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Review Article

The chick chorioallantoic membrane (CAM) as a versatile patient-derived xenograft (PDX) platform for precision medicine and preclinical research

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Abstract: Patient-derived xenografts (PDX) are an increasingly valuable tool in oncology, providing biologically faithful models of many different cancer types, and potential platforms for the development of precision oncology approaches. However, PDX have primarily been established in immunodeficient rodent models, with accompanying cost and efficiency constraints that pose barriers to more widespread adoption. The chicken egg chorioallantoic membrane (CAM) is an alternative *in vivo* PDX model. We provide here a comprehensive review of studies that grafted primary human tissue, as opposed to cell lines, onto the CAM. Twenty publications met our criteria of having inoculated patient-derived tumor tissue onto the CAM. Successful engraftment has been reported for over a dozen tumor subtypes, supporting the appropriateness of the CAM as a PDX platform. Resemblance of xenografts to the original patient tumor, increased vascularity of the CAM following engraftment, and micrometastasis into the chick mesenchyme were frequently reported. Application of standard or experimental cancer therapies to xenografts has also been undertaken, with the discovery of both synergistic drug effects and positive associations between the assay and clinical outcome. The CAM provides opportunities for RNA and DNA based sequencing of patient tumors, and the ability to efficiently (in 5-10 days) test multiple targeted therapies on fragments derived from the same tumor. While routine use of the CAM-based PDX model would benefit from a more-complete understanding of the stromal environment of CAM xenografts and interaction with the developing avian immune system, current literature supports the model's potential as an efficient, scalable precision medicine platform.

Keywords: Chick chorioallantoic membrane, patient-derived xenograft models, precision medicine, cancer drug development, angiogenesis, metastasis, preclinical models, preclinical assays, *in vivo* models

Introduction

'Precision medicine' in oncology has the goal of reducing morbidity and mortality by selecting subsets of patients that are most likely respond to treatment regimens [1]. Patient-derived xenografts (PDX), which retain key histologic and genetic characteristics of their donor tumor, are increasingly used in translational cancer research for this purpose [2]. The PDX platform is potentially useful for characterizing the pathophysiology, molecular features, and drug responsiveness of an individual tumor before the patient is subjected to therapy. While studies incorporating PDX models have traditionally favored the use of immunodeficient rodents, the chick chorioallantoic membrane (CAM) assay is complementary to this method and even presents unique advantages in some areas. In this review, we describe advantages and limitations of rodent-versus CAM-based PDX models, efforts to engraft patient-derived tumor tissue onto the CAM, and current and potential future applications of this method. This review does not focus on the extensive history of cell-line-based work in CAM models, which despite limitations as a "complete" cancer model, has still contributed enormously to preclinical research and cancer biology [3-5].

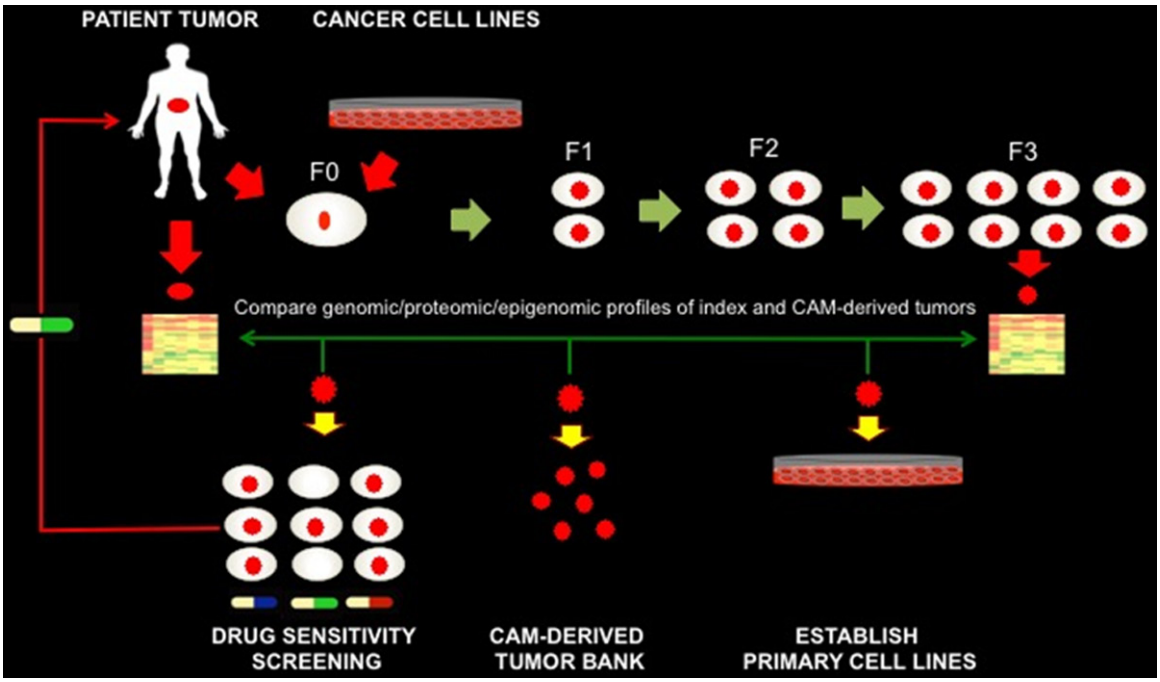

Figure 1. Potential utility of CAM-based PDX models in oncology. RNA and DNA based sequencing of patient tumors can provide a number of potentially relevant mutations and targets that may be prioritized following results of the assay. The CAM-based model allows the ability to efficiently (in 5-10 days) test multiple targeted therapies on tumor fragments from a given patient, with the goal of successfully predicting the sensitivities of an individual patient's cancer to specific treatments.

Practical benefits of patient-derived xenografts in oncology

Preclinical research and drug development have historically benefited from the use of cancer cell lines (CCL) [6]. While the propagation of these immortalized cell populations in simple media is straightforward and thus invaluable for experimentation, they also possess inherent shortcomings that result in limited translation of findings into patient benefit [6, 7]. CCLs undergo new mutations during adaptation to growth in culture and generally fail to retain the morphology, cellular heterogeneity, and molecular profiles of the original donor tissue [1, 8]. Furthermore, positive drug performance in xenografts from CCLs is not typically highly predictive of clinical efficacy [9]. The use of freshly tissue for xenografting more closely captures patient features by maintaining heterogeneity and pathophysiology of the original tumor [10, 11]. PDX models are thus particularly useful in assessing therapies for cancers driven by rare populations of highly aggressive cells or those with a high variance in molecular alterations between patients [12, 13]. The success of new drugs in oncology requires preclinical models that capture the heterogeneity and pathophysiology of patient tumors; for this reason, PDX models are thought to more accurately mimic drug effects in humans in comparison to CCL models [11, 14]. In fact, patient-derived xenografts in rodents have been used to establish most of the current models in pediatric oncology and have been useful for developing drugs like topotecan and irinotecan for solid tumors [11]. In studying the genetics of acquired resistance, use of PDX models has the advantage that discovered alterations in tumors actually occurred clinically (as opposed to developing resistance models from cell lines) [15]. Personalized study through PDX models ultimately has the potential to accelerate the development of new therapeutic compounds in oncology once promising candidates advance beyond preclinical testing [16].

In addition to accurately representing tumor biology, PDX models also have the theoretical advantage of efficiency (**Figure 1**). They can feasibly allow preclinical trials (also called "PDX clinical trials") in which a panel of tumors (derived from a single patient) is assigned to individual arms of a study for direct comparison

of treatment strategies [1]. PDX models are also useful for patients who are ineligible for clinical trials due to deteriorating health or other disqualifiers [16]. There are, however, some theoretical limitations of the PDX platform that need further assessment. Non-ideal tissue selection could conceivably be a limiting factor, based on the untested hypothesis that not all tumor foci are equally lethal [17]. Representation of metastasis has also been a challenge for certain malignancies [18]. In a recent study of 1,110 PDX samples across 24 cancer types, copy number alterations (CNAs) obtained during PDX passaging were found to differ from those acquired during tumor progression in patients, raising the question of whether genomic alterations may negatively impact modeling [19]. Finally, intratumoral heterogeneity has led to a lack of concordance among several models of pancreatic cancer [20]. These observations highlight the challenges of utilizing PDX as a preclinical discovery model for cancer therapeutics.

Predominance of rodent models in the PDX platform

Rodent PDX models are a mainstream modality in clinical oncology, and their numerous applications have been described recently [11, 16]. Since emerging in the 1980s, their utility has spanned prediction of responses and resistance to both first-line and experimental drugs, development of biomarkers, and identification of effective treatment regimens [6, 21]. Concurrently, enhanced understanding of the human and mouse genomes has facilitated appropriate genetic manipulation of mice and expedited the PDX model's effectiveness [22]. Recent studies have revealed greater activity of experimental drugs compared to standard ones and even guided optimal, nonobvious treatment choices in a small cohort of patients with advanced cancer [17, 23]. Several rodent PDX models have been established for colorectal cancer [11], in addition to models recapitulating of human metastatic sites in orthotopic transplant models of breast cancer [1]; models representing of the entire spectrum of ovarian cancer [24]; and models which have contributed to the identification of potential cellular targets prior to onset of incurable disease in prostate cancer [17]. Additionally, decades of murine research have fueled commercialization of rodent PDX models, with over a dozen companies offering a range of services [16]. Rodent models currently occupy the lion's share of scientific resources devoted to investigation on the PDX platform.

Despite their utility, rodent models have a number of practical and scientific limitations. Prohibitive cost and immense resources are required to maintain what are essentially 'live tumor banks'. The use of live mammals itself is labor-intensive, time-consuming, and raises ethical issues for some [6]. PDX mouse avatars proposed as a platform for precision medicine are not covered by health insurance [11, 16]. Another practical detriment to the efficiency of rodent PDX models is their low take rate (the fraction in which successful engraftment occurs). Certain malignancies (e.g. breast, melanoma, head and neck squamous cell carcinoma) have take rates consistently under 50% even with the use of the most severely immunosuppressed mice, and others (e.g. nasopharyngeal carcinoma) are nearly impossible to become established in mice [11, 25, 26]. Successful engraftment typically requires up to 4 months, and engraftment failure often cannot be determined until 4-6 months post-implantation [27]. Another scientific limitation is the potential for inaccurate representation of patient characteristics due to the gradual replacement of human stromal components in the tumor microenvironment with murine stroma [14]. This could impact determination of prognosis, identification of appropriate cellular and molecular targets, and drug-responsiveness. Increased occurrence of various niche changes may relate to both initial propagation in the mouse and cumulative changes during continued passage; collectively, these have the consequence that the initial tumor biopsy may significantly differ from the state of the patient's tumor at the time that treatment is initiated based on murine data [16]. It is difficult to study immunologic drugs in the immunodeficient mouse strains used in most PDX models without first undergoing an elaborate and resource-intensive process of "humanization" to establish an immune system [11]. Finally, drug metabolism differs between humans and rodents, and satisfactory modeling of clinically relevant dose (CRD) levels of standard agents is challenging [14, 25].

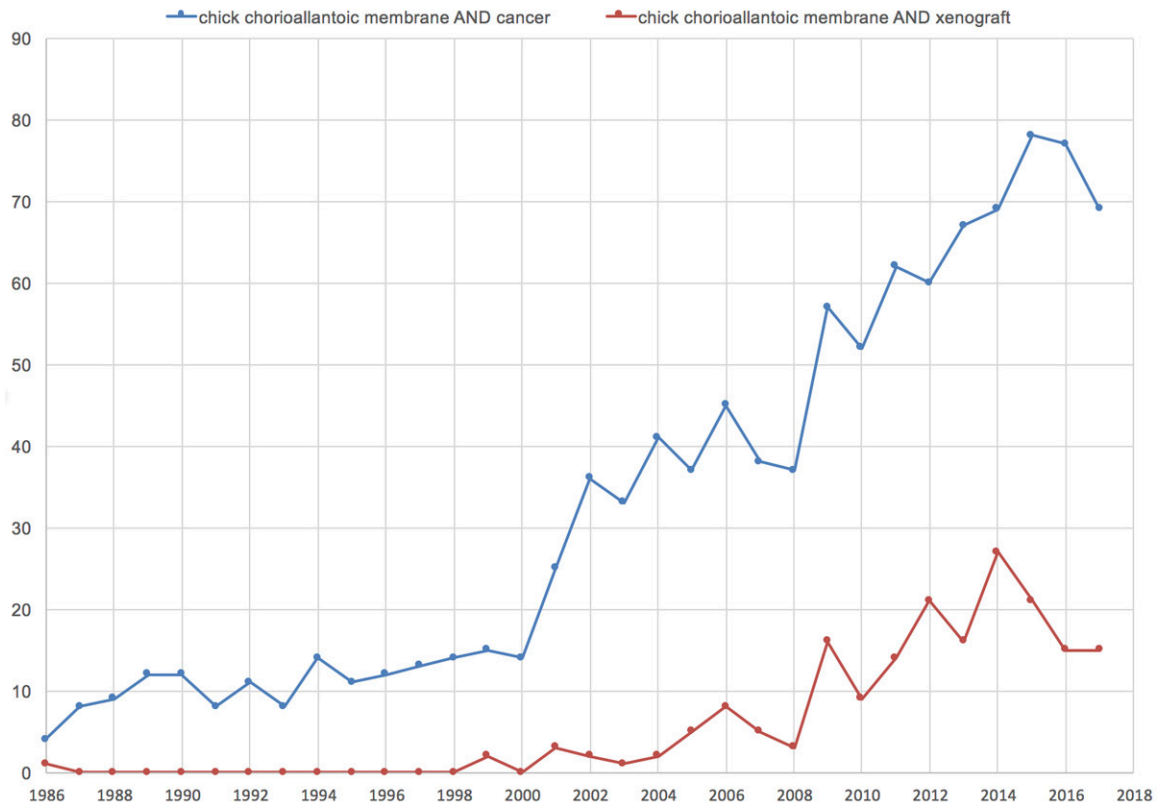

Figure 2. Number of PubMed results for search terms related to application of the CAM in oncology, by year.

The chick chorioallantoic membrane (CAM): an established assay

A well-established *in vivo* assay, the chick chorioallantoic membrane (CAM) model has contributed to fundamental scientific discoveries including the link between oncogenes and the formation of cancer [28]. The CAM is a highly vascularized extra-embryonic membrane connected to the embryo through a continuous circulatory system, easily accessible for experimental manipulation such as intravenous injection of compounds and direct visualization of local responses [29]. While T cells and B cells can be detected in the developing chick immune system by Day 11 and Day 12 of embryonic development, respectively, the embryo is not fully immunocompetent until Day 18 [30]. This feature makes it ideal for grafting of foreign tissue prior to immune competence. The CAM has historically been a favored system for the study of angiogenesis [31]; Folkman's experiments revealed that a tumor requires a newly formed vasculature, and the development of drugs to inhibit angiogenesis has since become an attractive approach to cancer therapy [32, 33]. The CAM is a low-cost model that allows simultaneous screening of large numbers of pharmacologic agents, with one limitation being the potential for development of nonspecific inflammatory response after 15 days of incubation [34]. Growth of cancer cell lines on the CAM is well-established and has been used regularly in pre-clinical screenings to assess the efficacy of anticancer drugs on tumor growth [35]. The CAM assay is significantly faster than most mammalian models, as tumor grafts become vascularized by chick vessels within 2-5 days following inoculation [36]. Techniques which have been used to visualize or detect tumor cells in the CAM assay include: *in vivo* videomicroscopy, detection of human urokinase plasminogen activator, GFP-labeled cells, PCR amplification of human sequences, viral nanoparticles, PET/CT imaging, and immunohistochemistry [30, 37]. The past decades have seen increasing interest in the CAM as a model to study neoplastic growth and the maintenance of foreign tissue (**Figure 2**).

Currently, the typical neoplastic cell assay involves lowering ("dropping") the membrane

by forming an air pocket between the separated shell membrane and the CAM itself. Tumor cells are then grafted as an inoculum introduced through a small window made in the shell above the CAM [37]. The ideal time for inoculation has been postulated to be day 9 of embryonic development, and the ideal time for harvest to be day 16 (7 days after inoculation) [38]. Another paradigm is to simply harvest the day before tumor rejection would likely occur at day 18 [39]. While the in ovo method has historically been more popular, ex ovo methods are also possible via controlled extrusion of the egg content, a modification that is proposed to enable easy *in vivo* documentation of effects and increased embryonic survival rates from ca. 30% to over 50% [40]. Ribatti reviewed both methods and found that the advantages of in ovo experimentation include high survival rate, reflection of physiological conditions and the ability to reach hatching, and easy methodology, while the advantages of ex ovo include a larger CAM area available for testing, direct visualization of the entire structure, and evaluation of several samples in a single embryo [41].

Focus of studies grafting fresh human tumor tissue on the CAM angiogenesis

Existing literature involving the engraftment of fresh human tumor tissue onto the CAM has highlighted the study of angiogenesis. Klagsbrun *et al.* cultured glioblastoma and meningioma cells (including both cell lines and fresh samples obtained by trypsinization of brain tumors), and tested the ability of their supernatant solutions to produce a hypothetical Tumor Angiogenesis Factor (TAF) [42]. At this time, few investigators were working on tumor angiogenesis, and virtually no companies were interested in developing anti-angiogenic drugs [43]. While all of the tumor-derived cells produced CAM vascularization, the fresh tissue samples exerted the most potent effect. The authors concluded that a correlation exists between the vascularity of a tumor *in vivo* and the potency of TAF *in vitro*, setting a precedent for a highly qualitative method of reporting angiogenic changes on the CAM model [42]. The first identification of a specific TAF molecule did not occur until 1984, when basic fibroblast growth factor isolated from a chondrosarcoma was found to be mitogenic for capillary endothelial cells [44].

Mostafa *et al.* induced vascularization with neoplastic lymphoid cells, both from cell lines and fresh biopsies, on the CAM. Angiogenic activity was dose-dependent on cell volume, and a previously unknown relationship between host monocyte chemotaxis and generation of vascularization was observed [45]. This was one initial recognition of the role played by the developing avian immune system in the CAM assay. Later, Balciuniene *et al.* reported both enlargement and increase in vascularization in the area of the CAM directly under the transplant. This phenomenon was theorized to be a common response to neoplastic transplants, and not indicative of a more complex interaction such as anastomosis between host vessels and the existing vascular network of the graft [46]. Thickening of the mesenchyme and increased vascularization under the implant were previously attributed to both growth factors from the implanted tumor and the nonspecific inflammatory reaction of the CAM [47, 48]. Uloza *et al.* reported that laryngeal squamous cell carcinoma (LSCC) implants induced both thickening of the CAM (mean increase of 401%), and increase in CAM vascularization (higher mean number of blood vessels per constant length of the membrane) versus the control group [49].

Petruzzelli *et al.* studied vascularization on the CAM stimulated by head and neck squamous cell carcinoma [50]. An important contribution from this study was a unique, subjective method of quantifying angiogenesis using blinded evaluators, as per Vu *et al.* [51]. Survival rates of tumor explants did not appear to be related to mean angiogenesis scores of individual tumors, suggesting that take rates are not significantly enhanced by a tumor's ability to stimulate new blood vessels [50].

Marzullo *et al.* modified a morphometric method of 'point counting', originally devised by Elias and Hyde, and reported a highly quantitative measurement of angiogenic response to tumor grafts on the CAM [52, 53]. Ribatti *et al.* subjected biopsies of neuroblastoma and endometrial adenocarcinoma to fenretinide treatment on the CAM and computed vascular response with a previously-developed planimetric method of point counting, which involved analyzing serial sections from a specimen through a fine mesh inserted into the eyepiece of the microscope [54, 55]. Their second method of quanti-

fying angiogenic response involved evaluating expression of VEGF and FGF-2 receptors and highlighting endothelial cells (and thus blood vessel formation) with an anti-factor VIII polyclonal antibody [54]. Staining intensity of receptor immunoreactivity was graded on a scale of 0 to 3 that represented a modification of Takahashi *et al.* [56]. This represented the most nuanced attempt yet to measure angiogenic response.

Sys *et al.* scored a total of 77 CAMs for macroscopic angiogenesis towards xenografts of sarcoma, using a scale of 0-2 originally developed by Knighton [38, 57]. Biotinylated *Sambucus nigra* (SNA) bark lectin, which binds exclusively to chick endothelium and causes avian blood vessels to appear brown, is another effective method [58, 59]. Visualization techniques such as these enable more accurate quantification of angiogenesis, independent of the specific counting method. A recent technique has perfused vessels penetrating breast CCL tumor grafts with certain polymers and then tracked blood flow with microCT scans; this method has the advantage of confirming the functionality of vessels while also approximating the average vessel diameter and total vascular density within the graft [60].

As an easily-visualized model, the CAM has facilitated highly reproducible studies of aggressive malignancies such as glioblastoma and pancreatic adenocarcinoma [61, 62]. While over half of experimenters attempted to quantify angiogenesis in response to fresh tumor material (**Table 1**), it remains to be seen whether angiogenic response alone may have translational significance with regards to tumor behavior in the patient. With regards to investigations of anti-angiogenic drugs, quantification of the primary response can be difficult due to a secondary vasoproliferative response from nonspecific inflammatory reactions following grafting [30]. This highlights the need to develop both an enhanced understanding of the chick immune system's role and an agreed-upon method of quantifying angiogenesis.

Concordance with parent tumor

Shortly after discovery that the CAM could support foreign tissues, Stevenson attempted the first engraftment of fresh human tumor onto the medium in 1918. No growth or mitotic figures were observed following the inoculation of tissue from 8 tumors [63]. Hurst *et al.* grafted 17 human tumors onto both duck and chicken eggs, with sectioning revealing that neoplastic cells were able to survive and multiply on the CAM [64]. Although a comparatively high proportion of healthy graft (take rate of 53% for surviving embryos) was reported for its time period, tumor take was said to have been achieved merely if some of the cellular elements were preserved, even if a majority of the graft had undergone necrosis by the time of sectioning. Hurst *et al.* noted that while cells typically appeared as healthy as those in the parent tumor, there was no growth approximating that in previous experiments with other mammalian tumors [64]. These initial experiments underscore the difference between describing subjective similarity with the histologic appearance of the parent tumor, and establishing true concordance via molecular markers or genetic profiling techniques available in the modern era.

Earlier studies typically included a brief mention of histologic similarity to the original tumor, even if primarily focused on other parameters such as drug sensitivity or angiogenic change [50, 65]. The first study exclusively focused on establishing concordance was performed by Balciuniene *et al.*, who transplanted fresh glioblastoma samples and sectioned specimens at 24-hour time intervals up to 7 days post-engraftment. This allowed the researchers to continually monitor interaction between the membrane and neoplastic tissue [46]. The glioblastoma fragments survived with all cytologic features intact; however, expression of intermediate filaments diminished alongside tumor growth, and Ki67 (a strong marker for proliferation) was found in only a few transplants. Transplanted tumors only survived up to 6 days and were limited by drying of the nourishing membrane, with more numerous necrotic zones in the graft as incubation progressed [46]. The finding of nutritional limitation was a significant departure from the expectation of most CAM experiments that tumor proceeds to grow until the chick immune system became mature [66].

Balke *et al.* demonstrated the first *in vivo* model for giant cell tumor of bone (GCT), for which attempts had previously failed. Although tumor samples cultured on the CAM displayed compo-

Table 1. Outcomes of studies that grafted fresh human neoplastic tissue onto the CAM

	Tumor Tissue	Inoculation Technique	Embryo Survival; Tumor Take Rate	Concordance with Patient Histopathology	Quantification of Angiogenesis	Micro-metastasis	Therapeutic Treatment of Xenograft
Stevenson (1918)	Carcinomas of breast, squamous cells, testicle, and trophoblasts	Doses of 0.003 g	35%; ~0%	Cells resembling those of parent tumor in a few sections, with multinucleation	Not performed	N/A	N/A
Hurst *et al.* (1939)	Carcinomas of breast and stomach, melanoma, ovarian cystoma	0.1-0.2 cc of minced tumor in Tyrode's solution	51/99 (52%); 27/51 (53%)	Confirmed with microscopy; frequent mitotic cells	Not performed	N/A	N/A
Sommers *et al.* (1952)	Miscellaneous tumors	1 cc injections of minced tumor in a penicillin solution	327/620 (53%); 74/327 (23%)	Degenerative changes much more frequent than mitosis	Not performed	N/A	N/A
Kaufman *et al.* (1956)	Melanoma; glioma; schwannoma; thyroid, breast, lung, kidney, bladder carcinoma; sarcoma	Fragments of 1-3 mm^2	Unknown; 147/295 (50%) for primary PDX	Generally well-preserved histologic characteristics	Not performed	N/A	N/A
Mostafa *et al.* (1980)	Lymphoma (Hodgkin's and non-Hodgkin's), Glioma, Lymph node metastases	Fragments of 1 mm^3 (up to 4 per egg), directly on the CAM or on millipore paper	Poor growth in general	Unknown	Considered strong if many new dilated and tortuous vessels present	No; cell survival and mitotic activity in only a few instances	N/A
Shoin *et al.* (1991)	Glioma (including GBM)	Minced 100 µl fragments	57/57 (100%); 57/57 (100%)	Yes	Not performed	Not assessed; more malignant tumors grew more quickly	ACNU and MCNU injections into CAM veins
Petruzzelli *et al.* (1993)	Head and neck squamous cell carcinoma (HNSCC)	Minced 100 mg fragments	44/80 (55%); 30/44 (68%)	Yes (cellular pleomorphism, mitoses, and keratinization)	Semi-quantitative grading on a scale of 0 to 4 by blinded evaluators	Yes; 'pushing borders' of xenografts onto the mesenchyme of the CAM	N/A
Marzullo *et al.* (1993)	Hepatocellular carcinoma (HCC)	Fragments of 1-2 mm^3	Unknown	Unknown	Morphometric method of 'point counting'	No; viable cells either adhered to the chorion or were enclosed within the mesenchyme	N/A
Ismail *et al.* (1999)	Ovarian adenocarcinoma	Fragments of diameter 3-8 mm and thickness 2-4 mm inoculated into a silicon ring	Some eggs excluded due to embryo mortality	Unknown	Qualitative observation of pattern, density, and size of blood vessels	Not assessed	Photodynamic therapy (PDT) with methylene blue (MB) formulations as a photosensitizer
Ribatti *et al.* (2001)	Neuroblastoma (NB) and endometrial adenocarcinoma	Fragments of 1-2 mm^3	Unknown	Unknown	Planimetric method of 'point counting'	Not assessed	Fenretinide pipetted directly onto the xenograft
Marimpietri *et al.* (2005)	Neuroblastoma (NB)	Fragments of 1-2 mm^3	Unknown	Unknown	Planimetric method of 'point counting'	Not assessed	Vinblastine and rapamycin admixed with tumor fragments, solo or combined
Balciuniene *et al.* (2009)	Glioblastoma (GBM)	Fragments of unknown size	178/200 (89%); 158/200 (79%)	Yes (GFAP, vimentin, and Ki67 protein intact)	Not performed	No; tumors survived as 'histologically isolated' units with no invasion by avian cells	N/A

Balke *et al.* (2011)	Giant cell tumor of bone (GCT)	Homogenized cells suspended in medium and inoculated into plastic rings	56/125 (45%); 60/69 (87%)	Somewhat (fewer giant cells and less mononuclear components)	Not performed	No; tumors appeared to grow on the CAM in an implant-like pattern rather than invading it	N/A
Sys *et al.* (2012)	Musculoskeletal sarcomas	Fragments of 1-3 mm in diameter	168/210 (80%); unknown	Yes, with chicken stroma largely replacing human stroma	Density and length of vessels towards the xenograft scored from 0 to 2	Not assessed	N/A
Fergelot *et al.* (2013)	Clear cell renal cell carcinoma (CCRCC)	Fragments of 2×2×3 mm	6/6 (100%); 6/6 (100%)	Yes (cell nests and fibrous axes, epithelial staining, and human stroma maintained)	Penetrating chick vasculature seen with India ink and confocal microscopy	Not assessed	N/A
Sys *et al.* (2013)	Sarcoma	Fragments of maximum 1 mm^3	Up to 75%; unknown	Yes (essential features and immunohistochemical characteristics)	Not performed	Yes; infiltration of the CAM mesenchyme by tumor cells and graft by chick fibroblasts	N/A
Xiao *et al.* (2015)	Nasopharyngeal carcinoma (NPC)	Fragments of 1.5×1.5 mm inoculated into a silicon ring	Unknown; 35/35 (100%)	Yes (morphology and poor differentiation with EBV genome intact)	Dividing area of blood vascularization by the total area of the CAM	Yes; intravasated tumor cells seen with confocal microscopy and qPCR amplification in organs	N/A
Uloza *et al.* (2015)	Laryngeal squamous cell carcinoma (LSCC)	Fragments of 8 mm^3	Unknown	Yes (retained original characteristics and expressed 3 markers of LSCC)	Mean number and area of blood vessels per constant length of the CAM	Yes; 2 of the 6 xenografts invaded the chorionic epithelium of the CAM	N/A
Ferician *et al.* (2015)	Renal cell carcinoma (RCC)	Fragments of 2×2 mm (tumor tissue and stroma) inoculated into a silicon ring	20/20 (100%); 20/20 (100%)	Yes (human-specific endothelial markers), with human stroma maintained	Viability of intratumoral blood vessels verified by presence of chick erythrocytes	Not assessed	Endostatin pipetted directly onto the xenograft
Uloza *et al.* (2017)	Recurrent respiratory papilloma (RRP)	Fragments of at least 0.5×0.5×0.5 cm	127/174 (73%); unknown	Yes (essential features and staining for cytokeratin, Ki67, and PCNA)	Number of SNA-stained vessels per constant length of section within "sight fields"	Yes; papilloma sprouts onto the chorionic epithelium	N/A

nents of GCT, giant cells were less numerous and contained fewer nuclei than in the original tumors [67]. While this may question the ability to culture a truly representative giant cell tumor on the CAM, the successful generation of PDXs on the model highlights its utility in the study of rare tumors that currently suffer from lack of suitable models. A recent novel model to study bone metastases in prostate cancer found that, on the CAM, metastatic cells preferentially colonized bovine trabecular bone xenografts that were artificially coated with the extracellular matrix protein tenascin-C, which is known to be deposited in the reactive stroma response [68]. Recapitulating exact features of a parent tumor may not always be an essential part of the assay, so long as the method can reliably replicate key biological processes in the patient that may have value as a therapeutic target.

Sys *et al.* evaluated whether xenografted tumor tissue of musculoskeletal origin could retain hallmarks of the original tumors. Diverse tumor fragments were grafted on the CAM and each retained the original tumor morphology; however, tumor-associated stroma from the human samples was largely replaced by chicken-derived stroma in the grafts [39]. This was the first reported instance of grafting human stroma alongside tumor tissue, a possible strategy for replicating original tumor microenvironment in PDX models [16]. The replacement of human stroma suggests that human stromal supplementation may be critical for truly representative models. In comparing tumor types, metastatic types were significantly more viable, infiltrative, and less necrotic than the benign samples, the primary malignant samples treated with chemotherapy, and the primary malignant samples not treated with chemotherapy. Viability was also significantly associated with the patient's disease progression [39]. These results demonstrated the model's ability to assess differences between tumor subtypes, and even between treated and untreated tumors with chemotherapy. While this relationship can't be assumed for every cancer type, it highlights the possible prognostic use of the model as an *in vivo* tool to assess tumor aggressiveness. This would be particularly beneficial in determining when a particular patient's cancer might require more aggressive therapy than initially thought.

Fergelot *et al.* validated the CAM as a model for the clear cell subtype of renal cell carcinoma and its interactions with the surrounding stroma. Penetration of chick vasculature into the grafts was visible 4 days after implantation via injection of India ink into a CAM vessel [58]. Formerly, penetration of avian vessels was either identified by IHC of chick erythrocytes as a marker in human vessels or not consistently observed (**Table 1**). Vessel phenotype was characterized by confocal microscopy, detecting human vessels with antibodies for human CD31 and CD34 and chicken vessels by the SNA lectin stain. The authors concluded that tumor vessels were maintained, anastomosed to host vessels, and then perfused [58]. This was the most detailed indication so far of a hybrid vessel formation between host and human capillaries in the CAM-based PDX model. Uloza *et al.* similarly injected the CAM's vessels with fluoresceinated anionic dextran and used biomicroscopy to confirm vascularization of the xenograft *in vivo* [59].

The study of nasopharyngeal carcinoma (NPC) has been hampered by a lack of suitable *in vivo* models. Furthermore, while NPC is strongly associated with Epstein-Barr virus (EBV), most of the cell lines that have been developed were rendered EBV-negative in prolonged culture, and thus have unknown relevance [69]. Several PDX lines that retain morphology and harbor the EBV genome have been developed; however, transplantation of primary tumor tissue into nude mice is not efficacious due to rapid replacement by murine stroma [70]. Xiao *et al.* established an *in vivo* CAM model from a total of 35 NPC primary tumor biopsies. Transplanted tumors retained morphology and poor histologic differentiation; notably, the EBV genome was also maintained [26]. This was the first description of the successful preservation of a cancer-associated virus in PDX culture on the CAM, highlighting the potential use of this assay to study viral-associated tumor biology with relatively simple visualization and verification. Future studies on the CAM-based PDX model could incorporate tissues harboring other cancer-associated viruses that collectively contribute to 10-15% of cancers worldwide, including human papillomavirus (HPV), hepatitis B and C viruses, human T-cell lymphotrophic virus (HTLV-1), and Kaposi sarcoma-associated herpes virus (HHV-8) [71].

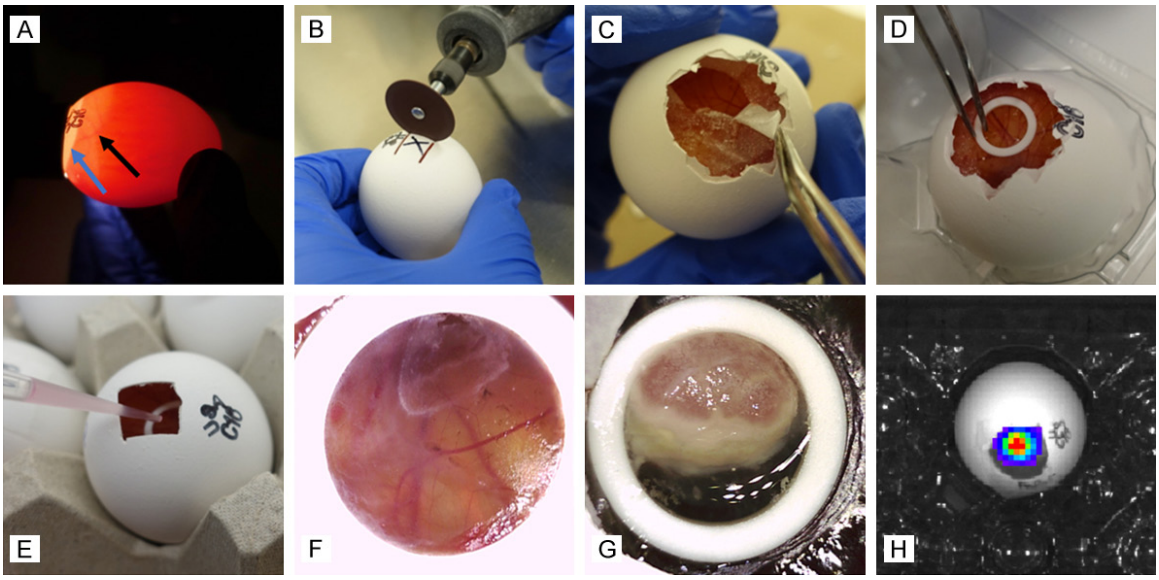

Figure 3. CAM Assay. A. Identification of the vascularization (black arrow) and the air sac (blue arrow) using the Egg Candler. B. Opening a window in the shell using a rotary tool. C. Removing the outer membrane from the CAM. D. Silicone ring being placed on top of the CAM. E. Cell line, FaDu, seeded into silicone ring. F. Day 1 of growth, FaDu. G. Day 5 of growth, FaDu. H. IVIS image of FaDu.

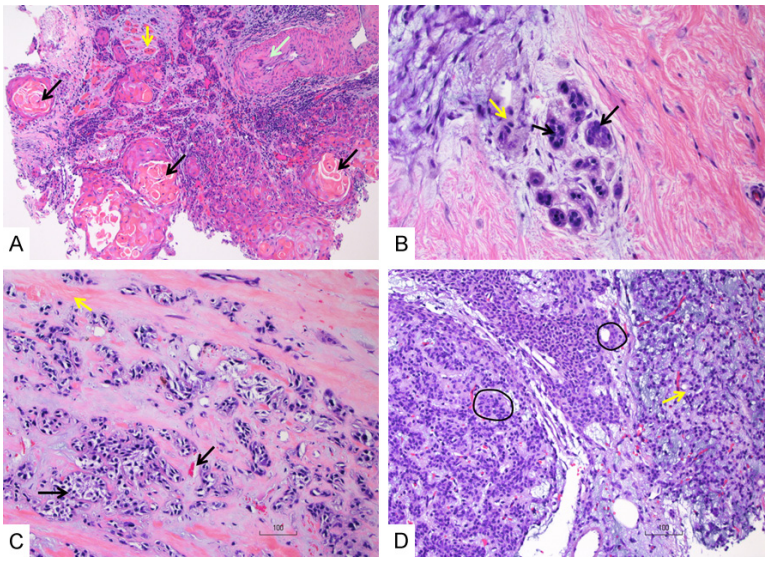

Figure 4. *In vivo* histologic appearance of experimental PDX on CAM. A. Oral squamous cell carcinoma with keratin pearls (black arrows), multinucleated giant cell reaction at the tumor-CAM interface (yellow arrow), and a large vessel (white arrow). B. Papillary thyroid carcinoma with enlarged, elongated, and overlapping nuclei of cells contained within discrete follicles (black arrows) and scattered, infiltrating neoplastic cells (yellow arrow). C. Medullary thyroid carcinoma with nests of hyperchromatic polygonal tumor cells separated by fibrovascular stroma (black arrows) and amorphous, eosinophilic amyloid deposition extracellularly. D. Pleiomorphic adenoma of the parotid with myoepithelial proliferation (black circle) and gland formation in a chondromyxoid background on the CAM (yellow arrow).

Our laboratory routinely cultures both cancer cell lines and primary patient-derived tumors on CAM (**Figure 3**), and our unpublished data closely recapitulates histologic hallmarks of four different types of head and neck cancer (**Figure 4**). The study of head and neck cancers on xenograft models has been constrained by low take rates and availability; for example, slow tumor growth and engraftment rates of medullary thyroid cancer (MTC) present serious challenges to the development of murine PDX models [72]. After obtaining informed consent, our own laboratory has successfully established patient-derived xenografts from frozen sections of various head and neck cancers, including MTC, with take rates of 70-80%. Our unpublished data closely recapitulates histologic hallmarks of four different types of head and neck cancer (**Figure 4**). Each of the stained sections in the panel has been scored by a pathologist and found to represent maintenance of the parent tumor on the CAM.

Establishing concordance between patient derived xenografts and original patient tumors is a gold standard for most models. As existing cell lines have failed to simulate tumorigenic phenotypes for laryngeal squamous cell carcinoma (LSCC), a CAM-based PDX model was recently developed. Uloza *et al.* grafted tumor tissue onto 120 CAMs and harvested 15 specimens, with Ki67 staining indicating generally well-preserved proliferative capacity and all grafts retaining characteristics and appropriate markers of the original tumor. Notable was an observed increase in LSCC angiogenesis, which parallels a feature of LSCC that may be positively correlated with lethal outcome [49]. The CAM-based PDX model is likely a suitable platform for further investigation of this subtype of HNSCC.

Micrometastasis

An oft-cited limitation of the CAM-based PDX model is that tumor cells cannot produce macroscopically visible colonies in secondary organs due to the short timeframe of the assay, which forces the detection of 'micrometastasis' into local areas in order to assess metastatic potential [73]. Mostafa *et al.* assessed tumor growth and expansion in the CAM-based PDX model; tumor implants grew poorly in general, with evidence of cell survival and mitotic activity in only a few instances [45]. Petruzzelli *et al.* first visualized metastatic potential, in head and neck squamous cell carcinoma (HNSCC), on the CAM [50]. Morphologically, its basement membrane simulates that of the oral mucosa and makes it well-suited for studying invasion in epithelial cancers [74]. This was an important milestone, as micrometastasis is a potential indicator of aggressiveness for parent tumors of PDX grown on the CAM. For at least some human cancers, engraftment onto the CAM seems to allow genuine expansion rather than mere survival.

Subsequent studies found less-than-convincing examples of micrometastasis. Marzullo *et al.* found that viable tumor cells either adhered to the chorion without invading the mesenchyme or were enclosed within the mesenchyme [53], in contrast to the previous demonstration of 'pushing borders' by HNSCC on the CAM mesenchyme [50]. Balciuniene *et al*. also demonstrated poor progression for glioblastoma xenografts on the CAM. The use of nucleated chicken erythrocytes as a marker, hematoxylin and eosin (H&E) stain, and IHC revealed that the tumors survived as isolated units, with no invasion by either cells or vessels into the mesenchyme or graft, and the tumor cells likely survived only by diffusion of oxygen and nutrients from the CAM [46]. Micrometastasis was also not visualized in the *in vivo* model for GCT, in which tumors appeared to grow on the membrane in a flat pattern rather than invade. Ki67 staining revealed a significantly lower proliferative fraction (<1% of cells) than in the original tumor, which was attributed in part to the short 6-day time span of tumor growth [67]. While simulating the early phase of tumor seeding has practical utility, it may also be advantageous to use serial passaging methods on the CAM-based PDX model to assess whether proliferation rate increases for subsequent generations [75].

Despite early difficulties, micrometastasis was demonstrated in 4 of the 5 most recent studies on the CAM-based PDX model (**Table 1**). Sys *et al.* observed revascularization of sarcoma grafts and infiltration of both the CAM mesenchyme (by tumor cells) and the graft itself (by chick fibroblasts) [38]. Xiao *et al.* quantified micrometastasis of an NPC cell line on the CAM by making observations with confocal microscopy 48 hours after inoculation. Their technique involved visualizing fluorescently labeled intravasated tumor cells within large vessels and then assessing invasion depth of micro-tumors in a 3D pattern; qPCR amplification of the human beta globin gene from frozen heart and lung tissues was also performed in order to quantify micrometastasis to distant organs [26]. Daily and noninvasive quantitative monitoring of microscopic spread of engineered tumors from prostate cancer and osteosarcoma cell lines has also been achieved with bioluminescence imaging (BLI) in the CAM assay [76]. Although macroscopically visible metastasis to chick tissue has not yet been achieved due to the short assay time, these proven methods to assess micrometastasis could potentially overcome the difficulty of measuring metastatic potential in CAM models. The fluorescent labeling, qPCR amplification, and BLI methods should be replicated in future studies with the use of primary biopsy tissue and then compared to retrospective analyses of parent tumor metastases in order to determine translational significance.

Visualization of micrometastasis could also elucidate the mechanism by which particular neoplasms disseminate. Uloza *et al.* demonstrated this while developing the first experimental model of recurrent respiratory papillomatosis (RRP) on the CAM. Sectioning of the PDX revealed a "crawling film" of acellular material with newly formed papilloma sprouts infiltrating onto the outer chorionic epithelium; this thin film likely acted as a scaffold by which tumor cells migrated onto previously healthy tissue [59]. The ability to simulate tumor behavior (such as the local spread of RRP within the respiratory tract) in the clinical setting is a major opportunity for future investigation.

Assessment of cancer therapies

Despite the CAM's versatility as a xenograft model, only five PDX studies evaluated its ability to predict response to cancer therapies (**Table 1**). Shoin *et al.* assessed its efficacy as a chemosensitivity predication model for two established drugs (ACNU and MCNU) in the treatment of malignant gliomas and found a high degree of positive association between the chick embryo assay and clinical outcome [65]. While it is difficult to quantity how faithfully the dosage administered to the tumor grafts represented that administered to the patients, the reasonably close tracking of clinical outcome suggests that dosage approximation was satisfactory. These results highlight the potential role that relatively cheap, fast assays such as the CAM can play in the screening of new anticancer agents [77].

Photodynamic therapy (PDT), which involves the use of a photosensitizing drug and activating light, is used as adjuvant treatment for patients with residual tumors or peritoneal metastasis following surgery. Ismail *et al.* used methylene blue (MB) as a photosensitizer in PDT of freshly biopsied malignant ovarian tumors cultivated on the CAM and visualized real-time changes in vasculature. Some treated tumors were transplanted onto new CAMs as a second generation at 8 days after PDT, and complete remission (if not seen in the first generation) was observed by 4 days after implantation [75]. This was another successful instance of serial passaging of PDX onto the CAM forty years after Kaufman *et al.* passaged both sarcoma and astrocytoma onto three consecutive embryos [78], and in both cases the short timeframe of the assay was overcome in order to continue morphological study. The later study also concluded that PDT using methylene blue has the potential to achieve complete eradication of visible ovarian tumors in patients with superficial lesions. Methylene blue has continued to be used as a photosensitizer in studies assessing treatments for ovarian cancer [79, 80]. Treatment approaches for other cancers using photodynamic therapy with various photosensitizers have been studied recently [81]; they might greatly benefit from preclinical testing in the CAM-based PDX model.

Ribatti *et al.* continued the trend towards assessment of possible clinical benefits by observing the effects of an experimental synthetic compound on a CAM-based PDX model for neuroblastoma (NB). The authors noted that a high vascular index in NB correlates with poor prognosis, making the CAM particularly appropriate for this experiment as an established model for angiogenesis [54]. Fenretinide (HPR) is a synthetic retinoid (a class suggested to have anti-angiogenic activity) that has been shown to inhibit carcinogenesis in animals and had also been used to treat cervical carcinoma cell lines in culture [82]. Findings from its application to the CAM a ssay supported the notion that fenretinide might provide new opportunities for neuroblastoma therapy [54]. At least one Phase I trial of this compound was completed over a decade later [83], and attempts to make HPR more efficacious through targeted delivery are ongoing [84].

Marimpietri *et al.* evaluated for a synergistic anti-angiogenic effect on NB for a low dose of vinblastine (VBL) and rapamycin (RAP), established compounds in oncology and transplant rejection prevention. Treating fresh biopsies or cell line xenografts with combination treatment, compared to single-drug exposure, significantly enhanced angiostatic activity [85]. This comparison is notable, because fresh NB tumor biopsies responded similarly to NB cell lines with respect to drug treatment. The researchers later replicated this synergistic effect in mice [86]. In an early testament to the predictive power of the CAM-based PDX model, a Phase I clinical trial nearly a decade later showed the safety, reduction in the circulating angiogenic factor VEGFR2, and existence of clinical responses with this drug combination

[87]. The CAM has also been shown to be a suitable model for acute drug toxicity screenings [88]. Taken together, these findings are an indication that engraftment techniques for many tumors are well-established and that the CAM-based PDX model as a whole is prepared for assessment of more specific clinical benefits.

Ferician *et al.* made the most recent attempt to study therapeutic compounds in the model, performing a comparative analysis of tumor cells and blood vessels from renal cell carcinoma (RCC) on endostatin-treated and control CAM implants by assessing endoglin, VEGF, and smooth muscle actin expression [89]. While endostatin is a well-known endogenous inhibitor of angiogenesis in the CAM assay, its use as an inhibitory agent in a CAM-based PDX model for RCC was previously unreported. Given that therapeutic resistance to targeted therapies for patients with this type of cancer often begins early [89], the CAM assay may be an ideal model for early quantification of tumor resistance to anti-angiogenic drugs.

With regards to particular therapeutic methods, the easy of accessibility of the CAM has enabled both the topical and intravenous administration of anticancer drugs [88], derivation of the optimal irradiation conditions in photodynamic therapies [90], and the potential for testing drug candidates in multiple tumor samples following exome sequencing [91]. Studies using the CAM-based PDX model to investigate established or experimental therapies have been promising. While further investigation is needed to determine the degree of concordance with clinical outcome for various malignancies and therapies, a retrospective analysis of drugs that have achieved remission in patients on an individual basis would be a way forward. This strategy has already been used to support superior predictability of clinical outcome in rodent PDX models [14]. In comparison to rodent models, the CAM-based model also has the potential for significantly lowering drug testing costs and lengthy development times [92]. Its rapid readout could improve treatment by shortening the time interval between tumor engraftment and the results of chemotherapy becoming available [25].

The short timeframe of the assay imparts inherent limitations; for example, it is difficult to track drug resistance or perform toxicology studies in only one week [92]. This is compounded by the fact that chick organs are fundamentally less similar to human organs than those of other mammals. However, drug resistance may arise at higher frequencies if tumors are serially passaged from one CAM to another. This would theoretically allow the assessment of acquired resistance but would also require verification that resistance is independent of changes resulting from subsequent grafting. Analyses could also be performed to quantify toxicity in chick organs for compounds known to have adverse effects in humans. Finally, the closed system of the CAM allows for experimental molecules to have a half-life relatively longer than in rodents, allowing efficient use of compounds that may be expensive or available in small quantities [93]. Because clinically-relevant dose (CRD) has not typically been established for drug delivery onto the CAM, further work needs to be done to ensure that representative dosages are being used to elicit effects on tumor growth.

Embryo survival and take rate

While embryo survival and take rate may not carry direct implications for patient outcome, they are helpful metrics for determining the efficiency of any PDX model. The procedure used to graft fresh tumor tissue onto the CAM has evolved over the past century, with the notable result that take rates (the fractions at which successful engraftment of viable tumor occurs) have steadily increased (**Table 1**). An early attempt to increase the rate of graft survival by irradiating eggs prior to inoculation was made by Sommers *et al.* While this technique did not improve take rates (23% in normal embryos versus 21% in irradiated embryos), the observation was made that the injection of cell suspensions directly into the CAM resulted in a proportional increase in embryo mortality with increasing number of injections [94]. Subsequent investigators have generally used less invasive techniques for inoculation that minimize embryo mortality (**Table 1**).

Sommers *et al.* also hypothesized that since first generation growth was generally the most difficult to obtain, prior heterotransplantation of tumor graft in other media might facilitate improved tissue adaptation and take rates [94]. This was later disproven by Kaufman *et*

al., who transplanted fresh biopsies after maintaining the tissue *in vitro* for 10 to 21 days. Compared to a cumulative take rate of 50% for PDX that were immediately transferred from the parent tumor, only 14 of 66 (21%) xenografts that were first maintained *in vitro* survived on the CAM after a period of 3 to 11 days [78]. Interestingly, both Sommers *et al.* and Kaufman *et al.* described that tumors of connective tissue origin were transplanted more successfully; in the case of the former, one sarcoma even survived a third transfer and 27 days total on the CAM assay [78, 94]. This congruence was one of the first indicators that take rates for particular neoplasms depends more on intrinsic properties of their unique tumor biology than differences in experimental techniques. There has been increased efficiency in the grafting process, with tumor take rates of 100% recently reported for nasopharyngeal carcinoma [26] and renal cell carcinoma [58, 89]. The CAM-based PDX model thus represents a potentially extremely efficient method of making the most scientific use of each patient's tumor tissue following biopsy or resection.

Embryo mortality (the fraction of eggs that perish at any time during the cultivation period following engraftment) is probably more a function of the gross number and type of inoculated tumor cells as opposed to procedural variation. For example, in the Shoin *et al.* experiment, all glioblastoma specimens had a take rate of 100% on the CAM, and no embryos died during the incubation period from either tumor cell dissemination or from toxicity at the dosage of anticancer drugs tested [65]. While this initially appears to be an impressively high take rate for its time period, it is likely explained by the fact that the quantity of implanted tissue was a miniscule 100 μL. Similarly, while tumor fragments derived from glioblastoma [46], sarcoma [39], and renal cell carcinoma [89] have produced high take rates of 80% and above, those from neoplasms such as head and neck squamous cell carcinoma [50] and giant cell tumor of bone [67] have only approached 50%. High mortality rates have been speculated to be caused by tumor cell dissemination or the secretion of coagulative factors resulting from hypoxia and serum deprivation [67].

The CAM is capable of supporting *in vivo* models of most types of malignancies from fresh tumor material (**Table 1**). Even tumors that have historically proven difficult, such as those of the musculoskeletal system, have been recapitulated on the CAM [38, 67]. While the potential for bias against publication of negative results should be kept in mind, there are few reports of inabilities to culture particular tumor types onto the CAM, although failures include attempts for acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) [92]. It should be noted that this model also may not be appropriate for cancers in which a loss of tumorigenicity is seen when cultured, as was the case with Lewis lung carcinoma in previous experiments [95]. Ideally, engraftment of these neoplasms will be attempted again with the use of fresh tumor tissue in order to reevaluate their use. While the contribution of rodent models to precision medicine should not be understated, features of the CAM-based PDX model give it the potential to fill unique gaps. Unsatisfactory take rates in immunodeficient rodents across a range of malignancies represents a major barrier to cost reduction and efficiency [11, 16, 96]. In contrast, engraftment of viable tumor has been achieved at progressively higher rates in the CAM-based PDX model over the past four decades. Tumor fragments are generally grown more successfully on the CAM as compared to the low take rates seen in some rodent models (**Table 1**).

Discussion

The advantages and limitations of the CAM as a versatile *in vivo* model are well-described [30, 97]. Overall, the CAM provides a simple, inexpensive, and quantifiable alternative to other *in vivo* techniques [51]. Its high vascularity stimulates tumor growth and facilitates analysis of angiogenic effects, and the immaturity of its immune system allows implantation of varied cell types from a range of species [37]. A paradigm shift to patient-derived xenograft studies with chick embryos would address widely accepted guidelines aimed at reduction of vertebrate animal numbers and suffering in research [62]. A great opportunity for the PDX platform is the ability to use a combination of genetic data and experimentation to predict and test sensitivity of an individual patient's cancer to specific treatments. The ability to test multiple targeted therapies on tumor fragments from a given patient in the CAM-based model could potentially guide prioritization of a num-

ber of potentially relevant mutations and targets following exome sequencing [91]. Despite having expanded knowledge of the human genome, there are still challenges to overcome prior to the full integration of genomics into precision medicine. In comparing primary human tumors and patient-derived xenografts, significant differences have been found at the protein level despite the concordance of genomic and transcriptomic data [20]. This should cause medical scientists to err on the side of caution when designing targeted therapies in the absence of information about the stromal component of the tumor. PDX models should be complementary to other preclinical models (e.g. genetically engineered mice) that have proven to be powerful tools for studying drug-responses within a well-defined genetic background [11, 98]. The initial sequencing of the chicken genome over a decade ago provides equivalent opportunities for creating genetically engineered CAM-based PDX models [99]. It has been proposed that use of 'Mouse Avatars' (in which therapies that have demonstrated efficacy in humanized mice also prove beneficial to the patient) could one day be a standard paradigm in the treatment of cancer [16]. A similar concept of chicken egg-based 'Ovotars' may also be considered for patients in clinical trials. Given its low cost, ease of manipulability, and promising track record of past research, the CAM-based PDX model could develop into a major player in the field of precision medicine.

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Disclosure of conflict of interest

MTL is a limited partner in StemMed LTD, a manager in StemMed Holdings LLC, and holds an equity stake in Tvardi Therapeutics Inc. AGS holds patents BAYM.P0196US.P1 and BAYM.P0172US.P1, based on CAM-related technologies.

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