Comprehensive Description of the Proposed Solution

Abstract

Proper organogenesis relies on the orchestrated spatial and temporal presentation of graded stimuli for cell fate commitment and maturation. This represents a major obstacle to deriving complex tissue structures, like the fully developed retina, in the laboratory. Typically, in-vitro culture systems deliver uniform stimuli to induce homogenous tissue differentiation at established time points; but spatial distribution of differentiation cues relies mainly on interactions occurring within the culture environment, which are not solely adequate for proper differentiation of all retinal layers. To overcome this obstacle, we propose a novel tissue bioreactor system that allows for the compartmentalization and gradation of stimuli designed to separately induce the maturation of inner and outer retinal cell phenotypes. Our bioreactor includes an air-tight upper chamber which allows the establishment of physiologically-relevant oxygen tension gradients across the developing retina. Thus, we can recapitulate the normal physiology in which the retina is exposed to a steep gradient of oxygen tensions across the highly oxygenated outer retina, and the hypoxic inner retina. We will use this solution to model Retinoblastoma (Rb), which is one of the leading causes of childhood cancer death worldwide. Rb is not an age-related degenerative disorder, but rather a developmental disease, making it ideal for studies in a platform of retinal development. Using CRISPR/Cas9 technology, we have established RB1-knockout iPSC lines in our laboratory (RB1KO). The availability of wildtype and RB1KO cells from the same parental cell lines, lets us model somatic and germline manifestations of the disease, and study how retinoblast cells interact with normally-developing retinal tissues. This approach allows us to characterize the phenotype(s) from which Rb tumors originate, which is still a matter of debate. Similarly, using our custom bioreactor system, we can study how retinoblastoma tumors transition from oxygen-dependent growth to their most aggressive phenotype, hypoxic-adapted vitreal seeds.

Background

All developmental processes rely on temporospatial presentation of graded stimuli. This is true throughout embryogenesis, from the establishment of body axes through the maturation of cellular phenotypes. In the retina, intrinsic factors play a big role in the specification and delineation of the prospective neural retina from other ocular tissues. However, final lamination and maturation of neural retinal phenotypes also depends on extrinsic stimulation arising from adjacent tissues such as the lens placode, vitreous body, and retinal pigmented epithelium (RPE) (Figure 1, 2). Among the known pathways driving correct retinal lamination and maturation are the sonic hedgehog (shh), bone morphogenetic protein (BMP), notch, wnt, and fibroblast growth factor (FGF) pathways (Aoto et al., 2009; Ashery-Padan, Marquardt, Zhou, & Gruss, 2000; Borday et al., 2012; Fujimura, Taketo, Mori, Korinek, & Kozmik, 2009; Hernandez-Bejarano et al., 2015; Matt et al., 2005; D. Murali et al., 2005; Picker & Brand, 2005; Sakuta et al., 2006).

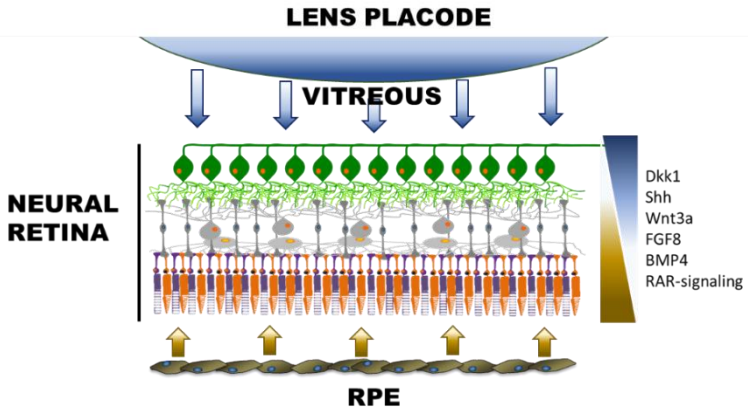

Figure 1: Schematic of extrinsic influences on retinal patterning and final lamination arising from the developing lens placode, vitreous body, and retinal pigmented epithelium (RPE). Gradients of known signaling pathways modulated by these tissues is presented.

Similarly, oxygen levels and hypoxic signaling play a critical role in retinal morphogenesis (Caprara et al., 2011). The fully-developed retina possesses active mechanisms that establish steep oxygen gradients and variations across its layers (K. Murali et al., 2016; Yu & Cringle, 2004; Yu, Cringle, & Su, 2005). Starting early in retinogenesis and through adulthood, the inner and outer retinas comprise two distinct domains in terms of oxygen perfusion levels (Cringle, Yu, Su, & Yu, 2006; Wangsa-Wirawan & Linsenmeier, 2003), and respond differently to inspired oxygen variations and vascular permeability modulation (Linsenmeier, 1990; Ruzafa, Rey-Santano, Mielgo, Pereiro, & Vecino, 2017; Shakoor, Blair, Mori, & Shahidi, 2006). The inner retina is kept in a hypoxic state, with partial pressures of oxygen maintained constantly below 25 mmHg (or 3% w/w) (Yu & Cringle, 2001). The oxygenation levels of the retina rapidly increases towards outer retina and bruch's membrane, reaching a maximum of approximately 60 mmHg (8% w/w) at the choroid (Yu & Cringle, 2001; Yu et al., 2005). Even under hyperoxic ventilation, the hypoxic maintenance mechanisms within the inner retina avoid significant increases in oxygen perfusion, while the outer retina and choroid, devoid of oxygen self-regulation, are allowed to experience oxygenation increases commensurate with the level of hyperoxic intake (Yu et al., 2005). However, until these mechanisms are fully formed, the developing inner retina is especially susceptible to high oxygen levels and oxidative stress, which can result in oxygen induced retinopathy (Mezu-Ndubuisi et al., 2013). In mice, inner retinal hypoxia is evidenced early in retinogenesis and can be identified by the expression of hypoxia inducible factor 1-alpha (HIF-1A) in the innermost retinal layers (Figure 2). In fact, retinopathy of prematurity (ROP) is a devastating clinical complication arising from hyperbaric oxygenation of premature infants with poorly developed retinas (Cringle & Yu, 2010; Hartnett, 2015). Controlling the level of oxygen available to the differentiating inner and outer retina segments can, thus, achieve functional differentiation and structural guidance to the various retinal phenotypes concurrently.

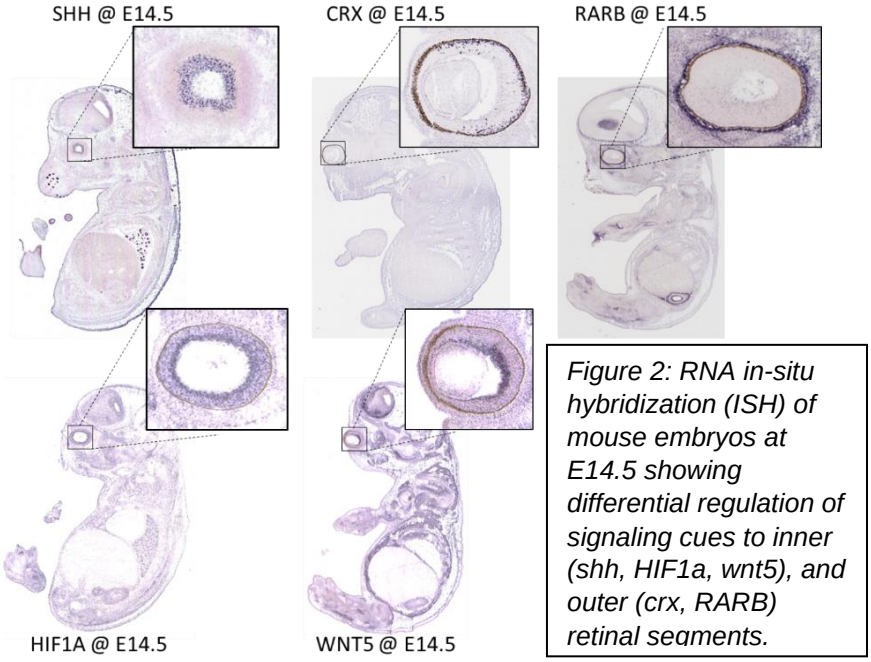

Figure 2: RNA in-situ hybridization (ISH) of mouse embryos at E14.5 showing differential regulation of signaling cues to inner (shh, HIF1a, wnt5), and outer (crx, RARB) retinal segments.

The **main challenge** in attaining structurally and functionally mature retinal constructs in laboratory culture vessels is the sole reliance on intra-organoid modulation of signaling cues to achieve proper retinogenesis (Eiraku et al., 2011). External stimulation protocols through media supplementation have been developed to induce either inner (Aparicio et al., 2017; K. Li et al., 2017; Nakano et al., 2012; Riazifar, Jia, Chen, Lynch, & Huang, 2014; Teotia et al., 2017), or outer (Chen, Kaya, Dong, & Swaroop, 2016; Parfitt et al., 2016; Reichman et al., 2017; Zhong et al., 2014) retinal maturation in retinal organoids with some success. We have successfully designed and tested our own formulation of media designed to induce retinal ganglion cell (RGC) maturation and maintenance (RGCM) in our laboratory (see Appendix). But in order to achieve full-thickness retinal lamination and maturation in-vitro, a way of concurrently compartmentalizing these stimulation cues and oxygenation requirements needs to be implemented.

Hence, we have designed and prototyped a novel tissue bioreactor system that can be used in any cell culture facility and allows for the compartmentalization of 2 differentiation medias on opposing side of the organoids, as well as for the establishment of physiologically-relevant oxygen tension gradients

across developing retinal organoid at atmospheric pressures. The use of bioreactor systems to study hypoxic growth and adaptation has been reported before (Acosta et al., 2014; C. Li et al., 2016; Shiwa, Uchida, & Tsukada, 2012). Building on these studies, and our own expertise in bioreactor construction and use in stem cell differentiation, we present our novel bioreactor system as a solution to this retinal organoid challenge competition.

Bioreactor Attributes (Figure 3):

1. Easily manufactured using 3D-printing: The bioreactor chamber assembly is designed to be printed in a 3-Dimensional (3D) fused deposition modeling (FDM) technology printer, thus allowing anyone with access to a 3D printer to generate their own bioreactor chamber array based on our computer aided drawing (CAD) files, which we plan on making available if given this award.
2. Designed to fit onto commonly used 12-well plate formats: Dimensions were modeled to fit Falcon® 12-well plate formats (cat #353043). This chamber array assembly allows for 12 separate media combinations and hypoxic conditions to be tested concurrently, each with 4 organoid holding compartments (32 samples run at any one time under 12 varying conditions).
3. Media chamber separation (compartmentalization): Only fluid exchange between chambers is through organoid holding aperture which is diffusion limited through a hydrogel due to balanced hydrostatic forces between the chambers.
4. Air tight upper chamber: Lower chamber remains open to incubator ambience and oxygen level. This allows to establish the desired gradient of oxygen tensions. Exhaust of upper chamber gas mixture is done to normal atmospheric pressures (outside air) so as to not create hydrostatic pressure buildup on any one chamber of the bioreactor.
5. Organoid loading and retrieval system: Retinal organoids are loaded into commercially available pipet tips (large orifice 200ul Fisher Scientific tips, cat #02-707-134) in Hyaluronic acid hydrogels. This maintains sterility, avoids excessive manipulation of organoids, and allows for loading and unloading to be done fast without need for specialized equipment.
    a. Use of inert and physiologically-relevant hydrogel scaffolds with known diffusion parameters in liquid permeation helps establish the required stimulation and oxygen gradients across the organoid. Hyaluronic acid (HA) was chosen based on the composition of the vitreous body, which is primarily made up of HA.
    b. Easy removal and manipulation: Having the retinal organoids in plastic pipet tips permits the researcher to easily collect them for sectioning, or downstream organoid recovery for imaging or analytical processing
6. Fully autoclavable: Use of biocompatible 3D printing polymers for the build, such as ABS-M30i (Stratasys), allows for sterilization and reusable chamber assemblies if needed. A number of 3D printing materials can be used as long as they are biocompatible and have an approved method for their sterilization.
7. Recapitulation of Biological complexity of developing retina: Most importantly, this bioreactor approach allows us to provide the developing retina with stimulation that would normally arise from external tissues such as the lens placode, cranial neural crest, vitreous body, and retinal pigmented epithelia. These stimuli are normally compartmentalized by the vasculature, the different cavities of the eye, and the membranous barriers that line the retina.
    a. Compartmentalization of Retinal Development stimuli into inner- and outer-retina stimulus. We can thus employ ventralization signals and lamination stimulation gradients through this media compartmentalization.
    b. Steep oxygen gradients and control to mimic physiologic oxygen tensions in the developing retina. We plan to use a custom mixture of 1% Oxygen balanced with 5% Carbon Dioxide, and 94% Nitrogen for our inner retina stimulation; and a 10% O_2, 5% CO_2, and 85% N_2 for the outer/ambient chambers.

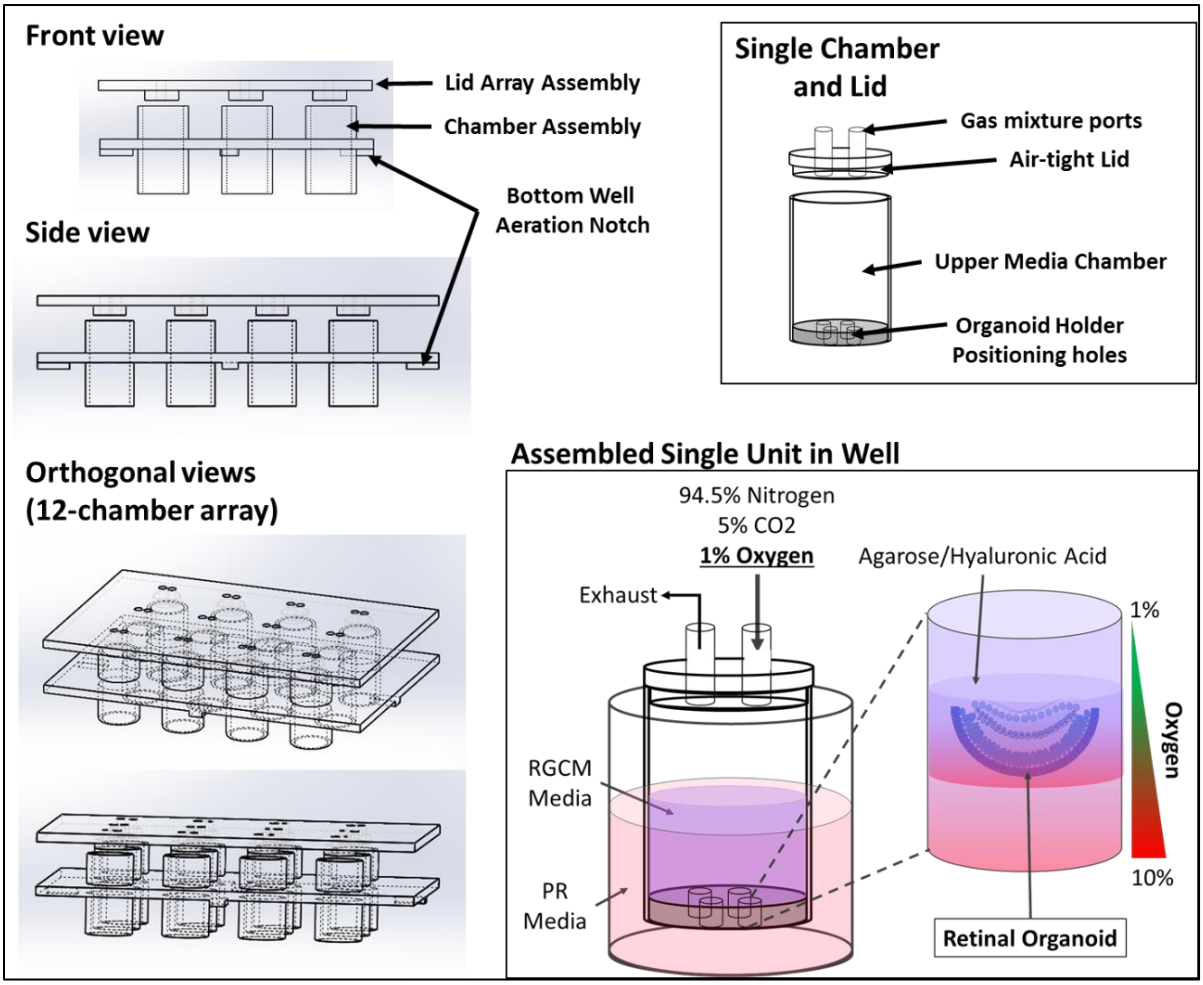

Figure 3: Computer aided drawings (CAD) of novel bioreactor chamber assembly for retinal organoid morphological differentiation and maturation. (Left): CAD projections of 12-chamber and lid array assemblies. (Right, top): Single chamber drawing outlining functional aspects of bioreactor chamber. (Right, bottom): Assembled chamber depicted inside tissue culture well with RGC maintenance (RGCM) media, and photoreceptor (PR) maturation media

Proposed working bioreactor protocol

To achieve an early stage of retinal differentiation, induced pluripotent stem cells (iPSC) will be subjected to a stage-wise differentiation protocol as described by Zhong et al., (Zhong et al., 2014). Briefly, iPSCs will be detached and dissociated into small clumps. Then, they will be cultured in suspension to induce aggregate formation. They will initially undergo a neuro induction stage where they will be cultured using a neural induction medium containing DMEM/F12, N2 supplement, non-essential amino acids and heparin. On day 7 of differentiation, aggregates will be transferred to a two-dimensional culture and they will be cultured on matrigel coated culture plates. On day 16, the culture medium will be switched to a retinal differentiation medium containing 3:1 DMEM/F12, B27 supplement and non-essential amino acids. On the fourth week of differentiation cells will be detached from the 2D culture to allow for the development of a three-dimensional culture. The cells will continue to be cultured under the retinal differentiation medium until week five. After specification of the prospective Neural Retina (NR) is achieved, the retinal organoids are transferred to a 5% (w/v) hyaluronic acid solution (reconstituted in the retinal induction media). A large orifice pipet is used to aspirate single retinal organoids under direct visualization through the microscope. The pipet volume is then adjusted to bring the organoid-hydrogel plug to the distal opening of the tip

(without expelling it from the tip). The pipet tip is removed from the pipettor and cut using sterile nail nippers right above the upper edge of the organoid-hydrogel plug. This organoid holding construct is then placed into an empty organoid holder positioning hole (Figure 3) in the bioreactor chamber using sterile tweezers. Once all positioning holes have been plugged with organoid holders (containing organoids or blank hydrogel controls), the bioreactor chamber and tissue culture plate well are filled with their respective media (for inner and outer retinal maturation, respectively). RGC maintenance media (see Appendix) will be used to promote inner retinal maturation. Outer retinal maturation will be achieved by implementing the photoreceptor maturation protocol described by Zhong et al (Zhong et al., 2014). Media volumes used should ensure that both media levels/heights are equal to eliminate hydrostatic-driven flow of solutes in any direction (thus limiting solute exchange to diffusion). The bioreactor lid assembly is then placed over the chamber assembly, gas fittings are connected, and the custom gas mixture (1% O_2, 5% CO_2, 94% N_2) is flowed through the upper air-tight chamber at a constant rate of 1cm^3/min, resulting in 1 full volume exchange of gas per minute (air volume of upper chamber is approximately 900uL). The entire assembly is placed in a cell culture incubator with ambient oxygen levels set at 10% (v/v). Once the experimentation period has concluded, organoid holders can be removed using sterile tweezers and processed for sectioning and imaging, or the organoid can be expelled from the holder and processed for PCR, Western Blot, or other downstream analytical technique.

Modeling of Retinoblastoma Progression and Hypoxic Adaptation in Novel Tissue Bioreactor

Retinoblastoma (Rb) is the most common primary ocular tumor in children and a leading cause of childhood cancer death worldwide (Dimaras et al., 2012; Mendoza & Grossniklaus, 2015). The tumors initiate from a biallelic mutation in the retinoblastoma gene (RB1), the first-ever described tumor suppressor (Mendoza & Grossniklaus, 2015; Sage, 2012). However, it is well documented that RB1 mutation by itself does not lead to malignant progression of Rb tumors (Dimaras et al., 2008; Mendoza & Grossniklaus, 2015). Over the years, researchers have sought to elucidate the molecular framework behind retinoblastoma tumor progression in hopes of understanding advanced retinoblastomas' most pervasive feature: a shift from oxygen-dependent growth to a hypoxic adaptation, which allows it to sprout tumor seeds into the vitreous and metastasize. Yet despite the efforts of many laboratories, to date there is no mutation identified (aside from the initiating RB1 mutation), or specific pathway dysregulation described that can cluster advanced stage retinoblastomas, and account for tumor vitreal seeding and progression (Mendoza & Grossniklaus, 2015).

As a developmental disease of the retina, retinoblastoma is an ideal disease to model in iPSC-derived retinal organoids. The use of iPSCs and organoid cultures for disease modeling has gained significant traction in recent years (Avior, Sagi, & Benvenisty, 2016; Clevers, 2016; Fatehullah, Tan, & Barker, 2016). Similarly, gene editing and CRISPR/Cas9 has revolutionized the applicability of iPSC in disease modeling and regenerative medicine (Hendriks, Warren, & Cowan, 2016; Hockemeyer & Jaenisch, 2016). Using CRISPR/Cas9 technology, we have established an RB1-knockout iPSC lines in our laboratory (RB1KO) and subjected them to the retinal organoid differentiation protocols (see Appendix). Along with our novel bioreactor system, we can establish a platform to study the biological complexities of retinoblastoma tumor initiation and progression as outlined below.

Elucidating Biological Complexities of Retinoblastoma:

- *Germline vs. somatic RB1 mutations:* Retinoblastomas can occur from germline (inherited) or somatic mutations in the RB1 gene. Hereditary Rb are bilateral and generally occur earlier in life than somatic, unilateral tumors (Dimaras et al., 2012; Mendoza & Grossniklaus, 2015). The availability of wildtype and RB1KO cells from the same parental cell lines, lets us model somatic and germline manifestations of the disease without the additional confounding variables of genetic background variations.
    - *Germline:* Use of 100% RB1KO iPSCs
    - *Somatic:* Use of 5-10% RB1KO with 90-95% WT iPSC
    - Studies into how non-mutated cells interact or stimulate RB1 mutant cell growth: RB1 mutations results in benign retinomas prior to their malignant transformation into retinoblastoma (Dimaras et

al., 2008). It is unclear as to how much influence wildtype cells have on the rate of malignant transformation of retinomas, and whether RB1 wildtype cells contribute to the later appearance of retinoblastomas in the somatic form of the disease. We can select out RB1KO cells from our retinal organoids by their expression of GFP from our CRISPR/Cas9 inserted cassette (see Appendix), and compare them to non-GFP expressing cells (RB1 wildtypes) for gene expression profiling, and next generation sequencing at various developmental stages.

- *Role of RB1 function in retinal phenotype terminal differentiation:* Our CRISPR/Cas9 gene editing approach inserts a GFP cassette that is driven by the native RB1 promoter. This allows us to study activation of the RB1 gene through retinal development as cell exit cell cycle to terminally differentiate (see Appendix). We can dissociate the retinal organoids at various developmental stages, select GFP-positive cells out (those that are trying to express the RB1 protein), and perform gene expression profiling to elucidate their retinal genotype.
    - Determine retinal phenotypes dependent on RB1 function for terminal differentiation: it is crucial to distinguish between neurogenesis and differentiation of retinal phenotypes (Engerer et al., 2017). Even in germline bilateral Rb tumors, some retinal phenotypes always achieve terminal differentiation without neoplastic growth. However, as the nature of the Rb tumor shows, some cellular phenotype(s) in the retina is/are dependent on RB1 function to terminally differentiate. By studying GFP-positive RB1KO cells at various time points, we can elucidate which retinal phenotypes are more susceptible to developmental dysfunction by RB1 mutations.
    - Cell of origin study for Retinoblastoma: While generally regarded as arising from a cone photoreceptor precursor (Xu et al., 2014), there is still some debate as to the retinal cell(s) of origin for these tumors (Dimaras et al., 2015; McEvoy et al., 2011). Our RB1KO iPSC and bioreactor platform are ideally suited to help shed light on retinal phenotypes from which these tumors evolve.

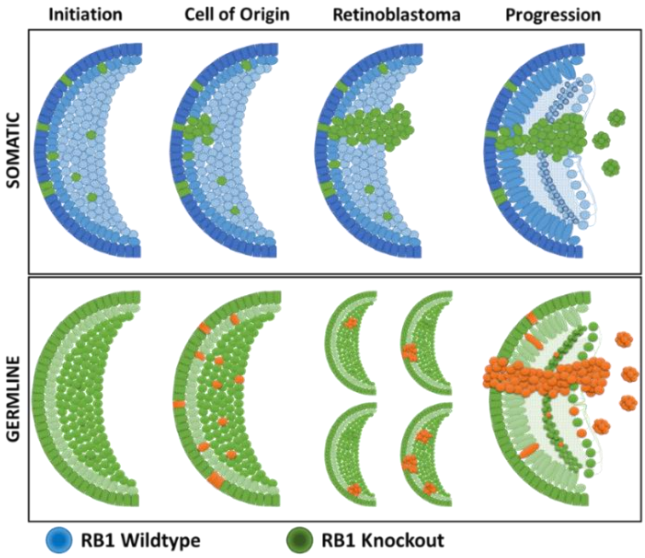

Figure 4: Schematic depicting the biological complexities of retinoblastoma tumors that can be explored using the proposed solution: Tumor initiation, Rb cell of origin, malignant transformations, hypoxic adaptation and progression.

- *Hypoxic adaptation of Retinoblastoma cells:* Once Rb tumors have seeded into the hypoxic vitreous, clinical management can become problematic. At this stage, systemic and intra-arterial chemotherapy become ineffective, and the need to remove the eye becomes more imminent (Dimaras et al., 2015). Our bioreactor system with a hypoxic chamber allows us to study how RB1 mutant cells may acquire adaptive mechanisms to thrive in low oxygen environments and, hopefully, provide novel therapeutic targets that can aid ocular oncologists in the fight against advanced Rb cases.

Innovation Statement:

The presented solution to the 3-D ROC competition is highly innovative and employs the latest techniques in terms of retinal induction of iPSCs, the use of the most cutting edge molecular biology methods like CRISPR/Cas9 gene editing, and the use of novel culture bioreactor systems to achieve the goals of the challenge. Furthermore, this application is comprised of a multidisciplinary team, bringing together experts in the fields of cell biology, developmental biology, stem cell biology, biomedical engineering, molecular genetics, and ocular oncology.