TISSUE ENGINEERING: Part B
Volume 00, Number 00, 2020

DOI: 10.1089/ten.teb.2020.0048

termis
Tissue Engineering
& Regenerative Medicine
International Society

Chorioallantoic Membrane Assay as Model for Angiogenesis in Tissue Engineering: Focus on Stem Cells

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Tissue engineering aims to structurally and functionally regenerate damaged tissues, which requires the formation of new blood vessels that supply oxygen and nutrients by the process of angiogenesis. Stem cells are a promising tool in regenerative medicine due to their combined differentiation and paracrine angiogenic capacities. The study of their proangiogenic properties and associated potential for tissue regeneration requires complex *in vivo* models comprising all steps of the angiogenic process. The highly vascularized extraembryonic chorioallantoic membrane (CAM) of fertilized chicken eggs offers a simple, easy accessible, and cheap angiogenic screening tool compared to other animal models. Although the CAM assay was initially primarily performed for evaluation of tumor growth and metastasis, stem cell studies using this model are increasing. In this review, a detailed summary of angiogenic observations of different mesenchymal, cardiac, and endothelial stem cell types and derivatives in the CAM model is presented. Moreover, we focus on the variation in experimental setup, including the benefits and limitations of *in ovo* and *ex ovo* protocols, diverse biological and synthetic scaffolds, imaging techniques, and outcome measures of neovascularization. Finally, advantages and disadvantages of the CAM assay as a model for angiogenesis in tissue engineering in comparison with alternative *in vivo* animal models are described.

Impact Statement

The chorioallantoic membrane (CAM) assay is an easy and cheap screening tool for the angiogenic properties of stem cells and their associated potential in the tissue engineering field. This review offers an overview of all published angiogenic studies of stem cells using this model, with emphasis on the variation in used experimental timeline, culture protocol (*in ovo* vs. *ex ovo*), stem cell type (derivatives), scaffolds, and outcome measures of vascularization. The purpose of this overview is to aid tissue engineering researchers to determine the ideal CAM experimental setup based on their specific study goals.

Keywords: angiogenesis, stem cells, chorioallantoic membrane assay, tissue engineering

Introduction

The tissue engineering field has been a booming and strong evolving research branch of regenerative medicine during the last decades. Its ultimate goal is the complete structural and functional restoration of the native tissue or organ after injury by an interdisciplinary approach of engineering and life sciences.[1] Organ transplantation could be a potential treatment for patients suffering from organ failure, but its application is limited by the strong imbalance between the limited supply and high demand, resulting in long waiting lists of multiple years.[2,3] In addition, although the presence of diverse adult stem cell types has been widely acknowledged, the intrinsic regenerative capacity of most tissues is very

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limited.[4] Therefore, new therapeutic strategies should be developed to reduce the huge social and economic impact associated with this major health care issue.

Autologous or allogeneic transplantation of stem cells is able to replace injured cells by their differentiation capacity into diverse cell types. In addition, their paracrine properties even assume a more important role in regenerative medicine by boosting endogenous repair.[5–7] Besides the formation of new functional tissue, one of the greatest challenges in tissue engineering is the immediate vascular supply of these newly formed tissues to deliver oxygen and nutrients, eliminate waste products, and prevent necrosis.[8] Successful survival of transplanted cells depends on the rapid activation of angiogenesis, which is the formation of new blood vessels from preexisting ones. This form of neovascularization is characterized by degradation of the extracellular matrix (ECM), proliferation, migration, tube formation of endothelial cells, and maturation and stabilization of new blood vessels by the attraction and attachment of perivascular cells.[9] The process is strictly regulated by a balance between proangiogenic and antiangiogenic factors captured in the ECM and expressed by diverse cell types, including endothelial cells.[10–12] Several types of stem cells have also been found to regulate angiogenesis by the production of proangiogenic molecules.[13–16]

The angiogenic capacity of growth factors, cytokines, and cells is usually studied by *in vitro* assays, simulating the distinct steps of the process. The activity of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) can be tested by zymography as indication for ECM degradation.[17,18] Direct cell counting,[19] DNA labeling with bromodeoxyuridine (BrdU)[20] or propidium iodide (PI),[21] and measuring cell metabolic activity with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[21] are useful tools for cell proliferation, while endothelial cell migration is studied by Boyden transwell[22] or scratch experiments.[23,24] Finally, vessel network formation capacity is measured in the tube formation assay.[25,26] However, the relevance of these *in vitro* assays remains questionable, due to the lack of a functional blood flow, complex ECM, and cells.

Improvement of current angiogenic strategies for tissue regeneration requires the availability of more sophisticated *in vivo* models that could evaluate the impact of test substances on the angiogenic process in its entirety, including hind limb ischemia[27–29] and matrigel plug animal models.[30,31] These assays are often expensive, time-consuming, and associated with severe animal suffering. An alternative regularly used screening tool for angiogenesis is the chick embryo chorioallantoic membrane (CAM) assay. For this experiment, test substances are seeded in scaffolds and placed on the highly vascularized extraembryonic CAM of fertilized chicken eggs to evaluate their induction of functional blood vessel formation.[32,33]

This review focuses on the potential of the CAM assay as model to study the angiogenic capacity of stem cells and their consecutive use in tissue engineering. An overview of studies that cover distinct stem cell types, scaffolds, study protocols, and outcome measurements is given, with particular attention to the differences between *in ovo* and *ex ovo* approaches. In addition, the benefits and limitations of this CAM model are discussed and compared with those of alternative *in vivo* angiogenic models.

Chorioallantoic Membrane During Chicken Embryo Development

The chicken embryo has extensively been used by developmental biologists as a reference model to unravel essential morphological and molecular mechanisms of the general embryonic development. Because of its easy accessibility and manipulation, every developmental stage has thoroughly been documented. The first head-to-tail axis is the primitive streak that appears from 6 to 7 h of incubation and achieves its full length after 18–19 h. In the following hours and days, somites and the primordia of different structures, including limbs, eyes, ears, heart, and associated vasculature, are formed. This process is associated with the growing and bending of the embryo. Feather germs start to develop at 6.5 days, but only become visible after 8 days. During the second half of the embryonic development, organ systems further mature, till the chicken hatches on day 20–21.[34]

Respiration of the chicken embryo depends on the extraembryonic CAM. This highly vascularized membrane develops by the fusion of the mesodermal layers of the allantois and chorion. The resulting tri-layered CAM consists of, starting from the eggshell, the chorionic ectoderm, the fused mesenchyme layer with the major blood vessel network, and the allantoic endoderm (Fig. 1). Two allantoic arteries and one allantoic vein ensure the interaction with the embryonic circulation, analog to the umbilical arteries and veins in mammals.[35] CAM development starts on embryonic day 4 as a small vesicle at the height of the head, after which it enlarges strongly with a threefold increase in size between day 8 and 12. At this time, the CAM is completely covering the embryo and a plateau phase is reached, which is characterized by differentiation and expansion of the different layers.[36]

The associated vasculature of the CAM is continuously remodeled to meet the increasing needs of the chicken embryo. On day 8, the blood vessel network is restricted to small arterioles and venules in the mesenchymal layer, which make contact with capillaries located just beneath the chorionic ectoderm. These capillaries invade the chorionic ectoderm and form a complex capillary plexus in close contact with the eggshell membrane on day 14.[36] Respiration is mediated by the transport of venous blood from the embryo through the allantoic arteries and mesodermal afferents toward the chorion capillaries for gas exchange. The oxygenated blood flows back through the mesodermal efferents and allantoic vein to the embryo.[37]

The first embryonic blood vessels develop by vasculogenesis, starting after 42 h of incubation. During this process, angioblasts derived from the mesodermal wall of the yolk sac form a compact plexus, which is further differentiated and luminized to form a network of primitive tubes with endothelium, red blood cells, and blood plasma. This vascular system in the yolk sac is functional after 48 h.[38,39] Next, new blood vessels are formed by the proliferation of endothelial cells, causing the lengthening of existing vessels by the process of sprouting angiogenesis.[36,40] Growth factors, especially fibroblast growth factors (FGFs), in the chorioallantoic fluid are responsible for this mitogenic effect.[41] After day 10, growth factor concentrations decrease and endothelial cells stop proliferating, mature, and become flattened near the eggshell with their nucleus and cytoplasm

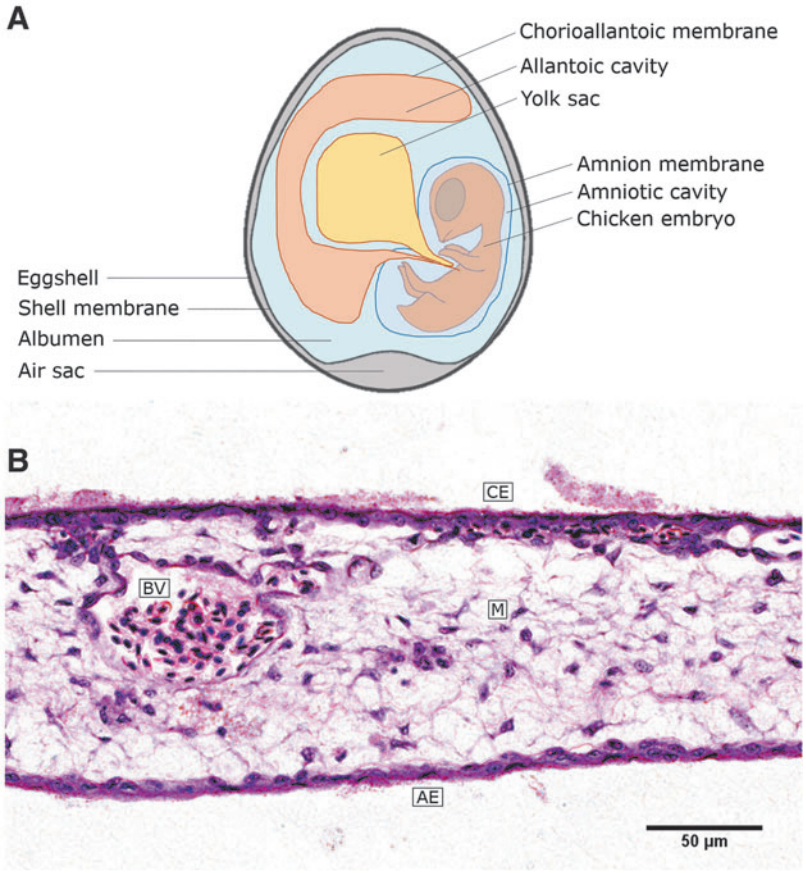

FIG. 1. General structure of the CAM of the chicken embryo. **(A)** Schematic overview of the general anatomy of the fertilized chicken egg with the embryo and its extraembryonic structures. **(B)** Histological hematoxylin-eosin staining of a 14-day-old CAM, showing the trilayered structure consisting of the CE, central mesenchyme layer (M) rich in BV, and AE. The figures are original work by our research groups. CAM, chorioallantoic membrane; CE, chorionic ectoderm; AE, allantoic endoderm; BV, blood vessels. Color images are available online.

situated on the opposite side to minimize the distance between oxygen and red blood cells, and to allow optimal exchange of oxygen and waste products.[36,42] During this stage, neovascularization shifts to the process of intussusceptive angiogenesis, which allows a strong rise in capillary numbers in just a few hours. This fast blood vessel formation is needed to meet the steep increasing metabolic needs of the embryo. In this process, existing capillaries are being split by the formation of transluminal pillars consisting of collagen fibril bundles, surrounded by endothelial cell processes. While the total number of endothelial cells remains stable, they are thinned out and remodeled to increase the surface of the vascular network. Several factors, including shear stress and vascular endothelial growth factor-A (VEGF-A), could initiate this form of neovascularization.[40,43–46] From day 18, the membrane and its associated blood vessel network degenerate and the respiratory activity is gradually taken over by the lungs.[37,47,48] In addition to this function, the CAM plays an essential role in calcium metabolism by transporting calcium from the eggshell to the embryo in an active way.[49] Finally, since the embryonic kidney excretes into the allantois, the CAM also stores the embryo's waste products, such as ammonia, urea, and uric acid.[50]

CAM as Experimental Model for Angiogenesis

Initially, the highly vascularized CAM has been exploited by scientists as an *in vivo* model to investigate tumor biology and angiogenesis. The very first described study dated from 1911, which successfully employed the extraembryonic membrane to grow implanted chicken sarcoma tumors.[51] Two years later, it was demonstrated that the CAM is also suitable for the transplantation of xenogeneic tissues without significant impact on their intrinsic morphological and biological characteristics. Grafting of rat sarcoma was performed between day 5 and 7 of incubation and allowed to grow until embryonic day 18, while tumor-induced angiogenesis was examined.[52] Both studies were conducted by the research group of Rous and Murphy, who stated that the outer extraembryonic chicken membrane was beneficial to use because of its location just underneath the shell membrane, its expanded vascular network, and the possibility of grafting with minimal associated trauma. Moreover, they suggested the absence of a significant defensive response against the allogeneic or xenogeneic tissue.[51,52]

Based on these first results, the CAM assay has been further adapted and exploited as an experimental model to study tumor biology,[53] angiogenesis,[54,55] invasion,[56,57] metastasis,[58,59] and antitumor therapeutics.[60,61] During the subsequent years, the CAM model was also used for transplantation of other allogeneic and xenogeneic (embryonic) tissues.[62–65] The cultivation of micro-organisms, including viruses and bacteria, started in 1930s.[66–68] Application of the CAM assay for vascular-related issues was initiated in the early 1970s. The proangiogenic and antiangiogenic capacity of diverse molecules, including tumor angiogenesis factor,[69] angiogenin,[70] FGF,[70] protamine,[71] and their associated carrier materials,[72] have been evaluated using the

CAM model. In addition, induction of CAM neovascularization by growth factor-secreting cells and their derivatives, seeded in diverse scaffolds, has also been described and is further elaborated in this review.

Different Culture Methods of the Chicken Embryo

The vasculature of the CAM can either be investigated inside the eggshell (*in ovo*) or the entire egg content can be grown in a recipient (*ex ovo*). The latter is also referred to as the shell-less culture technique or the *in vitro* method for chick embryo cultivation[73] (Fig. 2). In addition to the cultivation method, CAM protocols also differ in other culture parameters, including experimental timeline and incubation settings.

In ovo *CAM technique*

For the *in ovo* approach, eggs are rotated during the first 72 h of incubation to prevent attachment of the embryo to the eggshell. After 3 days, eggs are placed on the side and able to set under static conditions for short incubation times. Afterward, 2–3 mL albumen is removed and embryos are kept in their original egg structure in a humidified incubator with restricted access to the CAM through a small window that is made in the eggshell with sterile tweezers, drill, or electric engraving tools. In this way, pressure inside the egg is changed and sticking of the CAM to the shell membrane is prevented. Care should be taken to avoid shell dust falling down on the CAM, which could disrupt its native vascular development. To prevent dehydration and contamination of the embryo, holes are sealed with cellophane tape, Parafilm, or one half of a Petri dish. At a later phase, the window can be enlarged to facilitate access to the CAM.[74–78]

Ex ovo *CAM technique*

In the *ex ovo* model, the eggshell is gently cracked and the egg content is transferred to a recipient. The embryo is then further cultured in an incubator with high humidity and the developing CAM automatically grows superficially. Time of explantation is critical for embryonic survival and is ideally after 60–72 h of incubation. At this early embryonic stage, the CAM is not yet attached to the shell. When explanted after that period, the embryo's survival rate drastically decreases since the yolk sac tends to adhere to the CAM, leading to an increased chance of rupture when opening the shell.[79] In contrast, earlier cracking, as early as 48 h after the start of incubation, has also been described.[80] Nevertheless, based on our experience, this also leads to a lower survival rate (unpublished data).

In addition to the critical timing, embryo transfer is only successful when there is no damage to any of the embryonic structures or extraembryonic membranes, which would result in a decline of the embryo's viability as well. The yolk sac membrane is extremely prone to rupture when cracking is not performed gently. Even with careful cracking, small microscopic ruptures can occur, which remain unnoticeable the first days after *ex ovo* culture and can ultimately lead to release of yolk and death of the embryo. A frequently used method is to crack the egg on a sharp object, such as the edge of a knife, glass, or Petri dish.[79,81,82] Nevertheless, the shock of this action may cause the yolk membrane to break.

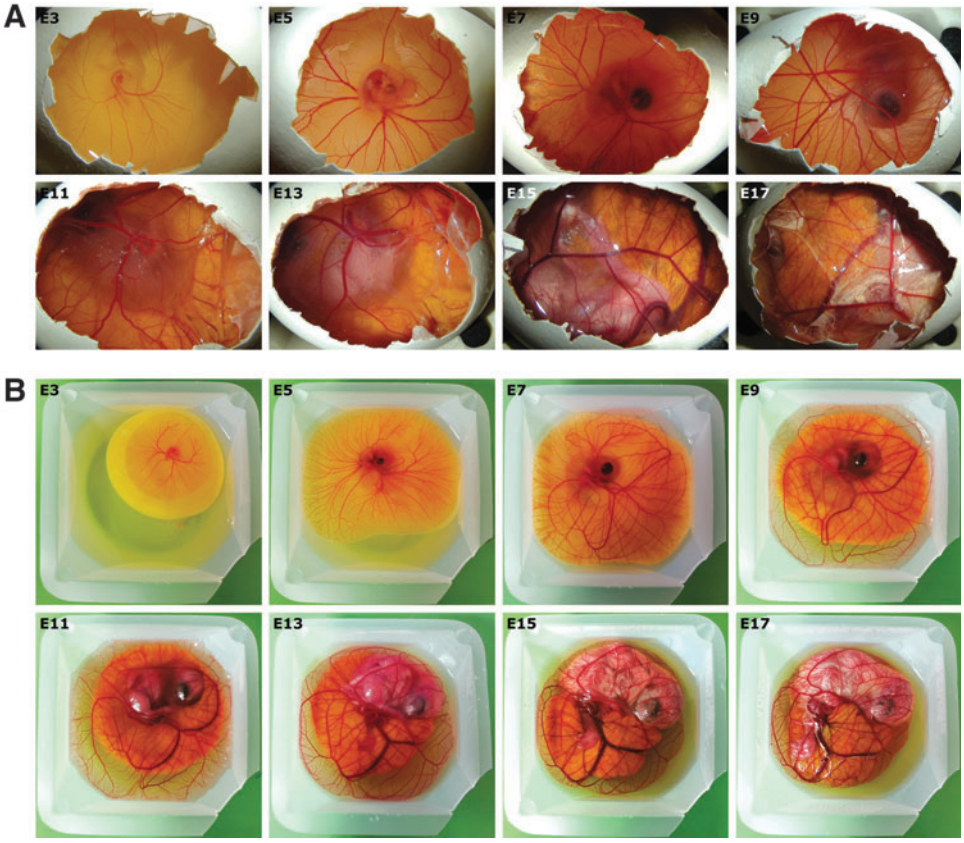

FIG. 2. Comparison of the *in ovo* and *ex ovo* embryonic development of the chicken. Fertilized chicken eggs were cultivated *in ovo* **(A)** or under shell-less **(B)** conditions in a humidified incubator. For the *in ovo* culture protocol, 2–3 mL albumen was removed after 3 days of incubation (embryonic day E3) and a window was made in the shell to visualize the embryo. *Ex ovo* cultured embryos were transferred to a weigh boat after an incubation period of 62 h. Embryonic development was followed up every 2 days, starting from E3, with a digital camera within in our research institutes. Color images are available online.

A second method of cracking the egg is by using a cutoff wheel (dremel tool) or small jigsaw to make small indents in the mineral part of the shell, perpendicular to its longitudinal axis. The egg can then be opened by applying gentle pressure, resulting in a better overall survival.[80,83]

Finally, the shape and material of recipient also influence the survival rate of the embryos. Various recipients are applied, of which round Petri dishes (80–100 mm in size) and square weigh boats of about the same size are the most commonly used containers.[33,73,79,81,83] These recipients are suitable for studying angiogenesis because of their stability and limited size, allowing easy manipulation and visualization by means of stereomicroscopy. In recipients with less depth (e.g., Petri dishes) the yolk sac is not completely immersed, which causes increased gravitational pressure on the yolk sac membrane, more frequent rupturing, and increased embryonic death. While weigh boats are deeper, allowing the yolk sac to be completely immersed in the albumen, round Petri dishes are still commonly used, since they are easily available and usually already sterilized.[73] An alternative recipient that has been described is a sort of hammock made from cling film that is fixed around the rim of a glass or plastic cup, used to cradle the embryo in.[80,82] However, this type of container is less convenient for studying the surface of the CAM because of its larger size.

Advantages and disadvantages of both culture models

The risk of contamination is an important issue for both *in ovo* and *ex ovo* culture methods, but is higher for the latter, because of the associated extra manipulations and larger surface of the egg content that is exposed. Contamination not only decreases embryo's survival rate but is also detrimental for a valid angiogenesis assay, because it can lead to a false positive angiogenic response. Specific precautions to minimize contamination include standard sterile handling procedures, such as rubbing the hands with antiseptic products, cleaning all used materials with 70% ethanol, and wiping the bottom part of the eggs with either 70% ethanol or povidone-iodine. The latter is preferred since it works instantaneously on both bacteria and fungi and is not absorbed through the eggshell, allowing a more thorough cleansing of the eggshells.[84] If possible, working in a laminar air flow hood minimizes contamination, but is not necessary to obtain good results.

The major disadvantages of the *ex ovo* CAM assay in comparison to the *in ovo* method are caused by the higher number of manipulations and materials needed. In addition, more precautions are required to maintain high survival rates. The *in ovo* approach is a sealed system, which permits the study of compounds with a short half-life,[74] while the large exposed surface of the *ex ovo* CAM may lead to excessive evaporation. Therefore, a stable and high humidity must be kept at all times, which can be achieved by working with individual boxes with holes to allow air circulation and containing an additional water source. It should be noted that the absence of the eggshell could cause preterm death, since older bird embryos rely on partial breakdown of the eggshell for essential nutrients, such as calcium and phosphates.[85] Studies that require embryos to survive past day 18 of incubation demand the addition of these nutrients.[86]

Despite these practical issues, the *ex ovo* CAM model has also a number of advantages. First of all, this approach results in a larger CAM area for experimental procedures compared to the *in ovo* method. Therefore, the amount of technical replicates per embryo can be increased, which minimizes interembryo variation in experimental settings and number of animals needed. Second, (real time) visualization of the CAM is much easier when the embryo has been brought *ex ovo* and the relatively easy access to the CAM opens a new range of otherwise impossible experimental designs.[87,88] These benefits of better visualization and manipulation have made the shell-less culturing technique a popular model.

Comparative studies between both cultivation systems could not demonstrate significant differences in size and weight of chicken embryos on embryonic day 10, 13, and 15. Moreover, chondrification, ossification, and nerve myelination run in parallel between *in ovo* and *ex ovo* cultures during the first 15 days of embryonic development.[80] These data suggest that isolation of the embryo from its normal eggshell structure has no significant impact on its native maturation. Nevertheless, Kirchner *et al.* showed a different course of the fractal dimension and vascular density of the CAM in time for both culture methods.[76] Therefore, the choice of cultivation system should be well considered and depends on the used experimental setup. Both methods have already been successfully applied to study the angiogenic potential of stem cells (Table 1).

Other culture variables

Various chicken strains from diverse breeders have been used, including white[22,75,89] and brown[90] leghorn eggs. Although studies comparing the CAM vasculature and angiogenesis between breeds are lacking, a significant impact on study outcome could be expected. For example, variation in sensitivity for viruses has already been described.[91] Furthermore, eggs are cultured in diverse incubators with various temperature and humidity settings ranging from 37°C to 38.5°C and 55% to 80%, respectively.[75,77,92–94]

Application of stem cells or their derivatives on the CAM has already been described starting from embryonic day 5, but might also be postponed to day 11. Starting the experiment in the initial slow-growing phase of the CAM or in its steep phase between embryonic day 8–12 will have a strong impact on measured background and stimulated angiogenesis levels. Most authors prefer this latter phase to induce a stronger angiogenic response compared to baseline values. Exposure time of the CAM to the test substance ranges from 24 h to 10 days (Table 1). The ideal incubation period might depend on several parameters, including stem cell type, start day of incubation, egg culture conditions, physical and chemical properties of the scaffold, and associated release and breakdown rate of angiogenic components. To prevent a decrease in the angiogenic action of stem cells or their secreted factors, repeated administration on a regular basis can be performed.[75,94] The CAM assay allows the application of multiple scaffolds per egg, which could reduce the number of animals needed.[95] Nevertheless, mutual interference between distant scaffolds cannot be excluded.

Testing the Angiogenic Capacity of Stem Cells and their Derivates in the CAM Assay

The potential of numerous stem cell types to induce neovascularization in the CAM assay has already been

Table 1. Overview of Publications Testing the Angiogenic Capacity of Stem Cells and Their Derivatives in the Chorioallantoic Membrane Assay

Test substance	*Test concentration*	*Time frame*	*Culture protocol*	*Scaffold*	*Outcome measure*	*Angiogenic effect*	*Reference*
Stem cells							
hBM-MSCs	0.2–2 million	E7-15	*In ovo*	GF-reduced Matrigel	Angiogenic scale 0–4	Increased angiogenesis	Oskowitz *et al.*[89]
	20,000	E5-6	*In ovo*	GF-reduced Matrigel-coated coverslips	IF	Capillary network formation by transplanted cells	Ball *et al.*[102]
	500,000	E11	*In ovo*	Intravenous injection	Real-time measurement of blood flow	Rolling and adhesion of cells to endothelium	McFerrin *et al.*[104]
rBM-MSCs	unknown	E11-14	*In ovo*	Not mentioned	Histology, IF	Invasion of transplanted cells into chicken embryo	Lu *et al.*[103]
hAT-MSCs	50,000	E7–10	*In ovo*	Bioglass® scaffolds and hernia (fibrous polypropylene) meshes	Number of nodes and bifurcations of ingrown blood vessels	Increased ingrowth of blood vessels	Handel *et al.*[106]
	5 million or 10 million	E7–16	*In ovo*	Fibrin matrix	Histology, IHC	Deep ingrowth of perfused blood vessels	Strassburg *et al.*[92]
	2 million	E9–12	*In ovo*	Chitosan and chitosan/gelatin hydrogel	Capillary area and number of blood vessel branch points	Increased number and branching of blood vessels	Cheng *et al.*[105]
	1 million	E10–13	*Ex ovo*	Nylon mesh	IF	Invasion of transplanted cells	Strong *et al.*[107]
hWJ-MSCs	1.5 million	E8-12	*In ovo*	Plastic ring	Number of blood vessel branches	Increased blood vessel number	Prieto *et al.*[93]
	500,000	E8-12	*In ovo*	Integra® Matrix	Number of blood vessel branches	Increased blood vessel number	Zavala *et al.*[109]
	unknown	E8-11	*In ovo*	GF-reduced Matrigel	Number and length of blood vessel branches	No significant effect on blood vessel formation	Kaushik and Das[90]
hPl-MSCs	100,000	E10-12	*In ovo*	Straw disk	Number of fine blood vessel branch points, IHC, IF	Increased blood vessel number and incorporation of transplanted cells in blood vessels	Lee *et al.*[111]
hDPSCs	50,000	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Bronckaers *et al.*[22]
	50,000	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Hilkens *et al.*[15]
	500,000	E7-14	*In ovo*	Silk fibroin scaffold + silicone ring	Scaffold perfusion by MRI, histology, number of blood vessels	Penetration of perfused blood vessels, but no significant differences with gingival fibroblasts	Woloszyk *et al.*[78]
	500,000	E7-14	*In ovo*	Silk fibroin scaffold + plastic ring	Scaffold perfusion by MRI, histology, IHC, scoring of number of blood vessels	Penetration of perfused blood vessels, but no significant increase in blood vessel number	Kivrak Pfiffner *et al.*[108]

(*continued*)

Table 1. (Continued)

Test substance	*Test concentration*	*Time frame*	*Culture protocol*	*Scaffold*	*Outcome measure*	*Angiogenic effect*	*Reference*
hSCAPs	50,000	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Hilkens *et al.*[15]
hMSCs	20,000	E10-17	*In ovo*	Silicone ring	Histology, IHC	Mutual interaction with chicken MSCs and induction of sprouting angiogenesis	Comsa *et al.*[112]
Human cardiopoietic stem cells	1 million	E7-14	*In ovo*	Optimaix-3D™ scaffold + plastic ring	Scaffold perfusion by MRI, histology, IHC	Increased perfused blood vessel density	Wolint *et al.*[95]
hCASCs	50,000	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Fanton *et al.*[16]
hNSSCs	300,000–500,000	E10-20	*In ovo*	Confetti membranes	Number of fine blood vessel branch points, histology, IHC	Increased blood vessel number and incorporation of transplanted cells in blood vessels	Vishnubalaji *et al.*[110]
hEPCs	2 million	E10-18	*In ovo*	Plastic ring	IF	Incorporation of transplanted cells in blood vessels	Sharma *et al.*[122]
	1 million/mL (hOECs)	E10-13	*Ex ovo*	Alginate hydrogel	Perfusion by Laser Doppler, number of blood vessels	Increased blood vessel number and perfusion	Campbell *et al.*[124]
mEPCs	400,000	E9-14	*In ovo*	hESC-qualified Matrigel + silicone ring	Vessel density, total branching points, total vessel network length, total segments	Increased angiogenic potential after angiopoietin-1 transfection	Steinle *et al.*[123]
Conditioned medium							
hBM-MSCs	concentrated by 10 kDa ultrafiltration	E8-12	*Ex ovo*	Gelatin sponges	Histology	Growth and infiltration of blood vessels toward the sponges	Gruber *et al.*[13]
	25X	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Merckx *et al.*[127]
mBM-MSCs	1X	E8-11	*In ovo*	Not mentioned	Number of class I (≥1 mm) and II (<1 mm) blood vessels	Increased blood vessel number of class II	Liu *et al.*[125]
	1X	E8-16	*In ovo*	Not mentioned	Number of class I (≥1 mm) and II (<1 mm) blood vessels	Increased blood vessel number of class I and II after inflammatory stimulation	Yang *et al.*[126]

(*continued*)

Table 1. (Continued)

Test substance	*Test concentration*	*Time frame*	*Culture protocol*	*Scaffold*	*Outcome measure*	*Angiogenic effect*	*Reference*
Canine BM-MSCs	1X	E6-9	*In ovo*	Silicone ring	Number of blood vessels and branches, angiogenic gene expression	Increased blood vessel density, overall increase in proangiogenic and decrease in antiangiogenic gene expression	Humenik *et al.*[77]
hAT-MSCs	1X	E7-14 (daily administration)	*In ovo*	Silicone ring	Total vessel density, total vessel network length, total branching points	No significant effect on blood vessel formation	Beugels *et al.*[75]
	1X	E10-13 (daily administration)	*Ex ovo*	Filter paper	Distribution and density of blood vessels by arbitrary values	Higher relative proangiogenic response	Wahl *et al.*[94]
hPl-MSCs	1X	E3-4	*In ovo* (yolk sac membrane assay)	Not mentioned	Number of blood vessels	Increased blood vessel number after prestimulation of cells with omega-3 fatty acids	Mathew and Bhonde[128]
hDPSCs	25X	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	No significant effect on blood vessel formation	Merckx *et al.*[127]
hCASCs	20X	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	Increased blood vessel number	Fanton *et al.*[16]
Extracellular vesicles							
hBM-MSCs	15 μL	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	No significant effect on blood vessel formation	Merckx *et al.*[127]
hUC-MSCs	50 μL	E10-15	*In ovo*	Filter paper	Number of blood vessels	Increased blood vessel number	Ma *et al.*[136]
hDPSCs	15 μL	E9-12	*In ovo*	GF-reduced Matrigel	Number of blood vessels crossing concentric circles	No significant effect on blood vessel formation	Merckx *et al.*[127]

E3-20, embryonic day 3–20; GF, growth factor; hAT-MSC, human adipose tissue-derived mesenchymal stem cell; hBM-MSC, human bone marrow-derived mesenchymal stem cell; hCASC, human cardiac atrial appendage stem cell; hDPSC, human dental pulp stem cell; hEPC, human endothelial progenitor cell; hESC, human embryonic stem cell; hMSC, human mesenchymal stem cell; hNSSC, human neonatal foreskin stem cell; hOEC, human outgrowth endothelial cell; hPl-MSC, human placenta-derived mesenchymal stem cell; hSCAP, human stem cell of the apical papilla; hUC-MSC, human umbilical cord-derived mesenchymal stem cell; hWJ-MSC, human Wharton's Jelly-derived mesenchymal stem cell; IF, immunofluorescence; IHC, immunohistochemistry; mBM-MSC, mouse bone marrow-derived mesenchymal stem cell; mEPC, mouse endothelial progenitor cell; MRI, magnetic resonance imaging; rBM-MSC, rat bone marrow-derived mesenchymal stem cell.

thoroughly documented. However, direct comparison between these studies is compromised by inconsistencies in type and number of transplanted stem cells, their tested paracrine secretion fractions, and the experimental setup, including egg culture conditions, experimental timeline, used scaffolds, and outcome measures for blood vessel formation (Table 1). All these parameters might influence the observed results on neovascularization and the preferred study protocol depends on the aimed research goals.

Test substance

Stem cells

Mesenchymal stem cells. The most extensively studied stem cell type in the field of tissue engineering is the mesenchymal stem cell (MSC), which is also reflected in the number of studies testing their angiogenic properties in the CAM. This cell population is characterized by its plastic adherence, fibroblast morphology, expression of several markers, including CD73, CD90, and CD105, and the absence of endothelial and hematopoietic surface markers such as CD34 and CD45. In addition, they possess a trilineage differentiation potential toward adipoblasts, chondroblasts, and osteoblasts.[96] MSCs can be isolated from various tissue sources, including bone marrow,[97] adipose tissue,[98] placenta,[99] Wharton's Jelly of the umbilical cord,[100] and soft tissues associated with teeth.[101] Proangiogenic properties have already been assigned to these stem cells in diverse *in vitro* and *in vivo* animal models, including the CAM assay.[13–15,22] Table 1 gives a detailed overview on the tested MSCs and the observed results in this latter assay.

Several research groups showed that application of bone marrow-derived MSCs (BM-MSCs) on the CAM induced neovascularization.[89,102–104] Oskowitz *et al.* demonstrated that this effect was enhanced after serum deprivation of the cells during 30 days.[89] Similarly, MSCs isolated from adipose tissue (AT-MSCs), Wharton's Jelly (WJ-MSCs), placenta (Pl-MSCs), newborn skin (NSSCs), and soft tissues in and around the tooth penetrated the chicken embryo and increased functional blood vessel growth in the CAM[15,22,78,92,93,105–111] (Table 1). These effects are mediated by their incorporation into newly formed blood vessels or paracrine secretion of proangiogenic factors that stimulate the host chicken endothelial cells. In case of NSSCs, Pl-MSCs, and WJ-MSCs, the angiogenic response was stronger when the cells were differentiated into endothelial cells before their implantation.[90,110,111] In contrast, Kaushik and Das could not detect a significant impact of WJ-MSCs on the number and length of blood vessels in the CAM, but the exact amount of transplanted cells was not mentioned in this study.[90]

Finally, Comsa *et al.* demonstrated a strong mutual interaction between implanted human MSCs (hMSCs) and chicken MSCs (cMSCs) present in the CAM. Transplanted hMSCs formed complex clusters in the chorionic epithelium with the induction of new vessel sprouts deriving from existing mesodermal vessels. In addition, cMSCs were promoted to organize into cord-like and capillary-like complexes in the chorionic epithelium and mesoderm. In parallel, hematopoiesis of cMSCs could be observed within these structures. Nevertheless, the tissue source of the used hMSCs was not clear in this study.[112]

Cardiac stem cells. Although present at very low percentages, different resident stem cell types have successfully been isolated from the adult cardiac tissue. Based on their expression of Islet-1,[113] c-kit,[114] and stem cell antigen-1,[115] three different types of cardiac stem cells (CSCs) have been discriminated. In addition, cardiosphere-derived cells[116] and cardiac atrial appendage stem cells (CASCs)[16] are defined by their isolation from self-assembling multicellular clusters of stem and progenitor cells or cardiac atrial appendages, respectively. CASCs express a plethora of proangiogenic factors and promote *in vitro* endothelial cell proliferation, migration, and differentiation. Moreover, transplantation of CASCs on the CAM promoted *in ovo* blood vessel formation.[16] In addition, hBM-MSCs differentiated toward cardiopoietic stem cells have been tested in the CAM assay by Wolint *et al.* Cell-seeded scaffolds were infiltrated with perfused blood vessels in a time-dependent way. Culturing these stem cells in 3D microtissues could improve their *in ovo* proangiogenic effect, resulting in higher blood vessel density and better perfusion efficacy.[95]

Endothelial progenitor cells. Vasculogenesis is associated with the formation of new blood vessels by differentiation of endothelial progenitor cells (EPCs) or angioblasts, in contrast to angiogenesis. This neovascularization process is dominant in the embryonic phase. Circulating EPCs were first isolated from the peripheral blood by Asahara *et al.* in 1997.[117] These cells are attracted to angiogenic foci in mouse and rabbit hind limb ischemia and myocardial infarction models, resulting in postnatal vasculogenesis.[117,118] In addition to their differentiation capacity, EPCs secrete proangiogenic factors and exert positive paracrine effects on endothelial cell survival, proliferation, migration, and tube formation.[119–121] In the CAM assay, GFP-labeled EPCs induced neovascularization and actively incorporated into the newly formed vasculature.[122] Transfection of murine embryonic EPCs with angiopoietin-1 mRNA improved their *in ovo* proangiogenic potential.[123] Application of umbilical cord-derived outgrowth endothelial cells, a specific subpopulation of EPCs, increased the number of functional CAM blood vessels and associated perfusion.[124]

Stem cell-derived conditioned medium.

As mentioned in the previously cited studies, most stem cells induce angiogenesis mainly in a paracrine way. This hypothesis has also been confirmed by applying solely conditioned medium (CM), which is cell culture medium incubated with stem cells for several hours to days and containing all secreted factors.

Mesenchymal stem cells. BM-MSC-derived CM from different animal species successfully enhanced neovascularization.[13,77,125,126] When these BM-MSCs were prestimulated with a combination of proinflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α for 12 h, a stronger angiogenic response was induced.[125,126] Application of canine BM-MSC-derived CM also resulted in higher blood vessel density in the CAM and was associated with an increase in the expression of chicken proangiogenic genes, including MMPs, FGF-1 and angiogenin, and inflammatory mediators, such as IFN-γ.[77]

Our comparative study of the angiogenic potential of human dental pulp stem cells (hDPSCs) and hBM-MSCs confirmed the *in ovo* proangiogenic effect of 25X concentrated CM of hBM-MSCs. In contrast, concentrated hDPSC CM did not induce neovascularization in the CAM, despite the angiogenic capacity of the cells themselves.[127] These data suggest that hDPSC-secreted factors are not sufficient to induce an *in ovo* proangiogenic response, possibly caused by the limited half-life of secreted growth factors.

Similar results were obtained for nonconcentrated hAT-MSC-derived CM, which only enhanced angiogenesis in the CAM assay when parental cells were electrically stimulated for 72 h. This pulsed electrical stimulation upregulated the secretion of proangiogenic and antiangiogenic proteins VEGF-A, thrombospondin-1 (TSP-1), TIMP-1, and monocyte chemoattractant protein-1 (MCP-1), while Serpin-E1 was significantly downregulated.[75] CM derived from hAT-MSCs cultured on collagen-glycosaminoglycan (GAG) matrix had a stronger angiogenic effect compared to medium originating from hAT-MSCs cultured on other scaffolds (i.e., chitosan films and fibrin matrices).[94]

The angiogenic capacity of CM derived from hPl-MSCs has only been studied in the chick yolk sac membrane assay and prestimulation of these cells with a combination of omega-3 polyunsaturated fatty acids increased their *in ovo* angiogenic potential.[128]

Cardiac stem cells. Medium derived from CASCs increased the number of CAM blood vessels growing toward the scaffold after 3 days, when concentrated 20 times.[16] Up to now, other CSC types have not been tested in the CAM assay.

Stem cell-derived extracellular vesicles. Besides their secretion of soluble mediators, stem cells release extracellular vesicles (EVs) in their CM. These nanoscale membrane-bound particles play an essential role in cell-to-cell communication by transferring proteins, miRNAs, and DNA between cells. Based on their size and origin, three different particle types have been discriminated, namely apoptotic bodies, microvesicles, and exosomes.[129,130] MSC EVs from different tissue sources contain a plethora of proangiogenic and antiangiogenic mediators. Moreover, they induce *in vitro* endothelial cell proliferation, migration, and tube formation and *in vivo* functional improvements in animal models of diseases characterized by disturbed vascularization, such as wound healing, stroke, and myocardial infarction.[131–135] However, our research group did not observe an angiogenic response in the CAM assay for human MSC EVs derived from the bone marrow or from the dental pulp.[127] In contrast, the exosomal subpopulation of human umbilical cord-derived MSCs (hUC-MSCs) increased the number of CAM blood vessels. Akt overexpression in the parental cells improved the angiogenic capacity of these exosomes.[136]

Scaffolds

To apply stem cells or their derivates on the CAM, diverse scaffolds have been used (Table 1). Selection of scaffold should be based on several important criteria including biocompatibility, bioactivity, and porosity. In addition, preservation of the viability and biological functions of stem cells should be guaranteed. Based on the density of the scaffold material, the release rate of cells and their associated secreted factors could vary. Moreover, some scaffolds might stimulate or inhibit baseline neovascularization themselves in the chicken membrane. The best choice is also depending on the desired outcome measure, such as blood vessel infiltration of the scaffold or general stimulation of neovascularization in the embryonic membrane[137–143] (Table 2).

The most frequently used scaffolds consist of ECM materials that resemble the *in vivo* natural 3D environment to preserve stem cell characteristics and functional properties and improve their survival. Matrigel is a basement membrane extract derived from mouse Englebreth-Holm-Swarm (EHS) sarcoma. This complex mixture of structural proteins (mainly laminin, collagen IV, and enactin), growth factors, and associated binding proteins is extensively used in the CAM assay[15,16,22,89,90,102] and other *in vitro* and *in vivo* angiogenic assays because of its rich cell-supporting composition.[144] Nevertheless, batch-to-batch variation and presence of intrinsic angiogenic mediators should be taken into consideration when interpreting the results.[145,146] To limit the intrinsic angiogenic capacity of this scaffold, the levels of growth factors, such as epidermal growth factor, insulin-like growth factor-1, and platelet-derived growth factor, are decreased in a commercially available 'Growth factor-reduced Matrigel'.[145] Other widely applied carriers are collagen-based scaffolds, such as Gelfoam sponges of collagen-degraded gelatin,[13] Optimaix-3D™ biodegradable sponges of porcine collagen,[95] and porous Integra® Matrix consisting of cross-linked bovine tendon collagen and GAGs.[109] In addition, fibrin stem cell-seeded matrices of polymerized fibrinogen and thrombin or collagen type I have been tested in the CAM assay.[92,147]

Membranes made of various synthetic materials have also regularly been used as support to place stem cells or their derivates on the chicken membrane. Straw disks,[111] filter paper,[94,136] confetti (hydrophilic polytetrafluoroethylene) membranes,[110] biologically inert elastic Optilene® (hernia fibrous polypropylene),[106] and nylon meshes[107] are possible successful alternatives for the more complex ECM-based scaffolds. The bioactive glass-ceramide composite 45S5 Bioglass® is mainly studied in the field of bone tissue engineering, because of its positive effect on bone formation.[148] In addition, this 3D scaffold is biocompatible with no effect on chicken embryonic development, neovascularization, or inflammation.[149] Therefore, it is an ideal carrier to study the angiogenic potential of stem cells in the CAM model as demonstrated by Handel *et al.*[106]

Biological materials are also promising candidates as delivery vehicles of stem cells to the CAM. Biocompatible and biodegradable silk fibroin scaffolds are derived from silkworms by chemical processing.[150] Kivrak Pfiffner *et al.* and Woloszyk *et al.* applied this hDPSC-seeded carrier system in the CAM assay, which resulted in the infiltration of perfused blood vessels in the scaffold.[78,108] Chitosan is a natural substance obtained from chitin present in the exoskeleton of different animals, including Crustacean. Chitosan-based scaffolds are biodegradable and biocompatible and their porosity can be regulated to optimize them for cell seeding.[151] Cheng *et al.* used a chitosan hydrogel to test the *in ovo* angiogenic potential of hAT-MSCs.[105]

Table 2. Overview of Scaffolds Used to Deliver Stem Cells and Their Derivatives on the Chorioallantoic Membrane

General characteristics	*Scaffold examples*	*Stem cells*	*References*
Natural materials			
Natural ECM proteins, resembling the *in vivo* native 3D environment	GF-reduced Matrigel	BM-MSCs, WJ-MSCs, DPSCs, SCAPs, CASCs	15,16,22,89,90,102,127
Support stem cell characteristics, survival, proliferation, adhesion, and biological functions	Gelfoam (gelatin) sponges	BM-MSCs, AT-MSCs	13,105
	Optimaix-3D™ sponges	Cardiopoietic stem cells	95
	Integra® matrices	WJ-MSCs	109
Biocompatible, biodegradable, bioactive	Fibrin matrices	AT-MSCs	92
Less controllable morphology, physical and chemical properties	Silk fibroin scaffolds	DPSCs	78,108
	Chitosan hydrogels	AT-MSCs	105
Poor mechanical strength	Alginate hydrogels	EPCs	124
High lot-to-lot variability			
Less scalability			
Strong purification needed to prevent contamination and immune reactivity			
Costly			
Fast degradation			
Synthetic materials			
Controllable, reproducible, high-scale production	Straw disks	Pl-MSCs	111
	Filter paper	AT-MSCs, UC-MSCs	94,136
High manipulation of degradation rate, morphology, mechanical, physical, and chemical properties	Confetti membranes	NSSCs	110
	Optilene® (hernia) meshes	AT-MSCs	106
	Nylon meshes	AT-MSCs	107
Less biocompatible			
Biologically inert			
Potential host immunological response			
Modifications needed to enable cell supporting/adhesion			
Ceramics			
Bioactive, biocompatible	45S5 Bioglass® scaffolds	AT-MSCs	106
Low immune reactivity			
High mechanical strength			
High porosity			
Less degradability			

AT-MSC, adipose tissue-derived mesenchymal stem cell; BM-MSC, bone marrow-derived mesenchymal stem cell; CASC, cardiac atrial appendage stem cell; DPSC, dental pulp stem cell; ECM, extracellular matrix; EPC, endothelial progenitor cell; GF, growth factor; NSSC, neonatal foreskin stem cell; Pl-MSC, placenta-derived mesenchymal stem cell; SCAP, stem cell of the apical papilla; UC-MSC, umbilical cord-derived mesenchymal stem cell; WJ-MSC, Wharton's Jelly-derived mesenchymal stem cell.

Finally, silicone or plastic rings have been placed on the CAM to define the location wherein stem cells or their derivates are administered.[75,77,93,112] This method allows the fast distribution of stem cells and their secreted factors over the embryonic membrane, which is in contrast with the previously mentioned viscous scaffolds that permit the gradual release of angiogenic factors. Some researchers combine these rings with other scaffolds to flatten the CAM around the delivery site.[78,95,108]

Comparative studies using more than one scaffold type emphasize the impact of the chemical, physical, and biological properties of these carriers on the angiogenic potential of stem cells or their derivates. While cell-free 45S5 Bioglass and hernia meshes had no intrinsic effect on CAM neovascularization, hAT-MSCs in hernia scaffolds induced a stronger blood vessel infiltration compared to stem cells seeded on the bioactive glass, probably caused by their higher proliferation rate and VEGF secretion.[106] In addition, a supporting hydrogel consisting of both gelatin and chitosan was needed for hAT-MSCs to increase the number and branching of CAM blood vessels, since cell-seeded chitosan scaffolds had no effect.[105] Gelatin supplementation mimics the *in vivo* ECM and improved stem cell adhesion and proliferation.[152]

Wahl *et al.* compared the effect of three different scaffolds (i.e., chitosan films, fibrin matrices, and collagen-GAG matrices) on the distribution, attachment, survival, and protein secretion profile of hAT-MSCs. Cell attachment was significantly lower in chitosan films compared to other scaffolds with the accumulation of stem cells at the seeding side. In the fibrin matrices, cells were mainly concentrated in the core, while a gradient from the seeding surface to the opposite side was observed for the collagen-GAG matrices in which cell viability was the highest. Moreover, variation in secretion of angiogenic proteins, such as VEGF, interleukin-8 (IL-8), and placental growth factor, was detected between the scaffolds. This resulted in significant differences in the proangiogenic potential of their CM in the CAM assay. CM from hAT-MSCs cultured on collagen-GAG matrices had the strongest effect on *ex ovo* blood vessel formation, followed by fibrin and chitosan matrices.[94]

Outcome measures of neovascularization

To describe the angiogenic response induced by stem cells in the CAM assay, diverse outcome measures have

been applied, including methods to quantify the blood vessel network, (immune)histology, RNA and protein analysis, and advanced imaging techniques (Table 1).

Methods to quantify blood vessels and their properties. The most commonly used method is to evaluate the morphology, distribution, and amount of blood vessels near the applied treatment on microscopic images (Fig. 3A). Based on these parameters, an arbitrary angiogenic score is given per egg.[89,94] Blood vessel density is also expressed as area percentage or absolute number of blood vessels that invade the cell-seeded construct,[90] which are present in a selected test area,[77,93,105,106,109–111,125,126,128,136] or that cross a concentric circle around the test substance.[15,16,22] Some studies discriminate the blood vessels based on their diameter[125,126] or determine the total length or shape of the CAM vasculature.[90,95] Although these analyses are standardly performed double blinded, this analysis method is not completely accurate and observations are not always reproducible. A possible solution is automatic analysis programs, such as WimCAM software that allows the measurement of total vessel density, vessel network length, and branching points.[75] Kirchner *et al.* successfully applied fractal analysis for the objective measurement of angiogenesis during CAM development.[76] This automation requires high-quality pictures of the vasculature with low background noise, which could be achieved by cutting out the CAM,[15,16,22] adding cosmetic white cream or zinc oxide suspension underneath the membrane,[75,93,109] injecting colloidal gold particles into the vasculature,[40] or digitally adjusting the contrast by ImageJ software.[77]

However, the biggest challenge to measure neovascularization is to discriminate between existing and new blood vessels. Therefore, some researchers prefer to take pictures before and after the treatment, but this is not always possible in each experimental setup.[77,128] An alternative solution, proposed by Nguyen *et al.*, is the application of a double nylon mesh-seeded scaffold. This standardized technique allows easy identification of new invaded blood vessels by focusing on the top mesh, while preexisting CAM vessels are limited to the bottom mesh (Fig. 3B, C).[33]

Histological and immunohistological analysis. Histological analyses and immunostaining provide in-depth evaluation of the angiogenic reaction of the CAM vasculature. Hematoxylin and eosin staining is a valuable method to study the general morphology of the connective tissue and associated blood vessels.[13,110,112] This technique has been used to evaluate the angiogenic effect of MSC-containing constructs[78,92,108] and collagen matrices loaded with human cardiopoietic stem cells.[95] Immunohistochemical and immunofluorescent staining for endothelial and pericyte markers, such as CD31, von Willebrandt factor, and α-smooth muscle actin (α-SMA), are useful tools to analyze the CAM vasculature.[108,112] Furthermore, immune-based staining against human antigens is applied to determine the viability and potential incorporation and differentiation of the stem cells into newly formed blood vessels.[92,110–112] Another regularly used method to track transplanted stem cells in the CAM is their prelabeling with fluorescent dyes such as CellTracker Green[104,107,111] or Fluoresbrite carboxylate microspheres.[103]

Advanced imaging techniques. In contrast to the aforementioned end-point analyses on tissue sections, other more advanced imaging techniques allow *in vivo* visualization of CAM vasculature. Injection of a radio-opaque contrast agent into the blood vessels and consecutive measurement of the X-ray signals by angiography offer a high-resolution real-time image of the heart and vascular network with low background noise. Assessed parameters are vessel density, diameter, and length in time.[153] Magnetic resonance imaging (MRI) can be performed on sedated chicken embryos with paramagnetic contrast agents such as Gadolinium-DOTA (Gd-DOTA) to measure perfusion capacity at different layers in the cell-seeded scaffolds.[78,95,108]

Kurz *et al.* made high-resolution 3D images of the CAM vasculature with a dual staining for α-SMA and YoPro-1 by

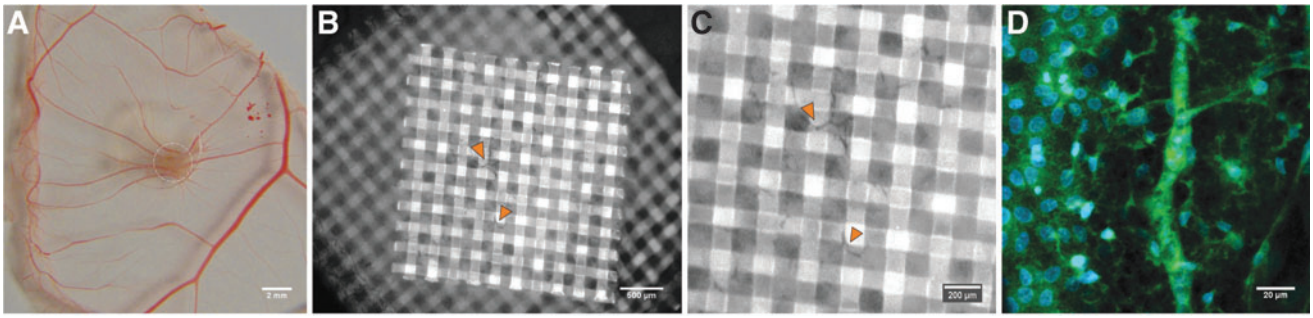

FIG. 3. Different methods to evaluate induced neovascularization in the CAM assay. **(A)** Application of a proangiogenic substance induces a typical spoke-wheel pattern of CAM blood vessels growing toward the scaffold. **(B, C)** Focusing on the *top* mesh of a double nylon mesh scaffold enables the evaluation of newly formed blood vessels, which can be distinguished from the preexisting vessels in the *bottom* mesh. Micrograph taken in the DAPI channel (excitation 385 nm and emission 463 nm), which shows the *dark* blood vessels (indicated by *arrows*) that are growing over the highly autofluorescent mesh. **(D)** The CAM vasculature can easily be visualized in 12-day-old chicken embryos by two-photon microscopy without additional fluorescent staining due to the autofluorescence of the blood vessel network (elastin). In addition, associated perivascular cells can be seen due to additional DAPI staining of their nuclei. The image shows the *green* blood vessels (excitation 800 nm and emission 500–560 nm) and the DAPI-stained nuclei. All data were collected within our research groups. Color images are available online.

confocal imaging. Arteries and veins were distinguished, since veins are devoid of erythrocytes during the fixation process.[154] Miller *et al.* demonstrated that confocal microscopic analysis of blood vessels injected with FITC-dextran was more objective and sensitive compared to standard evaluation of vascular density based on light microscopic pictures.[155] Confocal microscopy uses high-intensity excitation lasers with photons of low wavelength, which could damage chicken embryo development and limit its use in long-time follow-up CAM experiments.

A better alternative could be two-photon microscopy, which is based on excitation by two photons of half the energy (double the wavelength) compared to confocal microscopy. The longer wavelength of the photons induces less tissue injury. At the same time, two-photon microscopy allows deeper tissue penetration, since tissue scattering of high-wavelength photons is much lower, which could be very useful for studying the CAM. The 3D capabilities of two-photon and confocal microscopy are similar, although in two-photon microscopy, the resolution at higher depth is maintained, while in confocal microscopy, resolution rapidly worsens.[156] Therefore, our research group visualized the CAM vasculature without additional fluorescent staining (based on autofluorescence) by this microscopic technique (Fig. 3D). While our data were obtained on fixed CAM tissues, they do demonstrate that two-photon microscopy is a promising tool to monitor real-time neovascularization without additional manipulation due to the autofluorescence of the blood vessel network. Moreover, in such *in ovo* systems, the injection of fluorescent markers against blood vessel components, such as endothelial cells and pericytes, could allow more complex analyses. Such intravital imaging of blood vessels using two-photon microscopy has been used before in the mouse carotid region,[157] skin,[158] and brain.[159]

Analysis of RNA and protein expression. In addition to direct visualization of the CAM vasculature, angiogenesis can be studied by measuring the expression of proangiogenic and antiangiogenic factors in the CAM tissue. Humenik *et al.* extracted total RNA from CAMs incubated with CM from canine BM-MSCs. A polymerase chain reaction (PCR) array for 89 different angiogenesis-related genes was performed to compare their expression between treated and nontreated CAMs.[77] Protein levels can be measured by CAM-layered expression scanning. For this assay, proteins present in CAM tissue samples are transferred onto capture membrane stacks. By incubation of these membranes with fluorescently labeled antibodies against the proteins of interest, relative expression levels are determined based on the fluorescence intensity signals.[155]

Other Applications of the CAM Assay

Besides the study of the angiogenic capacity of stem cells and derivates, the CAM assay is frequently used to investigate tumor growth, morphology, vascularization, and molecular mechanisms.[160–166] Moreover, *in vivo* invasion of the epithelial basement membrane and successive metastasis by tumor cells can be simulated, in contrast to other animal models where this first barrier is bypassed by directly injecting cells into connective tissue.[161,167,168] In addition, tumor formation is established within a few days due to the nutrient-rich embryonic membrane and can easily be monitored in real time.[160,169] In this way, the CAM model enables to test the efficacy and side effects of potential anticancer therapeutics in a patient-personalized way, called ''Patient-derived chicken egg model'' (PDcE model). Tumors grafted onto the chicken membrane closely resemble the original patient tumor with a high transplantation success rate and can rapidly be screened for diverse therapeutic options.[169]

In addition to tumor grafts, other allogeneic and xenogeneic tissue fractions, including skin, liver, bone, and adrenal gland fragments, have successfully been transplanted onto the CAM to induce their growth, remodeling, and vascularization.[64,170–172] These effects arise from the mutual interaction between tissue graft and chicken components.[170] Tissue reperfusion is accomplished by the formation of peripheral anastomoses between graft and host vasculature and the ingrowth of CAM blood vessels.[171,173] The resulting tissue construct expresses strong similarities with its original morphology and offers opportunities to study physiological processes in tissue regeneration and remodeling *ex vivo*.[64,170,172]

Testing the suitability of diverse biomaterials for tissue engineering purposes is another potential application of the CAM assay. Several parameters, including cell infiltration, incorporation, angiogenesis, and inflammation, induced by biological and synthetic scaffolds that differ in chemical and physical properties, have been monitored in time.[142,143,174–176] The responses in the CAM are similar to those observed in mammalian animal models, which confirms the strong added value of this low-cost model for biomaterial screening.[175] In general, rough materials, such as collagen/elastin and filter membranes, result in neovascularization and inflammation, while smooth polymers inhibit angiogenesis.[142] The induced chicken embryonal inflammatory reaction is consistent to that of mammalians with an initial acute inflammation that progressively evolves into a chronic reaction associated with fibrosis.[174]

In addition to biomaterials, new potential drugs and chemicals are screened for their toxicity and biodistribution in the CAM model.[177] The most regularly used toxicity test is the Hen's egg test-CAM assay (HET-CAM). In general, the CAM is exposed to the test substance for 20 s and the vascular response is visually evaluated for 5 min. Based on different parameters, including hemorrhage, lysis, and coagulation, an irritation score is assigned ranging from nonirritant to severe irritation. This test is performed to predict conjunctiva irritation, since the highly vascularized mucous CAM resembles the eye membranous structure.[178] Moreover, toxicity studies on diverse substances, including pesticides, antimicrobiotics, and vehicles, demonstrated similar results in the HET-CAM and conventional Draize rabbit eye test.[179,180] Although other researchers questioned this correlation,[181] these data suggest that the HET-CAM test could serve as a fast and painless prescreening tool to reduce animal number and suffering. Besides ocular toxicity,[182–184] this model is also suitable to evaluate phototoxicity,[185,186] carcinogenicity,[187] skin toxicity,[188–190] and detrimental effects on tissue growth and development.[191]

Associated biodistribution of test substances is investigated by intravenous injection in the CAM vasculature. Tropism and accumulation of new potential anticancer therapeutics and diagnostic markers, such as photosensitizers

and drug-loaded nanocarriers, in tumor grafts can be confirmed in the CAM model.[192–194] In addition, new therapeutic targets that are accessible through the bloodstream might be identified.[195] Studying the cellular and organ engraftment of viral infections or cancer cells is another application of the CAM assay by histology, immunostaining, and real-time PCR of chicken embryonic tissues.[196,197] Moreover, biodistribution can also be analyzed in a noninvasive way using *in ovo* MRI after labeling of the test compounds with contrast agents.[198]

Benefits and Limitations of the Model

The CAM model is associated with numerous advantages in comparison with other *in vivo* models employed within the angiogenesis and vascular biology research field. One of the major gains is the cost-effectiveness of the model,[199] since the price of a fertilized egg and the associated care is significantly lower compared to a mouse or rat. Accordingly, the lower costs of the system and the rapid vascular growth of the CAM provide the opportunity for large sample sizes and rapid large-scale screening.[74,199,200] Moreover, multiple test compounds might be simultaneously implemented within one CAM as reported, for example, by Wolint *et al.*[95] The model also provides benefits in terms of technical simplicity, easy accessibility, and reproducibility.[200,201] Interestingly, the CAM is characterized by a slowly developing immune system up to day 15 postfertilization. Therefore, the study of regenerative processes induced by transplantation of xenogeneic cells, tissues, or (bio)materials is of particular interest, since immunological rejection might be restricted.[74,199,201,202]

Furthermore, the possibility for real-time *in vivo* monitoring puts the CAM assay to advantage. In particular, as previously mentioned, *ex ovo* shell-less CAM culture is much more accessible for live imaging. Several reports showed the usage of a wide range of imaging techniques reaching from conventional microscopy to photoacoustic microscopy and bioluminescence imaging.[203–206] Moreover, positron-emission tomography (PET), MRI, and computed tomography (CT) have been demonstrated to be useful tools for visualization of the CAM.[198,206–208] Nonetheless, X-ray-based procedures, such as PET and CT, might impact the CAM vasculature and interfere with observed angiogenic effects.[209,210]

The choice for this model could also be affected by other practical matters, including easy availability of equipment and skilled personnel and limited ethical considerations. Since the CAM is noninnervated, experiments are not associated with pain perception by the chicken embryo, which exempts this model from ethical approval for animal experimentation.[201] Besides these ethical advantages, the CAM assay also offers other benefits compared to mammalian experimentation, such as easy housing accommodations. Moreover, animals do not have to be restrained.[200]

Nevertheless, there are also several limitations associated with the CAM model. Although several outcome measures have been described to quantify angiogenesis, discriminating newly formed capillaries from preexisting ones is still challenging, since the CAM possesses a well-built expanded, actively growing baseline vascular network. Therefore, the angiogenic response induced by the test stimulus might be overestimated in this embryonic model, in comparison to the more physiologically relevant situation in which the vasculature is fully grown and quiescent.[74,199] In addition, the system only allows a short posttreatment observational period as physiological and morphological changes occur rapidly.[74,200] Moreover, the advantageous immune-deficient properties are limited to embryonic day 15 and a nonspecific inflammatory response may occur thereafter.[201] Moreno-Jimenez *et al.* explored a possible alternative approach to prolong the incubation period on the CAM. Their so-called double CAM system consists of harvesting the graft tissue and reimplanting it onto a second CAM.[206]

Even though the possibility for large sample sizes is advantageous, it is crucial to decrease interegg variability, which makes this technique labor-intensive. In addition, one of the major challenges of the CAM assay is the usage of measurable outcome methods and the lack of standardization in protocol, which has previously been illustrated (Table 1). Importantly, since the CAM model is a non-mammalian system, alternative drug metabolism might be present compared to other *in vivo* models.[74] Therefore, caution must be exercised when interpreting results. An additional downside of the CAM system is the high sensitivity to environmental factors, such as contamination, changes in pH or oxygen tension, and other irritants. These factors could impede growth of the embryo and might be accountable for an eventual false positive angiogenic response as mentioned above.[74,199,211] Finally, commercially available products, such as antibodies, cytokines, primers, and microarrays, for avian species are not widely available.[199]

Conclusion

Rapid and adequate angiogenesis is a crucial process within the field of tissue engineering, particularly when this involves transplantation of scaffolds with (stem) cells. Without the supply of the essential oxygen and nutrients, the engrafted cells do not survive. Therefore, numerous research efforts are focused on enhancing this type of blood vessel formation and need reliable *in vivo* models to investigate the potential of new scaffolds and (stem) cells. Traditional rodent angiogenesis models, such as the mouse matrigel and hind limb ischemia assays, are time-consuming, costly, and accompanied with ethical issues. Despite the fact that the CAM assay is used since more than a century, this type of experiment is still an excellent tool to study blood vessel formation as it is simple and inexpensive. While not mammalian, transplantation of cells derived from other species is possible since the chicken embryo is immune incompetent during the first 15 days of development. It also provides an answer in personalized medicine as it is feasible to graft patient-derived tumors on the CAM. In addition, a wide variety of scaffolds ranging from silicon, collagen, silk, and chitosan to bioactive glass have already been successfully applied on the CAM, proving its versatility. As most CAM assays allow a readout after several days, it may also speed-up the preclinical research process. Depending on the research question, it is important to consider the *in ovo* or *ex ovo* approach of this assay as described in this review. Most important disadvantages include the possibility of only short incubation times, interegg variability, and the non-mammalian drug metabolism. Nevertheless, the latest developments in automatic programs to calculate different

parameters of blood vessel formation and advanced imaging techniques, such as two-photon microscopy, make this tool an innovative and quantifiable assay, suitable for the tissue regeneration challenges of the 21st century.

Disclosure Statement

No competing financial interests exist.

Funding Information

This study was funded by ''Bijzonder Onderzoeksfonds'' of Hasselt University (BOF, BOF16DOC06) and Research Foundation Flanders (FWO, FWO1522518).

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Received: February 14, 2020
Accepted: March 23, 2020
Online Publication Date: May 1, 2020