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The chick embryo as an expanding experimental model for cancer and cardiovascular research

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Abstract

A long and productive history in biomedical research defines the chick as a model for human biology. Fundamental discoveries, including the description of directional circulation propelled by the heart and the link between oncogenes and the formation of cancer, indicate its utility in cardiac biology and cancer. Despite the more recent arrival of several vertebrate and invertebrate animal models during the last century, the chick embryo remains a commonly used model for vertebrate biology and provides a tractable biological template. With new molecular and genetic tools applied to the avian genome the chick embryo is accelerating the discovery of normal development and elusive disease processes. Moreover, progress in imaging and chick culture technologies is advancing real-time visualization of dynamic biological events, such as tissue morphogenesis, angiogenesis and cancer metastasis. A rich background of information, coupled with new technologies and relative ease of maintenance suggest an expanding utility for the chick embryo in cardiac biology and cancer research.

Keywords

Chicken embryo; chick model history; cancer metastasis; cardiac development; cell motility; in vivo imaging; chick CAM

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I. Introduction – Historical support for the chick as a cardiology and cancer model

Centuries of experimentation with the chicken built a foundation of knowledge that facilitates its use today for understanding human development and disease (Table 1). Two areas that are significantly enabled by the chick model are cardiac and cancer biology. Studies in cardiac biology originally stemmed from early investigations into development. Cancer studies came much later, but were facilitated by well-established information on chick development and immunology and open-egg handling techniques. Aristotle began the first recorded experiments on chicken eggs as long ago as c. 330 B.C. (Mason, 2008). As he watched chick development, he reported on the chronology of morphological changes in *Historia Animalium*. His fundamental theories shed new light on tissue development and demonstrated that the chick embryo shared many fundamental characteristics with humans.

Conserved characteristics are evident in the chick's use in understanding human development, including the genesis of the cardiovascular system. Early chick studies identified components of the hematogenous circulatory system and recognized the heart as a central pump pushing blood directionally through a network of vessels (Harvey, 1847 (published after death)). In these studies, William Harvey revealed that the heart began pumping even before blood development. He also recognized the functional difference between arteries and veins (Harvey, 1628). Inspired by Harvey's work, Marcello Malpighi used the chick to define and describe capillary vessels (Malpighi, 1672). The easy maintenance and relatively large size of the developing chick embryo allowed these significant observations using the simple tools of the 17th century.

Around 1750, German scientist, Beguelin, introduced the technique of cultivating chick embryos in an open egg, which allowed scientists to follow a single chick embryo throughout its development. By cutting a hole in the eggshell and covering it with a piece of shell from another egg to prevent dehydration, he was able to follow sequential developmental changes in the germinal disk (Romanoff, 1943). The Russian scientists, Karl Ernst von Baer and Heinz Christian Pander, used Beguelin's technique to describe the germ layers that form the embryo during development; the ectoderm, mesoderm, and endoderm (Romanoff, 1943).

As embryology advanced, more complex histological studies were carried out using the chick egg, leading Mathias Marie Duval (1844-1907) to publish the first complete morphological atlas of chick morphology, *Atlas d'embryologie*, in 1889 (Duval, 1889). These early developmental studies eventually provided the foundation for the Hamburger-Hamilton stages of development (Hamburger and Hamilton, 1951), which are still widely utilized. Viktor Hamburger and Howard Hamilton described 46 morphologically distinct stages of chick development beginning with a freshly laid egg and ending with a fully developed and hatched chick (Hamburger and Hamilton, 1951). These stages help provide consistency and coordination between the various areas that use the chick embryo model (Figure 1).

The immune system of the chick and chicken has also contributed to its tractability as a cancer model. In the early 1900s, infection of chickens with the Rous Sarcoma virus demonstrated that viruses could cause cancer (Rous, 1911). This led to the discovery of viral oncogenes; genes that were harnessed by infecting virus to expand their host cell populations. Many early-recognized viral oncogenes were identified using avian model systems (Javier and Butel, 2008). In 1976, Michael Bishop, Harold Varmus and their colleagues demonstrated that oncogenes were induced by mutations to proto-oncogenes. Their work showed that proto-oncogenes exist in most organisms, suggesting parallel disease processes in humans and potential model organisms (Ringold et al., 1976; Stehelin et al., 1976a; Stehelin et al., 1976b). This fundamentally changed our understanding of the genesis and growth of cancer and reinforced the applicability of chicken research to human health. Since the early studies by Rous, chicks have been used in a wide array of oncology studies to evaluate the causes of tumor initiation and cancer growth, as well as the mechanisms of tumor cell invasion, metastasis and angiogenesis (Stern, 2005; Zijlstra et al., 2006; Liu et al., 2013; Mu et al., 2013)

While the adult chicken helped discern the fundamental genetic underpinnings of cancer, current oncology research focuses on the chick embryo. Early experiments used the chick to evaluate host response to grafted tissues and identified characteristics that would allow the chick become a useful model for cancer research. James Murphy addressed immunological questions by transplanting various tissues into adult and embryonic chickens. Murphy showed that rat tissues could not grow in adult chickens while transplants of rat tissue could grow on the vascular chorioallantoic membrane (CAM) of chicks up until developmental day 18 (Murphy, 1914a; Murphy, 1914b). This demonstrated the natural immunodeficiency of the developing chick. In fact, its immune system does not begin to function until about 2 weeks into its development (Jankovic et al., 1975). This characteristic makes the chick amenable to tumor xenografting (Stevenson, 1918) and the CAM is a valuable model for tumor angiogenesis and cancer metastasis (Zijlstra et al., 2002; Zijlstra et al., 2008; Fein and Egeblad, 2013) Murphy's method of culturing competent immune cells from an adult chicken on the CAM of a developing embryo was soon expanded to an experimental system for analysis of transplant immune responses (Coppleson and Michie, 1965). Importantly, this lack of a developed immune system enables the chick CAM as a culture platform for the culture of transplanted human tumors (Figure 2) (Zijlstra et al., 2002).

Utility of the chick as a biological model was accelerated its physical attributes. The developing animal is naturally stationary and self-contained making it readily adaptable to complex investigative work requiring extensive manipulation with continued observation. The egg is self-sufficient and its normal development at 37°C & 60% humidity, ensures consistent viability of animals without artificial support media or complex culture requirements. Within the egg, the *in ovo* chick is a highly controlled, yet accessible and relatively transparent model in which normal physiology, disease pathology and the consequences of experimental manipulation can be visualized. Its relatively large size is particularly advantageous for analyzing the differentiation and behavior of cardiac cells (Patten, 1920; Hamburger and Hamilton, 1951; Wainrach and Sotelo, 1961). The ability to decant the embryo from its eggshell and culture the animal *ex ovo* provides a window with

unsurpassed transparency to view the biology and the consequences of experimental manipulation (to visualize this process see (Cho et al., 2011; Palmer et al., 2011)). The CAM is an established biological platform for carcinogenesis (Bader et al., 2006), tumor xenografting (Dagg et al., 1954; Easty et al., 1969; Murphy and Rous, 1912; Nicolson et al., 1978; Ossowski and Reich, 1983), tumor angiogenesis (Eliceiri et al., 1998), and cancer metastasis (Chambers et al., 1998; Chambers et al., 1982; Gordon and Quigley, 1986; Zijlstra et al., 2002).

II. The chick as a cardiac model

Given the aforementioned advantages of size and accessibility it is not surprising that the chick has been used to describe the early formation, septation, and vascularization of the heart (Patten, 1920; Hamburger, 1951; Wainrach, 1961). These same advantages also allowed investigators to develop *in ovo* and *in vitro* approaches that provided important insight into the development of physiological responsiveness of the heart (Galper et al., 1977; Galper and Catterall, 1978; Barnett et al., 1990; Barnett et al., 1993). Here we will focus on examples where the chick model has provided critical insight in three prominent events in cardiovascular development: coronary vasculogenesis, valve development, and neural crest contributions to outflow tract development, where the chick continues to hold great promise as a model organism.

Coronary Vasculogenesis

Our understanding of the origin and formation of coronary vessels has been shaped by experiments performed in the chick. The origins of coronary vessels have been attributed to endocardial cells entrapped in the myocardium during trabeculation of the myocardium (Grant, 1926; Viragh and Challice, 1981), outgrowth of the aorta via angiogenesis (Bennett, 1936; Goldsmith JB, 1937) or, more recently, derivation from the proepicardium (PE)/ epicardium (EP) (Mikawa and Fischman, 1992; Poelmann et al., 1993). Much of our current understanding of the now generally accepted role of the proepicardium (PE) in coronary vessel formation comes from studies in chick embryos. These studies revealed that coronary vessel development begins when mesothelial cells of the PE move from the liver primordium to the heart surface where they differentiate into a variety of cell lineages that make up distinct components of the heart (Manner, 1993; Manner et al., 2001; Olivey et al., 2004; Tomanek, 2005). In chicks, the PE arises from mesothelial cells along the caudal border of the pericardial cavity that are well defined, readily isolated by light microscopy, and amenable to experimental manipulation (Ho and Shimada, 1978; Tomanek et al., 2006; Lavine et al., 2008; Xiong, 2008). Labeling PE cells with vital dye or β-galactosidase (β-gal) produces mature chicks with labeled smooth muscle cells or coronary arteries (Mikawa and Fischman, 1992), demonstrating that coronary arteries arise directly from the PE. Preventing PE cells from attaching to the heart in chicks prevents coronary vessel development, further supporting that PE cells are necessary for coronary vessel formation (Goldsmith JB, 1937; Perez-Pomares et al., 2002; Manner et al., 2005). Despite the gains made in our understanding of coronary vessel development, the origin of cardiac endothelial cells is debated and unique properties of the chick system make it a useful model for addressing this question. When labeled quail PE is grafted into a developing chick embryo, quail cells

supply smooth muscle cells and fibroblasts to the host chick heart, but no quail-derived endothelial cells are seen in the coronary endothelium. Production of quail-derived endothelial cells in the host embryo from a quail PE graft requires a co-graft of quail liver (Poelmann et al., 1993). These studies suggest that although the PE can contribute to non-endothelial lineages, endothelial cells may arise from the liver and migrate with PE cells to the heart. More recent experiments in the mouse also suggest a nonPE origin of endothelial cells (Red-Horse et al., 2010). Tissue grafting, cell labeling and photoablation experiments in the relatively large and accessible chick embryo will continue to be useful for understanding these complex questions regarding the origins of specific cell types that make up the coronary vessels.

Explant culture of epicardial cells from chick embryos revealed some of the regulators driving epicardial cell epithelial to mesenchymal transition (EMT) and cell differentiation required for coronary vessel development. During development, cells undergo EMT during a critical stage of reprogramming, which results in changes to many of the cell's physical properties: including morphology, polarity and motility. Experiments in the chick helped define chemokine function during EMT in the heart. For example, in the chick, FGF and VEGF expression patterns support a role for each in epicardial transformation (Morabito et al., 2001; Molin et al., 2003). *In vitro* culture of chick PE or EP explants have also shown that Transforming Growth Factor Beta (TGFβ) induces smooth muscle differentiation in cells from the PE (Olivey et al., 2006) and the EP (Compton et al., 2006). The ability to culture explanted PE and EP cells allowed this observation to be further supported and the mechanism elaborated. Serum Response Factor (SRF), a transcription factor associated with smooth muscle cell differentiation, was shown to be expressed in the PE and subepicardial mesenchyme *in vivo* and in EP-derived cells *in vitro* (Landerholm et al., 1999). Chick-quail chimera experiments suggest that SRF levels are regulated upstream by PDGF-BB activation of rhoA and p160rho kinase (Lu et al., 2001). Inhibition of p160rho kinase in quail PE explanted into chick embryos inhibits SRF transcription and disrupts mesenchyme formation in the myocardium (Lu et al., 2001), suggesting that p160rho kinase is required for the migration or survival of mesenchyme in the myocardium. Culture of chick PE and EP explants should continue to aid in revealing how these molecular cues regulate the cell transformation and differentiation required for coronary vessel development.

Endocardial Cell Heterogeneity and Early Valve Formation

Experiments in the chick provided key insight into the earliest stages of heart valve development and revealed the presence of endocardial cell heterogeneity in the embryonic heart. Structural analysis of the embryonic heart identified endocardial cell transformation in the matrix-rich, valve-forming regions of the heart, the endocardial cushions (Markwald et al., 1975; Markwald et al., 1977). The development of a system for the *in vitro* culture and scoring of embryonic valve-forming tissue (Bernanke and Markwald, 1979) led to a clearer description of the process of endocardial cell transformation and provided a system for the screening and identification of morphogens that regulate cell transformation. This *in vitro* assay depends upon the identification, isolation, and culture on a collagen gel of specific regions of the heart tube where endocardial cells undergo transformation to initiate valve development and regions that do not. The vast majority of studies use the region of the heart

tube that lies between the common atrium and ventricle, referred to as the atrioventricular cushion (AVC), where the inflow valves will form. Transformation of the explanted AVC has been studied extensively in avian systems (reviewed in (Barnett and Desgrosellier, 2003; Delaughter et al., 2011; Lencinas et al., 2011)). Experiments using this system demonstrated that the endocardium of the cushions differs functionally from the endocardium overlaying the ventricle (Mjaatvedt et al., 1987; Delaughter et al., 2011). The description of endocardial cell heterogeneity using the chick has been critical to understanding early valve development and directed experimental approaches in other organisms.

The use of this *in vitro* explant system led to the identification of several key regulators of endocardial cell transformation (Barnett, 2003; Schroeder et al., 2003; Butcher et al., 2007) (de Vlaming et al., 2012). A prototypic example is the identification of a role for members of the TGFβ family and the use of this system to reveal signaling mechanisms downstream of an atypical TGFβ receptor. The addition of ligands, neutralizing antisera, or antisense oligonucleotides to this chick explant model identified specific TGFβ ligands and receptors that regulate endocardial cell transformation ((Potts and Runyan, 1989; Potts et al., 1991) reviewed in (Lencinas et al., 2011)). A significant adaptation of the *in vitro* explant assay was coupling the assay with viral gene transfer techniques to introduce genes into either AVC or ventricular endocardial cells to perform gain and loss of function experiments in order to analyze the function of specific molecules that may regulate endocardial cell behavior. Initial experiments used incubation of explants with viral-containing solutions to introduce genes into endocardial cells which resulted in useful, but inefficient, infection of endocardial cells. This approach was initially used to identify a unique and nonredundant role for the atypical Type III TGFβ receptor (TGFβR3) in endocardial cell transformation (Brown et al., 1999). Later modifications of this method took advantage of new culture techniques (Selleck, 1996; Chapman et al., 2001) that allowed embryos to be removed from the egg so that the viral-containing solution could be injected into the lumen of the heart tube at a stage of development prior to the joining of the heart tube to the vasculature (Desgrosellier et al., 2005). Injection of a solution containing adenovirus results in highly efficient infection of endocardial cells throughout the heart tube. Embryos are cultured for 24 hours after which AVC or ventricular explants may be cultured and subsequently scored for gain or loss of function by counting the number of virally infected endocardial cells that undergo transformation. The ability to score for gain-of-function and loss-of-function of candidate molecules provides a powerful system to assay for candidate molecules that may regulator transformation (Lai et al., 2000; Desgrosellier et al., 2005; Okagawa et al., 2007; Kirkbride et al., 2008; Townsend et al., 2008). Additional experiments used adenoviral gene transfer to probe the function of the TGFβR3 in endocardial cells and serves as a model for how this approach may be used to provide insight into the functions of specific molecules. For example, the only functional assay for TGFβR3 activation is the stimulation of endocardial cell transformation in endocardial cells. Overexpression of this receptor in ventricular endocardial cells that normally lack TGFβR3 results in transformation after the addition of TGFβ ligand. This approach allowed for the identification of additional ligands for the receptor (Kirkbride et al., 2008) and identified signaling pathways downstream of TGFβR3 that are distinct from the canonical TGFβ signaling pathway(Desgrosellier et al., 2005; Okagawa et al., 2007; Townsend et al., 2008; Townsend et al., 2011; Townsend et al.,

2012). These experimental studies in the chick identified key signaling molecules that regulate endocardial cell transformation and catalyzed the development and characterization of an *in vitro* system in the mouse to complement studies performed in the chick (Camenisch et al., 2002; Stevens et al., 2008). The continued interest in endocardial and endothelial cell transformation in both valve development and, more recently, in disease processes and the past successes of the explant system in revealing the mechanisms that underlie endocardial cell transformation suggest that contributions to our understanding of endocardial and endothelial cell behavior will still derive from studies in the chick.

Neural Crest Contributions to Outflow Tract Development

Neural crest cells (NCC) are multipotent, embryonic cells derived from the developing neural tube ectoderm in all vertebrates including amphibians, fish, avians, and mammals (Bronner-Fraser, 1993). NCC migrate away from the neural tube along its length, populate different areas of the embryo, and terminally differentiate to contribute to the formation of many different organs and organ systems (Nakamura and Ayer-le Lievre, 1982; Ziller and Smith, 1982). Studies in the chick have contributed significantly to our comprehension of the diversity and roles of NCC. Insights gained from elegant studies in the chick revealed that although all NCC are morphologically similar when they begin to migrate, discrete populations are not equally capable of generating specific tissues (reviewed in(Bronner-Fraser, 1995)). NCC in specific locations of one embryo were surgically ablated and replaced with neural crest grafted heterotopically from a donor embryo. These experiments demonstrated that cell fate is limited by the timing of cell migration out of the neural tube rather than by the location of the NCC transplant (Noden, 1975; Nakamura and Ayer-le Lievre, 1982). Of particular interest to cardiovascular development was the identification of a specific population of NCC, the cardiac neural crest, that contributes to the development of the outflow tract (Kirby et al., 1983). Analysis of chick-quail chimeras showed that NCC from the regions of somite 1-3 migrated into the outflow tract and that ablation of these cells resulted in outflow tract malformations. Since this initial report, there has been much debate in the literature over the nature and cause of defects associated with neural crest ablation. The most consistently observed defect as a result of cardiac neural crest ablation is Persistent Truncus Arteriosus (PTA), where the outflow tract fails to form a septum dividing it into a left ventricular outlet (aorta) and a right ventricular outlet (pulmonary artery). Secondary outflow tract defects are common in neural crest ablation in the chick and mouse, including overriding aorta and double outlet right ventricle, which complicate phenotyping (Kirby et al., 1985) (Waldo et al., 1998; Yelbuz et al., 2002). However, the manifestations of NCC ablation are more similar between chicks and mice than other vertebrate models, such as zebrafish or Xenopus (Snider et al., 2007). Although transgenic mouse models of cardiac developmental defects have provided important insights into the nature of congenital malformations and defined new molecules and signaling pathways important during cardiac development, mouse models displaying complex cardiac phenotypes present a challenge to investigators attempting to tease apart how and where gene products act during cardiogenesis. The embryonic lethality associated with cardiovascular defects coupled with the poor accessibility of mammalian embryos suggests that experimental manipulations in the chick will continue to be a fruitful approach to reveal the roles of NCC in complex

developmental events such as outflow tract remodeling due to the ease of accessibility and monitoring.

III. The chick as a model for cancer biology

There is considerable overlap between mechanisms governing cell survival and motility during embryogenesis and in cancer formation and metastasis. Identification of the first transcription factor regulating the epithelial-mesenchymal transition facilitated our understanding of gastrulation and neural crest emigration and ultimately cancer metastasis (Nieto et al., 1994). It stands to reason that the chick model would find utility in both of these highly related arenas. The chick embryo is a unique model that overcomes many limitations to studying the biology of cancer *in vivo*. The accessibility of the chorioallantoic membrane (CAM), the well-vascularized extra-embryonic tissue located underneath the eggshell, and its acceptance of xenografted tumor cells all make it easy to use (Figure 2). Consequently, the CAM has a very successful history as a biological platform for the molecular analysis of cancer including viral oncogenesis (Rous, 1911), carcinogenesis (Bader et al., 2006), tumor xenografting (Murphy and Rous, 1912; Dagg et al., 1954; Easty et al., 1969; Nicolson et al., 1978; Ossowski and Reich, 1983), tumor angiogenesis (Eliceiri et al., 1998), and cancer metastasis (Chambers et al., 1982; Gordon and Quigley, 1986; Chambers et al., 1998; Zijlstra et al., 2002). Since the chick embryo is naturally immunodeficient, the CAM readily supports the engraftment of both normal and tumor tissues (Zijlstra et al., 2002). A selection of tumor cell lines effectively cultured on the CAM is presented in Table II. Most importantly, the avian CAM successfully supports most cancer cell characteristics including growth, invasion, angiogenesis, and remodeling of the microenvironment. This makes the model exceptionally useful for investigating the molecular pathways of oncogenesis (Zijlstra et al., 2002; Bobek et al., 2004; Fergelot et al., 2013; Liu et al., 2013; Mu et al., 2013).

Cancer cell motility and metastasis

In recent years, particular emphasis has been placed tumor cell motility and its contribution to cancer metastasis (Palmer et al., 2011). We have successfully adapted the CAM as a model to quantify the rate limiting steps of metastasis using species-specific and quantitative Alu-PCR for the detection of disseminated human tumor cells in secondary tissues (25 cells/tissue) (Zijlstra et al., 2002; Arpaia et al., 2011). The detection of disseminated cells by Alu-PCR makes it possible to quantitatively assess metastasis to organs that are colonized by as few as 25 cells (Zijlstra et al., 2002; Zijlstra et al., 2008; Palmer et al., 2011). This approach was used to demonstrate the role of matrix metalloproteinases (MMPs) (Kim et al., 1998) and allowed for the quantitative differentiation among tumor cell variants with divergent metastatic abilities (Zijlstra et al., 2002). This strategy has been used more recently to quantitatively define the contribution of CD151 to metastasis, a molecular scaffolding protein that regulates tumor cell motility (Zijlstra et al., 2008).

To document the consequences of disrupting tumor cell motility, a novel intravital strategy was developed around the avian embryo (MacDonald et al., 1992; Zijlstra et al., 2008; Leong et al., 2010; Cho et al., 2011). Microscopic evaluation of tumor cells in the CAM revealed an incredibly dynamic cellular microenvironment in which tumor cells propelled

themselves rapidly through the tumor tissue as well as through the adjacent stroma (Zijlstra et al., 2008). Direct evaluation of the immobilization caused by the metastasis-inhibiting antibody to CD151 revealed that cells failed to detach in the rear. This inhibition of detachment blocked movement of the tumor cells out of the primary tumor thereby preventing tumor cell intravasation and subsequent metastasis. Intravital imaging also revealed detail outside the tumor that implied significant involvement of the vasculature and the stromal cells (Lewis et al., 2006; Pink et al., 2012; Ruhrberg, 2012; Fein and Egeblad, 2013). Recent work in the laboratory of Harold Moses used the chick model to demonstrate that stromal cells can drive the outward migration of tumor cells (Matise et al., 2012).

Hemodynamics and Angiogenesis

The vascular supply of normal or neoplastic tissues is necessary for tissue survival. It is then not accidental that the chick played an important role in the early descriptions of the vertebrate vascular system made by both William Harvey and Marcello Malpighi. The vessels in the developing chick and the extraembryonic membranes are well defined and their superficial nature makes them readily available for observation and manipulation (Figure 3). *In ovo* observations of tumor-induced vascularization (angiogenesis) were made in the CAM at the turn of the 20th century (Murphy, 1913) and early studies of visualization continued in the egg. With modern noninvasive, intravital imaging systems (Lewis et al., 2006; Zijlstra et al., 2008) the chick embryo provides a robust *in vivo* model to monitor the vasculature (Figure 3). A novel class of viral nanoparticles enabled the visualization of newly formed vasculature in expanding tumors (Leong et al., 2010) and monitoring of targeted-delivery to the tumor in the CAM (Cho et al., 2011). This approach to evaluating cancer as a comprehensive (micro)environment is increasingly becoming the standard approach to investigate both the physiology of tumors, the molecular mechanisms that drive them, and therapies that can intervene (Botkjaer et al., 2012)

When the developing chick is decanted from the egg and cultured *ex ovo*, the CAM naturally expands across the albumin and yolk sac, exposing its vasculature and providing an easy platform for long-term imaging experiments (Lewis et al., 2006). The unprecedented access to the vasculature offered by the CAM was recognized by many but perhaps utilized most famously by Judah Folkman who implemented the CAM routinely to evaluate factors controling vascular growth (Auerbach et al., 1974; Klagsbrun et al., 1976; Kusaka et al., 1991; Hanahan and Folkman, 1996; O'Reilly et al., 1997). This work in the CAM revealed that a tumor required a newly formed vasculature and demonstrated that angiogenesis inhibitors could block tumor growth. Early work led to the discovery of a fungal derived inhibitor (Kusaka et al., 1991) and the revelation that a cryptic peptide, endostatin, released during proteolytic remodeling of the extracellular matrix, is a negative regulator of vascular outgrowth (O'Reilly et al., 1997). In more recent years advances in the CAM assays (detailed in (Pink et al., 2012)) led to the identification of hemopoietic cells that contributed the proteases MMP9 and MMP13 required for matrix remodeling during angiogenesis (Zijlstra et al., 2004; Zijlstra et al., 2006).

Advances in imaging technologies have made it possible to visualize vascular perfusion, vascularization of the CAM and the distinct steps of angiogenesis (Lewis et al., 2006; Leong

et al., 2010; Pink et al., 2012). New contrast and imaging agents that selectively label developing vessels promote visualization of specific vascular structures at the microscopic level (Lewis et al., 2006; Leong et al., 2010). Since tumors grow easily on the CAM surface and induce the growth of supportive host blood vessels, this is a useful model to visualize real-time tumor blood flow *in vivo*. High resolution imaging of CAM supported human tumors reveal fluid and small molecule dynamics within tumors (Cho et al., 2011; Steinmetz et al., 2011). Following small nanoparticles through the vascular beds that feed solid tumors can predict how intravenously administered drugs will localize within tumors and their surrounding tissues. Microfabrication techniques allow for near-microscopic control over vascular formation in the CAM (Jeong et al., 2012). Imaging tumor vascular dynamics in the CAM is faster, easier and less expensive than in mammals, promoting its utility for screening drugs or new designs for drug carriers and potential targeting agents. Current research is testing a variety of targeting strategies that might be used in conjunction with drug carriers to target them more specifically to cancer cells (Bobek et al., 2004; Zijlstra et al., 2008; Botkjaer et al., 2012; Busch et al., 2013; Fein and Egeblad, 2013)

IV. Future Directions

A major advantage of the chicken embryo (*gallus gallus domesticus*) as a current and future model of development results from the accumulated knowledge about the developing chick built over centuries of study. Description of basic development in gastrulation, neurulation, and organogenesis is available in whole mount (Hamburger and Hamilton, 1951), with RNA expression (http://geisha.arizona.edu), and by electron microscopic analysis (Bellairs, 1979; Meier, 1980; Hiruma and Hirakow, 1985; Bellairs et al., 2005). There is also a developing Wikipedia page for the chick embryo, where new tools and reagent information can be shared among researchers who work in the chick model. Development of new resources like these, along with the growing power of imaging (Lewis et al., 2006; Sweetman et al., 2008; Zijlstra et al., 2008; Song et al., 2011) and the expanding ability for genetic modification (discussed below) will all influence the future of the chick model.

In 2004, the genome of *Gallus gallus* was sequenced by Sanger shotgun sequencing (ICGSC, 2004) and mapped with extensive BAC contig-based physical mapping (Wong et al., 2004). This not only made the chicken available for broad genetic analysis, it also enabled full-genome comparison to humans and other models systems. Despite the differences, 70 million bp of the chicken sequence is highly conserved with humans, both within coding gene segments and outside of coding regions (ICGSC, 2004). The conserved base pairs outside of coding regions may be regulatory elements, which are often located at great distances from the genes they control (Schmutz and Grimwood, 2004). By focusing on the conserved sequences, comparative genomics projects have already revealed some key functional elements in the human genome (Birney et al., 2007). With the availability of full chick genome, the model is well situated for implementation of system-wide analysis. In the past year alone, a dynamic atlas of chick heart development was created as a reference to connect cell differentiation with organ function (Al Naieb et al., 2012) and a three-dimensional map of chick gene expression was made during development and placed in the public domain (Wong et al., 2013). There is a growing repository of anatomical and genetic information that continues to expand the tractability of the chick model.

Publication of the chicken genome also enables expansion of transgenic techniques within the chick model system. Current genetic approaches in chick embryos primarily include transient methods, such as *in ovo* electroporation (Funahashi et al., 1999; Funahashi and Nakamura, 2008; Nakamura and Funahashi, 2013) and retrovirus mediated protein expression (see (Bronner-Fraser, 2008) and references therein). This is primarily because adult chickens are much more challenging to maintain and handle. In contrast, chick embryos are stationary, self-contained and readily cultured. This tractability contributed in no small part to the fact that the majority of the discoveries described here were made using the developing embryo model. Recent advances in electroporation techniques facilitate gain-of-function and loss-of-function experiments to define gene function during early embryonic development and can be confined to the embryo model. Retrovirus mediated transient transfection also allows for mis-expression of genes in the embryo and has been used to determine gene function in development (Logan and Tabin, 1998). Replication-competent viruses may also deliver genes for the analysis of their impact on development and organogenesis. For example, Fekete & Cepko combined retroviral infection with tissue transplantation to limit gene transfer (Fekete and Cepko, 1993) and Morgan, et al used virus to target the mis-expression of Hox4.6 in the limb and described homeotic transformation (Morgan et al., 1992). Replication-competent virus was used to over-express the hedgehog gene product in the developing chicken forelimb to show that hedgehog was an important component of the zone of polarizing activity (ZPA) regulating anterior/posterior identity of developing digits and distal structures (Tabin and McMahon, 2008; Gros et al., 2009). However, stable gene integration can be achieved with transposons and expression can be regulated with tetracycline-inducible systems for short-term or long-term experiments. Confining experiments to the chick embryo, a single stage of life and a single generation is limiting and the publication of the chicken genome inspires research toward the establishment and characterization of transgenic animals (Heo et al., 2011; Lyall et al., 2011). Thus, the future of the model for scientific research likely includes both the chicken and the egg.

Utilization of the chick model for genetic manipulation such as knockout, TALENs, CRISPRs, Zinc-finger nuclease technology has not yet come to fruition. However, considering the viability of the chick embryo as a model, many groups are rapidly developing strategies for implement genetic analysis using these tools. Transgenic techniques in avian species are becoming more common and successful (for reviews see (Ishii and Mikawa, 2005; Stern, 2005; Logan and Francis-West, 2008; Sauka-Spengler and Barembaum, 2008). The main challenges toward broad implementation of genetically modified avian model systems have been the cost and availability of animal husbandry. However, the increasing universality of affordable and reliable genetic tools such as TALENs and fluorescent tracking is undoubtedly going to expand the genetic utility of avian models. Cardiovascular development and tumor progression are examples of highly dynamic processes that involve complex interplay that spans all dimension from the individual cells to the organ tissue and the intact organism itself. Understanding these processes requires investigation of the intact animal in a manner made possible by the chick embryo. Future studies will combine modern molecular and genetic techniques with the classical techniques in the chick embryo to provide insight pertaining to gene regulation, cell fate, and tissue

specification in development and cancer models (example (Ohta et al., 2003). With time, this branch of experimental intervention will become commonplace for those investigating the dynamics of complex biological systems.

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Highlights

- History of the chick in cardiac biology and cancer research
- Utility of the chick as an experimental model system
- The chick embryo for the investigation of cancer biology
- The chick embryo for the investigation of cardiac biology
- Future directions for the chick in heart and cancer studies

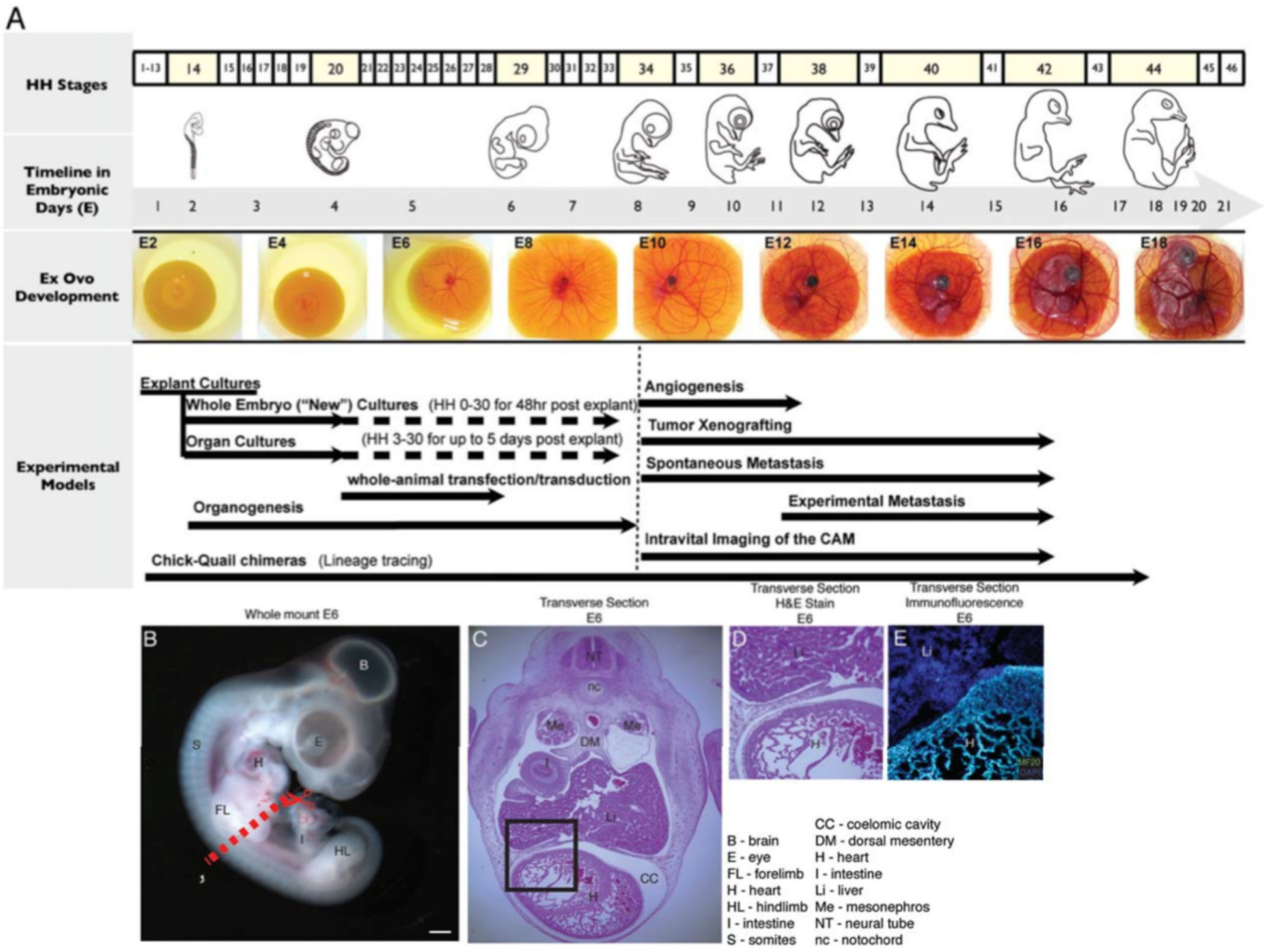


Figure 1. A) Chick embryo staging and the experimental timeline

A developmental timeline of the chick embryo in days is matched with the Hamburger-Hamilton stages using selected images (Hamburger and Hamilton, 1951) and time-matched images from *ex ovo* culture. Common experimental models are matched to the time line shown at the top. **B)** Whole mount of E6 embryo. Dotted line indicates approximate transected views shown in C-E. **C)** H&E staining of transverse section taken from E6 embryo. Developing structures in heart **(D)** were visualized using immunofluorescent staining with matching H&E staining (**E)** respectively.

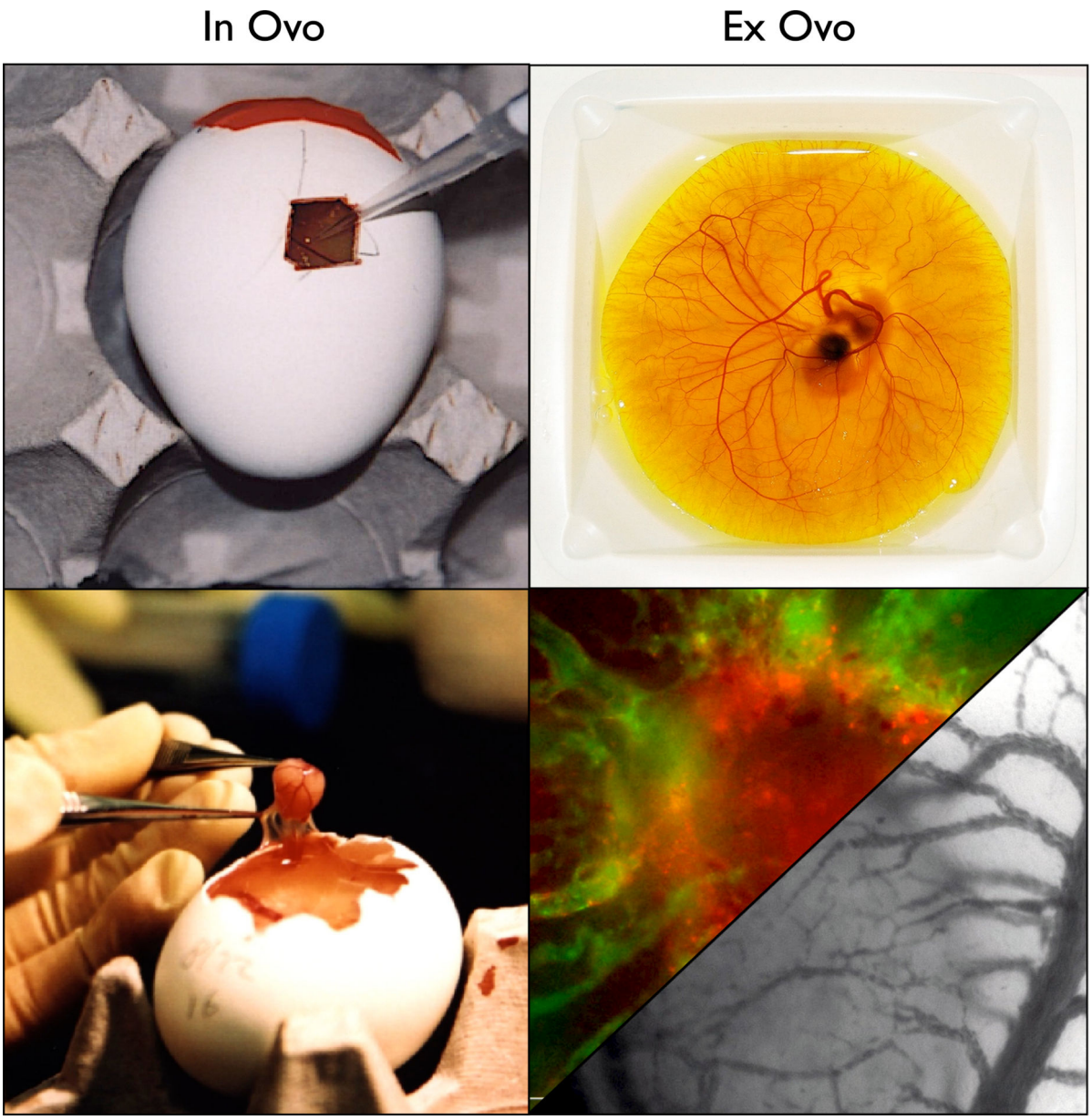


Figure 2. Tumor xenografting onto the chick CAM

Images demonstrate *in ovo* (left column) and *ex ovo* (right column) xenograft models of the CAM. Tumor cells grafted *in ovo* can be harvested for traditional procession. Tumors grafted *ex ovo* are more readily accessible for in situ analysis including direct observation through intravital imaging (bottom right).

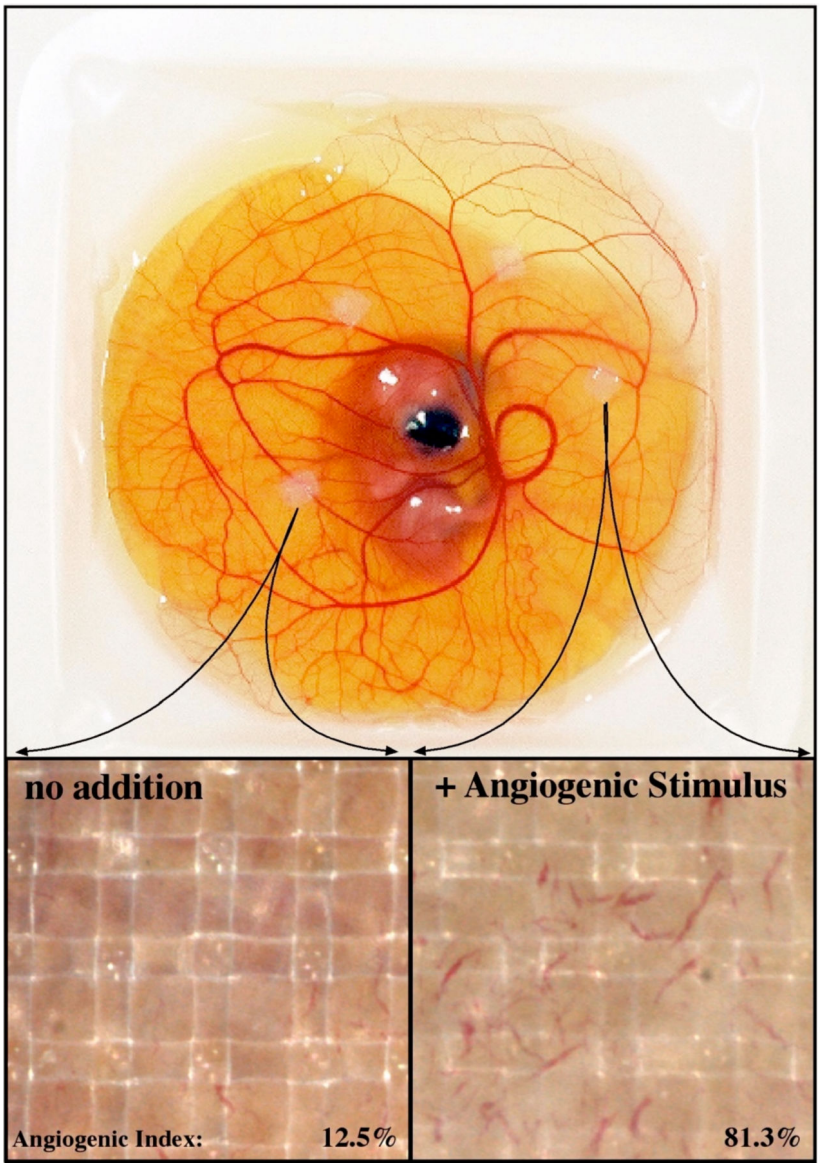


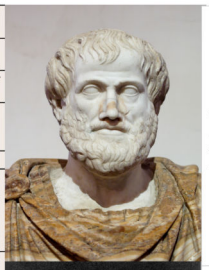
Figure 3. Modeling angiogenesis in the chick CAM

A day 13 chick embryo bearing 4 angiogenesis onplants is shown. The insets show a control onplant that lacks angiogenic growth factors and an angiogenic onplant that contains both Vascular Endothelial Cell Growth Factor (VEGF) and Basic Fibroblast Growth Factor (FGFb).

Table I

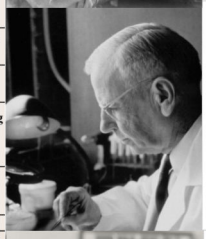
Historical timeline of significant discoveries made with the chick embryo.

Field	Time	Discovery
Development	1400 BCE	Egyptians are the first time artificially incubate chicken eggs, during the 18th dynasty.
	350 BCE	Aristotle begins work with chick embryos to study development (leads to major principles and mistakes) and is the first to actually dissect the embryo.
	1400s	Albert Magnus composes treatises on chick embryology that serve as the filler between Aristotle and the Renaissance.
	1567	Volcher Coiter publishes work on the development of the chick embryo, and compares this development to that of reptiles, humans, and other birds. This makes the field of comparative anatomy take off.
	1570	Volcher Coiter identifies the blastoderm using chick embryos.
	1628	William Harvey discribes the formation of blood islands and circulation, including functional differences between veins and arteries in chick embryos. He studies heart formation and commencement of beating in ovo using a magnifying lens. Previous to this, it was thought that the heart did not beat until birth/hatching.
	1651	William Harvey publishes findings that the generation of a chick is the result of epigenesis not metamorphosis. Rebukes Aristotles belief that chick eggs can grow without male fertilization.
	1671	Malpighi, through his studies of frogs and chicks, publishes work describing the role of capillaries.
	1672-1675	Malpighi discovers function of neural tubes and somites through the study of chick embryos. He describes the chick blastoderm, neural genesis and early heart development.
	1749	Beguelin perfects the window in the shell technique for chick observation as the embryo develops.
	1759	Casper Friedrich Wolff publishes "The Theory of Generation". His paper indicates that body organs develop in the embryo through a series of steps and challenged contemporary thought that organisms were preformed. His arguments sparked new interest in embryogenesis.
	1817-1828	Heinz Christian Pander, a follower of Wolff, and Karl Ernst von Baer discover and identify germ layers in the forming chick embryo.
	1826	Karl Ernst von Baer is the first to identify the mammalian ovum and notochord. He used the light microscope to extend Pander and von Baer's germ layer discovery, showing that it is universally present in vertebrates. Before him, it was suspected that changes between species in the stages of development represented progressive evolution. His findings supposedly influenced Darwin's thinking.
	1859	Darwin's publishes 'On the Origin of Species' and demonstrating correlations between organisms.
Immunology and Cancer	1906	Levaditi introduces the chick embryo as a model to study infection.
	1907-1913	Goldman and Murphy graft human tumors onto the CAM and recognize the vascular response necessary for successful engraftment.
	1911	Peyton Rous identifies the retrovirus Rous Sarcoma virus (RSV) in chicken embryos. He won the Nobel prize for his work in 1966.
	1931	Francis Ernest Goodpasture and Alice Woodruff publish their groundbreaking paper on their cultivation of viruses on the chick embryo, using the chick embryo for the cultivation of viruses becomes a common method.
	1932	Waddington developes a procedure to remove the chick blastoderm and culture it ex ovo. This technique is improved by New (1955) and becomes a valuable experimental model for development.
Genetics	1936	Frederick Hutt publishes the first genetic map of the chicken.
Cancer	1945-1955	Dagg, Karnofsky and Toolan perform routine serial transplantation of human tumors and initiat therapeutic trials on tumor bearing chicks.
Neurology	1952	Rita Levi-Montalcini – nobel prize winner for discovering nerve growth factors. Most of her defining work involved nerve development in the chick.
	1967	Michel Abercrombie discovers the cellular process of contact inhibition through his studies on the chick embryo, this process is now used to distinguish between normal and cancerous cells.
Cancer	1974	Folkman publishes CAM assay as a model to study vascularization.
	1983	Schwartz,Tizard and Gilbert determine the 9312 nucleotide sequence for the Rous sarcoma virus (RSV).
		Bishop reviews 25 known oncogenes. Nine are from domestic fowl.
		Ossowski, Chambers, and Quigley establish the chick as a model for metastasis.
genetic model for human disease	1991	Tiersch and Wachtel discover that the genome of birds, specifically gallus gallus, is one third the size of mammals, indicating the chickas a simple genetic model.
	2004	Avain flu moves from chicken to human infection (starting in Vietnam and Thailand) causes a world-wide focus on avian biology and disease.
		Richard Wilson's group (Washington University) publish a full avian genome sequence.
Intravital imaging model	1996	Chambers monitors single-cell behavior in the CAM using In vivo video
	2006	Lewis implements viral nanoparticles to image CAM and tumor vasculature intravitally.
	2008	Zijlstra uses intravital imaging to demonstrate correlation between cell migration in the primary tumor and metastasis to distant organs.


Aristotle


Marcello Malpighi


Dr. Francis Peyton Rous


Dr. Ernest Goodpasture


Dr. Rita Levi-Montalcini


Dr. Judah Folkman

Table II

tumor cell lines effectively cultured on the CAM

Origin	Cell Line	Source	Citation
Human	HEp3	Head and Neck Carcinoma	(Dagg et al., 1954), (Zijlstra et al., 2002)
	HT1080	Fibrosarcoma	(Rasheed et al., 1974), (Zijlstra et al., 2002)
	PC3	Prostate Carcinoma	(Deryugina et al., 2009)
	MDA MB231	Breast Carcinoma	(Unpublished, Zijlstra Lab)
	HeLa	Cervical Carcinoma	(deRidder et al., 1977)
	BLM	Melanoma	(Unpublished, Zijlstra Lab)
	SW480	Colorectal Carcinoma	(Unpublished, Zijlstra Lab)
Mouse	PyV-mT	Breast Carcinoma	(deRidder et al., 1977)
	4T1	Breast Carcinoma	(Unpublished, Zijlstra Lab)
	B16	Melanoma	(Nicolson et al., 1978)
	LLC/3LL	Lung Carcinoma	(Li et al., 1990)