Establishment of Patient-derived Xenografts of Retinoblastoma & Choroidal Melanoma on the Avian Chorioallantoic Membrane

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Research Article

Keywords: Eye cancer, Ocular cancer, Retinoblastoma, Uveal melanoma, Choroidal melanoma, Chorioallantoic membrane, CAM, patient-derived xenograft

Posted Date: June 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1769766/v1

Abstract

Background: To develop a viable in vivo chorioallantoic membrane (CAM) model to study the growth and invasion of patient-derived Retinoblastoma (RB) and Choroidal Melanoma (CM) xenografts (PDXs). The study utilizes primary tumor samples instead of cancer cell lines which provides a more authentic representation of tumors due to conserved morphology and heterogeneity.

Methods: Fertilized chicken eggs were procured, windowed, and their CAM layers were dropped. On embryonic development day (EDD) 10, freshly cut patient-derived CM and RB tumors were implanted on the CAM layer and the set-up was incubated for 7 days. The tumor-embedded CAM layer was harvested on EDD-17 and the extracted tumor samples were subjected to Hematoxylin and Eosin staining and Immunohistochemical analysis to evaluate the extent of tumor invasion.

Results: Significant changes in the vascularity around the RB and CM PDXs were observed indicating an angiogenic environment. The cross-sectional histological view of the tumor implant site revealed the invasion of both the tumor types into CAM. Invasion of CM to CAM mesoderm was visualized in the form of pigmented nodules and of RB was indicated by synaptophysin and Ki-67 positivity in IHC.

Conclusion: The CAM xenograft model was successfully able to support the growth of CM and RB PDXs and their invasion in CAM, thus presenting as a feasible alternative to mammalian models for studying tumorigenicity and invasiveness of ocular tumors. Moreover, this model can further be utilized to develop personalized medicine by inoculating patient-specific tumors for pre-clinical drug screening.

Introduction

Uveal melanoma (UM), arising from the melanocytes of the uveal tract, is the most common cause of primary malignant intraocular neoplasm in the western world(Fiorentzis et al. 2019). It originates from iris (4%), ciliary body (6%), or choroid (85-90%), the latter being the most prevalent form(Shields et al. 2009). Uveal melanoma is characteristically distinct from cutaneous melanoma which originates from the melanocytes confined within the basal layer of the epidermis(van der Kooij et al. 2019). Prognosis and metastasis of all UM are majorly affected by mutations in GNAQ and GNA11 genes(Coupland et al. 2013). Nearly 50% of the afflicted individuals succumb to metastasis within 10 years of diagnosis(Kaliki, Shields, and Shields 2015).

Choroidal melanoma (CM) accounts for more than 70% cases of uveal melanoma rendering it the second most predominant form of malignant tumors. Despite the advancement in therapeutics, the median survival of patients with choroidal melanoma is less than a year(Kaliki, Shields, and Shields 2015). Its common metastatic sites are the liver (90%), lungs (24%), and bones (16%) owing to its heterogeneous spread(Fiorentzis et al. 2019). Depending on the extent of tumor growth, its treatment could vary from brachytherapy, endoresection, exoresection to the complete removal of the eyeball(Hamza and Elhusseiny 2018; Brady and Hernandez 1992). High metastatic potential and low survival incidence of choroidal melanoma make it more pertinent to oncology research.

Amongst children, Retinoblastoma (RB) is the most widespread intraocular tumor, arising in both heritable and non-heritable forms, accounting for approximately 8000 cases annually(Dimaras et al. 2012; 2015). It is caused by a mutation in both RB1 alleles in a retinal cell; early lesions of RB are found in the inner nuclear layer of the retina(Cocarta et al. 2019; Bremner and Sage 2014). Like UM, it is also metastatic in nature and if left untreated, it is highly likely to spread outside the eye and invade the central nervous system via the optic nerve. Through metastases in the bloodstream, it can also spread in regional lymph nodes, bones, lungs, and liver, making it very important to study its characteristics(Kletke et al. 2019; Ishaq and Patel 2022).

Extensive efforts have been devoted to developing an ideal model to study metastasis of Uveal melanoma and Retinoblastoma. This is primarily done using established UM and RB cancer cell lines on nude mice models and transendothelial, transwell, and matrigel invasion assays(Yang, Cao, and Grossniklaus 2015; Richards et al. 2020; Tschulakow et al. 2016; Liu et al. 2018; Nair et al. 2017). However, each of these models have their own disadvantages like the matrigel invasion assay which is extensively attributed to variability and the nude mice model which needs to be artificially immunocompromised and fails to lay out a natural environment for tumor development(Lei et al. 2016; Justus et al. 2014). The chorioallantoic membrane assay (CAM) offers to be an admissible alternative to the above-mentioned models and can be employed to study tumor invasion in real-time and it, together with extracellular matrix protein (ECM), imitates the true microenvironment of the basement membrane, allowing for tumor development(Staton, Reed, and Brown 2009). Chick CAM is an extra-embryonic structure identical to the placenta in mammals. It serves as the primary site for the gaseous exchange and also protects the chicken embryo during its development(Metcalfe and Stock 1993). CAM is formed due to the fusion of mesodermal layers of chorion and allantois. Its rich vascular network and immune-naïve nature make it an exemplary substrate for tumor cultivation and angiogenesis study. Its high reproducibility, short incubation time, simple set-up, and cost-effectiveness make it more ideal as compared to conventional animal models(Lokman et al. 2012).

Recent studies have underlined the implication of utilizing xenograft in understanding metastasis. Most of this research involves the use of uveal melanoma cell lines namely OMM1, 92.1, and retinoblastoma cell lines namely- RB- Y79, WERI-RB1, RB-355(Kalirai et al. 2015; Busch et al. 2015). However, the xenograft model of Choroidal melanoma and Retinoblastoma on CAM has been less utilized. Solid primary tissue has an edge over cell lines as it is a true representation of the actual tumor and conserves heterogeneity of tumor cell subpopulation(Miserocchi et al. 2017). Furthermore, results from xenograft of Hepatocellular carcinoma, oral and laryngeal squamous cell carcinoma on CAM have established its success to study tumor invasion(Li et al. 2015; Uloza et al. 2015; Kauffmann et al. 2018).

This research aims to study the survival and tumor invasion of Choroidal melanoma and Retinoblastoma xenograft on CAM and provides a basis for a potential future approach about their invasiveness and metastatic potential.

Materials And Methods

Ethics approval

Tumors were obtained after approval from the institutional ethics review board of All India Institute of Medical Sciences (AIIMS), New Delhi, India (IEC-299/01.06-2018) and the study followed the tenets of The Declaration of Helsinki. All patients provided written informed consent.

Approval to work on fertilized chicken eggs in the study was obtained from the ethical and safety committee of Shivaji College, University of Delhi, New Delhi, India (Bioethics and biosafety committee report, SCS2019).

Egg Procurement and Preparation

An overview of the protocol has been described in Figure 1.

Fertilized eggs of *Gallus gallus domesticus* were procured from Chattarpur Farms, New Delhi, India. In the research laboratory, these eggs were cleaned using sterile water and paper towels. Following this, the eggs were maintained in an upright position under suitable conditions of 37.5°C temperature and 55-60% relative humidity in order to induce embryogenesis. The study was carried out after approval from the institutional ethical and safety committee of Shivaji College, University of Delhi, New Delhi, India (Bioethics and biosafety committee report, SCS 2019).

The CAM assay was performed between the chick embryonic development day (EDD) 9 and 17. On EDD 9, the CAM tissue was identified. Further, the eggs were cleaned with distilled water, followed by which the air sac was punctured using a 19 G needle, and a suction force was applied for the dropping of CAM. A window of 1.5 cm diameter was established on the upper surface of the eggs under aseptic conditions, and all the embryos were examined for any signs of local bleeding. Next, sterile silicon -o- ring (inner and outer diameter 8mm and 10 mm respectively) was placed onto the CAM in an area with visible vascularization.

Tumor Procurement and Preparation

Fresh tumor tissues of Choroidal Melanoma (CM) and Retinoblastoma (RB) were obtained from two patients undergoing enucleation from the Operation Theatre of Dr. R.P. Centre, All India Institute of Medical Sciences (AIIMS), New Delhi after written and informed consent from donor patients and approval from the institutional ethics review board of AIIMS, New Delhi, India (IEC-299/01.06-2018). The samples were transported from the Department of Pathology in isotonic saline solution at ambient temperatures of 18-20°C.

The tumor sample procured from the patient with Choroidal melanoma was divided into three tissue segments (replicates) of approximately 4-5 mm length, and the same step was followed for the tumor sample procured from the patient with Retinoblastoma. The replicates were excised with minimal disruption of the ocular architecture using a scalpel and blade. The rest of the tumor tissue was formalin-fixed and paraffin-embedded for light microscopy and immunohistochemistry. The patient-derived

xenografts were then subjected to CAM assay in separate set ups. On EDD 10, each excised replicate of CM and RB was placed separately in the middle of a sterile silicon -o- ring on the CAM of the egg after 45-60 minutes of enucleation. The egg windows were resealed with sterile tape.

Patient-Derived Xenografts and CAM Assay

The six inoculated eggs containing the patient-derived xenograft were placed back in the incubator, maintaining them in an undisturbed state for 7 days until EDD 17. On EDD 17, the chick was euthanized by inducing hypothermia. Following this, the CAM tissue bearing the patient-derived xenograft was extracted and washed with PBS to remove the remnants of yolk and extraembryonic membranes. The observations were then made under a stereoscopic microscope and photographs were taken. Shortly after this, the extracted tissue bearing the tumor sample was fixed in 3.7% paraformaldehyde for further processing.

Histopathological Investigation

Following fixation and processing of the CAM tissue bearing the tumor, standard protocols were utilized to accomplish Hematoxylin and Eosin (H/E) staining on the sections. Sections were mounted and imaging was performed using light microscopy. H/E stained sections were then evaluated by an ophthalmic pathologist (S.S) and two observers. The tumor invasion was shown to be represented by the occurrence of invasive cancer cells in the mesoderm of CAM on histological sections analysis.

Immunohistochemistry

Paraffin-embedded and formalin-fixed tissue segments were mounted on poly L lysine microscope adhesion slides. Next, deparaffinization was performed by utilizing xylene, followed by which, rehydration was achieved using a series of graded alcohols. After this, antigen retrieval was performed by incubating tumor sections for approximately 20 minutes at a temperature of 90°C in a citrate buffer of pH 6.0. The slides were then allowed to cool, after which they were washed with Tris-Buffered Saline of pH 7.5 and the endogenous peroxidase activity of the tumor sections was stopped by incubating them in H_2O_2 (0.3% v/v) for 20 minutes. The retinoblastoma xenograft tissue and associated CAM tissue sections were then incubated with monoclonal anti- synaptophysin antibody (MA5-14532, Thermo Fisher) and anti-Ki-67 antibody (MA5-14520, Thermo Fisher) at dilution of 1:200. The immunoreactive signals were identified using the substrate DAB peroxidase. Hematoxylin was used as a counterstain for 30 seconds, following which the tissue sections were dehydrated and DPX was utilized for mounting them. The results were demonstrated using light microscopy.

Results

Retinoblastoma and Choroidal Melanoma PDXs were successfully grafted onto the CAM in all three replicates and were viable throughout the duration of the experiment i.e. until EDD 17 after which the CAM tissue bearing the tumor was excised. Gross visual assessment post-H/E staining demonstrates the

development of dense vasculature around Retinoblastoma and Choroidal Melanoma PDXs post-incubation indicating an angiogenic microenvironment. The morphology of RB cells invading CAM resembles the native patient-derived tumor on gross visual microscopic observation, indicating successful invasion. In the case of melanoma cells, the spindle-shaped morphology of the surface cells of the CAM-invading tumor is the same as the original tumor xenograft.

Growth of blood vessels and high vascularization is observed over the side and underside of the tumor (Fig. 2 and 3). The cross-sectional histological view of the control sample which lacks the tumor implant reveals a three-layered structure composed of ectoderm, mesoderm, and endoderm (Fig. 2). H/E staining of choroidal melanoma tumor cell mass implanted on CAM further revealed that tumor cells adhere to CAM ectoderm and show prominent invasion to the mesoderm (Fig. 2). The invading cells in the mesoderm were visualized due to the pigmentation in melanoma cells and appear as cleaving pigmented tumor nodules (Fig. 2). H/E staining of Retinoblastoma cells was similar to Choroidal melanoma cells with the bulk of tumor cells adhering to CAM ectoderm and certain cells invading the CAM mesoderm (Fig. 3). The only difference observed was in the degree of invasion which was lesser compared to choroidal melanoma cells.

Immunohistochemical analysis was performed using synaptophysin and Ki-67 antibodies to confirm the identity of invading human Retinoblastoma cells. Invading cells in CAM mesoderm were positive for synaptophysin in the membrane and Ki-67 in the nucleus, thus confirming that invading cells are patient-derived and retain the hallmarks of retinoblastoma cells (Fig. 3). Overall, both Retinoblastoma and Choroidal melanoma PDXs demonstrated a consistent adherence and cell invasion in all the samples.

Discussion

Invasion and metastasis are frequent and often a life-threatening transformation for Retinoblastoma and Choroidal Melanoma patients. The chick chorioallantoic membrane fosters tumor growth and administers an assisting microenvironment with vascularized stromal tissue. CAM assay is a well-established assay that has long been used as an in-vivo environment to study tumor angiogenesis, invasion, and metastasis. Recent experimental findings have demonstrated that the patient-derived xenograft model utilizing CAM assay enables tumor progression and metastasis.

The application of CAM assay to investigate the invasive properties of aggressive ocular tumors are limited. So far most published papers have relied on tumor cell lines instead of fresh patient-derived tumor xenografts. In the study presented here, we observed that both CM and RB fresh tumor tissue, when placed over CAM, resulted in high vascularization in the surrounding region followed by the migration and invasion into the mesoderm PDX in the CAM assay has been reported for many malignancies, including RCC, Sarcoma, pancreatic adenocarcinoma, ovarian carcinoma(Fergelot et al. 2013; Sys et al. 2013; Rovithi et al. 2017; Hu et al. 2019; Ismail et al. 1999). Although any papers utilizing CM and RB tissue grafts have not been reported in CAM to the best of our knowledge.

In this study, we have established successful growth of patient-derived CM and RB tissue and their invasion in CAM. Both the tumor tissue grafts evinced a consistent adherence on the endodermal surface of CAM and cell division in all setups. The invasion was more prominent in CAM carrying CM PDXs. The tumor mass appeared to have invaded the CAM ectoderm and even formed tumor nodules in the CAM mesoderm. In RB PDXs CAM close adherent connection between RB tumor and CAM vasculature was grossly examined. Neural cell marker synaptophysin positivity substantiated the presence of invasive RB cells. The invasion resulting in the destruction of the ectoderm followed by migration and invasion into the mesoderm has also been evidenced in various types of solid tumors such as ovarian carcinoma and nasopharyngeal carcinoma(Ismail et al. 1999; Xiao et al. 2015). The successful relocation of tumor cells in these tumor types was employed to examine the metastatic dissemination of cancer cells in vivo.

Recent work has ascertained the expansion of RB cells arising from the primary tumor on CAM in order to evaluate the effect of drugs. However, the invasive properties of RB cells were not demonstrated. Successful display of metastatic properties of uveal melanoma has been illustrated using GFP labeled, OMM2 & 92.1 cell line on CAM(Kalirai et al. 2015). A recent publication has also utilized CAM assay to explore the metastatic properties of RB cell lines WERI-Rb1, Y-79, and RB 355)(Busch et al. 2018). The use of tumor fragments from patients offers numerous benefits over cell lines. PDX retains morphological similarity and conserves heterogeneity of tumor cell subpopulations. On the other hand, cell lines harbor negligible resemblance and therefore cause vast disparities between preclinical efficacy and clinical responses(Dagogo-Jack and Shaw 2018). Studies exercising laryngeal squamous cell carcinoma have revealed that the CAM invading cells preserve the original tumor characteristics and did not show any signs of necrosis till EDD 17(Uloza et al. 2015).

The histological analysis of the CM-CAM graft uncovered that the tumor nodule within the mesoderm had retained phenotypic characteristics implying that both the implanted tumor tissue and the invasive cells retained the properties of the original tumor. Similarly, the invasive RB cell in CAM also expressed synaptophysin indicating the immunophenotypic character of the RB tumor. The aggressiveness of the RB tumor on the other hand is indicated by the strong Ki-67 immunopositivity.

The successful grafting and migration of CM & RB PDXs in the chick CAM assay make it a robust technique to monitor the invasion of aggressive ocular tumors. Comparison between CAM-invading tumor and original tumor xenograft, and their properties can be made definitive in subsequent research using metrics like gene expression patterns, copy number alterations, and other investigative techniques. Hence, this study shows that the CAM assay can be utilized to analyze fresh CM and RB samples as a xenograft model.

Conclusion

The current study allowed us to successfully envision the establishments of CM and RB PDXs on CAM for the period between EDD 10 and EDD 17, post which, histopathological and immunohistochemical analysis was performed. This analysis revealed high vascularization, adherence of tumor cells to CAM

ectoderm, as well as invasion by tumor cells into the CAM mesoderm, however, the degree of invasion was more in case of CM when compared to RB. These outcomes feature the remarkable potential of CAM assay over conventional models: the chorioallantois is immunodeficient till EDD 13-14 allowing the growth of tumor xenografts without eliciting an immune response, the set-up is time and cost effective, and poses almost no ethical objections. Furthermore, the resemblance of CAM assay to the patient tumor microenvironment, with use of patient-derived tumors, makes it a potential model to develop personalized medicine for patients suffering from ocular cancer.

Abbreviations

CAM: Chorioallantoic membrane

RB: Retinoblastoma

CM: Choroidal Melanoma

PDX: Patient-derived xenograft

EDD: Embryonic development day

H/E: Hematoxylin and Eosin

IHC: Immunohistochemistry

UM: Uveal Melanoma

ECM: Extra-cellular matrix

DPX: Dibutylpthalate polystyrene xylene

DAB: 3,3’-Diaminobenzidine

RCC: Renal Cell Carcinoma

GFP: Green fluorescent protein

Statements And Declarations

Contributions

Concept and design (PJ), methodology and data collection (PJ, NK, SS, HR, PK, SD, PC, and MG), data interpretation (PJ, NK, HR, PK, SD, PC, and MG), data curation (PJ and SS), literature search and writing the article (HR, PK, SD, PC, and MG), images (SD and PC), critical revision of the article (PJ, NK, and SS). All authors reviewed and approved the final manuscript.

Funding

The authors report no grants from any funding agency.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgment

The authors are grateful to Shivaji College, University of Delhi for providing laboratory facilities and Dr. R.P. Centre, All India Institute of Medical Sciences for providing tumor specimens and permission to use instruments for imaging and analytical testing. The authors also extend their sincere appreciation to the Principal of Sri Venkateswara College and the Principal and laboratory staff of Shivaji College.

Funding

The authors report no grants from any funding agency.

DECLARATION OF INTEREST

None

Consent for Publication

Not applicable, as no identifying information about the subjects are disclosed.

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Figures

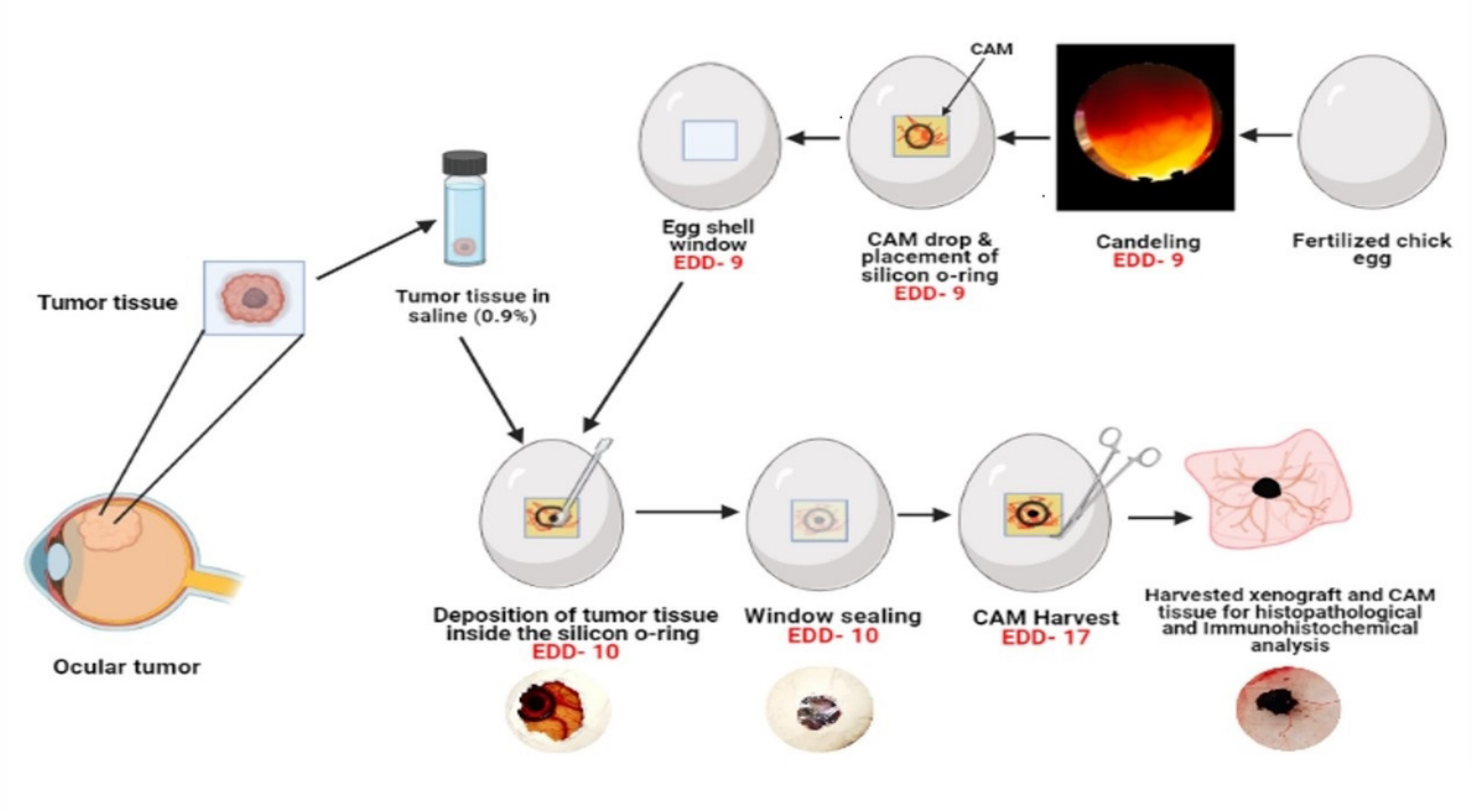

Figure 1

A Schematic representation of the protocol followed to study CM and RB Xenografts on CAM model.

Figure 2

(A) Gross macroscopic photograph of heavily pigmented choroidal melanoma. (B) CM xenograft induced angiogenesis (arrow) on the area of implantation on the chick CAM (bottom view) (C) gross appearance of CM xenograft tumor tissue on top of CAM (top view) with surrounding blood vessels (arrow) (D) Hematoxylin and Eosin stained section of normal CAM revealing three-layered structure composed of ectoderm (ET), mesoderm (M), and endoderm (ED) (H/E stain, × 200). (E) H/E stained section showing the attachment of CM tumor mass on the ectodermal surface of CAM (arrow) at the site of xenograft implantation (× 200). (F) Representative choroidal melanoma tissue sample with spindle shaped tumor cells arranged in fascicles with a moderate amount of cytoplasmic melanin pigment in most of the cells (H/E stain, × 400). (G) Histological section revealing the pigmented choroidal melanoma invasion into the mesoderm of the chick CAM membrane (arrow) (H/E stain, × 200).

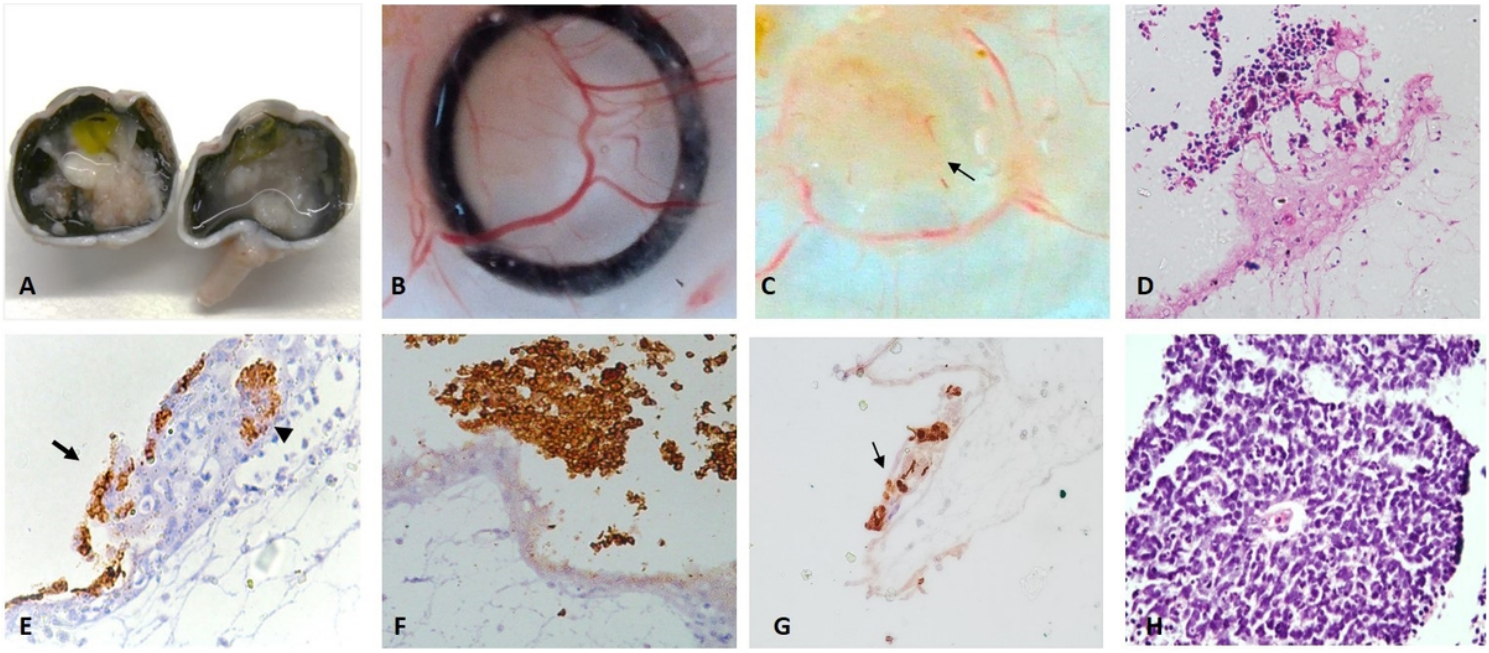

Figure 3

(A) Gross macrophotograph of human retinoblastoma (B) Gross appearance of CAM along with retinoblastoma xenograft showing high vascularity under the tumor implant site (bottom view). (C) Gross appearance of the implanted retinoblastoma xenograft (arrow) on the chick CAM (D) Hematoxylin and eosin stained section showing retinoblastoma cells adhered to the ectodermal surface of CAM at the site of tumor implantation (H/E stain, × 200). (E) Anti-human synaptophysin positivity revealing human retinoblastoma cells attached to the ectoderm of the CAM (arrow) and invading into the CAM mesoderm (arrowhead) (× 200). (F) Synaptophysin positivity in retinoblastoma xenograft on the surface of CAM (IHC, × 200). (G) Positive Ki-67 staining in retinoblastoma PDX (arrow) on CAM epithelium (IHC, × 200). (H) Hematoxylin and eosin stained section of poorly differentiated retinoblastoma sample (H/E stain, × 400). ET = Ectoderm. M = mesoderm. ED = endoderm.