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Laryngeal Carcinoma Experimental Model Suggests The Possibility Of Tumor Seeding To Gastrostomy Site

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Abstract

Some studies state that laryngeal squamous cell carcinoma (LSCC) is associated with possible direct tumor cell seeding to percutaneous endoscopic gastrostomy (PEG) site. However, there is a lack of experimental proof that LSCC tumor tissue can adhere and grow in distant sites. Therefore, we aimed to investigate the growth pattern of LSCC implants on chicken embryo chorioallantoic membrane (CAM) and evaluate possible associations between clinical course of the disease and behavior of experimentally implanted LSCC tumors. Our results show that implanted LSCC tissue survives on CAMs in 95% of cases while retaining essential morphologic characteristics and proliferative capacity of the original tumor. We identified the increased CAM vascularization, an infiltrative growth pattern of the implant and formation of distant isolated metastatic nodes on the CAMs. LSCC tumors with worse differentiation degree (G2 or G3) adhered to the experimental CAMs significantly better than G1. These results facilitate the understanding of tumor biology and allow presuming that dissemination and direct implantation of LSCC cells into the stomal wall during the *pull* PEG procedure might be possible.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is the most common malignant tumor of the upper respiratory tract. The reported incidence of the disease is 2.76/100 000 and prevalence 4.33/100 000 cases per year[1]. The standard treatment for LSCC, i.e. surgery alone or in combination with chemoradiation therapy is generally accepted[2]. Despite the modern treatment options and advanced surgical techniques, the 5- year survival rate of LSCC in Europe does not change significantly over the last decades[1]. Those patients who undergo chemoradiotherapy for the LSCC and other head and neck malignancies treatment quite often ultimately require and undergo a percutaneous endoscopic gastrostomy (PEG) tube placement to ensure alternate route for nutrition[3]. However, several complications when using the PEG technique are given in the literature[2,3]. For instance, the head and neck tumor spreading to a PEG site due to PEG placement using “pull” technique and/or to tracheostomy wound is described, but as iatrogenic one[4]. These complications may be considered as a possible outcome of direct implantation of the detached malignant cells into an enteral access site of the PEG tube or in the tracheostomy wound, however the pathogenesis of this process is not known[4].

Experimental models of LSCC would provide a better understanding of the biological mechanisms that control progression and spreading of LSCC in distant areas including seeding to gastrostomy. Establishing concordance between the patient derived xenografts and original patient tumors is a gold standard for most of experimental models. However, current *in vivo* models for human LSCC investigation and existing cell lines have failed both to simulate enough tumorigenic phenotypes for LSCC and establish sufficient experimental data about carcinogenesis and metastatic potential of this type of tumor in a live medium[5–7].

Therefore, a chicken embryo chorioallantoic membrane (CAM) based experimental model for this type of tumor was recently developed as a relevant host medium for implanting fresh tissues of the LSCC, that reveals numerous unique properties and advantages[8]. Generally, the chicken embryo CAM model has been used for experimental purposes for decades, therefore this assay is precisely described in the literature and some evident benefits of the CAM assay have been recognized[9–11]. The chicken embryo CAM is an experimental medium that is known for good vascularization and lack of immune response. Therefore, the transplanted tissues are more likely to survive and grow on the CAM[12]. According to previous studies the chicken CAM model has got similar advantages as rodent ones. This experimental model is cost effective and not time consuming for research of many tumors[9]. Results of the recent study revealed that the LSCC implants adhere to the host chicken embryo CAM and induce significant morphological changes of it, however retaining the original morphology of the tumor and allowing visualizing microscopically the behavior of implanted tumor cells[8,13,14].

We hypothesized that the growth pattern of LSCC implants on chicken embryo CAM was associated with clinical course of the disease, and that the behavior of experimentally implanted LSCC tumor could be similar to its clinical behavior, thus supporting the possibility of LSCC dissemination and direct implantation to distant sites.

The aim of the present study was to investigate the growth pattern of LSCC implants on chicken embryo CAM and evaluate the correlations between clinical course of the disease and behavior of experimentally implanted LSCC tumors.

Results

Primary LSCC tumor growth (in vivo biomicroscopy and histological evaluation)

LSCC tumor implants adhered the host CAMs in 95% (n=228) of cases (Fig. A, B). Histological evaluation of the implants revealed typical histological features of LSCC tumor (atypical epithelial cells, irregular nuclei and increased number of nucleoli, atypical keratinized cells, i.e. “carcinoma pearls” in parenchyma and loose connective tissue with different level of monomorphonuclear cells in stroma) (Fig. B). Starting from the third day of implantation, the LSCC tumor implants showed an infiltrative growth pattern on CAMs in 22% (n=49) of cases; 2% (n=5) of LSCC tumor implants infiltrated mesenchyme of the host CAMs (Fig. C). Besides, 22% (n=49) of LSCC tumor implants formed distant isolated metastatic nodes on CAMs, (one, two or three nodes per case).

Visualization of tumor growth and angiogenesis with fluorescein

In vivo biomicroscopy with fluorescinated anionic dextran exhibited evident vascularization of the CAMs and vascular orientation towards the implants - so called *spoked-wheel* formation in LSCC tumor implants. Starting from the 6th day of implantation LSCC tumor implants were supplied by chicken embryo blood (as confirmed by the presence of distinct fluorescence of the implants. (Fig. D).

Histomorfometric parameters of CAMs after LSCC implantation

Histomorphometric analysis on experimental CAMs was conducted on the 10th day of eggs' incubation. The results reveal that CAMs under the LSSC implants were up to 478% thicker due to thickening of CAM mesenchyme (p=0.001) when compared to control group. However, mean thickness of the CAM epithelium was statistically significantly higher up to 250% (p=0.001) in the LSCC group versus control group. The results are presented in Table 1.

The comparison of manifestations of LSCC on chicken embryo CAM and in human larynx

No statistically significant associations between LSCC tumor stage and LSCC survival rates on chicken embryo CAMs were observed in our series (p=0.845). However, the LSCC tumors with worse differentiation degree (G2 or G3) adhered the experimental CAMs significantly better than G1 (p=0.001) (Table 2).

The LSCC tumors that formed distant metastases in patients also formed metastatic nodes on chicken embryo CAMs more often than those who did not spread in human body (p=0.002). This phenomenon was noticed starting from the 4th day of tumor implantation (Table 3).

Discussion

The hypothesis of possibility of direct tumor cell seeding to distant sites of patient's body is discussed in scientific literature. The new case reports of head and neck carcinoma spreading to a PEG site were described recently[3]. However, the precise mechanism of this type tumor spreading still remains unclear[15].

Three possible mechanisms for PEG site metastasis have been considered in the literature: a) tumor cells could be disrupted and directly implanted into the PEG site or esophagus when the PEG tube is placed from upper digestive tract to a stoma in the abdominal wall; b) disrupted tumor cells could be disseminated hematogenously and implanted at the PEG site; c) PEG-site metastases could be random events from hematogenous distribution of tumor cells[15–21].

Results of the prospective study of M. Ellrichmann et al may be considered as a support of direct implantation of disrupted tumor cells. The authors obtained cytology samples from brushings of PEG site and identified the presence of malignant cells initially after the procedure in 22.5 % and at 6 months of follow up in 9.4% of cases[21]. Another concept of malignant tumor seeding phenomenon is known as a stomal recurrence of carcinoma after total laryngectomy. As the risk factor for tumor transmission a prelaryngectomy tracheostomy is described. Therefore some strategies to prevent tumor cell seeding such as prophylactic radiation before tracheostomy procedure or tumor-cell attachment inhibitors usage during these procedures are discussed[3].

The *pull* and the *push* techniques for the placement of a PEG tube are commonly used in clinical practice. In the pull technique, the PEG tube is pulled through the oropharynx into position in the stomach by an

endoscopically placed guide-wire which runs from the mouth to the stomach and through the anterior abdominal wall. Contrary to the *pull* technique, the *push* technique allows for insertion directly into the abdominal wall, avoiding contact between the gastrostomy tube and the tumor[15,16]. Consequently, the *push* technique for PEG placement is associated with a lower incidence of PEG-site metastases and an overall lower complication rate when compared to the pull technique[15,20,22]. Generally, the incidence of PEG site metastasis or stomal recurrence is low; therefore, the presence of tumor seeding is suggested as a marker of tumor malignancy and aggressive behavior[3].

Results of the present study possibly could explain some mechanisms of the tumor dissemination. We used an alive medium of chicken embryo CAM for tumor implantation. The CAM is known as an immunodeficient host that has a well-established bloodstream. The chicken embryo and the CAM grow fast therefore the results of the experiment are evident in few days and external factors do not interrupt the implant growth[9,10]. It is possible to observe through the process of tumor growth directly all day long[10]. Most of the studies were completed with implantation of tumor cells' cultures on chicken embryo CAM but not with fresh tumor tissues[10]. However, it does not reveal the real environment of tumor seeding in PEG or tracheostomy procedure in LSCC patients. Therefore, we implanted fresh pieces of LSCC tumors onto chicken embryo CAMs. Results of our study showed that 95% of the implants adhered the experimental membranes and induced the evident changes of them.

The studies analyzing different tumor cells growth pattern on CAM show, that inoculated cell cultures form a solid tumor in 2 to 5 days after implantation. Further those cell conglomerates grow similar to maternal tumors[10]. Our study reveals that LSCC tumors induced the thickening of both epithelium and mesenchyme of the host CAMs. The blood supply of the newly growing implants was confirmed when performing *in vivo* biomicroscopy with fluorescinated anionic dextran. The histological evaluation of experimental CAMs disclosed that 22% of LSCC implants manifested with the infiltrative growth pattern through the host membranes or infiltrated the mesecnchyme of the CAMs. Those tumor implants that represented the worse differentiation degree G2 or G3, adhered chicken embryo CAMs significantly better than G1 ones. It is well known from clinical practice that worse differentiation degree of the tumor leads to worse outcomes of the malignant disease. Results of the present study confirmed association between LSCC differentiation degree and the tumors adhesion rate. Moreover, those LSCC tumors that formed distant metastases in patients were more likely to disseminate in experimental CAMs after the implantation. This phenomenon was observed starting from the 4th day of LSCC tumor implantation. It is naturally, because the first 24 hours are known as prevascular phase when the implanted tumor arouses the vascular proliferation of the CAM. The next 24-48 hours the newly formed vessels of CAM penetrate the implant. Afterwards the tumor grows and disseminates on CAM[10,23,24].

Results of the present study support presumption that LSCC tumor implants show similar behavior in both human and experimental membranes. The LSCC implants in our series induced both thickening of the CAM and increase in CAM vascularization (higher mean number of blood vessels per constant length of the membrane), thus establishing a concordance between patients derived xenografts and original

patient tumors. An observed notable increase of angiogenesis in LSCC implants on experimental CAMs paralleled a feature of LSCC that may be correlated with worse outcome of the malignant disease.

Conclusion

To summarize, these experimental results can be translated to clinical settings and allow presuming, that dissemination and direct implantation of LSCC cells into stomal wall during PEG procedure is possible. In this respect, it supports preference of *push* PEG method against the *pull* method, because *push* PEG method may prevent a possible tumor seeding into the PEG site. Therefore, use of other percutaneous techniques that do not involve traversing the hypopharynx with the catheter may help to prevent laryngeal/hypopharyngeal tumors' translocation[17].

At the same time, results of the present study allow better understanding of LSCC biology and clinical features. Therefore, further development of chicken embryo CAM based LSCC model may help searching of new selective therapeutic agents to limit spread of LSCC.

Material And Methods

Investigations in the present study were performed in accordance with the principles outlined in the Declaration of Helsinki and approved by Kaunas Regional Bioethics Committee (P1-BE-2-34/2007), Kaunas, Lithuania. LSCC tissue samples were acquired in accordance with the protocol approved by the Institutional Review Board of Lithuanian University of Health Sciences (LUHS), Kaunas, Lithuania. Written Informed Consent was obtained from the patients before surgery and patients' identifiers were removed to ensure anonymity.

Patients

Twelve male patients with histologically verified LSCC participated in this study. The mean age of the patients was 54±7 years. Distant metastases of LSCC were diagnosed in 5 cases. Four patients passed away during the 5 years follow up period after LSCC diagnosis. Data about the differentiation grade and the stage of the LSCC is given in Table 4.

The five-year follow up data from the group of LSCC patients starting from the experimentation day were analyzed according to the differentiation grade and the stage of the disease. All patients were evaluated for the recurrence of LSCC and distant metastasis every 3 months during the first 2 years and every 6 months during the next 3 years. Those patients who did not survive to the end of experimentation were noted too.

Incubation and egg opening

Fertilized hen eggs (*Cobb-500*) (N=20) were incubated at 37.7 °C temperature and 59-60 % relative humidity for each experiment, in total 240 ones. On the day 3 of incubation albumen was removed and

window in the egg shell was opened. Embryos that showed signs of vitality were prepared for further incubation by covering egg-shell windows with the sterile transparent tape in the same conditions for 96 to 144 hours when the fresh LSCC tumor tissues were implanted.

LSCC tissue implantation onto the CAM, tissue sampling and histological evaluation

Fresh 219 LSCC tissue samples were obtained from 12 male patients who underwent laryngeal surgery because of LSCC in the Department of Otorhinolaryngology, LUHS. Tumor samples were placed in isotonic saline solution for transportation to Department of Histology and Embryology, LUHS, and then implanted onto CAMs within 45 minutes. Each patient's LSCC tissue sample was sliced in small pieces of about 0.8 mm^3 and gently placed on the outer surface of the CAM (1 piece per egg) [13].

In vivo biomicroscopy was performed for each embryo every 48, 72, 96, 120, 144 and 168 hours after tumor implantation. The random two eggs at each of the reported hours were injected with 10 µl of a 20 mg/ml 70- kDa fluoresceinated anionic dextran (Eugene, OR, USA) in phosphate-buffered saline (PBS) into the biggest apparent vessel of the CAM[14]. The newly formed micro-vascular network on the CAM was evaluated with OLYMPUS SZX 16 stereomicroscope (Olympus Life Science Europa GmbH, Hamburg, Germany). CAMs with the ingrowing implants were excised, fixed in formalin, cut out and embedded into paraffin blocs for the period of 5 days. The embedded membranes were sliced and stained with hematoxylin and eosin (H&E) for histological examination. Histological evaluation of the samples stained with H&H was performed with the cold light microscope OLYMPUS BX40F4 (Olympus Opticae co. LTD., Japan) under 10x magnification using CellSensDimention1.9 Digital Imaging Software for Research Applications (Olympus Corporation of the Americas, USA). The thicknesses of the CAM and of the chorionic epithelium per constant length of the CAM section were measured under the implanted tumors on the images obtained with Olympus digital camera (Olympus U-CMAD3, Philippines).

Twenty-two CAMs that proceeded under the same protocol, however without LSCC implantation constituted the control group.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. (Armonk, NY: IBM Corp. Software). Data were presented as the mean ± standard deviation (SD). The Student's *t* test was used for testing hypothesis about equality of the mean. The size of the differences among the mean values of the groups was evaluated by estimation of type I and type II errors (α and β) of the tests. The difference was considered to be significant if $\beta \leq 0.2$ and $\alpha = 0.05$. The level of statistical significance by testing statistical hypothesis was 0.05.

Declarations

Author contributions:

The following have made substantial contributions to conception and design: IU, IB, AV, AK, VU, JP.

Acquisition of data, analysis and interpretation of data: IB, AV, AK, VU, JP, IU.

The following have been involved in drafting the manuscript or revising it critically for important intellectual content: AK, VU, AV.

The following have given final approval of the version to be published: VU, AV.

All of the authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of interest: The authors declare that they have no conflict of interest.

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Tables

Table 1. The histomorphometric parameters of experimental CAMs under LSCC implants

LSCC			Control group		Difference			
Feature	Mean µm	SD	Mean µm	SD	**µm**	**%**	***p value***	**Observed power***
Thickness of CAM	214	124	37	11	**183**	**478**	**0.001**	**0.90***
Thickness of CAM epithelium	21	10	6	1	**15**	**250**	**0.001**	**0.90***

LSCC - laryngeal squamous cell carcinoma; SD - standard deviation.

* Computed using α=0.05

Table 2. The LSCC tumor adhesion rate on chicken embryo CAM according to tumor differentiation degree.

The compared differentiation degree of implanted LSSC	The adhesion rate % of LSCC on CAM	p
G1/G2	73/97	0.001
G1/G3	73/98	0.001
G2/G3	97/98	0.798
G4	-	-

LSCC - Laryngeal squamous cell carcinoma; CAM - chicken embryo chorioallantoic membrane.

* Computed using α=0.05

Table 3. The comparison of LSCC metastases rate in patients and on chicken embryo CAMs.

Days after LSCC implantation	Number of samples	The mean rate of LSCC metastases on CAM	The mean rate of LSCC metastases in patients	p
2	30	0.25	0.75	0.398
3	50	0.44	0.56	0.889
4	40	0.47	0.27	0.002

LSCC, Laryngeal squamous cell carcinoma, CAM, chicken embryo chorioallantoic membrane.

* Computed using α=0.05

Table 4. Characteristics of LSCC patients (n=12)

	No.
Males	12
Age (mean±SD)	54±7
Differentiation grade G1	4
Differentiation grade G2	6
Differentiation grade G3	2
Stage I	0
Stage II	2
Stage III	6
Stage IV	4
Not survived	4
Patients with distant metastases	5

Figures

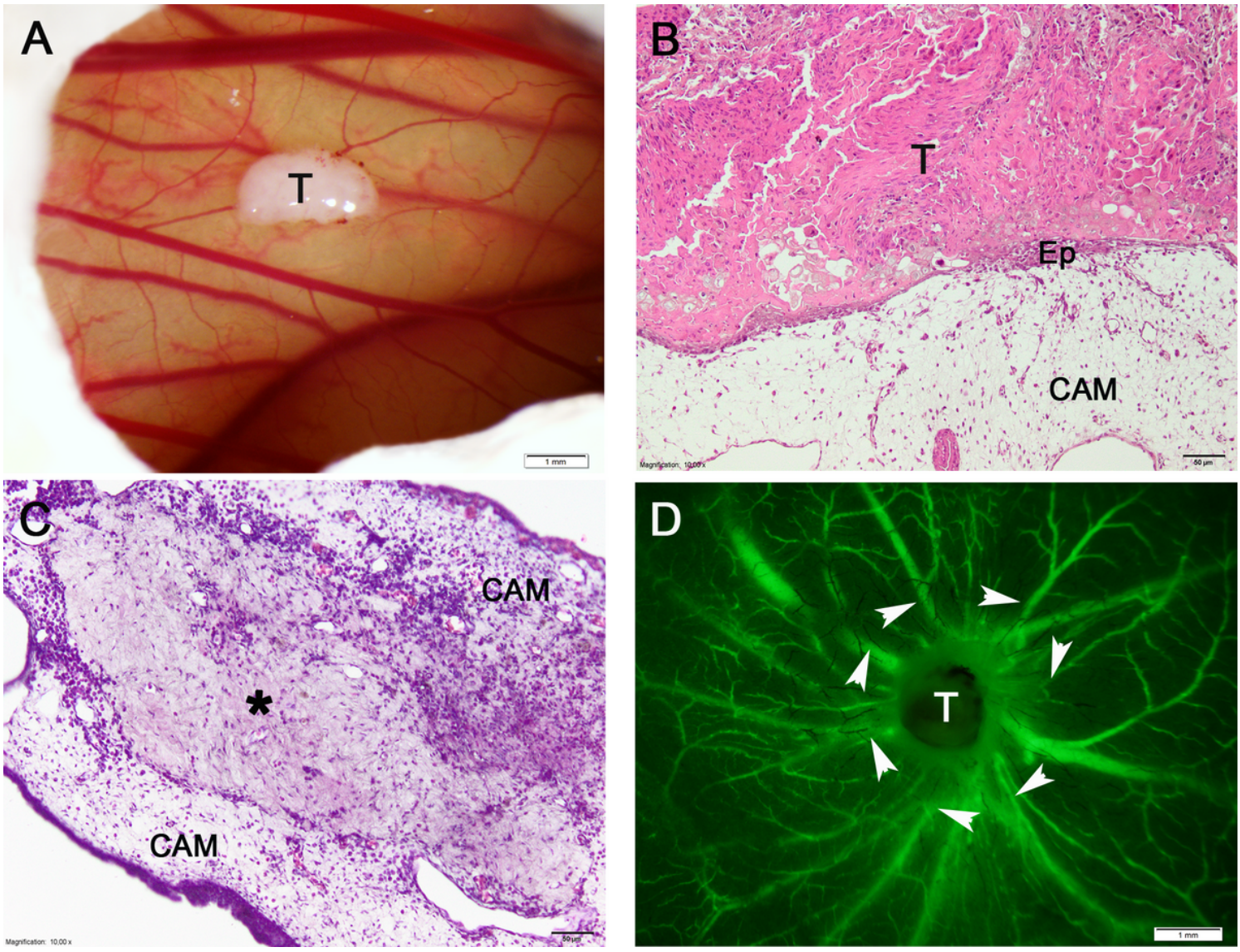
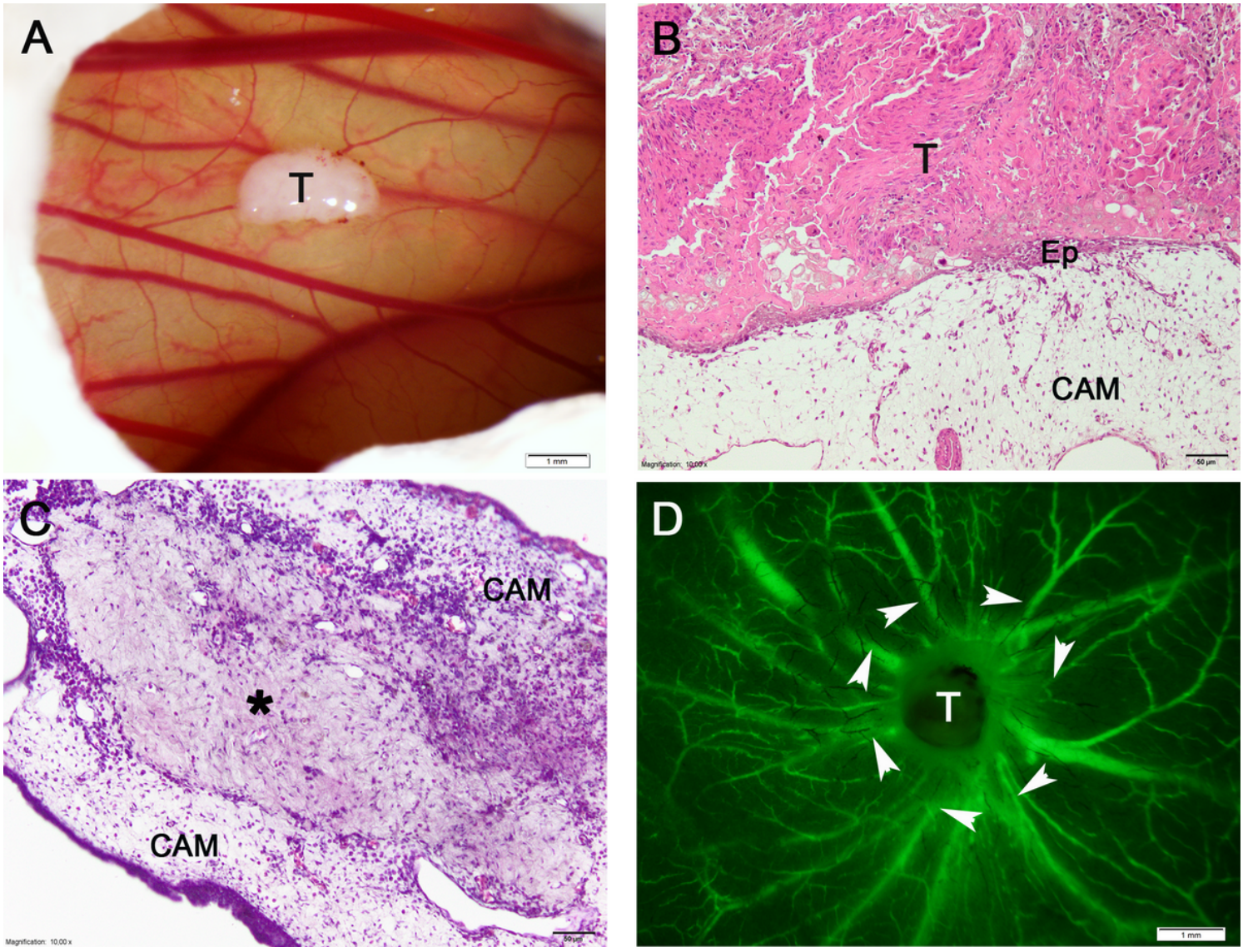


Figure 1

In vivo biomicroscopy and histological evaluation of LSCC tumor growth A, B - LSCC tumor implants adhered the host CAMs (A - in vivo biomiocroscopy; view through the opened window in the egg shell; B – histological view of the CAM with adhered LSCC implant, thickening of the CAM under the LSCC implant is visible too); C - LSCC tumor showed an infiltrative growth pattern on the CAM and infiltrated mesenchyme of the host CAM (asterisk); D – in vivo biomicroscopy with fluoresceinated anionic dextran revealed a spoked-wheel formation in the CAM around the LSCC tumor implant (arrowheads). Ep – CAM epithelium; T – tumor; scale: A, D – 1 mm; B, C – 50 μm



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In vivo biomicroscopy and histological evaluation of LSCC tumor growth A, B - LSCC tumor implants adhered the host CAMs (A - in vivo biomiocroscopy; view through the opened window in the egg shell; B – histological view of the CAM with adhered LSCC implant, thickening of the CAM under the LSCC implant is visible too); C - LSCC tumor showed an infiltrative growth pattern on the CAM and infiltrated mesenchyme of the host CAM (asterisk); D – in vivo biomicroscopy with fluoresceinated anionic dextran revealed a spoked-wheel formation in the CAM around the LSCC tumor implant (arrowheads). Ep – CAM epithelium; T – tumor; scale: A, D – 1 mm; B, C – 50 μm