

Review

Chick Embryo Chorioallantoic Membrane (CAM) Model for Cancer Studies and Drug Evaluation

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Abstract

The chick embryo chorioallantoic membrane (CAM) model is gaining increasing attention from cancer researchers worldwide. Its affordability, short experimental duration, robustness, and ease of tumor xenograft visualization make it a valuable tool in cancer research. This review explores recent advancements and potential applications of the avian CAM model, including the following: (1) studying tumor growth and metastasis, (2) investigating mechanisms of tumor chemoresistance, (3) optimizing drug delivery methods, (4) improving bioimaging techniques, (5) evaluating immuno-oncology drug efficacy, (6) examining tumor-extracellular matrix interactions, (7) analyzing tumor angiogenesis, and (8) exploring the roles of microRNAs in cancer. Additionally, we compare the *in ovo* CAM model with other *in vivo* animal models and *in vitro* cell culture systems. Positioned between *in vitro* and *in vivo* models in terms of cost-effectiveness and accuracy in cancer recapitulation, the CAM model enhances both preclinical and translational research. Its expanding use in cancer studies and therapy development is expected to continue growing.

Keywords: chick embryo chorioallantoic membrane (CAM); *in ovo*; cancer metastasis; drug development; radiotherapy; chemotherapy; miRNA; tumor-microenvironment interactions; immuno-oncology; immune-vascular dynamics

1. Introduction

The earliest experiments on chicken embryos date back to the 1750s when German scientist Beguelin observed successive developmental changes in the germinal disk by creating a window in the eggshell [1,2]. By the 1890s, Mathias Marie Duval published the first comprehensive morphological atlas of chicken embryo development [1]. In the early 20th century, studies on chickens infected with the sarcoma virus led to the discovery of viral carcinogenesis [1], prompting researchers to investigate the feasibility of chicks as an animal model for human tumor studies. By the latter half of the 20th century, chicken embryos were increasingly used in cancer research [3–5]. More recently, the chick embryo chorioallantoic membrane (CAM) model has gained attention as a cost-effective *in ovo* model for studying various aspects of tumorigenesis and metastasis (Fig. 1).

Table 1 (Ref. [1,2,6–20]) presents a compelling timeline that juxtaposes historical advancements with recent breakthroughs in CAM research.

The development of anticancer drugs is a complex process that involves preclinical trials requiring a large number of mice for pharmacological analysis [21]. Ethical considerations play a crucial role in these experiments, which must adhere to the 3R principles (Replacement, Reduction, and Refinement) [22]. The CAM model aligns

with these principles, offering an ethically favorable alternative to traditional mammalian models. In many countries, chicken embryos are not classified as live animals before embryonic day 17 (E17), allowing CAM experiments conducted before this stage to be exempt from ethical oversight, such as Institutional Animal Care and Use Committee (IACUC) review. This exemption streamlines procedures while minimizing animal suffering [23,24]. For experiments extending beyond E17 or involving invasive procedures, the use of anesthetics (e.g., isoflurane) and adherence to humane endpoints—such as limiting tumor burden or preventing excessive bleeding—are essential to meet refinement standards [25]. Regulatory acceptance of the CAM model varies globally. The European Union Directive 2010/63/EU provides specific protections for embryonic models, while in the United States, experiments conducted before hatching may not be subject to animal welfare regulations [26,27]. These regulatory considerations enhance the model's credibility and support its broader adoption within the scientific community.

The CAM model serves as an intermediate system between *in vitro* and *in vivo* models, offering distinct advantages over both (Fig. 2). Compared to *in vitro* models, the CAM provides a physiologically relevant microenvironment, including vascularization and dynamic tissue interactions, enhancing its translational potential [23]. While



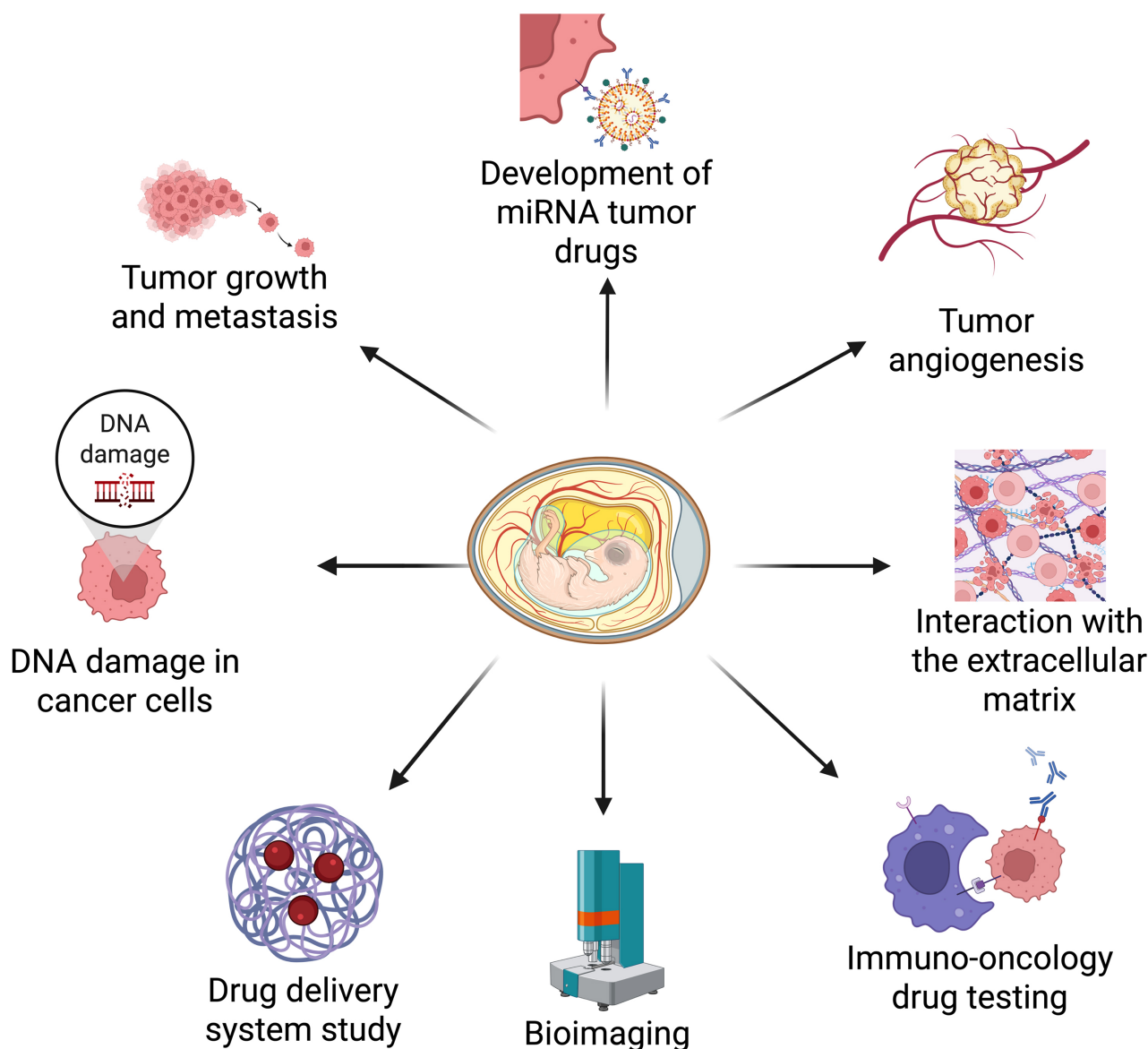


Fig. 1. The chick embryo chorioallantoic membrane (CAM) oncogenesis model is widely used to investigate various aspects of tumorigenesis, metastasis, and drug delivery. These applications include the following: (1) tumor growth and metastasis, (2) genomic DNA damage in cancer cells, (3) drug delivery optimization, (4) bioimaging advancements, (5) immuno-oncology drug testing, (6) tumor-extracellular matrix (ECM) interactions, (7) tumor angiogenesis, and (8) the role of microRNAs in cancer. Created with [BioRender.com](https://www.biorender.com).

mouse and rat models remain the gold standard in cancer research due to their ability to replicate complex tumor-host interactions, growing evidence supports the CAM model as a viable alternative or complement to traditional animal systems [28]. Recent study demonstrates that enhanced permeability and retention (EPR)-dependent macromolecules exhibit intra-tumoral accumulation and retention kinetics in CAM xenografts that closely resemble those in mouse models [29]. Additionally, Pinto *et al.* [30] successfully adapted the limiting dilution assay (LDA) from mouse models to develop the CAM-LDA system, validating its robustness for assessing breast cancer stem cell tumorigenicity. Rousset *et al.* [31] further demonstrated that CAM mod-

els could effectively replace mouse-derived circulating tumor cells, advancing applications in personalized medicine. This unique balance makes the CAM model an ethically favorable and scientifically robust platform for preclinical cancer research.

This review decisively highlights the emerging utility of the CAM model in anti-tumor microRNA (miRNA) research, tumor-matrix interactions, and immuno-oncology drug screening, distinguishing it from previous reviews that provided a more generalized perspective of the model's applications. Traditional models have notable limitations: *in vitro* assays lack physiological complexity, while conventional animal models often present challenges in ef-

Table 1. Historical milestones in the development and biomedical applications of the CAM model.

| Time period | Research advancement | Key findings/applications |
|---------------------------------|--|---|
| 1750s | Early embryonic observation | German scientist Beguelin [1,2] introduced a technique to cultivate chick embryos by opening a window in the eggshell, enabling sequential observation of developmental changes in the germinal disk. |
| 1890s | Morphological atlas publication | Mathias Marie Duval [1] published the first comprehensive morphological atlas of chick embryo development, enhancing the understanding of avian embryology. |
| First half of the 20th century | Discovery of Rous sarcoma virus | Peyton Rous [6] identified a transmissible agent causing sarcoma in chickens, establishing a foundational model for studying viral oncogenesis. |
| | Tumor transplantation studies | James B. Murphy [7] demonstrated that foreign tissues could survive in the CAM, facilitating tumor transplantation research. |
| | Viral cultivation techniques | Alice Miles Woodruff and Ernest Goodpasture [8] developed a method using chicken eggs to propagate viruses, advancing virology research. |
| | Standardization of developmental stages | Viktor Hamburger and Howard Hamilton [9] developed the Hamburger-Hamilton stages, a standard framework for chick embryo development still in use today. |
| Second half of the 20th century | Angiogenesis research | The CAM model was emerging as a promising platform for investigating tumor angiogenesis [10]. |
| | Metastasis studies | Researchers used the CAM model to study how tumors metastasize, providing important insights into cancer progression [11]. |
| 21st century | Drug testing and nanotechnology | The CAM model serves as a valuable platform for evaluating anticancer drugs and nanomaterials because of its vascular characteristics and ease of observation [12–15]. |
| | Immuno-oncology applications | Researchers employed the CAM model to assess immune responses and the efficacy of immunotherapeutic agents against tumors [16–18]. |
| | Precision medicine and patient-derived xenografts (PDXs) | The CAM model has been refined to facilitate swift testing of individual tumor responses, significantly enhancing personalized cancer treatment strategies [19,20]. |

iciency and cost. The CAM model addresses these issues with its intrinsic vascular network, enabling rapid, cost-effective, and physiologically relevant assessments of miRNA-mediated regulation, tumor-stroma interactions, and immune-vascular dynamics. Furthermore, we discuss recent technological advancements that enhance the model's translational relevance and precision, solidifying its role as a critical bridge between simplified *in vitro* systems and traditional *in vivo* models.

2. Development of the Avian Embryo and the Extra Embryonic CAM

Histologically, the CAM consists of three layers: an external epithelial layer of ectodermal origin, an intermediate stromal layer, and an internal epithelial layer of endodermal origin (Fig. 3) [32]. CAM development occurs between embryonic day 5 (E5) and E21. From E5 to E12, the chorion and allantois fuse, and the CAM expands to cover the entire egg surface. Between E13 and E18, the CAM undergoes rapid growth, differentiation, and expansion, with chorionic capillaries occupying the superficial layer and the number of allantoic layers increasing to four. Proliferation

and differentiation peak at E18, followed by gradual degradation from E18 to E21 [33]. CAM angiogenesis occurs in three stages [34], with structural maturation during the third stage (E13–E14) [35]. Since rapidly proliferating tumors favor intussusceptive angiogenesis for nutrient acquisition [34], the optimal time for tumor transplantation is after E8, when this process begins.

On days E4–E5, the CAM attaches to the surface of the eggshell removing calcium and thus accessing oxygen through the porous eggshell [32]. The general structure of the chicken embryo is shown in (Fig. 4).

The lymphatics of the CAM develop at E6–E9 and grow by blind-ended sprouting [36]. The CAM lymphatic vascular endothelial cells express vascular endothelial growth factor receptor 2 and 3 (VEGFR-2 and VEGFR-3) molecules, which resemble the mammalian lymphatic system [32,37]. Studies [38,39] have demonstrated a strong link between lymphatic vessels and cancer metastasis. The structural and functional similarities between the avian CAM and mammalian lymphatics further support the CAM's suitability as an *in vivo* oncogenesis model [40].

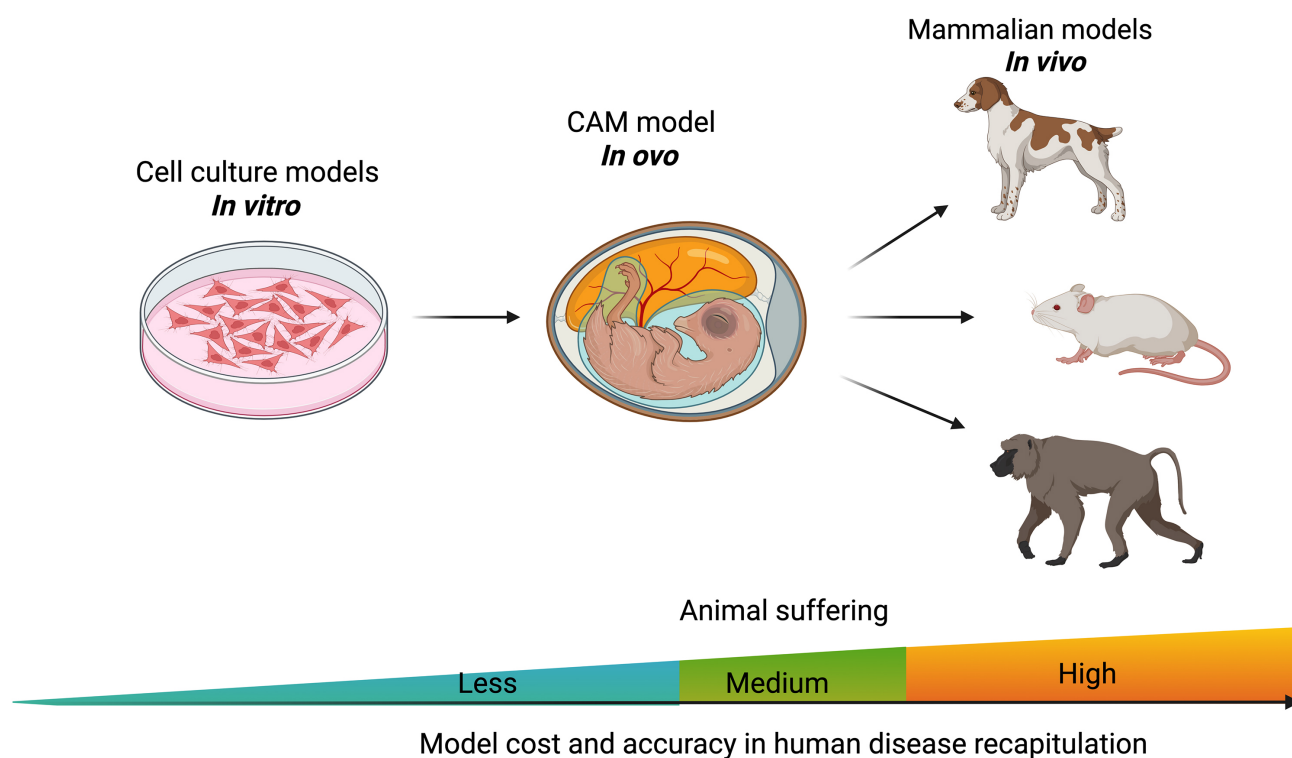


Fig. 2. The chicken embryo CAM model is placed between *in vitro* cell culture and *in vivo* animal models. Created with [BioRender.com](https://www.biorender.com).

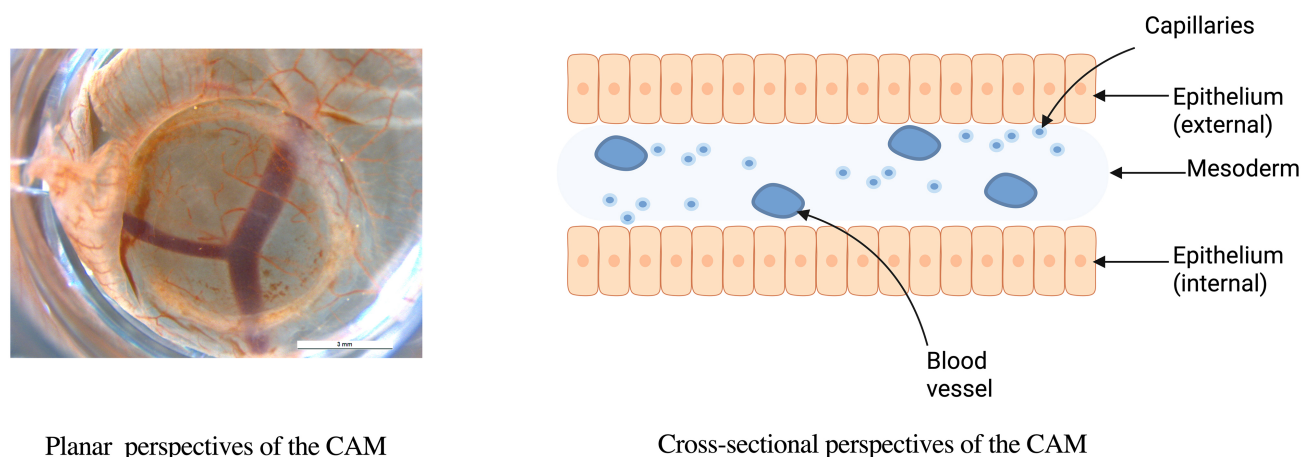


Fig. 3. Schematic diagram of the CAM. Scale bar = 3 mm. Created with [BioRender.com](https://www.biorender.com).

3. Potential Applications of CAM Assays in Oncology Research and Drug Development

3.1 CAM Model to Monitor Tumor Growth and Metastasis

The progression of cancer-related health deterioration often begins with metastasis. The complexity of this process, which involves multiple tissues and organs and occurs deep within the body, makes continuous observation challenging in traditional animal models. The *in ovo* model provides a robust experimental system that mimics all stages of the metastatic cascade, facilitated by the continuous circu-

latory connection between the extraembryonic membranes and the avian embryo, allowing for easy manipulation. While existing 3D *in vitro* models fail to accurately replicate the intricate structure of blood vessels—particularly capillaries and post-capillary venules, which are key sites of cancer invasion—rodent models also present limitations due to the rarity of metastasizing tumors. The CAM's rich vascularization offers an optimal platform for studying both experimental and spontaneous metastasis (Fig. 5), enabling comprehensive investigations of the metastatic process [41]. Notably, the chicken CAM model has been ap-

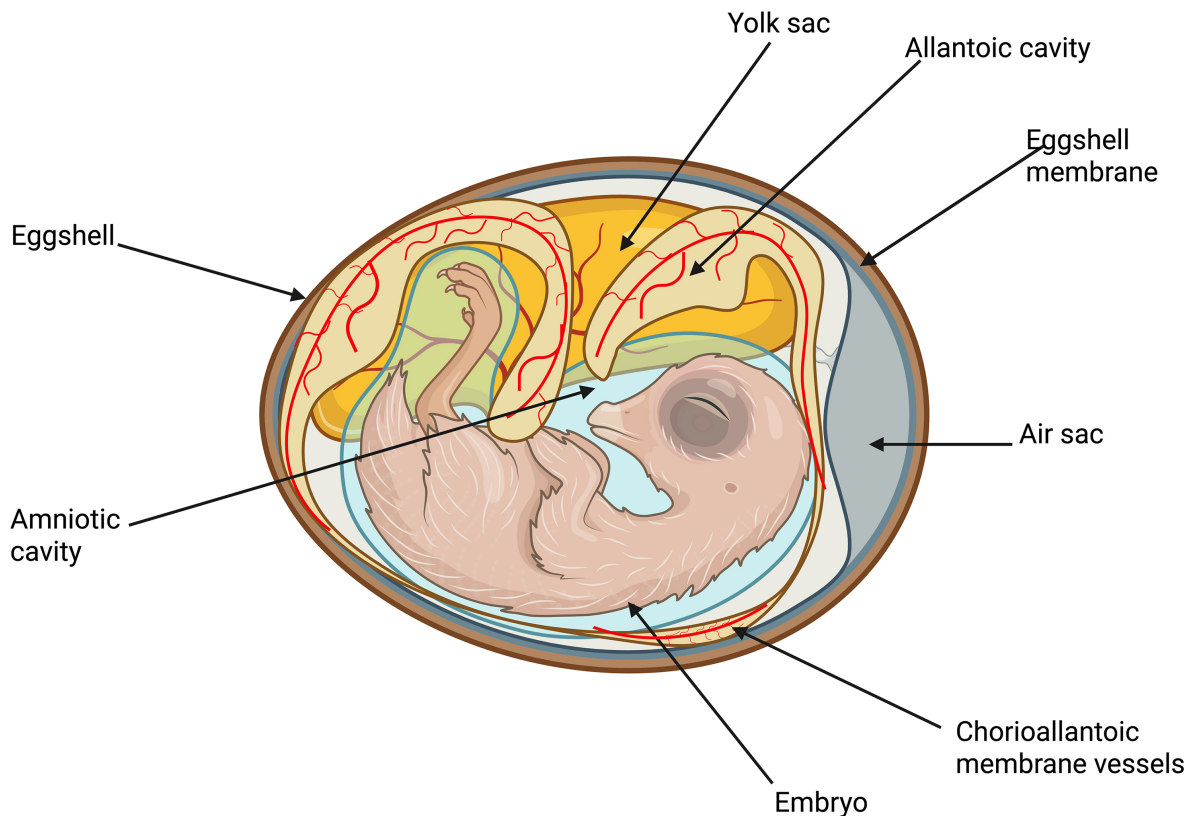


Fig. 4. Schematic diagram of the intra-egg of the pre-developing chicken embryo. Created with [BioRender.com](#).

plied to study cancer cell motility and invasion *in vivo*, in multiple studies [42–46].

Cancer cell invasion, extravasation, and metastatic colonization have been investigated *in ovo* using a combination of detailed morphological assessments, selective outgrowth of metastasized cells in controlled environments, and advanced imaging techniques for detecting microscopic tumor nodules [14]. Additionally, several biomarkers, including human urokinase plasminogen activator (uPA) activity [1,43] and those linked to epithelial-mesenchymal transition (EMT) [47], have been analyzed in the *in ovo* model using methods such as enzyme-linked immunosorbent assay (ELISA), tissue staining, fluorescence-activated cell sorting (FACS), and polymerase chain reaction (PCR). A significant limitation of conventional mouse models for studying experimental metastasis is that most injected tumor cells die in the microcirculation before they can successfully extravasate. In the innovative chicken CAM metastasis model, extensive research has demonstrated that an impressive 80% of tumor cells, when injected into the allantoic vein, successfully survive the initial phase and manage to extravasate within a timeframe of just 1 to 3 days [48]. The ability of these cells to not only survive but also navigate toward these vessels is a crucial aspect of understanding cancer metastasis. This model serves as a vital tool in cancer research, providing insights into the dynamics of tumor behavior in relation to vascular structures.

A recently reported intravital microscopy platform enables high-resolution time-lapse imaging of human tumor growth, cell migration, and extravasation *in ovo*, allowing for longitudinal monitoring and quantitative analysis of tumor cell metastatic capacity following CAM implantation [14,49]. In this approach, chicken blood vessel cells were labeled with fluorescent *Lens culinaris* agglutinin, and blood flow was visualized using a fluorescent dye. Fluorescently labeled cancer cells were then injected into the vitelline vein to track tumor cell movement and examine the role of invadopodia in metastasis over a 24-hour period [49]. Additionally, the study has investigated spontaneous metastasis following tumor cell grafting onto the CAM by injecting labeled cells that can be traced in organs, blood, and the amnion in both *in ovo* and *ex ovo* models [50,51].

Detecting metastatic colonization in different organs is challenging due to the low number of tumor cells and the limited timeframe of experimental studies, which often prevents the formation of macroscopic nodules. However, the human genome's unique enrichment in Alu sequences, occurring at a frequency of 5%, enables the sensitive detection and quantification of metastasized human tumor cells using Alu PCR assays [52,53]. These powerful assays can identify and quantify as few as 50 cells [54].

Last, but not least, a standardized method for evaluating the ability of human cancer cells to degrade the basement membrane—specifically their delamination

Tumor cell injection (Experimental metastasis analyse)

Tumor cell implantation on CAM (Spontaneous metastasis analyse)

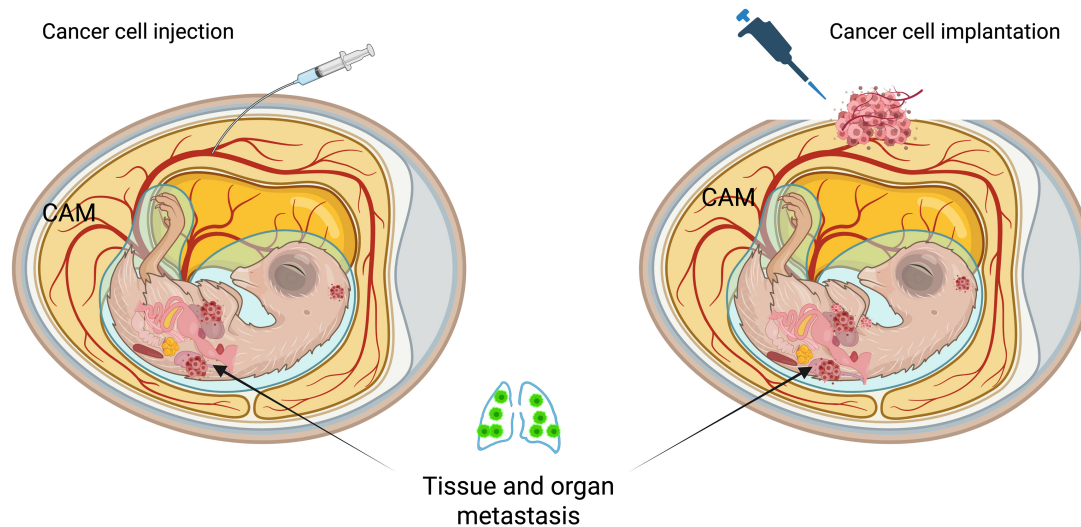


Fig. 5. Tumor metastasis models in chicken embryos. Left—the experimental metastasis model; Right—the spontaneous metastasis model. Created with [BioRender.com](https://www.biorender.com).

capacity—is crucial for assessing metastatic aggressiveness. The so-called “CAM-Delam” assay has demonstrated, through a variety of human cancer cell lines, that the capacity for delamination can be classified into four distinct categories [55]. This classification not only quantifies metastatic aggressiveness but also highlights the assay’s vital role in measuring delamination potential. Moreover, it aids in uncovering the molecular mechanisms that govern delamination, invasion, micro-metastasis formation, and the modulation of the tumor microenvironment. This approach has the potential to greatly enhance both the pre-clinical and clinical analysis of tumor biopsies, as well as facilitate the validation of compounds that could boost survival rates in metastatic cancer [55].

3.2 The CAM Assay for Analysis of Angiogenesis-associated Effects

Deregulated angiogenesis, now recognized as a fundamental hallmark of cancer, plays a critical role in tumor progression by enabling malignancies to develop their own blood supply [56]. The highly vascularized CAM, which functions as the gas exchange organ in birds, is an ideal model for angiogenesis research. Traditionally, pharmacological evaluations of pro- and anti-angiogenic compounds have been conducted *in ovo*, with growing interest in applying this approach to preclinical oncology. Following tumor inoculation, the tumor xenografts begin to manifest and become clearly visible within just 2 to 3 days. During this time, they establish a robust vascular network, primarily derived from the chick CAM, which rapidly penetrates deep into the tumor tissue. This efficient blood supply not only

supports the tumor’s growth but also facilitates the delivery of essential nutrients and oxygen. As a result, the developing tumor can quickly adapt and proliferate, leading to observable increases in size and mass [15].

This model can be applied to the evaluation of any factor influencing angiogenesis as well as to probing connections between angiogenesis and other processes or conditions such as cancer. Vascular growth inhibition as a mechanism of cooper chelators’ anticancer effects was also demonstrated in the chicken CAM model [57]. In the other study [58], different cancer cell grafts in the CAM were subjected to functional gas challenge, which is the exposure of cancer cell grafts to various combinations of atmosphere gases. Magnetic resonance imaging (MRI) was used to evaluate functional gas challenge effects on cancer cell grafts. Different responses were observed for different cancer cells depending on the formed graft size and its vascular density [58].

The growing number of angiogenesis studies on the CAM model has provoked the development and refinement of corresponding methods. Numerous qualitative and quantitative methodologies have been extensively documented to assess *in ovo* angiogenic response. The CAM assay was optimized for quantitative measurement of changes in vessel density, endothelial proliferation, and protein expression after the application of anti-angiogenic agents [59]. Recently, an artificial intelligence method of the CAM cancer model angiogenesis quantification was developed. This method aims to provide technical support for therapeutic options targeting cancer angiogenesis [60].

Recent breakthroughs in imaging technologies, alongside enhancements in contrast and imaging agents, now enable the precise visualization of vascular perfusion and the selective labeling of vascular structures at microscopic scales. Histological and immunohistochemical analyses serve as the benchmark for reference standards. These analyses routinely utilize hematoxylin-eosin (HE) staining, along with staining for vascular and endothelial markers, including CD31 and von Willebrand factor (vWF) [61,62]. Chicken endothelium can be specifically stained by *Sambo negro* agglutinin (SNA) [63]. Micro-computed tomography (micro-CT) combined with polymer injection has proven effective for assessing three-dimensional vascular remodeling *in ovo* [64].

Vascular dynamics have been monitored longitudinally using digital photos captured in controlled lighting and examined with image analysis software [65]. Intravital vascular architecture and fluid dynamics can be monitored by viral nanoparticles carrying reporter genes [66]. As a label-free alternative, the Doppler mode measurements of vascular structure can be used to expand pre-described ultrasonography used for the assessment of tumor growth suppression by anti-Chloride Intracellular Channel Protein 1 (CLIC1) [67]. However, because of tissue clutter, this method is mostly restricted to monitoring big vessels. The small size of tumors and the radiopaque eggshell, which restricts monitoring to a top-down perspective, present challenges for conventional imaging methods. High-frequency ultrasensitive ultrasound microvessel (UMI) imaging has enabled a more detailed characterization of slow-flow vessels within the microvasculature, as demonstrated in a renal cell carcinoma model following the administration of two Food and Drug Administration (FDA)-approved anti-angiogenic drugs [21]. Ultrasonography is cost-effective and widely accessible, making it a valuable tool for *in ovo* tumor imaging. Combined with the affordability and simplicity of CAM assays, this approach allows for high-throughput, quantitative imaging while facilitating longitudinal monitoring of tumor progression. Consequently, the CAM assay serves as a viable alternative to mouse xenograft models, improving reproducibility in *in vivo* tumor testing. One significant drawback is that data interpretation may be impacted by the inability to differentiate tumor-related neoangiogenesis from innate embryonic neovascularization or increased vascular density brought on by the rearrangement of preexisting arteries [68]. Angiogenic investigations must be timed carefully to prevent confounding variables. Hence, angiogenic investigations in the CAM model are commonly conducted between E10/11 and E14/15 since the endothelium mitotic index and the overall complexity of the CAM diminish around E11 [48,69].

Overall, the CAM model's suitability for cancer angiogenesis research supports its rapid development and broad application in the field.

3.3 The Collagen I-rich Extracellular Matrix (ECM) of the CAM Resembles the Aggressive Cancer Stroma

The CAM model, with its collagen I-rich ECM, provides an excellent platform for mimicking the *in vivo* conditions of aggressive tumors [70]. One of the key characteristics of many aggressive cancer types, such as breast cancer, pancreatic cancer, and osteosarcoma, is their dense collagen I-rich extracellular matrix [71–74]. This matrix not only supports the structural integrity of the tumor but also plays an active role in promoting cancer cell migration, invasion, and metastasis. By using the CAM model, researchers can replicate this specific ECM environment and observe how cancer cells interact with the collagen-rich matrix, offering a highly relevant *in vivo*-like setting for tumor studies. In aggressive cancers, collagen crosslinking and increased ECM stiffness are critical factors that enhance cellular adhesion, elevate invasive potential, and activate mechano-transduction pathways, all of which contribute to tumor growth and metastasis [75]. The CAM is highly vascularized and readily promotes angiogenesis when tumor cells are grafted onto its surface, creating a realistic model for studying how cancer cells influence and are influenced by the surrounding vascular network. This process is closely tied to the ECM, as ECM components, such as collagen, interact with angiogenic factors like vascular endothelial growth factor (VEGF) to promote new vessel formation [76]. The CAM's ability to mirror both the ECM and the angiogenic environment allows a comprehensive analysis of tumor-ECM-vasculature interactions.

3.4 The CAM Model as a Promising Alternative for In Vivo Studies in Immuno-oncology Drug Development

Currently, the most commonly used animal models for preclinical immuno-oncology research can be broadly categorized into two types: (1) xenograft models, where human cancer cell lines are transplanted into immunodeficient mice; and (2) syngeneic models, where mouse-derived cancer cell lines are transplanted into immunocompetent mice. Humanized mice are one of the more suitable models for pre-clinical immunotherapy studies. While these models have advanced immuno-oncology research to some extent, they exhibit significant limitations [77].

In this context, the CAM model emerges as an ideal choice. During the early stages of embryonic development (E0–E12), the immune-deficient state of the embryo enables stable xenograft transplantation of human tumor cell lines, resulting in reliable tumor formation. As the immune system matures in the mid-to-late stages of embryonic development (E12–E18), the CAM model becomes a valuable platform for studying the efficacy of various immunotherapeutic agents. Building on the various humanization models used in mice, two recent extensive studies [78,79] propose the dual engraftment of human immune and tumor cells in fertilized chicken embryos. This approach offers a xenogenic system to explore immunotherapies in a more

thorough yet cost-effective semi-*in vivo* model. The brief experimental duration presents a significant limitation, as it constrains our capacity to perform long-term evaluations of cancer immunity *in ovo*. Consequently, the dual role of the immune system in the context of immunoediting remains unexplored. Although avian and mammalian lymphoid organs are different, there are some functional similarities between chicken and mammalian immune systems. Thus, CAM model experiments can be used as a transition between cell culture and complex mammalian model experiments [51]. Different functional immune cells such as T cells, B cells, and Natural Killer (NK) cells can be detected at later stages of avian embryonic development [48,80,81]. Importantly, immune checkpoints similar to that in human immuno-oncology therapy, including Programmed Cell Death Protein 1 (PD-1), Programmed Death-Ligand 1 (PD-L1), Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4), and Lymphocyte-Activation Gene 3 (LAG-3), were identified in chickens [80], suggesting the suitability of the CAM model for immune-oncology studies. Applicability of the CAM model for immune checkpoint inhibitor (ICI) studies was directly demonstrated in a recent study, where the efficacy of the FDA-approved ICI drug Pembrolizumab was successfully verified on the CAM model [16]. These studies point to the great potential of the chicken embryonic tumor model for promising immuno-oncology studies including cancer immune drug testing.

3.5 CAM Model in Bioimaging

Non-invasive assessment of tumor biodistribution is very useful for cancer studies and the development of new anti-cancer drugs. Several recent studies have revealed the feasibility of intra-oval imaging in the CAM model [82,83]. Some research groups have optimized MRI compounds for intra-oval imaging, and they suggest that using isoflurane to chill the embryo can reduce artifacts and improve resolution [84–86]. Other studies have reported the use of chicken embryos in positron emission tomography imaging [87–91]. In a study of a CAM tumor model for colorectal cancer, researchers evaluated a new radiotracer, Ga-Pentixafor, for preclinical trials by performing simultaneous positron emission tomography (PET) and magnetic resonance (μ PET/MR) scans [92]. Recently, the PET imaging capabilities of radiolabeled peptides, specifically the ^{68}Ga -labeled human epithelial growth factor receptor-2 (HER2) affibody, ^{68}Ga -MZHER, have been effectively validated for tracking tumor-targeting potential and pharmacokinetic profiles in chicken CAM tumor models of human breast cancer [93]. Furthermore, ^{18}F -labelled prostate-specific membrane antigen (PSMA) ligand, [^{18}F]F-siPSMA-14, PET/MRI was successfully used to image PSMA-positive and negative tumors both *in ovo* (CAM model) and *in vivo* [94].

Nevertheless, significant challenges persist in this field, such as the variability in tumor growth rates, the com-

plexities of cannulating the CAM vessels for dynamic imaging, and the necessity to cool the egg to immobilize the embryo. This cooling process adversely affects the delivery, internalization, and metabolism of radiotracers. Recently, a simple method for vessel cannulation and the use of liquid narcotics for chick immobilization enabled direct comparison studies showing that the chick CAM may reduce the reliance on subcutaneous tumor-bearing mice. The non-small cell lung cancer (NSCLC) tumor uptake patterns of ^{18}F -2-fluoro-2-deoxy-D-glucose (^{18}F -FDG) were similar in both *in ovo* and *in vivo* studies. However, the radioactivity levels associated with tumors cultivated on the CAM were notably higher throughout the 60-minute imaging duration. Additionally, (4S)-4-(3- ^{18}F -fluoropropyl)-L-glutamate (^{18}F -FSPG) proved to be an early biomarker for assessing treatment response to external beam radiotherapy and targeted inhibition *in ovo* [95]. Therefore, CAM models could serve as an excellent platform for assessing the effectiveness of pharmacological compounds, particularly radiopharmaceuticals [96]. Further development of bioimaging techniques can be anticipated for the CAM model, given the increasing interest in this model from researchers.

3.6 The CAM Model for Investigating Anti-cancer Therapies

3.6.1 CAM Model to Study Tumor Chemoresistance Mechanisms

A key focus in the study of tumor chemoresistance is the creation of cost-effective and user-friendly realistic three-dimensional (3D) *in vitro* and *in vivo* models tailored to individual patient tumors [97]. The CAM (*in ovo*) model presents a compelling alternative to traditional *in vivo* models for tumor engraftment. It presents a compelling alternative to conventional 3D spheroids [98], organoids [99], and costly patient-derived xenograft (PDX) or the cell line-derived xenograft (CDX) mouse models [100]. *In ovo* experiments can be executed within a concise timeframe of just 18 days. Additionally, this low-cost approach allows for straightforward retrieval of the tumors formed, enhancing the feasibility of research endeavors [46].

Cancer stem cells (CSCs) are fundamental to the processes of cancer initiation, progression, and the unfortunate relapse of the disease following treatment. These unique cells possess the ability to self-renew and differentiate, contributing to the heterogeneity found within tumors. Their resilience and adaptability often complicate treatment efforts, making them a significant focus in cancer research and therapy development. The molecular mechanisms underlying the radio- and chemo-resistance of CSCs are largely due to their improved ability to repair DNA double-strand breaks (DSBs) [101,102]. Understanding the mechanisms by which CSCs operate is crucial for improving cancer treatment outcomes and preventing recurrences.

The CAM model was evaluated alongside the immunodeficient mice model to assess the tumorigenic potential of

CSCs derived from patients. The CAM model has proven to be a suitable alternative for this assay, standing alongside the immunodeficient mice model, which is regarded as the gold standard in cancer stem cell research. In fact, xenograft tumors developed significantly faster and exhibited enhanced stability and reproducibility in naturally immunodeficient chicken embryos compared to engineered immunocompromised mice [30]. Two recent studies have consistently demonstrated the successful application of the CAM model to assess the tumorigenic potential of circulating cancer cells in patients with breast, prostate, and lung cancers [31,103]. The dependable and consistent development of PDX tumors highlights the CAM model's effectiveness for drug evaluation studies. In fact, the CAM model, utilizing ovarian cancer PDXs, has proven successful in assessing the efficacy, toxicity, biodistribution, and delivery of doxorubicin-loaded nanovesicles [13]. Consequently, the CAM model is equally effective as the immunodeficient mice model for PDX studies, particularly in evaluating chemoresistance.

The CAM model has proven effective in evaluating the aggressiveness and tumorigenic potential of chemoresistant retinoblastoma (RB) cell lines. Etoposide-resistant RB cells demonstrate more aggressive behavior in the CAM model compared to their original tumor counterparts, potentially increasing the risk of local relapse. In contrast, cisplatin-resistant RB cells exhibit a markedly reduced tumorigenic potential [104].

3.6.2 CAM Modeling for Assessing the Efficacy of Chemotherapeutic Drug Delivery Materials

Chemotherapy stands as a primary strategy in cancer treatment. Nevertheless, the majority of chemotherapeutic agents lack specificity and necessitate high dosages to attain the desired therapeutic effect. As a result, this not only escalates treatment costs but also aggravates adverse side effects [105,106]. Cancer-targeting drug delivery systems are developed to overcome these limitations. Animal research is essential for the development of effective drug-delivery systems.

The United States FDA has granted official approval for the CAM model, recognizing it as a highly effective alternative to conventional animal models for the evaluation of oncology drugs [107]. This model assesses drug activity, biodistribution, and pharmacokinetics, while also evaluating toxicity and side effects at various developmental stages of the late embryo [12,108]. This sheds light on the growing popularity of the model among scientists.

Nanocarriers for drug delivery are defined as particles with diameters ranging from 1 to 1000 nm, encompassing various forms such as nanoparticles, nanoemulsions, carbon-based nanomaterials, and nanofiber composites [109,110]. The CAM model was applied to assess drug delivery with nanocarriers in multiple studies. Efficacy, toxicity, biodistribution, and delivery of

doxorubicin-loaded nanovesicles were successfully evaluated in the CAM model with ovarian cancer PDX [13]. Numerous studies have employed the CAM model to investigate the anti-angiogenic effects of various nanomaterials, frequently incorporating active ingredients within these materials. Anti-angiogenic effects of imiquimod-loaded nanoparticles [111], nanoemulsions with resveratrol [112], graphite, multi-walled carbon nanotubes, fullerenes [113], and paclitaxel-loaded poly(d,l-lactide)/polyethylene glycol (PLA/PEG) micro/nanofibers [114] were reported after testing on the CAM model. A recent study investigated the tumor uptake of modified viral nanoparticles using high-resolution *in vivo* imaging techniques within the CAM model featuring human tumor xenografts. This study made significant advancements by enhancing the CAM model, enabling continuous, high-resolution visualization of nanoparticle distribution within the tumor and its surrounding blood vessels [115].

Recent advancements include studies on hybrid nanostructures that combine chemotherapy with photothermal therapy. For example, polydopamine nanoparticles have been shown to synergize chemotherapy and photothermal therapy for liver cancer treatment, demonstrating efficacy in both CAM and *in vitro* models [116]. Similarly, hybrid nano-architectures loaded with metal complexes have been explored for the co-chemotherapy of head and neck carcinomas, emphasizing the versatility of CAM assays for evaluating complex nanomaterial-based therapies [117]. Furthermore, CAM modeling has been employed to explore platinum-based drugs and their impact on tumor metabolism, revealing insights into their antitumor and antimetastatic activity as well as their influence on amino acid metabolism [118].

Alongside nanocarriers, hydrogels play a crucial role in the delivery of anti-cancer medications. The antitumor and anti-angiogenic effects of bevacizumab-loaded alginate hydrogel were effectively demonstrated using the CAM model featuring tumor grafts [119].

In summary, the CAM model serves as an exceptional tool for studying chemotherapy delivery, a fact corroborated by numerous publications from various independent research teams.

3.6.3 Harnessing the Capabilities of the CAM Model in Radiation Biology and Oncology Research

The CAM model is an exceptional animal model that creates an optimal external environment for tumor growth. As a result, it provides a valuable platform for assessing tumor responses to ionizing radiation and variations in tumor vascularity (Fig. 6, Ref. [68]) [120]. Conversely, the tumor CAM model presents a cost-effective and more practical alternative for radiation oncology research when compared to the pricier mouse models. The National Institutes of Health (NIH) and the IACUC have determined that chick embryos do not possess the capacity to perceive pain before the 14th

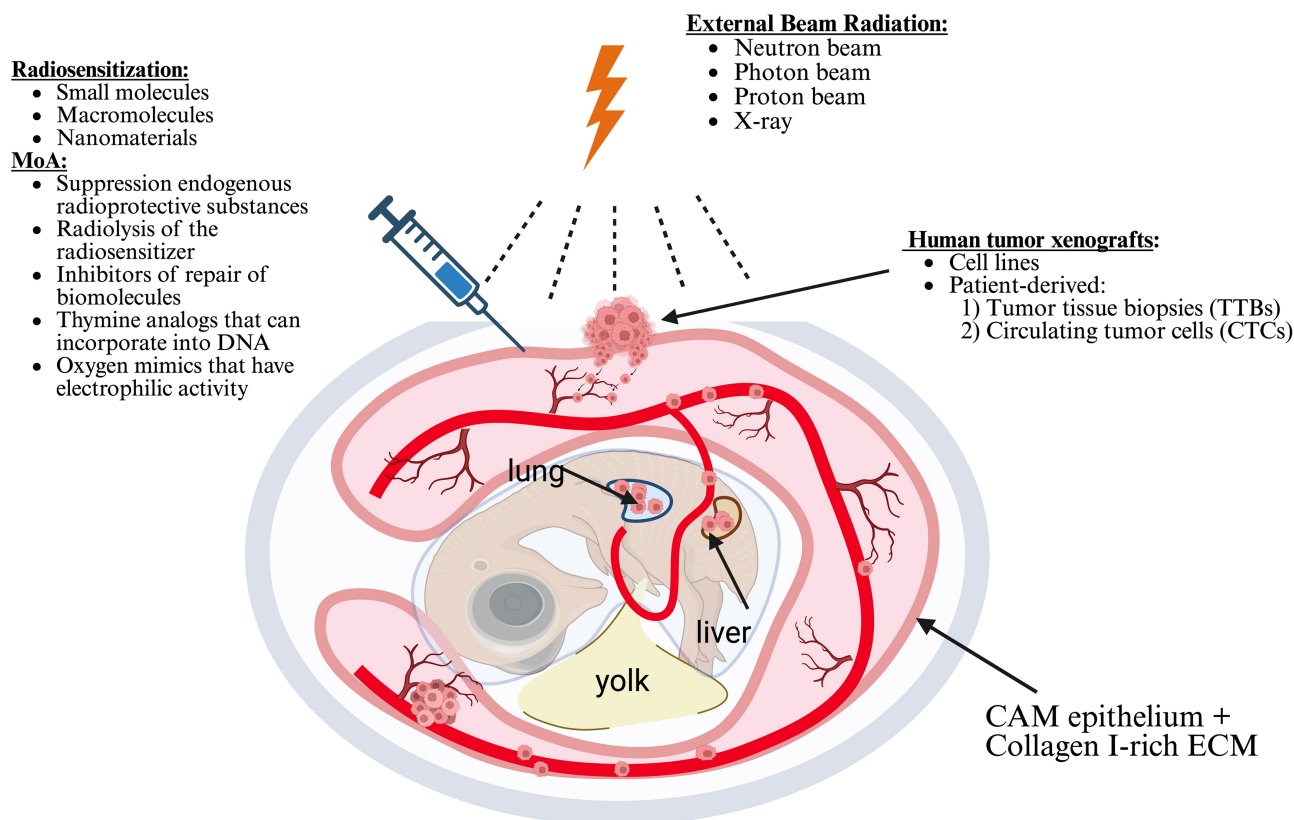


Fig. 6. Schematic diagram of radiotherapy and chemoradiotherapy in a tumor chicken embryonic model. Drawn based on [68]. Created with [BioRender.com](https://www.biorender.com/).

day of gestation [121,122], which allows their use without ethical concerns. These benefits render CAM models a highly appealing choice in cancer research, particularly for evaluating the efficacy of cancer radio- and chemotherapy, conducting drug screenings, and deepening our understanding of the molecular mechanisms underlying tumor growth and metastasis.

The efficacy of radiosensitizers in cancer cells can also be investigated using the CAM model. For instance, the effectiveness of etanidazole has been successfully assessed on the EMT6 mouse breast cancer cell line and the Colon26 mouse colorectal cancer cell line utilizing chicken embryos [123]. The combination of etanidazole and 8 Gy X-ray irradiation results in a significant 35% reduction in the growth of solid tumors in the CAM model, highlighting the radiosensitizing effects of etanidazole [124]. It has also been shown that implanting C6 glioma cells into a CAM model subjected to X-ray radiation leads to a significantly higher rate of neoangiogenesis in tumors compared to unirradiated controls. This observation appears to be closely linked to the tumors' resilience against radiation therapy [125].

A recent study reveals that various radiation modes inflict varying levels of damage on CAM capillaries. Specifically, a radiation dose of 20 Gy leads to the collapse of the capillary network when the CAM is subjected to a

continuous radiation pattern. In contrast, the alterations caused by the microbeam radiation mode, even at a higher dose of 200 Gy, remain reversible. This finding underscores the promising potential of microbeam radiation as an innovative treatment approach for antitumor angiogenesis [126]. The research conducted by Kardamakis and colleagues [125] reveals that combining paclitaxel with ionizing radiation significantly inhibits neoangiogenesis in the CAM model. This finding opens up exciting new avenues for enhancing tumor radiotherapy.

Recent research suggests that the CAM model serves as an effective tool for the preclinical evaluation of chemoradiotherapy using small molecule signal transduction inhibitors. The combination of radiotherapy with sunitinib, a receptor tyrosine kinase (RTK) inhibitor that targets multiple angiogenesis-related receptors, faces clinical challenges due to infrequent but severe side effects and inconsistent clinical benefits. To enhance the therapeutic potential of this combination treatment approach, various scheduling regimens and dose modifications for both sunitinib and radiotherapy were explored within the CAM model. Sunitinib, which was administered over four days at a dosage of 32.5 mg/kg per day, significantly enhanced the inhibitory impact of ionizing radiation (4 Gy) on angiogenesis while also effectively suppressing tumor growth. When radiation

therapy was administered prior to sunitinib, it nearly eradicated tumor growth. In contrast, administering radiation therapy concurrently was less effective, and applying it after sunitinib yielded no further impact on tumor progression. Additionally, with optimal scheduling, it was possible to reduce the sunitinib dosage by 50% while still achieving similar antitumor effects. The CAM model conclusively shows that optimal treatment regimens allow for reduced doses of angiogenesis inhibitors, potentially minimizing the side effects associated with combination therapy in clinical practice [64].

The chick embryo presents a promising alternative model for assessing acute drug toxicity, offering an efficient preliminary screening option before conducting comprehensive toxicological studies in rodents during the development of anticancer drugs [12]. The *in ovo* carcinogenicity assay (IOCA) has emerged as an efficient and cost-effective non-animal approach for testing carcinogenicity and conducting experimental investigations into the mechanisms of carcinogenesis [127]. Chemically induced alterations in mitochondrial DNA (mtDNA) could serve as a significant marker for assessing the carcinogenic potential of various substances, as the mitochondrial genome tends to exhibit greater sensitivity to DNA-damaging effects than nuclear DNA. CAM treatment with diethylnitrosamine caused a dose-dependent transition in mtDNA conformation, shifting from a supercoiled to a relaxed state. This change suggests a potential induction of single-strand breaks [128,129]. Understanding the mechanisms behind mitochondrial impairment could provide valuable insights into potential therapeutic targets for age-associated conditions and cancers.

Briest *et al.* [128] effectively demonstrated the therapeutic potential of DNA damage modifiers used alongside radiation therapy for well-differentiated gastroenteropancreatic neuroendocrine tumors in a CAM model. Che and colleagues [129] developed a groundbreaking biodegradable ultra-small nanostructure (NAs-cisPt) encapsulated in a silica shell, which contains a cisplatin prodrug, serving as a sensitizer for radiotherapy in a CAM model. Their findings revealed that the integration of NAs-cisPt with radiotherapy significantly amplified DNA damage and promoted apoptosis in pancreatic ductal adenocarcinoma (PDAC) cell lines, ultimately leading to a reduction in tumor growth rate [130]. Daniluk *et al.* [131] demonstrated that employing graphene as a carrier for melittin (M), a potent and toxic component of bee venom, significantly improved the targeting of cancer cells in a CAM model of breast cancer cell lines. This approach not only amplifies DNA damage in cancer cells but also elevates the levels of proteins linked to the suppression of tumor progression [132].

Polyvinylpyrrolidone-coated platinum nanoparticles (PtNPs) emerge as highly promising alternatives to cisplatin (CDDP), demonstrating remarkable efficacy in inhibiting tumor growth and metastasis in the MDA-MB-231 breast

cancer xenograft using the CAM model. This study highlights the CAM assay as an essential model, showcasing the ability of PtNPs to inhibit angiogenesis, reduce amino acid levels, and disrupt different sets of enzymes of the tricarboxylic acid (TCA) cycle [118].

In conclusion, the CAM model serves as an effective model for assessing reoxygenation, DNA damage repair, cell cycle redistribution, cellular radiosensitivity, and cellular repopulation—five biological concepts that are crucial for accurately predicting the responses of both tumor and normal tissues to radiotherapy and chemoradiotherapy. A recent systematic review has identified the CAM assay as a groundbreaking technique that offers a straightforward and efficient method for inducing tumors, evaluating treatment efficacy, conducting metastasis research, performing patient biopsy grafts, and advancing personalized medicine [133].

4. CAM Model for Cancer-related miRNA Studies

4.1 CAM Model to Assess miRNA Functions in Oncogenesis

MicroRNAs are small non-coding RNA molecules with a size range of 18–25 nucleotides [130]. These molecules regulate the expression of multiple protein-encoding genes at the post-transcriptional level through repression of translation with consequent mRNA destabilization. MicroRNAs are involved in every biological process and deregulation of their expression also contributes to oncogenesis [132,134,135]. Research on microRNAs' roles in carcinogenesis has been very intensive for the last two decades [136]. The CAM model is a very convenient *in vivo* tool for this research. Research publications, where the CAM model is applied to study functions of microRNAs in cancer, have begun to emerge. For example, the anti-angiogenesis effects of miR-1 overexpression were demonstrated on the CAM model with xenografts of prostate cancer cells [137]. Similarly, anti-angiogenic and anti-metastatic effects of miR-101 overexpression in nasopharyngeal carcinoma cells were detected in the CAM model with xenografts of nasopharyngeal carcinoma cells [138]. The CAM model was also applied to confirm a reduction in angiogenic properties of ovarian cancer cells upon miR-21 activity inhibition. Particularly, a reduction in pro-angiogenic effects of conditioned medium from ovarian cancer cells upon miR-21 inhibition was detected in the CAM model of angiogenesis [139]. These studies clearly demonstrate the usefulness of the avian CAM model to decipher mechanisms, which involve microRNAs in carcinogenesis.

4.2 CAM Model to Study miRNA-Mediated Tumor Chemoresistance

Multiple studies [140,141] have linked deregulation in the expression of microRNAs in cancer to cancer chemore-

sistance. One such study identified an association between the upregulation of miR-153 expression and colorectal cancer aggressiveness. Further functional assays demonstrated that miR-153-mediated down-regulation of Forkhead box O3a (*FOXO3a*) expression increased the invasiveness of colorectal cancer cells as well as their resistance to oxaliplatin and cisplatin [142]. It is also published that alterations in let-7i, miR-16, and miR-21 levels significantly sensitize cancer cells to several anticancer drugs. In addition, a list of about thirty different microRNAs, which are associated with cancer chemosensitivity, is reported in the same study [143].

Mechanisms behind microRNA effects on cancer cell chemoresistance were discovered for some microRNAs. For example, inhibition of miR-106a in the ovarian cancer OVCAR3 cell line upregulates the programmed cell death protein 4 (*PDCD4*) gene expression, which sensitizes this cell line to the cisplatin treatment [144]. It is also demonstrated that miR-451 upregulation in doxorubicin-resistant Michigan Cancer Foundation-7 (MCF-7) breast cancer cells restores the doxorubicin sensitivity of these cells. Doxorubicin sensitivity in breast cancer cells is restored through expression upregulation of a miR-451 target—the Multiple drug resistance 1 (*MDR1*) gene encoding the P-glycoprotein [145], which belongs to the ATP-binding cassette (ABC) family of drug transporter proteins [146]. As we already discussed in Section 3.6, many chemotherapeutic agents kill tumor cells by inducing DNA damage, which leads to apoptosis in these cells. Noteworthy, elevated expression of the high mobility group A2 (HMGA2) protein triggers a genotoxic response to DNA double-strand breaks in cells. High expression of HMGA2 in tumor cells increases the doxorubicin sensitivity of these cells [147]. Consistently, miRNA-98 expression upregulation reduces the expression of HMGA2, which is a miR-98 target, and sensitizes carcinoma cells to doxorubicin treatment [148].

miRNA-338-3p appears to play a dual role in cancer chemotherapy, functioning either as a tumor promoter or as a tumor suppressor. Experimental evidence highlights its anti-tumor properties, suggesting that enhancing the expression of miRNA-338-3p is crucial for effective cancer treatment. Consequently, elevating the expression of miRNA-338-3p is crucial for developing effective cancer treatments. Although the recent CAM assay-based study did not directly test the hypothesis that the overexpression of MicroRNA-338-3p is linked to increased sensitivity to radiotherapy and chemotherapy [149], the findings highlight the role of miR-338-3p in promoting angiogenesis in hepatocellular carcinoma by targeting *MACC1*, β -catenin, and VEGF [150].

A groundbreaking pharmacological study has showcased a novel bioluminescent CAM model that enhances the speed of *in vivo* screening for therapeutic agents while also allowing for the examination of potential epigenetic factors influencing chemosensitivity [151]. Notably, this

pioneering research presents the first evidence that primary cancer cells engrafted in the CAM can be effectively treated with both standard chemotherapy and targeted agents. This study focused on a model characterized by the overexpression of c-Met due to copy number gain, a condition found in approximately 45% of patients with PDAC [152]. The combination of the c-Met/anaplastic lymphoma kinase (ALK) inhibitor crizotinib and gemcitabine resulted in a remarkable decrease in the growth of PDAC CAM tumors. Furthermore, the investigation into miRNA modulation confirmed two synergistic mechanisms: the downregulation of miR-21 and the upregulation of miR-155. The expression of miR-21 has been linked to gemcitabine resistance, resulting in a decrease in apoptosis induction [153]. Consequently, the blockade of c-Met by crizotinib, which has been shown to downregulate miR-21 in NSCLC models [154,155], may enhance the cytotoxic effects of gemcitabine. Conversely, the increase in miR-155 levels may be attributed to a feedback mechanism triggered by the inhibition of c-Met, which is crucial for the interactions between tumors and fibroblasts. Recent research indicates that PDAC cells may stimulate nearby normal fibroblasts through the release of microvesicles rich in miR-155 [156]. These discoveries established the groundwork for a research pipeline that facilitates the effective cultivation of tumors derived from pre-treatment biopsies on the CAM. This streamlined workflow is designed to maximize productivity while minimizing the need for extensive resources and facilities. This approach has the potential to provide insights into specific tumor characteristics within just 2 to 3 weeks. It can pave the way for personalized cancer treatments and testing drug sensitivity in individual patients, while also offering a deeper understanding of the miR-mediated mechanisms behind the effects of the therapies being tested. In perspective, it could significantly accelerate *in vivo* screening processes for anticancer drugs, ultimately reducing both development time and costs for innovative compounds.

Overall, the effects of microRNAs on cancer chemoresistance and the mechanisms underlying these effects attract significant interest from cancer researchers. We expect the CAM model, which is ideally suited for studying both microRNA and chemoresistance, to become a standard *in vivo* model for this type of research.

5. Recent Advances in the CAM Model for Preclinical Drug Development

The CAM model has gained significant traction in assessing anti-cancer therapies, due to its remarkable capability to replicate *in vivo* tumor growth and drug responses. In ovarian cancer research, Chitcholtan *et al.* (2024) [157] demonstrated the inhibitory effects of resveratrol on tumor growth using a CAM model implanted with ovarian cancer cells. Their findings not only validated the therapeutic potential of resveratrol but also showcased the CAM model's capability to provide rapid and cost-effective in-

sights into drug efficacy in a dynamic tumor microenvironment [157]. Similarly, Wang *et al.* (2024) [158] applied the CAM model to study Cluster of Differentiation 19 (CD19)-targeting Chimeric Antigen Receptor T cells (CAR-T cells) for lymphoma treatment. Their innovative approach demonstrated the CAM model's utility in immunotherapy research, highlighting its ability to assess both therapeutic efficacy and tolerability in a preclinical setting [158]. These studies underscore the CAM model's growing influence as a reliable tool in preclinical drug screening, facilitating the transition from *in vitro* studies to clinical trials.

A significant advancement lies in the integration of the CAM assay with innovative platforms like the IKOSA platform (<https://www.kolaido.com/our-offerings/ikosa-ai/>), designed for quantifying vasculature. This platform harnesses artificial intelligence, enabling a more comprehensive and accurate assessment of antiangiogenic strategies, especially in the area of cancer research [159]. This method not only amplifies the assay's effectiveness but also plays a crucial role in minimizing animal usage in research by delivering more accurate data from *ex ovo* models. A significant advancement is to develop a culture method that harnesses the advantages of both *in ovo* and *ex ovo* techniques. Recently, Huang *et al.* [160] introduced an innovative cubic artificial shell that effectively overcomes the limitations of existing methods. The shell, constructed from polycarbonate and PDMS, features intricately designed microchannels on its surface that guide CAM blood vessels to grow precisely along the intended pathways dictated by these channels. The embryo developed a form reminiscent of that found within a natural shell, which significantly enhanced its survival rate to an impressive 80% after 14 days of incubation. In contrast to the *ovo* method, this approach features a fully transparent structure that allows for a broader view of the CAM, akin to the *ex ovo* method. This innovative approach holds great promise for tissue engineering by facilitating the delivery of drugs, degradation products, or even the testing of hydrogels. It allows for a thorough evaluation of their impact on angiogenesis through strategically placed openings in the external layer of the artificial shell.

6. CAM Model Limitations

Limitations of the CAM model should be taken into account along with its advantages, when the model is considered for a particular research study. These limitations can be divided into two groups: technical and fundamental. The technical limitations include the following: (1) vulnerability of the fertilized egg and its sensitivity to environmental factors such as temperature and humidity; (2) susceptibility of the chicken embryo to bacterial infection; (3) density and quick development of the CAM vascular network, which complicates studies of pro-angiogenic factors and effects; (4) limited repertoire of available reagents such as primers, cytokines, and antibodies, which are suit-

able for CAM and chicken embryo studies [32,161,162]. The obvious fact that chickens stand evolutionary farther from humans than mice is one of the most important fundamental limitations of the CAM model. Profound differences in embryo versus adult physiologies should be regarded as the other fundamental limitation of this model. In addition, the CAM model permits only short-term experiments and cannot be applied to study long-term processes such as cancer recurrence and therapy resistance development. The limited duration of the experimental timeline imposes constraints on our ability to conduct a thorough long-term evaluation of cancer immunity in the chick embryo *in ovo* model. This limitation particularly hinders our understanding of the nuanced dual role that the immune system plays in the process of immunoediting. Consequently, without extended observation periods, we cannot fully assess how immune responses evolve over time in the presence of tumors. However, research conducted on animal models has demonstrated that tumor growth during the first seven days following engraftment is a strong indicator of overall survival and the effectiveness of treatment [163]. Furthermore, the short experimental time frame prevents the CAM model from experiencing substantial genetic evolution, ensuring that the harvested CAM tumor remains remarkably similar to the original tumor. In-depth studies are essential to define and assess appropriate protocols for investigating cancer immunity *in ovo*. Critical aspects that require clarification include the optimal timing for immune cell grafting, the most effective administration routes, the co-administration of cytokines and growth factors, and the variations depending on cell lineage. One potential solution that merits consideration involves the use of additional irradiation aimed at targeting and destroying the developing avian immune system. This approach could help prevent the maturation of the immune response in birds, ultimately extending the timeframe of immuno-oncology research. However, it is important to evaluate thoroughly this method to understand its implications and effectiveness in practical applications. An additional limitation is that while the designated compound can be detected *in situ* in tumor tissues and in the circulation of chickens following topical application, this does not accurately represent the systemic turnover and modification of the drug. Conversely, due to the isolated nature of the CAM, the half-life of various molecules, including small peptides, is significantly extended compared to mammalian models. This extended half-life enables the effective study of compounds that are available in limited quantities.

Despite these challenges, the CAM model remains a highly versatile and cost-effective system, particularly for preliminary investigations of cancer biology, angiogenesis, and drug screening. Its accessibility and rapid experimental timelines make it an attractive alternative to more complex *in vivo* models, especially during proof-of-mechanism testing or for high-throughput screening. Beyond improving

the accuracy of angiogenesis assessments, advanced imaging technologies could be employed to enhance the visualization and dynamic monitoring of tumor cell metastasis patterns *in vivo*, and the development of reagents and tools specific to chicken embryos could expand the scope of molecular studies. Additionally, integrating the CAM model with organoids or mammalian models offers the added benefit of accelerating research processes by bridging the gap between *in vitro* and *in vivo* mouse experiments. This integration not only provides a seamless transition but also allows for the early validation of hypotheses, reducing reliance on more resource-intensive mammalian models during the initial experimental stages. Such an approach leverages the complementary strengths of each model, facilitating a more efficient and comprehensive exploration of complex biological phenomena, including post-treatment tumor recurrence, drug resistance, and distant metastasis. The CAM model of human-derived tumor cells allows transcriptomic analysis by human and chicken gene expression to determine the genetic profile of the host stroma and tumor [48]. Since neither genetic background nor gender of the chicken embryo model is standardized in any way, this model is expected to be more robust and closer to the natural genetic heterogeneity of the human cancer patient population. We can assume that this should contribute to the reproducibility of preclinical data obtained on the chicken embryo in clinical trials, although such an assumption requires rigorous verification.

In summary, while the CAM model has inherent limitations, its unique advantages justify continued exploration and refinement. Future advancements in methodologies and cross-species validation could further enhance the applicability and reliability of this model, solidifying its role as a valuable tool in preclinical and translational research.

7. Conclusions and Prospects

The CAM tumor xenograft-bearing model is an inexpensive *in vivo* model suitable for both basic cancer research and the development of cancer therapies. Cancer research directions, which can be pursued with the chicken CAM model, are numerous and discussed in this review. The low costs of this *in vivo* model permit large experimental and control groups, which creates high statistical power of CAM model experiments. The short experimental time allows quick repeats of the CAM experiments, which further enhances the robustness of generated experimental data. The lack of nociception in the early chicken embryo drastically simplifies compliance with the ethical 3R regulations. A relative disadvantage of the CAM is the absence of direct comparisons between mouse models and CAM models for specific applications, which complicates the validation and adoption of these models in research and development processes. In addition, researchers adhering to traditional approaches perceive enhanced hetero-

geneity and reduced standardization of the CAM as a disadvantage. Together, these issues significantly impede the progress and effectiveness of CAM implementations in various scientific fields. In this context, our current review wholeheartedly endorses the invaluable proposal to establish a dedicated organization for CAM researchers and regulators within a CAM society [164]. This initiative aims to effectively tackle the challenges identified in CAM research. The discussed advantages of the CAM model over the other *in vivo* models suggest that applications of the *in ovo* model will become more prominent in cancer research. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis conducted in a recent proteomic study revealed significant enrichment in terms related to endocytosis, focal adhesion, and gap junctions [165]. These findings underscore the critical structure-function relationships among the constituents of CAM proteins, suggesting their potential to broaden biomedical applications of CAM models [166,167]. The recent comprehensive review suggests that the integration of various models can be a highly advantageous strategy to enhance both basic and translational research efforts [168]. The discussed advantages of the chick and ostrich CAM models compared to other *in vivo* models indicate that the *in ovo* model is poised for increased utilization across various research fields, including cancer studies, biomaterials evaluation, tissue engineering, drug delivery and toxicity assessment, dental biology, and regenerative medicine. The avian embryo model is anticipated to significantly enhance the validity of *in vivo* research data by complementing existing studies.

Drawing from this review, we suggest categorizing the innovative technological applications of CAM models into three key areas.

(1) Molecular and genetic research. This category encompasses studies that leverage the CAM model to investigate molecular and genetic mechanisms underlying cancer development and progression. The CAM platform allows rapid genetic manipulation and high-throughput screening, providing distinct advantages over traditional animal models. Notably, recent advancements have expanded the CAM's application in exploring miRNA-mediated tumor regulation, gene silencing, and oncogene/tumor suppressor gene functions. Researchers have used the CAM model to quickly validate miRNA targets *in vivo*, confirming their impact on tumor growth, spread, and resistance to chemotherapy. The CAM is an ideal model for molecular oncology research due to its easy genetic manipulation and short experimental timeframe, providing a unique, efficient, and cost-effective alternative to mammalian systems.

(2) Tumor-microenvironment interaction studies. The CAM model effectively mimics important features of the tumor microenvironment, such as blood vessels, ECM components, and interactions between stromal and tumor cells, which are often poorly represented in traditional *in vitro* models like two-dimensional cell cultures or even three-

dimensional matrices (e.g., organoids and spheroids). Conventional *in vitro* systems do not replicate the complexity of natural ECM structures and cellular interactions, and mouse models are costly and slow. CAM models provide a vascularized and accessible microenvironment, allowing for quick observation of tumor-stroma interactions, ECM remodeling, angiogenesis, and tumor cell invasion. These attributes improve studies on the interaction between tumor cells, stromal cells, and ECM, leading to a better understanding of metastasis and chemoresistance.

(3) Immuno-oncology and therapeutic screening studies. Effective immunotherapy research requires a healthy and functional blood system to transport immune cells, nutrients, and treatments to tumors. Standard *in vitro* assays do not capture vascular complexity well, and traditional mouse models, while more relevant to physiology, can be costly, ethically problematic, and time-consuming. The CAM model circumvents these limitations by inherently possessing a robust, functional vascular system accessible for intravascular administration of therapeutic agents, including immunotherapeutic drugs and engineered immune cells (e.g., CAR-T cells). Recent studies highlight the CAM's potential for evaluating immunotherapy efficacy, immune cell infiltration, and real-time therapeutic responses [51,77–81]. The CAM offers a fast and cost-effective way to screen immuno-oncology drugs, helping to connect lab studies with clinical applications.

Despite the advantages of CAM models, such as rapid results, cost efficiency, and strong statistical power, challenges related to experimental variation and lack of standardization have restricted their use. International collaboration is needed to create uniform CAM methodologies and regulations. This will help overcome current challenges ensuring consistency in cancer research and clinical studies. The CAM model has significant potential to advance oncology through its integration with precision medicine, such as PDX and novel imaging technologies. Recent proteomic study [165] has linked the CAM to key cancer pathways like endocytosis, focal adhesion, and gap junction signaling, highlighting its importance in understanding cancer biology and treatment responses. Integrating CAM-based platforms with cutting-edge technologies is crucial for enhancing the impact and translational value of future preclinical and clinical research.

Author Contributions

YW—Conceptualization, Reference Collection and Analysis, Writing—Original Draft Preparation, Writing—Review & Editing. WX—Software, Visualization, The acquisition of literature, Writing—Review & Editing. MP—Design of the work, Writing—Review & Editing DNA—damaging therapeutics in cancer section. DVK—The acquisition of literature, Project Administration, Funding Acquisition, Writing—Review & Editing. SL—Conceptualization, Project Administration, Resources, Su-

pervision, Writing—Review & Editing. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The micrograph presented in this study on Fig. 3 was obtained from chick embryos at day 9 of gestation. According to the guidelines of the National Institutes of Health (NIH) and IACUC, chick embryos do not develop the capacity to perceive pain before day 14 of gestation; therefore, the use of embryos at this stage does not require ethical approval.

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Kain KH, Miller JWI, Jones-Paris CR, Thomason RT, Lewis JD, Bader DM, *et al.* The chick embryo as an expanding experimental model for cancer and cardiovascular research. *Developmental Dynamics: an Official Publication of the American Association of Anatomists.* 2014; 243: 216–228. <https://doi.org/10.1002/dvdy.24093>.
- [2] Mason I. The avian embryo: an overview. In Sharpe PT, Mason I (eds.) *Molecular embryology: Methods and protocols* (pp. 223–230). Humana Press: Totowa, New Jersey. 2009. https://doi.org/10.1007/978-1-60327-483-8_14.
- [3] Chambers AF, Shafir R, Ling V. A model system for studying metastasis using the embryonic chick. *Cancer Research.* 1982; 42: 4018–4025.
- [4] Ossowski L, Reich E. Changes in malignant phenotype of a human carcinoma conditioned by growth environment. *Cell.* 1983; 33: 323–333. [https://doi.org/10.1016/0092-8674\(83\)90414-2](https://doi.org/10.1016/0092-8674(83)90414-2).
- [5] Dagg CP, Karnofsky DA, Toolan HW, Roddy J. Serial passage of human tumors in chick embryo: growth inhibition by nitrogen mustard. *Proceedings of the Society for Experimental Biology and Medicine.* Society for Experimental Biology and Medicine (New York, N.Y.). 1954; 87: 223–227. <https://doi.org/10.3181/00379727-87-21341>.
- [6] Rous P. A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells. *The Journal of Experimental Medicine.* 1911; 13: 397–411. <https://doi.org/10.1084/jem.13.4.397>.
- [7] Murphy JB. Transplantability of Tissues to the Embryo of Foreign Species: Its Bearing on Questions of Tissue Specificity and Tumor Immunity. *The Journal of Experimental Medicine.* 1913; 17: 482–493. <https://doi.org/10.1084/jem.17.4.482>.
- [8] Woodruff AM, Goodpasture EW. The Susceptibility of the Chorio-Allantoic Membrane of Chick Embryos to Infection with

the Fowl-Pox Virus. The American Journal of Pathology. 1931; 7: 209–222.5.

- [9] Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Journal of Morphology. 1951; 88: 49–92.
- [10] Folkman J. Proceedings: Tumor angiogenesis factor. Cancer Research. 1974; 34: 2109–2113.
- [11] Chambers AF, MacDonald IC, Schmidt EE, Koop S, Morris VL, Khokha R, *et al.* Steps in tumor metastasis: new concepts from intravital videomicroscopy. Cancer Metastasis Reviews. 1995; 14: 279–301. <https://doi.org/10.1007/BF00690599>.
- [12] Kue CS, Tan KY, Lam ML, Lee HB. Chick embryo chorioallantoic membrane (CAM): an alternative predictive model in acute toxicological studies for anti-cancer drugs. Experimental Animals. 2015; 64: 129–138. <https://doi.org/10.1538/expanim.14-0059>.
- [13] Vu BT, Shahin SA, Croissant J, Fatiev Y, Matsumoto K, Le-Hoang Doan T, *et al.* Chick chorioallantoic membrane assay as an in vivo model to study the effect of nanoparticle-based anticancer drugs in ovarian cancer. Scientific Reports. 2018; 8: 8524. <https://doi.org/10.1038/s41598-018-25573-8>.
- [14] Pawlikowska P, Tayoun T, Oulhen M, Faugueroux V, Rouffiac V, Aberlenc A, *et al.* Exploitation of the chick embryo chorioallantoic membrane (CAM) as a platform for anti-metastatic drug testing. Scientific Reports. 2020; 10: 16876. <https://doi.org/10.1038/s41598-020-73632-w>.
- [15] Ribatti D. The Chick Embryo Chorioallantoic Membrane in the Study of Angiogenesis and Metastasis: The CAM assay in the study of angiogenesis and metastasis. Springer Science & Business Media: Berlin. 2010. <https://doi.org/10.1007/978-90-481-3845-6>.
- [16] Wang Y, Rousset X, Prunier C, Garcia P, Dosda E, Lepilus E, *et al.* PD-1/PD-L1 Checkpoint Inhibitors Are Active in the Chicken Embryo Model and Show Antitumor Efficacy *In Ovo*. Cancers. 2022; 14: 3095. <https://doi.org/10.3390/cancer14133095>.
- [17] Ribatti D. The chick embryo chorioallantoic membrane as an experimental model to study immune checkpoint inhibitors. Internal and Emergency Medicine. 2025. (Online ahead of print) <https://doi.org/10.1007/s11739-025-03904-8>.
- [18] Nipper AJ, Warren EAK, Liao KS, Liu HC, Michikawa C, Porter CE, *et al.* Chick Embryo Chorioallantoic Membrane as a Platform for Assessing the In Vivo Efficacy of Chimeric Antigen Receptor T-cell Therapy in Solid Tumors. ImmunoHorizons. 2024; 8: 598–605. <https://doi.org/10.4049/immunohorizons.2400059>.
- [19] DeBord LC, Pathak RR, Villaneuva M, Liu HC, Harrington DA, Yu W, *et al.* The chick chorioallantoic membrane (CAM) as a versatile patient-derived xenograft (PDX) platform for precision medicine and preclinical research. American Journal of Cancer Research. 2018; 8: 1642–1660.
- [20] Pion E, Karnosky J, Boscheck S, Wagner BJ, Schmidt KM, Brunner SM, *et al.* 3D In Vivo Models for Translational Research on Pancreatic Cancer: The Chorioallantoic Membrane (CAM) Model. Cancers. 2022; 14: 3733. <https://doi.org/10.3390/cancers14153733>.
- [21] Eckrich J, Kugler P, Buhr CR, Ernst BP, Mendler S, Baumgart J, *et al.* Monitoring of tumor growth and vascularization with repetitive ultrasonography in the chicken chorioallantoic-membrane-assay. Scientific Reports. 2020; 10: 18585. <https://doi.org/10.1038/s41598-020-75660-y>.
- [22] Russell WMS, Burch RL. The principles of humane experimental technique. 1959. Available at: https://hero.epa.gov/hero/index.cfm/reference/details/reference_id/3512337 (Accessed: 20 January 2025).
- [23] Ribatti D, Annesse T. Chick embryo in experimental embryology and more. Pathology, Research and Practice. 2023; 245: 154478. <https://doi.org/10.1016/j.prp.2023.154478>.
- [24] Sarogni P, Mapanao AK, Marchetti S, Kusmic C, Voliani V. A Standard Protocol for the Production and Bioevaluation of Ethical *In Vivo* Models of HPV-Negative Head and Neck Squamous Cell Carcinoma. ACS Pharmacology & Translational Science. 2021; 4: 1227–1234. <https://doi.org/10.1021/acspstsci.1c00083>.
- [25] Sharrow AC, Ishihara M, Hu J, Kim IH, Wu L. Using the Chicken Chorioallantoic Membrane In Vivo Model to Study Gynecological and Urological Cancers. Journal of Visualized Experiments: JoVE. 2020; 10.3791/60651. <https://doi.org/10.3791/60651>.
- [26] Redmond C. When is an alternative not an alternative? Supporting progress for absolute replacement of animals in science. Animal experimentation: working towards a paradigm change (pp. 654–672). Brill: Leiden. 2019. https://doi.org/10.1163/9789004391192_028.
- [27] Wu T, Yu GY, Xiao J, Yan C, Kurihara H, Li YF, *et al.* Fostering efficacy and toxicity evaluation of traditional Chinese medicine and natural products: Chick embryo as a high throughput model bridging in vitro and in vivo studies. Pharmacological Research. 2018; 133: 21–34. <https://doi.org/10.1016/j.phrs.2018.04.011>.
- [28] Misericocchi G, Spadazzi C, Calpona S, De Rosa F, Usai A, De Vita A, *et al.* Precision Medicine in Head and Neck Cancers: Genomic and Preclinical Approaches. Journal of Personalized Medicine. 2022; 12: 854. <https://doi.org/10.3390/jpm12060854>.
- [29] Hilbrig C, Löffler J, Fischer G, Scheidhauer E, Solbach C, Huber-Lang M, *et al.* Evaluation of the EPR Effect in the CAM-Model by Molecular Imaging with MRI and PET Using ⁸⁹Zr-Labeled HSA. Cancers. 2023; 15: 1126. <https://doi.org/10.3390/cancers15041126>.
- [30] Pinto MT, Ribeiro AS, Conde I, Carvalho R, Paredes J. The Chick Chorioallantoic Membrane Model: A New In Vivo Tool to Evaluate Breast Cancer Stem Cell Activity. International Journal of Molecular Sciences. 2021; 22: 334. <https://doi.org/10.3390/ijms22010334>.
- [31] Rousset X, Maillet D, Grolleau E, Barthelemy D, Calattini S, Brevet M, *et al.* Embryonated Chicken Tumor Xenografts Derived from Circulating Tumor Cells as a Relevant Model to Study Metastatic Dissemination: A Proof of Concept. Cancers. 2022; 14: 4085. <https://doi.org/10.3390/cancers14174085>.
- [32] Nowak-Sliwinska P, Segura T, Iruela-Arispe ML. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. Angiogenesis. 2014; 17: 779–804. <https://doi.org/10.1007/s10456-014-9440-7>.
- [33] Makanya AN, Dimova I, Koller T, Styp-Rekowska B, Djonov V. Dynamics of the Developing Chick Chorioallantoic Membrane Assessed by Stereology, Allometry, Immunohistochemistry and Molecular Analysis. PloS One. 2016; 11: e0152821. <https://doi.org/10.1371/journal.pone.0152821>.
- [34] Vimalraj S, Saravanan S, Anuradha D, Chatterjee S. Models to investigate intussusceptive angiogenesis: A special note on CRISPR/Cas9 based system in zebrafish. International Journal of Biological Macromolecules. 2019; 123: 1229–1240. <https://doi.org/10.1016/j.ijbiomac.2018.11.164>.
- [35] Schlatter P, König MF, Karlsson LM, Burri PH. Quantitative study of intussusceptive capillary growth in the chorioallantoic membrane (CAM) of the chicken embryo. Microvascular Research. 1997; 54: 65–73. <https://doi.org/10.1006/mvre.1997.2022>.
- [36] Parsons-Wingerter P, McKay TL, Leontiev D, Vickerman MB, Condric TK, Dicorleto PE. Lymphangiogenesis by blind-ended vessel sprouting is concurrent with hemangiogenesis by vascular splitting. The Anatomical Record. Part A, Discoveries in Molecular, Cellular, and Evolutionary Biology. 2006; 288: 233–247. <https://doi.org/10.1002/ar.a.20309>.
- [37] Oh SJ, Jeltsch MM, Birkenhäger R, McCarthy JE, Weich HA,

- Christ B, *et al.* VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Developmental Biology*. 1997; 188: 96–109. <https://doi.org/10.1006/dbio.1997.8639>.
- [38] Shayan R, Achen MG, Stacker SA. Lymphatic vessels in cancer metastasis: bridging the gaps. *Carcinogenesis*. 2006; 27: 1729–1738. <https://doi.org/10.1093/carcin/bgl031>.
- [39] Alitalo A, Detmar M. Interaction of tumor cells and lymphatic vessels in cancer progression. *Oncogene*. 2012; 31: 4499–4508. <https://doi.org/10.1038/ncr.2011.602>.
- [40] Oliver G, Detmar M. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes & Development*. 2002; 16: 773–783. <https://doi.org/10.1101/gad.975002>.
- [41] Palmer TD, Lewis J, Zijlstra A. Quantitative analysis of cancer metastasis using an avian embryo model. *Journal of Visualized Experiments: JoVE*. 2011; e2815. <https://doi.org/10.3791/2815>.
- [42] MacDonald IC, Schmidt EE, Morris VL, Chambers AF, Groom AC. Intravital videomicroscopy of the chorioallantoic microcirculation: a model system for studying metastasis. *Microvascular Research*. 1992; 44: 185–199. [https://doi.org/10.1016/0026-2862\(92\)90079-5](https://doi.org/10.1016/0026-2862(92)90079-5).
- [43] Ossowski L. In vivo invasion of modified chorioallantoic membrane by tumor cells: the role of cell surface-bound urokinase. *The Journal of Cell Biology*. 1988; 107: 2437–2445. <https://doi.org/10.1083/jcb.107.6.2437>.
- [44] Ossowski L. Plasminogen activator dependent pathways in the dissemination of human tumor cells in the chick embryo. *Cell*. 1988; 52: 321–328. [https://doi.org/10.1016/s0092-8674\(88\)80025-4](https://doi.org/10.1016/s0092-8674(88)80025-4).
- [45] Deryugina EI, Zijlstra A, Partridge JJ, Kupriyanova TA, Madsen MA, Papagiannakopoulos T, *et al.* Unexpected effect of matrix metalloproteinase down-regulation on vascular intravasation and metastasis of human fibrosarcoma cells selected in vivo for high rates of dissemination. *Cancer Research*. 2005; 65: 10959–10969. <https://doi.org/10.1158/0008-5472.CAN-05-2228>.
- [46] Lokman NA, Elder ASF, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *International Journal of Molecular Sciences*. 2012; 13: 9959–9970. <https://doi.org/10.3390/ijms13089959>.
- [47] Freund E, Spadola C, Schmidt A, Privat-Maldonado A, Bogaerts A, Von Woedtke T, *et al.* Risk evaluation of emt and inflammation in metastatic pancreatic cancer cells following plasma treatment. *Frontiers in Physics*. 2020; 8: 569618. <https://doi.org/10.3389/fphy.2020.569618>.
- [48] Ribatti D. The chick embryo chorioallantoic membrane (CAM). A multifaceted experimental model. *Mechanisms of Development*. 2016; 141: 70–77. <https://doi.org/10.1016/j.mod.2016.05.003>.
- [49] Leong HS, Robertson AE, Stoletov K, Leith SJ, Chin CA, Chien AE, *et al.* Invadopodia are required for cancer cell extravasation and are a therapeutic target for metastasis. *Cell Reports*. 2014; 8: 1558–1570. <https://doi.org/10.1016/j.celrep.2014.07.050>.
- [50] Mapanao AK, Che PP, Sarogni P, Sminia P, Giovannetti E, Voliani V. Tumor grafted - chick chorioallantoic membrane as an alternative model for biological cancer research and conventional/nanomaterial-based theranostics evaluation. *Expert Opinion on Drug Metabolism & Toxicology*. 2021; 17: 947–968. <https://doi.org/10.1080/17425255.2021.1879047>.
- [51] Miebach L, Berner J, Bekeschus S. *In ovo* model in cancer research and tumor immunology. *Frontiers in Immunology*. 2022; 13: 1006064. <https://doi.org/10.3389/fimmu.2022.1006064>.
- [52] Mira E, Lacalle RA, Gómez-Moutón C, Leonardo E, Mañes S. Quantitative determination of tumor cell intravasation in a real-time polymerase chain reaction-based assay. *Clinical & Experimental Metastasis*. 2002; 19: 313–318. <https://doi.org/10.1023/a:1015563031769>.
- [53] Zijlstra A, Mellor R, Panzarella G, Aimes RT, Hooper JD, Marchenko ND, *et al.* A quantitative analysis of rate-limiting steps in the metastatic cascade using human-specific real-time polymerase chain reaction. *Cancer Research*. 2002; 62: 7083–7092.
- [54] Kim J, Yu W, Kovalski K, Ossowski L. Requirement for specific proteases in cancer cell intravasation as revealed by a novel semiquantitative PCR-based assay. *Cell*. 1998; 94: 353–362. [https://doi.org/10.1016/s0092-8674\(00\)81478-6](https://doi.org/10.1016/s0092-8674(00)81478-6).
- [55] Palaniappan TK, Šlekienė L, Jonasson AK, Gilthorpe J, Gunhaga L. CAM-Delam: an in vivo approach to visualize and quantify the delamination and invasion capacity of human cancer cells. *Scientific Reports*. 2020; 10: 10472. <https://doi.org/10.1038/s41598-020-67492-7>.
- [56] Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000; 100: 57–70. [https://doi.org/10.1016/s0092-8674\(00\)81683-9](https://doi.org/10.1016/s0092-8674(00)81683-9).
- [57] Heuberger DM, Wolint P, Jang JH, Itani S, Jungraithmayr W, Waschki CF, *et al.* High-Affinity Cu(I)-Chelator with Potential Anti-Tumorigenic Action-A Proof-of-Principle Experimental Study of Human H460 Tumors in the CAM Assay. *Cancers*. 2022; 14: 5122. <https://doi.org/10.3390/cancers14205122>.
- [58] Buschmann J, Heuberger DM, Kivrak Pfiffner F, Wolint P, Jang JH, Jungraithmayr W, *et al.* Probing Vasoreactivity and Hypoxic Phenotype in Different Tumor Grafts Grown on the Chorioallantoic Membrane of the Chicken Embryo In Ovo Using MRI. *Cancers*. 2022; 14: 3114. <https://doi.org/10.3390/cancers14133114>.
- [59] Miller WJ, Kayton ML, Patton A, O'Connor S, He M, Vu H, *et al.* A novel technique for quantifying changes in vascular density, endothelial cell proliferation and protein expression in response to modulators of angiogenesis using the chick chorioallantoic membrane (CAM) assay. *Journal of Translational Medicine*. 2004; 2: 4. <https://doi.org/10.1186/1479-5876-2-4>.
- [60] Oliinyk D, Eigenberger A, Felthaus O, Haerteis S, Prantl L. Chorioallantoic Membrane Assay at the Cross-Roads of Adipose-Tissue-Derived Stem Cell Research. *Cells*. 2023; 12: 592. <https://doi.org/10.3390/cells12040592>.
- [61] Amirian ES, Marquez-Do D, Bondy ML, Scheurer ME. Anti-human-cytomegalovirus immunoglobulin G levels in glioma risk and prognosis. *Cancer Medicine*. 2013; 2: 57–62. <https://doi.org/10.1002/cam4.44>.
- [62] Ames JJ, Henderson T, Liaw L, Brooks PC. Methods for Analyzing Tumor Angiogenesis in the Chick Chorioallantoic Membrane Model. In Cao J (ed.) *Breast Cancer. Methods in Molecular Biology*, vol 1406 (pp. 255–269). Humana Press: New York, NY. 2016. https://doi.org/10.1007/978-1-4939-3444-7_22.
- [63] Fergelot P, Bernhard JC, Soulet F, Kilarski WW, Léon C, Courtois N, *et al.* The experimental renal cell carcinoma model in the chick embryo. *Angiogenesis*. 2013; 16: 181–194. <https://doi.org/10.1007/s10456-012-9311-z>.
- [64] Kleibeuker EA, Ten Hooften MA, Castricum KC, Honeywell R, Griffioen AW, Verheul HM, *et al.* Optimal treatment scheduling of ionizing radiation and sunitinib improves the antitumor activity and allows dose reduction. *Cancer Medicine*. 2015; 4: 1003–1015. <https://doi.org/10.1002/cam4.441>.
- [65] M HR, Ghosh D, Banerjee R, Salimath BP. Suppression of VEGF-induced angiogenesis and tumor growth by Eugenia jambolana, Musa paradisiaca, and Coccinia indica extracts. *Pharmaceutical Biology*. 2017; 55: 1489–1499. <https://doi.org/10.1080/13880209.2017.1307422>.
- [66] Javed S, Soukhtehzari S, Fernandes N, Williams KC. Longitudinal bioluminescence imaging to monitor breast tumor growth and treatment response using the chick chorioallantoic mem-

- brane model. *Scientific Reports*. 2022; 12: 17192. <https://doi.org/10.1038/s41598-022-20854-9>.
- [67] Radu-Cosnita AD, Nesi A, Berzava PL, Cerbu S, Cosma A, Comsa S, *et al*. Anti-chloride Intracellular Channel Protein 1 (CLIC1) Antibodies Induce Tumour Necrosis and Angiogenesis Inhibition on *In Vivo* Experimental Models of Human Renal Cancer. *Anticancer Research*. 2022; 42: 1313–1325. <https://doi.org/10.21873/anticancer.15599>.
- [68] Dünker N, Jendrossek V. Implementation of the Chick Chorioallantoic Membrane (CAM) Model in Radiation Biology and Experimental Radiation Oncology Research. *Cancers*. 2019; 11: 1499. <https://doi.org/10.3390/cancers11101499>.
- [69] Chu PY, Koh APF, Antony J, Huang RYJ. Applications of the Chick Chorioallantoic Membrane as an Alternative Model for Cancer Studies. *Cells, Tissues, Organs*. 2022; 211: 222–237. <https://doi.org/10.1159/000513039>.
- [70] Papadimitriou E, Unsworth B, Maragoudakis M, Lelkes P. Time course and quantification of extracellular matrix maturation in the chick chorioallantoic membrane and in cultured endothelial cells. *Endothelium*. 1993; 1: 207–219. <https://doi.org/10.3109/10623329309102698>.
- [71] Nazemi M, Yanes B, Martinez ML, Walker HJ, Pham K, Collins MO, *et al*. The extracellular matrix supports breast cancer cell growth under amino acid starvation by promoting tyrosine catabolism. *PLoS Biology*. 2024; 22: e3002406. <https://doi.org/10.1371/journal.pbio.3002406>.
- [72] Elenjord R, Allen JB, Johansen HT, Kildalsen H, Svineng G, Maelandsmo GM, *et al*. Collagen I regulates matrix metalloproteinase-2 activation in osteosarcoma cells independent of S100A4. *The FEBS Journal*. 2009; 276: 5275–5286. <https://doi.org/10.1111/j.1742-4658.2009.07223.x>.
- [73] Forciniti S, Dalla Pozza E, Greco MR, Amaral Carvalho TM, Rolando B, Ambrosini G, *et al*. Extracellular Matrix Composition Modulates the Responsiveness of Differentiated and Stem Pancreatic Cancer Cells to Lipophilic Derivate of Gemcitabine. *International Journal of Molecular Sciences*. 2020; 22: 29. <https://doi.org/10.3390/ijms22010029>.
- [74] Di Molfetta D, Cannone S, Greco MR, Caroppo R, Piccapane F, Carvalho TMA, *et al*. ECM Composition Differentially Regulates Intracellular and Extracellular pH in Normal and Cancer Pancreatic Duct Epithelial Cells. *International Journal of Molecular Sciences*. 2023; 24: 10632. <https://doi.org/10.3390/ijms241310632>.
- [75] Chin L, Xia Y, Discher DE, Janmey PA. Mechanotransduction in cancer. *Current Opinion in Chemical Engineering*. 2016; 11: 77–84. <https://doi.org/10.1016/j.coche.2016.01.011>.
- [76] Winkler J, Abisoye-Ogunniyan A, Metcalf KJ, Werb Z. Concepts of extracellular matrix remodelling in tumour progression and metastasis. *Nature Communications*. 2020; 11: 5120. <https://doi.org/10.1038/s41467-020-18794-x>.
- [77] Li QX, Feuer G, Ouyang X, An X. Experimental animal modeling for immuno-oncology. *Pharmacology & Therapeutics*. 2017; 173: 34–46. <https://doi.org/10.1016/j.pharmthera.2017.02.002>.
- [78] Khabipov A, Käding A, Liedtke KR, Freund E, Partecke LI, Bekeschus S. RAW 264.7 Macrophage Polarization by Pancreatic Cancer Cells - A Model for Studying Tumour-promoting Macrophages. *Anticancer Research*. 2019; 39: 2871–2882. <https://doi.org/10.21873/anticancer.13416>.
- [79] Miebach L, Freund E, Clemen R, Kersting S, Partecke LI, Bekeschus S. Gas plasma-oxidized sodium chloride acts via hydrogen peroxide in a model of peritoneal carcinomatosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2022; 119: e2200708119. <https://doi.org/10.1073/pnas.2200708119>.
- [80] Garcia P, Wang Y, Viallet J, Macek Jilkova Z. The Chicken Embryo Model: A Novel and Relevant Model for Immune-Based Studies. *Frontiers in Immunology*. 2021; 12: 791081. <https://doi.org/10.3389/fimmu.2021.791081>.
- [81] Alkie TN, Yitbarek A, Hodgins DC, Kulkarni RR, Taha-Abdelaziz K, Sharif S. Development of innate immunity in chicken embryos and newly hatched chicks: a disease control perspective. *Avian Pathology: Journal of the W.V.P.A.* 2019; 48: 288–310. <https://doi.org/10.1080/03079457.2019.1607966>.
- [82] Zuo Z, Syrovets T, Wu Y, Hafner S, Vernikouskaya I, Liu W, *et al*. The CAM cancer xenograft as a model for initial evaluation of MR labelled compounds. *Scientific Reports*. 2017; 7: 46690. <https://doi.org/10.1038/srep46690>.
- [83] Wittig R, Rosenholm JM, von Haartman E, Hemming J, Genze F, Bergman L, *et al*. Active targeting of mesoporous silica drug carriers enhances γ -secretase inhibitor efficacy in an in vivo model for breast cancer. *Nanomedicine (London, England)*. 2014; 9: 971–987. <https://doi.org/10.2217/nmm.13.62>.
- [84] Zuo Z, Syrovets T, Genze F, Abaei A, Ma G, Simmet T, *et al*. High-resolution MRI analysis of breast cancer xenograft on the chick chorioallantoic membrane. *NMR in Biomedicine*. 2015; 28: 440–447. <https://doi.org/10.1002/nbm.3270>.
- [85] Bain MM, Fagan AJ, Mullin JM, McNaught I, McLean J, Condon B. Noninvasive monitoring of chick development in ovo using a 7T MRI system from day 12 of incubation through to hatching. *Journal of Magnetic Resonance Imaging: JMIR*. 2007; 26: 198–201. <https://doi.org/10.1002/jmri.20963>.
- [86] Heidrich A, Würbach L, Opfermann T, Saluz HP. Motion-artifact-free in vivo imaging utilizing narcotized avian embryos in ovo. *Molecular Imaging and Biology*. 2011; 13: 208–214. <https://doi.org/10.1007/s11307-010-0355-4>.
- [87] Zlatopolskiy BD, Zischler J, Schäfer D, Urusova EA, Guliyev M, Bannykh O, *et al*. Discovery of 7-[18F] fluorotryptophan as a novel positron emission tomography (PET) probe for the visualization of tryptophan metabolism in vivo. *Journal of medicinal chemistry*. 2018; 61: 189–206. <https://doi.org/10.1021/acs.jmedchem.7b01245>.
- [88] Freesmeyer M, Kuehnle C, Opfermann T, Niksch T, Wiegand S, Stolz R, *et al*. The Use of Ostrich Eggs for In Ovo Research: Making Preclinical Imaging Research Affordable and Available. *Journal of Nuclear Medicine: Official Publication, Society of Nuclear Medicine*. 2018; 59: 1901–1906. <https://doi.org/10.2967/jnumed.118.210310>.
- [89] Steinemann G, Dittmer A, Schmidt J, Josuttis D, Föhling M, Biersack B, *et al*. Antitumor and antiangiogenic activity of the novel chimeric inhibitor animacroxam in testicular germ cell cancer. *Molecular Oncology*. 2019; 13: 2679–2696. <https://doi.org/10.1002/1878-0261.12582>.
- [90] Warnock G, Turtoi A, Blomme A, Bretin F, Bahri MA, Lemaire C, *et al*. In vivo PET/CT in a human glioblastoma chicken chorioallantoic membrane model: a new tool for oncology and radiotracer development. *Journal of Nuclear Medicine: Official Publication, Society of Nuclear Medicine*. 2013; 54: 1782–1788. <https://doi.org/10.2967/jnumed.112.117150>.
- [91] Würbach L, Heidrich A, Opfermann T, Gebhardt P, Saluz HP. Insights into bone metabolism of avian embryos in ovo via 3D and 4D 18F-fluoride positron emission tomography. *Molecular Imaging and Biology*. 2012; 14: 688–698. <https://doi.org/10.1007/s11307-012-0550-6>.
- [92] Benčurová K, Friske J, Anderla M, Mayrhofer M, Wanek T, Nics L, *et al*. CAM-Xenograft Model Provides Preclinical Evidence for the Applicability of [68Ga] Ga-Pentixafor in CRC Imaging. *Cancers*. 2022; 14: 5549. <https://doi.org/10.3390/cancers14225549>.
- [93] Wang L, Yan J, XinyuWang, Xu Y, Pan D, Chen C, *et al*. Evaluation of chicken chorioallantoic membrane model for tumor imaging and drug development: Promising findings. *Animal Models and Experimental Medicine*. 2025; 8: 287–294.

<https://doi.org/10.1002/ame2.12380>.

- [94] Löffler J, Hamp C, Scheidhauer E, Di Carlo D, Solbach C, Abaei A, *et al.* Comparison of quantification of target-specific accumulation of [18F] F-siPSMA-14 in the HET-CAM model and in mice using PET/MRI. *Cancers*. 2021; 13: 4007. <https://doi.org/10.3390/cancers13164007>.
- [95] Smith LM, Greenwood HE, Tyrrell WE, Edwards RS, de Santis V, Baark F, *et al.* The chicken chorioallantoic membrane as a low-cost, high-throughput model for cancer imaging. *Npj Imaging*. 2023; 1: 1. <https://doi.org/10.1038/s44303-023-00001-3>.
- [96] Haller S, Ametamey SM, Schibli R, Müller C. Investigation of the chick embryo as a potential alternative to the mouse for evaluation of radiopharmaceuticals. *Nuclear Medicine and Biology*. 2015; 42: 226–233. <https://doi.org/10.1016/j.nucmedbio.2014.10.010>.
- [97] Clohessy JG, Pandolfi PP. Mouse hospital and co-clinical trial project—from bench to bedside. *Nature Reviews. Clinical Oncology*. 2015; 12: 491–498. <https://doi.org/10.1038/nrclinonc.2015.62>.
- [98] Lee KH, Kim TH. Recent Advances in Multicellular Tumor Spheroid Generation for Drug Screening. *Biosensors*. 2021; 11: 445. <https://doi.org/10.3390/bios11110445>.
- [99] Nagle PW, Plukker JTM, Muijs CT, van Luijk P, Coppes RP. Patient-derived tumor organoids for prediction of cancer treatment response. *Seminars in Cancer Biology*. 2018; 53: 258–264. <https://doi.org/10.1016/j.semcancer.2018.06.005>.
- [100] Pauli C, Hopkins BD, Prandi D, Shaw R, Fedrizzi T, Sboner A, *et al.* Personalized *In Vitro* and *In Vivo* Cancer Models to Guide Precision Medicine. *Cancer Discovery*. 2017; 7: 462–477. <https://doi.org/10.1158/2159-8290.CD-16-1154>.
- [101] Schulz A, Meyer F, Dubrovskaya A, Borgmann K. Cancer Stem Cells and Radioresistance: DNA Repair and Beyond. *Cancers*. 2019; 11: 862. <https://doi.org/10.3390/cancers11060862>.
- [102] Pustovalova M, Blokhina T, Alhaddad L, Chigasova A, Chuprov-Netochin R, Veviorskiy A, *et al.* CD44+ and CD133+ Non-Small Cell Lung Cancer Cells Exhibit DNA Damage Response Pathways and Dormant Polyploid Giant Cancer Cell Enrichment Relating to Their p53 Status. *International Journal of Molecular Sciences*. 2022; 23: 4922. <https://doi.org/10.3390/ijms23094922>.
- [103] Pizon M, Schott D, Pachmann U, Schobert R, Pizon M, Wozniak M, *et al.* Chick Chorioallantoic Membrane (CAM) Assays as a Model of Patient-Derived Xenografts from Circulating Cancer Stem Cells (cCSCs) in Breast Cancer Patients. *Cancers*. 2022; 14: 1476. <https://doi.org/10.3390/cancers14061476>.
- [104] Busch M, Papior D, Stephan H, Dünker N. Characterization of etoposide- and cisplatin-chemoresistant retinoblastoma cell lines. *Oncology Reports*. 2018; 39: 160–172. <https://doi.org/10.3892/or.2017.6100>.
- [105] Estanqueiro M, Amaral MH, Conceição J, Sousa Lobo JM. Nanotechnological carriers for cancer chemotherapy: the state of the art. *Colloids and Surfaces. B, Biointerfaces*. 2015; 126: 631–648. <https://doi.org/10.1016/j.colsurfb.2014.12.041>.
- [106] Jain A, Jain SK, Ganesh N, Barve J, Beg AM. Design and development of ligand-appended polysaccharidic nanoparticles for the delivery of oxaliplatin in colorectal cancer. *Nanomedicine: Nanotechnology, Biology, and Medicine*. 2010; 6: 179–190. <https://doi.org/10.1016/j.nano.2009.03.002>.
- [107] Vargas A, Zeisser-Labouëbe M, Lange N, Gurny R, Delie F. The chick embryo and its chorioallantoic membrane (CAM) for the in vivo evaluation of drug delivery systems. *Advanced Drug Delivery Reviews*. 2007; 59: 1162–1176. <https://doi.org/10.1016/j.addr.2007.04.019>.
- [108] Rupp T, Legrand C, Hunault M, Genest L, Babin D, Frogget G, *et al.* A Face-To-Face Comparison of Tumor Chicken Chorioallantoic Membrane (TCAM) In Ovo with Murine Models for Early Evaluation of Cancer Therapy and Early Drug Toxicity. *Cancers*. 2022; 14: 3548. <https://doi.org/10.3390/cancers14143548>.
- [109] Vittorelli FD, Cardoso VMDO, Ferreira NN, Calixto GMF, Fontana CR, Baltazar F, *et al.* Chick embryo chorioallantoic membrane as a suitable in vivo model to evaluate drug delivery systems for cancer treatment: A review. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E.V.* 2020; 153: 273–284. <https://doi.org/10.1016/j.ejpb.2020.06.010>.
- [110] Dobson P, Jarvie H, King S. Nanoparticle. *Encyclopedia Britannica*. 2024. Available at: <https://www.britannica.com/science/nanoparticle> (Accessed: 20 January 2025).
- [111] Dias MF, Figueiredo BCPD, Teixeira-Neto J, Guerra MCA, Fialho SL, Silva Cunha A. In vivo evaluation of antitumoral and antiangiogenic effect of imiquimod-loaded polymeric nanoparticles. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*. 2018; 103: 1107–1114. <https://doi.org/10.1016/j.biopha.2018.04.079>.
- [112] Pund S, Thakur R, More U, Joshi A. Lipid based nanoemulsifying resveratrol for improved physicochemical characteristics, in vitro cytotoxicity and in vivo antiangiogenic efficacy. *Colloids and Surfaces. B, Biointerfaces*. 2014; 120: 110–117. <https://doi.org/10.1016/j.colsurfb.2014.05.016>.
- [113] Murugesan S, Mousa SA, O’connor LJ, Lincoln DW, 2nd, Linhardt RJ. Carbon inhibits vascular endothelial growth factor- and fibroblast growth factor-promoted angiogenesis. *FEBS Letters*. 2007; 581: 1157–1160. <https://doi.org/10.1016/j.febslet.2007.02.022>.
- [114] Hobzova R, Hampejsova Z, Cerna T, Hrabeta J, Venclikova K, Jedelska J, *et al.* Poly(D,L-lactide)/polyethylene glycol micro/nanofiber mats as paclitaxel-eluting carriers: preparation and characterization of fibers, in vitro drug release, antiangiogenic activity and tumor recurrence prevention. *Materials Science & Engineering. C, Materials for Biological Applications*. 2019; 98: 982–993. <https://doi.org/10.1016/j.msec.2019.01.046>.
- [115] Cho CF, Ablack A, Leong HS, Zijlstra A, Lewis J. Evaluation of nanoparticle uptake in tumors in real time using intravital imaging. *Journal of Visualized Experiments: JoVE*. 2011; e2808. <https://doi.org/10.3791/2808>.
- [116] Emanet M, Lefevre MC, Ceccarelli MC, Battaglini M, Carmignani A, Schiavone F, *et al.* Polydopamine Nanoparticle-Based Combined Chemotherapy and Photothermal Therapy for the Treatment of Liver Cancer. *ACS Applied Materials & Interfaces*. 2024; 16: 40695–40713. <https://doi.org/10.1021/acsami.4c08491>.
- [117] Santi M, Frusca V, Ermini ML, Mapanao AK, Sarogni P, Gonnelli A, *et al.* Hybrid nano-architectures loaded with metal complexes for the co-chemotherapy of head and neck carcinomas. *Journal of Materials Chemistry. B*. 2023; 11: 325–334. <https://doi.org/10.1039/d2tb01930b>.
- [118] Mitrevska K, Merlos Rodrigo MA, Cernei N, Michalkova H, Splichal Z, Hynek D, *et al.* Chick chorioallantoic membrane (CAM) assay for the evaluation of the antitumor and antimetastatic activity of platinum-based drugs in association with the impact on the amino acid metabolism. *Materials Today. Bio*. 2023; 19: 100570. <https://doi.org/10.1016/j.mtbio.2023.100570>.
- [119] Ferreira NN, M B Ferreira L, Miranda-Gonçalves V, Reis RM, Seraphim TV, Borges JC, *et al.* Alginate hydrogel improves anti-angiogenic bevacizumab activity in cancer therapy. *European Journal of Pharmaceutics and Biopharmaceutics: Official Journal of Arbeitsgemeinschaft Fur Pharmazeutische Verfahrenstechnik E.V.* 2017; 119: 271–282. <https://doi.org/10.1016/j.ejpb.2017.04.019>.

- [120] Abe C, Uto Y, Nakae T, Shinmoto Y, Sano K, Nakata H, *et al.* Evaluation of the in vivo radiosensitizing activity of etanidazole using tumor-bearing chick embryo. *Journal of Radiation Research*. 2011; 52: 208–214. <https://doi.org/10.1269/jrr.10122>.
- [121] Couto M, Cates C. *Laboratory Guidelines for Animal Care. Methods in Molecular Biology* (Clifton, N.J.). 2019; 1920: 407–430. https://doi.org/10.1007/978-1-4939-9009-2_25.
- [122] Leland SE, Straeter PA, Gnadt BJ. The Role of the IACUC in the Absence of Regulatory Guidance. *ILAR Journal*. 2019; 60: 95–104. <https://doi.org/10.1093/ilar/ilz003>.
- [123] Giannopoulou E, Katsoris P, Hatziaepostolou M, Kardamakis D, Kotsaki E, Polytarchou C, *et al.* X-rays modulate extracellular matrix in vivo. *International Journal of Cancer*. 2001; 94: 690–698. <https://doi.org/10.1002/ijc.1535>.
- [124] Sabatasso S, Laissue JA, Hlushchuk R, Graber W, Bravin A, Bräuer-Krisch E, *et al.* Microbeam radiation-induced tissue damage depends on the stage of vascular maturation. *International Journal of Radiation Oncology, Biology, Physics*. 2011; 80: 1522–1532. <https://doi.org/10.1016/j.ijrobp.2011.03.018>.
- [125] Kardamakis D, Hadjimichael C, Ginopoulos P, Papaioannou S. Effects of paclitaxel in combination with ionizing radiation on angiogenesis in the chick embryo chorioallantoic membrane. A radiobiological study. *Strahlentherapie und Onkologie: Organ der Deutschen Röntgengesellschaft...[et al.]*. 2004; 180: 152–156. <https://doi.org/10.1007/s00066-004-1140-6>.
- [126] Witt A, Ahr HJ, Brendler-Schwaab S, Enzmann H, Steinke W. Carcinogen-induced Mitochondrial DNA Damage in the In Ovo Model. *Toxicology in Vitro: an International Journal Published in Association with BIBRA*. 1998; 12: 329–333. [https://doi.org/10.1016/s0887-2333\(97\)00112-4](https://doi.org/10.1016/s0887-2333(97)00112-4).
- [127] Enzmann H, Brunnemann KD. The in ovo carcinogenicity assay (IOCA): a review of an experimental approach for research on carcinogenesis and carcinogenicity testing. *Frontiers in Bioscience: a Journal and Virtual Library*. 1997; 2: c30–c39. <https://doi.org/10.2741/a168>.
- [128] Briest F, Koziol EJ, Albrecht J, Schmidt F, Bernsen MR, Haack J, *et al.* Does the proteasome inhibitor bortezomib sensitize to DNA-damaging therapy in gastroenteropancreatic neuroendocrine neoplasms? - A preclinical assessment in vitro and in vivo. *Neoplasia* (New York, N.Y.). 2021; 23: 80–98. <https://doi.org/10.1016/j.neo.2020.11.004>.
- [129] Che PP, Mapanao AK, Gregori A, Ermini ML, Zamborlin A, Capula M, *et al.* Biodegradable Ultrasmall-in-Nano Architectures Loaded with Cisplatin Prodrug in Combination with Ionizing Radiation Induces DNA Damage and Apoptosis in Pancreatic Ductal Adenocarcinoma. *Cancers*. 2022; 14: 3034. <https://doi.org/10.3390/cancers14123034>.
- [130] Bravo-Vázquez LA, Méndez-García A, Rodríguez AL, Sahare P, Pathak S, Banerjee A, *et al.* Applications of nanotechnologies for miRNA-based cancer therapeutics: current advances and future perspectives. *Frontiers in Bioengineering and Biotechnology*. 2023; 11: 1208547. <https://doi.org/10.3389/fbioe.2023.1208547>.
- [131] Daniluk K, Lange A, Wójcik B, Zawadzka K, Bałaban J, Kutwin M, *et al.* Effect of Melittin Complexes with Graphene and Graphene Oxide on Triple-Negative Breast Cancer Tumors Grown on Chicken Embryo Chorioallantoic Membrane. *International Journal of Molecular Sciences*. 2023; 24: 8388. <https://doi.org/10.3390/ijms24098388>.
- [132] Inoue J, Inazawa J. Cancer-associated miRNAs and their therapeutic potential. *Journal of Human Genetics*. 2021; 66: 937–945. <https://doi.org/10.1038/s10038-021-00938-6>.
- [133] Mesas C, Chico MA, Doello K, Lara P, Moreno J, Melguizo C, *et al.* Experimental Tumor Induction and Evaluation of Its Treatment in the Chicken Embryo Chorioallantoic Membrane Model: A Systematic Review. *International Journal of Molecular Sciences*. 2024; 25: 837. <https://doi.org/10.3390/ijms25020837>.
- [134] Seto AG, Beatty X, Lynch JM, Hermreck M, Tetzlaff M, Duvic M, *et al.* Cobomarsen, an oligonucleotide inhibitor of miR-155, co-ordinately regulates multiple survival pathways to reduce cellular proliferation and survival in cutaneous T-cell lymphoma. *British Journal of Haematology*. 2018; 183: 428–444. <https://doi.org/10.1111/bjh.15547>.
- [135] Yan LX, Wu QN, Zhang Y, Li YY, Liao DZ, Hou JH, *et al.* Knockdown of miR-21 in human breast cancer cell lines inhibits proliferation, in vitro migration and in vivo tumor growth. *Breast Cancer Research: BCR*. 2011; 13: R2. <https://doi.org/10.1186/bcr2803>.
- [136] Lee TJ, Yuan X, Kerr K, Yoo JY, Kim DH, Kaur B, *et al.* Strategies to Modulate MicroRNA Functions for the Treatment of Cancer or Organ Injury. *Pharmacological Reviews*. 2020; 72: 639–667. <https://doi.org/10.1124/pr.119.019026>.
- [137] Reuter A, Sckell A, Brandenburg LO, Burchardt M, Kramer A, Stope MB. Overexpression of MicroRNA-1 in Prostate Cancer Cells Modulates the Blood Vessel System of an *In Vivo* Hen's Egg Test-Chorioallantoic Membrane Model. *In Vivo* (Athens, Greece). 2019; 33: 41–46. <https://doi.org/10.21873/in.vivo.11436>.
- [138] Tang XR, Wen X, He QM, Li YQ, Ren XY, Yang XJ, *et al.* MicroRNA-101 inhibits invasion and angiogenesis through targeting ITGA3 and its systemic delivery inhibits lung metastasis in nasopharyngeal carcinoma. *Cell Death & Disease*. 2017; 8: e2566. <https://doi.org/10.1038/cddis.2016.486>.
- [139] Javanmardi S, Abolmaali SS, Mehrabanpour MJ, Aghamaali MR, Tamaddon AM. PEGylated nanohydrogels delivering anti-MicroRNA-21 suppress ovarian tumor-associated angiogenesis in matrigel and chicken chorioallantoic membrane models. *BioImpacts*. 2022; 12: 449–461. <https://doi.org/10.34172/bi.2022.23263>.
- [140] Geretto M, Pulliero A, Rosano C, Zhabayeva D, Bersimbaev R, Izzotti A. Resistance to cancer chemotherapeutic drugs is determined by pivotal microRNA regulators. *American Journal of Cancer Research*. 2017; 7: 1350–1371.
- [141] Magee P, Shi L, Garofalo M. Role of microRNAs in chemoresistance. *Annals of Translational Medicine*. 2015; 3: 332. <https://doi.org/10.3978/j.issn.2305-5839.2015.11.32>.
- [142] Zhang L, Pickard K, Jenei V, Bullock MD, Bruce A, Mitter R, *et al.* miR-153 supports colorectal cancer progression via pleiotropic effects that enhance invasion and chemotherapeutic resistance [published correction in *Cancer Research*. 2022; 82: 1669. <https://doi.org/10.1158/0008-5472.CAN-22-0501>]. *Cancer Research*. 2013; 73: 6435–6447. <https://doi.org/10.1158/0008-5472.CAN-12-3308>.
- [143] Blower PE, Chung JH, Verducci JS, Lin S, Park JK, Dai Z, *et al.* MicroRNAs modulate the chemosensitivity of tumor cells. *Molecular Cancer Therapeutics*. 2008; 7: 1–9. <https://doi.org/10.1158/1535-7163.MCT-07-0573>.
- [144] Li H, Xu H, Shen H, Li H. microRNA-106a modulates cisplatin sensitivity by targeting PDCD4 in human ovarian cancer cells. *Oncology Letters*. 2014; 7: 183–188. <https://doi.org/10.3892/ol.2013.1644>.
- [145] Kovalchuk O, Filkowski J, Meservy J, Ilnytsky Y, Tryndyak VP, Chekhun VF, *et al.* Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Molecular Cancer Therapeutics*. 2008; 7: 2152–2159. <https://doi.org/10.1158/1535-7163.MCT-08-0021>.
- [146] Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. *FEBS Letters*. 2006; 580: 998–1009. <https://doi.org/10.1016/j.febslet.2005.12.060>.
- [147] Boo LM, Lin HH, Chung V, Zhou B, Louie SG, O'Reilly

- MA, *et al.* High mobility group A2 potentiates genotoxic stress in part through the modulation of basal and DNA damage-dependent phosphatidylinositol 3-kinase-related protein kinase activation. *Cancer Research*. 2005; 65: 6622–6630. <https://doi.org/10.1158/0008-5472.CAN-05-0086>.
- [148] Hebert C, Norris K, Scheper MA, Nikitakis N, Sauk JJ. High mobility group A2 is a target for miRNA-98 in head and neck squamous cell carcinoma. *Molecular Cancer*. 2007; 6: 5. <https://doi.org/10.1186/1476-4598-6-5>.
- [149] Mirzaei S, Zarrabi A, Asnaf SE, Hashemi F, Zabolian A, Hushmandi K, *et al.* The role of microRNA-338-3p in cancer: growth, invasion, chemoresistance, and mediators. *Life Sciences*. 2021; 268: 119005. <https://doi.org/10.1016/j.lfs.2020.119005>.
- [150] Zhang T, Liu W, Zeng XC, Jiang N, Fu BS, Guo Y, *et al.* Down-regulation of microRNA-338-3p promoted angiogenesis in hepatocellular carcinoma. *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*. 2016; 84: 583–591. <https://doi.org/10.1016/j.biopha.2016.09.056>.
- [151] Rovithi M, Avan A, Funel N, Leon LG, Gomez VE, Wurdinger T, *et al.* Development of bioluminescent chick chorioallantoic membrane (CAM) models for primary pancreatic cancer cells: a platform for drug testing. *Scientific Reports*. 2017; 7: 44686. <https://doi.org/10.1038/srep44686>.
- [152] Avan A, Caretti V, Funel N, Galvani E, Maftouh M, Honeywell RJ, *et al.* Crizotinib inhibits metabolic inactivation of gemcitabine in c-Met-driven pancreatic carcinoma. *Cancer Research*. 2013; 73: 6745–6756. <https://doi.org/10.1158/0008-5472.CAN-13-0837>.
- [153] Giovannetti E, Funel N, Peters GJ, Del Chiaro M, Erozcenci LA, Vasile E, *et al.* MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Research*. 2010; 70: 4528–4538. <https://doi.org/10.1158/0008-5472.CAN-09-4467>.
- [154] Chen RL, Zhao J, Zhang XC, Lou NN, Chen HJ, Yang X, *et al.* Crizotinib in advanced non-small-cell lung cancer with concomitant ALK rearrangement and c-Met overexpression. *BMC Cancer*. 2018; 18: 1171. <https://doi.org/10.1186/s12885-018-5078-y>.
- [155] Li B, Ren S, Li X, Wang Y, Garfield D, Zhou S, *et al.* MiR-21 overexpression is associated with acquired resistance of EGFR-TKI in non-small cell lung cancer. *Lung Cancer (Amsterdam, Netherlands)*. 2014; 83: 146–153. <https://doi.org/10.1016/j.lungcan.2013.11.003>.
- [156] Pang W, Su J, Wang Y, Feng H, Dai X, Yuan Y, *et al.* Pancreatic cancer-secreted miR-155 implicates in the conversion from normal fibroblasts to cancer-associated fibroblasts [published correction in *Cancer Science*. 2021; 112: 945. <https://doi.org/10.1111/cas.14779>]. 2015; 106: 1362–1369. <https://doi.org/10.1111/cas.12747>.
- [157] Chitcholtan K, Singh M, Tino A, Garrill A, Sykes P. Effects of Resveratrol on In Vivo Ovarian Cancer Cells Implanted on the Chorioallantoic Membrane (CAM) of a Chicken Embryo Model. *International Journal of Molecular Sciences*. 2024; 25: 4374. <https://doi.org/10.3390/ijms25084374>.
- [158] Wang Y, Rousset X, Prunier C, Dosda E, Gutierrez-Guerrero A, Abrial P, *et al.* An innovative in vivo model for CAR-T cell therapy development: Tolerability and efficacy evaluation of CD19-targeting CAR-T cells on human lymphoma using the chicken CAM assay. *Cancer Research*. 2024; 84: 4018. <https://doi.org/10.1158/1538-7445.AM2024-4018>.
- [159] Faihs L, Firouz B, Slezak P, Slezak C, Weißensteiner M, Ebner T, *et al.* A Novel Artificial Intelligence-Based Approach for Quantitative Assessment of Angiogenesis in the Ex Ovo CAM Model. *Cancers*. 2022; 14: 4273. <https://doi.org/10.3390/cancers14174273>.
- [160] Huang W, Itayama M, Arai F, Furukawa KS, Ushida T, Kawahara T. An angiogenesis platform using a cubic artificial eggshell with patterned blood vessels on chicken chorioallantoic membrane. *PloS One*. 2017; 12: e0175595. <https://doi.org/10.1371/journal.pone.0175595>.
- [161] Vimalraj S, Renugaa S, Dhanasekaran A. Chick embryo chorioallantoic membrane: a biomaterial testing platform for tissue engineering applications. *Process Biochemistry*. 2023; 124: 81–91. <https://doi.org/10.1016/j.procbio.2022.11.007>.
- [162] Gow NAR, Knox Y, Munro CA, Thompson WD. Infection of chick chorioallantoic membrane (CAM) as a model for invasive hyphal growth and pathogenesis of *Candida albicans*. *Medical Mycology*. 2003; 41: 331–338. <https://doi.org/10.1080/13693780310001600859>.
- [163] Gopinathan A, Morton JP, Jodrell DI, Sansom OJ. GEMMs as preclinical models for testing pancreatic cancer therapies. *Disease Models & Mechanisms*. 2015; 8: 1185–1200. <https://doi.org/10.1242/dmm.021055>.
- [164] Fischer D, Fluegen G, Garcia P, Ghaffari-Tabrizi-Wizsy N, Gribaldo L, Huang RYJ, *et al.* The CAM Model-Q&A with Experts. *Cancers*. 2022; 15: 191. <https://doi.org/10.3390/cancers15010191>.
- [165] Ahmed TAE, Cordeiro CMM, Elebute O, Hincke MT. Proteomic Analysis of Chicken Chorioallantoic Membrane (CAM) during Embryonic Development Provides Functional Insight. *BioMed Research International*. 2022; 2022: 7813921. <https://doi.org/10.1155/2022/7813921>.
- [166] Patiño-Morales CC, Jaime-Cruz R, Ramírez-Fuentes TC, Villavicencio-Guzmán L, Salazar-García M. Technical Implications of the Chicken Embryo Chorioallantoic Membrane Assay to Elucidate Neuroblastoma Biology. *International Journal of Molecular Sciences*. 2023; 24: 14744. <https://doi.org/10.3390/ijms241914744>.
- [167] Danes D, Raval P, Singh A, Pillai L, Balakrishnan S. Exposure to a sublethal dose of technical grade flubendiamide hampers angiogenesis in the chicken chorioallantoic membrane. *Toxicological Research*. 2024; 40: 627–638. <https://doi.org/10.1007/s43188-024-00254-z>.
- [168] Giusti V, Miserocchi G, Sbanchi G, Pannella M, Hattinger CM, Cesari M, *et al.* Xenografting Human Musculoskeletal Sarcomas in Mice, Chick Embryo, and Zebrafish: How to Boost Translational Research. *Biomedicine*. 2024; 12: 1921. <https://doi.org/10.3390/biomedicine12081921>.