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Establishment of an animal replacement model for pancreatic cancer therapy studies

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To my parents

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Establishment of an animal replacement model for pancreatic cancer therapy studies

1A. SUMMARY

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal malignancies and new therapeutic options are urgently needed. Personalized tumor models derived from xenotransplantation of fresh patient tissue to mice are emerging as a promising tool for therapy studies, but ethical concerns, long experimental duration and high costs limit the efficacy. In the present study, we asked if the xenotransplantation of freshly resected PDA tissues to the chick chorioallantoic membrane (CAM) might be superior. Neoplastic tissue of pancreatic origin from 42 patients, including 23 PDA tumors, was transplanted to the CAM, which resulted in the growth of solid, neovascularized tumors. The median grafting efficiency of PDA tumors was 70 %, which was higher than in a murine model. The time until tumor growth became evident on the CAM (latency) was on average 3 days and thus shorter than that of tumors cultivated in mice. Importantly, the morphology with a pronounced tumor stroma resembled the primary tumors. The immunohistochemical analysis of the established markers for cancer stem cells (CSCs), k-Ras and fibronectin revealed that the histological features of the original tumors remain stable in their corresponding egg xenografts. Also, this model is suited for personalized therapeutic evaluation as shown by our data measuring tumor take, tumor volume and proliferation of untreated, gemcitabine and dexamethasone pre-treated tumors, as well as tumor volume and proliferation under *in ovo* treatment by intratumoral and intravenous injection of gemcitabine. Furthermore the cultivation of giant cell tumor of bone tumors (GCTBs), derived from stromal cells of 8 different patients and osteosarcoma tumors, derived from three cell lines, was established on the CAM. The replacement of rodent models with the CAM model may also contribute to a more ethical experimental technique. Fertilized chicken eggs are widely used in the biomedical research and have been suggested as an alternative to mammalian models. Unfortunately, it is mostly not taken into account, that the chick embryo is susceptible to pain from day 7 of breeding. In my view, this model is only in accordance with the "3 R" principles of ethical experimentation, if an appropriate anaesthesia of the chick embryo in potentially painful procedures is provided. Although many experimental

approaches are performed on the non-innervated CAM, the euthanasia of the embryo strongly requires a more humane technique than freezing at -20° C, decapitation or *in ovo* fixation with paraformaldehyde without prior anaesthesia. These methods are commonly applied and are not acceptable. However, protocols regarding feasible and ethical methods for anaesthesia and euthanasia of avian embryos are currently not available. Therefore, we established an easy and readily achievable method for the euthanasia and short-term anaesthesia of the chick embryo.

In summary, the CAM is a promising model to accelerate data acquisition in personalized medicine and to promote progress towards a more ethical biomedical research.

Etablierung eines Tierversuchersatzmodells für Therapieversuche beim Pankreaskarzinom

1B. ZUSAMMENFASSUNG

Das duktale Adenokarzinom des Pankreas (PDA) ist eine der fatalsten Krebsarten und neue Therapieoptionen werden dringend gebraucht. In letzter Zeit kristallisierte sich heraus, dass personalisierte Tumormodelle, die auf der Xenotransplantation von frischem Patientengewebe auf Mäuse basieren, ein vielversprechendes Werkzeug für Therapiestudien darstellen. Jedoch stellen ethische Bedenken, lange Versuchsdauer und hohe Kosten wesentliche limitierende Faktoren dieser Modelle dar. In der vorliegenden Studie untersuchten wir, ob die Xenotransplantation von frisch entfernten PDAs auf die Chorioallantoismembran (CAM) des bebrüteten Hühnereies das Mausmodell ersetzen kann. Neoplastisches Gewebe pankreatischen Ursprungs von 42 Patienten, darunter 23 PDAs, wurde auf die CAM transplantiert, was in der Bildung solider, neovaskularisierter Tumore resultierte. Die mittlere Anwachsrate der PDA Tumore betrug 70 %, was höher ist als in vergleichbaren Mausmodellen. Die Latenzzeit, welche der Zeit bis zur sichtbaren Bildung des Tumors auf der Membran entspricht, betrug im Mittel 3 Tage und war somit kürzer als in der Maus. Darüber hinaus blieben die morphologischen Charakteristika der Originaltumore in den Eiertumoren erhalten. Die immunohistochemische Analyse zeigte ferner, dass die Expressionsprofile der etablierten Marker für Krebsstammzellen (CSCs), das extrazelluläre Matrixprotein Fibronectin, sowie das Proto-Onkogen k-Ras in den auf Eiern gewachsenen Tumoren die der Patiententumore widerspiegeln. Das Modell eignet sich ebenfalls für personalisierte Therapiestudien, wie unsere Messungen der Tumoranwachsrate, des Tumorvolumens und der Proliferation von mit Gemcitabine und Dexamethasone vorbehandelten Tumoren, sowie des Tumorvolumens und Proliferation von *in ovo* intratumoral und intravenös mit Gemcitabine behandelten Tumoren demonstrieren. Ferner wurde die Kultivierung von Riesenzelltumoren des Knochens, die aus Stromazellen von 8 verschiedenen Patienten stammten, sowie von Osteosarkomen, die aus drei Zelllinien stammten, auf der CAM etabliert. Das Ersetzen von Nagermodellen mit dem CAM Modell mag ebenso einen Schritt in Richtung einer ethisch besser vertretbaren Experimentierkultur bedeuten. Die

Verwendung befruchteter Hühnereier ist in der biomedizinischen Forschung weit verbreitet und wird oft als Alternative zu Versuchen an Säugern angesehen. Leider wird jedoch meistens außer Acht gelassen, dass der Hühnerembryo bereits ab dem 7 Bebrütungstag Schmerzempfindlichkeit entwickelt. In meinen Augen erfüllt dieses Modell aber nur dann die Anforderungen der „3 R“ Prinzipien der humanen Experimentiertechnik, wenn in potenziell schmerzhaften Eingriffen für eine angemessene Anästhesie des Hühnerembryo gesorgt wird. Wenn auch in vielen Versuchsansätzen nur die nicht innervierte CAM verwendet wird, muss die Euthanasie des Embryos am Ende der Experimente mit einer humaneren Technik durchgeführt werden, als die üblicherweise verwendeten Methoden, wie Einfrieren bei -20° C, Enthauptung, oder die *in ovo* Fixierung des nicht narkotisierten Embryos mit Paraformaldehyd. Bedauerlicherweise existieren zur Zeit keine Protokolle zur Anästhesie und Euthanasie von Vogelembryonen. Aus diesem Grund entwickelten wir eine einfache und verlässliche Methode für die Kurzzeitanästhesie und Euthanasie des Hühnerembryos. Zusammenfassend birgt das CAM Modell großes Potenzial die Datenerhebung in der personalisierten Medizin zu erleichtern und den Fortschritt in Richtung einer ethisch orientierten Forschung zu beflügeln.

2. ABBREVIATIONS

3 R	Replacement, Reduction and Refinement
AEC	3-amino-9-ethyl-carbazole
AVMA	American Veterinary Medical Association
BALB	Bagg Albino
CAM	Chorioallantoic membrane
CSC	Cancer stem cells
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
EMT	Epithelial to mesenchymal transition
FCS	Fetal calf serum
GCTB	Giant cell tumor of bone
GEMM	Genetically engineered mouse model
H&E	Hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPMC	Intraductal papillary mucinous carcinoma
IPMN	Intraductal papillary mucinous neoplasia
NET	Neuroendocrine tumor
PDA	Pancreatic ductal adenocarcinoma
PDTX	Patient-derived tumor xenografts
UICC	Union for international cancer control

3. INTRODUCTION

3.1. Pancreatic cancer

Pancreatic cancer lists among the leading causes of cancer-related deaths in the European Union and the USA. Despite extensive research efforts in preclinical and clinical science, there hasn't been much progress concerning mortality reduction since three decades and the relative 5-year survival rate is still less then 6 %. The predominant histological type of exocrine pancreatic neoplasms is PDA, which accounts for over 80 % of the cases.[1,2] It distinguishes itself by a notoriously malignant phenotype, which is highly resistant to cytotoxic therapy. A dense desmoplastic stroma and pronounced hypovascularity is characteristic for PDA and unique among solid tumors.[3-6] Extensive local invasion and early systemic dissemination contribute to the dismal prognosis of this disease. Normal pancreatic duct epithelium progresses to infiltrating cancer through a series of histologically defined precursors, the PanINs.[7] PDA involves multiple mutations that trigger the tumor progression cascade. Most predominant are point mutations in the k-Ras gene, which are present in 90 % of PDA patients.[3,8] Recent findings suggest that a distinct subset of cancer cells, the CSCs and a phenomenon known as epithelial to mesenchymal transition (EMT) play a pivotal role in the tumorigenesis and progression of pancreatic cancer. EMT is a process of transformation of epithelial cells into cells with a mesenchymal phenotype, which leads to increased migratory and invasive properties.[9] It is presumed that the EMT process may give rise to CSCs, or at least cells with stem-cell-like properties.[10] CSCs are suspected to possess self-renewal potential and the ability to differentiate.[11-13] In addition they are likely to promote tumor growth, therapy resistance, invasion and metastasis.[14] Most PDAs are considered sporadic, whereas hereditary conditions account for 10 % of the cases.[2] Even though the etiology of pancreatic cancer is not well understood there are several factors known to increase the risk. The factors include tobacco exposure, alcohol use, consumption of dairy and meat products, obesity, the presence of diabetes, a high-fat diet and low fruit and vegetable intake.[15-20] Early stage PDA is typically asymptomatic. When symptoms do occur, in the majority of the patients the tumor has usually already spread to surrounding tissues or distant organs. Symptoms vary based on the location of the lesion. In the case of a mass in the head of the pancreas that compresses the bile duct, patients present with painless jaundice. Neoplastic masses in the body and tail, however, can remain asymptomatic until late stage of the disease. Unexplained weight loss, abdominal pain, back pain, pruritus, new-onset diabetes and

depression are common late symptoms.[2,8] Surgical removal has a low success rate, but offers the only likelihood of cure.[21,22] Unfortunately, due to lack of early symptoms and efficient screening tools the majority of cases is metastatic at presentation.[2] Gemcitabine is the standard chemotherapy in this setting but its benefits are only modest.[23,24] Besides PDA, also cystic neoplasms of the exocrine pancreas are described. The most common among these lesions are serous cystadenomas, mucinous cystic neoplasms and intraductal papillary mucinous neoplasms (IPMNs). Serous cysts are nearly always benign, whereas mucinous tumors exhibit variable malignant potential and are considered precursor lesions of PDA. The grade of epithelial dysplasia is used to classify these neoplasms as benign, borderline or malignant. Malignant IPMNs are also called intraductal papillary mucinous carcinomas (IPMCs). The treatment of cystic tumors has yet not been established, but surgical resection is indicated in the case of malignity and for symptomatic patients.[25-30] Also, neuroendocrine tumors (NETs) of the pancreas are described, which are composed of cells showing neuroendocrine cell differentiation. NETs, also referred to as islet cell tumors, are rather uncommon and comprise < 2 % of all pancreatic tumors. These tumors grow in an indolent fashion, but may produce hormones, such as insulin, gastrin or glucagon and cause symptoms due to the overproduction of these substances. They are usually slowly growing, but they can be life threatening, as they have the potential to spread. Therapy for NETs is primarily surgical resection and somatostatin analogues, which reduce symptoms of hormone overproduction. Systemic treatment options for advanced disease continue to be limited.[31-34] In summary, there is a strong need for new therapeutic modalities to improve the survival time of patients suffering from pancreatic cancer.

3.2 Laboratory *in vivo* models for pancreatic cancer therapy studies

Laboratory *in vivo* models of pancreatic cancer are consistently used to develop and qualify new therapeutic agents for study in clinical trials. The most used models include murine tumors grown in genetically engineered mice (GEMM) and xenografts of human tumors grown in immunodeficient mice.[35] In GEMMs tumors arise in transgenic and/or knockout mice engineered to recapitulate various genetic alterations, which are suspected to be causative for specific types of cancer in humans. They have the advantage of allowing study of the earliest stages of tumorigenesis.[36] However, the value of GEMMs in predicting clinical results remains to date, poor.[35] Disadvantages of GEMMs include major physiological differences between mice and humans, high costs and the fact that some models require crossing

of three or more lines of mice, which is time consuming. Also, many GEMMs are based on the activation or inactivation of genes in the embryonic pancreas to initiate tumor development, which is different to tumorigenesis in humans.[36] GEMMs are dependent on a few critical genetic lesions, such as k-Ras, p53 and cdkn2a/p16, which does not mirror the genetic diversity that exemplifies human PDA.[37] It is becoming clear that pancreatic cancer tumors are genetically highly heterogeneous. A genomic analysis of 24 advanced pancreatic adenocarcinomas revealed that the tumors contain on average 63 genetic alterations.[38] This heterogeneity may explain the pronounced resistance of this cancer type to chemotherapy and provide indications why targeted therapies to specific tumor mutations are unlikely to succeed in clinical trials.[3]

Other approaches to the preclinical investigation of pancreatic cancer therapies rely on the cultivation of human tumors grown in immunodeficient mice. Traditionally, the standard toolkit for cancer biologists were established monocellular layers of tumors maintained in serum-based growth media and mouse xenografts derived from those cells. These cell line xenograft models are of undoubted value as a quick "first pass" assessment of potential pharmacological properties of an agent, but they cannot generally predict the behaviour of a given substance in the clinical setting. It is not uncommon for new drugs to be highly effective in cell-line-derived mouse tumors. Frustratingly, such preclinical results are often followed by failure in clinical trials, or the benefits of the drug are only modest and improve patient survival by at best some months.[39,40] Cell-line-derived tumors may not fully mirror the human disease for various reasons. Cancer cell lines have been adapted to grow in the laboratory and they do not recapitulate the tumors from which they were derived. Cells cultured on plastic for decades undergo substantial changes in behavior and histopathology, which deviate from the original tumor.[36] The selective pressure of cell culture allows the least differentiated cells to thrive, which results in irreversible losses of biological properties.[41] Patterns of gene expression are reduced in complexity during the transition to the cell culture and are not regained when the tumors are re-established as secondary xenografts.[42] Also, the stroma is underrepresented in these models and data suggest that stroma plays an important role in the mechanisms of cancer cell survival, particularly in pancreatic cancer.[37] The most prominent concern is, however, that a particular cell line derived from one individual patient, is homogenous and therefore does not recapitulate the heterogeneity evident amongst pancreatic cancer patients. Human malignancies are complex and heterogenic both between patients, as well as between cancer cells of one individual.[40] In view of this, it is not surprising that anticancer agents selected in established cell line models do not succeed in clinical trials. Moreover, even if a drug is effective, the trials define the best treatment for an average patient, but this might not be the optimum therapy for an individual

patient. When it comes to developing anticancer drugs the "one size fits it all" approach may be not the best way. Undisputable cell line based models are indispensible for preliminary drug screening, but there is a strong need for individual tumor models. To address this need personalized tumor models are made from patient-derived tumor tissue. In this approach, fresh surgically removed tumor samples from patients are transplanted into immunodeficient mice. Patient-derived tumor xenografts (PDTXs) preserve histological and phenotypic features of the original tumor.[43] There is no selection and all cellular components, including CSCs, are preserved.[44] It has been demonstrated recently, that freshly resected pancreatic cancers from 14 patients transplanted into mice maintained their fundamental genotypic features despite serial passages.[45] Also, the clinical outcome of a patient with gemcitabine-resistant PDA, who responded to DNA damaging agents on the basis of activity of these drugs against a personalized xenograft generated from the patient`s surgically resected tumor was reported. Overall, there was a remarkable correlation between drug activity in the model and in the patient, both in terms of resistance and sensitivity.[46] In another study, 69 pancreatic cancer tumors were implanted into mice and enabled prediction of poor survival and resistance to gemcitabine.[37] Similarly, it has been shown, that tumors derived from women with breast cancer authentically reflected the pathology, growth, metastasis and disease outcomes.[47] In view of this, PDTXs are emerging as an important model for the validation of anticancer drug sensitivity and the prediction of patient prognosis. However, this model has also some drawbacks. PDTXs maintained in mice are expensive and their passage requires more specialized skills, than does the maintenance of cultured cell lines. Moreover, PDTX models can suffer from long tumor latency periods. Tumor latency, measured as the time between transplantation and tumor development in mice, range from 2 to 12 months. Also, the engraftment rates can vary between 23 % and 75 %, depending on the tumor type.[41,43] In summary, PDTX is certainly a very promising tool for personalized cancer therapy studies, but improvements in terms of the shortening of tumor graft latency and cost reduction are desirable.

3.3. The chorioallantoic membrane of the chick embryo as an alternative to mammalian cancer models

Laboratory *in vivo* models of cancer, which employ rodents, give causes for major ethical concerns. The animals are submitted to tumor injections and have to tolerate the pain, which is caused by the mass extension of the growing tumor and is often

associated with ulcers. Living in unnatural conditions in a very limited space and handling of the animals also causes stress.[48-50] In 1959, Russell and Burch published "The Principles of Humane Experimental Technique ". They postulated that if animals were to be used in experiments, every effort should be made to replace them with non-sentient alternatives, to reduce to a minimum the number of animals used, and to refine experiments which used animals so that they caused the minimum pain and distress. These guiding principles, the "3Rs", are now established as essential considerations when animals are used in research.[51,52] The CAM of the chick embryo may circumvent the ethical issues of experiments performed on mammals. During chick development, the CAM forms from day 4 by fusion of the mesodermal part of the allantois with the mesodermal layer of the chorion.[53,54] The CAM is an extra-embryonic tissue, located adjacent to the inner egg shell membrane and until day 8 it develops an extremely dense vessel system, which is connected to the embryonic circulation. Until hatching at day 21, the CAM serves as respiratory organ, waste reservoir for storage of embryonic excrements and for absorption of calcium from the eggshell for bone development.[53,55] The CAM is not innervated and allows experiments, which are not associated with pain of the embryo.[56] The first use of the CAM in biomedical research dates to over 100 years ago.[57] The list of topics under investigation, in which this model is employed, includes tumor biology [58], angiogenesis [59], pharmacology [60,61], regenerative medicine [62], teratology [63], infectiology [64] allergology [65] and many more. The CAM provides an excellent natural substrate for all types of cancer cells. Tumor cells seeded onto the CAM form vascularized, three-dimensional tumors. As chick embryos are naturally immunodeficient the tumor grafts are not rejected.[66,67] Recent studies demonstrate the successful CAM transplantation of established tumor cell lines derived from glioblastoma,[68] osteosarcoma,[69] prostate cancer[70] and pancreatic cancer,[71,72] and the transplantation of freshly resected, patient-derived tissue from thyroid carcinoma, adenocarcinoma of the breast, squamous cell carcinoma of the lung[73] giant cell tumor of bone[74] and of other mucosceletal tumors.[75] The use for xenotransplantation of freshly resected pancreatic tumors has not been reported. Yet, this certainly very promising model also implicates some ethical issues, which need to be taken into account. Nociception in birds is similar to that in mammals [76] and there is a consensus among scientists that avian embryos have the ability to experience pain at a certain point of development. The exact time point is not defined, as this capacity develops stepwise, beginning at day 7 of incubation.[56] It can be assumed, that at day 13 of development, the chick neural tube has developed into a functional brain and that the animal is fully conscious a few days prior to hatching.[77,78] In view of this, experiments on chick embryos can only be characterized as an alternative method to

mammalian models, when special attention to minimize pain and distress of the embryo is paid. Investigations in which embryos older then day 7 and especially older than day 13 are used require the consideration of appropriate anaesthesia. Moreover, even if experiments are only performed on extraembryonal structures (like the CAM), which are not innervated, at the end of the experiments the animal should be euthanized with a humane method. According to the AVMA guidelines on euthanasia[79] the applied technique should result in rapid loss of consciousness followed by cardiac or respiratory arrest and finally a loss of brain function. Pain and distress prior to the loss of consciousness should be minimized. The commonly applied procedure to end experiments on fertilized eggs, is freezing at -20° C of the whole egg containing the embryo. This technique does not list among the acceptable methods in the AVMA guidelines for embryos older than day 10 of incubation. Bird embryos that have attained > 50 % incubation should be euthanized by similar methods used in avian neonates such as anaesthetic overdose, decapitation, or prolonged (> 20 minutes) exposure to CO_2.[79] However, concerns are now being raised, whether decapitation fulfills the criteria for a gentle and easy death, as it has been shown for lambs, rats and mice that conscious awareness may persist for up to 29 seconds in the disembodied heads.[80] Until the proof of the contrary, it is the interest of animal welfare to assume that conscious awareness can also persist for this amount of time in the brain of a decapitated chick embryo and therefore this technique is not acceptable. While CO_2 exposure has long been used as a method for euthanasia, questions have arisen that this practice may not be characterized as a humane method, as there is sufficient evidence that exposure to CO_2 is painful and may cause onset of asphyxia while the animal is still conscious [81]. Also, the required CO_2 dose may be difficult to determine, as neonatal birds are acclimated to high CO_2 concentrations.[79] In some publications the fixation of the CAM with paraformaldehyde without previous anaesthesia or euthanasia of the embryo is reported. In these cases, there is emerging evidence that the embryo dies a painful death. According to the AVMA guidelines animal welfare should be the main factor taken into consideration when choosing an appropriate method of euthanasia. In view of this, an anaesthetic overdose should be the method of choice. Unfortunately, protocols on how to anaesthetize and/or euthanize avian embryos in a practicable and humane way are not available. Recently, the application of 2,2,2-tribromoethanol and urethane/α-chloralose directly onto the CAM to prevent motion of the embryo was reported [82]. However, anaesthesia was not an objective of this study and there is no scientific evidence of whether the administration of these agents in this particular way results in a deep anaesthetic state of the embryo. Heidrich et al. (2011) also used isoflurane, but the use of this agent requires expensive equipment, which may be not feasible for

experimental approaches in which anaesthesia is not the crucial objective. In summary, the CAM is an ethically more tolerable model than those which use mammals, but a humane method for euthanasia is needed.

4. AIMS OF THE STUDY

The primary aim of the present study was to develop an alternative *in vivo* pancreatic cancer model for preclinical drug screenings, as well as for personalized medicine, which is ethically better tolerable and more practical than current rodent and CAM models.
The specific aims were:

1) Establishment of the cultivation of pancreatic cell-line-derived tumors on the CAM.
2) Development of a method for the cultivation of pancreatic patient-derived tumors on the CAM.
3) Establishment of the CAM as a system for the evaluation of potentially cancer affecting compounds.
4) Development of a method for an ethical euthanasia of the chick embryo *in ovo*.

A secondary aim was the establishment of the cultivation of 2 types of cell-line-derived tumors of the musculosceletal system on the CAM, namely GCTBs and osteosarcomas.

5. MATERIALS AND METHODS

5.1. Eggs, reagents and incubation equipment

Fertilized white Leghorn chicken eggs from a local ecological hatchery (Geflügelzucht Hockenberger, Eppingen, Germany), were stored for a maximum of 5 days at 10 ± 1° C and prior to incubation washed with 40 ± 5° C 70 % ethanol. The eggs were incubated at 37.8 ± 0.2° C and a relative humidity of 50 ± 5 % in digital motor breeders Type 168/D (Siepmann GmbH, Herdecke, Germany).

5.2. Pancreatic cancer cell lines and reagents

The established human pancreatic cancer cell lines BxPc-3, MIA-PaCa2 and AsPC-1[83-86] were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were authenticated throughout the culture by the stable morphology of the cells *in vitro*, stable tumorigenic potential in eggs, a stable morphology of the tumors grown in eggs and a stable expression of the diagnostic markers Cyt-19, Ki-67, Vimentin and E-cadherin of the tumors grown in eggs, which was evaluated by immunofluorescence staining. In BxPc-3 cells a cuboidal epithelioid morphology, high nucleus-to-cytoplasma ratio and an adherent, confluent growth pattern was preserved throughout the culture. AsPC-1 and MIA-PaCa2 cells showed an epithelioid, yet pleomorphic morphology with huge cells, abundant cytoplasm and the tendency to grow in colonies. An adherent growth pattern is typical for AsPC-1, whereas MIA-PaCa2 cells have a tendency to “round up” on top of others and then become free in suspension Cells were cultured in DMEM (PAA, Pasching, Austria) and supplemented with 10 % heat-inactivated FCS (Sigma, Deisenhoffen, Germany) and 25 mmol/L HEPES (PAA). To maintain authenticity of the cell lines, frozen stocks were prepared from initial stocks and every 3 months, a new frozen stock was used for the experiments. Monthly testing ensured mycoplasma-negative cultures.

5.3. GCTB stromal cells and reagents

Giant cell tumor of bone (GCTB) stromal cells from 8 different patients were obtained from the Orthopedic University Hospital Heidelberg (AG Dr. J. Fellenberg)

and authenticated throughout the culture by their typical plump spindle-shaped morphology.[74] Cells were cultured in DMEM (PAA, Pasching, Austria) and supplemented with 10 % heat-inactivated FCS (Sigma, Deisenhoffen, Germany) and 25 mmol/L HEPES (PAA).

5.4. Osteosarcoma cell lines

The established human osteosarcoma cell lines MG-63, HOS and Saos-2[87-90] were obtained from the Orthopedic University Hospital Heidelberg (AG Dr. J. Fellenberg) and subsequently transplanted to eggs.

5.5. Patient tissue

Surgical samples were obtained under the approval of the ethical committee of the University of Heidelberg after written informed consent of patients. The diagnoses were established by conventional clinical and histological criteria according to the World Health Organization. All surgical resections were indicated by the principles and practice of oncological therapy. The tumor types and the tumor stages were determined by a clinical pathologist in accordance with the UICC guidelines for classification of malignant pancreatic cancer tumors.[91] The freshly resected non-diagnostic neoplastic specimen of pancreatic and duodenal origin were stored on average for one hour and a maximum of 5 hours in 5 mL Oncostore medium (Oncoscience AG, Wedel, Germany) at 4 ± 1° C and thereafter transplanted to eggs, which at that time point where at day 9 – 12 of embryonic development (see the results section 6.2 for details). Grafts were monitored daily to evaluate vascularization, as well as the latency of tumor growth, which is defined as the time until a tumor was first visible after the transplantation. The grafting efficiency for each tumor type was calculated as follows: N1 × 100/N2 (N1 = number of patient tumors of a particular tumor type, which formed at least one xenograft on eggs, N2 = total number of patient tumors of this type, which were transplanted to eggs). Between days 17 and 18 of embryonic development the grafts were resected and the tumor size was measured with calipers. Tumor volumes were determined by the formula: Volume = $4/3 \times p \times r^3$ ($r = 1/2 \times$ (square root of diameter 1 × diameter 2)).[68,74] The tumors were embedded in Tissue Tek O.C.T. Compound (Sakura, Zoeterwoude, The Netherlands) and stored on dry ice. Tumors from eggs containing dead embryos were excluded from further analysis.

5.6. Treatment of pancreatic cancer cells *in vitro*

The treatment of pancreatic cancer cells was performed by Dr. Frank Schönsiegel. A gemcitabine solution (Eli Lilly, Indianapolis, IN, USA) was diluted in the cell culture medium. 48 hours later the cells were transplanted to eggs. Alternatively the cells were incubated with dexamethasone (Sigma Aldrich, Taufkirchen, Germany) for 7 days and thereafter transplanted to eggs. The combined treatment was carried out by treating the cells with dexamethasone for 5 days and additional 2 days with gemcitabine and dexamethasone. The final concentrations of the solvents in media were 0.1 and 0.005 % for dexamethasone and gemcitabine, respectively. At the time point of transplantation the eggs were between day 9 and 12 of embryonic development. Tumor take was calculated by the formula: N1 × 100/N2 (N1= number of eggs with tumor, N2 = number of eggs containing living embryos). The handling of tumors and the calculation of tumor volumes was carried out as described for patient tissue.

5.7. Intratumoral treatment of egg xenografts

BxPc-3 cells were transplanted to eggs (see the results section 6.1 for details). On days 5 and 6 after transplantation, 50 µL of 50 ηM gemcitabine were injected in 3 regions of each tumor. Injections of DMEM served as control. 3 days later the tumors were sampled and the relative increase in volume was calculated using the following formula: (N1 × 100/N2) – 100 (N1 = tumor volume on the day of excision in mm^3, N2 = tumor volume on day 5 of incubation in mm^3). Tumor samples were handled as described above.

5.8. Intravenous treatment of egg xenografts

A primary pancreatic cancer specimen was transplanted to eggs. On days 6 and 7 after transplantation, 250 µL of 100 ηM gemcitabine (treatment group) or DMEM (control) were injected into a blood vessel of the CAMs, which were hosting the xenografts. 3 days later the tumors were resected and handled as described above.

5.9. Immunohistochemistry and immunofluorescence staining

Endogenous biotin was blocked using the Avidin/Biotin blocking kit (Vector, Burlingame, CA) according to the instructions of the manufacturer. Endogenous peroxidase was quenched in 0.3 % methanol. Primary antibody of the immunohistochemical staining was rabbit polyclonal Ab against human Ki-67 (Thermo Scientific, Rockford, IL, USA). Biotinylated goat anti-rabbit or anti-mouse IgG (Vector) was used as a secondary Ab. The signal was amplified using the ABC Elite kit (Vector, Burlingame, CA). AEC (3-amino-9-ethyl-carbazole) was used as a chromogen. Samples were counterstained with hematoxylin (Mayer) and mounted in Pro Tags Aqua mount (Quartett, Berlin, Germany). Omission of the primary Ab served as a negative control. The signal was detected at ×400 magnification using a Leica DMRB fluorescence microscope (Leica, Wetzlar; Germany). Primary antibodies for the immunofluorescence staining were rabbit polyclonal Abs against the not species-specific fibronectin (Acris, Herford, Germany), human CD44 (Genetex, Eching, Germany), Sox2 (Abcam) and c-Met (Enzo, Lörrach, Germany) and rabbit monoclonal Ab against human Ki-67 (Abcam) and mouse monoclonal Abs against human Cyt-19 (Abcam), CD24 (P. Altevogt), CD133 (Millipore, Billerica, Massachusetts), k-Ras (Abcam) and EpCAM (G. Moldenhauer). Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, 1 µg / mL). Goat anti-rabbit Alexa Fluor 488 IgG, and goat anti-mouse Alexa Fluor 594 (Invitrogen, Camarillo, CA, USA) were used as secondary Abs. Images of representative fields were captured using a SPOT^{TM} FLEX 15.2 64 Mp shifting pixel digital colour camera (Diagnostic, Instruments, Inc. USA) and analysed with SPOT Basic/Advanced 4.6 software.

5.10. Statistical analysis

The significance of data was analysed using Student's *t*-test, chi-quadrat-test, Fisher-Exact-Test and Mann-Whitney test. $P < 0.05$ was deemed to be statistically significant.

6. RESULTS

6.1. Establishment of the cultivation of pancreatic cell-line-derived tumors on the CAM

The establishment of the cultivation of pancreatic cell-line-derived tumors on the CAM was performed as described [74,92] with few modifications. To gain access to the extraembryonic circulation the eggs were opened on day 4 of embryonic development. At that time point the formation of the extraembryonic vascular network is already visible, as well as the heart of the growing chick, which allows the identification of viable embryos. On the 5th day of embryonic development, the CAM attaches to the inner eggshell membrane[93,94] and opening of the egg without rupturing this structure is no longer possible. The eggs were washed with warm (40 ± 5° C) 70 % ethanol and placed on a six well plate in a horizontal position. 2.5 × 5 cm of Leukosilk® tape (BSN medical, Hamburg, Germany) were attached to the eggshell, covering the middle part and the rounded pole of the egg (Fig. 1A). A hole, circa 1 mm in diameter, was created at the round end of the egg (which is the area where the air sac is located underneath the eggshell) by knocking and gently drilling the eggshell with delicate curved scissors (Fig. 1B). Next, 3 – 4 mL of albumen were aspirated with a 5 mL syringe and an 18G × 1 ½ needle. To avoid injury to the embryo, the needle should be directed downwards (Fig. 1C). This step results in detaching and sinking of the embryonic and extraembryonic egg contents. Needles and syringes were changed regularly to prevent infections. Afterwards, a hole was created in the middle part of the egg, followed by enlarging the hole with scissors to a diameter of circa 1.5 cm × 2.5 cm in the taped area, to avoid cracks in the eggshell (Fig. 1D). Viable embryos were identified by clear blood vessels and a beating heart (Fig. 1E). 1 – 2 mL of the previously removed albumen were re-injected into the egg and the window was sealed with tape. The egg was placed back into the incubator and kept in a horizontal position (Fig. 1F).

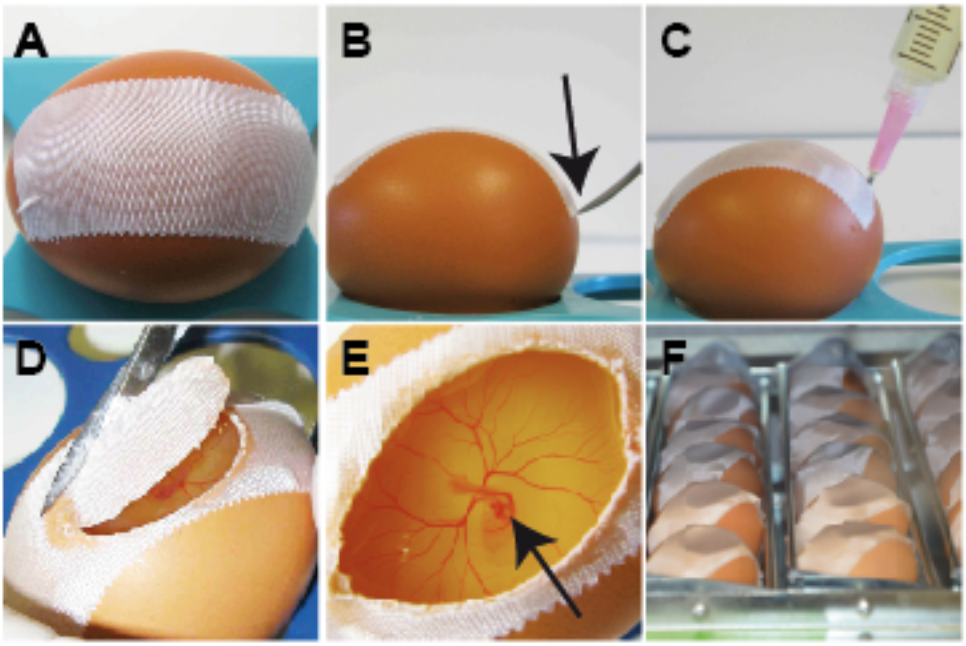


Fig. 1. ***Opening of eggs on day four of embryonic development.*** To gain access to the extraembryonic vascular system fertilized chicken eggs were opened at day four of incubation. Representative photographs in which the procedure is demonstrated, are shown. **(A)** Leukosilk® tape is attached to the eggshell. **(B)** The puncturing of the egg is performed in the middle of the rounded pole, precisely in the area of the air sac. The correct position of the scissors is demonstrated (**arrow**) **(C)** 3 – 4 mL of albumen are aspirated, which creates a false air sac between the egg contents and the shell. The needle should point to the bottom of the egg and away from the embryo. (**D**) A window in the eggshell is created with delicate curved scissors. (**E**) After removal of the eggshell, viable embryos can be recognized by a beating heart (**arrow**). **(F)** The eggs continue the incubation in horizontal position.

At day 9 of embryonic development pancreatic tumor cells from the established cell lines BxPc-3, AsPC-1 and MIA-PaCa2 were transplanted to the CAM of 235, 30 and 23 eggs, respectively. Hand-made rings from Thermanox™ cover slips (Thermo Scientific, Waltham, USA) were placed on the CAM and the epithelial layer of the membrane was gently lacerated (Fig. 2 A – C). Afterwards 5×10^5 pancreatic tumor cells (the cell number was calculated using the Neubauer chamber) in DMEM mixed with Matrigel™ Basement Membrane Matrix (Becton Dickinson, Heidelberg, Germany) in 1 : 1 ratio to a total volume of 25 µL were dropped into the rings (Fig. 3D). At the end the eggs were sealed with tape and placed back into the incubator.

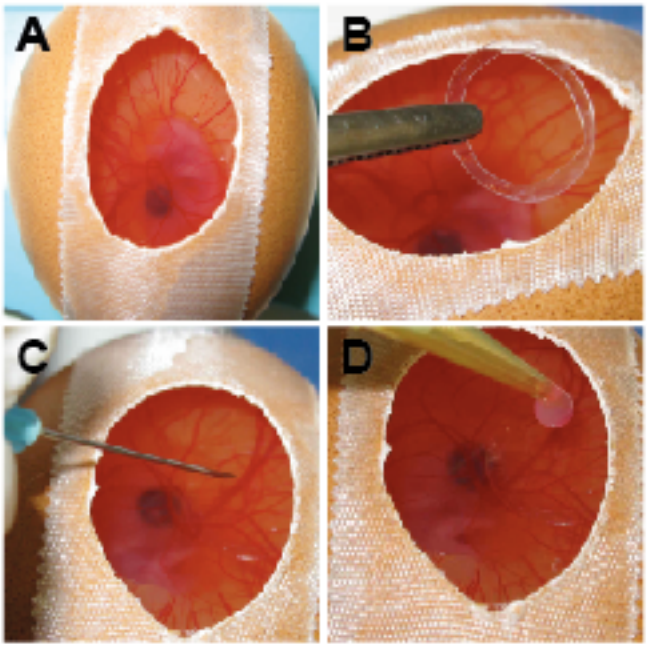


Fig. 2. ***Transplantation of pancreatic tumor cells from established cell lines to the CAM.*** At day 9 of embryonic development BxPc-3, AsPC-1 and MIA-PaCa2 cells were transplanted to the CAM. Representative photographs, in which the procedure is demonstrated, are shown. **(A)** Egg day 9 of embryonic development. **(B)** Hand-made rings from Thermanox™ cover discs are placed on the CAM. **(C)** The epithelial layer of the membrane is lacerated with a 27G × ¾ needle. **(D)** The cell suspension is applied into the ring.

Cells from all employed cell lines formed solid 3-dimensional tumors on the CAM (Fig. 3A and B). On day 18 of embryonic development the tumor take was determined by the following formula: N1 × 100/N2 (N1= number of eggs with tumor, N2 = number of eggs containing living embryos). Thereafter the tumors were resected using delicate curved scissors and tissue forceps and photographed. The size was measured with a caliper and the volume was calculated using the following formula: Volume = 4/3 × p × r^3 (r = 1/2 × (square root of diameter 1 × diameter 2)).[68,74] The tumor take was positively correlated with the aggressiveness of the cell line. Transplanted cells from the very aggressive CSC-high cell line MIA-PaCa2 formed tumors in 96 % of the eggs. The tumor take of the less aggressive CSC-medium cell line AsPC-1 was 78 % and that of the CSC-low cell line BxPc-3 only 70 % (Fig. 4A). Likewise, the mean tumor volume of MIA-PaCa2-derived grafts was 191 mm^3, whereas BxPc-3 and AsPC-1 formed tumors with an average volume of 82.2 and 21 mm^3, respectively (Fig. 4B).

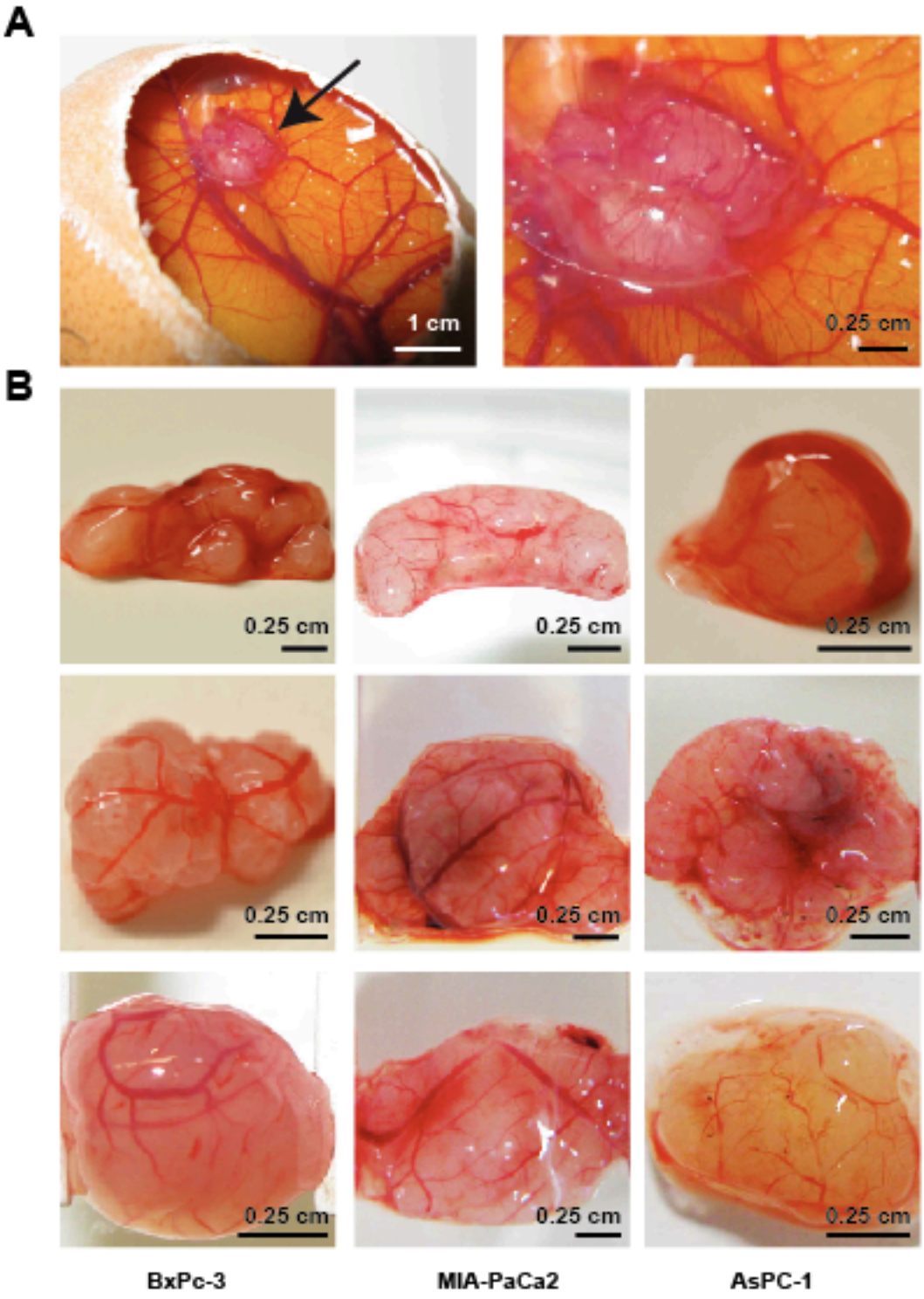


Fig. 3. ***Tumor cells from established cell lines form solid tumors on the CAM.*** BxPc-3, AsPC-1 and MIA-PaCa2 cells were transplanted to the CAM (5×10^5 cells per egg). At day 18 of embryonic development the thereof formed tumors were resected and photographed. Representative photographs are shown. (**A**) Egg with a BxPc-3 tumor (**arrow**). The bars indicate 1 cm and 0.25 cm, respectively. (**B**) BxPc-3, MIA-PaCa2 and AsPC-1 tumors after resection. The bars indicate 0.25 cm.

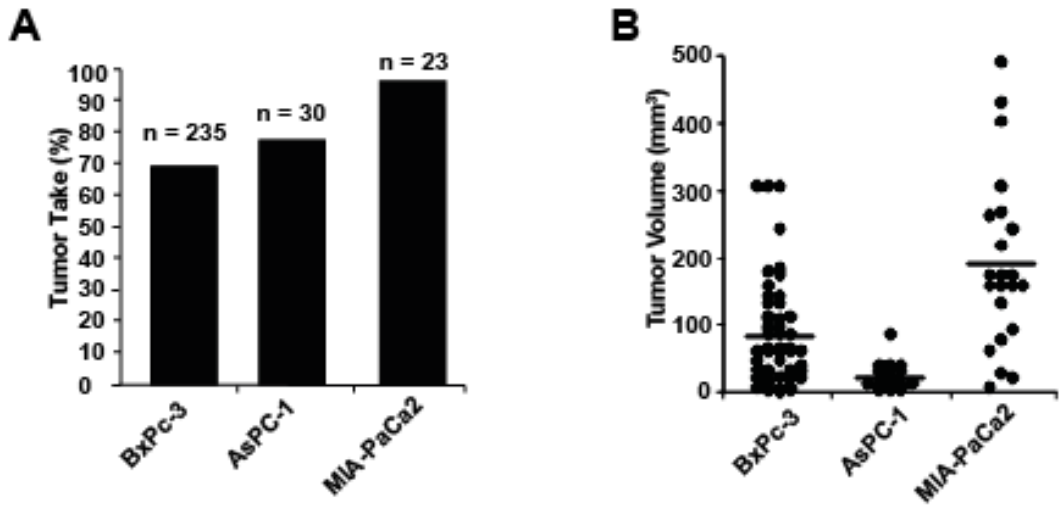


Fig. 4. ***Tumor take and tumor volume of cell-line-derived tumors correlate with the aggressiveness of the cell line.*** BxPc-3, AsPC-1 and MIA-PaCa2 cells were transplanted to the CAM of 235, 30 and 23 eggs, respectively. 9 days later the tumor take was determined by the following formula: N1 × 100/N2 (N1= number of eggs with tumor, N2 = number of eggs containing living embryos). Thereafter the tumors were resected followed by measurement of size with calipers and calculation of volume using the following formula: Volume = 4/3 × p × r^3 (r = 1/2 × (square root of diameter 1 × diameter 2)). (**A**) Diagram in which the mean tumor take rates of each cell line are presented. (**B**) The tumor volumes of cell-line derived tumors are shown. The size of each tumor is presented as a black dot. If no tumor grew, the volume was set to 0. The bars indicate the means of the tumor volumes.

6.2. Development of a method for the cultivation of pancreatic patient-derived tumors on the CAM

To create a pancreatic cancer model, which is closer to the patient, a method for the transplantation of patient-derived tumor tissue to the CAM was developed. The procedure, which finally led to successful engraftments, is described in the following. Freshly resected surgical non-diagnostic specimens of pancreatic origin were transported in 5 mL Oncostore medium (Oncoscience AG, Wedel, Germany) on ice to the laboratory, where they have been processed in sterile conditions. After photo documentation, the volumes were determined by calipers, and necrotic tissues were removed. One quarter of each sample was embedded in Tissue Tek O.C.T. Compound (Sakura, Zoeterwoude, The Netherlands) and stored on dry ice for immunohistochemical analysis. The residual tissue was mechanically minced with sterile scissors and 0.5 mL Oncostore medium was added. After sedimentation of the tissue pieces (ca. 1 min), the supernatant was transferred to another tube. This procedure was repeated 3 times followed by centrifugation. Thereafter, Oncostore medium, mixed with Matrigel™ Basement Membrane Matrix (Becton Dickinson, Heidelberg, Germany) at a ratio of 1 : 1, was added to the pellet. At day 9 – 12 of

embryonic development, 50 µL of the tumor supernatant mixture were transplanted to eggs analogical to the procedure for established cell lines (see section 6.1 and Fig. 2 for details). Tissue pieces with an average size of 200 mm^3 were transplanted onto 6 eggs.

6.3. Patient-derived pancreatic cancer xenografts form solid, neovascularized tumors on the CAM

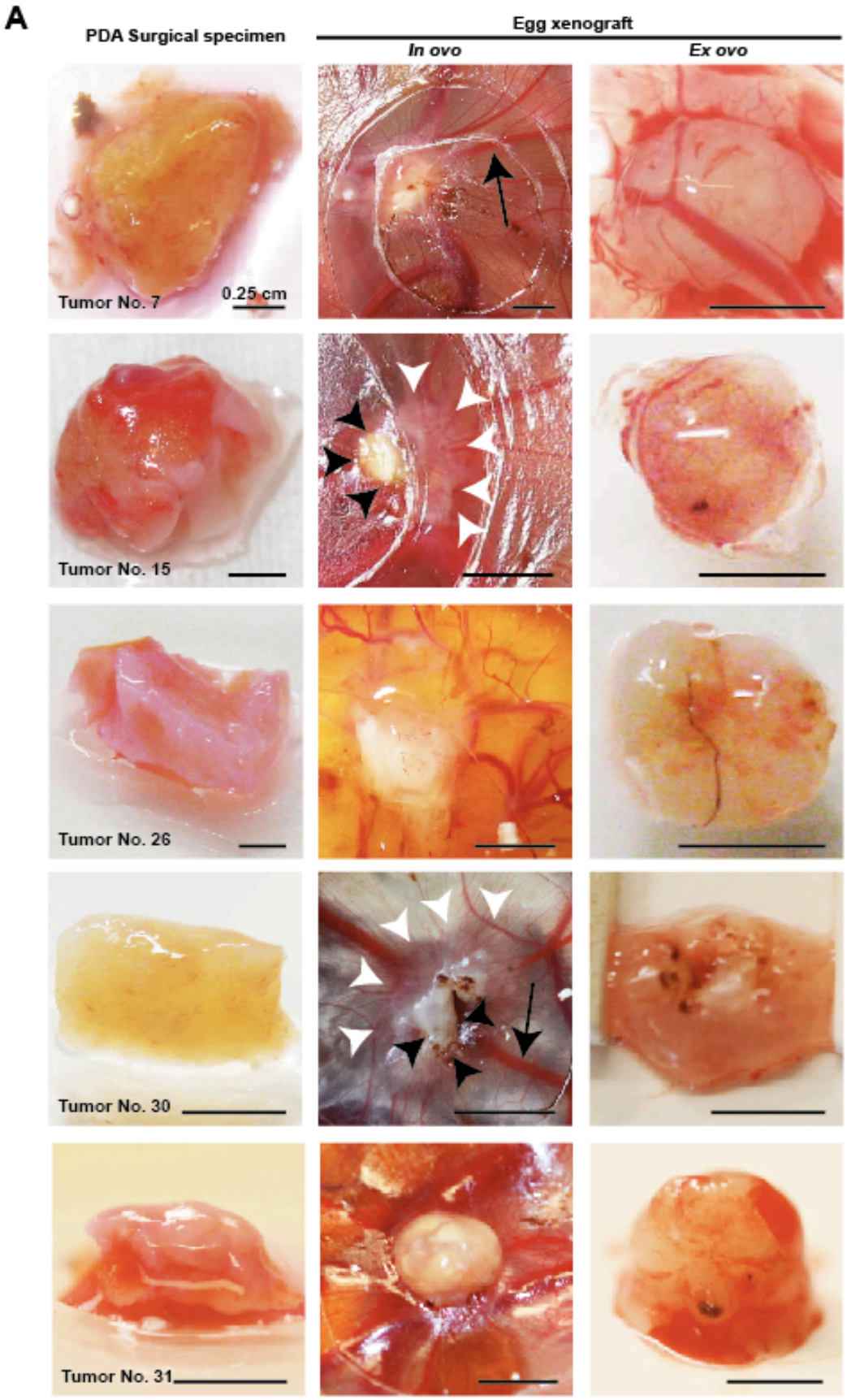
Forty-two freshly resected malignant and benign pancreatic neoplasms of exocrine and endocrine type (Table 1) were transplanted to fertilized chicken eggs as described in section 6.2. 52.4 % of all transplanted specimens formed solid 3-dimensional tumors on the CAM. The latency of tumor growth (which is the time until a tumor nodule became first visible after transplantation) was 2 to 4 days. The grafts showed a homologous growth pattern, independent from their pathological classification as exemplified for xenografts from PDAs and NETs (Fig. 5A and B). They penetrated all layers of the CAM and continued growing on both sides of the CAM: on the bottom, inside the allantoic sac, as well as on its upper side. In most cases the strongest tumor growth was evident on the underside of the CAM. Macroscopically, the parts of the neoplastic nodules located close to the CAM, as well as those growing on its bottom, appeared pale-pink, shining, regular-shaped and had a fleshy consistence. In contrast, the parts growing on the upper side and especially in the middle of the tumor, appeared pale-white, dry, irregular-shaped, had a gelatinous consistence and frequently contained small black areas in the centre, which we interpreted as necrotic changes in these tumor regions. Unlike cell monolayers, solid neoplasms show a 3-dimensional growth pattern and central parts are often found to be necrotic, due to the nutrient deficiency and hypoxia in this region.[95] At day 2 after seeding, a vascular reaction started to be macroscopically visible on the CAM. New branches of vessels were recruited and continued growing towards the xenograft and penetrating it, mostly in the areas close to the CAM and on its bottom. This supports the theory of the heterogeneous nutrient accessibility inside the tumor. Tumor volumes were determined after resection by the formula: Volume = $4/3 \times p \times r^3$ ($r = 1/2 \times$ (square root of diameter 1 $\times$ diameter 2)). Patient-derived egg xenografts were on average smaller than those from established cell lines. The mean tumor size was 34 mm^3 without remarkable deviations among the various tumor types.

Table 1. Patient characteristics - donors of pancreatic cancer tissue, which was transplanted to eggs.

No.	Gender	Age at Resection	Tumor Type	TNM Stage	UICC Stage	Engraftment	Latency (d)
1	f	52	PDA	T3, N1 (11/24), G3, M0, R1	2b	✔	4
2	m	72	PDA	T3, N0, G2, M0, R1	2a	✔	4
3	f	37	PDA	Metastasis, M1, R2	4	✔	2
4	f	73	PDA	T1, N0, G2, M0, R0	1a	✗	
5	f	65	PDA	T3, N1 (3/27), G4, M0, R1	2b	✔	4
6	f	58	IPMN	-		✗	
7	m	69	PDA	T3, N1 (10/20), G3, M0, R0	2b	✔	2
8	m	62	IPMC	TiS, N0, M0, R0	0	✔	3
9	f	60	PDA	T3, N1 (12/49), G3, M0	2b	✗	
10	f	74	PDA	T3, N0, G2, M0, R0	2a	✔	3
11	m	75	PDA	T2, N0, G3, M0	1b	✔	4
12	m	64	IPMN	-	0	✗	
13	m	57	PDA	T3, N0, R1	2a	✔	4
14	f	73	IPMN	-	-	✗	
15	m	67	PDA	M1, R2	4	✔	2
16	f	24	Cystadenoma	-	-	✗	
17	m	76	PDA	T3, N1, G3, M0, R1	2b	✔	3
18	m	77	Cystadenoma	-	-	✗	
19	f	62	Cystadenoma	-	-	✗	
20	m	57	NET	T3, N1 (2/12), G2, M1, R0	4	✔	2
21	m	73	PDA	T4, N1 (3/24), G4, R1	3	✗	
22	m	81	IPMC	TiS, N0	0	✗	
23	f	65	IPMN	-	-	✗	
24	f	73	Cystadenoma	-	-	✗	
25	m	59	PDA	T3, N1 (4/20), G2	2b	✗	
26	m	76	PDA	T3, N1 (3/20), G2	2b	✔	2
27	f	62	IPMC	TiS, N0	0	✗	
28	f	73	PDA	T3, N1	2b	✗	

				(14/42), G2, R1			
29	m	32	NET	T3, N1 (8/21), G2, R0	2b	✗	
30	m	60	PDA	T3, N1 (6/89), G3, R1	2b	✔	3
31	f	82	PDA	T3, N1 (5/15), G3, R1	2b	✔	3
32	f	57	Cystadenoma	-	-	✔	3
33	f	65	IPMN	-	-	✔	3
34	m	71	PDA	T3, N0, G2, R0	2a	✔	2
35	f	66	PDA	T3, N1 (9/24), G3, R1	2b	✔	3
36	m	85	PDA	T3, N0, G4, R0	2a	✗	
37	f	64	NET	T3, N1 (1/8), G2, R0	2b	✔	2
38	m	61	PDA	T3, N1 (1/10), G3, R1	2b	✔	4
39	f	72	Cystadenoma	-	-	✔	4
40	f	66	Cystadenoma	-	-	✗	
41	m	46	Cystadenoma	-	-	✗	
42	m	65	PDA	TiS, N0, R0	0	✗	

f: female; **m:** male; **PDA:** Pancreatic ductal adenocarcinoma; **NET:** Neuroendocrine tumor; **IPMN:** Intraductal papillary mucinous neoplasia; **IPMC**: Intraductal papillary mucinous carcinoma; **Tis:** Carcinoma in situ; **T1:** Tumor limited to the pancreas, 2 cm or less in greatest dimension; **T2:** Tumor limited to the pancreas, more than 2 cm in greatest dimension; **T3:** Tumor extends beyond pancreas; **T4:** Tumor involves coeliac axis or superior mesenteric artery; **N0:** No regional lymph-node metastasis; **N1:** Regional lymph-node metastasis; **M0:** No distant metastasis; **M1:** Distant metastasis; **G1:** Well-differentiated; **G2:** Moderately differentiated; **G3:** Poorly differentiated; **UICC:** Union for international cancer control; ✔: Engraftment; ✗: No engraftment.


A
PDA Surgical specimen
Egg xenograft
In ovo
Ex ovo
Tumor No. 7
0.25 cm
Tumor No. 15
Tumor No. 26
Tumor No. 30
Tumor No. 31

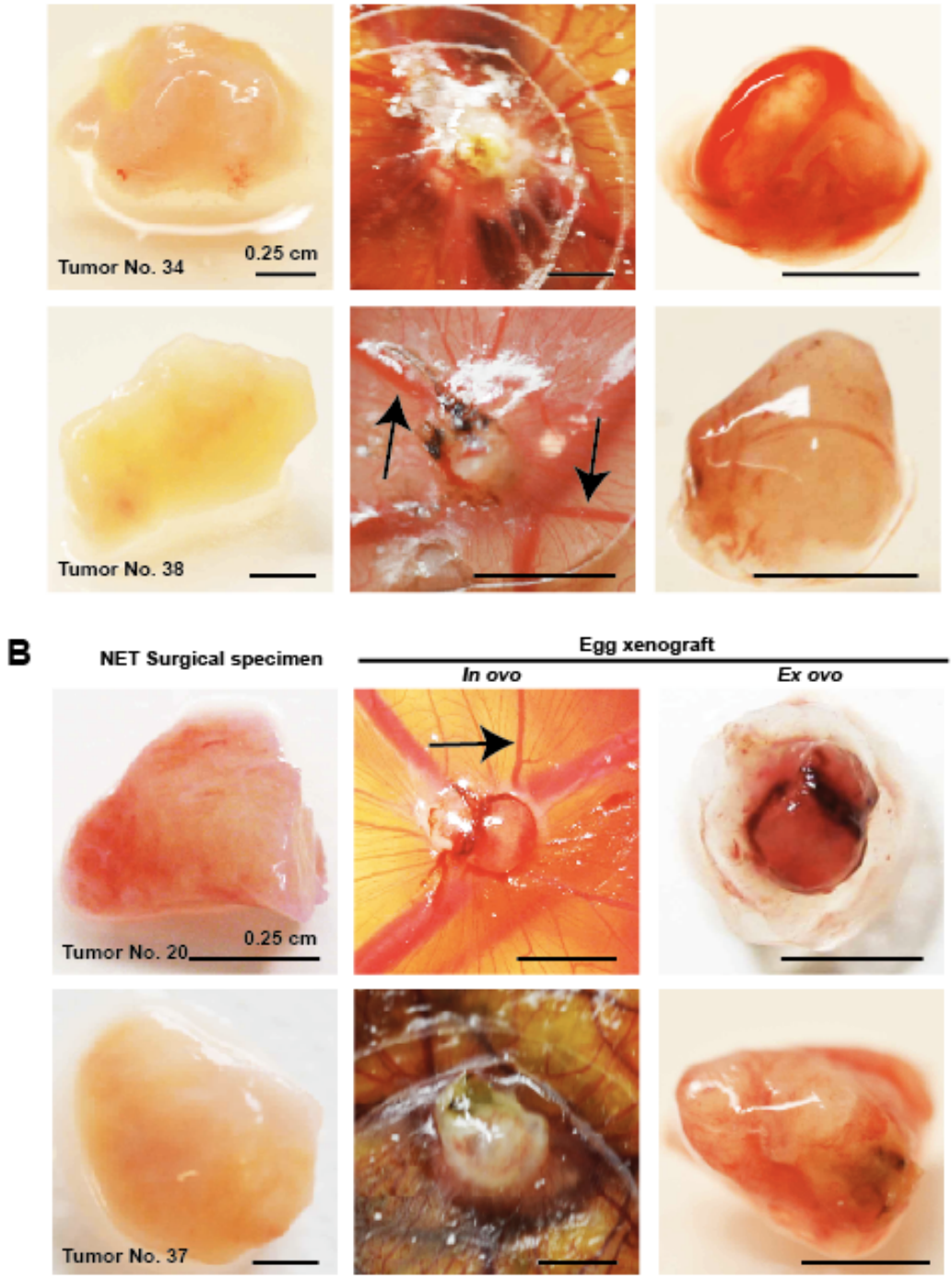


Fig. 5. ***Patient-derived pancreatic cancer xenografts form solid tumors on the CAM.*** Surgical specimens from patients with pancreatic neoplasms were transplanted to the CAM of fertilized chicken eggs. Photographs of patient material and the egg xenografts on day 18 of embryonic development are presented. **(A)** PDA and **(B)** NET surgical specimens (**left column**), their corresponding egg xenografts *in ovo* (**middle column**) and after resection (**right column**). Note the pale less vascularized parts of the xenografts on the upper side of the CAM (**black arrowheads**). The majority of the viable well-vascularized tumor parts are growing on the bottom of the CAM (**white arrowheads**) and are visible after resection. New branches are formed from chick vessels and continue growing towards the human graft (**arrows**). The bars indicate 0.25 cm.

6.4. The grafting efficiency of patient-derived pancreatic cancer egg xenografts correlates with the aggressiveness of the original lesions

Surgical specimens of the original lesions, which were transplanted to the CAM were examined by a clinical pathologist and the tumor type was determined. Additionally, the malignant tumors were classified by tumor stage according to the UICC guidelines for classification of malignant pancreatic cancer tumors.[91] Among the transplanted tissues, 29 tumors were malignant including 23 PDAs, 3 NETs and 3 IPMCs. Thirteen tumors were benign including 5 IPMNs and 8 cystadenoma tumors (Table 1). The transplantation of malignant tumors was successful in 66 % of all cases, whereas benign tumors had a significantly lower grafting efficiency of 23 % (Fig. 6A). The grafting efficiency of PDA tumors was 70 % (n = 23), that of NET tumors was 67 % (n = 3) and that of IPMC tumors was 33 % (n = 3). IPMN and cystadenoma tumors were successfully transplanted in 20 % (n = 5) and 25 % (n = 8) of all cases, respectively (Fig. 6B). Furthermore, the grafting efficiency of malignant tumors positively correlated with the anatomic disease extent (stage) of the original lesion. Transplantation of stage 4 lesions resulted in a 100 % xenograft formation, followed by stage 2a with 80 % and stage 2b with 73 % (Fig. 6C). In contrast, carcinomas in situ (stage 0) and stage 1 lesions had a grafting efficiency of only 33 %. The only available and transplanted stage 3 tumor did not form a xenograft on eggs.

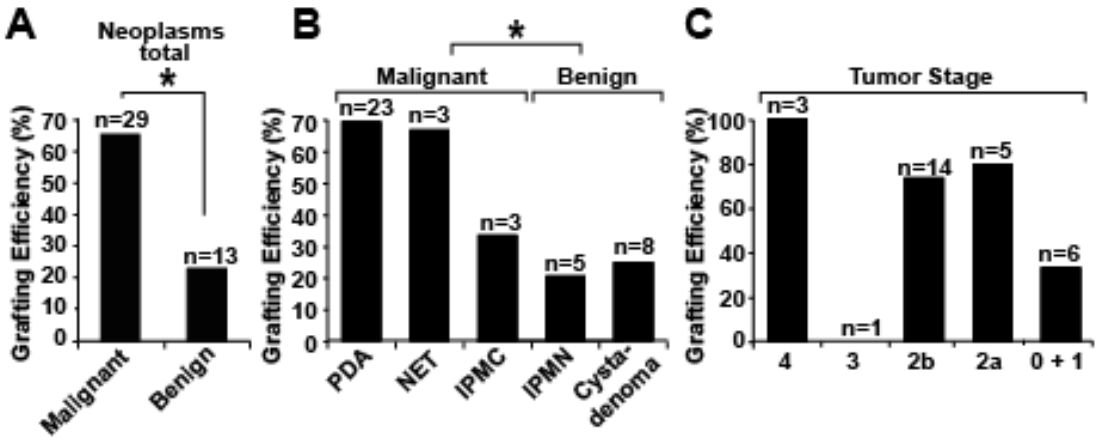


Fig. 6. ***The grafting efficiency of patient-derived egg xenografts correlates with the aggressiveness of the original lesion.*** Forty-two surgical pancreatic cancer specimens were transplanted to the CAM. The patient material was examined by a clinical pathologist and the tumor type was determined. The malignant neoplasms included 23 PDA, 3 NET and 3 IPMC tumors. 5 IPMN tumors and 5 cystadenoma tumors listed among the benign lesions. Furthermore, the tumor stage (anatomic disease extent) according to the UICC guidelines for classification of malignant pancreatic cancer tumors was determined. 3 tumors were classified as stage 4 cancer, 1 as stage 3, 14 as stage 2b, 5 as stage 2a and 6 as stage 1 or 0 (carcinoma in situ). On day 18 of embryonic development the success of the

transplantation was evaluated. The grafting was identified as being successful in the case when a 3-dimensional, vascularized tumor was formed on the CAM and further if this neoplasm resembled the histological features of the original tumor and was positive for human markers, which was evaluated with histological stainings (see figure 7 and 8 for details). The grafting efficiency was calculated by the following formula: (N1 × 100／N2 (N1 = number of patient tumors of a particular tumor type, which formed at least one xenograft on eggs, N2 = total number of patient tumors of this type, which were transplanted to eggs). Tumors from eggs containing dead embryos were excluded from further evaluation. (**A)** Diagram shows the grafting efficiency of malignant and benign neoplasms. **(B)** Grafting efficiency of different tumor types is shown. **(C)** The grafting efficiency of malignant neoplasms in regard to the tumor stage is presented. *$p < 0.05$.

6.5. The morphology and marker expression of primary patient tumors is retained in the egg xenografts

To determine whether the morphology is preserved after passaging on the CAM, H&E-staining from 8 primary patient tissues and their corresponding egg xenografts was performed. 4 high-grade, poorly differentiated PDAs (No. 30, 1, 31 and 17) and 4 low-grade, moderately differentiated tumors from a PDA (34), 2 NETs (37, 20) and a benign IPMN (33) were used (Fig. 7A and B). In PDAs, ductal structures – more or less chaotically organized – among clusters of atypical cells with large bizarre nuclei, embedded in dense tumor stroma, occurred in both the primary tumors and the derived egg xenografts. In NETs, the original histological characteristics (islet-like structures embedded in dense stroma) were transferred to the tumor copies on eggs, where small, infiltrating chick cells contribute to the tumor stroma. The benign IPMN 33 appears as atrophy of the acinar parenchyma with islets of Langerhans and this morphology appears in both, the primary tumor and its derived egg xenograft. Moreover, vessels filled with nucleated avian erythrocytes were detected (Fig. 7C).

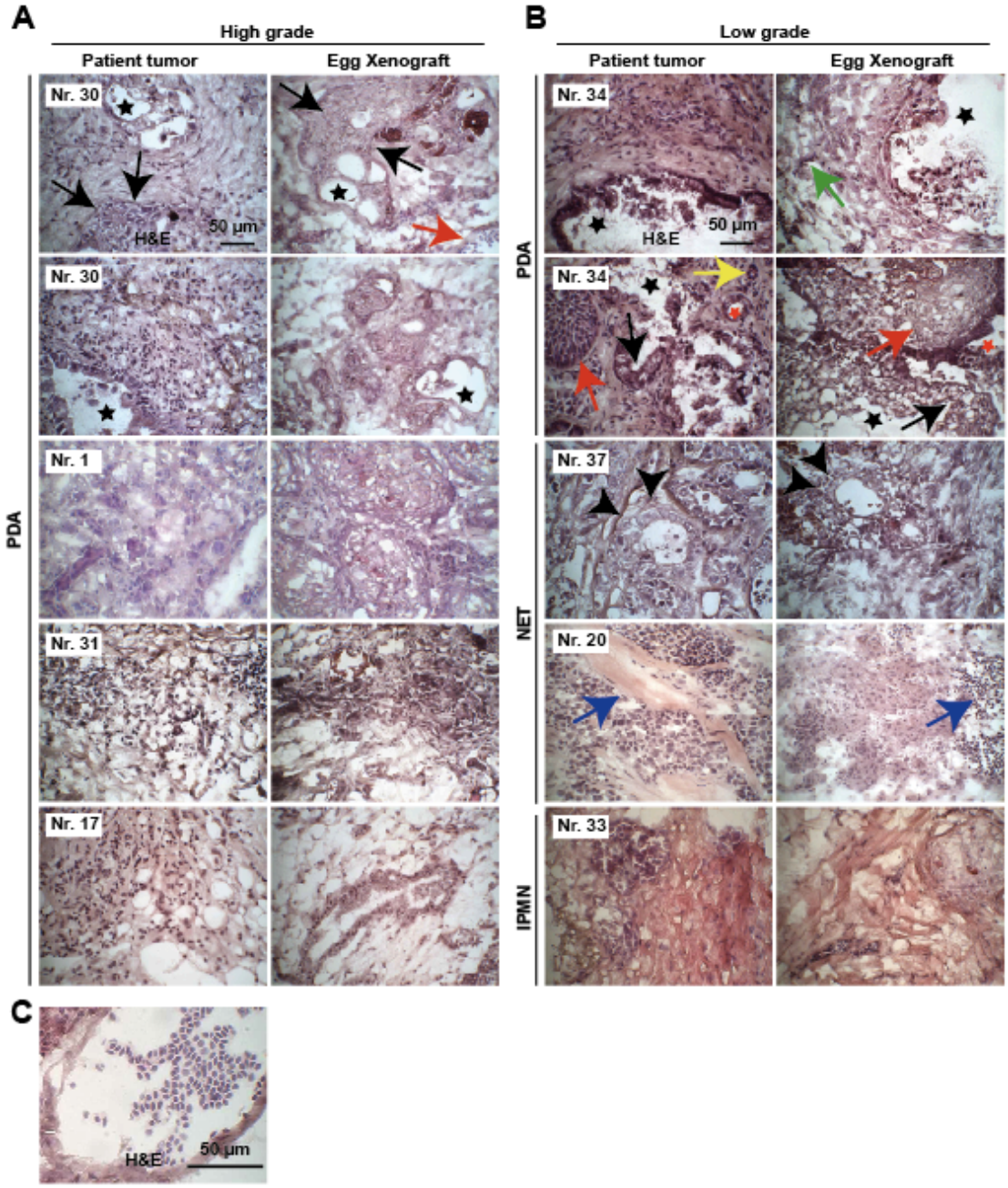


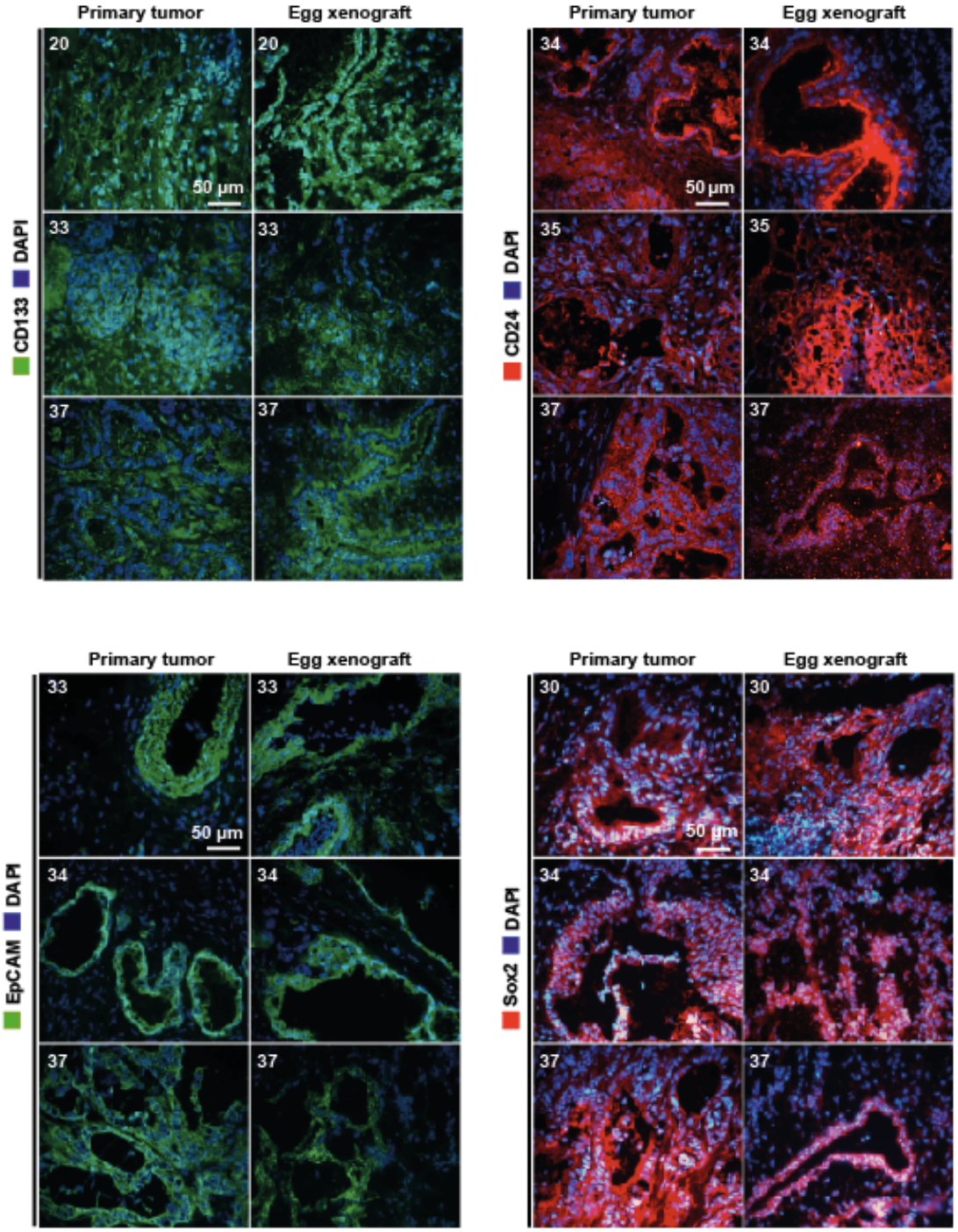
Fig. 7. ***The morphology of primary patient tumors is retained in the derived egg xenografts.*** **(A)** H&E staining of frozen sections from 4 poorly differentiated PDA Grade 3 patient tumors (**left column**) and their corresponding egg xenografts (**right column**). PDA 30: Irregularly shaped duct-like neoplastic structures (**asterisks**) adjacent to undifferentiated clusters of atypical cells with large bizarre nuclei (**black arrows**) are visible in the original lesions and the derived egg xenograft. Note the presence of nucleated avian erythrocytes in the blood vessel of the egg xenograft (**red arrow**). **(B)** H&E staining of frozen sections of moderately differentiated patient tumors derived from one PDA Grade 2, two NETs and one IPMN (**left column**) and their corresponding egg xenografts (**right column**). PDA 34: Large duct-like structures (**black asterisks**) next to smaller neoplastic glands (**red asterisks**) and tumor cell nests (**red arrows**) embedded in dense stroma. Ducts are lined by cells with clear cytoplasm, which differ in size and have a frequently condensed hyperchromatic nucleus (**black arrows**). Note the smaller chicken cells in the stroma of the egg xenograft (**green arrow**). In patient material remnants of acini and normal ducts are present (**yellow arrow**). NET 37: Islet-like structures (**black arrowheads**). NET 20: Dense stroma, which is formed in the egg xenograft by small, infiltrating chick cells (**blue arrows**). (**C**) H&E staining of a frozen egg xenograft section containing an avian blood vessel is presented. Magnification ×400. The bars indicate 50 µm.

To examine whether also the expression of progression markers is preserved in egg xenografts, 8 primary patient tissues and their corresponding egg xenografts were analysed by immunostaining and fluorescence microscopy for the expression of markers for CSCs CD133,[96] CD24,[97] EpCAM,[97] Sox2,[98] c-Met,[99,100] CD44[97] the extracellular matrix protein fibronectin[101] and the proto-oncogene k-Ras.[102,103] The analysis revealed a huge variety among the different tissues (Table 2), but the expression profiles of individual primary tumor tissues reflected those of the derived egg xenografts, with only few exceptions, as shown for representative examples (Fig. 8). In egg xenografts, the tumor cells were accompanied by small cells, which were negative for human markers, indicating that these are infiltrating chick cells, which form the tumor stroma.

Table 2. Expression of progression markers in primary pancreatic cancer patient tissue and egg xenografts.

No.	Tumor Type	Marker Expression															
		Fibronect.		k-Ras		Sox2		c-Met		CD24		CD44		CD133		EpCAM	
		Tu	Xg	Tu	Xg	Tu	Xg	Tu	Xg	Tu	Xg	Tu	Xg	Tu	Xg	Tu	Xg
20	NET	✔	✔	✗	✗	✔	✔	✗	✔	✔	✗	✗	✗	✔	✔	✗	✗
30	PDA	✔	✔	✔	✔	✔	✔	✔	✔	✔	✗	✔	✔	✔	✔	✔	✔
31	PDA	✗	✗	✔	✔	✗	✗	✔	✔	✗	✗	✔	✔	✗	✗	±	✗
32	Cystadenoma	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔
33	IPMN	✔	✔	✔	✔	✗	✗	±	✔	✔	✔	✔	✔	✔	✔	✔	✔
34	PDA	✗	✗	✗	✗	✔	✔	✔	✔	✔	✔	✔	✔	✗	✗	✔	✔
35	PDA	✗	✗	✔	✔	✗	✗	✔	✔	✔	✔	✔	✔	✗	✗	✔	±
37	NET	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔	✔

PDA: Pancreatic ductal adenocarcinoma; **NET:** Neuroendocrine tumor; **IPMN:** Intraductal papillary mucinous neoplasia; **Tu**: Primary patient tumor; **Xg**: Patient-tumor-derived egg xenograft; **✓:** Expression; ✗: No expression; ±: Unclear expression/weak signal; **Fibronect**.: Fibronectin


Primary tumor
Egg xenograft
20
20
50 µm
33
33
37
37
CD133 DAPI
Primary tumor
Egg xenograft
34
34
50 µm
35
35
37
37
CD24 DAPI
Primary tumor
Egg xenograft
33
33
50 µm
34
34
37
37
EpCAM DAPI
Primary tumor
Egg xenograft
30
30
50 µm
34
34
37
37
Sox2 DAPI

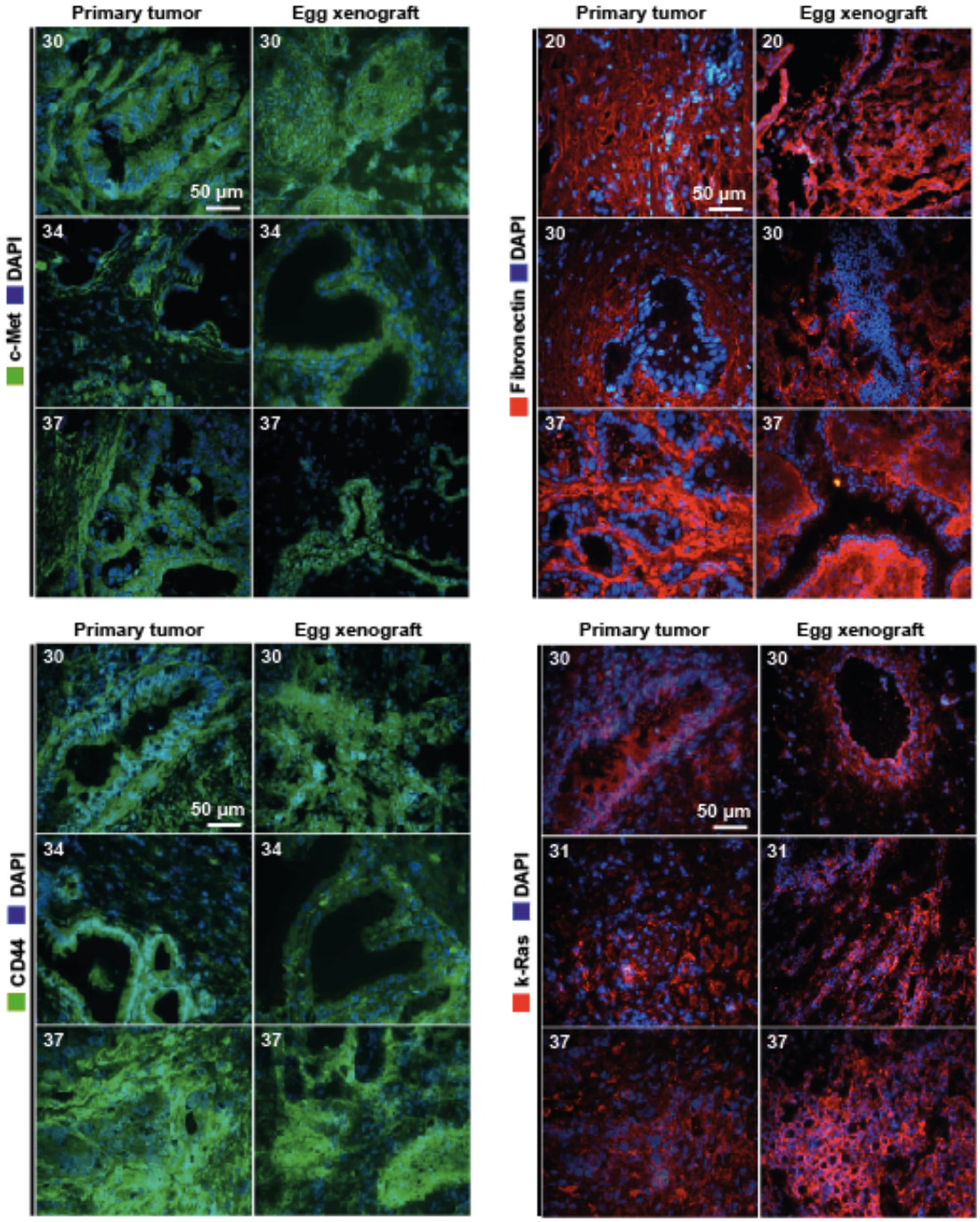
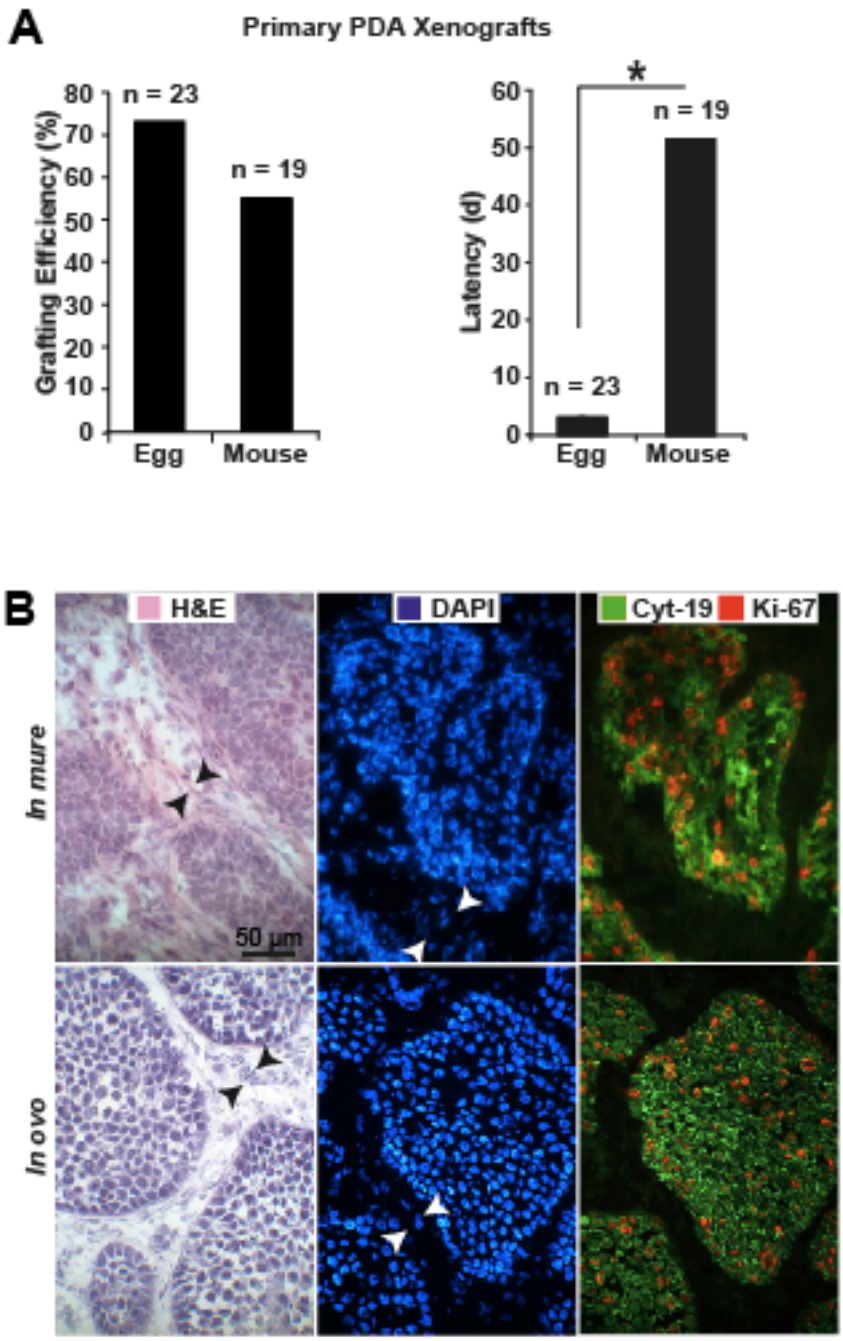


Fig. 8. ***The expression of progression markers in primary patient tumors remains stable in the egg xenografts.*** Representative immunofluorescence stainings of frozen tumor sections of original pancreatic patient tumors (20, 30, 31, 33, 34, 35 and 37) and their corresponding egg xenografts. The tissues were stained with markers for CSCs CD133, CD24, EpCAM, Sox2, c-Met, CD44 the extracellular matrix protein fibronectin and the proto-oncogene k-Ras. Positive cells appear green and red, respectively. The nuclei were counterstained with DAPI (blue). Magnification ×400. The bars indicate 50 µm.

6.6. Pancreatic ductal adenocarcinoma engrafts faster and with a higher percentage in eggs than in mice with comparable morphology

In another study,[104] which was performed by Dr. Sabrina Labsch in the same laboratory, PDA tumors derived from the same surgical clinic were transplanted to mice. Our data obtained from transplantation of PDAs to the CAM were compared to the data of Sabrina Labsch, which were obtained from transplantation of PDAs to mice. All data obtained from mouse experiments were derived and analysed by Dr. Sabrina Labsch and are presented here with her kind permission. Nineteen PDA tumors were transplanted subcutaneously into the flanks of immunodeficient 6-week-old BALBc (nu/nu) female mice. This resulted in a grafting efficiency of 52 %, and thus was lower compared to the egg model, in which 23 PDA tumors were transplanted and a grafting efficiency of 70 % was received. The mean latency of egg xenografts was 3 days, whereas tumors in mice were first palpable on average after 51 days (Fig. 9A). Thus, the avian model is superior to the mouse xenograft model regarding grafting efficiency and latency. To further compare mouse and egg xenografts, the human established PDA cell line BxPc-3 was transplanted onto eggs and mice. H&E staining of frozen xenograft sections demonstrated a similar morphology as indicated by large clusters of tumor cells and a sparse tumor stroma. Furthermore, subsequent staining with the human-specific markers Cyt-19[105] and Ki-67[106] revealed that islets of human tumor cells are surrounded by stroma of chick or mouse origin (Fig. 9B). Finally, the morphology of the primary PDA patient tissues 1, SL22 and SL29 and their corresponding egg and mouse xenografts was compared. The PDA tumor 1 was transplanted as freshly resected tissue from a patient directly into eggs and mice. The tumors SL22 and SL29 were transplanted into mice first and after serial passages in mice transplanted to eggs. Here as well a similar morphology in H&E staining was detected (Fig. 9C). These data suggest that primary PDA tumors engraft better and grow faster on eggs than on mice with similar morphology and a pronounced tumor stroma, which is formed by host cells.


A
Primary PDA Xenografts
Grafting Efficiency (%)
80
70
60
50
40
30
20
10
0
n = 23
n = 19
Egg
Mouse
Latency (d)
60
50
40
30
20
10
0
*
n = 19
n = 23
Egg
Mouse
B
H&E
DAPI
Cyt-19
Ki-67
In mure
In ovo
50 µm

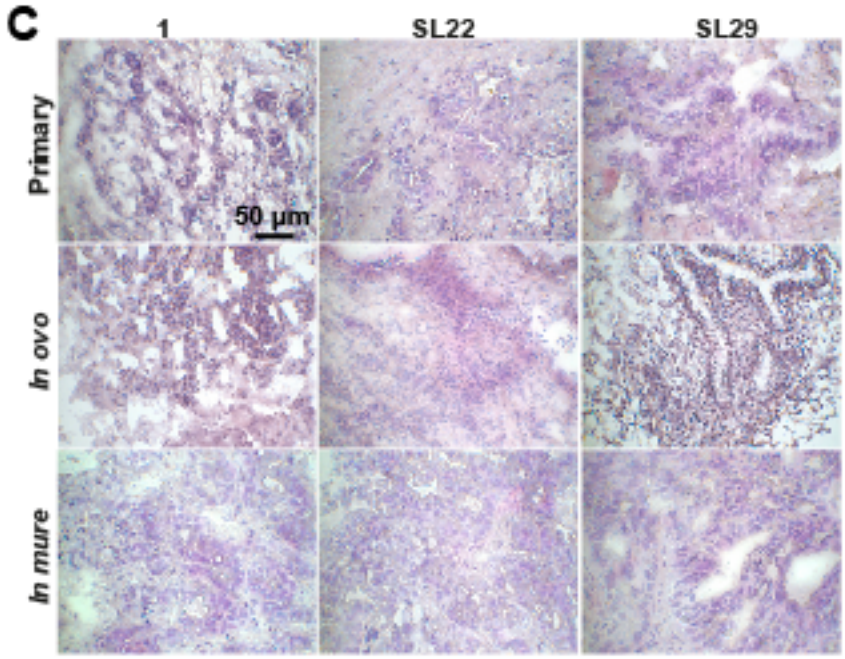


Fig. 9. ***PDA engrafts faster and with a higher percentage in eggs than in mice with comparable morphology.*** **(A)** Freshly resected PDA surgical specimens were transplanted onto eggs (n = 23) and subcutaneously into the flanks of immunodeficient 6-week-old BALBc (nu/nu) female mice (n = 19), which resulted in successful tumor grafting of 70 % within 3 days in eggs but only 52 % within 51 days in mice. The latency was defined as the days (d) until a first xenograft growth could be detected and the mean values are presented. *p< 0.05. **(B)** BxPc-3 cells were transplanted onto eggs and mice and frozen tumor xenograft sections were stained with H&E, DAPI, and double stained with the human markers Cyt-19 (green) and Ki-67 (red). Note the small murine/avian cells in the tumor stroma of H&E-stained sections (**black arrowheads**), which are stained with DAPI (**white arrowheads**), but are negative for the human markers. **(C)** H&E stainings of frozen tissue sections from primary PDA patient tumors (1, SL22 and SL29) and their corresponding xenografts in eggs and mice. Magnification ×400. The bars indicate 50 µm.
All mouse experiments and their analysis were performed by Dr. Sabrina Labsch. All stainings of mouse-derived tumor tissue, as well as the primary and egg-derived tumor SL29 and their photo documentation were performed by Jury Gladkich. These data are presented here with their kind permission.

6.7. Establishment of the egg model as a system for the evaluation of cancer drugs

To upgrade the CAM to a model in which potentially cancer targeting substances can be tested, the effect of gemcitabine and dexamethasone on tumors grown on the CAM was evaluated. Gemcitabine is at the moment the standard drug for the chemotherapy of pancreatic cancer[23] and glucocorticoids (such as dexamethasone) are suspected to promote the progress of diverse cancer types.[107] The pancreatic cancer cell lines BxPc-3, MIA-PaCa2 and AsPC-1 were transplanted to 407 fertilized chicken eggs, either untreated (control group), with previous gemcitabine treatment, dexamethasone treatment or combined dexamethasone and gemcitabine treatment. The effect of the

treatment was evaluated by the tumor take rate, the volume of developed tumors and by the expression of the proliferation marker Ki-67, determined by immunohistochemistry of frozen tumor sections. Tumor take rates and tumor volumes were reduced in gemcitabine treated groups compared to the controls (Fig. 10A). The strongest therapeutic effect was evident in the least aggressive, CSC-low cell line BxPc-3. Here a 19-times lower tumor take and a 6-times reduced tumor volume was detected in the gemcitabine group than in the control. The more malignant CSC-medium cell line AsPC-1 and CSC-high cell line MIA-PaCa2 were less affected by the treatment. In the dexamethasone group the tumor take was significantly higher in AsPC-1 and BxPc-3 cell lines, compared to the control group. In the MIA-PaCa2 cell line the tumor take of the control and the treated group were nearly equal, what is not surprising, due to the fact that this is a very aggressive cell line, thus tumor take in the control group was already nearly 96 % and could hardly be increased. The tumor volume was significantly increased in all tumor types, especially in AsPC-1. Here the xenografts from the glucocorticoid treated group revealed a 10-times higher mean volume than in the control. The combined treatment with dexamethasone and gemcitabine resulted in a higher tumor volume and a higher tumor take compared to the cells, which were treated with gemcitabine alone. Proliferation was significantly increased in the dexamethasone treated tumors, whereas in those treated with gemcitabine the opposite effect was detected. The combined therapy resulted in a marked higher proliferation rate than the sole gemcitabine treatment (Fig. 10B and C).

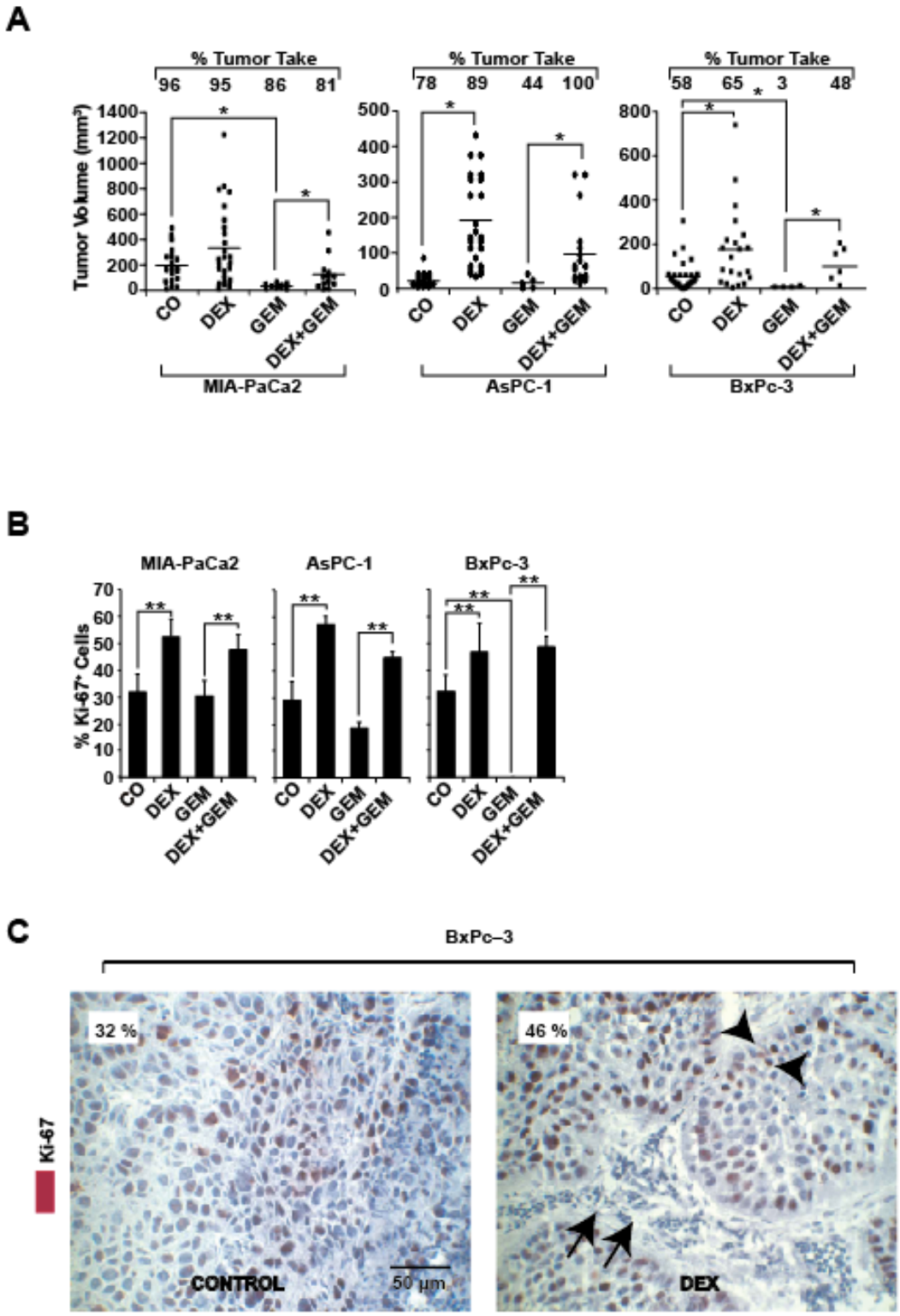


Fig. 10. ***Gemcitabine and dexamethasone treatment of pancreatic cancer cells transplanted to the CAM.*** MIA-PaCa2, AsPC-1 and BxPc-3 cells were transplanted to the CAM of fertilized chicken eggs on day 9 of embryonic development, either untreated (CO), with previous gemcitabine (GEM) treatment, dexamethasone (DEX) treatment or combined dexamethasone and gemcitabine treatment (see the "Materials and methods" section for details). On day 18 the tumors were resected followed by measurement of size with calipers. Furthermore the tumor take was determined as the percentage of grown tumors. In (**A**) the tumor take and the tumor volume in each treatment group are shown. The size of each tumor is presented as a black dot. If no tumor grew, the volume was set to 0. The bars indicate the mean of the tumor volumes. (**B**) The amount of cells positive for the human proliferation

marker Ki-67 was evaluated with immunohistochemistry. The number of positive cells was quantified in ten vision fields under ×400 magnification and the means ± SD are shown. (**C**) Immunohistochemistry staining for the expression of Ki-67 in a frozen BxPc-3 tumor section. Representative photographs of the control and dexamethasone treated group are shown. Positive cells appear dark red. Note that the majority of proliferating cells is located on the outer layer of tumor cell nests (**arrowheads**). The smaller cells of avian origin populate the tumor stroma and are Ki-67 negative (**arrows**). Magnification ×400. The bar indicates 50 μm. *p< 0.05, **p< 0.01.

To prove whether direct *in ovo* treatment matches similar results, BxPc-3 cells were seeded into eggs first, followed by treatment on day 5 and 6 after transplantation by injecting gemcitabine into the growing tumors. Here gemcitabine significantly reduced tumor growth and proliferation compared to the untreated tumors (Fig. 11A and B). Furthermore the intravenous treatment with gemcitabine was tested on a patient-tumor-derived egg xenograft. A primary surgical specimen was transplanted into 3 eggs. On day 6 and 7 after transplantation, one tumor was treated with gemcitabine via intravenous injection into a blood vessel of the CAM. DMEM was administered intravenously into the CAMs hosting the other 2 tumors, which served as the control. No difference in size between control and gemcitabine-treated tumors was detected, which might be due to the slow growth of primary grafts compared to the growth of tumors from established cell lines. However, the staining of tissue sections with Ki-67, followed by the evaluation of the percentage of positive cells, showed that gemcitabine strongly reduced the proliferation to 1 %, compared to 20 % in the control. Interestingly, the percentage of Ki-67-positive cells in the primary patient tumor tissue was 6 %, indicating that xenotransplantation to eggs leads to a faster tumor growth than in the human body (Fig. 11C).

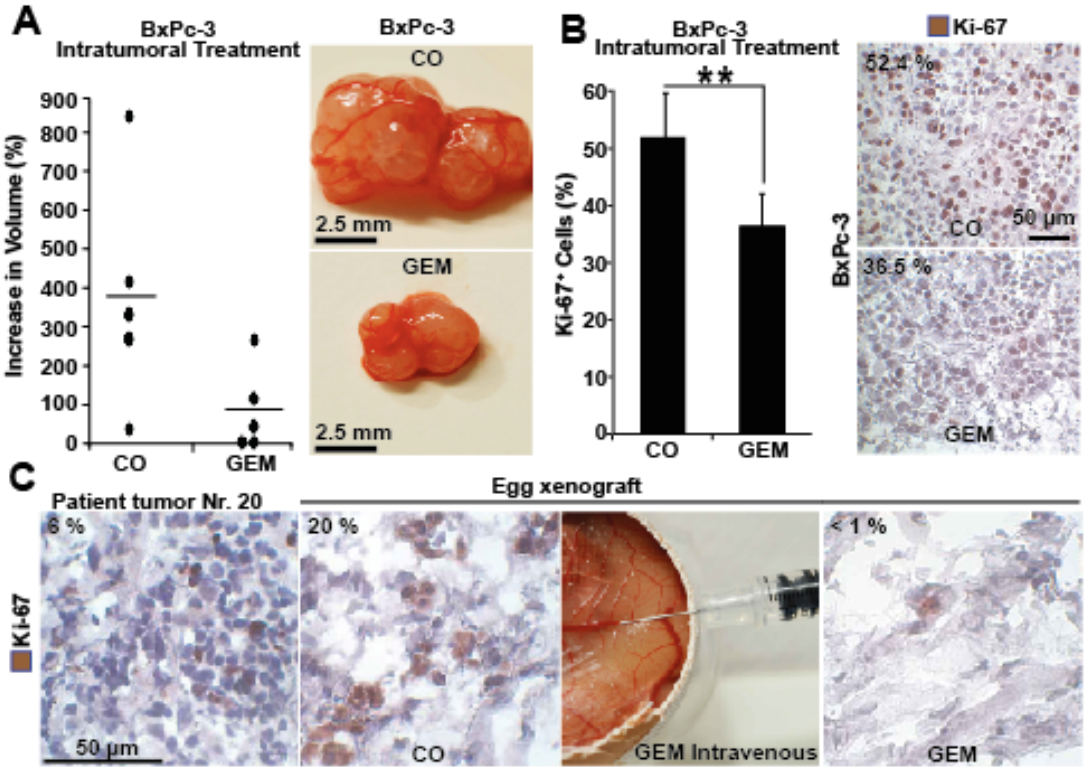


Fig. 11. ***Gemcitabine treatment of tumors in ovo.*** (**A**) BxPc-3 cells were transplanted to the CAM. On day 5 and 6 after transplantation the volume of each tumor was measured *in ovo* and gemcitabine was injected in 3 regions of each tumor. Alternatively, DMEM was injected, which served as a control. 3 days later the tumors were sampled and the percentage increase in volume was calculated using the following formula: (N1 × 100 / N2) – 100 (N1 = tumor volume on the day of excision in mm^3, N2 = tumor volume on day 5 of incubation in mm^3). Each dot represents the increase in volume in % of each tumor and the bars indicate the means of both. Additionally, photographs of representative tumors are presented. The bars indicate 2.5 mm. (**B**) The amount of cells positive for the human proliferation marker Ki-67 was evaluated with immunohistochemistry in frozen tumor sections. The number of positive cells was quantified in ten vision fields under ×400 magnification and the diagram visualizes the means ± SD of each treatment group. The photographs visualize representative immunohistochemistry stainings for the detection of Ki-67 positive cells (dark red). Sections of untreated, as well as gemcitabine treated tumors are shown. Magnification ×400. The bar indicates 50 µm. (**C**) A patient-derived NET of the pancreas was transplanted to the CAM of 3 eggs. On day 6 and 7 after transplantation gemcitabine was injected into a blood vessel of the CAM hosting one xenograft. DMEM was injected intravenously in blood vessels of the CAM on which 2 other grafts were located. 3 days later the tumors were harvested and the amount of proliferating cells in frozen tumor sections of the egg xenograft, as well as of the primary patient tumor was detected by labelling Ki-67 positive cells by immunohistochemistry. Photographs of representative areas of the Ki-67 staining in patient material, one untreated egg xenograft and the gemcitabine treated egg xenograft are shown. The number in the left upper corner of the photographs expresses the mean of positive cells, which was quantified in ten vision fields under ×400 magnification. Positive cells appear dark red. The bar indicates 50 µm. Additionally, the procedure of the intravenous treatment is demonstrated. *$p < 0.05$, **$p < 0.01$.

6.8. Establishment of the cultivation of tumors of the musculosceletal system on the CAM

To create a model in which therapeutic studies on GCTBs can be performed, the transplantation of GCTB stromal cells to the CAM was established (Fig. 12A). The transplantation was carried out analogical to the procedure, which was employed for pancreatic cell lines. However, a higher number of cells is needed for successful tumor growth, namely $1 – 1.5 \times 10^6$ cells per egg. Moreover, the grafting efficiency of GCTBs was under 10 %, which is much lower than that of pancreatic tumors. The H&E staining revealed, that large GCTB tumor cells are accompanied by smaller chicken cells, which form the stroma (Fig. 12B). The positive staining for the human CSC marker c-Met confirmed the human origin, as exemplified for 2 patients (Fig. 12C).

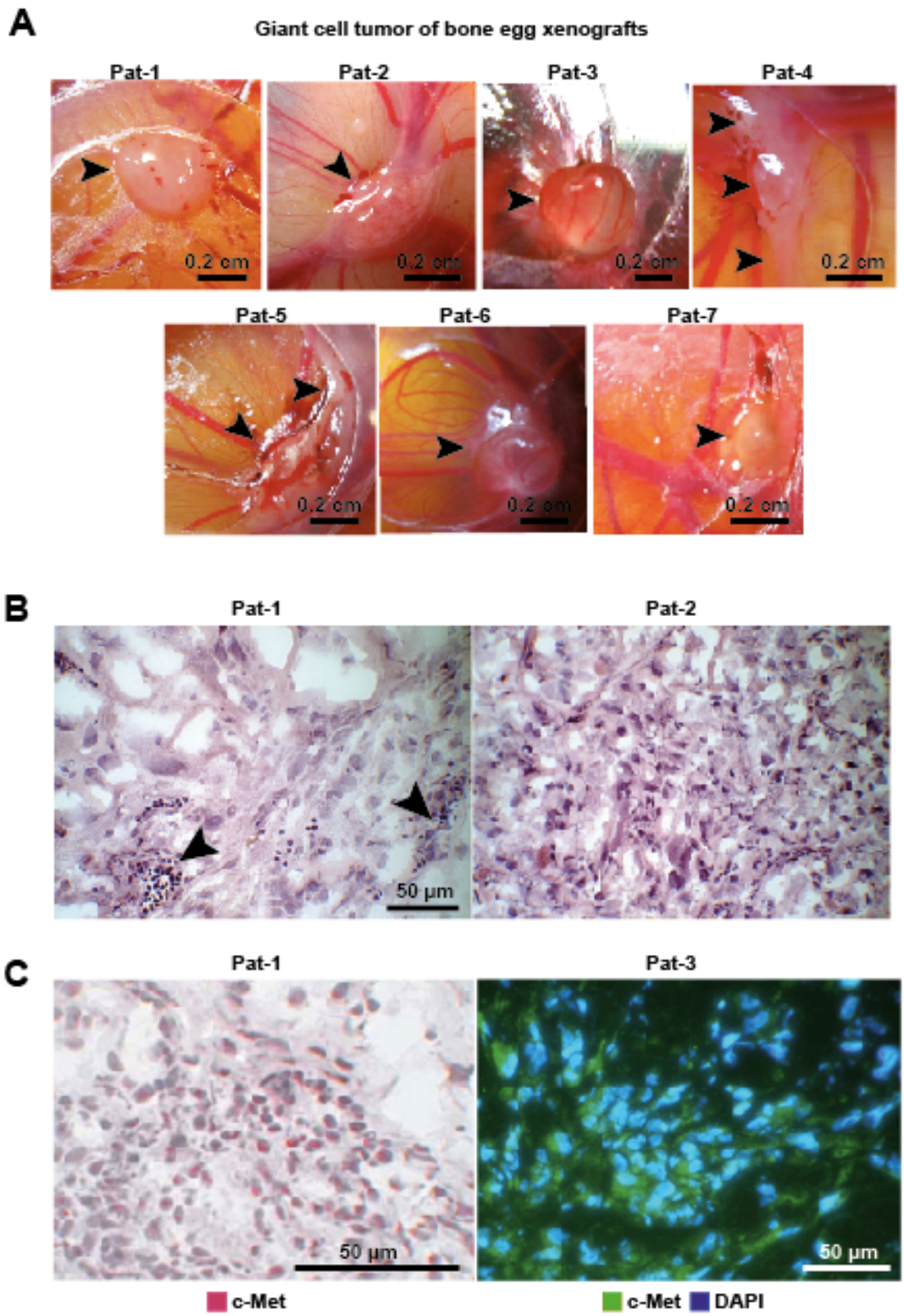


Fig. 12. ***GCTB stromal cells form solid tumors on the CAM.*** (**A**) GCTB stromal cells from 7 patients were transplanted to the CAM of fertilized chicken eggs (1 – 1.5 × 10^6 cells per egg). Between days 16 – 18 of embryonic development the thereof formed tumors (**arrowheads**) were resected and photographed. Representative photographs are shown. The bars indicate 0.2 cm. (**B**) H&E staining of representative frozen xenograft sections derived from patients 1 and 2. Small chicken cells form the tumor stroma (**arrowheads**). (**C**) Staining with c-Met antibody of frozen tumor sections derived from patient 1 and 3. The tissue of patient 1 was stained with immunohistochemistry. Positive cells appear violet. In the tumor of patient 3 c-Met was detected with immunofluorescence (green) and counterstained with DAPI (blue). Magnification ×400. The bars indicate 50 µm.

Furthermore, the cultivation of osteosarcoma tumors on the CAM was established. The osteosarcoma cell lines HOS, MG-63 and Saos-2 were seeded into eggs (1×10^6 cells per egg), which resulted in formation of 3-dimensional tumors (Fig. 13). The tumor take rates were 50, 80 and 60 % for HOS, MG-63 and Saos-2, respectively. However, these results are not statistically significant, due to the limited number of available cells and small group sizes (5 eggs per cell line).

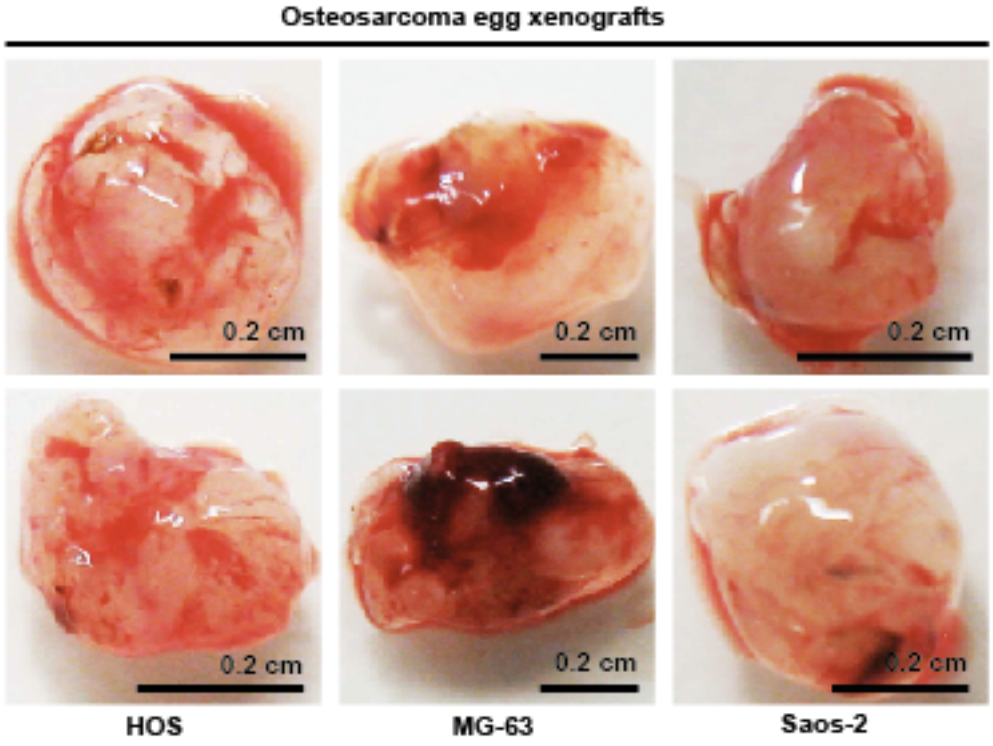


Fig. 13. ***Osteosarcoma cells form solid tumors on the CAM.*** Osteosarcoma cells from the established cell lines HOS, MG-63 and Saos-2 were transplanted to the CAM of fertilized chicken eggs (1×10^5 cells per egg). On day 18 of embryonic development the thereof formed tumors were resected and photographed. Representative photographs are shown. The bars indicate 0.2 cm.

6.9. Cultivation of patient-derived non-Hodgkin`s lymphoma and duodenal carcinoma tumors on the CAM

Although it was not an aim of this study to cultivate other tumors than of pancreatic and mucosceletal origin on the CAM, inadvertently 2 other tumor types were transplanted to the CAM. Those tumors were first classified as PDAs, but the

pathological analysis revealed that one tumor was a non-Hodgkin`s lymphoma and the other a carcinoma of the duodenum. The tumors were transplanted to the CAM freshly after surgical resection from the patient as described for the primary pancreatic cancer xenografts. Both tumors formed 3-dimensional tumors on the CAM. Interestingly, the lymphoma tumor formed multiple nodules on the CAM, which is mirroring the situation in the human body.[108,109] The histological analysis of the xenograft revealed that the tumor cells express the human CSC marker CD133 and that the stroma was positive for the marker for fibronectin. The tumor was also examined for the expression of CD24, EpCAM, Sox2, CD44, c-Met, CD44 and k-Ras, but here the results were negative. The duodenal carcinoma tumor was negative for all the above-mentioned markers, except for CD44 where a weak signal could be detected (Fig. 14 A and B).

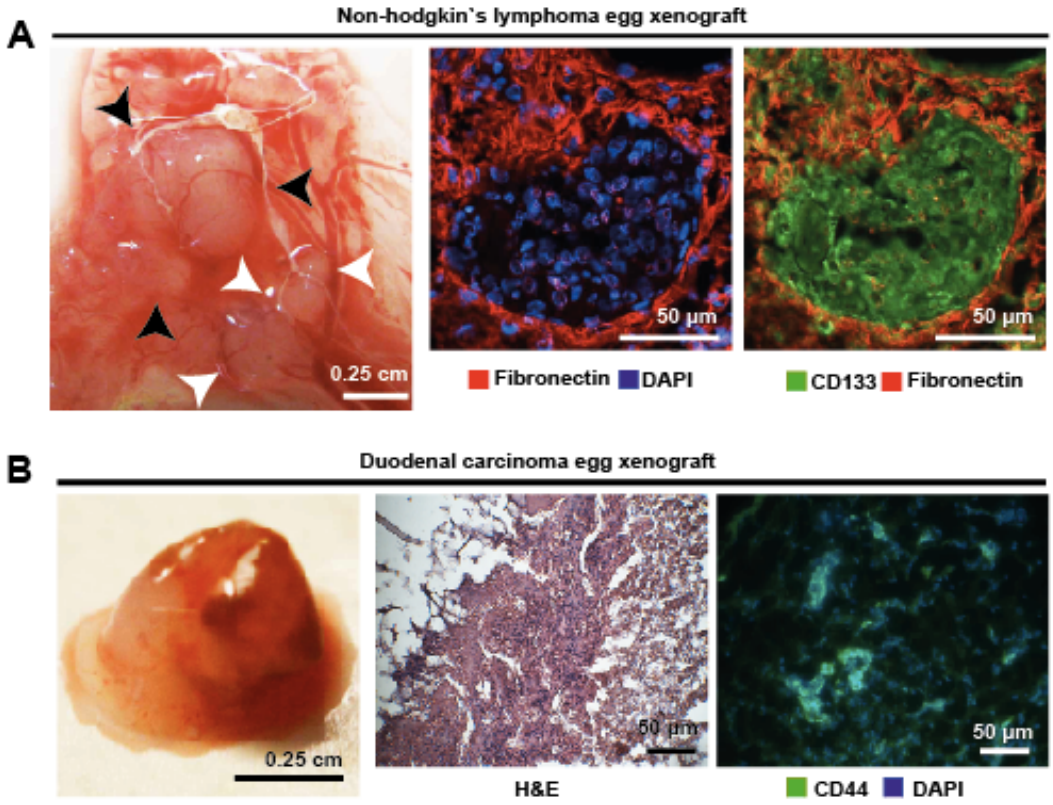


Fig. 14. ***Patient-derived non-Hodgkin's lymphoma and duodenal carcinoma xenografts form solid tumors on the CAM.*** (**A**) A freshly resected surgical non-Hodgkin`s lymphoma specimen was transplanted to the CAM. The transplant formed multiple tumor nodules on the CAM (**left, arrowheads**). The bar indicates 0.25 cm. After resection immunofluorescence staining for fibronectin (red), CD133 (green) and a counterstaining with DAPI (blue) of the frozen tissue was performed. Representative photographs are presented (**middle and right**). Magnification ×400. The bars indicate 50 µm. (**B**) A freshly resected surgical duodenal carcinoma specimen was transplanted to the CAM. 10 days later the egg xenograft was resected and photographed (**left**) the bar indicates 0.25 cm. The frozen tissue was stained with H&E (**middle**) and the presence of the marker CD44 was detected with immunofluorescence staining (green) and DAPI (blue) counterstaining (**right**). Representative photographs are shown. Magnification ×400. The bars indicate 50 µm.

6.10. Development of a method for a short-term anaesthesia and an ethical euthanasia of the chick embryo *in ovo*

According to the AVMA guidelines on euthanasia[79] the intravenous injection of a barbituric acid derivative is the quickest and most reliable means of euthanizing birds. To provide a method for an ethical euthanasia of the chick embryo the effect of the administration of pentobarbital into the extraembryonic vascular system was tested. Between days 11 and 18 of embryonic development Narcoren® (sodium pentobarbital 16 g⁄100 mL, Merial, Hallbergmoos, Germany) was injected into the chorioallantoic vascular system. The anaesthetic effect and the survival time following the drug administration were determined in 112 embryos. Prior to the injection of the narcotic, the window in the eggshell was widened until the point where the CAM meets the inner eggshell membrane. Next, blood vessels suitable for the administration of the barbiturate were identified on the CAM. Chorioallantoic blood vessels can be easily distinguished by colour: light-red vessels are under low pressure and are suitable for drug injection. Dark-red vessels are under high pressure and here the administration of substances is not possible, as this results in extensive bleeding (Fig. 15A). Thereafter, 0.02 – 0.05 mL of Narcoren® were injected into a vessel of the CAM of each egg using a fine dosing 30G × 12 mm Omnican® insulin syringe with an integrated needle (B. Braun, Melsungen, Germany) (Fig. 15B). As dosage recommendations for chick embryos are not available, the dose used is based on our experience. However, it is meant only as an orientation, as pentobarbital should not be given strictly in accordance with dosage recommendations, but in accordance with the responses of the animal.[110] Therefore, the embryo inside the egg should be observed carefully, while the intravenous drug administration is performed. Subsequently after the narcotic was given, voluntary movements of the embryos, as well as the pulsation of dark-red blood vessels were monitored inside the egg (Fig. 15C). All embryos stopped moving inside the egg immediately after the injection of pentobarbital. In almost one third of the eggs the injection of the drug resulted in an immediate interruption of the pulsation of the extraembryonic blood vessels. Consequently, cardiac arrest in those embryos occurred immediately after this procedure. The embryos were observed in the egg for 1 min and thereafter the CAM was lifted quite high to ensure that no parts of the embryo have been caught as well (Fig. 15D) and ruptured using delicate forceps. Finally, the embryo was gently taken out of the egg (while care was taken not to rupture the navel) and placed in lateral position (Fig. 15E). Because the bird might be still fully conscious it should not be positioned in dorsal recumbency, as this impedes the bird's respiratory activity.[76] Occasionally, the intravenous injection is not feasible due to the lack of accessible

vessels on the CAM. In this case the bird was taken out of the egg and immediately euthanized via intracoelomic injection of 0.05 mL Narcoren®. To avoid injections in the air sac and ensure fast absorption of the drug the intracoelomic injection was performed in the abdominal region of the bird (Fig. 15F).

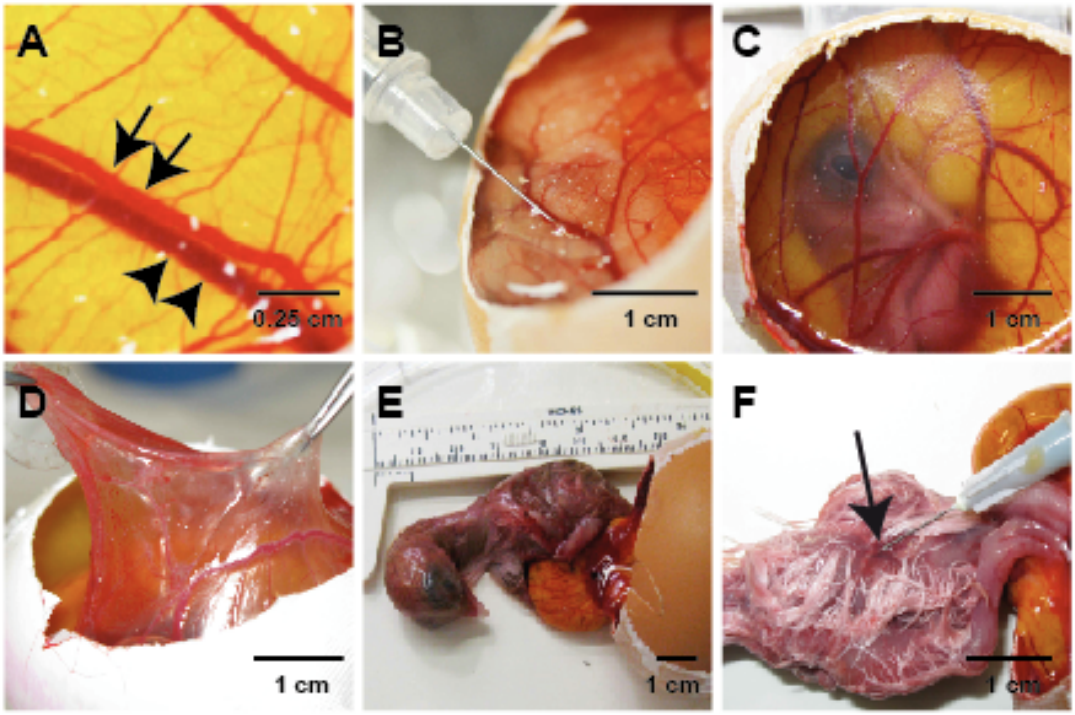


Fig. 15. ***Administration of pentobarbital into the extraembryonic vascular system.*** Narcoren® was administered intravenously into the chorioallantoic vascular network of 112 eggs containing embryos at day 11 – 18 of embryonic development. **(A – E)** Representative photographs, in which the procedure is demonstrated, are shown. **(A)** CAM on day 18 of embryonic development. Light-red blood vessels carry oxygen-rich blood, are under low pressure and are suitable for the injection of liquids (**arrow**). Dark-red blood vessels are under high pressure and are not suitable for drug administration, as this results in heavy bleedings (**arrowheads**). The bar indicates 0.25 cm. (**B**) Pentobarbital is administered intravenously in the vascular system of the CAM using a fine dosing insulin syringe with an integrated needle. The needle should be injected flat and parallel to the CAM. (**C**) After pentobarbital was given, the embryo is monitored inside the egg. In case of successful administration the voluntary movement of the bird will interrupt. (**D**) The CAM is lifted with delicate forceps and ruptured. Care should be taken not to injure the embryo while performing this procedure. (**E**) The embryo is taken out of the egg and placed in lateral recumbency. Dorsal positioning of the embryo is to be avoided, as this impedes the bird's respiratory activity. (**F**) If the intravenous injection is not possible due to the lack of accessible vessels on the CAM, the bird is euthanized via intracoelomic injection of pentobarbital. To ensure rapid absorption of the substance, the injection should be performed in the abdominal region of the animal. The correct position of the needle is demonstrated (**arrow**). (**B – F**) The bars indicate 1 cm.

The depth of anaesthesia, as well as the vitality of the remained embryos were monitored every 5 min until death. The cardiac activity was monitored via palpation of the bird`s chest. The presence or the absence of corneal, palpebral and pedal reflexes, voluntary movements and responses to postural changes served as assessment criteria for the level of anaesthesia. The examination revealed good

muscle relaxation, as well as the absence of the above-mentioned reflexes in all embryos. Among the embryos, which were in 15^{th}, 17^{th} or 18^{th} day of incubation, 40 % survived longer than 5 min, whereas 10 min after drug administration a cardiac arrest could be determined in 93 % of the cases. Seven percent of the embryos survived for 15 min or longer (Fig. 16A). Among the 11 – 14 day old embryos the survival time was shorter. Cardiac arrest was detected in 88 % of all embryos 5 min after the barbiturate was given (Fig 16B). However, one 14-day-old embryo survived for up to 20 min (Table 3).

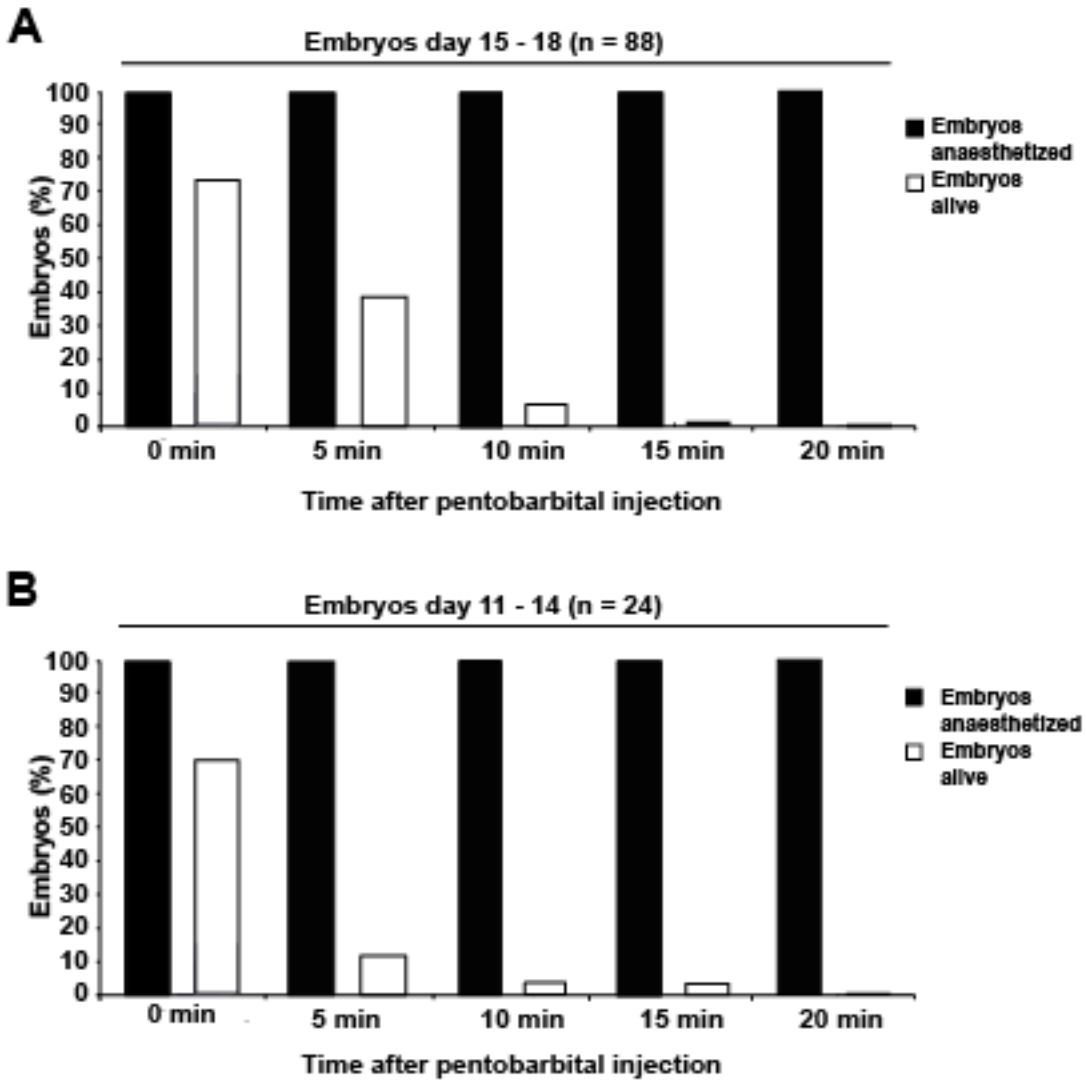


Fig. 16. ***The intravenous in ovo administration of pentobarbital is a suitable method for euthanasia and short-term anaesthesia of the chick embryo.*** Narcoren® was injected into a vein of the CAM in 112 eggs hosting chick embryos in the 15th, 17th or 18th (**A**) and the 11th, 12th, 13th or 14th (**B**) day of embryonic development. Subsequently after the administration of the drug the depth of anaesthesia, as well as the vitality of the embryos were monitored every 5 min until the time of death. The embryos were considered as being anaesthetized in the case of the absence of the corneal, palpebral and pedal reflexes, voluntary movements and responses to postural changes. The bird's cardiac activity was determined via palpation of the chest. The diagrams visualize the percentage of anaesthetized and living embryos at different time points after the drug administration.

Table 3. Survival time of anaesthetized chick embryos after intravenous injection of pentobarbital.

		Number of embryos with cardiac arrest at different time points after pentobarbital administration				
Day of incubation	Embryos total	0 min	5 min	10 min	15 min	20 min
18	77	15	42	71	76	77
17	1	0	1	1	1	1
15	10	8	10	10	10	10
14	9	4	6	8	8	9
13	9	3	9	9	9	9
12	3	0	3	3	3	3
11	3	0	3	3	3	3

7. DISCUSSION

There is a strong need to improve the survival time of patients suffering from pancreatic cancer. For the development of optimal therapeutic options the selection of potential agents in *in vivo* models, which are based on established cell lines, is an essential step. When it comes to the specific treatment strategy for an individual patient, the selection of appropriate drugs in patient-derived personalized tumor models may give more accurate indications. Laboratory models, in which rodents are involved, have been rising ethical concerns, are expensive and time consuming. Furthermore, concerning the short survival time of patients with pancreatic cancer, a limiting step of mouse models is that a primary patient tumor needs several months to form a xenograft. The CAM of the fertilized chicken egg may be the solution to the above-mentioned problems. In the present study we have established the cultivation and treatment of patient- and cell-line-derived pancreatic cancer tumors on the chick CAM. Pancreatic tumor cells from the established cell lines BxPc-3, AsPC-1 and MIA-PaCa2 were transplanted to the CAM of 235, 30 and 23 eggs respectively. Cells from all three cell lines formed solid tumors on the CAM. The tumor take rates, as well as the tumor volumes were positively correlated with the CSC-content of each specific cell line. In general the tumor take rates were high, ranging from 70 % in the CSC-low cell line BxPc-3 to 96 % in the CSC-high cell line MIA-PaCa2, indicating that the CAM model is highly efficient and economical.

In the following step, a method for the transplantation of fresh patient-derived pancreatic tumors to the CAM was developed and eventually established. Forty-two surgically resected malignant and benign pancreatic neoplasms of exocrine, as well as of endocrine type were transplanted to the CAM. The specimens included 23 PDAs, 3 NETs, 5 IMPNs, 3 IPMCs and 8 cystadenomas. 52.4 % of all tumors formed solid tumors on the CAM within 2 to 4 days, accompanied by a vascular reaction. New vessels were recruited from the CAM to vascularize the graft.

I was able to achieve a rate of 70 % viable engraftments for PDA tumors. In another study, performed in the same laboratory, 19 PDA tumors derived from the same surgical clinic were transplanted into mice.[104] The grafting efficiency in this model was 52 %, whereas the mean latency was 51 days. Garrido-Laguna et al. transplanted 69 fresh PDA tumors into mice and 61 % engrafted.[37] These data suggest that the egg model is superior to the mouse model with regard to the grafting efficiency and latency of tumor growth. Also, in terms of practicability the CAM appears superior to the murine model. When performing experiments with mice, trained staff, an animal experiment application and a sterile animal room are required. Moreover, the costs for

an immunodeficient mouse are 50 – 200 €. For experiments with fertilized chicken eggs only a simple egg incubator is necessary and the price of one egg is on average 0.25 €. Furthermore, the CAM model allows unlimited access to the graft throughout the whole duration of the experiment. A disadvantage of the avian model may be the shorter cultivation period (until day 18 of embryonic development), which results in a limited increase in volume of the tumors. However, for personalized therapeutic studies it may be more beneficial to use a model in which tumor formation is fast, than a model, in which the tumors can be maintained longer. Concerning the short expected survival time of pancreatic cancer patients at the time of surgical intervention, it is crucial to perform drug screenings as soon as possible. Besides this, the grafting efficiency of the CAM model was positively correlated with the aggressiveness of the original lesion. Malignant lesions formed tumors more likely than benign. Among the malignant samples high-stage neoplasms showed a higher grafting efficiency than the low-stage tumors. Macroscopically and microscopically the morphology of the egg-derived grafts closely resembled the features of the original lesions and high-grade, low differentiated tumors could be distinguished from low-grade, moderately differentiated tumors. In view of the fact, that the anatomic disease extent (stage) and the histological grade are important determinants for cancer treatment and prognosis,[111] the CAM may be used as a therapeutic and prognostic device in clinical terms. In contrast to tumors from established cell lines, patient-derived tumor cells were surrounded by a pronounced stroma. This fact is of major value, since recent work has revealed that the stroma supports tumor growth and metastasis and serves as a physical barrier to drug delivery, especially in pancreatic cancer.[112-115] Thus, the effectiveness of therapeutic agents can only by truly verified when the tumor microenvironment is simulated as close to reality as possible, even though the stroma is derived from the chick host. Also, vessels filled with nucleated avian erythrocytes were detected. In conclusion, the blood vessels were exclusively of avian origin. According to the classification by Ausprunk, this type of vascular ingrowth is defined as vascularization of tumor type (defined as the penetration of implants by proliferating host blood vessels).[116] In contrast to Sys et al.,[75] I did not detect vascularization of embryonic type (defined as anastomosis between graft vessels and the host circulation). This was to be expected, since no tissue pieces with intact human vessels were transplanted. The expression profiles of CSC, k-Ras and fibronectin markers differed among patients, which shows again their heterogeneity and highlights the need for individual tumor models. It is crucial, however, that the characteristics of the original tumors were retained in the corresponding egg copies. A growing number of studies implicate the critical role of CSCs in the progression process of pancreatic cancer and novel therapeutic strategies aim at targeting these

cells.[117-119] Thus, it is of highest importance that all cellular components are preserved in the egg xenografts.

Furthermore, the presented results suggest that the CAM is a suitable system for the testing of substances, which potentially affect pancreatic cancer. Pre-treatment of cells, as well as direct *in ovo* treatment of PDA tumors with gemcitabine resulted in significant reduction of tumor take, tumor volumes and proliferation. The strongest therapeutic effect was obtained in the CSC-low cell line BxPc-3. The CSC-medium cell line AsPC-1 and the CSC-high cell line MIA-PaCa2 were less affected by the treatment, most probably due to their CSC dependent resistance to chemotherapy. Treatment with dexamethasone led to the opposite effect and so does the combined dexamethasone and gemcitabine treatment. Dexamethasone is suspected to induce resistance to cytotoxic drugs in diverse cancer types,[107] which might explain the effects of the combined treatment. In three recent studies[120-122] on the effect of other substances (such as sulforaphane, TRAIL and triptolide) on pancreatic and prostatic cancer cell lines, my colleagues and me demonstrated that results obtained from the CAM model are confirmed by our *in vitro* and *in vivo* experiments. In view of this, drug screenings in eggs can be performed on a huge scale in a standardized, reproducible fashion. The exploitation of this method may also allow personalized therapeutic prediction for individual patients, as the intravenous treatment of a patient-derived NET resulted in a reduced proliferation. However, further studies are required to prove this strategy.

Additionally, the cultivation of giant cell tumors of bone, derived from 7 cell lines and the cultivation of osteosarcoma tumors, derived from 3 cell lines, was established on the CAM. Also, the accidental growth of a patient-derived non-Hodgkin`s lymphoma and duodenal carcinoma on the CAM was shown. These findings emphasize the high potential of this model as a fully adequate alternative to mammalian models.

In contrast to murine models, the CAM model is not associated with pain to the bird, due to the absence of nerves in the CAM. Nevertheless, the point must also be made, that to address the CAM model as a humane animal replacement method, at the end of the experiment the embryo should be euthanized with a painless technique. According to the AVMA guidelines on euthanasia[79] the intravenous injection of a barbituric acid derivative is the quickest and most reliable means of euthanizing birds. Thus, here we established a fast and simple method for the *in ovo* euthanasia of the chick embryo via administration of pentobarbital into the extraembryonic vascular system. The intravenous administration of pentobarbital resulted in a prompt interruption of motility, loss of muscle tension and reflexes in all embryos. According to Lierz`s and Korbel`s determination of anaesthetic stages in birds [76] the absence of the palpebral,

pedal and corneal reflexes in combination with good muscular relaxation are evidences for deep surgical anaesthetic state. In conclusion, the application of this method resulted in an immediate surgical anaesthetic state in all examined embryos, which was maintained up to the time of death. Interestingly, 40 % of the embryos, which at that time point were in the 15th, 17th or 18th day of embryonic development, were anaesthetized, but alive for longer then 5 min. In view of this, this new technique can also be used for short-term anaesthesia for experimental procedures in the living embryo. Pentobarbital leads to a rapid loss of consciousness in warm-blooded animals,[110,123] is readily available in approved solutions (e.g. Narcoren®) and is less expensive than many other euthanasia agents. One disadvantage may be the bureaucratic burden, since the use of barbiturates is strictly regulated by national legislation in most countries. However, in practice this basically means that the barbiturate has to be stored in a place not accessible for unauthorized persons and the amount used has to be registered in a protocol, which is a manageable effort. The skill of the intravenous administration of drugs into the CAM requires some training. However, based on my experience, learning this technique requires only one day of practice. From day 9 of development onwards, the chorioallantoic vessels are large enough for intravenous administration of substances, which in trained hands takes no more than a minute per egg. In rare cases, the intravenous injection was not feasible, due to the lack of exposed vessels on the CAM. These birds were euthanized via intracoelomic injection only. Prior to drug administration these embryos showed active voluntary movements particularly of the beak and caudal extremities. This could be observed for up to 5 min after the intracoelomic injection of pentobarbital. Consequently, the intravenous route is the preferred option and should be performed whenever possible. If pentobarbital is not available, the use of other injectable anaesthetic agents may be considered, such as a combination of ketamine and an alpha-2-agonist medetomidine or xylazine. It must be noted that none of the above-mentioned drugs is to be used as a monoanaesthetic agent, due to the insufficient analgesic potency in birds.[76,124] Based on my experience, in an average 18 day 10 g chick embryo the combination of 2 mg ketamine and 0.2 mg xylazine applied into the breast muscle leads to a good anaesthesia and a rapid death within a few minutes. For longer survival times and/or intravenous administration routes the dose should be carefully adjusted based on the effect on the animal. While dosage recommendations for unhatched birds do not exist, it is always to be taken into account that young and small animals need relatively higher doses compared to adult ones. However, these alternatives to pentobarbital are not authorized for euthanasia, are more expensive and the necessity of mixing two substances prior to the application is less practical. Finally, the point must be made, that the positioning of the embryo after removal from

the egg is an important issue. As the intravenous injection is not always successful the embryo might be still fully conscious and choking is to be avoided. Based on my experience, the ability of chick embryos to breathe is controversially discussed. However, according to Burton and Tullet (1985), regular respiratory movements of the avian embryo can be detected two to three days before hatching. Under natural conditions, lung respiration is initiated when the beak penetrates the inner shell membrane at day 19 of incubation and the lungs fill with gas from the air space. The lungs then increasingly take over the function of gaseous exchange whilst the contribution from the chorioallantois decreases.[125] It is not described in the literature how the gas exchange works when the incubation of chick embryos is interrupted in an unnatural way, as described in this study. But according to my observations, the chick embryos do perform respiratory movements when they are taken out of the egg on day 18 and also at earlier stages. As described by Lierz and Korbel the cycle of inspiration and expiration is a procedure closely bound to muscular activity, which is difficult when the bird is placed in dorsal position[76] Thus, dorsal positioning of the embryo may result in choking of the embryo, which is contradictory to animal welfare.

Below the line, there is no doubt that my method implies some administrative and time-consuming burdens and to skip the anaesthesia is the less complicated way. Still, to characterise experiments with avian embryos as alternatives to animal models, the minimization of animal pain and distress should be of higher value than the minimization of human effort. The intravenous administration of anaesthetics into the CAM is a feasible method for euthanasia and short-term anaesthesia of the avian embryo, which still maintains the balance between the ideals in medical ethics and the every day reality in the laboratories.

Summing up, the CAM is a highly effective pancreatic cancer model, which allows the cultivation and treatment of patient- and cell-line-derived tumors. In regard to efficiency and practicability the CAM is superior to mouse models. If the here presented method for euthanasia is applied, it can be characterized as an appropriate alternative to mammalian experiments.

8. CONCLUSIONS

The CAM is an adequate system for the cultivation of patient- and cell line-derived pancreatic cancer xenografts. The exploitation of this experimental tool may speed up preclinical data collection and support progress towards personalized medicine. Moreover, the reasonable use of this model allows a considerable reduction and/or replacement of animal experiments in accordance with Russell's and Burch's principles of humane experimental technique.[51]

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11. DECLARATION BY THE CANDIDATE

I hereby declare that this thesis is my own work and effort.
Where other sources of information have been used,
they have been indicated or acknowledged.

12. APPENDIX

Publications of Ewa Aleksandrowicz

1 **Aleksandrowicz, E**. and Herr, I.
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