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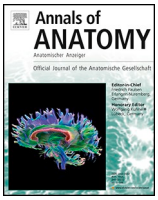

An easy-to-perform protocol for culturing primary murine lung tumor cells as organoids

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ABSTRACT

Cancer research involves significant animal consumption and suffering. Tumor cells can be differentiated *in vitro* into three-dimensional organoids that resemble the primary tumor. In basic cancer research, however, tumor organoids are usually only used alongside animal experiments. We have established an easy-to-perform protocol that allows to culture KRAS-driven lung tumor cells as organoids for extended periods of time. Like the corresponding tumors in mice, the organoids produce surfactant protein C but no markers of airway epithelial cells (e.g. SCGB1A1, KRT5). The organoids can be passaged as single cell suspensions. Our organoid model contributes to replace animal experiments with cell culture systems and can be used for drug testing or functional studies in cancer research.

1. Introduction

Lung cancer is a deadly disease and responsible for millions of deaths worldwide every year (Schabath and Cote, 2019). Despite intensive efforts, it has not yet been possible to develop therapies that significantly improve the prognosis of most lung cancer patients. Basic research therefore continues to deal intensively with the pathogenesis of lung cancer and possible therapeutic approaches, with mouse models being primarily used here. However, lung cancer models in which tumor growth is caused by carcinogens, genetic engineering, or transplanted cancer cells involve significant animal suffering and consumption (Guerin et al., 2020).

About 25 % of primary lung tumors are associated with a KRAS mutation (Riely et al., 2008). Johnson et. al developed a mouse strain (here, KRAS mice) in which lung cancer spontaneously occurs due to the activation of an oncogenic Kras allele (Johnson et al., 2001). Lung tumors develop in KRAS mice via morphological stages, such as mild hyperplasia and alveolar adenomas. This is accompanied by an average survival rate of approximately 200 days, depending on the mouse strain.

KRAS mice have been widely used in the past to understand the role of inflammation in tumorigenesis and progression. For this purpose, genes for inflammatory mediators or cell signaling are often additionally deleted in KRAS mice and pulmonary inflammation is induced with smoke or bacteria (Brooks et al., 2016; Chang et al., 2014; Ritzmann et al., 2022; Takahashi et al., 2010). While such studies provide important basic knowledge about lung cancer, they are associated with a significant consumption of mice.

Organoids that differentiate from isolated cells in cell culture and resemble tissues regarding structure and composition are a promising approach to reduce the need for animal experiments and are useful for functional studies and drug development. Patient-derived tumor organoids that reflect various properties of tumor tissue, such as genomic changes and molecular properties, are used for personalized cancer treatment and drug development (Drost and Clevers, 2018; Xu et al., 2022a). Tumor organoids are also used in basic cancer research, but usually only in addition to animal experiments (Wang et al., 2020; Xu et al., 2022b). In a current study, for example, KRAS-driven tumor cells were isolated from reporter mice using complex FACS protocols. Single cell analysis here showed that alveolar epithelial progenitor cells expressing oncogenic KRAS have reduced expression of mature lineage identity genes (Dost et al., 2020). Tien et al. dissociated tumor nodules from KRAS mice and seeded them in Matrigel. They showed that the lack

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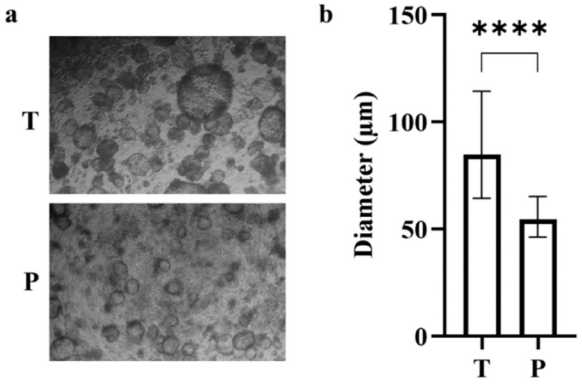

Fig. 1. Size of tumor and lung organoids 8 days after seeding. Organoids were differentiated from tumor cells from KRAS mice (T) or parenchymal cells from wild-type mice (P) and cultured for eight days. **a** Representative phase contrast images. **b** Diameter of the organoids (n = 548 for T, n = 460 for P). Data were compared by Mann-Whitney test and are shown as the median with interquartile. $p < 0.0001$.

of Argonaute 2 (AGO2) resulted in smaller organoids. The organoids themselves have not been further characterized here (Tien et al., 2021).

In this study, we present an easy-to-perform protocol that allows tumor cells from KRAS mice to be cultured as 3D organoids for extended periods of time.

2. Methods

2.1. Isolation of tumor cells

Organ harvesting was approved by the Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland in agreement with the national guidelines for animal treatment. The study was reported in accordance with the recommendations of the ARRIVE guidelines. The mice were euthanized with an intraperitoneally injected overdose of 525 mg/kg ketamine hydrochloride (Ketanest; Pfizer, Germany) and 35 mg/kg xylazine hydrochloride (Rompun; Bayer, Germany). 150–200 days old B6.129S-Krastyj Kras mice were euthanatized, the lungs were removed and 12–15 visible tumor nodules per mouse were isolated, taking care not to isolate adjacent parenchyma. For the control cultures, the same amount of tumor-free parenchyma was isolated from wild-type mice. To obtain single cell solutions the isolated tissues were enzymatically digested by using a lung dissociation kit (Miltenyi Biotec, Germany) under gentle agitation for 20 minutes at 37°C. For an additional mechanical dissociation, the obtained cell solutions were passed through a 27 G needle. After two washes with prewarmed PBS, the cells were resuspended in modified organoid culture

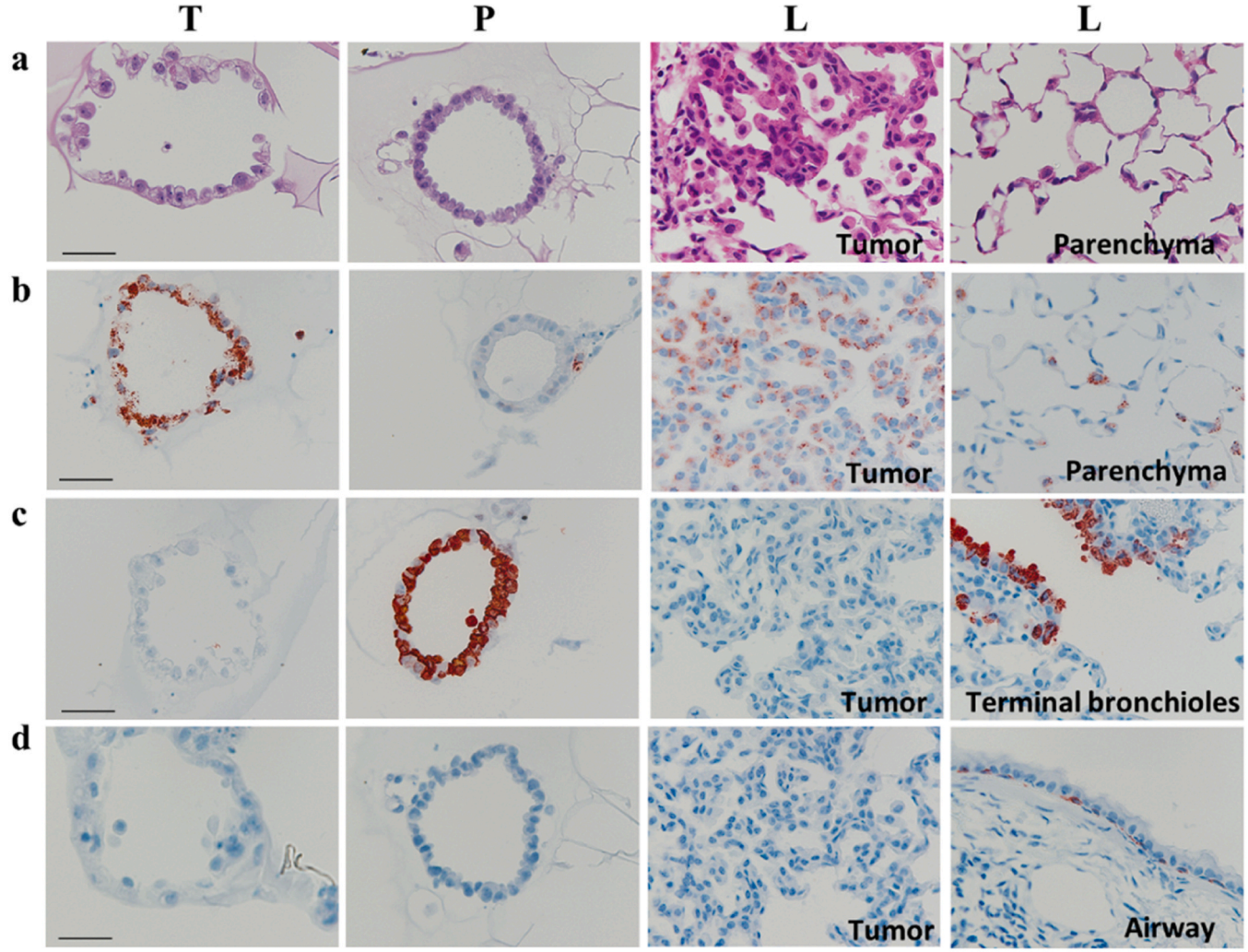

Fig. 2. Expression of epithelial markers in organoids 8 days after seeding. Organoids were differentiated from tumor cells (T) or parenchymal cells (P) and cultured for eight days. **a** Representative H&E staining of organoids. Immunohistochemistry for **b** SFTPC, **c** SCGB1A1, and **d** KRT5 (scale bar: 20 µm). Lungs of KRAS mice (L) served as control.

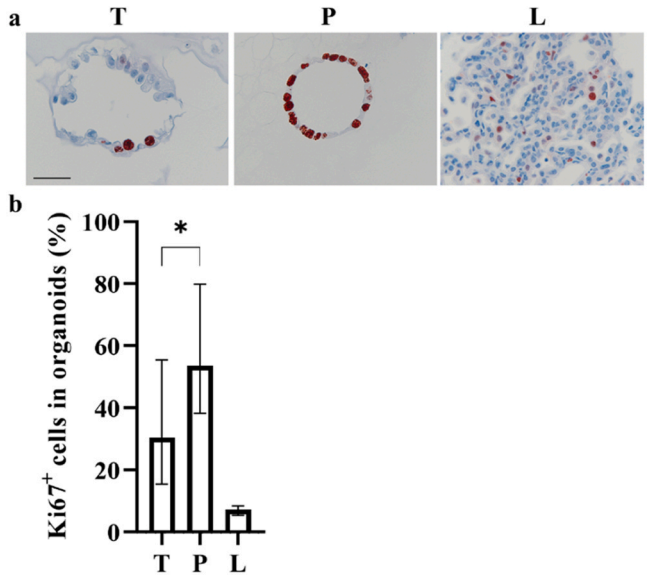

Fig. 3. Proliferation of organoids 8 days after seeding. Organoids were differentiated from tumor cells (T) or parenchymal cells (P) and cultured for eight days. **a** Representative IHC analysis of Ki67 (scale bar: 20 μm.). **b** Ki67 index (n = 33 for T, n = 16 for P,). Data were compared by Mann-Whitney test and are shown as the median with interquartile range. $p < 0.05$. Lungs of KRAS mice (L, n = 3) served as control.

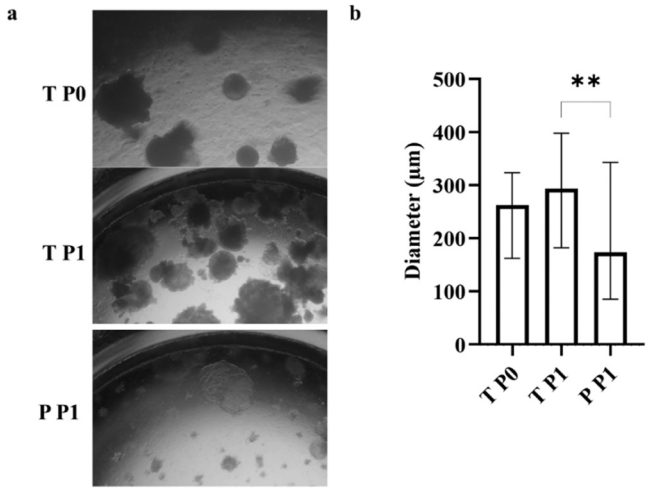

Fig. 4. Size of organoids 30 days after seeding. Passage 0 (P0) and passage 1 (P1) tumor organoids (T) and passage 1 parenchymal organoids (P P1) were cultured for 30 days. **a** Representative phase contrast images. **b** Diameter of the organoids (n = 65 for T PO, n = 80 for T P1, n = 42 for P P1). Data were compared by Mann-Whitney test and are shown as the median with interquartile range. $p < 0.01$.

medium (OCM) (Tien, et al., 2021). OCM was composed of DMEM/F12 (Gibco, USA), supplemented with 1x ITS (Insulin,Transferrin and Selenium, Invitrogen, USA), 0,5 % BPE (bovine pituary extract, Gibco), 25 ng/ml mEGF (murine epiderman growth factor, Pepro Tech, USA), 100 nM retinoic acid (Cayman, USA), 10 μM Y-27632 inhibitor (ROCK inhibitor, StemCell, Canada) and 0,1 % Primocin (Invivogen, USA). Cell solutions were passed through a 70 μm cell strainer and cell concentration as well as viability was determined using trypan blue staining.

2.2. Tumor organoid cultures

For organoid cultures, 4 $\times 10^4$ cells in 50 μl OCM were mixed with 50 μl growth factor reduced Matrigel (Corning, USA) (Tien, et al., 2021). 100 μl of cell-Matrigel mixture were plated on top of a 6.5 mm transwell insert (Corning). 500 μl OCM was added into the basolateral compartment and changed every other day. Growth and morphology of the cultures were documented on a Zeiss Axiovert 25 microscope.

2.3. Co-culture with macrophages

The isolation of alveolar macrophages was performed as described before (Ritzmann et al., 2021). In brief, mouse lungs were lavaged with 1 ml PBS, centrifuged and resuspended in RPMI supplemented with 10 % FCS, 100 U/ml penicillin and 100 U/ml streptomycin. 2 $\times 10^4$ cells per well were seeded in a 24-well plate and incubated at 37° C and 5 % CO^2 for 24 hours in order to achieve adhesion of the macrophages on the bottom of the well. Non-adherent cells were washed aspirated and washed using OCM. For co-culture experiments 500 μL of OCM and transwells inserts with freshly seeded cancer cells were added to the wells.

2.4. Staining of organoids

Organoids from each group were collected by removing Matrigel with Cell Recovery Solution (CRS, Corning). In brief, 200 μl CRS was added in each Transwell insert and incubated for 10 Minutes at 4°C. Organoids from one group were pooled and embedded in paraffin as described before (Sprott et al., 2020). Sections of 2 μm thickness were stained with hematoxylin–eosin (H&E). For immune histochemistry, epitopes were retrieved by heat in TRIS-EDTA buffer. The samples were permeabilized by incubation in TBS-T buffer (0002 % Tween-20 in TBS). After blocking with 2 % BSA in PBS, the sections were incubated with primary antibodies against Ki67 (Abcam, Cambridge, UK) SFTPC, SCGB1A1, an KRT5 (Cell Signaling Technology, USA) in PBS overnight at 4°C. Signals were detected by enhanced chemiluminescence (BioRad, Dreieich, Germany) using appropriate peroxidase-conjugated secondary antibodies (Agilent, DAKO, Santa Clara, CA, USA).

2.5. Ki67 index

The Ki67 index was evaluated using the open source software ImageJ. For this purpose, overview images of the Ki67 stained organoid sections were taken. With the help of the ImageJ cell counter, nuclei positive for Ki67 and the total number of nuclei per organoid were counted. The organoid-specific Ki67 index was calculated with the ratio between Ki67 positive nuclei and the total number of nuclei counted per organoid.

2.6. Statistics

Comparisons were tested by Mann-Whitney or Student's t-test (two-sided) when data were normally distributed using the software Prism (GraphPad Software, San Diego, CA). The results were considered statistically significant for $p < 0.05$.

3. Results

3.1. Primary tumor cells isolated from tumor nodules form organoids negative for airway epithelial markers

For the cultivation of the tumor cells, tumor nodules were prepared from KRAS mice, enzymatically digested, filtered and seeded onto transwell inserts in Matrigel. As a control, lung parenchyma from WT mice was processed and seeded in the same way. From both tumor and parenchymal cells, organoids could be formed (Fig. 1a). With a median diameter of 80 μM eight days after seeding, the organoids from tumor cells were significantly larger than those from parenchymal cells (Fig. 1b).

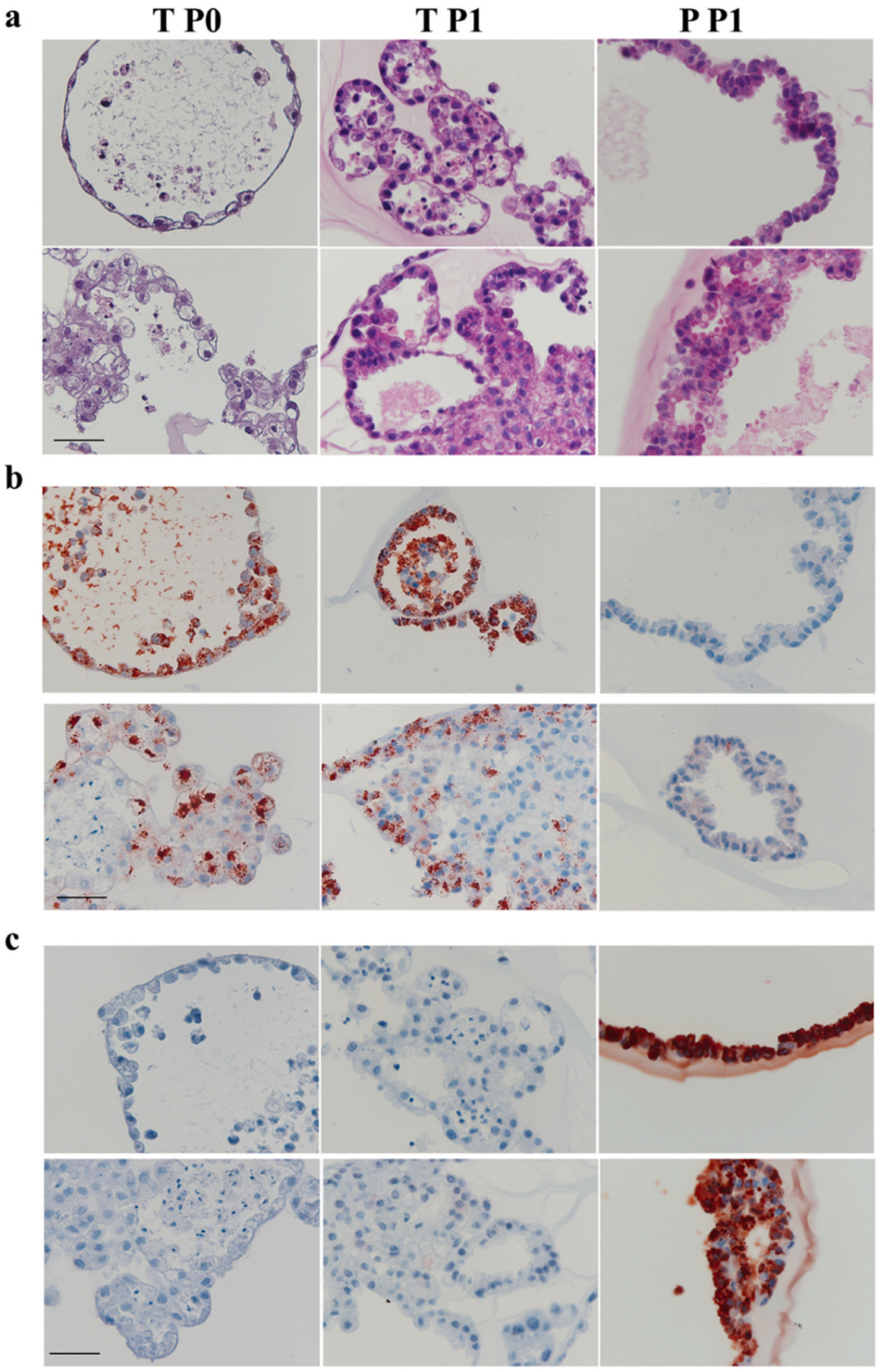

Fig. 5. Expression of epithelial markers in organoids 30 days after seeding. Passage 0 (P0) and passage 1 (P1) tumor organoids (T) and passage 1 parenchymal organoids (P P1) were cultured for 30 days. **a** Representative H&E staining of organoids. Immunohistochemistry for **b** SFTPC and **c** SCGB1A1 (scale bar: 20 μm.).

H&E overview staining showed that the organoids had a cystic appearance (Fig. 2a). Adenocarcinomas in KRAS mice have been described as positive for surfactant C (SFTPC), a marker for type 2 pneumocytes, and negative for SCGB1A1 (also called CCSP), a marker for club cells (Jackson et al., 2001; Moghaddam et al., 2009). Immunohistochemical staining showed that all tumor organoids as well as lung tumors in mice stained positive for SFTPC (Fig. 2b) and negative for SCGB1A1 (Fig. 2c) and the basal cell marker KRT5 (Fig. 2d).

In contrast, organoids from parenchymal cells were positive for SCGB1A1 and partially also positive for SFTPC. Thus, cells from lung parenchyma formed organoids consisting of club cells. We also examined tumor cell proliferation by immunostaining for Ki67 (Fig. 3a). About 30 % of the tumor cells and 55 % of the parenchymal cells were positive for Ki67 (Fig. 3b).

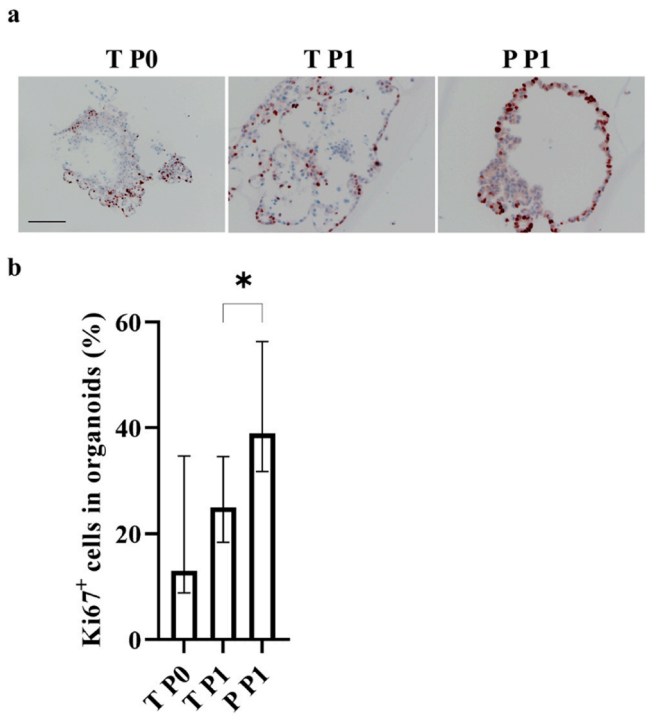

Fig. 6. Proliferation of organoids 30 days after seeding. Passage 0 (P0) and passage 1 (P1) tumor organoids (T) and passage 1 parenchymal organoids (P P1) were cultured for 30 days. **a** Representative IHC analysis of Ki67 (scale bar: 50 μm). **b** Ki67 index (n = 9 for T P0, n = 21 for T P1, n = 7 for P P1). Data were compared by Mann-Whitney test and are shown as the median with interquartile range. $p < 0.01$.

3.2. Tumor organoids can be passaged

Next, we investigated whether organoids can be passaged. For this purpose, passage 0 organoids were cultured for 8 days, dissociated, seeded again in Matrigel and cultivated again for 30 days. With a median diameter of 293 μM, the organoids from tumor cells were significantly larger than those from parenchymal cells (Fig. 4a and b).

In the overview staining, the tumor organoids appeared as spheres with partially denser areas (Fig. 5a). Immunohistochemical staining showed that all passage 0 and passage 1 tumor organoids stained positive for SFTPC and negative for SCGB1A1. In contrast, all passage 1 organoids from parenchymal cells were positive for SCGB1A1 and negative for SFTPC (Fig. 5b and c).

Immunostaining for Ki67 showed around 15–20 % of the tumor cells and 40 % of the parenchymal cells were positive for Ki67 (Fig. 6a and b).

3.3. Coculture with macrophages increases the yield of tumor organoids

Studies showed that macrophages promote tumor proliferation (Casanova-Acebes et al., 2021). To test how macrophages support organoid formation, we cocultured tumor cells with alveolar macrophages placed in the basolateral compartment of the transwells. Co-culture with alveolar macrophages resulted in a significantly increased number of tumor organoids (Fig. 7a) without affecting their size (Fig. 7b). The tumor cystic organoids were positive for SFTPC and negative for SCGB1A1 (Fig. 7c to e).

4. Discussion

We have established an easy-to-perform protocol that allows to culture KRAS-driven tumor cells in 3D for extended periods of time. For this purpose, tumor nodules were prepared from KRAS mice, enzymatically digested and seeded in Matrigel. Within a week, spherical tumor

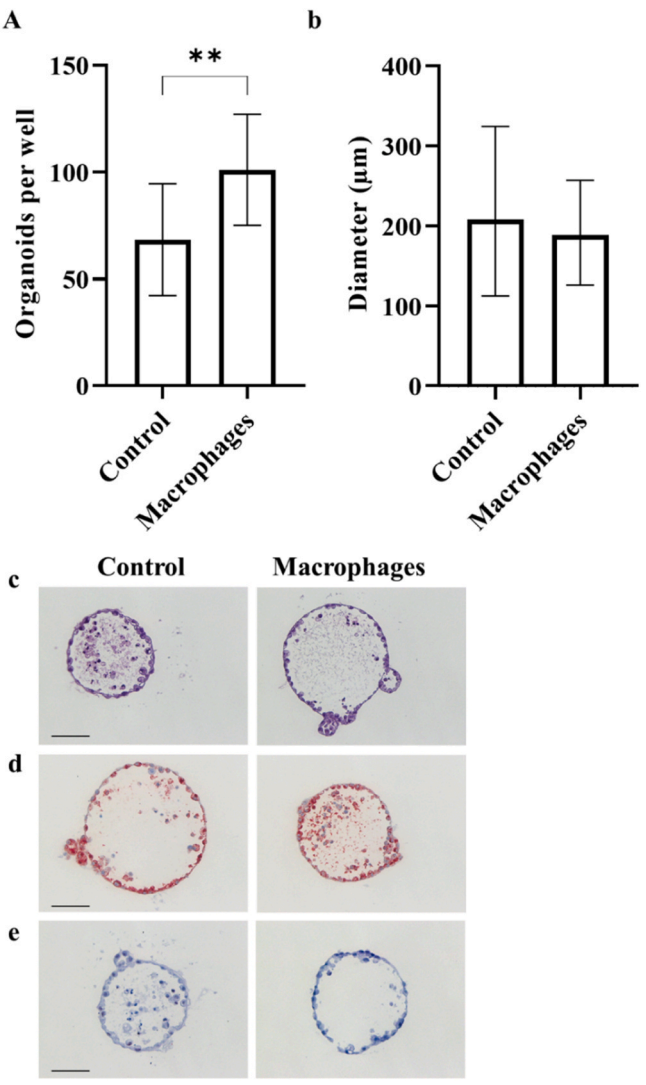

Fig. 7. Co-culture of tumor cells with alveolar macrophages. Passage 0 tumor organoids were cultured with or without macrophages placed in the basolateral compartment of the transwells for 30 days. **a** Number of organoids per well (n = 7 per group). Data were compared by Student's t-test and are shown as ±SD. $p < 0.01$. **b** Diameter of organoids (n = 141 for control, n = 311 for macrophages). Data are shown as the median with interquartile range. **c** Representative H&E staining of organoids. Immunohistochemistry for **d** SFTPC and **e** SCGB1A1. Scale bar: 50 μm.

organoids formed, which could be passaged as single cell suspensions and thus be expanded. As in previous work, we show by immunohistochemistry that tumor cells in KRAS mice are positive for the alveolar type 2 marker SFTPC and negative for the club cell marker SCGB1A1 (Jackson, et al., 2001, Moghaddam, et al., 2009). Like the primary tumors, the tumor organoids were positive for SFTPC and negative for SCGB1A1 in both passage 0 and passage 1. In contrast, parenchymal control organoids, which were cultivated like the tumor cells, were strongly positive for SCGB1A1 and negative for SFTPC. Thus, in our controls, no alveolar organoids formed, but organoids composed of club cells. Therefore, our tumor organoids closely resemble tumor lesions in KRAS mice and represent a model for early tumor development.

About 20 % of the cells per organoid were positive for Ki67 8 days after seeding. This shows that a significant proportion of the tumor cells were proliferating. Compared to tumors in mouse lungs, the proportion of proliferating tumor cells was about four times as high (Ritzmann et al., 2019). This may be because the medium used contained growth-promoting factors such as EGF and insulin. Moreover, club cell

organoids from control parenchyma showed significantly increased proliferation compared to tumor organoids. It is known that club cells have stem cell properties and proliferate vigorously in appropriate media (Lee et al., 2017). This underscores the potential for club cells to overgrow the cultures and the importance of examining the tumor organoids for markers of airway epithelial cells in this model.

Animal experiments are still necessary in cancer research and involve a considerable consumption of mice. In many countries, animal experiments must be reduced to an ethically justifiable level by law. It is therefore important to develop cell culture-based methodologies that can replace animal testing and be applied in basic research and drug development. We think that our model contributes to advance the use of cell culture models as a substitute for animal experiments in basic and translational cancer research. Since tumor cells from genetically modified mice can be integrated into our model, it is also very well suited for mechanistic studies. In addition, such models are also suitable for testing active substances.

5. Conclusion

In summary, we have characterized an easy-to-perform protocol that allows to culture KRAS-driven lung tumor cells in 3D for extended periods of time. The tumor organoids can be passaged. The co-culture with macrophages increased the yield of organoids and also showed that the system can be used for the interaction of tumor cells with inflammatory cells. We think that our model contributes to replacing animal experiments with cell culture systems in cancer research.

Funding

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Ethical statement

Organ harvesting was approved by the Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland in agreement with the national guidelines for animal treatment.

CRediT authorship contribution statement

Jannis Ludwig: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. **Felix Ritzmann:** Conceptualization, Investigation, Methodology, Validation, Writing – original draft. **Andreas Kamyschnikow**: Data curation, Mthodology. **Christian Herr:** Data curation, Writing – original draft. **Robert Bals:** Conceptualization, Writing – original draft. **Christoph Beisswenger:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data Availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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