using an assay for transposase-accessible chromatin using sequencing (ATAC-seq) has identified features of gene expression characteristic of human development, such as
expression of cadherin 7 (CDH7) solely in human neurons. One could envisage using similar comparative analysis to decipher regulatory networks for fovea/macula development, a primate-specific retina structure required for high acuity vision and affected in many retinal diseases.

Important but so far unexplored features of retinal organoids are their proteomic and metabolomic profiles compared to the native retina. Considering limited development of outer segment structures in retinal organoids, it is pertinent to comprehensively assess their levels of phototransduction-related proteins. Research on kidney organoids has uncovered the extracellular matrix (ECM) formed by stem cell-derived glomeruli and compared it with native tissue finding an immature ECM profile more consistent with fetal glomeruli. This study demonstrates the potential to characterize the protein composition of cellular compartments in organoids and suggests that an analogous approach could conceivably be used for retinal organoid proteome characterization. Given the small size, isolated nature, and distinct protein composition of the photoreceptor cilium compartment, determining the impact of mutations in ciliary proteins on cilia proteomic profile might shed light on their impact on the rest of the photoreceptor cell, eventually leading to its death. Efficient organoid protocols might allow performing such analysis with IRD patient-derived samples.

The retinal photoreceptors are highly metabolically active and, because of the nature of light detection machinery, highly enriched in metabolites related to the retinoid visual cycle. RPE, which plays critical roles in the metabolic processes of the visual cycle, is not correctly associated with photoreceptors in current retinal organoid protocols. Furthermore, vasculature does not form in organoids, potentially limiting oxygenation and impacting on oxidative phosphorylation. A study using human cortical organoids revealed higher cell stress markers in vitro and impaired molecular subtype specification. Cell stress signatures could be alleviated by transplantation into mouse cortex, presumably by improved oxygenation and/or exposure to vasculature-derived factors. Taking into consideration in vitro culture limitations, it would

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**FIGURE 4** Utility of retinal organoids in developing therapies for retinal diseases. Stem cell-derived retinal organoids are valuable models of retinal development and disease conditions for studies aiming to identify promising potential gene and cell therapies. Applying emerging technologies such as single-cell analysis and multi-omics may further inform about how faithfully organoids recapitulate native tissue and provide insights into molecular mechanisms of disease and therapy development.
be interesting to determine how well the metabolic profile of retinal organoids recapitulates the native tissue (for summary of novel analysis approaches, see Figure 4).

7 | CONCLUSION

Exciting fundamental advances in the differentiation of a plethora of cell lineages and tissues, including the retina, have unlocked new frontiers for deciphering underlying mechanisms of retinopathies and identifying potential therapies. Somatic cells of affected patients can now provide, via in vitro differentiation of iPSCs, retinal organoids, which recapitulate many aspects of gene expression and cell type organization of the native human retina. Broader incorporation of high-throughput and next-generation "omics" tools is expected to expand the utility of retinal organoids as disease models, facilitate elucidation of underlying cellular pathways, and assist in discovery of effective treatments.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

A.S.: conception and design, manuscript writing, final approval of manuscript, and financial support and supervision; K.K.: conception and design, manuscript writing.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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