Diploma Thesis

Influence of visible light on angiogenic features of melanocytes in CAM assay.

submitted by

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Declaration in lieu of oath

I, herewith, declare that I have written the following diploma thesis fully on my own and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself. Any thoughts directly or indirectly taken from somebody else's sources are made discernible as such.

Graz, 03.11.2021 *Attila Ismayilov eh.*

Acknowledgments

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Content

Glossary and Abbreviations

BF	brightfeld
bFGF	basic fibroblast growth factor
CAM	chorioallantoic membrane
DAPI	4′,6-diamidin-2-phenylindol
DNA	deoxyribonucleic acid
e.g.	exempli gratia
EtOH	ethanol
Fl	fluorescence
HE	haematoxylin and eosin
IOP	intraocular pressure
LCA	leukocyte common antigen
Nr	number
OCT	optical coherence tomography
P/S	penicillin streptomycin
PBS	phosphate-buffered saline
PFA	paraformaldehyde
pH	potential of hydrogen
RPE	retinal pigment epithelium
U/mL	units per milliliter
UV	ultraviolet

Table of Figures

Table of tables

Zusammenfassung

Melanozyten produzieren das Pigment Melanin, welches vor allem in der Haut und in der Iris des Auges eine wichtige Schutzfunktion gegenüber schädlichen UV-Strahlen hat. Da Melanozyten auch im Gewebe welche nicht lichtexponiert sind (z.B.: dem Innenohr oder der Aderhaut des Auges), wird angenommen, dass diese Zellen auch weitere Funktionen haben. Ein Fehlen dieser Zellen im Innenohr führt zur Taubheit, ein Fehlen in der Aderhaut des Auges zu einem Mikrophthalmus. Der genaue Zusammenhang zwischen Melanozyten und diesen Pathologien ist jedoch noch nicht geklärt. Wir suggerierten, dass Melanozyten Angiogenese induzieren, welche wichtig für die Organentwicklung ist. Eben diese Funktion soll in dieser Arbeit experimentell beleuchtet werden.

Zielsetzung

Ziel dieser Arbeit ist es, die Hypothese, dass die Melanozyten unabhängig vom Licht ein angiogenetisches Potential haben, experimentell zu erforschen.

Material und Methoden

Für die Untersuchung der Angiogenese wird die Chorioallantois Membran des Hühnerembryo eingesetzt. Primäre Melanozyten werden aus der menschlichen Vorhaut isoliert und auf die Chorioallantois Membran des Hühnerembryo (CAM) kultiviert. Auf der CAM werden die Melanozyten ex-ovo mit und ohne Licht für 4 Tage inkubiert. Das auf CAM entstandene Gewebe wird histologisch aufgearbeitet, und mikroskopisch ausgewertet.

Resultate und Diskussion

Melanozyten wuchsen sehr gut auf CAM, in den histologischen Präparaten konnte man eine lichtunabhängige Melanozyten induzierte Angiogenese beobachten. Die Ergebnisse sind ein Grundstein für weitere Untersuchungen, zu einem besseren Verständnis der melanozytären Funktionen. Darüber hinaus würde das CAM Model eingesetzt werden können, um Erkrankungen, welche im Zusammenhang mit Melanozyten (z.B. Mb. Waardenburg) stehen, besser zu verstehen.

Abstract

Melanocytes produce the pigment melanin, which has an important protective function against harmful UV rays, especially in the skin and in the iris. Since melanocytes are also found in tissues that are not exposed to light (e.g. the inner ear or the choroid of the eye), it is suggested that these cells also have other functions. A lack of these cells in the inner ear leads to deafness, a lack in the choroid of the eye to microphthalmos. However, the exact relationship between melanocytes and these pathologies is not yet clear. We suggest in this work that melanocytes induce angiogenesis which is important for organ development. It is precisely this function that is to be experimentally elucidated in this work.

Aim of this study

The aim of this study is to define the extent of rightfulness of the hypothesis which states that melanocytes have angiogenic potential independent of light. It can be achieved through further exploration of the relationship between melanocytes and angiogenesis.

Material and Methods

Primary melanocytes are isolated from the human foreskin and cultured on Chick Chorioallantoic Membrane (CAM). On chicken CAM, melanocytes are incubated ex-ovo with and without light for 4 days. The tissue produced on CAM is histologically sectioned, stained and microscopically evaluated.

Results and discussion

It is a proven fact that melanocytes strongly participate in angiogenesis. Yet, results of the experiments revealed that melanocytes grown on the CAM induce angiogenesis which are independent from the light. The CAM model will be useful for further experiments and will lead to a better understanding of melanocytic functions. Furthermore, this model can be used to investigate melanocyte-related diseases (e.g., Mb. Waardenburg).

1 Introduction

1.1 Anatomy of the eye

The eye is a complex organ, three layers can be distinguished (1)(*Figure 1*):

- The external fibrous layer (cornea and sclera)
- The middle vascular layer – uvea (choroid, ciliary body, and iris)
- The internal layer (retina)

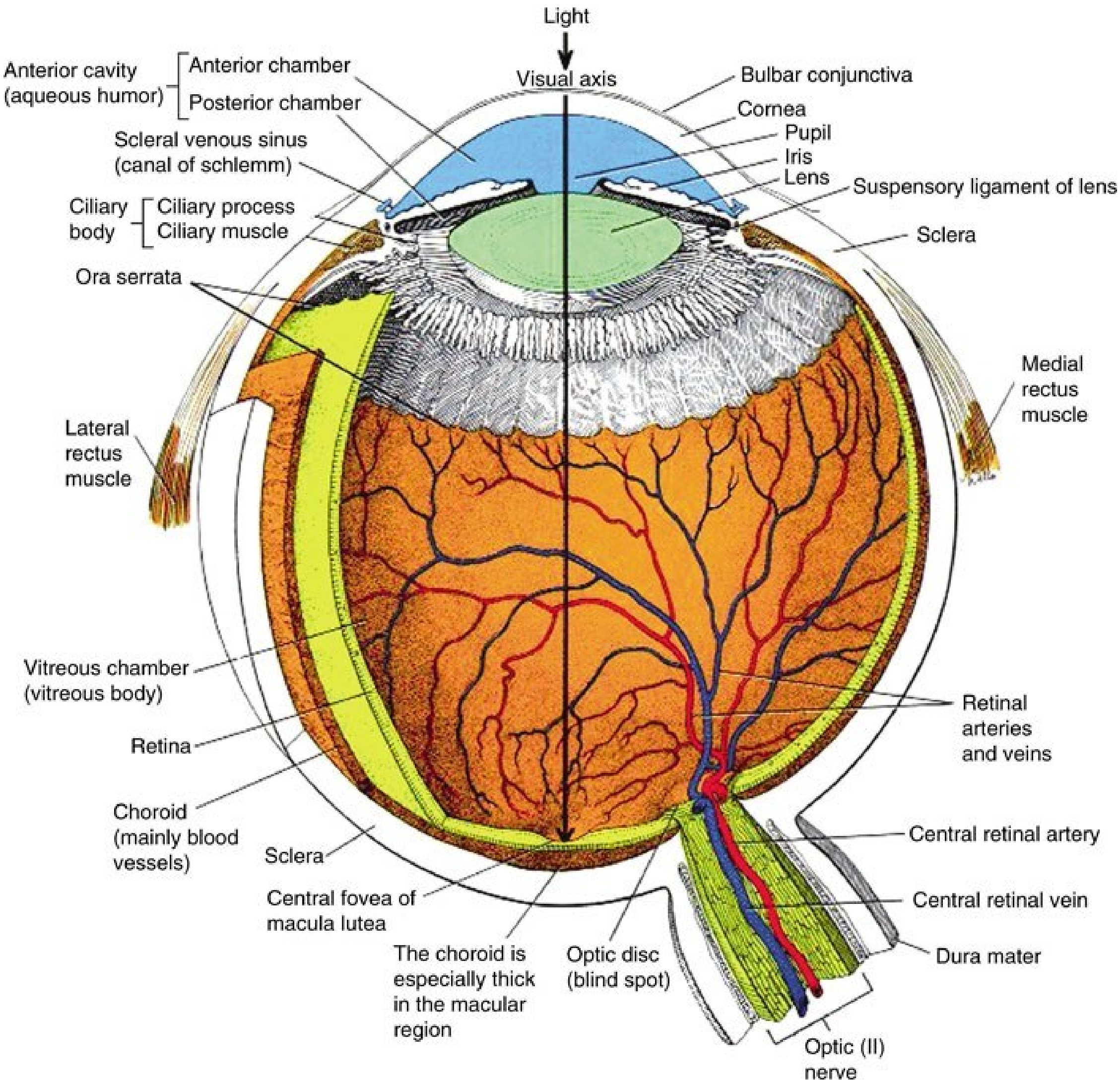


Figure 1: Anatomy of the eyeball from (2)

Regarding this study, the choroid - which will be discussed in the following - is from special interest.

1.1.1 Choroid

The choroid lays between the retina and the sclera (3). It is highly vasculated providing oxygen and nourishment to the outer retina. (Figure 2 (4)).

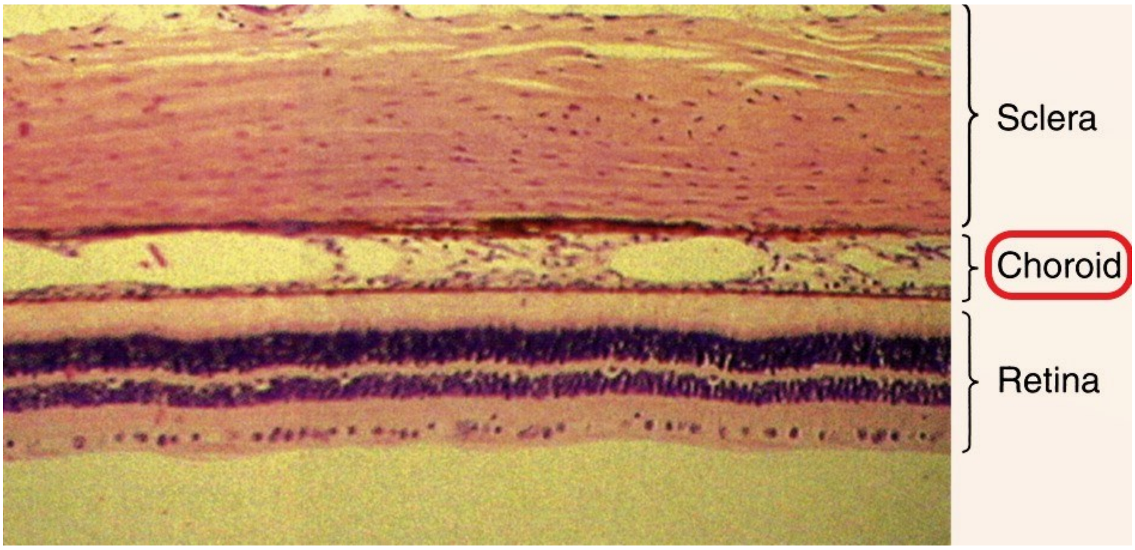


Figure 2: Photomicrograph of the three tunics at the back of the primate eye (4)

The choroid develops from the mesenchyme surrounding the two eye vesicles, which in humans sprout from the embryonic forebrain (prosencephalon) at the end of the first month and ultimately become the eyes. At about this time, melanocyte precursors migrate from the neural crest into the uvea; these do not differentiate until 7-8 months of gestation. The mesenchyme, which forms the choriocapillaris at about 2 months, is in contact with the developing retinal pigment epithelium (RPE) which seperates the choroid and the retina (5).

The choroid is composed of blood vessels, melanocytes, fibroblasts, resident immunocompetent cells, etc. It is one of the most vascularized tissues in the body, its main function is usually seen as supplying oxygen and nutrients to the outer retina (5). Other possible functions include absorption of the light (in species with pigmented choroid), the regulation of temperature through heat dissipation, and modulation of intraocular pressure (IOP) through vasomotor control of blood flow. The choroid also plays an important role in the bilge of aqueous humor from the anterior chamber via the uveoscleral pathway. Approximately 35% of the aqueous humor is drained by this pathway. This rate is higher in nonhuman primates, (between 40% and 60%), and much lower in cats and rabbits (3-8%) (5).

In humans, the central choroid is approximately 200 µm thick at birth and decreases to about 80 µm by age 90 (6). In non-human primates, the choroid is thinner, approximately 95 µm at the center, thinning to 55 µm at the periphery (7). In other species, such as birds, only sparse melanocytes are present in the outer choroid, resulting in lighter pigmentation (8).

The photoreceptors are extremely metabolically active. For this reason, over 90% of the oxygen supplied by the choroid is consumed by the photoreceptors, especially in darkness when the light-gated ion channels are open: Active transport of ions is necessary for ion homeostasis (9-11).

1.1.1.2 Choroidal Thickness

The thickness of the choroid depends on various factors and can change due to different mechanisms (12,13). Following, five hypotheses are presented which might explain the changes of choroidal thickness:

- *osmotically active molecules* - This hypothesis is based on the finding that choroidal membranes that thicken rapidly as the eyes recover from form deprivation-induced myopia synthesize significantly higher amounts of proteoglycans than choroidal membranes from normal eyes (14).
- *fluid flow from the anterior chamber to the choroid* - The choroid is part of the uveoscleral outflow pathway. Therefore, changes in the amount of aqueous humor shunted to the choroid by the ciliary muscle might be responsible for the increase in the size of lacunae that respond to myopic defocus in eyes (14,15).
- *movement of fluid through the RPE* - A regular flow of ions and water across the retina (16) and RPE into the choroid is well described. Modulations of this flow could modulate choroidal thickness.
- *the tonus of non-vascular smooth muscle* - It has been described that the choroid might thin very rapidly, approximately 100 µm in 3-4hours in younger chicks (17). Given that the choroid carries plentiful non-vascular smooth muscle, this finding might be suggested muscular, instead of osmotic mechanisms - probably due to sympathetic and parasympathetic signals. Abelsdorff et al. measured and compared the fluid exchange in the eye and supported the finding that the choroid thickness increases with decreasing the IOP (18). The reason for this hypothesis is that the lacunae

of the choroid are continually relatively hypertonic and generally tend to gather fluid. This tendency prevents the tone of non-vascular smooth muscle. If they contract, the choroid becomes thinner, and if they relax, the choroid turns into thicker. However, it is also possible that contraction of this muscle mass facilitates filling of the lacunae, because the nonvascular smooth muscle is not oriented perpendicular to the plane of the choroid (18).

- *vascular permeability* - Another possible factor influencing the choroidal thickening is osmotic pressure: Increased fluid flow from choroidal vasculature may result in increasing in capillary permeability, causing proteins to transport into the extracellular matrix and/or lymphatic vessels, followed by passive, osmotic fluid flow (19-21). It was found that protein content in suprachoroidal fluid was reduced in thinned (by prior mold deprivation) choroids and increased in choroids thickened by vision restoration. Further it was seen that light deprived chickens showed significant less fenestrations of the choriocapillaris (22).
- Furthermore, it was shown that number of choroidal melanocytes have also influence on the choroidal thickness: On the one hand, an increased number of melanocytes is associated with a thickened choroid. On the other hand, a decreased number of choroidal melanocytes is associated with a decreased choroidal thickness. It was proposed that melanocytes may play a role in choroid angiogenesis - therefore it was hypothesized that choroidal melanocytes influencing the number of choroidal capillaries and subsequently choroidal thickness (23,24).

At this point I would like to outline that both, melanocytes and light influence choroidal thickness (25). Since Melanocytes are light sensitive cells, we assume a linkage between light, melanocytes and choroidal thickness which I investigated in this study.

1.2 Function of the Melanocytes

Melanocytes, the pigment-producing cells of humans and animals, are descendants of the neuroectoderm (Figure 3)(26).

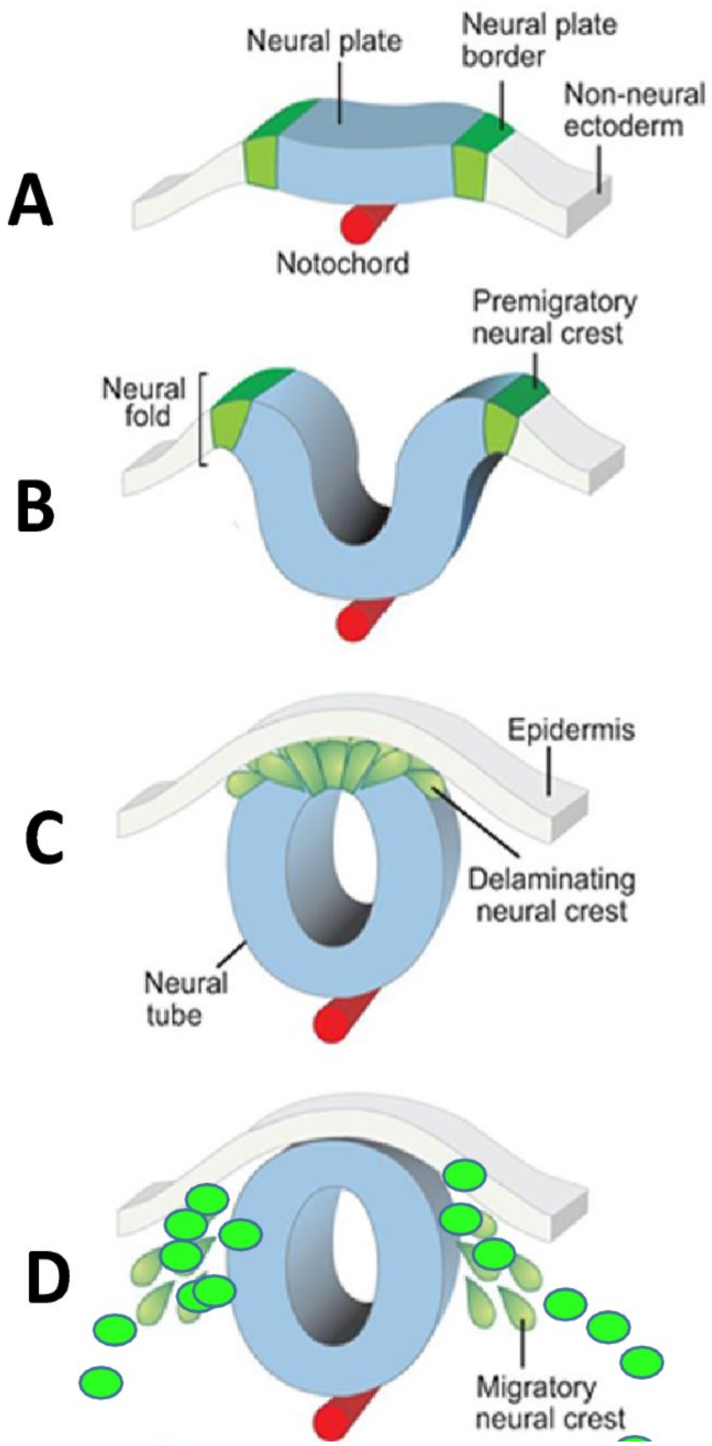


Figure 3: The development of the neural crest from (62)

During embryogenesis, these cells arise from neural crest cells - so called melanoblasts. These cells migrate along the nerve sheaths into various tissues (e.g., epidermis, choroid of the eye, stria vascularis of the inner ear or the brain) and differentiate to melanocytes (26-30). Melanocytes produce melanin, a pigment that can be found in the skin, eyes, and hair. The melanin production process is called *melanogenesis*. Melanogenesis determine skin tone, eye - and hair color. Melanogenesis activity is distinguished between the basal and activated levels.

For instance, basal melanogenesis is low in fair-skinned people. If a fair-skinned individuum is exposed to sunlight, melanogenesis is activated leading to a sun induced tan (31,32) . Melanin absorbs light in order to protect cells from harmful radiations (33).

However, this function does not explain why melanocytes are also located at certain parts of body, that do not receive light.

Beside their existence in the skin, melanocytes also present in the stria vascularis of the cochlea (34), in the leptomeninges (35), substantia nigra and locus coeruleus (36) of the brain, in the heart (37,38) and even adipose tissue (39).

Since the absorption of light is certainly not the main function in these tissues, we assume further melanocytic function additional to photoprotection.

1.2.1 Melanocytes in the eye

The melanocytes are located in the choroid, which is the middle layer of the eye. These cells are called uveal melanocytes (UM). Uveal melanocytes have the ability to grow very well and to produce melanin. In this process, the basic fibroblast growth factor (bFGF), cyclic adenosine monophosphate-increasing agents and serum play major roles (40).

There are several interesting studies that have determined the biological results of an absence of choroidal melanocytes.

The absence of melanocytes is called leucism. This should not be confused with albinism - a disease in which melanocytes are present but are not able to produce pigment. While in humans “only” partial leucism is described in the context of

Waardenburg syndrome, a large number of different mutations and syndromes have been described in animals which lead to more pronounced leucistic phenotypes. Several different mutations have been described in animals which can lead to leucism: Mutations of the endothelin receptor B, the paired box gene 3, the microphthalmia-associated transcription factor and the c-kit or steel locus or the Merle factor are mentioned here (41-43).
Some mutations lead to attenuated forms of leucism such as spotting of the coat or skin, but others - especially when homozygous - lead to far-reaching health impairments - many of these animals are deaf, often coupled with severe eye deformities such as microphthalmos and congenital cataract (41,42,44,45).
An older, histologic study of microphthalmic eyes from leucistic dogs demonstrated that the choroid had only very few vessels in the absence of melanocytes (46) - another possible hint for a linkage between melanocytes and angiogenesis (47).

1.2.2 Melanocytes in the inner ear

Some mutations lead to weakened forms of leucism such as spotting of the fur or the skin, but others - especially if these are homozygous - lead to far-reaching health impairments - many of these animals are deaf, often paired with severe eye malformations such as microphthalmos or nanophthalmos (46)
The deafness in leucistic animals can be explained due to distinct histological findings of the stria vascularis of the inner ear in mice. The stria vascularis is - similar to that of the choroid - a dense network of vessels in the inner ear with a high melanocyte content. Interestingly it has been shown that if melanocytes are missing in this tissue, hardly any vessels can be seen histologically (45).

1.3 Aim of the study

The aim of our study is to observe the effect of visible light on the angiogenic factors of melanocytes in experiments.
To study the angiogenic capability of different cells, the so-called Chick Chorioallantoic Membrane model (CAM) offers a feasible model to study angiogenesis *in vivo*.

1.4 CAM Model

The chorio-allantoic membrane (CAM) is an extraembryonic membrane of the developing chick. Its main function is gas exchange during the development of the chick embryo. The CAM is comparable to the allantois extending from the ventral wall of the endodermal hindgut. In early embryological stages the CAM it is, at the embryological age of 3 days it quickly develops a rich vascular plexus consisting of many arteries and veins (48).

Development of CAM Model

The CAM starts to grow from the 3rd day of embryonic development (49). It is the respiratory organ of developing avian embryos. Although most attention is given to the vascular system in our experiments, it is important to note that the CAM has a fully developed lymphatic system, which has important functional and molecular similarities to the mammalian lymphatic system (50).

It is formed by the fusion of the splanchnic mesoderm of the allantois and the somatic mesoderm of the chorion. An extensive network of extraembryonic capillaries forms, covering the entire surface by day 12. The vessels are important for respiration and excretion. The development of these vessels offers a great opportunity to study angiogenesis.

Since one century the CAM model became increasingly popular in research, especially in order the study of factors (e.g. cells or cytokins) influencing angiogenesis.

CAM model experiments enabled new intriguing insights regarding vascular development, angiogenesis, tumor growth and metastasis (51), respiratory properties (52), ion transport (53,54), selective vascular occlusion therapies, biocompatibility of engineered materials, drug distribution and toxicology.

For this reason, this method is very useful for us to look at angiogenesis obviously. Since the aim of our study is to investigate the angiogenic potency of melanocytes, the CAM model seems to be an appropriate scientific tool to do so.

In our study we hypothesise that will melanocytes induce angiogenesis in the CAM and the visible light may have an reinforcing effect. To test this hypothesis, we studied the angiogenetic potential of melanocytes cultivated in CAM models with and without the visible light.

2 Material and Methods

In our study isolated human foreskin melanocytes were incubated on CAM models.

The schema below *(Figure4)* describes chronological the steps of the experiment - main steps are highlighted with red arrows. This experiment was conducted twice under the same conditions.

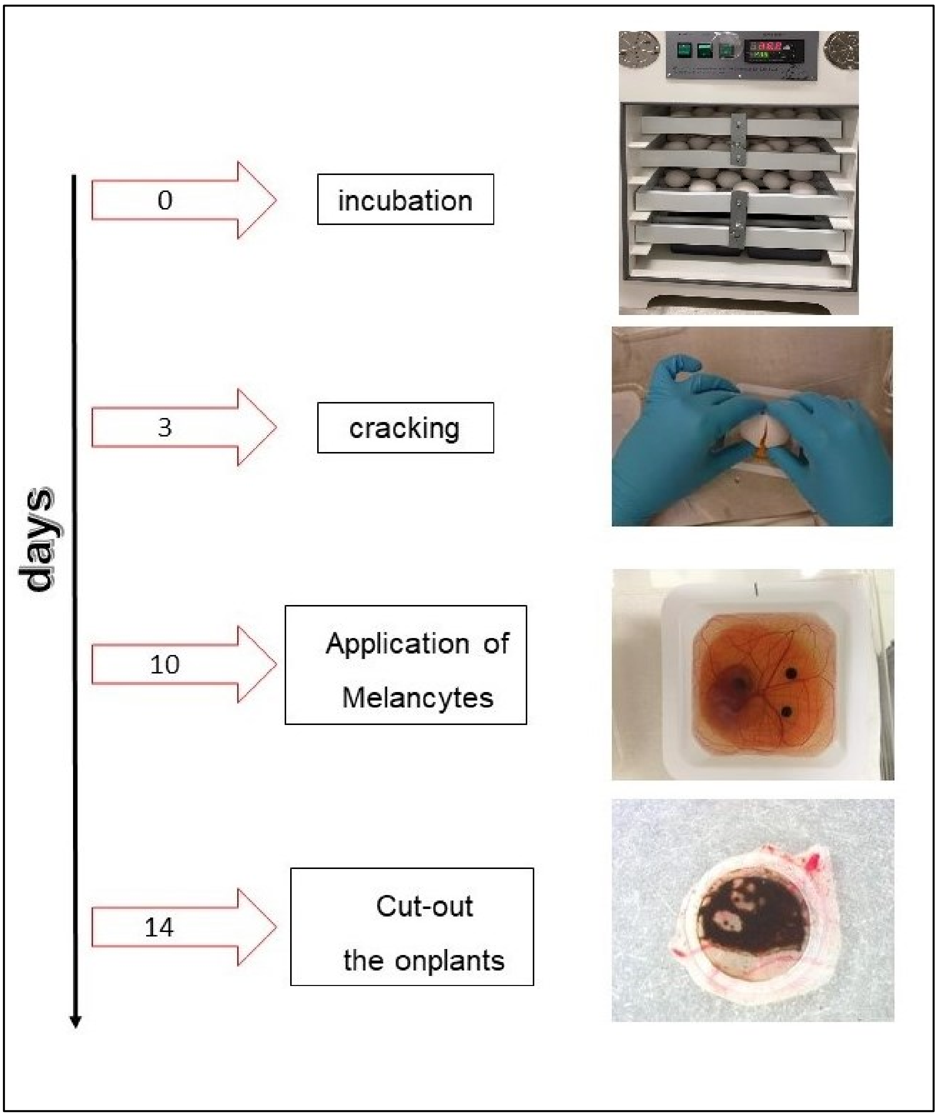


Figure 4:The setup of experiment until dehydration and paraffinization of the onplants

2.1 Isolation of human juvenile Melanocytes

We obtained the isolated human juvenile melanocytes from the Division of Biomedical Research.

2.1.1 Material

- TrypLE
- PBS
- Dispase, diluted to 2U/ml in PBS and sterile filtered, aliquots stored at -20°C
- Penicillin Streptomycin (P/S)
- Cell culture treated flask
- Scalpel
- Forceps
- Petri dish
- Melanocyte Growth Medium M2

2.1.2 Method

Culturing the explants

The cultivation of the cells is based on the method of Godwin et al. (55).

- As a sterile control, 5ml of the transport medium was taken and it was incubated in a T25 for 48h in a 37°C, 5% CO_2 humidified incubator.
- Then the explants were washed 3 times with PBS and were incubated in a 10% P/S solution (with PBS) at room temperature.
- The explants were cut into 1-2 mm thick slices and placed in a petri dishes or 6-well plate. The already inflamed pink-red coloured tissue was discarded.
- Optionally, cover glasses can be placed on explants.
- Dispase solution (2U/ml) was applied to the explants. The epidermis should be upside down and everything should be covered.
- The explants were incubated overnight (max. 16-18 hours) at 4°C.

- On the next day the supreme pinkish or brownish layer - epidermis was removed with forceps and transferred into 6 well plate or petri dish filled with PBS.
- Epidermis layer was transferred to a 6-well plate, was cut into small pieces and covered with a coverslip.
- 1-2ml of melanocyte medium was added per well and incubated in a 37°C, 5% CO_2 humidified incubator.
- After 24h must be replaced with fresh medium.
- For subcultivation cells were trypsinized and centrifuged at 250-300g for 4 minutes.
- The supernatant can now be discarded and the cell pellet is resuspended with 5ml of melanocyte medium, then the cell suspension is transferred to a 25cm2 flask and the flasks are placed in the CO_2 incubator.
- Melanocytes begin to round up and start floating when getting too confluent (Figure 5).

Note: Melanocytes can be subcultivated up to passage 6-8.

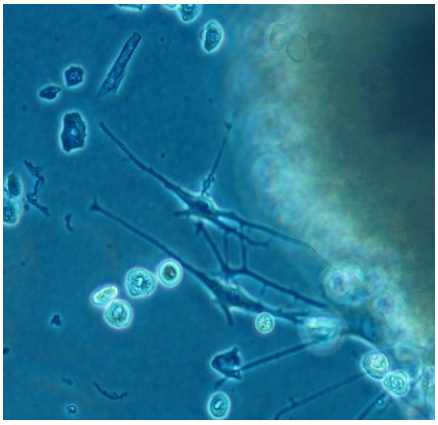


Figure 5: Foreskin melanocytes in culture phase contrast microscopy 200x

After three days of cultivation, melanocytes can be identified. The melanocytes are clearly characterised by their spindle-shaped body and their long processes. (Figure 6).

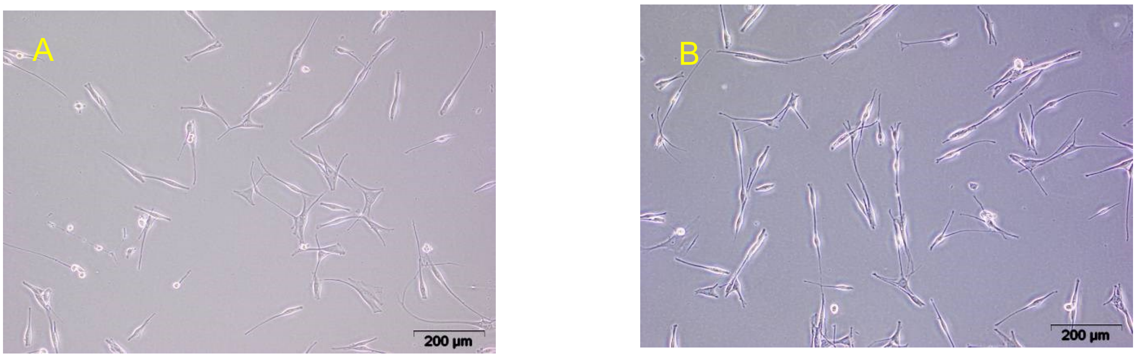


Figure 6: Melanocytes after 2 weeks of cultivation (A) and after passaging (B)

2.2 Preparation of the CAM

For our experiments, we used fertilized white Leghorn eggs from a local hatchery in Gloggnitz, lower Austria.

For the first experiment trial 50 eggs were used and for the second 70 eggs (total 120 eggs). All eggs were cleaned from visible dirt with a brush and after that washed. After drying, we sprayed 75% EtOH all over them to ensure sterile conditions. The eggs were then put into the incubator at 37.6°C and 75% humidity for 3 days. During this time, they got automatically slowly rotated by a special underlay, which is necessary for embryonic development.

2.2.1 Cracking Prosses

Materials:

- Scales pans
- Square petri dishes
- Angle Grinder
- 75% EtOH
- Container for unfertilized or dead eggs

- Container for the eggshells

Before the process was performed, the working place and the equipment was sterilized with 75% EtOH. As shown in the scheme on incubation day 3 of the eggs, the cracking process was performed (*Figure 4*).

Eggs were taken from the incubator; their lower parts were sterilized by 70% EtOH. At this stage it is important not to rotate the eggs. Because if the embryo is located on the upper side of the egg, it will not get hurt during the opening of the eggshell. With the angle grinder we cut the lower side of the eggs about 1 cm. Then the eggs were put into a weigh boat and was lightly pressed from the upper surface. Thus we were able to open the egg and transfer the embryo from in-ovo to ex-ovo (*Figure 7*).

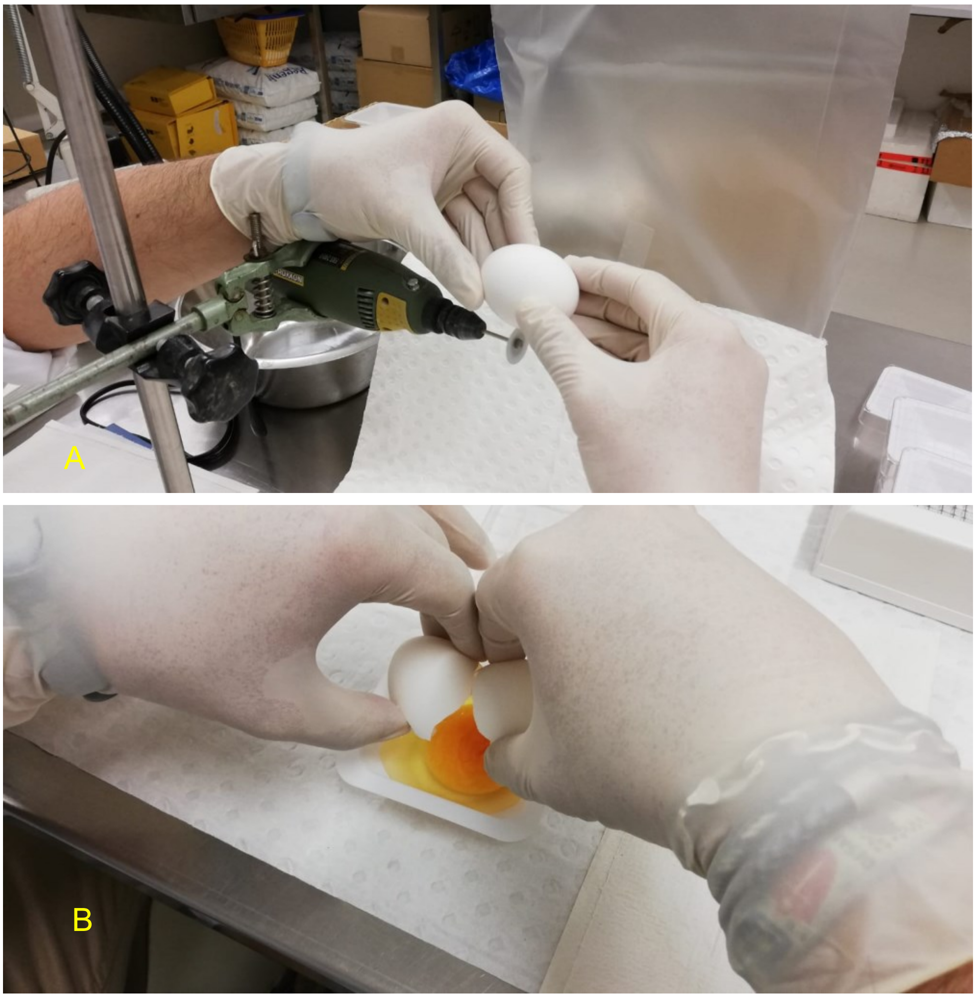


Figure 7: Cracking the egg with angle grinder (A) and opening the egg (B).

After the cracking and transfer the eggs were divided into two groups. For one week, the first group was incubated under the 1000 lux light for 10 hours (from 8AM to 6PM), while the other group was incubated in a dark environment around the clock. The ex-ovo eggs were checked every day, their development was documented and dead embryos were removed (*Figure 8*).

Figure 8: The development of ex-ovo egg: 1.day(a) and 4.day (b)

2.3 Transfer and Incubation of the Melanocytes

On the day 10 of the chick development, the melanocytes isolated from the human foreskin (30 $\cdot 10^6$ in total) were diluted with Matrigel Matrix and PBS, and centrifuged. Initially, we put silicon rings of 5mm in diameter on the CAM, where we planned to transfer the melanocytes, in order to concentrate the cells on a single spot. 20µl melanocytes – e.g. a mixture of melanocytes ($1x10^6$ cells/15µl) and Matrigel® (Table 1) were instilled into the silicon rings on the CAM (onplants) in both groups (*Figure 9*). The embryos were incubated again for 3 more days.

Table 1: Total volume of the melanocyte suspension

	1 ring
Cell suspension	15µl
Matrigel	5µl
Final volume	20µl

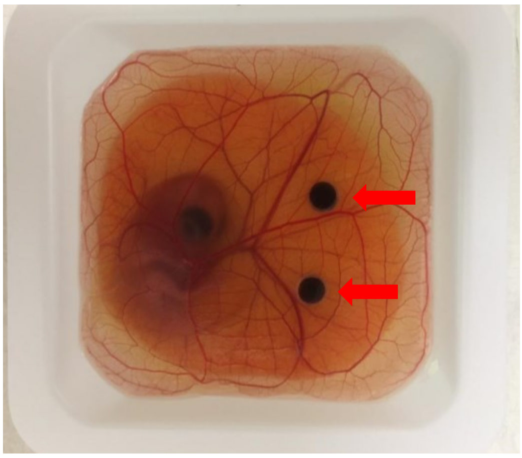


Figure 9: Ex-ovo embryo with melanocytes. Red arrows show the onplants

2.4 Preparation of the onplants

2.4.1 Excision of the onplants

The 14 days old chicken embryos were taken from the incubator and photo documentation was performed. The embryos were put on ice in order to get a sedation of them. The onplants were then very carefully cut out in the shape of a square and cleaned in PBS solution and again a photo documentation has been performed (*Table 5,6*).

After that we fixed the onplants in 4% Paraformaldehyd (PFA) over night. 24 hours later we took out the onplants from the PFA solution and placed them between two sheets of paper (4cmx5cm), which were placed in plastic grids and finally put into numbered plastic embedding cassettes and filled with 70% EtOH.

2.4.2 Dehydration and paraffinization of the onplants

In order to embed the onplants in paraffin, the tissue needed to be dehydrated and pass through the following alcohol series (Table 2).

Table 2: Dehydration of the tissues.

Steps	Time
1. 95% EtOH	30 min
2. 95% EtOH	15 min
3. 100% EtOH	30 min
4. 100% EtOH	15 min
5. Toluol solvent	30 min
6. Toluol solvent	30 min

Then the tissues were put into the melted paraffin wax in 60$°C$ (Table 3).

Table 3: Paraffinization of the tissues.

Steps	Time
1. Paraffin wax 6	30 min
2. Paraffin wax 9	30 min

2.4.3 Embedding

The cassettes were opened and the tissues were taken out. The silicon rings were removed. The tissues were placed perpendicularly on the metal shell and filled with melted paraffin. Then the metal shell was covered with a part of the plastic cassette (*Figure 10*).

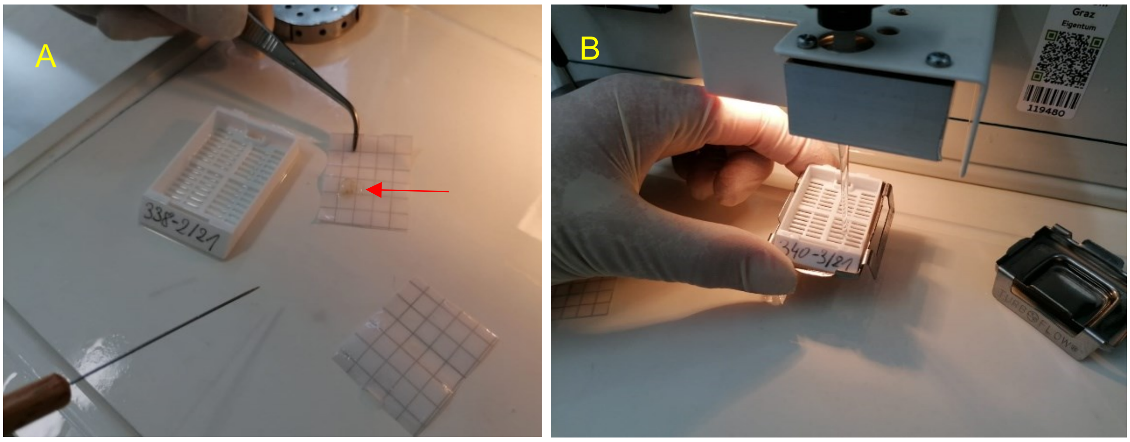


Figure 10: Opening the cassette and taking the tissue (marked with red arrow) (A), covering the tissue with the melted paraffin (B)

Then the metal shell together with tissue were quickly put on $-9°C$ cooled plate. Finally, we have covered the metal shell by the lids of the cassettes (*Figure 11*).

After the paraffin getting hard, metal shells were removed, the materials stored in the cold room under $-4°C$.

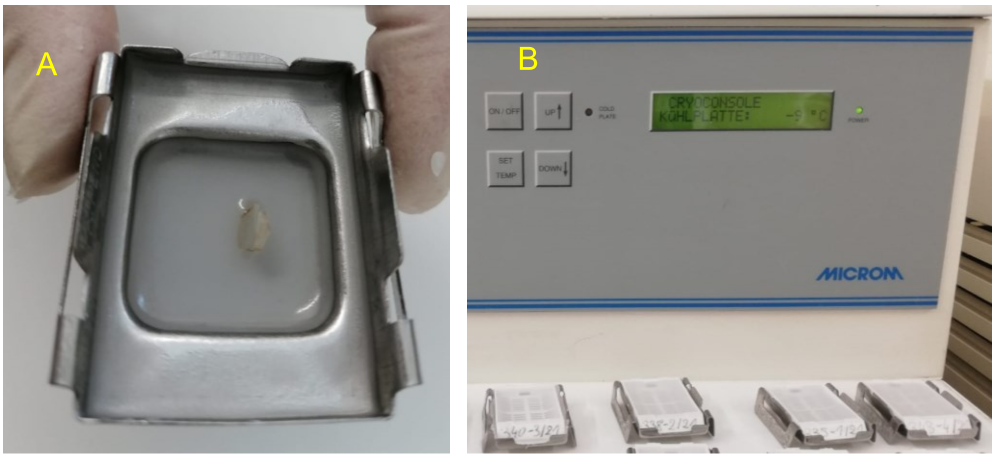


Figure 11 shows us the tissue in frozen paraffin (A) and metal shells with plastic covers on cooled plate(B).

2.4.4 Sectioning

After this process, the tissue samples were hardened and were able to cut. The plastic capsule with the adhering paraffin was cut all around by hand, and then clamped in the microtome (Leica RM2245 V2.4 rotary microtome; Leica Biosystems Nussloch GmbH), 5 µm thin sections were prepared (*Figure 12*).

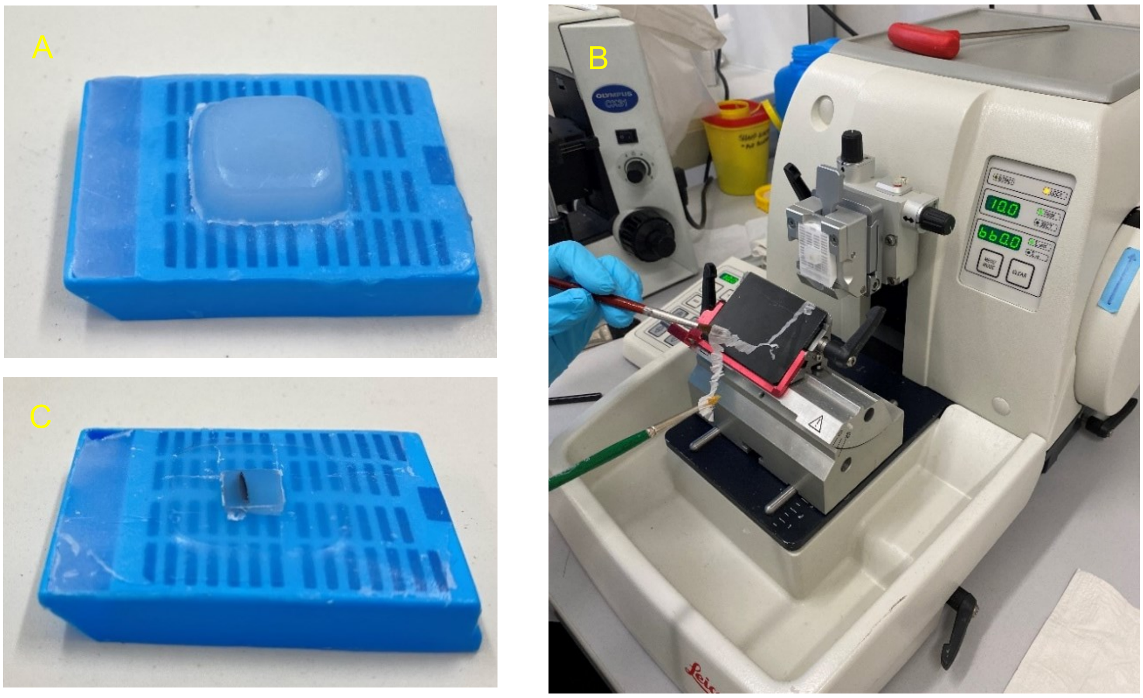


Figure 12 shows us the cassette before (A), during (B) and after (C) the preparation conducted by microtome

The thin cuts tend to roll up. They were therefore placed in a container of water filled up and heated to 26°C, where they spread out flat again due to the surface tension of the distilled water. Now the sections could be mounted on chrome alum-gelatin coated object slides.

Next the sections were placed on a hot plate at 32°C. The Paraffin softened and "sticked" to the slide. The section preparation is now ready and must be stored in a dustproof and shatterproof manner until stained (*Figure 13*).

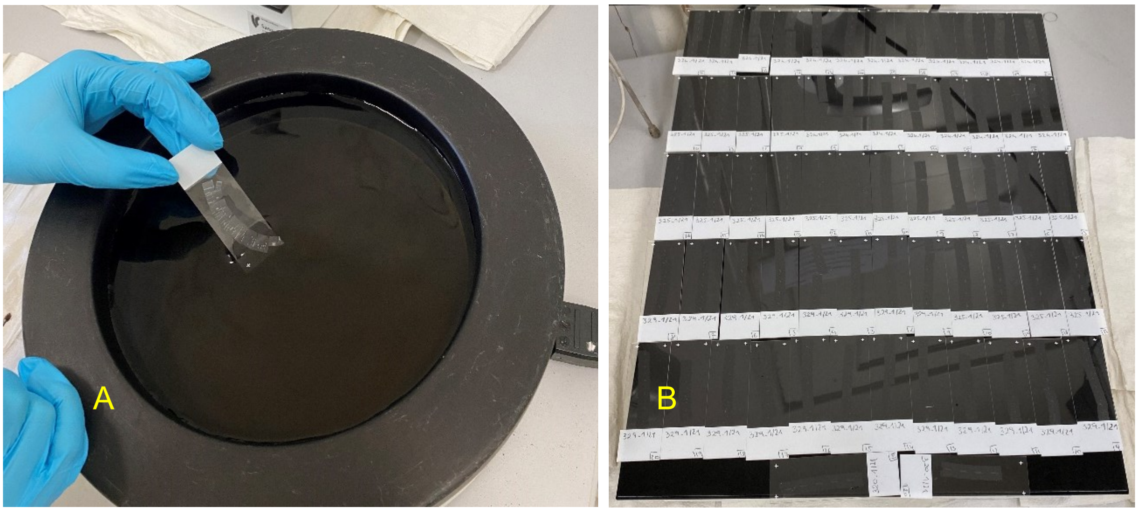


Figure 13: The tissues cut by microtome were placed on warm water 26°C and mounted on the object slide (A). Then the sections were placed on the hot plate 32°C (B).

2.4.5 Cell Staining

2.4.5.1 Material

- Xylene
- EtOH 100%
- EtOH 95%
- EtOH 80%
- Distilled water
- Mayer´s Haematoxylin
- Eosin G-solution
- Acid
- Hydrogen peroxide

Figure 14: Materials for HE staining

2.4.5.2 Principle of hematoxylin-eosin staining

Hematoxylin-eosin (HE) staining is a routine staining method for morphological studies. It is used for recognizing and differentiating various tissue types and morphologic changes (56).

Before the actual staining, the sections are deparaffinized by a Xylene series and rehydrated with an EtOH series that decreases in concentration.

Hematoxylin is a natural dye, consisting of aluminum ion (hemalaun), forming a metal-hematein complex with a positive charge at low pH. This hemalaun solution according to Mayer forms a bond with basophilic structures in an acidic environment, resulting a red-brownish staining. When the pH is raised above 3 by rinsing with water, the color changes to the typical blue-violet color.

Rinsing the sections with water also fixes the coloration, as it is poorly soluble at higher pH values. Eosin G solution 0.5 % aqueous is used for counterstaining (57).

After the actual staining, the sections are dehydrated with an EtOH series rising in concentration and clarified with Xylene (*Table 4*). Subsequently, the stained, dehydrated and clarified sections were covered by glass, which protects the sections from mechanical destruction and provides the optical prerequisite for clarity. After this process, the slides were finally ready for microscopy.

Table 4: HA Staining protocol

1.	**Deparaffinizing**	Xylene	2-3 min
		Xylene	2-3 min
2.	**Rehydration**	EtOH 100%	2-3 min
		EtOH 100%	2-3 min
		EtOH 90%	2-3 min
		EtOH 90%	2-3 min
		EtOH 80%	2-3 min
		dH_2O	2-3 min
3.	**Staining**	Hematoxylin	2 min
		Flowing water	
		Acid alcohol	Dip and pull
		Flowing water	
		Hydrogen peroxide	2 min
		Flowing water	
		EtOH 80%	1 min
		Eosin	10 sec
4.	**Dehydration**	EtOH 90%	1 min
		EtOH 90%	1 min
		EtOH 90%	1 min
		EtOH 100%	2 min
		EtOH 100%	2 min
5.	**Clarify**	Xylene	2 min
		Xylene	2 min

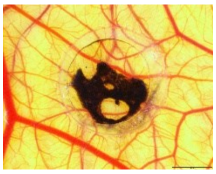
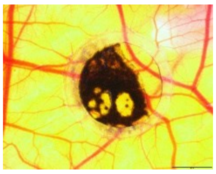
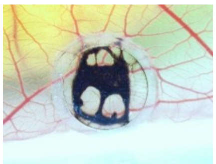
3 Results

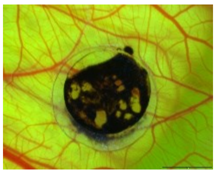
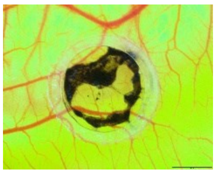
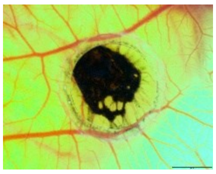
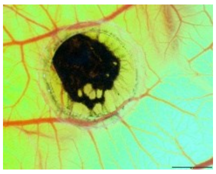
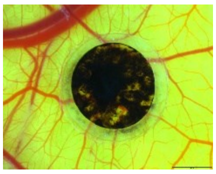
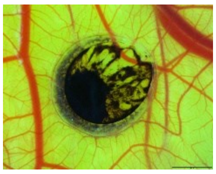
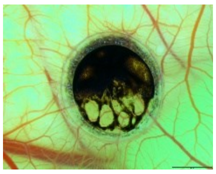
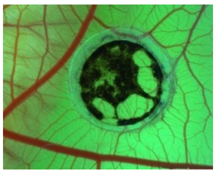
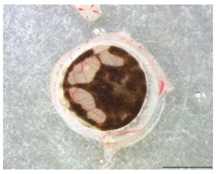
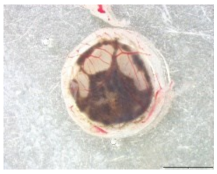
In order to precisely differentiate the influence of light on angiogenic potential of the melanocytes, we observed both the photos of the onplants and the histologic sections. The experiment was conducted twice. During both experiments, all incubated eggs were regularly checked, and dead eggs were removed.

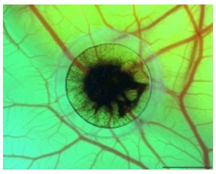
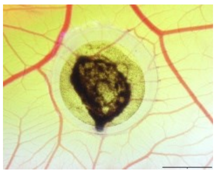
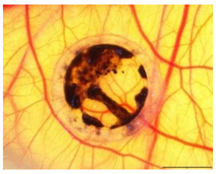
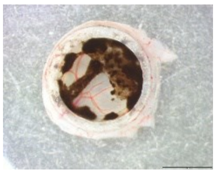
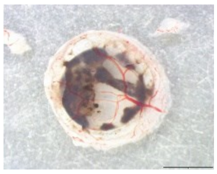
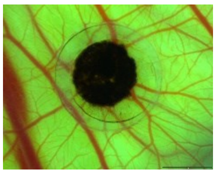
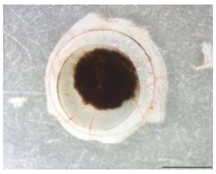
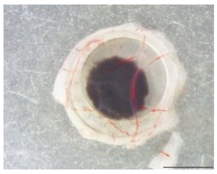
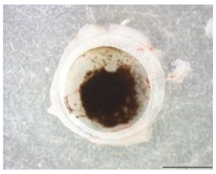
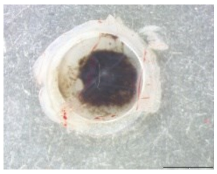
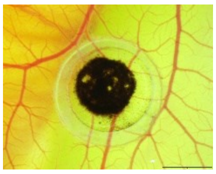
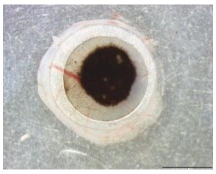
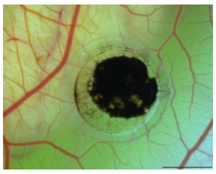
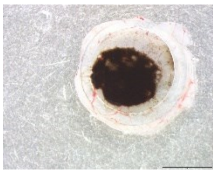
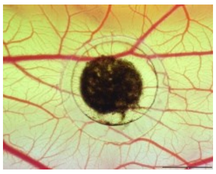
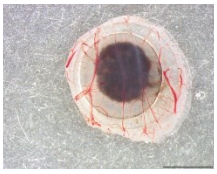
3.1 Macroscopic Observation

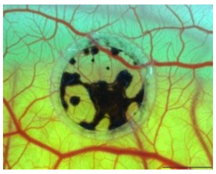
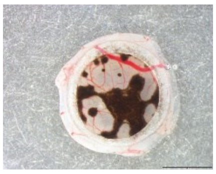
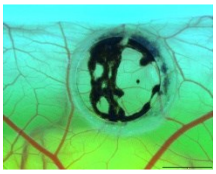
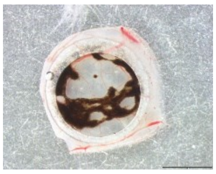
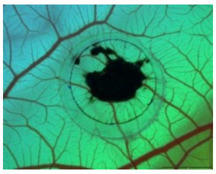
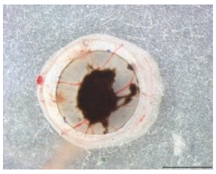
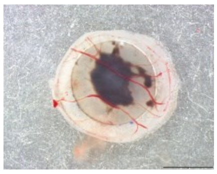
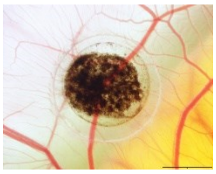
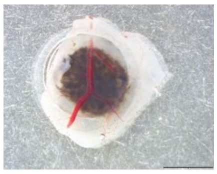
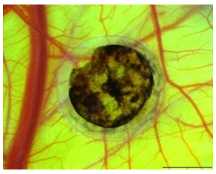
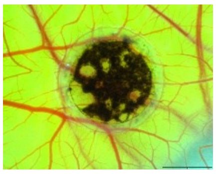
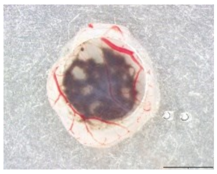
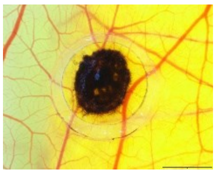
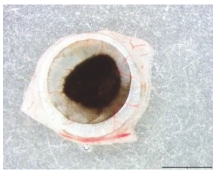
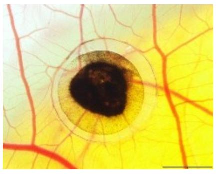
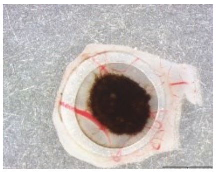
In the first experiment, 26 eggs were incubated ex ovo - 12 eggs (Nr. 358 – 364) were incubated with light and the remaining 14 eggs (Nr. 365 – 378) were incubated in the dark. The table below shows an overview of the process (*Table 5*).

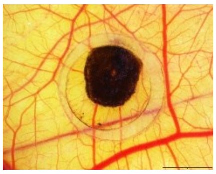
Table 5: Exposed (Nr. 358-364) and unexposed (Nr. 365-378) eggs from the first experiment on day 14 of egg incubation that survived until the end. Egg number 357 died.

Egg Nr.	Day 4	Day 4 – top	Day 4 - bottom
353			
354			
355			

356			
358			
359			
360			
361-1			
361-2			
362			
363			

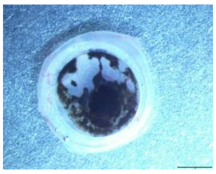
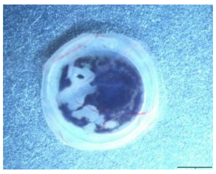
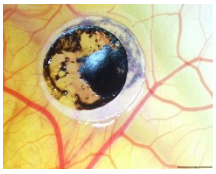
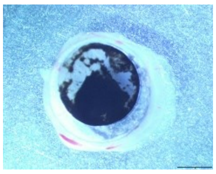
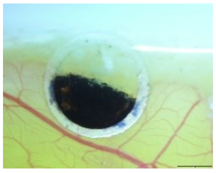
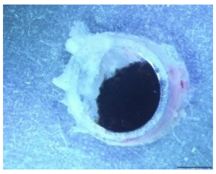
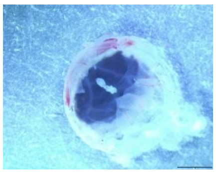
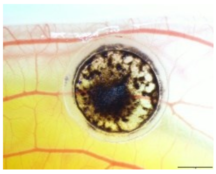
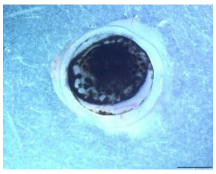
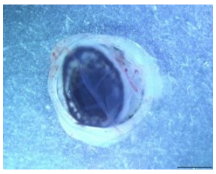
364			
365			
366			
367			
368			
369			
370			
371			

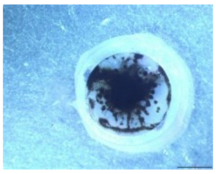
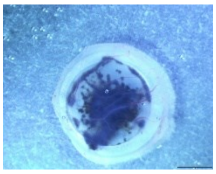
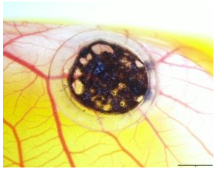
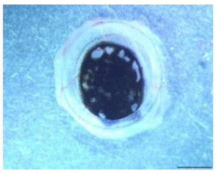
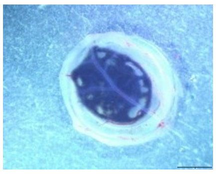
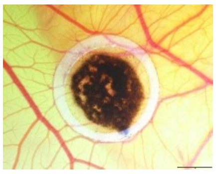
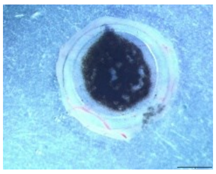
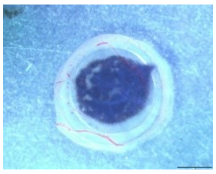
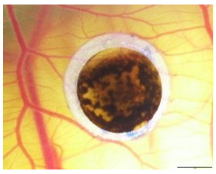
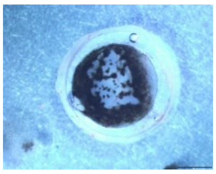
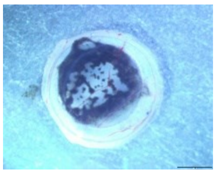
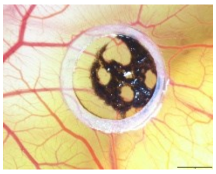
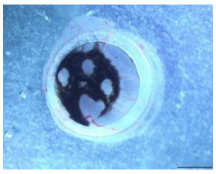
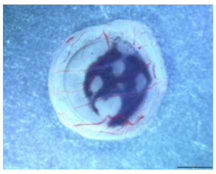
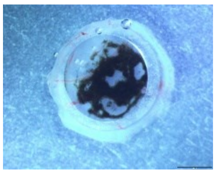
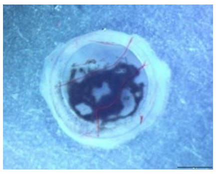
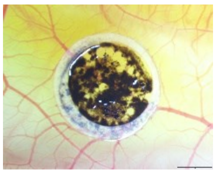
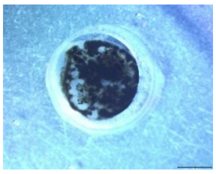
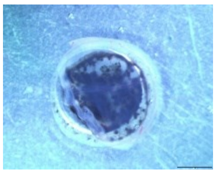
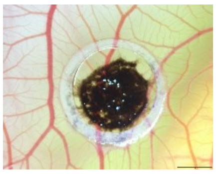
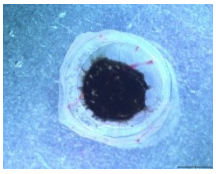
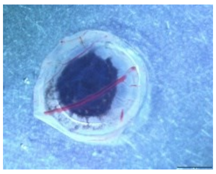
372			
373			
374			
375			
375-1			
376-2			
377-1			
377-2			

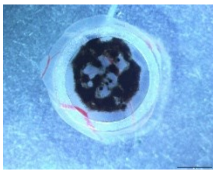
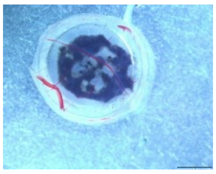
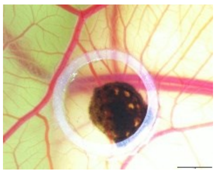
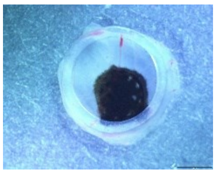
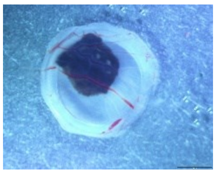
378			

To reduce random and systematic errors, we performed the same experiment twice under the same conditions. The second experiment was carried out with 16 eggs. 8 eggs (Nr. 379 – 386) were exposed and 8 eggs (Nr. 387 – 394) were incubated in the dark. (*Table 6*)

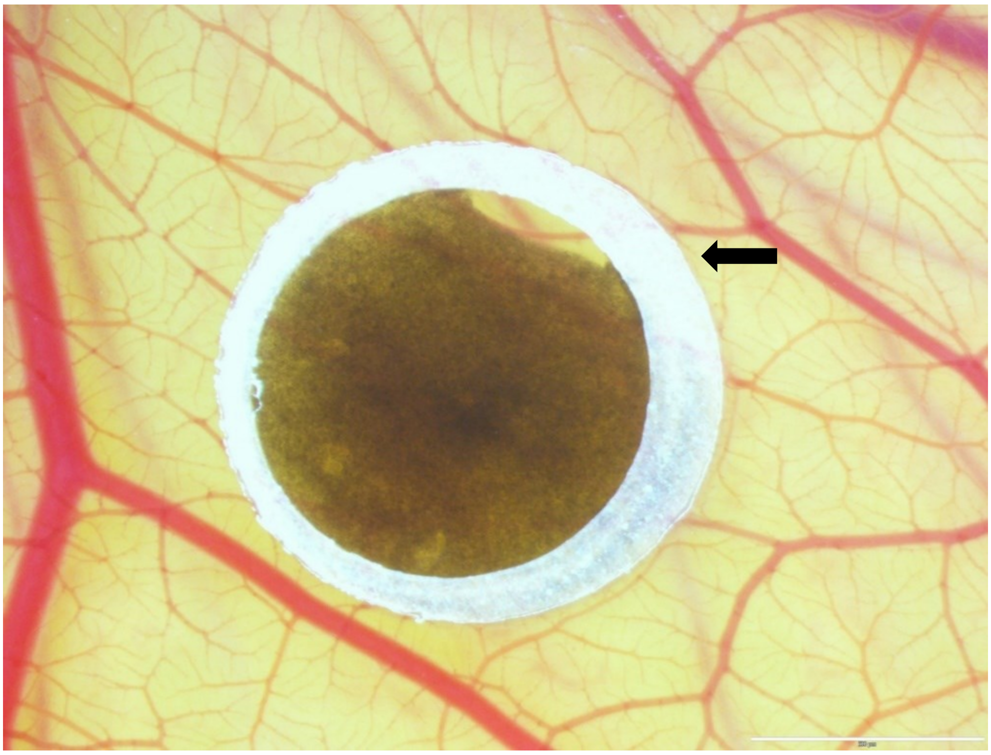
Table 6: Exposed (Nr. 379-386) and unexposed (Nr. 387-394) eggs from the second experiment on day 14 of egg incubation that survived until the end. Eggs Number 379 and 386 died

Egg Nr.	Day 4	Day 4 – top	Day 4 - bottom
380			
381			
382			
383			

384			
385			
387			
388			
389			
390			
391			
392			

393			
394			

After the melanocytes have been applied onto the CAM, the development of angiogenesis was photographically documented on the first and on the fourth day (*Figure 15-17*).



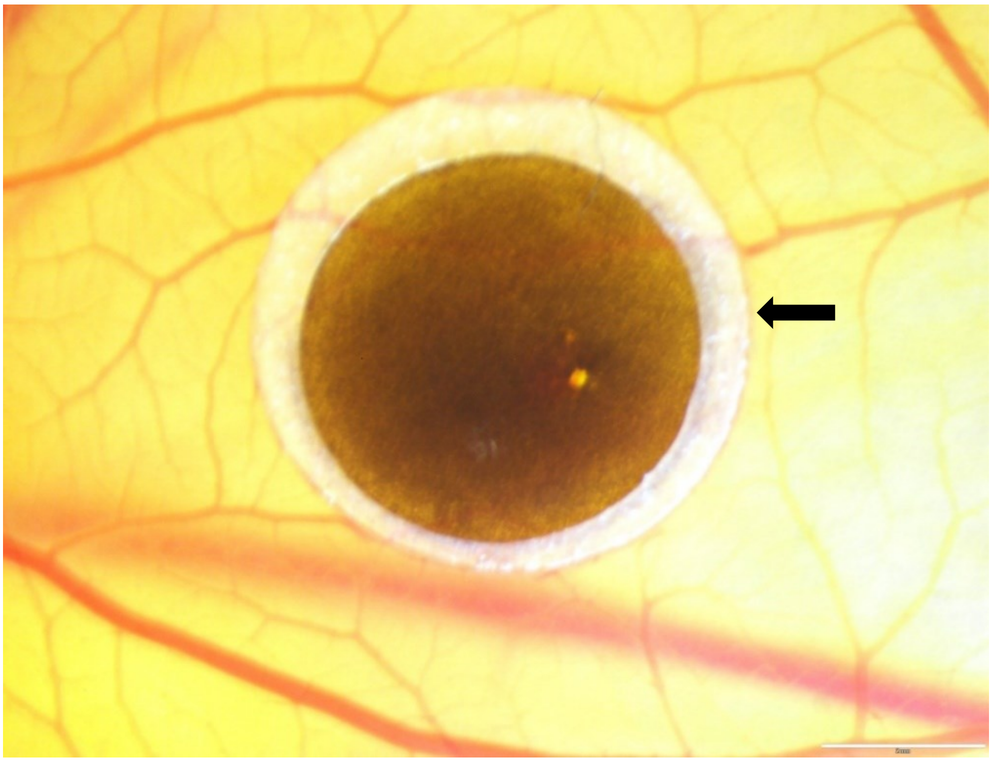


Figure 15 shows us exposed (A) and unexposed (B) onplants which have been advened with melanocytes. Silicone rings (labelled with black arrows) are situated on the bifurcation point of the thin blood vessels of the CAM.

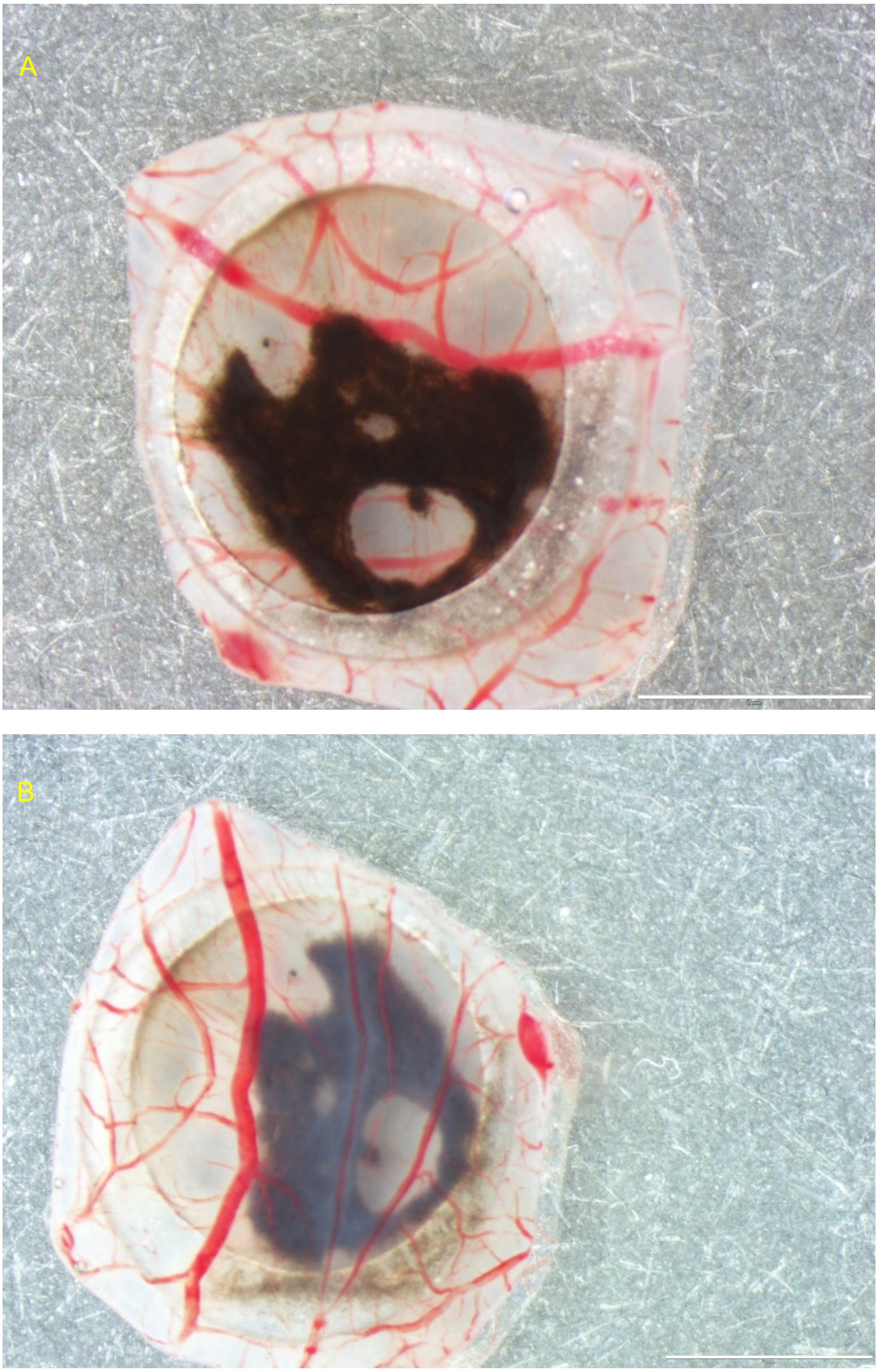


Figure 16 shows us upper (A) and lower (B) view of the melanocyte applied exposed, truncated onplants of the CAM at 4.day.

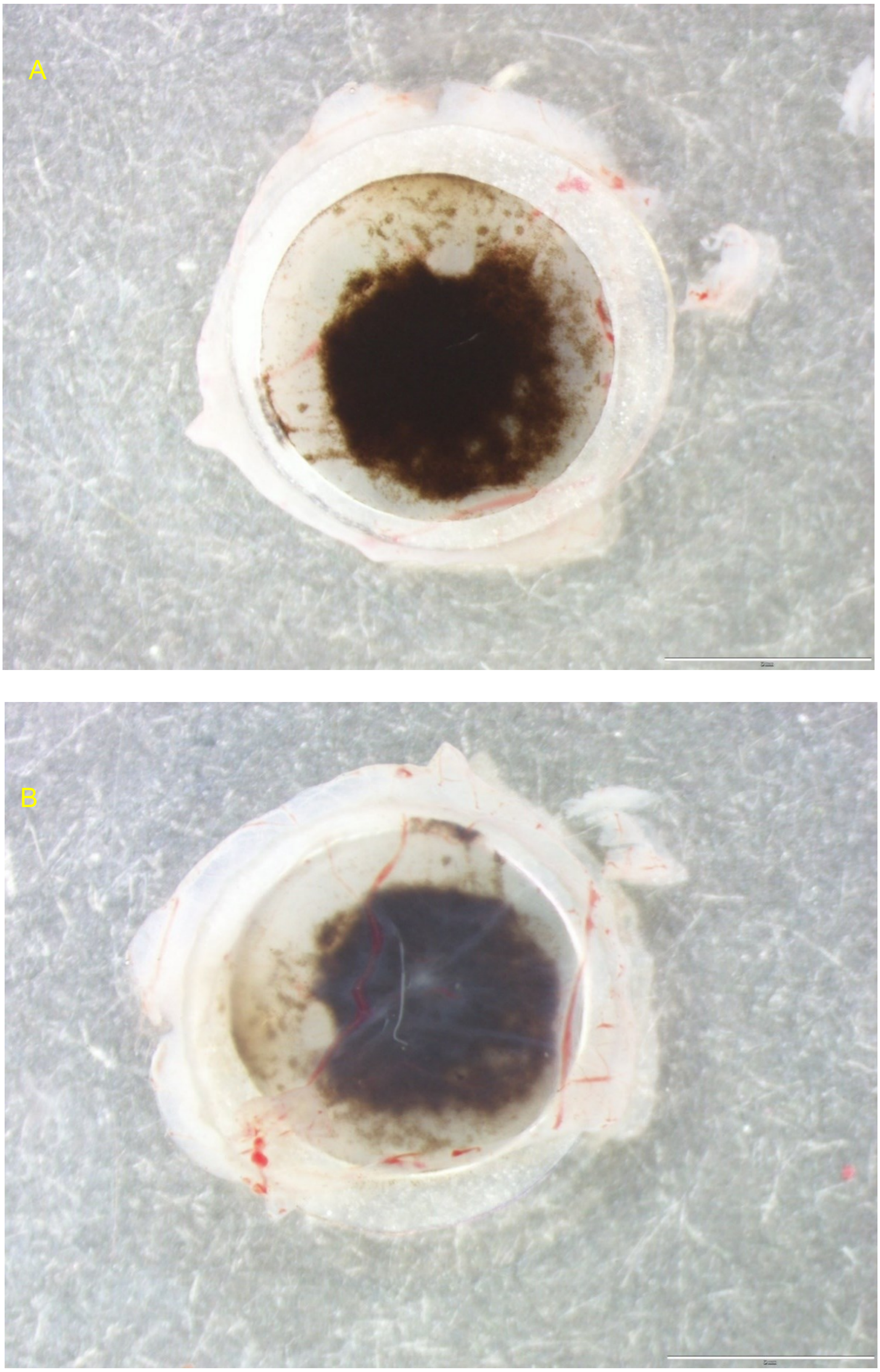


Figure 17 shows us upper (A) and lower (B) view of the melanocyte applied unexposed, truncated onplants of the CAM.at 4.day.

Melanocytes colonize on the vessel bifurcation in 4 days, see Figures 15-17. Nevertheless, we didn't see any noticeable difference between exposed and dark groups (*Figure 18*).

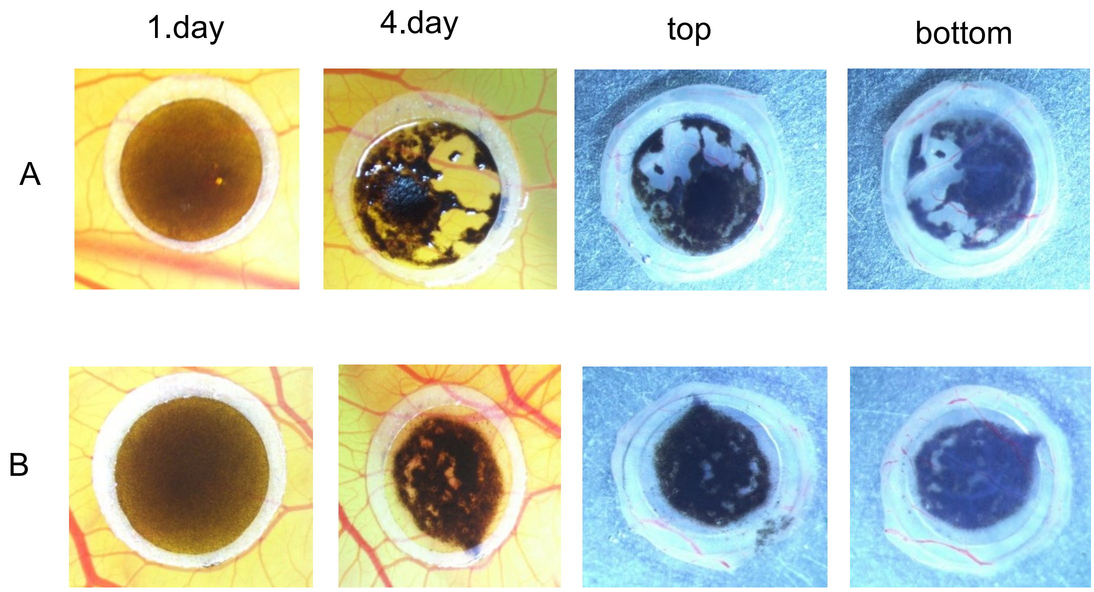


Figure 18: exposed (A) and unexposed (B) onplants before and after excision

3.2 Microscopic Observation

3.2.1 Hematoxylin and eosin staining of the CAM Model

In order to assess the relationship between light and the angiogenic features of melanocytes, histological sections were prepared and stained with the routine staining haematoxylin-eosin (HE) for overview. The following figure explains the most important components of the xenograft. These are the CAM tissue (marked with black arrows), its blood vessels (labelled with red arrows) and melanocytes (marked with a blue arrows) (*Figure 20*).

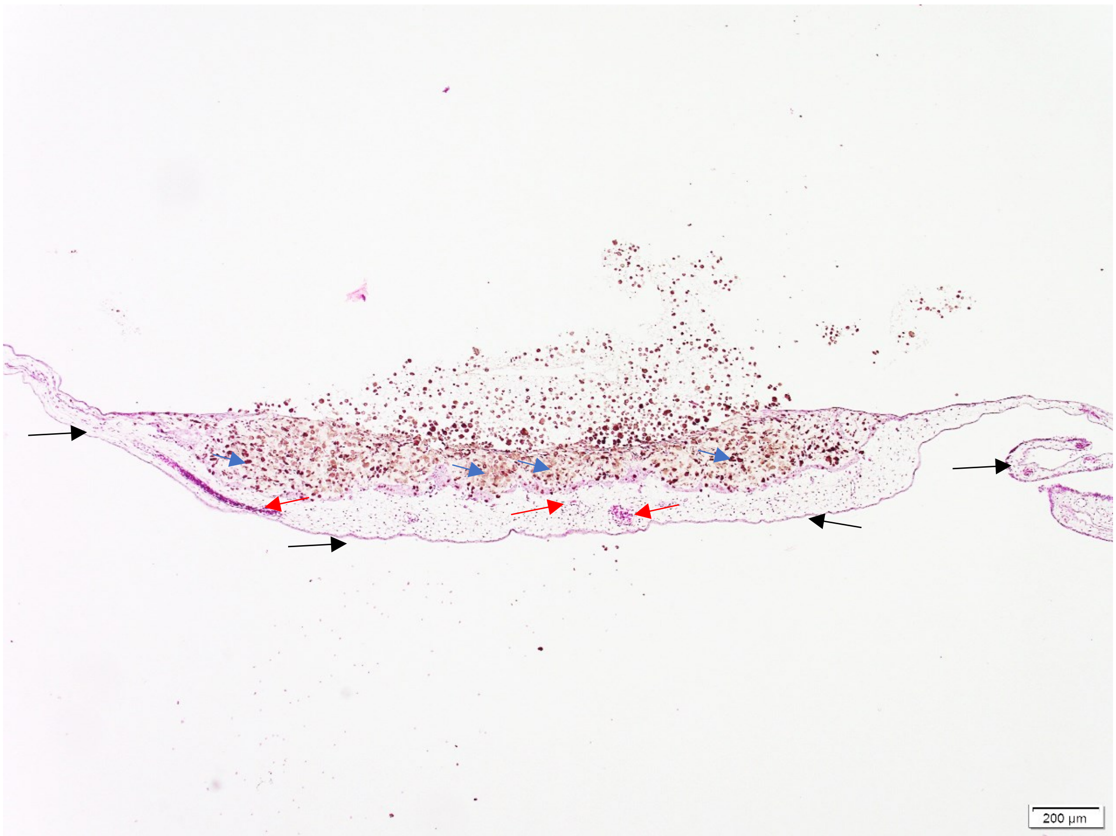


Figure 19: With HE method stained xenograft. Main components; CAM (black), melanocytes (blue), blood vessels (red) were marked with different arrows.

Paraffin blocks were cut through a microtome, were photographed and examined under microscope with different magnifications (*Table 7*). A total of 34 paraffinized onplants were cut histologically (*Table 8*).

Table 7: Results of H&E staining of exposed and unexposed tissue samples cut from CAM.

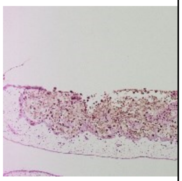
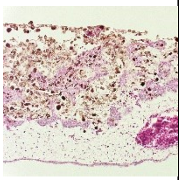
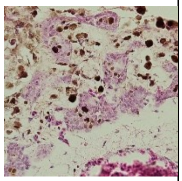
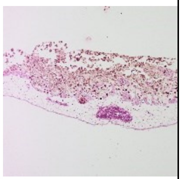
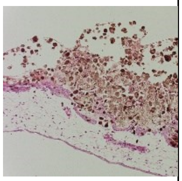
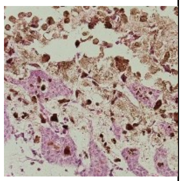
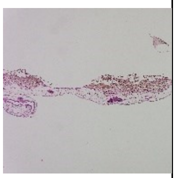
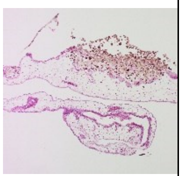
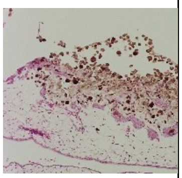
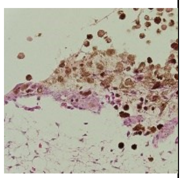
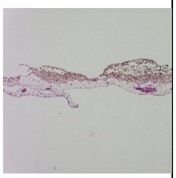
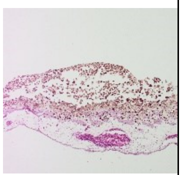
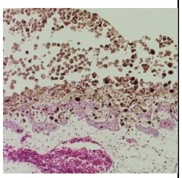
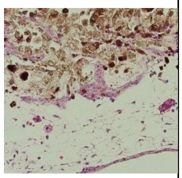
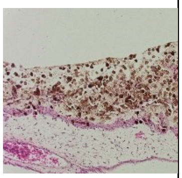
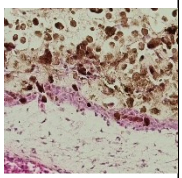
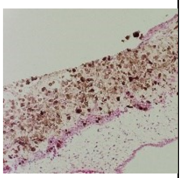
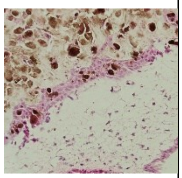
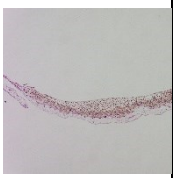
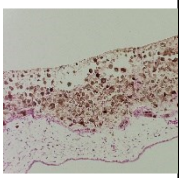
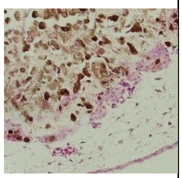
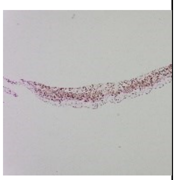
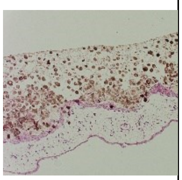
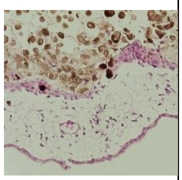
Slide Nr. \ Magnific.		4x	10x	20x	40x
exposed	1#				
	10#				
	20#				
	30#				
unexposed	1#				
	10#				
	20#				
	30#				

Table 8 shows us, when the tissues were cut and how many slides, we have obtained from them.

Egg Nr.	CAM#	cutting date	number of slides
353	162	14/07/2020	40
354	162	14/07/2020	36
355	162	14/07/2020	35
356	162	15/07/2020	25
358	162	15/07/2020	20
360-1	162	16/07/2020	40
360-2	162	16/07/2020	20
361-1	162	16/07/2020	20
361-2	162	16/07/2020	20
362-1	162	17/07/2020	20
362-2	162	17/07/2020	20
363	162	17/07/2020	20
364-1	162	17/07/2020	30
364-2	162	17/07/2020	30
365	162	17/07/2020	25
366	162	17/07/2020	25
368	162	20/07/2020	25
369	162	20/07/2020	25
370	162	21/07/2020	25
371	162	21/07/2020	20
373	162	03/08/2020	20
375	162	04/08/2020	30
376-1	162	04/08/2020	25
376-2	162	05/08/2020	30
377-1	162	05/08/2020	25
377-2	162	05/08/2020	30
378	162	05/08/2020	30
380	163	05/08/2020	25
382	163	07/08/2020	25
383	163	07/08/2020	20
384	163	10/08/2020	20
389	163	01/09/2020	25
392	163	02/09/2020	25
393	163	02/09/2020	25
394	163	02/09/2020	20

If we draw our attention to both images in Figure 21, we can see that angiogenesis process develops independently of the light (red arrows for vessels).

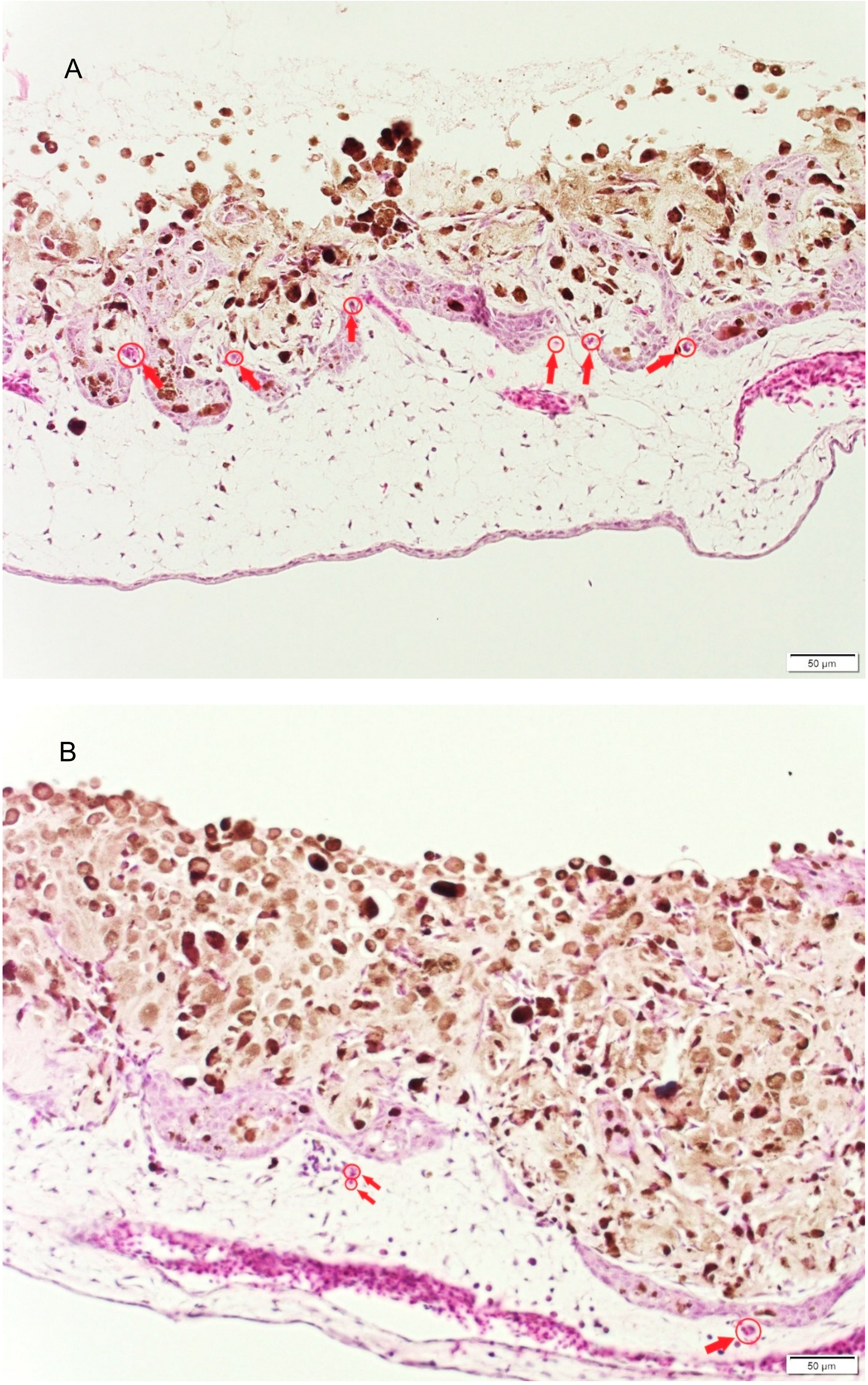
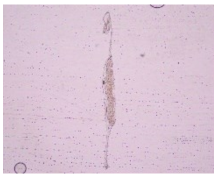
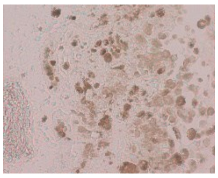
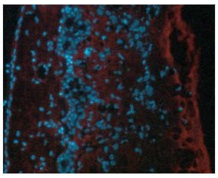
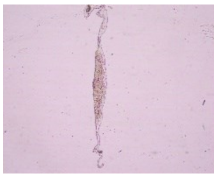
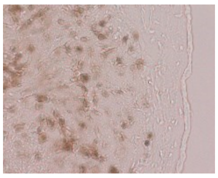
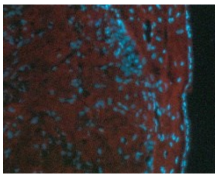
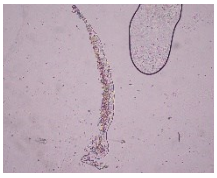
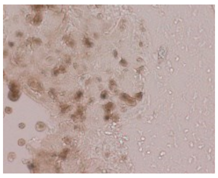
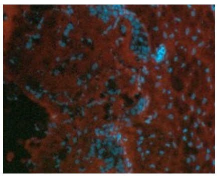
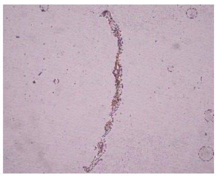
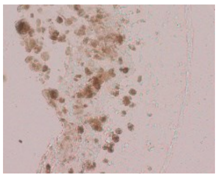
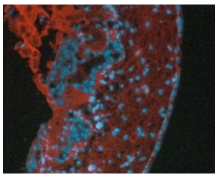


Figure 20: exposed (A) and unexposed (B) explants, 20x. Brown melanocytes and numerous blood vessels (marked with red arrows) are visible.

3.2.2 LCA/DAPI Staining

In order to identify the endothelial cells in the CAM more precisely, the remaining slides, where the melanocytes and blood vessels can be well visualised, were stained by leukocyte common antigen&4′,6-diamidin-2-phenylindol (LCA/DAPI method). The preparations are photo-documented under both brightfield (BF) and fluorescence (FI) microscopy (*Figure 22*). With this method it was difficult to determine the melanocytes.

Table 9: Results of LCA/DAPI staining of exposed and unexposed tissue samples from both experiments

Magnification / Egg Nr.		2x	20x - BF	20x - FI
exposed	364			
	384			
unexposed	376			
	392			

4 Discussion and Conclusion

So far, several angiogenic factors have been fully discovered and their amino acid sequences has been determined. These polypeptides include acidic and basic fibroblast growth factor, angiogenin and transforming growth factors alpha and beta (58). Other less well-characterized angiogenesis factors have also been isolated, some of which are lipids (58). The angiogenic factors have great importance in the growth regulation of the vascular system. It is important to investigate them further to understand the mechanism of both tumor and nonneoplastic diseases. The reason is that these diseases are dominated by pathological growth of capillary blood vessels (58).

Several studies give us the reason, that we hypothesize, that melanocytes also play a major role in angiogenesis. For example, the absence of melanocytes - leucism leads to some diseases in humans and animals whose relationship is not yet researched(47). Our work aims to find out whether melanocytes have an influence on angiogenesis.

In addition, the influence of light was also interesting for us, whether melanocytes may induce angiogenesis. It might also be helpful in the therapy of myopia - a common public health problem all over the world.

In this study, we investigated the influence of light on angiogenic features of melanocytes. As an intermediate stage between animal and human models, the chick chorioallantoic membrane model with xenografted foreskin melanocytes seems to be the ideal model for our experiment. Although the method has been published in many papers for years, it has not yet been used for human melanocyte transplantation. In my opinion, CAM assay was very suitable for our experiment. The criteria for the adhesion of our melanocytes were easy to recognize after 3 days of incubation, the vascular plexus showed a significantly thicker appearance, based on the angiogenic activity of the melanocytes.

The first step was to investigate with the relationship between angiogenesis and melanocytes. Distinct organs – such as the inner ear or the eye lose their main functions due to melanocyte deficiency.

We were able to successfully develop human foreskin melanocytes on the CAM, using ex ovo culturing conditions. The melanocytes grew very well on the CAM and formed round within three days. The xenografts show invasive behavior into the CAM and are highly angiogenetic.
After harvest, fixation, paraffin-embedding and immunohistochemical staining, the histological preparations were documented using the Olympus stereomicroscope and counted according to a manual counting protocol.
It has been suggested that melanocytes are involved in the process of angiogenesis. Through our experiment it was determined what possible function the melanocytes may have in these organs that do not receive light. Especially in the study by Schatz et al. the fundus examination of the leucistic dog using optical coherence tomography (OCT) shows us how thicker the choroid is, where the choroid has a lot of melanocytes - is darker. (47).
In addition, by macroscopic observation we see how melanin cells colonize where vascularization is high. This experiment helps us to prove the hypothesis of the relationship between melanogenesis and angiogenesis (47). By defining this relationship, the mechanism of human diseases, such as Waardenburg syndrome, deafness, microphthalmia, might be better explained.
The influence of light was the next target of our work. Some studies show that children who often engage in outdoor activities suffer from myopia less than others (59-61). For this reason, the influence of light on angiogenic factors of melanocytes was particularly important for us.
To observe the influence of the light, the eggs were divided into two groups – bright and dark, in a group of eggs that was exposed daily from 8 AM to 6 PM to visible light of 1,000 lux, in another group that was unexposed during the whole experiment. Both groups were cultivated under the same conditions.
The evaluated data show that the results are similar. The data show that the light in our experiments had no detectable effect on the melanocytes or rather on angiogenesis. All xenografts from the two groups give similar results in the angiogenesis assay. For the Verification, the experiment was conducted twice, and the results were significant.

However, the difference was not significant between “bright” and “dark” groups, because vessel development and melanocyte invasion were equal in both groups.
In our experiments neither by HE-staining nor by the LCA/DAPI method it was possible to detect an obvious influence of the light.
In conclusion, the CAM assay is a well-suited method for our study. It was well possible to successfully grow melanocytes on the CAM, excise and histologically process the xenografts, connect histological and immunohistochemical analyses and successfully perform the angiogenesis assay.
As mentioned before both experiments showed light-independent melanocyte-induced angiogenesis. There is no visible difference between exposed and non-exposed groups. Furthermore, in our experiment melanocytes from foreskin were cultivated instead of melanocytes from choroid. If the melanocytes were isolated not from foreskins but from eye - choroids, different results might be obtained.
Moreover, Lan W. has 3 days old chicken explored (25), while we have incubated chicken embryos. According to Lan's experiment, light certainly causes an effect on the thickness of the choroid.
Besides, it would be helpful to expose the "bright" group all day instead of 10 hours. This might certainly also be helpful for myopia prophylaxis in children.
In summary, our experiments provide strong evidence that although melanocytes are strongly involved in the angiogenesis process, visible light has no remarkable effect on this process. With the help of this research, the occurrence of human diseases such as leucism, myopia, deafness may be better explained or treated. We should not forget that it has already been researched and found that bright light (10,000 lux) increases the choroidal thickness and angiogenesis in three-day-old chickens (25). Therefore, for future tests it will be necessary to repeat the experiment with CAM assay not with 1000 lux but with even brighter light (10,000 lux). Extending the incubation time and the melanocytes from the choroid may also help to achieve different results.

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