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Transitioning into GMP-Compliance: Alternative Methods for Producing Retinal Organoids for Transplantation

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Three-dimensional retinal organoids derived from human induced pluripotent stem cells (hiPSCs) are gaining much attention as a possible source for cell transplantation to treat retinal degenerative conditions. However, the protocol for producing retinal organoids is time and labor intensive, involving a sequence of precise steps, and thus has yet to be successfully translated into a Good Manufacturing Practice (GMP)-compliant procedure. This review seeks to define the progress that has already been made in the pursuit of designing a GMP-compliant, streamlined, and automated protocol for retinal organoid production for optimal clinical success. The reviewed literature compares various approaches for cell culture automation, appropriate xeno-free conditions, and cell sources for iPSC line generation; yet, there are still important gaps for these three key considerations that remain to be addressed. Thus, the authors also discuss further potential strategies to successfully achieve GMP-compliant production of retinal organoids for eventual safe and efficient use in clinical trials.

Translational Relevance: Designing a GMP-compliant protocol for three-dimensional retinal organoid production is of urgent need in order to bring transplantation of hiPSC-derived retinal tissue and derived cells to clinical trials – and ultimately patient treatment – for retinal degenerative diseases.

Introduction

Transplantation of retinal tissue and retinal cells obtained from stem cell-derived retinal organoids is emerging as the future of clinical treatment for several retinal degenerative diseases. Thus far, clinical trials for stem-cell derived retinal transplants have been mostly limited to retinal pigment epithelial (RPE) cells in suspension or grafts.[1,2] In order for the stem cell-derived products to be used in therapeutic practice, the entire process must pass Good Manufacturing Practice (GMP) and design guidelines.[2] Three-dimensional retinal organoids present a new challenge, in that the precise and laborious protocol for producing the organoids must be adapted to GMP standards. Although much of the adaptation involves converting the protocol to xeno-free conditions, establishing a routine that will be the most successful during clinical trials also necessitates reducing the time of hands-on labor during organoid production, thus reducing the cost of GMP certified staff. Establishing the retinal transplant protocol for clinical trial also requires deciding between an allogeneic versus autologous approach, the latter of which would require growing induced pluripotent stem cells (iPSCs) specifically for each

individual patient. In this review, the authors will examine the strategies currently in use to translate retinal organoid production to GMP standards, including the use of automation to reduce "hands-on" labor, the type of iPSC lines in use, and the switch to xeno-free media and reagents. The goal is to determine which approaches are most efficient and effective to design the most ideal GMP compliant protocol for retinal organoid transplantation.

Automation

The scope of bioreactors and cell culture automation is extensive. For example, the term "bioreactor" includes a variety of systems, including hollow fiber perfusion systems, hydrogels, "organ-on-a-chip" microfluidic platforms, and polystyrene scaffold perfusion chambers, to name a few.[3] However, in this review, we will only focus on the bioreactors and automated cell culture technology that is relevant to organoid production.

The purpose of implementing a bioreactor in a GMP-compliant protocol is to reduce the time and manpower spent on growing and maintaining organoids in culture, while also ensuring the protocol is reproducible and reducing the risk of contamination. A GMP-compliant cell culture facility will be utilized, and costs can be reduced by automating the protocol as much as possible.[2] In all protocols currently used for retinal organoid production, the researcher must dedicate several hours each week to cultivating and refining the retinal organoids, as well as regularly changing media in many individual plates, as the organoids are cultured in small separate batches. Including a bioreactor eliminates human interaction with the retinal organoids during feeding via a perfusion mechanism, and a Rotating Wall Vessel (RWV) – a design originally developed at National Aeronautics and Space Administration (NASA) – or stirred-tank bioreactor that provides agitation may curtail the time demanded to refine and produce retinal organoids.[4] The Rotary Cell Culture System (RCCS) from Synthecon is a commercially available RWV, which features a perfusion mechanism as well as slowly revolving walls that suspend and rotate the contents using low shear movements (Synthecon, Inc.). Meanwhile, stirred tank bioreactors, such as those sold by Chemglass, tend to be a less expensive option, as they are a simpler design by nature and may be used with a magnetic stir bar.

Not only would implementing a bioreactor reduce the labor needed to grow organoids, but could also improve the quality and increase the yield of the product.[4,5] RWV and stirred-tank bioreactors keep organoids moving in suspension continuously through the media, resulting in a better nutrients exchange and oxygen flow, likely because the environment mimics the natural flow of blood circulation in the body.[3] Studies have reported that organoids grown in a bioreactor with continuously flowing media, whether in an RWV or stirred-tank bioreactor, have improved cell type organization and differentiation, increased photoreceptor yield, and often mature faster than those grown in static conditions.[4–6] DiStefano et al. reported via immunofluorescence that in RWV grown mouse retinal organoids cone and rod photoreceptors appeared as early as day 22, but in static conditions rods were not observed until day 25 and cones were scarce when they were finally observed at day 32.[5] However, the neural retinal tissue in the RWV organoids began degenerating after day 25, while the organoids kept in static plates did not begin to degrade until after day 32.[5] Ovando-Roche et al. observed via immunofluorescence that human retinal organoids grown in a stirred-tank bioreactor showed earlier appearance of rod photoreceptors, as early as week 11 in the bioreactor group and week 15 in the static control group.[6] However, using a bioreactor to grow retinal organoids presents real disadvantages, as well: the authors did cite that the bioreactor organoids sometimes stuck together, and the outer borders of the outer segments of these organoids were also compromised, likely due to the shear of rotation.[6] Likewise, mouse retinal organoids grown in the RWV bioreactor developed more abnormal rosettes than their static counterparts, which may have been a result of the continuous rotation of the organoids.[5] These results highlight that while current bioreactors may provide some advantages, as, for example, faster maturation and cell differentiation, they also may lead to damaged and/or abnormal organoids that will not meet the minimum requirements as a clinical product. Further studies are needed to develop more appropriate bioreactors with optimal physical properties (type of impeller, speed of rotation, and flow of media) to achieve appropriate balance between advantages and disadvantages of bioreactor systems for clinical applications.

Stirred-tank and RWV bioreactors only cut down labor during the organoid production phase after the organoids have been lifted and mostly formed. A more efficient protocol would also use automation technology to streamline the first few steps of the protocol, specifically during iPSC colony maintenance, passaging, and plating. Not only does automated technology ease the labor load, but also standardizes each step in the protocol, allows for cell growth and production at a much larger scale, and lessens the possibility of contamination via human contact.[7]

One option for "hands-off" iPSC differentiation is the TECAN Fluent (Tecan Trading AG). A multifunctional tool with a variety of options to completely automate the cell culture protocol, the Fluent can be outfitted with media changing, cell seeding, cell imaging, and plate washing functions, to name a few. Stem cells can be plated, passed, grown into colonies, and differentiated with complete sterility and reproducibility. Likely, the instrument could be designed to grow and plate neural aggregates (sometimes referred to as embryoid bodies). After full differentiation in the TECAN Fluent, retinal organoids could be lifted by hand and then transferred to a bioreactor, making the protocol almost entirely "hands-off." Other options for automated cell culture include the Biomek Workstation from Beckman Coulter, and the CompacT SelecT from Sartorius, both of which are equipped to automatically replace media and passage cell lines with complete sterility (Beckman Coulter Inc., Sartorius AG). However, the TECAN Fluent still seems to be the most robust automated cell culture system available.

Despite the benefits of mechanized cell technology, including bioreactors and automated cell culture systems, there are drawbacks that must be considered when choosing to implement one of the aforementioned systems or something similar. Mostly, the concerns are financial: automated cell culture systems and bioreactors are costly, and any laboratory equipment requires maintenance, an additional expense.[7] Therefore, the upfront and maintenance cost of the equipment must be compared to the cost of human labor, while also considering that using automated equipment allows for greater and more sterile production.

When choosing a bioreactor with the idea of increasing GMP-compliant efficiency in mind, the type of cell line in use must also be considered. If implementing a universal cell line which will supply retinal transplants to every patient, a large bioreactor that can culture and feed hundreds to thousands of retinal organoids may be the best option. Many commercial RWV bioreactors would best serve this purpose. However, these larger commercially available bioreactors are expensive to purchase and run, so they are only an efficient option when large batches of organoids are required.[3] Therefore, if an autologous, patient-specific approach is used and a different iPSC line is grown for each individual patient, a large bioreactor would be an expensive waste of reagents. A smaller bioreactor, such as the Spin∞, implements smaller spinning fans that fit into each well of a plate, so that smaller batches of organoids can be kept separately.[8]

Scale and Cell Lines

As stated above, the scale of retinal organoid production, and therefore the most appropriate bioreactor system largely depends on the stem cell type used. If a universal iPSC line is implemented, organoid production for transplantation should be performed on a large scale. A universal cell line is theoretically one that has been genetically modified to prevent triggering an immune response in the overall population, so that the line may be used safely for transplantation in any given patient.[9] An alternative, although less efficient option, may be using an allogeneic human leukocyte antigen (HLA)-matched cell line, which is derived from a generic donor specifically selected to provide HLA matching to large portions of the overall population. The advantage of an HLA-matched cell line is that it would prevent immune rejection caused when the HLA genes of the transplanted cells do not match those of the host cells.[10] It is unlikely, however, that therapies derived from HLA-matched cell lines will be completely immune-compatible with recipients, although it is expected that it will at least mitigate the degree to which immunosuppression is required.[1] Nonetheless, the risk of an allogeneic transplant should not be understated: an immune rejection process could pose serious risk to the patient, a particularly important consideration because many patients qualifying for stem cell-derived retinal transplant are elderly and may not respond well to immunosuppressive drugs.[11] Meanwhile, an autologous iPSC line is generated directly from each individual patient's cells. Therefore, using the autologous cell line method eliminates the concern of a potential immune response in the patient after transplantation. On the other hand, growing the cells for each patient's transplants individually requires much more media, multiple small bioreactors instead of one large bioreactor, and demands more hands-on labor than using one universal cell line for all of the transplants. As a result, a far greater number of transplantations are feasible in a given time frame using an allogeneic versus autologous approach.[11,12] In addition, using a universal cell line also ensures that the organoids are reproducibly generated while reducing the cost and time needed to manufacture the clinical product.[11] Although implementing a patient-specific cell line protects against immune rejection, there are safety concerns that have been raised as a result of autologous lines: the first clinical trial utilizing human induced pluripotent stem cells (hiPSCs) for RPE transplant by RIKEN in 2013 (UMIN000011929) eventually switched tactics from autologous cells to allogeneic after mutations were discovered in the patient-derived iPSC line.[2,13] Although these mutations may not have

resulted in serious complications, concern for safety is of the utmost importance and thus the autologous trial was halted.[2,13]

With concerns over simplifying the protocol and cutting production costs in mind, the simpler approach seems to be implementing an allogeneic strategy for retinal transplantation, but this approach is not devoid of important safety concerns either. It is well documented that pluripotent stem cells are prone to genetic instability and accumulation of genomic aberrations during long-term culturing and passaging.[14,15] These aberrations can include copy number variations, trisomy, amplifications and deletions of chromosomal regions, loss of heterozygosity, and epigenetic abnormalities. This highlights the need of a strict, systematic, and recurrent platform of quality controls of genome integrity and stability in banked iPSC lines intended for allogeneic therapeutic use. Guidelines for the development of human pluripotent stem cell seed stocks from The International Stem Cell Banking Initiative include: virological, sterility, and mycoplasma testing to assess microbiological hazards; and conventional GTG karyotyping, spectral karyotyping (SKY), fluorescent in situ hybridization (FISH), comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays, whole-genome sequencing, and standardized tumorigenicity testing for assessing genome integrity and stability.[15,16] A number of companies have begun developing a universal cell line, including Seattle's Universal Cells Inc., Tokyo-based Healios K.K., and Cynata Therapeutics, originally based out of University of Wisconsin-Madison.[10] However, a suitable line has yet to successfully pass through clinical trials and will require equivalent strict, systematic and recurrent quality controls as banked allogeneic cell lines.

The scale of the project is only one aspect of determining iPSC reprogramming and differentiation. The researchers also must determine from which source to derive the stem cells, with a variety of options at their fingertips: fibroblasts and blood cells among the most popular options. Fibroblasts certainly remain the most frequently utilized source of iPSCs, as they are cost effective and easy to use. However, there are significant drawbacks to relying on fibroblasts: a painful skin biopsy is required and causes undesired secondary effects to the patient, and the cells take a long time to reprogram and with very low efficiency.[17] Meanwhile, blood cell derived stem cells are a much more efficient option in terms of reprogrammability.[17] Previously, this method provided substantial obstacles due to the process of obtaining the blood. In a method similar to stem cell donation via bone marrow, the patient injects growth factors into him or herself to increase $CD34^+$ blood cells, and then connects to a machine that performs blood collection over many hours, contributing to a slow, expensive, painful process.[17] However, in recent years reprogramming via blood has made significant advances, and can now be performed via a simple fingerprick.[17,18] Outside of fibroblasts and blood cells, researchers have also reprogrammed iPSCs using patient urine and keratinocytes. These are useful options in that they involve the least invasive method of collection, but are also both less efficient than reprogramming via fibroblasts or blood cells.[17]

Last, the method of reprogramming the human samples into iPSCs must be defined. Originally, iPSCs were reprogrammed via retroviral or lentiviral transduction, which are both highly efficient and popular in many research laboratories. However, these viral methods of integrating transcription factors into the cell leaves leftover genomic sequences within the target cell.[17,19] Therefore, a reprogramming method that leaves no such residue must be implemented to derive stem cells for GMP compliant clinical use. Some nonintegrating reprogramming options include using a Sendai virus, which utilizes viral transduction but do not remain in the genome after the cells are reprogrammed, although it is costly and difficult to work with.[19,20] Another choice may be mRNA injection, which is very efficient yet again, difficult and laborious.[17,19,20] Using episomal vectors as a tool to delivery pluripotency genes has a similarly high success rate, but is more cost effective and easier to perform in the laboratory.[21,22] It has also been demonstrated that reprogramming proteins themselves can be inserted into the cells, but this method is challenging for the researcher and results in a low reprogramming efficiency.[17,19]

Reagents

In addition to converting the established retinal organoid protocol into a GMP-compliant pipeline, the reagents currently in use must be re-examined. At the moment, there are supplements used to grow retinal organoids that are animal-derived, mostly notably Matrigel and fetal bovine serum (FBS).[23] Matrigel is a mouse-derived basement membrane matrix used in culture of iPSCs and retinal organoids as a substrate with which to coat culture plates and promote iPSC and neuroretinal domain attachment while restricting iPSC spontaneous differentiation, whereas FBS provides key nutrients, such as growth factors needed for cell health.[24,25] However, if retinal organoids are to be used for human transplant, reagents from animal origin must be eliminated due to safety concerns.[24,26] Namely, human infection and immune response are

more likely to occur in transplants involving supplements developed in animals.[26] Another disadvantage of Matrigel and FBS is the variability between lots, waiving the ability of the researcher to ensure all aspects of the transplant are kept constant.[24,25] In this section of this review, the authors will explore alternative, GMP-compliant, xeno-free reagents that could be used in place of Matrigel and FBS in the retinal organoid production protocol.

Matrigel is a murine-derived supplement used to coat tissue culture plates for expansion of iPSCs. As previously addressed, it is animal-derived and thus not acceptable for use in human transplantation, and is also difficult to work with and expensive. There are a number of reagents commercially available that claim to foster similar successful stem cell expansion, such as recombinant human vitronectin (hv), human fibronectin (hf), Synthetax plates (Corning), and pooled human cryoprecipitate (hcpt), to name a few.[24,26–28] Recombinant hv, which seems to be the most popular xeno-free cell-adherent coating, is popular due to its availability and efficiency.[27] Nevertheless, recombinant hv, hf, and hcpt are all reliable options, as the mechanism of cell attachment is similar: they bind to a number of stem cell integrins, the receptors on cell surfaces that induce cell adhesion, thereby allowing for expansion.[27] Although recombinant hv, fibronectin, and cryoprecipitate are all natural alternatives to Matrigel, Synthemax cell culture plates are a synthetic replacement. Synthemax is a xeno-free, vitronectin-based cell-adherent peptide used to coat glass and plastic surfaces (Corning Inc.). Synthetic alternatives to Matrigel are advantageous in that they do not allow for batch variability, as is common with natural coating substrates, and are also easily sterilized via radiation techniques that cannot be used to sterilize biological substances due to risk of denaturing proteins.[24] However, replacing Matrigel with a GMP-compliant coating has not yet been studied in reference to the step in the retinal organoid protocol in which the retinal domains are plated and attached. Therefore, although they have been proven effective to promote stem cell attachment and expansion, further research must be done in order to determine if one of the aforementioned coatings will be sufficient to use throughout the entire retinal organoid production process.[27,28]

FBS, a widely available supplement used for its abundance of growth factors, is commonplace in many cell culture facilities. However, its use in human clinical trials is not GMP-compliant, as it has been linked to a variety of complications. FBS, like Matrigel, is lot-dependent so it cannot be held constant, and is also often cited as a source of contamination.[25] A few potential substitutions for FBS are human platelet lysate (hpl), human serum (hs), and commercially available low-serum Allegro Unison Medium (RoosterBio Inc.), all of which provide growth factors needed for stem cell proliferation. In a study performed using human muscle stem cells, the authors found that cells produced in hpl or hs enriched media had a similar growth rate to those cultured using FBS enriched media.[25] In a separate study done in hiPSCs, the authors produced similar findings: cells grown in hpl supplemented or Allegro low-serum media had similar properties to those grown using FBS, although with slightly slower proliferation found in those enriched with hpl.[29]

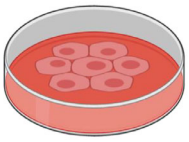
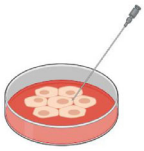
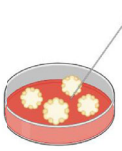
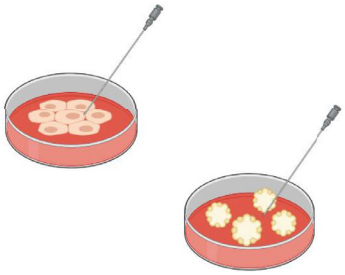
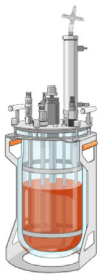
In addition to Matrigel and FBS, there are a few further reagents in the retinal organoid protocol that do not reach GMP standards, including tissue culture treated plates, dispase, and mFreSR (STEMCELL Technologies, Inc.), which is serum-free but not animal component-free.[23] As replacements, researchers can use nontissue culture treated plates, which have a hydrophobic surface that is conducive to hv coating, FreSR or Cryostor (STEMCELL Technologies, Inc.), neither of which contain animal components, in place of mFreSR to freeze and store iPSCs, and recombinant dispase or ReLeSr to replace dispase as an enzymatic tool with which to detach iPSC colonies.

Conclusion and Further Directions

Although the use of an automated cell culture tool, such as the TECAN Fluent, can produce neural aggregates with little human interaction, and a perfusion bioreactor that creates a gentle media flow could be used to change media and keep retinal organoids healthy, an efficient method of lifting retinal organoid domains has yet to be published (Table). Regent et al. observed that the entire plate of domains can be scraped off at once to eliminate the manpower needed to lift the retinal organoids, but many of these organoids remain fused together past the initial separation, and develop abnormal lamination with the presence of rosettes.[30] Furthermore, whereas it is a very practical start to refining the labor-intensive organoid protocol, scraping the retinal organoids still necessitates cleaning and separating them by hand to ensure healthy growth and proper cell type differentiation.

There are a variety of options for iPSC lines for use in retinal transplantation to prevent an immune response: autologous lines, grown specifically to match each patient, a universal cell line, which would theoretically match each patient, or allogeneic cell lines, which may be HLA-matched in order to match to a wider pool of patients than the autologous lines.

Table. Current Protocol and Reagents Required for Retinal Organoid Production in Comparison to Potential GMP-Compliant Automation and Reagents

Protocol	iPSC expansion	Neural aggregate* generation	Retinal domain recovery	Retinal organoid differentiation and maintenance
Current "hands-on" procedure				
GMP compliant automation	TECAN Fluent 		Two-step "hands-on" labor 	Bioreactor 
Current reagents in use	Dispase	Matrigel	FBS	
GMP compliant reagents	Recombinant dispase ReLeSr	Recombinant human vitronectin Human fibronectin Synthetax plates Pooled human cryoprecipitate	Human platelet lysate Human serum Allego Unison Medium	

*Sometimes referred to as embryoid bodies.

However, each strategy elicits a challenge, and there is still insufficient literature to support a standard approach that balances production time and cost with requirements for quality control and safety. Perhaps the most straightforward aspect of transitioning the retinal organoid protocol to GMP-compliant standards is the shift to xeno-free media (Table). Most compatible supplements used to replace FBS and Matrigel have already been tested in culture, and thus present the possibility of a smooth evolution into a completely xeno-free protocol.

There are numerous clinical trials utilizing human embryonic stem cells (hESCs), briefly described here. Astellas Institute for Regenerative Medicine has sponsored many trials utilizing hESC derived RPE from 2011 through 2018, focusing on RPE cell transplants in patients with AMD and Stargardt's Macular Dystrophy (SMD), a number of which have already been completed (NCT01469832, NCT02941991, NCT01345006, NCT01344993, NCT02445612, NCT02463344, and NCT03167203).[31] The Chinese Academy of Science has also launched two trials using hESC derived RPE cells to treat age-related macular degeneration (AMD; NCT02755428 and NCT03046407), as well as one trial aimed to treat retinitis pigmentosa (NCT03944239).[31] Regenerative Patch Technologies, LLC, is currently sponsoring a clinical trial to treat AMD with hESC derived RPE grown on a polymer membrane (NCT02590692), and the Federal University of Sao Paulo in Brazil performed transplantation using RPE in suspension (NCT02903576).[31] Moorsfield Eye Hospital NHS Foundation Trust in the UK launched a safety study using hESC derived RPE cells (NCT03102138), and Lineage Cell Therapeutics is sponsoring a safety study for OpRegen, a new RPE replacement therapy (NCT02286089).[31]

Although there are more clinical trials currently utilizing hESCs instead of iPSCs for retinal regeneration, using the latter has become more common in recent years due to expanded methods of inducing pluripotency. Despite the current challenges outlined in this review, there are iPSC-based therapies for AMD that have made it to clinical trials. The first of such was sponsored by the RIKEN Institute in Japan in 2013 and planned to transplant a sheet of autologous iPSC-derived RPE cells (UMIN000011929).[13] At 5 years postsurgery the RPE sheet, as well as the

patient's visual acuity, remains stable.[31] Yet, even after the first surgery presented no serious side effects, the trial was halted due to mutations in the iPSCs of the second patient.[13,32] This unfortunate event further draws into question if autologous cell lines are the proper course of action for retinal transplant, as additional expenses are incurred if a patient's line develops mutations, as well as the obvious concern for the patient's safety. The RIKEN Institute continues to approach its goal and thus initiated a second clinical trial in 2017, although it will use allogeneic HLA-matched cells in an attempt to ensure patient safety, with the added benefit of reducing cost and time (UMIN000026003).[13,32] However, this did not put an end to attempts to develop autologous RPE transplants. In 2015, Moorfields Eye Hospital in the UK launched another clinical trial, which similarly planned to use RPE cells grown on a polyester membrane derived from each patient's cells as a retinal transplant (NCT02464956).[13,31] Last, in 2019, the National Eye Institute, a division of the National Institutes of Health (NIH) in the United States, announced another trial to transplant a patient-derived RPE patch into each patient's retina (NCT04339764).[33] The iPSC-derived retinal cell production gained US Food and Drug Administration (FDA) approval after meeting GMP standards, although it is notable that the protocol was not for retinal organoid growth, only for RPE cell production.[33] The latter two of these trials remain ongoing, and although each trial does involve transplanting autologous iPSC-derived RPE cells, none of them involve transplanting retinal organoids themselves or their derivative cells. A clinical trial aiming to transplant organoid tissue or derived cells has yet to be initiated.

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