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Tumor grafts derived from sarcoma patients retain tumor morphology, viability, and invasion potential and indicate disease outcomes in the chick chorioallantoic membrane model

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ABSTRACT

The chick chorioallantoic membrane (CAM) assay was used to evaluate whether xenotransplanted sarcomas retain the histological characteristics and functional behavior of the original tumors. Metabolically active tumor tissue, identified by dynamic-contrast MRI, from 28 patients with a bone or soft-tissue tumors was applied to the CAM. Angiogenesis and graft and host behaviors were evaluated. The essential features and immunohistochemical characteristics of the original tumors were maintained, illustrating the diversity of sarcomas. Graft viability was inversely related to patient survival, but longer follow-up and more patients are needed to relate tumor graft behavior to natural history. We conclude that the CAM assay is a potential prognostic and predictive preclinical xenograft model for tumors that are difficult to culture in vitro, such as sarcomas; therefore, the use of the CAM assay may facilitate personalized medicine.

1. Introduction

Tumors of the musculoskeletal system are very rare and diverse. According to the WHO classification, there are more than 100 different tumor types. For clinical studies on the treatment of musculoskeletal tumors, international collaboration is required to collect sufficient data to yield significant results in statistical analyses. Such studies take several years, and therefore several years pass before treatment strategies can be altered. Laboratory experiments are crucial to speed up data collection, but the culturing of sarcomas can be difficult. In addition, there is a poor correlation between the behavior of single cell lines in vitro and tumors encountered in patients. Currently, prognostic markers are being studied extensively, but due to the heterogeneity of sarcomas even within the same tumor type, the clinical applicability of these markers is limited.

In this article, we describe the development of the chick chorioallantoic membrane (CAM) assay as an in vivo model for studying fresh tumor samples from sarcoma patients in a standardized, repeatable fashion. The preservation of the microenvironment of the heterogeneous tumor cell population and the associated matrix is a theoretical benefit of using fresh tumor samples. The use of mouse models is expensive and time consuming and requires the approval of an ethics committee. The CAM assay provides a cheap, quick, in vivo model for determining the rate of angiogenesis and the invasion and metastatic behaviors of tumor cells. However, CAM protocols vary among authors [1]. Studies evaluating the tissue grafts of different tumor types and of skin have been published, but to date the data on tumors derived from the musculoskeletal system are scarce [2–5].

We assessed whether the histological and immunohistochemical characteristics of fresh musculoskeletal tumor samples are preserved when these samples are grown on CAMs. Efficient grafting of the samples onto the CAM is essential for studying viability, migration, invasion and angiogenesis. The present study was designed to determine the suitability of this assay to study the behavior and characteristics of each individual tumor sample derived from the musculoskeletal system, regardless of tumor type and grade.

2. Materials and methods

2.1. Patient material

Fresh tumor material was obtained from 28 consecutive patients, 12 females and 16 males, with a tumor of the musculoskeletal system, including aggressive

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Table 1
Patient material: samples from fresh tumor material from 28 patients with a musculoskeletal tumor were placed on the CAM. The number of inoculated CAMs is noted in the column on the right.

	Age	Sex	Tumor type	Localization	Bone/ ST	B/ 1°noCT/ 1°CT/M	Current status	Viability (%)	Tumor cell infiltration (%)	Fibroblast infiltration (%)	Vascular infiltration (%)	Live/total number of eggs
1	36	F	Chondrosarcoma G1	Fibula	Bone	1° no CT	CDF	100	83	33	67	6/7
2	40	M	Recurrent GCT	Hip	Bone	B	CDF	50	0	0	50	2/3
3	50	F	Adenoca	Femur	Bone	M	AWD	100	100	0	100	2/2
4	54	F	Osteosarcoma	Fibula	Bone	1° CT	CDF	0	0	33	33	3/3
5	8	M	Angiomatous FH	Upper arm	ST	B	NED	67	33	33	33	4/5
6	65	M	Pleomorphic S	Trunk	ST	1° no CT	CDF	25	0	0	0	4/5
7	60	M	Plasmocytoma	Pelvis	Bone	1° no CT	AWD	67	67	0	100	3/5
8	67	M	Pleomorphic S	Gluteus	ST	1° no CT	Died 4,5 mo later	50	0	0	0	2/3
9	66	M	Adenoca	Femur	Bone	M	Died 10 mo later	0	0	50	75	4/4
10*	64	F	Osteosarcoma	Fibula	Bone	1° no CT	Died 13 mo later	75	63	75	75	8/9
11	6	F	Osteosarcoma	Fibula	Bone	1° CT	CDF	0	0	0	0	2/4
12	60	M	Chondrosarcoma G1	Hip	Bone	1° no CT	CDF	33	0	0	0	4/5
13	53	F	Synoviosarcoma	Trunk	ST	1° no CT	CDF	0	50	75	100	4/5
14	56	M	Chondrosarcoma G2 recurrence	Tibia	Bone	1° no CT	Metastatic : lung	0	0	33	33	4/6
15	49	M	Osteosarcoma	Knee	ST	1° no CT	Metastatic: lung	0	0	0	0	0/4
16	25	M	Osteosarcoma	Femur	Bone	1° CT	CDF	0	0	17	17	6/6
17	22	M	Chondromyxoid fibroma recurrence	Hallux	Bone	1° no CT	NED	20	10	70	80	10/10
18*	64	F	Osteosarcoma	Fibula	Bone	1° CT	Died 2,5 mo later	40	20	46	30	11/13
19	9	M	Ewing sarcoma	Calcaneus	Bone	1° CT	CDF	63	13	38	38	10/11
20	13	F	Osteosarcoma	Calcaneus	Bone	1° CT	CDF	50	0	75	87	10/12
21	54	F	Chondrosarcoma G1 recurrence	Femur	Bone	1° no CT	Progression G3 15 mo later	33	11	60	30	11/13
22	65	M	Chondrosarcoma	Thigh	ST	M (lung)	Progression lungM	12	15	100	100	13/17
23	38	M	Enchondroma	Humerus	Bone	B	NED	44	11	67	67	10/12
24	35	M	Chondrosarcoma G1 recurrence	Thigh	ST	1° no CT	Recurrence: resected	50	0	30	40	13/17
25	43	M	GCT tendon sheath	Subtalar	ST	B	CDF	50	33	50	50	6/6
26	62	M	Liposarcoma LG	Thigh	ST	1° no CT	CDF	14	14	71	71	8/9
27	63	F	Myxoid LipoS HG	Thigh	ST	1° no CT	CDF	0	0	17	67	8/9
28	14	F	Osteosarcoma	Femur	Bone	1° no CT	CDF	0	0	0	0	0/5
								42,7	18,5	49,3	56,3	168/210

Bone: lesion in bone.
G1: grade 1.
B: benign.
CDF: currently disease free.
ST: lesion in soft tissue.
G2: grade 2.
1° noCT: primary malignant, no chemotherapy before CAM.
AWD: alive with disease.
GCT: giant cell tumor.
LG: low grade.
1° CT: primary malignant, chemotherapy before CAM.
NED: no evidence of disease.
FH: fibrous histiocytoma.
HG: high grade.
M: metastasis.
* Two samples were taken from the same patient, one before and one after chemotherapy.

benign and malignant bone and soft-tissue tumors and bone metastases. Some samples originated from patients with primary malignant tumors who had already received neoadjuvant chemotherapy (Table 1). The only prerequisite for patient selection was the ability to adjust the date of intervention to the stage of development of the chick. A viable area of the tumor was identified by dynamic contrast MRI. After informed consent had been obtained from the patient, representative material was harvested at the time of the intervention, either a biopsy or the resection of the lesion. In the operating theatre, the material obtained was immediately placed in a sterile vial containing DMEM (Dulbecco's Modified Eagle Medium) and was transported to the lab (Fig. 1). Under sterile conditions, the contents of the vial were placed into a sterile Petri dish for further processing.

2.2. CAM assay

Fertilized eggs from a local commercial hatchery were incubated in an automatic incubator at 37.8 °C and 50% humidity. On day three, a window of one cm^2 was cut in the shell after lowering the level of the CAM by removing two ml of albumen through an 18 G needle, thus preventing damage to the chorioallantoic membrane while cutting the window. Afterwards, the window was sealed with a semipermeable film.

Under sterile conditions, several samples were taken from the fresh tumor material. These samples were measured and placed directly on the CAM on day 9 or 11 of incubation. The size of the samples varied between one and three mm in

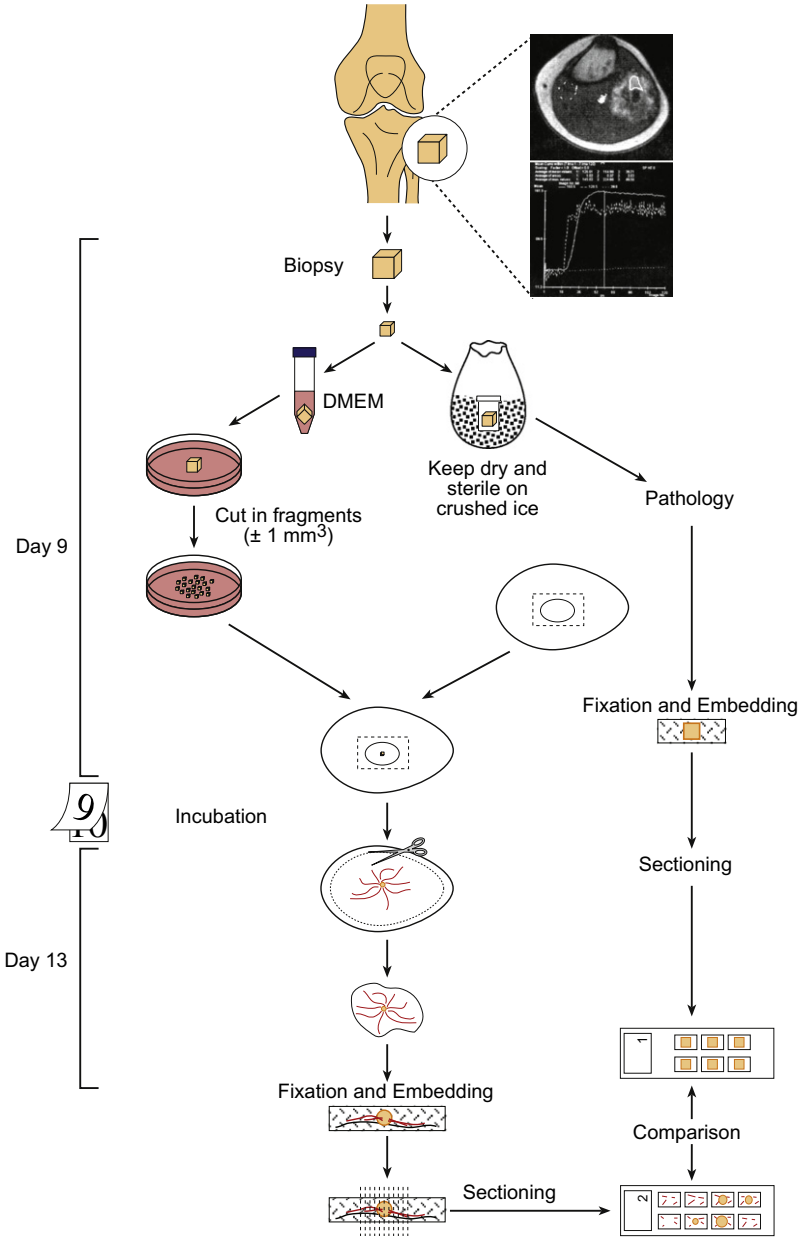

Fig. 1. Study sequence illustrating sample prelevation and processing both for pathological diagnosis and for the CAM assay.

diameter. Before the application of the tissue, the CAM was superficially scratched with a No. 11 scalpel to remove the epithelial cell layer, taking care not to inflict any damage. After inoculation, the shell window was sealed again.

On day 17, the CAMs were harvested. Samples on eggs in which the embryos died were excluded from the study. A photograph was taken in situ, and the membrane was fixed by the application of 300 µl of buffered formaldehyde to the intact CAM. After 5 min, the membrane was cut with a pair of sterile scissors at the border lying against the shell. Starting from patient 16, the severed membrane was placed in a labeled Petri dish containing buffered formalin, and the upper and lower sides were photographed macroscopically and microscopically (magnification 6, 12 and 25×) with a digital camera. Seventy-seven CAMs were used to score macroscopic angiogenesis towards the tumor graft (0 = no vascular alteration, 1+ = convergence of a few vessels towards the tumor implant, and 2+ = a greater density and length of vessels) [6].

2.3. Histological evaluation

Next, the membranes were embedded in paraffin and prepared for histological H&E examination. Additional stainings were performed for further clarification, if needed. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue sections of 3.5 µm in thickness using a Ventana Automated Slide Stainer (Benchmark XT, Ventana Medical Systems Inc., Arizona, USA) according to manufacturer's instructions. The following primary antibodies were used: a mouse monoclonal antibody to Ki-67 (clone MIB-1, dilution 1/100), a mouse monoclonal antibody to human CK20 (clone K_s20.8, dilution 1/100), a rabbit polyclonal antibody to S100 (dilution 1/3000) (Dako Denmark, Glostrup, Denmark), a mouse monoclonal antibody to alpha smooth muscle actin (α-SMA) (clone 1A4, dilution 1/400), a mouse monoclonal antibody to CK7 (clone OVTL, dilution 1/400) (Biogenex Laboratories, CA, USA), a mouse monoclonal antibody to human vimentin (clone SRL33, dilution 1/400, Novocastra, Leica Biosystems, Newcastle Upon Tyne, UK), a mouse monoclonal antibody to CD99 (clone 013, dilution 1/4, Covance, CA, USA), the CONFIRM™ anti-Estrogen Receptor antibody, a rabbit monoclonal antibody to human oestrogen receptor alpha (clone SP1, ready-to-use), the CONFIRM™ anti-Progesterone Receptor antibody, a rabbit monoclonal antibody to human progesterone receptor A and B (clone 1E2, ready-to-use) (Ventana Medical Systems Inc.), and a mouse monoclonal antibody to MDM2 (clone IF2, dilution 1/10, Invitrogen, CA, USA). Heat-induced epitope retrieval was performed using Cell Conditioning 1 buffer for all aforementioned antibodies except vimentin, for which Cell Conditioning 2 buffer was used, and CK7, for which an additional protease treatment lasting 4 min was added (Ventana Medical Systems Inc., Arizona, USA). For all antibodies,

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visualization was performed with the ultraView™ Universal DAB Detection Kit (Ventana Medical Systems Inc.) according to manufacturer's instructions. The dehydration of the tissue sections and the application of coverslips was performed using an automated coverslipper (Tissue-Tek Film Coverslipper, Sakura, Alphen aan den Rijn, The Netherlands).

Three observers evaluated the slides and scored tumor type, viability (morphology of nuclei: viable cells have nuclei with evenly dispersed fine chromatin), necrosis (complete or central necrosis with a vital rim against the CAM), infiltration of tumor into the CAM, infiltration of fibroblasts (elongated cells leaving the CAM and invading the tumor sample or fibrous septae between tumor cells), bleeding, CAM hyper/metaplasia, and vascular ingrowth. Vascular ingrowth was defined according to the Ausprunk classification into true neovascularisation (=tumor type, defined as the deterioration of the graft vessels and the ingrowth of proliferating chick vessels), revascularisation (=embryonic type, defined as anastomosis between graft vessels and the vessels of the CAM), or no vascular ingrowth (=adult type, defined as no vascular ingrowth or degeneration of the graft vessels) [7]. We also dichotomised vascular ingrowth into 'present' or 'not present' for comparison purposes, with the tumor type and the embryonic type making up the 'present' group in this analysis.

The chick embryos were dissected, and the liver and lungs were removed and placed in a labeled container with buffered formaldehyde. These tissues were embedded in paraffin and prepared for histological H&E examination.

For statistical analysis, tumor samples were grouped according to patient identification number, grouped as benign or malignant, or subclassified as benign, malignant with neoadjuvant chemotherapy, malignant without neoadjuvant chemotherapy or metastatic. All data were collected prospectively in an SPSS database (IBM SPSS 19) and were analyzed using unpaired non-parametric tests according to sample size (Kruskal–Wallis or chi-square/Fisher's exact tests). The significance level α was set at 0.05.

3. Results

3.1. Grafting efficiency from major sarcoma types

Metabolically active sarcoma samples taken from diverse types of musculoskeletal tumors (28 patients in total) were transplanted onto 210 eggs (Table 1). During the incubation period, 42 chicken embryos died, leaving 168 eggs eligible for study. Tumor take was macroscopically indicated by adherence of the tumor grafts to the CAM and microscopically by the embedding of the tumors in the tissue of the CAM membrane. The source of the tumor did not significantly predict successful engraftment. The superficial part of the tumor sample proved to be necrotic in large samples, while the area in contact with the CAM contained small and larger cells with nuclear atypia and a high nucleus-to-cytoplasm ratio. Ki67-positive tumor cells, which are actively engaged in cell cycle division, were found in regions in close contact with the CAM and in the vascularised regions of the tumor samples, confirming viability (Fig. 2). Viability was observed in 42.7% of our tumor samples, with a significant difference between patients ($P < 0.001$). There was no significant difference in viability ($P = 0.838$) between benign and malignant samples. However, after the further classification of malignant samples, metastases proved to be significantly more viable (72.2%) than the benign samples (60%, $P = 0.031$), the primary malignant samples treated with chemotherapy ($P = 0.010$) and the primary malignant samples not treated with chemotherapy ($P = 0.015$). Viability was significantly associated with patient's disease progression (death or metastasis) ($P = 0,039$). Partial necrosis was observed in 15.9% of the tumor samples, and complete necrosis was observed in 56.3% of the samples. Metastases proved to be significantly less necrotic than the primary malignant samples treated with chemotherapy ($P = 0.004$) and the primary malignant samples not treated with chemotherapy ($P = 0.002$). The infiltration of tumor cells into the CAM tissue was observed in 18.5% of tumor grafts and differed significantly between patients ($P < 0,001$). Primary tumors treated with chemotherapy showed markedly less infiltration into the CAM than primary tumors not treated with chemotherapy ($P = 0.04$).

3.2. Tumor grafts retain original tumor characteristics

A clinical sarcoma pathologist who was blinded to the identities of the samples evaluated H&E sections of the original tumors and the tumor grafts. All tumor grafts retained the original tumor morphology, and high-grade lesions could be distinguished from low-grade lesions for liposarcoma and chondrosarcoma (Figs. 3 and 4). Diagnostic immunohistochemical markers remained positive in most cases after passage on the CAM, and negative stains remained negative. The expression levels of ER, PR, CK7 and CK20 were evaluated for two patients (3 and 9) with metastatic breast carcinoma (Figs. 5 and 6); S100 expression was evaluated for high-grade and low-grade chondrosarcoma (cases 14 and 21) (Figs. 3 and 4); MDM2 expression was assessed for high-grade and low-grade liposarcoma (cases 26 and 27) (Fig. 7); vimentin expression was analyzed for a metastatic soft-tissue chondrosarcoma (case 22) (Fig. 8); and CD99 expression was assessed for monophasic synoviosarcoma (case 13) (Fig. 9).

3.3. Tumor grafts recruit the host environment

Infiltration into the tumor samples of elongated, spindle-shaped cells, assumed to be the chicken analogue of carcinoma-associated fibroblasts because they were human vimentin negative, was observed in 50.7% of samples and differed significantly between patients ($P < 0.001$). Accompanying vascular channels were observed, with a larger diameter at the base of the invasion cone, diminishing near the tip of the cone. These vascular channels were filled with nucleated chick erythrocytes. Thus, tumor-associated stroma from the human samples was largely replaced by chicken-derived stroma in the tumor grafts, with no significant differences ($P = 0.317$) between the benign and malignant samples. However, samples from metastases showed significantly more infiltration when compared with samples from other tumor types ($P = 0.008$). When only viable tumor samples were selected, the difference between metastases and primary tumors not treated

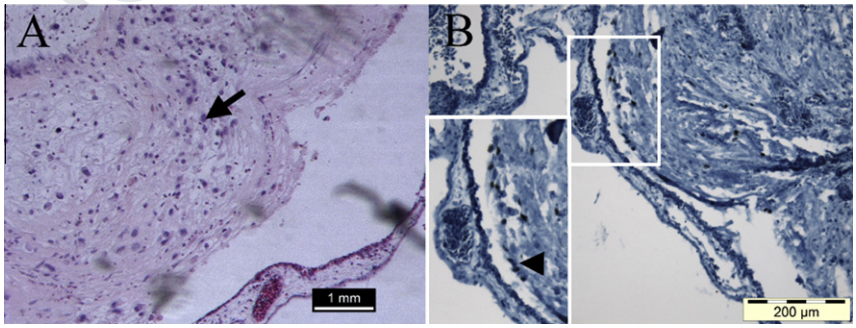

Fig. 2. High-grade chondrosarcoma: (A) cells with a high nucleus-to-cytoplasm ratio (arrow) can be observed close to the CAM and (B) positive Ki67 staining (arrowhead) in the same sample.

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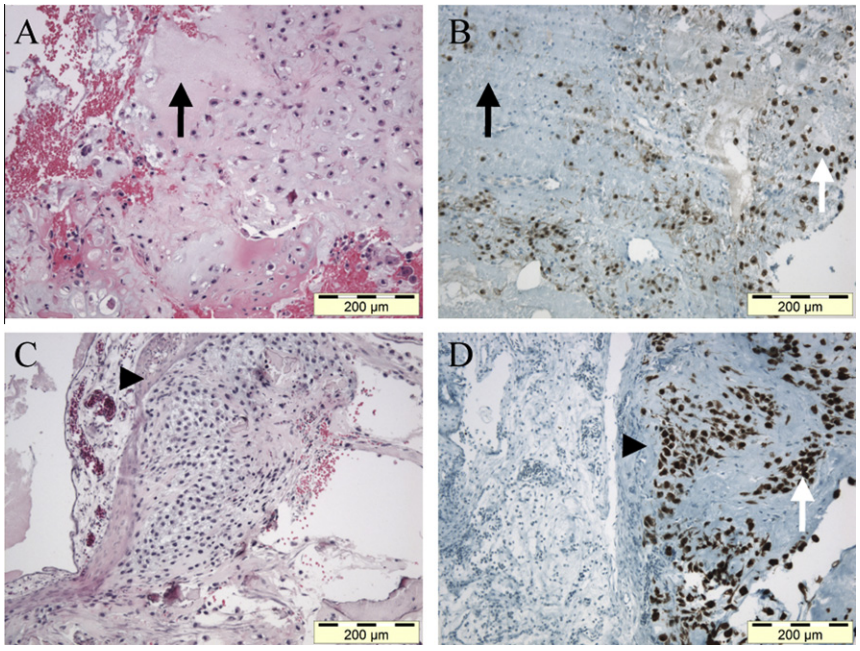

Fig. 3. Chondrosarcoma grade 1. (A and B) original tumor; (C and D) CAM material. (A and C) HE staining; (B and D) S100 staining, 100×. The S100 staining remains positive in the CAM sample (white arrows). Black arrows: matrix production, arrowheads: the CAM remained uninvaded by the tumor.

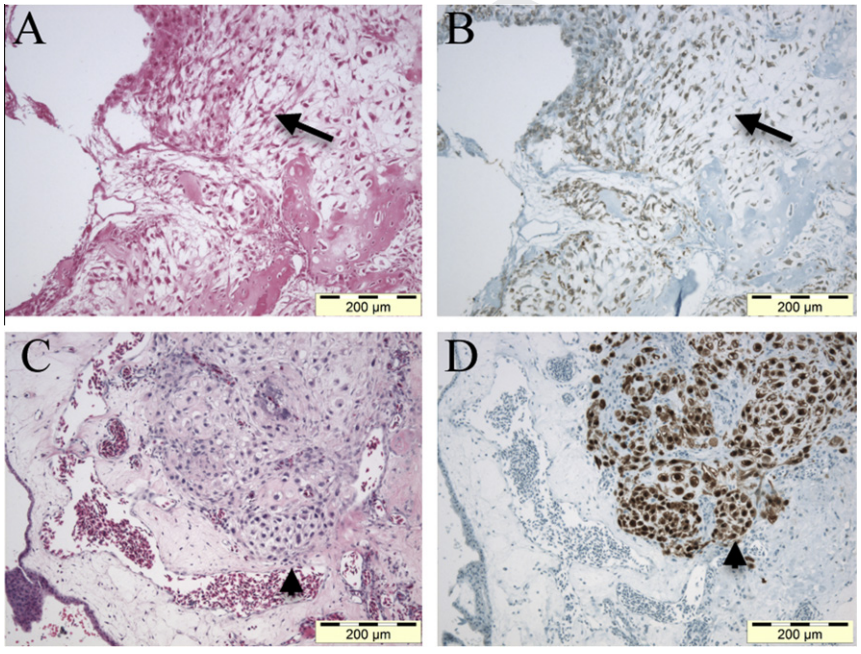

Fig. 4. High-grade chondrosarcoma: (A and B) original tumor; (C and D) CAM material. (A and B) HE staining; (C and D) S100 staining (100×, scale bar 200 µm). The nuclei become more irregular and pleomorphic. Less matrix production is observed compared with grade 1 chondrosarcoma (arrows). The CAM is invaded by tumor cells in the high-grade tumor, whereas the CAM remains intact in the low-grade tumor sample (arrow heads).

with chemotherapy was significant ($P = 0.013$). Tumor viability was significantly correlated with fibroblastic infiltration ($P = 0.003$), whereas necrosis was inversely correlated with fibroblastic infiltration ($P < 0.001$). In accordance with these results, fibroblast infiltration was significantly correlated both with neo- and revascularisation ($P < 0.001$).

We were able to compare a lipoma-like liposarcoma (low-grade liposarcoma) with a myxoid liposarcoma (high-grade liposarcoma, characterized by a rich vascular network). In the lipoma-like liposarcoma, a distinct fibroblast invasion cone containing vascular channels was observed. In the myxoid liposarcoma, no ingrowth of fibroblasts was observed; however, the original vascular network remained and was filled with chick erythrocytes. In other samples, such as osteosarcomas, a mixed population of chick and human erythrocytes was observed.

We found no vascular infiltration in 43.7% of samples, whereas revascularisation and neovascularisation occurred in 23.2% and 19.2% of samples, respectively. In 13.9% of samples, both types of vascular infiltration were observed. Benign and malignant tumors differed significantly with respect to the type of vascular ingrowth

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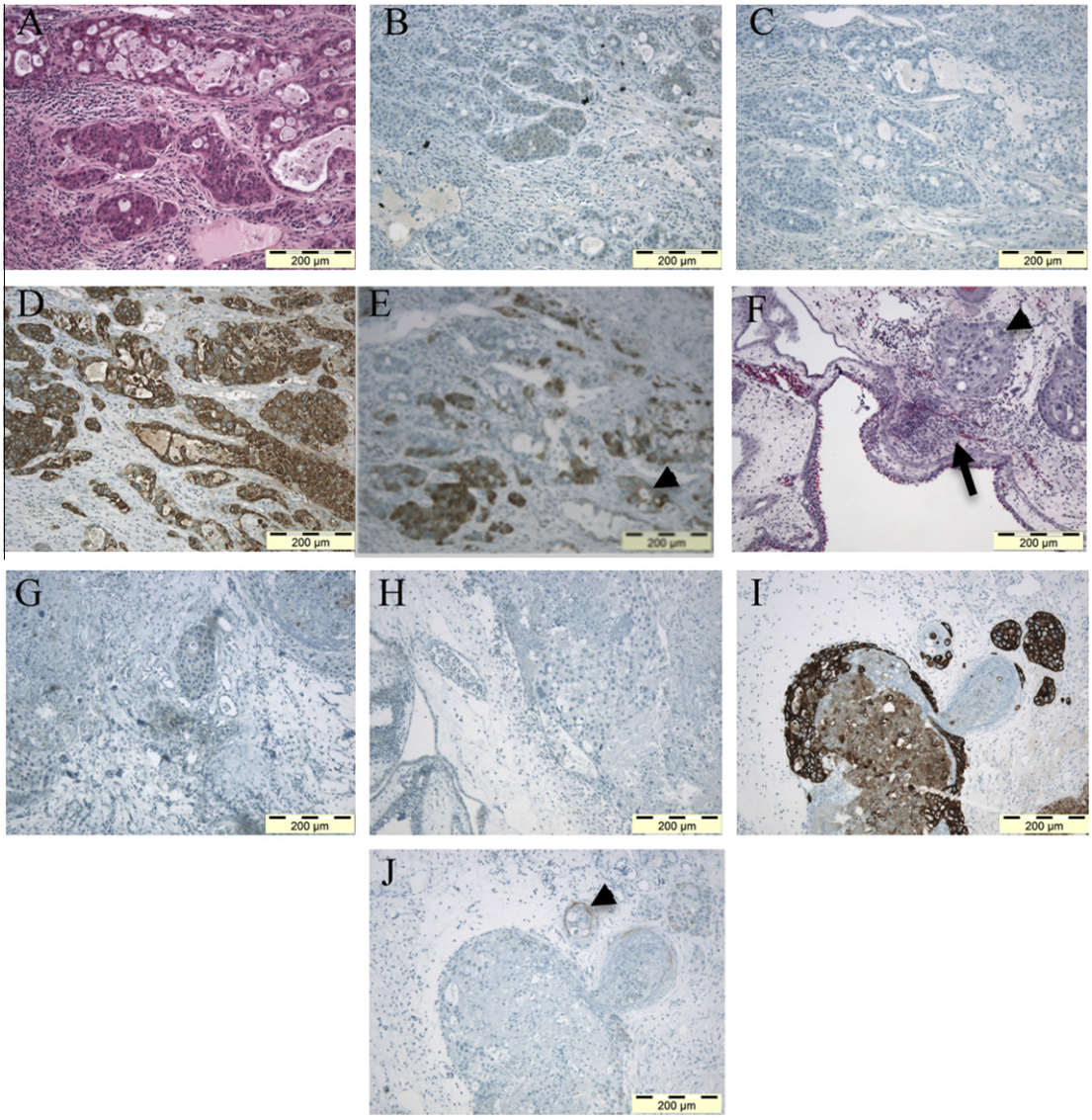

Fig. 5. Breast carcinoma metastasis in the bone (patient 3): ER and PR, negative; CK7, strongly positive; and CK20, focally positive (arrowhead) in both the original patient material and in the CAM material. Panel F illustrates the invasion of tumoral nodules (arrowhead) into the CAM tissue (arrow). A–E = original tumor; F–G = CAM material; (A and F) HE staining; (B and G) ER staining; (C and H) PR staining; (D and I) CK7 staining; (E and J) CK20 staining. (100×, scale bar 200 μm).

($P = 0.011$). Primary tumors treated with or not treated with chemotherapy were significantly less neo- and revascularised relative to metastasis ($P < 0.001$). When the vascular infiltration types were dichotomised, a significant difference was detected between patients ($P < 0.001$). The classification of tumor samples as benign or malignant did not result in significant differences ($P = 1.00$). However, after further classification of malignant samples, metastases again proved to be significantly more vascularised than other tumor types ($P = 0.001$). When only viable tumor samples were selected, all metastatic samples proved to be vascularised, resulting in significant differences relative to primary tumors treated with ($P = 0.039$) and not treated with ($P = 0.013$) chemotherapy. Viable samples showed significantly more vascular ingrowth (67.2%) than did non-viable samples (47.7%) ($P = 0.017$).

Macroscopically, a vascular reaction of the CAM was observed in most cases. In translucent tumor samples such as chondrosarcoma, new tortuous vessels were observed entering the sample, where they branched and anastomosed with one another (Fig. 10). Digital photographs of 77 samples were used for macroscopic angiogenesis evaluation by three independent observers: 2.6% of samples had a score of 0, 54.3% had a score of 1, and 43.1% had a score of 2. The interobserver variability for this score was fair (kappa > 0.41). No significant correlation was found between the macroscopic score and the microscopically observed vascular ingrowth ($P = 0.920$), even after dichotomising the different types of vascular ingrowth ($P = 0.507$). Thus, the macroscopic increase in vascularisation following transplantation is therefore merely an embryonic reaction and is distinct from the vascular ingrowth into tumors.

Bleeding of the CAM was observed in only three samples, each from a different patient. CAM hyperplasia was observed in 3.3% of samples, including hyperplasia of the stroma and of the basal layer. In some cases, even budding (rete-like focal irregular hyperplasia) of the basal layer was observed (Fig. 11). A marked infiltration of cells supposedly involved in a cellular immunologic reaction was observed histologically in the CAM at the periphery of certain tumor samples. CAM metaplasia was observed in 8.7% of samples. No significant difference between patients ($P = 0.064$) was found for either of the reaction types.

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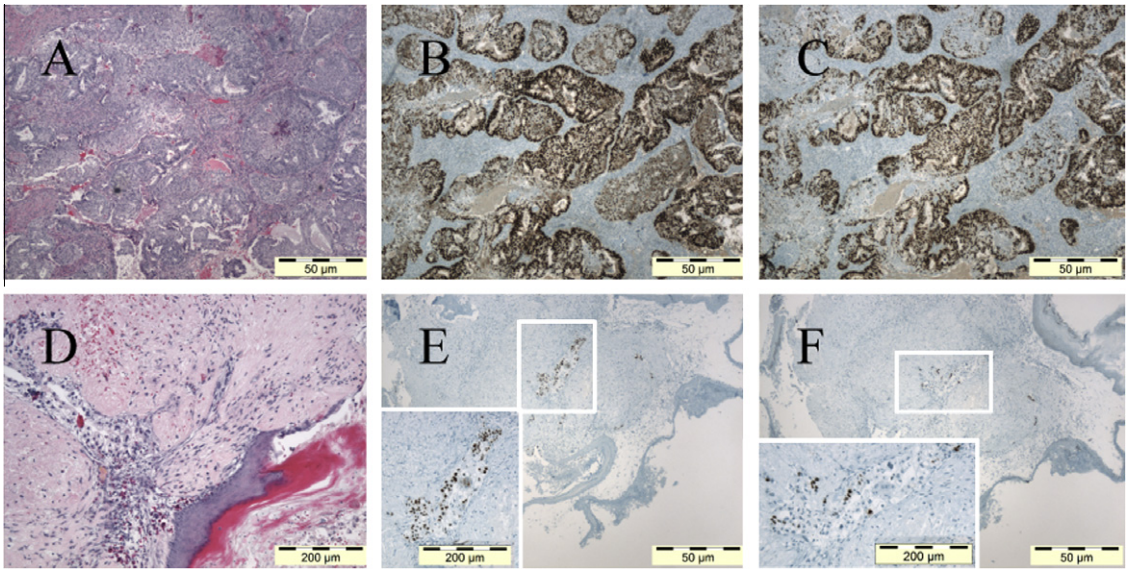

Fig. 6. Breast carcinoma metastasis in the bone (patient 9): stainings for ER and PR were positive in the original patient material but only focally positive in the CAM material (insets, panels E and F). A–C = original tumor; D–F = CAM material; (A and D) HE staining; (B and E): ER staining; (C and F) PR staining.

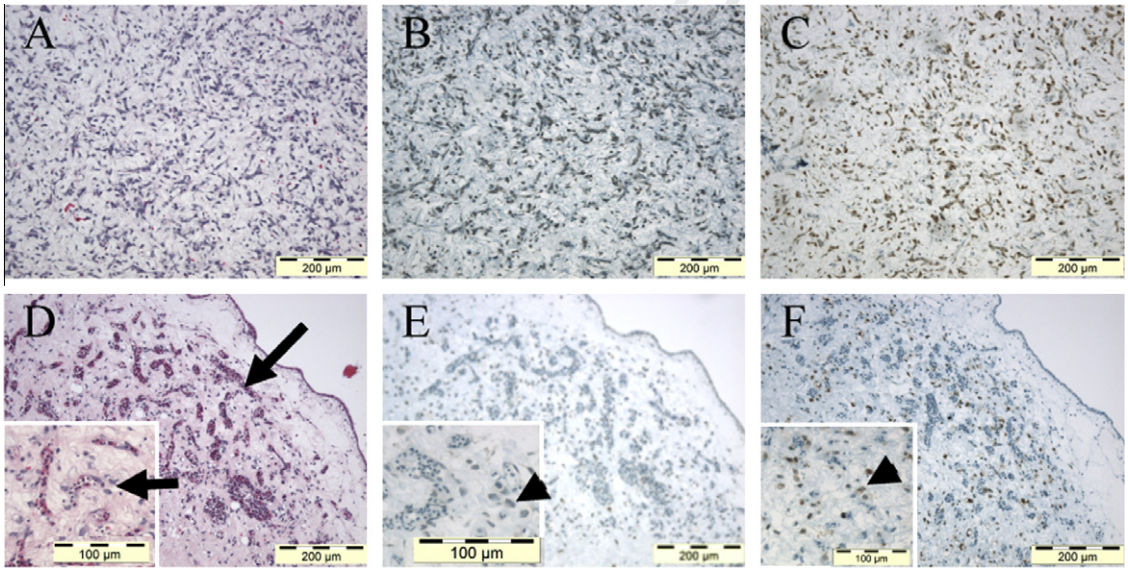

Fig. 7. High-grade liposarcoma: stainings for MDM2 and S100 were positive in both the original tumor material and the CAM material (arrowheads, inserts in panels E and F). Revascularization of the graft is demonstrated in panel D and insert (arrows). The capillaries are filled with nucleated chick erythrocytes: (A–C) patient material, (D–F) CAM material. (A and D) HE staining; (B and E) MDM2 staining; C and F: S100 staining. (100×, scale bar 200 µm; inserts, 200×, scale bar 100 µm).

4. Discussion

The use of fresh tumor samples preserves the original tumor microenvironment, allowing interactions between the different cell populations in the sample. However, theoretically, revascularisation of the sample is required for the sample's survival and growth. The tumor samples on CAMs in eggs in which the embryo died proved to be completely necrotic, supporting this theory. Among the samples with surviving embryos, we observed viable tumor tissue in 42.7% of the samples [7]. Larger samples tended to become rounded off at the surface, without obvious revascularisation. They also tended to show central necrosis except in cases of vascular anastomosis, indicating that smaller samples (1 mm^3) should be used if possible.

Some malignant and some benign tumor samples showed invasion into the CAM, which demonstrates the applicability of the CAM assay as an indicator for local tumor invasion potential. Tumor cell migration could not be observed macroscopically as no secondary macroscopically visible nodules were formed.

In contrast to Ribatti et al. [9], we did observe revascularisation of the tumor graft through anastomosis between host vessels and the existing vascular network of the graft, as confirmed by the presence of chick erythrocytes in human vessels. Apparently, this process depends on the histological tumor type and the extent of the pre-existing vascular network. In other grafts, marked ingrowth of elongated, spindle-shaped fibroblastic cells was observed from the periphery towards the central core of the grafts. We hypothesise that the ingrowth of fibroblasts from the CAM into the tumor samples precedes the formation of vascular channels. This hypothesis is supported by the observation of larger-diameter vessels at the base of the conical structure and smaller – diameter vessels at the tip of this cone. When the original tumor sample

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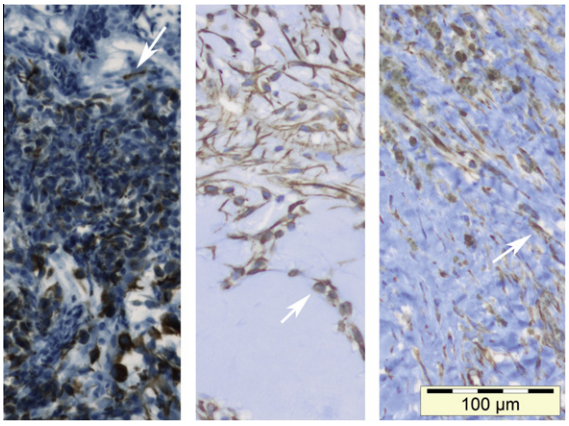

Fig. 8. Left: A chondrosarcoma metastasis sample on a CAM remains positive for vimentin, and positive cells have invaded the CAM (invading cells at the periphery of the tumor graft are indicated by the arrowhead). Middle: At the center of the original tumor metastasis, vimentin-positive cells are found in chondroid matrix (arrow). Right: Center of the original tumor of the thigh with vimentin-positive cells (arrow). Human-specific vimentin, scale bar 100 µm.

330 contained a pre-existing widespread vascular network, no invasion
331 of these fibroblastic cones was observed. This hypothesis is also supported by the correlation between the absence of vascular ingrowth and the absence of fibroblastic ingrowth, by the positive correlation with viability and by the inverse correlation with necrosis. These correlations were most pronounced in metastatic lesions. Other samples, such as those from chondrosarcomas, showed marked revascularisation throughout the full thickness of the graft. In these translucent tumors, scattered, punctiform bleeding could be discerned between the vessels, indicative of tumoral factors causing increased endothelial permeability.

Cellular immunity of the chick embryo starts at day 13, and immune cells reach the CAM at day 15 [10]. To avoid tumor rejection because of immune system development at day 18 [11], the CAM was harvested at day 17 of development. Tumor grafting induces marked reactions of the CAM, such as hyperplasia, fibrous nodules, edema and immune cell infiltration. Jakob et al. recommended the addition of hydrocortisone because they observed similar reactions after the application of non-cellular materials onto the CAM [12]. However, as neovascularisation is also suppressed by hydrocortisone, this strategy would negatively influence graft viability.

The subdivision of tumor samples is essential for the correct interpretation of the observations. Primary malignant tumors treated with chemotherapy tended to behave less invasively than equally aggressive tumors not subjected to chemotherapy. Metastatic lesions proved to be very viable and showed marked vascular

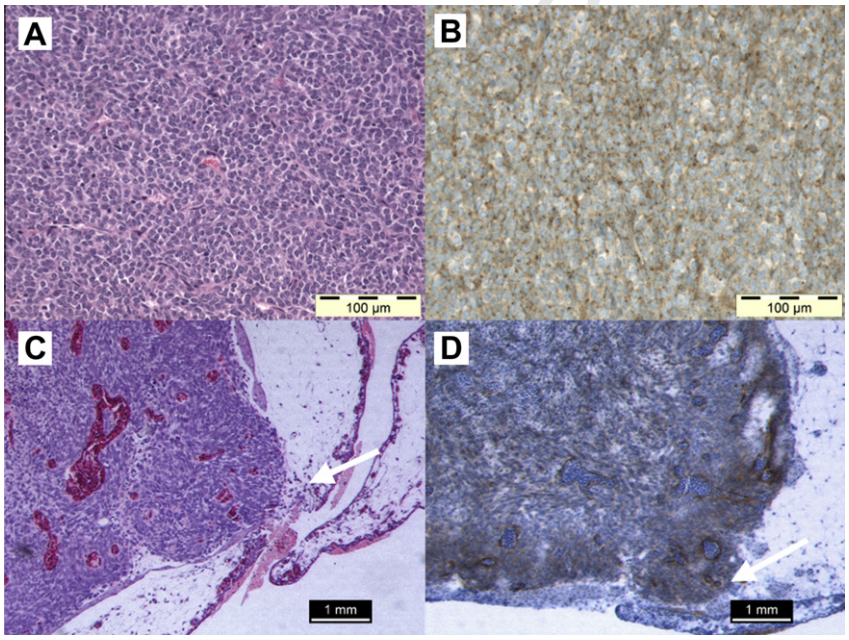

Fig. 9. Synoviosarcoma: Cell morphology and S100 positivity of the original tumor are retained in the CAM material. The CAM is invaded by the tumor (arrows) (A and B) original tumor; (C and D) CAM material; (A and C) HE staining; (B and D) S100 staining.

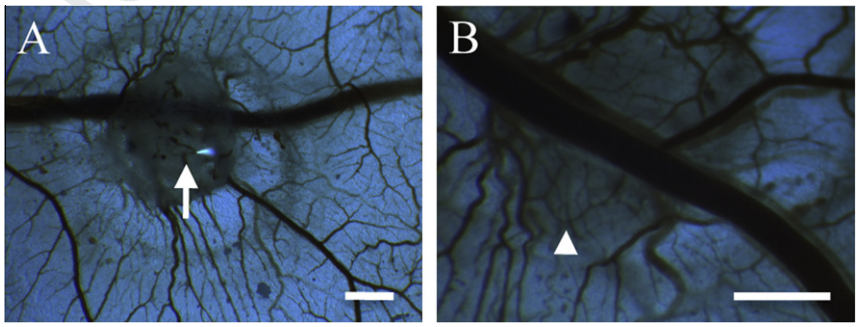

Fig. 10. Metastatic lesion of a soft tissue chondrosarcoma: (A) upper view, 12×: Vessel formation (arrow) can be discerned throughout the graft and (B) at the lower side, branching and anastomosing vessels are clearly observed (arrowhead).

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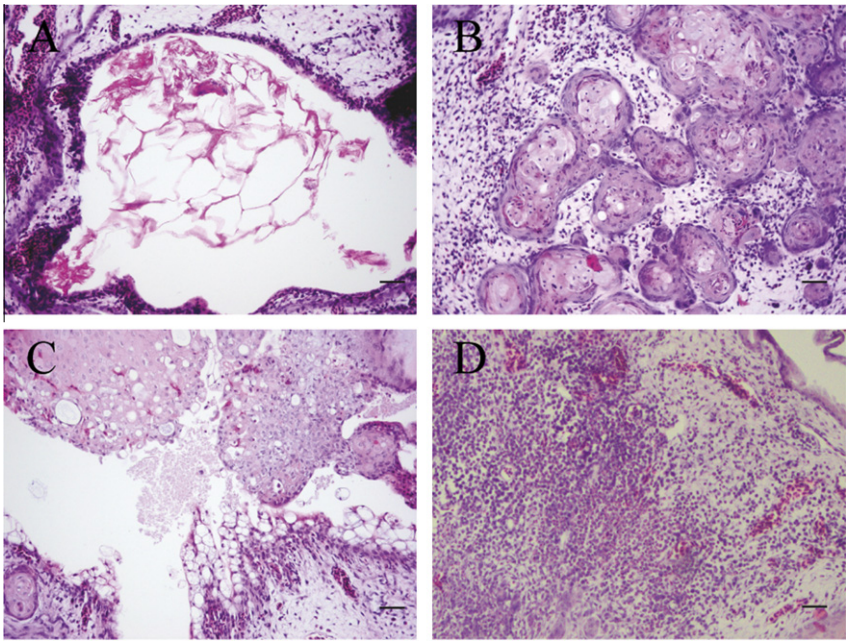

Fig. 11. (A) Cyst formation on the CAM, (B) hyperplasia with budding of the basal layer, and (C) CAM metaplasia, (D) cellular infiltration of the CAM. HE, 20×, scale bar 80 μm.

recruitment. Despite our short follow-up, the correlation between disease progression and graft vitality has important prognostic implications, as has also been described by DeRose [13]. Intrinsic genetic heterogeneity or regional clonal discrepancies may influence the overall tumor grafting; marked differences were indeed noted in samples originating from the same patient [14].

The results of this study may lead to the development of the CAM assay as an in vivo tool for assessing tumor aggressiveness and tumor responses to therapeutic agents. Preclinical validation of potential therapeutic targets using in vivo models is regarded as an essential step in anticancer drug development. Concerns are now being raised that what is still deemed a successful endpoint at the preclinical level – positive performance of a drug in xenografts of different human cancer cell lines – is in fact not predictive of compound efficacy in the clinical setting [15]. The obvious objection is that immortalized cancer cell lines, which are commonly employed in xenograft experiments, have a phenotype adapted to culturing on plastic substrates, and thus these cells differ genetically from the original cancers in patients. Furthermore, the catalogue of currently available cell lines is poor, especially for sarcomas. Experiments with cell lines cannot recapitulate the wide heterogeneity of human malignancies. One robust way to proceed with drug development at the stage of in vivo validation would be to perform preclinical population-based studies. To do this, we will make use of human sarcoma specimens directly transplanted onto the CAMs of developing chick embryos to generate a study population that could be randomised for prospective treatment with targeted agents. Others have used a similar xenopatient approach for glioblastomas and colorectal cancers in rodents [16,17]. We were able to achieve a rate of viable sample engraftments of 42% on CAMs. Notwithstanding their ectopic site of growth, sarcoma xenografts retained the morphological characteristics of the corresponding lesions in the patients, supporting the robustness and the predictive power of our strategy.

The results of our study show that the CAM assay can be used to analyze fresh material derived from tumors of the musculoskeletal system. All grafts examined retained critical characteristics of the original tumor specimens from living individuals with sarcoma. The CAM assay is a useful tool for the evaluation of tumor sample viability and of locally invasive behavior and can indicate disease outcomes.

5. Uncited reference

[8]. Q3

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