Chapter 33

Method to Study the Role of Galectins in Angiogenesis In Vivo Using the Chick Chorioallantoic Membrane Assay

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Abstract

Angiogenesis is a complex multi-process involving various activities of endothelial cells. These activities are influenced in vivo by environmental conditions like interactions with other cell types and the microenvironment. Galectins play a role in several of these interactions and are therefore required for proper execution ofin vivo angiogenesis . This chapter describes a method to study galectins during physiologic and pathophysiologic angiogenesis in vivo using the chicken chorioallantoic membrane (CAM) assay.

Keywords Chorioallantoic membrane (CAM) assay, Chicken, Angiogenesis, Galectin, Tumor graft, Blood vessel, Vasculature

1 Introduction

Angiogenesis is a complex multi-process involving different activities of endothelial cells. The function of endothelial cells can be influenced by environmental conditions like changing flow dynamics, interactions with other cell types, and interactions with specific extracellular matrix components [1, 2]. Thus, while in vitro assays can provide insights in the effects of molecules like galectins and/or galectin inhibitors on endothelial cell function, further assessment of their role in angiogenesis in vivo is important. A commonly used assay to study angiogenesis in vivo is the chick chorioallantoic membrane (CAM) assay. The CAM is a highly vascularized extra-embryonic membrane that mediates exchange of gas and nutrients during embryonic chick development. It is formed between embryonic day of development (EDD) 3–10 of the 21-day gestation period by fusion of the allantois mesodermal layer—extending out of the embryo—with the mesodermal layer of the chorion. Within the resultant double layer, a dense vascular network develops up to EDD11 after which endothelial cell proliferation drops rapidly allowing further maturation of the vascular bed [3–5].

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Apart from the rapid vascular development, there are numerous advantages that warrant the use of the CAM assay for in vivo angiogenesis studies. First, the assay is low in cost, reproducible, reliable, and fairly simple to perform [3]. Furthermore, there are a variety of methods for the application of testing compounds using CAM, and several methods are available to monitor the subsequent response in the vasculature. For example, we have used the CAM assay to study the effects of galectin-1 and galectin-9 on angiogenesis [6–8]. The CAM assay can also be used to test the effects of other treatment modalities like radiotherapy or photodynamic therapy [9, 10]. Finally, the model does not require a sterile work environment, and since the immune system of the chicken embryo is not fully developed until ± EDD18, the CAM assay can also be used for grafting xenograft cells and tissues. On the other hand, nonspecific reactions can occur due to contamination with egg shell itself or due to the use of reactive carrier vehicles [5]. In addition, CAM development is sensitive to alterations in environmental conditions like temperature, oxygen tension, and osmolarity. This indicates that experiments using the CAM assay should be carefully executed. In this chapter, we will describe a method for the topical application of soluble compounds (galectins and/or galectin inhibitors) on the CAM in order to study their effects on angiogenesis in vivo. In addition, we describe how galectins, galectin-inhibitors, or other compounds can be injected intravenously in the CAM vasculature, and we provide a method to graft tumor cells onto the CAM which can provide information on the role of galectins in tumor growth and tumor angiogenesis.

2 Materials

We use fertilized eggs of white leghorn chicken that are purchased from a local commercial supplier. The eggs can be stored for several days at 4 °C/39.2 °F but after more than 1 week, the quality and viability of the eggs decrease, affecting the quality of the data. Please be aware that depending on the legislation of your country regarding animal use in research experiments, a license might be needed to perform the described experiments.

2.1 Incubation of the Chicken Eggs

1. Milli-Q water (or equivalent).
2. Sterilized fine point tweezers (e.g., standard 15-cm college tweezers).
3. Scotch "Magic" adhesive tape (seeNote 1).

1. Non-latex elastic rings (seeNote 2).
2. Saline (from any commercial vendor).

2.2 Application of Galectins/Galectin Inhibitors onto the CAM

3. Galectin ofinterest and/or galectin inhibitor, either purchased or home-made.
4. 10–100 µL pipette with sterile filter tips.

2.3 Data Acquisition and Analysis

1. Fridge or cold room.
2. 20-mL syringe.
3. 21 Gauge injection needle.
4. Contrast solution (4 g of zinc oxide (Sigma-Aldrich) in 50 mL pure vegetable oil (from your local supermarket)).

2.4 Data Acquisition and Analysis

1. Image analysis software (HetCAM, DCIlabs) or Adobe Photoshop/MS Office.

2.5 Grafting Tumor Cells onto the CAM

1. Approximately 5×10^6 cells (seeNote 3).
2. Matrigel (seeNote 4).
3. Soft paper tissues.
4. Ice.
5. 100- µL pipette with sterile filter tips.
6. Ruler with mm scaling.
7. Small surgical scissors.
8. Balance.
9. Phosphate-buffered saline (PBS).
10. Fixative, e.g., 4% paraformaldehyde in PBS or zinc fixative.

2.6 Intravenous Injection of Labeling Agents or Cancer Cells

1. 33 Gauge point 4 Hamilton injection needle.
2. 100- µL Hamilton syringe.
3. Saline.

2.7 Special Equipment

1. Fan-assisted humidified (egg) incubator, 37.8 °C/100.04 °F (seeNote 5). We use a FIEM MG140/200 Rural which allows us to switch between tilting racks and non-tilting racks (see Note 6). The incubator should be well humidified throughout the whole experiment to prevent dehydration of the CAM. We achieve this by putting water basins on the floor of the incubator (seeNote 7).
2. Fiber optic illuminator. We use a Schott KL 1500 Electronic Light Source (seeNote 8).
3. Stereo microscope equipped with a camera (e.g., Leica M125 stereomicroscope with 12.5:1 zoom which is equipped with a Leica KL1500 LED ring illumination system and a Leica 5 Megapixel DFC425 CCD camera).

3 Methods

A schematic drawing of the chicken gestation period is shown in Fig. 1a. A typical CAM assay takes approximately 10 days and a CAM tumor graft experiment takes 17 days. Thus, depending on the type and frequency of treatment, careful planning of the experiments is important. The time schedules that are used in our lab for a normal CAM experiment as well as for a tumor graft CAM experiment are shown in Fig. 1b.

3.1 Incubation of the Chicken Eggs

1. Transfer the eggs from the cold storage to room temperature for at least 12 h prior to the incubation (seeNote 9).
2. Clean the shell of each egg with a paper wipe or tissue soaked with Milli-Q water.
3. Place the eggs horizontally on a 90 ° tilting rack (provided with the egg incubator), which rotates minimally six times per 24 h. Place the rack in a pre-warmed and humidified fan-assisted egg incubator at 37.8 °C/100.04 °F (seeNotes 5 –7). The starting day of the incubation is regarded as EDD0.
4. On EDD3, put the eggs in an upright position and make a small hole in the narrow end of the shell with fine tip tweezers. This will translocate the air compartment in the egg to the top of the egg. Seal the hole with adhesive tape using as little tape as possible (seeNote 10). Stop the rotation of the racks and place the eggs back in the incubator, with the sealed hole at the top.
5. On EDD6, check the eggs for fertilization. Point the fiber optic light source (seeSpecial equipment and Note 8) toward one side of the egg. Vasculature should become visible at the opposite side of the egg. If not, the egg is not fertilized and can be discarded.
6. Create a window of ± 1 cm^3 in the top of the shell with fine tip tweezers (see Note 11). The CAM vasculature can now be observed through the window.
7. Proceed with direct application of galectin or galectin ofinterest onto the CAM (Subheading 3.2) or with grafting of tumor cells onto the CAM (Subheading 3.5).
8. Intravenous injection into the CAM vessels is possible from EDD10 (Subheading 3.6).

3.2 Application of Galectins/Galectin Inhibitors onto the CAM

1. On EDD6, carefully place a sterilized non-latex plastic dental ring through the window on top and in the center of the CAM. Seal the window with adhesive tape (seeNote 10) and place the egg back in the incubator for at least 2 h. This allows the ring to settle down on the CAM (seeNote 12).

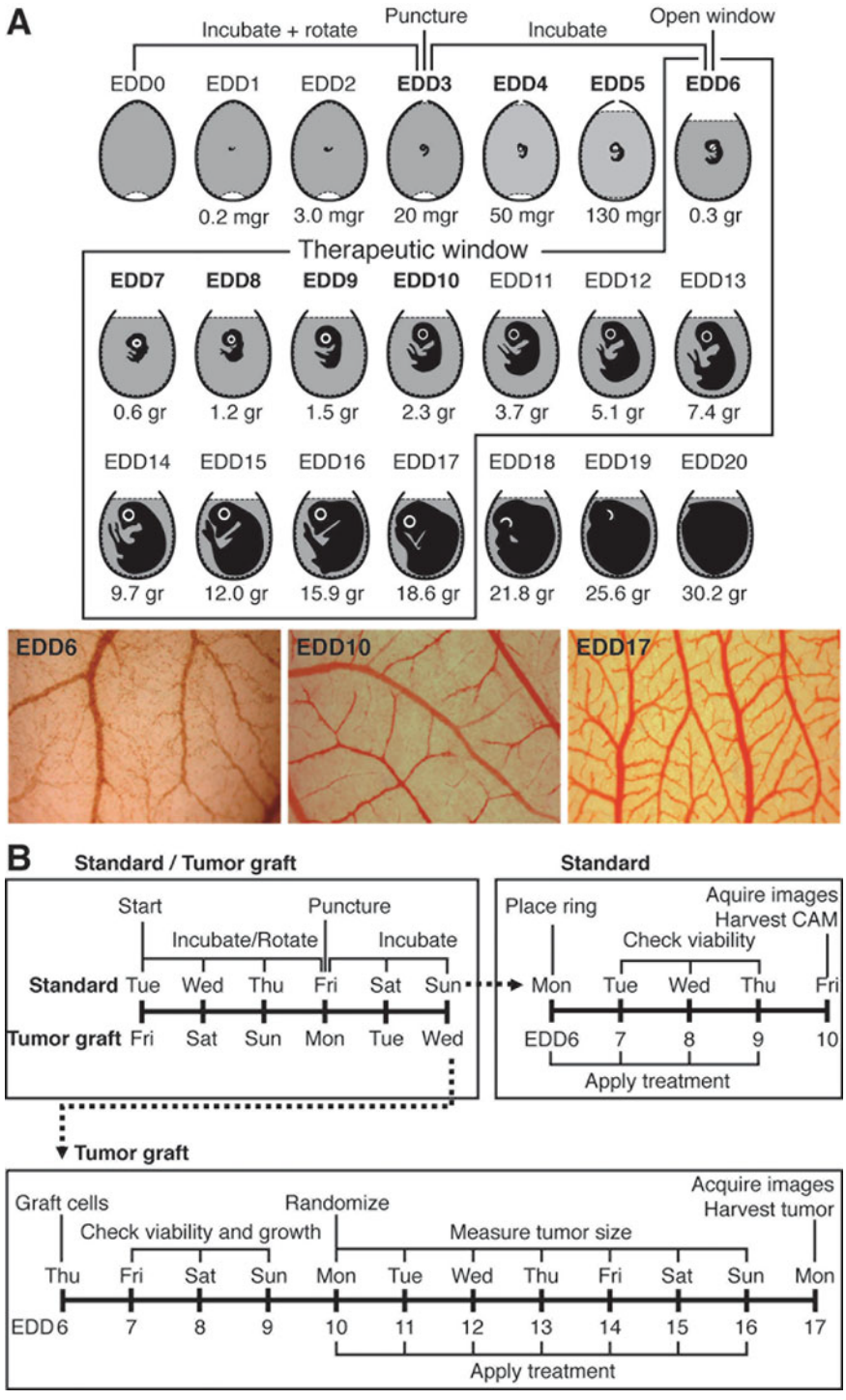

Fig. 1 Time schedule for the CAM assay. (a) Schematic representation of the CAM assay schedule during embryonic chicken development from embryonic day of development (EDD) 0 until EDD20, i.e., the day before hatching. The EDD during which extensive angiogenesis takes place in the CAM are shown in bold. The images show the CAM vasculature at different EDD. The number below the egg indicates the weight of the embryo in

2. Prepare the treatment solution by diluting the galectin of interest with or without the specific inhibitor in saline. Use a concentration range to identify the optimal concentration. For most galectins, this will likely be in the mid-micromolar range. The total amount of solution depends on the number of eggs, the diameter of the ring, and the duration/frequency of the treatment (see Note 13).
3. Take an egg out of the incubator. Open the sealed window and check if the embryo is still alive (you should see the heart beating). Apply 50–80 µL of galectin/galectin inhibitors at the desired concentration (usually in the mid-micromolar range) within the ring without touching the CAM itself. Reseal the window and place the eggs back in the incubator (see Note 10).
4. Repeat the addition of galectin/galectin inhibitors depending on your required treatment schedule. Usually, treatment is performed on a daily basis until EDD9.

3.3 Data Acquisition

1. On EDD10, place the eggs at 4 °C/39.2 °F for 30 min to induce hypothermia (see Note 14).
2. Prepare contrast solution by mixing 4 g of zinc oxide with 50 mL of pure vegetable oil in a 50-mL tube. Shake vigorously and leave it on a roller platform for 20 min.
3. Fill a 20-mL syringe with the zinc oxide/oil mixture. Make sure to remove any air bubbles.
4. Open the shell of a hypothermic egg as far as possible without disrupting the CAM.
5. Carefully inject ± 1 mL of the contrast solution directly under the CAM where the ring is located.
6. Use the microscope with camera to acquire images of CAM vasculature within the ring area (see Note 15).
7. If necessary, the treated CAM area can be collected for further analysis, e.g., gene expression, immunohistochemistry. Following acquisition of images, isolate the CAM area under the ring using fine tweezers and small surgical scissors. Wash the freshly isolated CAM in PBS and transfer it to the desired fixation buffer or liquid nitrogen. Further processing of the tissue is not described in this chapter.
8. Finally, euthanize the chicken embryo by transferring the egg to −20 °C/ −4 °F for 24 h.

Fig. 1 (continued) milligrams (mg) or grams (g). (b) Scheme of a standard CAM assay (upper panels) and of the tumor graft assay on the CAM (lower panel)

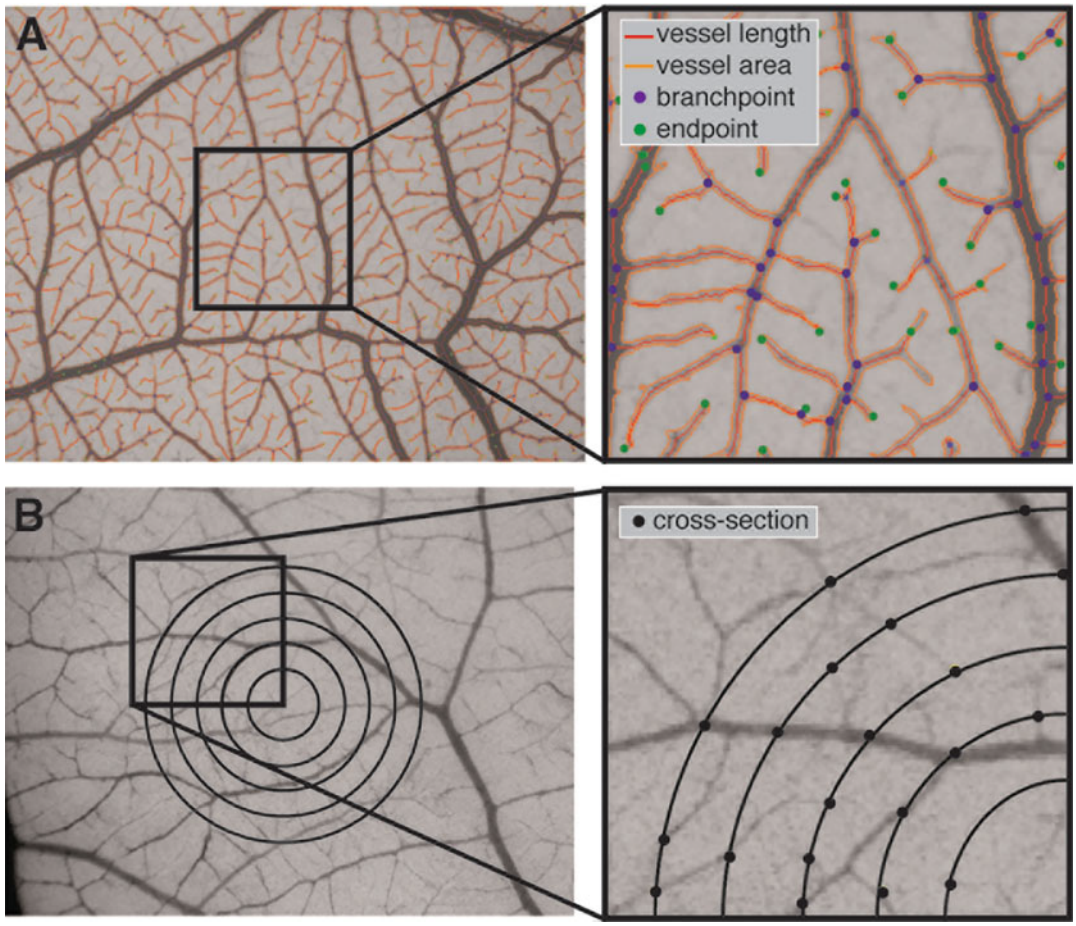

Fig. 2 Analysis of the CAM vasculature. (a) CAM analysis by skeletonization-based method. The HetCAM software (DCI labs) automatically analyses the skeleton length, the vessel area, the number of endpoints, and the number of branchpoints in each CAM picture. This method provides a highly objective and precise analysis of the vascular bed, which is quick and allows for high-throughput analysis. (b) Morphometric CAM analysis. In this method, five concentric rings are projected over the CAM image and the cross-sections of the vessels with the rings are counted. This will give insight in the vessel density of the CAM

3.4 Data Analysis

Several methods have been published to analyze the CAM images [5, 11–13]. Nowadays, software-based image analysis is often used for rapid, objective, and extensive image analysis. The software uses specific algorithms to recognize and skeletonize the vascular bed from which different vascular parameters can be extracted like vessel length, vessel branchpoints, vessel endpoints, total vessel area, etc. (Fig. 2a). We use HetCAM software (DCIlabs, Belgium), but other software packages might be used as well. However, we are aware that such software is expensive and not always available. Therefore, we here describe a widely accepted morphometric method to analyze images of the CAM, using software that is available in most

research labs, e.g., Adobe Photoshop or ImageJ (Fig. 2b) (see Note 16).

1. Open the desired CAM image in a graphics editing program like Adobe Photoshop (or any comparable software package).
2. Set the image to grayscale to enhance the contrast between the vessels and the background. If necessary, use the image auto-contrast function to improve contrast. Note that this should be performed for all images within a single experiment and that this should not be used to obscure or remove any unwanted data.
3. Place the CAM image in a graphic design program like Adobe Illustrator or a presentation program like PowerPoint.
4. Project 5 concentric rings over the CAM image and count the cross-sections between the vessels and the rings. The sum of these counts is a morphometric measurement for vessels density in the CAM.

3.5 Grafting Tumor Cells onto the CAM

While the method described above provides information on the direct effects of galectin/galectin inhibitors on angiogenesis, much galectin research is performed in the context of tumor biology. Consequently, it is important to determine how galectin expression in tumor cells or treatment with galectin-targeting compounds affects tumor growth and tumor angiogenesis. This can be readily studied using the CAM assay since it is possible to graft (human) tumor cells onto the CAM, most of which will rapidly grow into well vascularized tumors.

1. On EDD6, harvest the tumor cells. Count the cells and aliquot them in separate 2-mL tubes, each tube containing 5×10^6 cells and spin them down for 5 min at 400 rpm. Discard the medium.
2. On ice, mix 5×10^6 cells with 50 μL Matrigel (see Note 3).
3. Carefully "damage" a small area of the CAM by touching it with a soft tissue causing a small bleeding (see Note 17).
4. Transfer the Matrigel/cell mix onto the damaged area.
5. Close window and place egg back into incubator.
6. Check growth of tumor daily and measure size and width using a ruler (see Note 18).
7. If necessary, start treatment on EDD10 by applying the galectin/galectin inhibitor of interest topically onto the tumor or by direct injection into the tumor tissue or injection in the CAM vasculature (Subheading 3.6). The appropriate concentration should be determined for each specific galectin or inhibitor by using a concentration range, e.g., from high nM to high μM range.

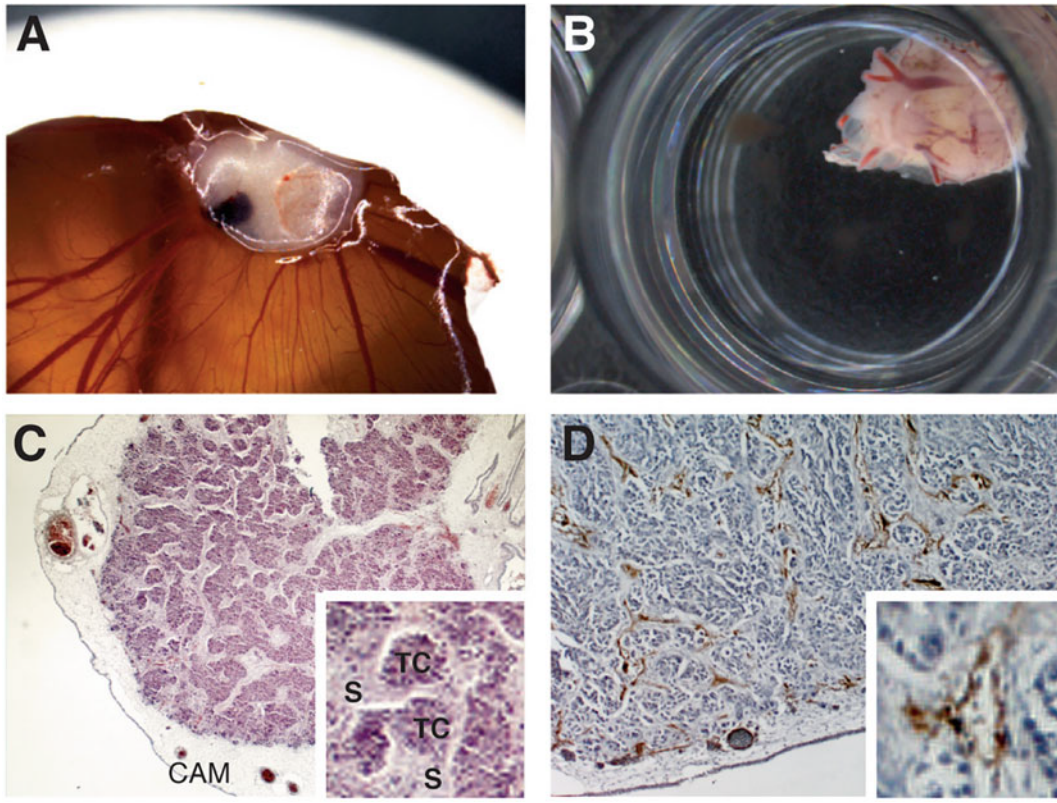

Fig. 3 Tumor Grafts on the CAM. (a) Image of a HT29 tumor on the CAM at EDD14. The tumor cells were grafted on EDD6. (b) HT29 tumor after resection. The tumor is well vascularized, indicating adequate tumor angiogenesis. (c) Image of hematoxylin/eosin staining on a standard paraformaldehyde-fixed and paraffin-embedded HT29 tumor graft. Both the CAM and nest of tumor cells (TC) surrounded by tumor stroma (S) are clearly visible. (d) Image of vessel staining (CD31, brown) on a paraformaldehyde-fixed and paraffin-embedded HT29 tumor graft

8. Measure size on a daily basis until EDD14 or maximally until EDD17 (seeNote 18).
9. At the end of the experiment, harvest, photograph, and weigh the tumor and subsequently place it in the appropriate fixative for further processing (Fig. 3).
10. Discard the eggs as described in Subheading 3.3 .

3.6 Intravenous Injection of Cancer Cells or Labeling Agents

If necessary, galectins, galectin inhibitors, or other compounds of interest can also be injected intravenously. Please note that this takes quite some practice and include some additional eggs to compensate for loss of egg due to bleedings. Of note, it is also possible to inject cells to analyze cell migration and metastatic growth [3], but this will not be described here. Again, optimal concentrations should be determined empirically.

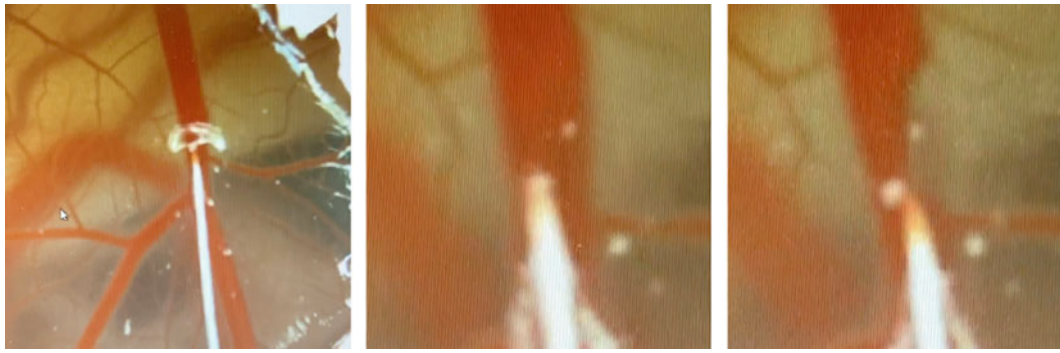

Fig. 4 Intravenous injection in the CAM vasculature. The left image shows an overview of the CAM vasculature to get a sense of the size of vessels that are appropriate to inject. The arrowhead shows the point of entry of the 33-gauge needle. The middle image shows an enlargement of the needle entry point and the right image shows a volume ofliquid entering the blood vessel (highlighted by dotted line)

1. On EDD6, open the eggs and close window and place eggs back in the incubator (seeNote 19).
2. Injection is possible from EDD10. On earlier time points, the vessels are too small and bleedings will more frequently occur reducing the efficacy of the experiment.
3. Under the stereomicroscope, carefully microinject 50 µL intravenous using a 33-Gauge Hamilton needle and 100- µL Hamilton syringe (seeNote 20 and Fig. 4).
4. Ensure that any air bubbles are removed prior to injection.
5. During injections, it is recommended to gently pulse the plunger. This will result in more uniform distribution of agents. Expect an average injection time of 2–5 min per embryo.
6. After injection, slowly pull needle out of the vessel. Clean up blood by light tapping with a soft tissue.
7. Proceed with the experiment as described in Subheading 3.2 or Subheading 3.5 .

4 Notes

1. Other brands of tape can be used, but we have good experience with the Scotch Magic tape because it is not too sticky which makes it easy to repeatedly open and close the window in the egg shell.
2. Non-latex rings are commercially available, or they can be custom-made. It is important that the weight of the ring is as low as possible to avoid nonspecific responses in the vasculature due to occlusion of vessels by the ring. We have found that orthodontic dental elastic bands (non-latex, Ø 9.5 mm) are a

good and cheap alternative. Varying the diameter of the rings allows larger area's to be treated but also requires more compound. In addition, with increasing diameter the weight of the ring also increases. A diameter of ±Ø 9.5 mm typically allows application of 50–80 µL solution.

3. The number of cells required for adequate grafting depends on the specific cell line and should be tested. However, we have found that increasing the cell number increases the success of grafting and most cell lines tested in our lab (colon, kidney) show successful grafting when 5 million cells are used. This is just a matter of trial and error. We usually start by testing 0.5×10^6, 1×10^6, 2×10^6 and 5×10^6 in five eggs per group to determine graft rate.
4. We have successfully used both normal and growth factor-reduced Matrigel. The latter is preferred if the angiostimulatory effect of cells is tested since normal Matrigel already contains more stimulatory factors by itself.
5. Temperature setting depends on the type ofincubator. For a still-air incubator (no fan): 38.5 °C (101.3 °F) measured at the top of the eggs. For a fan assisted incubator: 37.8 °C (100.04 °F) measured anywhere in the incubator.
6. If the incubator has no tilting racks, manual rotation of the eggs is also possible. Turn the eggs through 180 degrees by hand at least twice a day.
7. Humidity should be maintained at ±60% during incubation. Excessive humidity could result in an increased rate ofinfections in the eggs.
8. The fiber optic illuminator is used to check successful fertilization on EDD6. Vessel-like tube structures (orange/reddish) should be visible on the inner side of the egg shell. However, if no such device is available, successful fertilization can also be readily checked following opening of the egg shell.
9. We use anywhere between 8 and 10 eggs per treatment condition. For experienced users, a total number of about 80 eggs per experiment is manageable which thus allows for 8–10 different groups per experiment.
10. The adhesive tape prevents the CAM from dehydration. However, the tape also prevents gas exchange through the egg shell and should therefore be kept to a minimum.
11. The egg shell should be removed carefully since debris of the shell can induce a response in the CAM.
12. Instead of a ring to define the therapeutic area, some people prefer to use for example gelatin or methylcellulose discs [14, 15]. Also, other absorbent materials might be used as long as these do not induce a response in the CAM.

13. In general, 50–80 μL of compound is applied daily from EDD6 until EDD10. The solution can be prepared fresh daily or stored at 4 °C/39.2 °F. Note that long-term storage at 4 °C/39.2 °F of galectins in solution can affect protein stability and activity. Furthermore, solutions stored at 4 °C/39.2 °F should be allowed to return to room temperature before applying them to the CAM.
14. Movements of the embryo are very likely to disturb you while taking pictures of the CAM. The induced hypothermia will result in less movements, by slowing down the metabolism of the embryo. However, if the aim is to measure blood flow, hypothermia should not be applied.
15. The magnification will determine the level of detail that can be analyzed. At 25-fold magnification (2.5 × 10) mainly the larger, mature vessels will be visible while at 100-fold magnification (10 × 10) detailed images of the capillary bed can be obtained. We usually acquire images at both magnifications in order to distinguish between both vessel types.
16. This quantification method is laborious, less accurate and more sensitive to subjective errors. In addition, it will only provide information about the vascular density. Nevertheless, it is a cheap method and available to everyone.
17. Preferentially damage the small vessels above the embryo. The larger vessels less easy to damage and bleedings will more frequently occur reducing the viability of the embryo.
18. Be aware that not all tumors will grow on top of the CAM. We have observed that some tumors will grow just underneath the CAM.
19. We observe that more eggshell debris drops onto the CAM when embryos are opened at a later time point.
20. We use a 33-gauge Hamilton needle and 100- μL Hamilton syringe. It is also possible to use a pulled sodium borosilicate needle and 1-mL disposable syringes. Injection is most easy in one of the larger vessels or at a bifurcation of a larger vessels. Also try to inject in the direction of the blood flow.

References

1. Carmeliet P, Jain RK (2011) Molecular mechanisms and clinical applications of angiogenesis. Nature 473:298–307
2. Griffioen AW, Molema G (2000) Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. Pharmacol Rev 52:237–268
3. Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V (2001) Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 264:317–324
4. Ribatti D (2008) Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. Int Rev Cell Mol Biol 270:181–224

5. West DC, Thompson WD, Sells PG, Burbridge MF (2001) Angiogenesis assays using chick chorioallantoic membrane. Methods Mol Med 46:107–129
6. Aanhane E, Schulkens IA, Heusschen R et al (2018) Different angioregulatory activity of monovalent galectin-9 isoforms. Angiogenesis 21:545–555
7. Thijssen VL, Postel R, Brandwijk RJ et al (2006) Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. Proc Natl Acad Sci U S A 103: 15975–15980
8. Thijssen VL, Barkan B, Shoji H et al (2010) Tumor cells secrete galectin-1 to enhance endothelial cell activity. Cancer Res 70: 6216–6224
9. Nowak-Sliwinska P, van Beijnum JR, van Berkel M, van den Bergh H, Griffioen AW (2011) Vascular regrowth following photodynamic therapy in the chicken embryo chorioallantoic membrane. Angiogenesis 13:281–292
10. van Beijnum JR, Thijssen VL, Lappchen T et al (2016) A key role for galectin-1 in sprouting angiogenesis revealed by novel rationally designed antibodies. Int J Cancer 139(4):824–835
11. Irvine SM, Cayzer J, Todd EM et al (2011) Quantification ofin vitro and in vivo angiogenesis stimulated by ovine forestomach matrix biomaterial. Biomaterials 32:6351–6361
12. Nowak-Sliwinska P, Ballini J-P, Wagnieres G, van den Bergh H (2010) Processing of fluorescence angiograms for the quantification of vascular effects induced by anti-angiogenic agents in the CAM model. Microvasc Res 79:21–28
13. Rizzo V, DeFouw DO (1996) Mast cell activation accelerates the normal rate of angiogenesis in the chick chorioallantoic membrane. Microvasc Res 52:245–257
14. Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, Presta M (1995) Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. Dev Biol 170:39–49
15. Ribatti D, Nico B, Vacca A, Presta M (2006) The gelatin sponge-chorioallantoic membrane assay. Nat Protoc 1:85–91