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Tissue Engineering of a human 3D in vitro tumor test system
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Abstract:	Cancer is one of the leading causes of death worldwide. Current therapeutic strategies are predominantly developed in 2D culture systems, which inadequately reflect physiological conditions in vivo. Biological 3D matrices provide cells an environment in which cells can self-organize, allowing the study of tissue organization and cell differentiation. Such scaffolds can be seeded with a mixture of different cell types to study direct 3D cell-cell-interactions. To mimic the 3D complexity of cancer tumors, our group has developed a 3D in vitro tumor test system. Our 3D tissue test system models the in vivo situation of malignant peripheral nerve sheath tumors (MPNSTs), which we established with our decellularized porcine jejunal segment derived biological vascularized scaffold (BioVaSc). In our model, we reseeded a modified BioVaSc matrix with primary fibroblasts, microvascular endothelial cells (mvECs) and the S462 tumor cell line. For static culture, the vascular structure of the BioVaSc is removed and the remaining scaffold is cut open on one side (Small Intestinal Submucosa SIS-Muc). The resulting matrix is then fixed between two metal rings (cell crowns). Another option is to culture the cell-seeded SIS-Muc in a flow bioreactor system that exposes the cells to shear stress. Here, the bioreactor is connected to a peristaltic pump in a self-constructed incubator. A computer regulates the arterial oxygen and nutrient supply via parameters such as blood pressure, temperature, and flow rate. This setup allows for a dynamic culture with either pressure-regulated pulsatile or constant flow. In this study, we could successfully establish both a static and dynamic 3D culture system for MPNSTs. The ability to model cancer tumors in a more natural 3D environment will enable the discovery, testing and validation of future pharmaceuticals in a human-like model.

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Cover letter

Tissue Engineering of a human 3D in vitro tumor test system

Authors: Corinna Moll, Jenny Reboredo, Thomas Schwarz, Antje Appelt, Sebastian Schürlein, Heike Walles, Sarah Nietzer

The manuscript submitted introduces a novel three-dimensional (3D) *in-vitro* system for tumor models. The authors describe the multi-step set-up of such 3D tissue models, including the decellularization of porcine tissue as scaffold, the isolation of different tumor-associated primary cell types and bioreactor systems that allow static as well as dynamic culture conditions. The reasons why we think that our paper is a very good topic for the JOVE format is that we use very complex bioreactors in our work. So it would be much more catchy to see our bioreactors in a movie than to read a description of its construction plan and handling. Additionally, it would be very informative, to see the development, preparation as well as analysis of our 3D tumor test systems shown by a video. These short-takes of all of the steps during the tumor test tissue generation would illustrate our work optimally. We think that the new field of tissue engineering in developing test systems would be very suitable for being portrayed in your journal. Visualizing the individual experimental steps will render the topic accessible for scientists from different fields of study.

Human in-vitro tumor models will play a major role in future personalized therapeutic strategies and will have a significant impact on tumor drug research by providing a controlled environment mimicking human conditions. Additionally, *in-vitro* tumor models will help to reduce the need for animal testing.

The authors Jenny Reboredo and Thomas Schwarz worked as engineers on the development and construction of the bioreactors. The authors Antje Appelt and Sebastian Schürlein established the cell isolation and culture protocols. Corinna Moll applied the system for the establishment of a human nerve sheath tumor model under the supervision of Sarah Nietzer. Jenny Reboredo and Corinna Moll contributed equally to the work; Jenny Reboredo is listed as corresponding author.

Tissue Engineering of a human 3D *in vitro* tumor test system

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Short Abstract:

Methods to create human 3D tumor tissues as test systems are described. These technologies

are based on a decellularized Biological Vascularized Scaffold (BioVaSc), primary human cells and a tumor cell line, which can be cultured under static as well as under dynamic conditions in a flow bioreactor.

Long Abstract:

Cancer is one of the leading causes of death worldwide. Current therapeutic strategies are predominantly developed in 2D culture systems, which inadequately reflect physiological conditions *in vivo*. Biological 3D matrices provide cells an environment in which cells can self-organize, allowing the study of tissue organization and cell differentiation. Such scaffolds can be seeded with a mixture of different cell types to study direct 3D cell-cell-interactions. To mimic the 3D complexity of cancer tumors, our group has developed a 3D *in vitro* tumor test system.

Our 3D tissue test system models the *in vivo* situation of malignant peripheral nerve sheath tumors (MPNSTs), which we established with our decellularized porcine jejunal segment derived biological vascularized scaffold (BioVaSc). In our model, we reseeded a modified BioVaSc matrix with primary fibroblasts, microvascular endothelial cells (mvECs) and the S462 tumor cell line. For static culture, the vascular structure of the BioVaSc is removed and the remaining scaffold is cut open on one side (Small Intestinal Submucosa SIS-Muc). The resulting matrix is then fixed between two metal rings (cell crowns).
Another option is to culture the cell-seeded SIS-Muc in a flow bioreactor system that exposes the cells to shear stress. Here, the bioreactor is connected to a peristaltic pump in a self-constructed incubator. A computer regulates the arterial oxygen and nutrient supply via parameters such as blood pressure, temperature, and flow rate. This setup allows for a dynamic culture with either pressure-regulated pulsatile or constant flow.

In this study, we could successfully establish both a static and dynamic 3D culture system for MPNSTs. The ability to model cancer tumors in a more natural 3D environment will enable the discovery, testing and validation of future pharmaceuticals in a human-like model.

Introduction:

New pharmaceuticals must be validated in regard to their quality, safety and efficacy before market authorization. To date, animal experiments are the standard method for drug testing and validation. However, due to species-specific differences, animal experiments often do not comprehensively evaluate the effect of the compounds in humans[1]. For this reason, it is important to generate human tissue models that can be used for *in vitro* tests of new drugs and substances.

One of the focuses of our group is the creation of *in vitro* test models with our biological vascularized scaffold (BioVaSc)[2,3]. The BioVaSc can be used as a static or dynamic 3D matrix system. For static culture, the decellularized porcine jejunal segment (Small Intestinal Submucosa SIS-Muc) is placed in a metal insert for cell reseeding. Various cells, such as cancer and endothelial cells can be cultured on the scaffold.

For dynamic culture, the BioVaSc is attached to a bioreactor system that applies flow throughout the vasculature or across the surface of the scaffold. Current bioreactors implement biological, mechanical or electrical stimuli that act upon the differentiation or proliferation of cells[4]. For bioreactors in the field of Tissue Engineering, the basic concept is to simulate the conditions in the human body. Wherein, cells are provided a natural environment in which they can interact with each other and their surrounding extracellular matrix. For the production of *in vitro* test systems or transplants, the ability to mimic the natural environment of cells with an appropriate carrier structure and bioreactor system is critical[5]. Therefore, more complex and technically demanding devices must be developed in order to fulfill these tasks[6].

For the establishment of our tumor model, we used a scaffold derived from a porcine jejunal segment with preserved tubular structures, which include the feeding artery, vein and the connecting capillary bed. All porcine cells were removed by chemical, mechanical and enzymatic decellularization, and the scaffold was gamma-sterilized. The restored tubular vascular structures was subsequently reseeded with human microvascular endothelial cells using a recirculation perfusion bioreactor[7], which mimics the biomechanical and/or biochemical parameters such as pH, temperature, pressure, nutrient supply and waste removal[6]. The re-endothelialization of the tubular structures creates a human blood vessel equivalent within the collagenous scaffold[3,7]. In the following step, the surface of the former lumen (mucosa) was seeded with primary human cells to establish co-culture[3,7,8].

Protocol:

1.) Decellularization of the BioVaSc

1.1) Rinse the vascular system of the porcine jejunal segment via cannulated arterial access and the intestinal lumen with PBS⁻. Repeat until it is completely clean.

1.2) Prepare a 200 mm diameter glass tank with 4 adapters and connect them via silicon tubes to the peristaltic pump (Ismatec). The pressure controlling unit can be monitored via a pressure sensor that is connected to a sterile disposable dome (see figure 1).

1.3) Fill reservoir bottles with decellularization (DZ) solution, check the tubing system for possible air bubbles and pump 500 ml DZ solution into the red arterial access (figure 1B) of the vascular system.

1.4) Connect the intestinal lumen with cable ties to the glass connectors for luminal flow. Interrupt the pumping process every 15 minutes shortly to manually press out the entire intestinal lumen.

1.5) During the decellularization process, the pressure of the buffer solution should be between 80 - 100 mm Hg, modeled after the natural blood pressure.

1.6) Wash the BioVaSc with PBS⁻ until it is free of cell remnants ("completely white").

1.7) Fill the BioVaSc completely with DZ solution and incubate it submers in DZ solution over night at 4°C on rocking shaker.

1.8) Repeat step 1.6.

1.9) Place the BioVaSc in DNase solution and incubate over night at 4°C on rocking shaker.

1.10) Remove DNase solution and rinse with washing buffer.

1.11) γ-sterilization with 25 kGy

2.) The different cell types

2.1) Isolation of primary human dermal microvascular endothelial cells (mvECs) and fibroblasts

2.1.1) Cut skin biopsy (preferably preputium) into strips of 2-3 mm width with scalpel and rinse them 3x with PBS^- solution.

2.1.2) Cover the tissue with dispase solution and incubate it for 16 to 18 h at 4°C.

2.1.3) Separate epidermis from dermis with 2 tweezers and transfer both separately into petri dishes filled with PBS^+.

2.1.4) Rinse dermis strips 1x with Versene.

2.1.5) Add 10 ml Trypsin/EDTA solution to the dermis strips and incubate it in the incubator for 40 min.

2.1.6) Stop the enzyme reaction immediately with 1% FCS.

2.1.7) Transfer skin strips to a petri dish filled with VascuLife and scratch out each strip with the scalpel 8x each side, adding a little pressure.

2.1.8) Transfer the produced cell suspension via a cell strainer to a centrifuge tube and rinse cell strainer 3x with VascuLife.

2.1.9) Centrifuge the tube at 1200 U/min for 5 min and resuspend the cell pellet with VascuLife.

2.1.10) To isolate the fibroblasts, chop dermis strips into little pieces using the scalpel.

2.1.11) Add 10 ml of collagenase solution to dermis pieces.

2.1.12) Incubate it for 45 min in the incubator, then centrifuge it and carefully remove supernatant.

2.1.13) Wash pellet 1x with DMEM + 10% FCS + % PenStrep, centrifuge it and carefully remove supernatant.

2.1.14) Resuspend the pellet in culture medium and transfer it to a T75 culture flask to allow cells to grow out of the tissue.

2.2) Tumor cell line S462

The tumor cell line S462 (kindly provided by Dr. Nikola Holtkamp, Charité University Medicine Berlin) was generated from a malignant peripheral nerve sheath tumor of a female patient with the hereditary tumor predisposition syndrome neurofibromatosis type 1[9].

S462 are cultured in DMEM supplemented with 10% FCS. Medium has to be changed every 2-3 days. Once a week the cells have to be split.

3.) Tumor test system: static culture conditions compared with dynamic culture in bioreactor systems

3.1) Cut the tubular SIS-Muc open on one side and fix it between two metal rings (cell crowns, 10 mm diameter, self constructed). Cover the SIS-Muc in cell culture medium overnight.

3.2) Seed isolated cells in defined cell numbers (see below) on one or both sides of the SIS (mono- or co-culture set-up).

3.2.1) Seed 8,000 cells/cm² of primary mvECs in a total volume of 100 µl onto the basolateral surface of the SIS (former serosa). 3 hours later fill the well with medium to ensure a submersed culture.

3.2.2) Allow endothelial cells to adhere for 3 days. Flip the static culture system by 180° and transfer it to a 12-well plate.

3.2.3) Seed a mixture of primary dermal fibroblasts (8,000 cells/cm²) and tumor cells (15,000 cells/cm²) within a total volume of 500 µl on the apical surface of the SIS (the side of the former lumen).

3.2.4) Allow cells to adhere for 3 hours and fill the well with medium (submersed culture, medium: 50% Vasculife + 50% DMEM supplemented with 10% FCS).

3.2.5) The tumor test system is cultured under static conditions at 37°C, 5% CO_2 in the incubator for additional 14 days. Change culture medium every 2 - 3 days.

3.8) For the dynamic culture, fix the SIS-Muc between two metal rings and seed primary mvECs as described in 3.2.1. After 3 days remove the SIS-Muc from the metal rings and insert the

membrane into the flow reactor (see figure 2C/D). Apply the primary dermal fibroblasts and tumor cells with a syringe and cannula onto the matrix in the bioreactor, and allow the cells to adhere for 3 hours before filling the bioreactor system with culture medium.

3.9) On the following day dynamic culture conditions with constant medium flow (3.8 ml/ min, 37°C and 5% CO_2) can be initiated. The dynamic culture is maintained for 14 days, culture medium is changed after 7 days,

3.10) The dynamic test setup is cultured in a self-constructed incubator that provides media flow through a pump and the necessary temperature and CO_2 content.

4.) Characterization methods for analysis

4.1 Fixing and paraffin-embedding of the seeded collagenous matrix

4.1.1 For (immuno-) histological characterizations remove the culture medium and fix the tissue with 4% paraformaldehyde for 2 h.

4.1.2 Remove 4% paraformaldehyde and transfer the SIS from the metal insert to a tissue embedding cassette. Water the tissue to remove remaining fixative and dehydrate for paraffin infiltration.

4.1.3 Before embedding in a paraffin block, cut the SIS in 2 - 3 slices and place them in a paraffin-filled metal base mold so that the cut surfaces face downwards. Add the tissue cassette on top of the mold as a backing.

4.1.4 Cut 5 μm slices and float them on a 40°C water bath for straightening, then mount slides onto suitable glass slides. Uncoated slides are used for H&E-stains, polylysine-coated slides for immunohistological staining to improve attachment. Let slices dry thoroughly.

4.1.5 Melt paraffin, remove it with xylene and rehydrate slices for following staining.

4.2 Staining

4.2.1 The rehydrated slices can be stained with Hematoxylin/Eosin as a standardized overview staining.

4.2.2 For immunhistological staining, the fixed and paraffin-infiltrated tissue slices must undergo an antigen retrieval to allow antibodies to recognize and bind to their specific epitopes. Therefore, deparaffinized and rehydrated slides are placed in a steam cooker with heated citrate buffer (pH 6,0) for 20 min.

4.2.3 Transfer slides to washing buffer (0.5 M TBS buffer + 0.5% Tween) and circle slices with a PAP pen to minimize the required volume for staining solutions.

4.2.4 Place the slide in a moisture chamber. To secure a specific horseradish peroxidase-mediated visualization of antigen-antibody-binding, the endogenous peroxidase must be saturated with 3% hydrogen peroxide.

4.2.5 Primary antibody dilutions are applied to the slices, incubated for 1 h at RT and carefully washed off with washing buffer.

4.2.6 For detection of specific antigen-antibody bindings the Ultra Vision Quanto Detection System HRP DAB (Thermo Scientific) is used according to the recommended protocol.

4.2.7 Nuclei are counterstained with Hematoxylin for 1 min.

4.2.8 Slides are mounted with an aqueous medium, dried and imaged using an inverse microscope.

Representative Results:

As shown in figure 1B, we decellularized the porcine jejunal segment (about 2 m in length and 20 mm in diameter) with preserved tubular structures of the capillary network. After chemical, enzymatic and mechanical decellularization, we obtained a collagen I/ III scaffold, which can be used for 3D cell culture. A Feulgen test was performed to demonstrate the purity (no DNA remnants) of the matrix (data not shown).

Figure 2A and 2B show the static culture of SIS-Muc secured by the cell crowns. We fixed the SIS-Muc in an in-house designed bioreactor (figure 2C) for dynamic culture. Figure 2D illustrates the simulated dynamic flow through the chamber of the bioreactor. The bioreactor is placed in a self-constructed incubator system and connected with a peristaltic pump. This setup allows a dynamic culture with either pressure-regulated pulsatile flow or constant flow.

Figure 3 gives an overview of the statically cultured S462 tumor cell line in 2D monoculture (figure 3A) and in 3D coculture (figure 3B-D). Figure 3B shows the triple culture of tumor cells S462 and primary fibroblasts on the apical side of SIS-Muc (former inner lumen side) and mvEC on the basolateral side (former serosa side). The identification of different cell types is possible by staining cell-type specific markers, such as von Willebrand factor to label mvEC (figure 3C). The p53-positive S462 cells can be distinguished from the p53-negative primary fibroblasts (figure 3D) and the 3D distribution of cells can be analyzed. Figure 4 shows stainings equivalent to figure 3 of the dynamically cultured triple culture.

Tables and Figures:

Figure 1: DECELLULARIZATION SET-UP. (A) Bioreactor and pump set-up for decellularizing the BioVaSc, monitored by a PC. (B) Decellularized BioVaSc in glass tank. The lumen and the arterial inlet are connected to the adapters.

Figure 2: OVERVIEW OF THE DIFFERENT CULTURE SET-UPS. (A) CAD section view of static culture system in Microtiter well plate, (B) metal inserts for static culture, (C) medium flow simulation (velocity field [m/s]) for dynamic culture of the upper lid of the flow bioreactor, (D) flow bioreactor for dynamic culture connected to the peristaltic pump.

Figure 3: OVERVIEW OF THE IMMUNOHISTOLOGICAL CHARACTERIZATION OF THE STATIC 3D TUMOR MODEL. (A) Hematoxylin stain of the statically cultured 2D mono culture of S462 cells on a permanox slide, (B) H&E stain of the statically cultured 3D triple culture, arrows mark endothelial cells, (C) immunohistological staining for von Willebrand factor, (D) immunohistological staining for p53.

Figure 4: OVERVIEW OF THE IMMUNOHISTOLOGICAL CHARACTERIZATION OF THE DYNAMIC 3D TUMOR MODEL. (A) H&E stain of the dynamically cultured 3D triple culture, arrows mark endothelial cells, (B) immunohistological staining for von Willebrand factor, (C) immunohistological staining for p53.

Discussion:

When comparing 2D and 3D culture systems in tumor research, 3D systems, despite being the more expensive approach, have proven to mimic the conditions in biological microenvironments better. It could be shown that some tumor cells grow much slower in a 3D culture than in a common 2D culture 14, which is in accordance to the situation in a real tumor. Bissell and coworkers showed in their work that the behavior of carcinogenic breast cells reflects the *in vivo* situation, including cell morphology and signaling, more accurately when a 3D culture within a matrix offers cell-ECM interactions. Furthermore, they emphasized the importance of the extracellular environment in 3D by demonstrating that changes in the environmental interactions led to the reversion of the malignant cells to a normal phenotype. Additionally and most importantly, these outcomes could also be confirmed in *in vivo* animal models[10,11].

The direct comparison of *in vivo* animal experiments and *in vitro* tissue models reveals advantages and drawbacks in both systems. One advantage of *in vitro* models is the permission of a much better real-time or fixed imaging by microscopy. A limitation is that they mimic static or short-term conditions, whereas *in vivo* systems often progress. The current lack of vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions are further disadvantages of *in vitro* models[12]. Therefore, 3D *in vitro* systems as presented in this study offer a promising addition to animal experiments. They provide a better comparability to the human organism and therefore minimize experimental misinterpretations. Biomimetic *in vivo* model systems will hence become more relevant to study how cancer and metastatic spread is dependent on microenvironmental conditions that regulate

tumorigenesis[11].

Our study shows that the 3D environment provided by the SIS-Muc leads to a more tumor-like tissue formation of cells, which is not observed in the common 2D cell culture (see figure 3A). Moreover, the use of primary cells derived from tumor biopsies is a very important step towards personalized medicine, a discipline that aims at identifying the best treatment depending on a patient's individual needs. Incorporating primary patient-specific tumor cells isolated from biopsy material will allow in vitro testing of therapeutic strategies. Such test systems will make it possible to investigate different drugs and combinations thereof in a time- and cost-saving high-throughput screening. Additionally, the integration of tumor-associated stromal cells as shown in this study is important for the personalized approach, since a tumor's microenvironment influences tumor progression[13] and might prove as suitable therapeutic target.

Alternatively to a personalized approach, our tumor model can be modified to serve as a generalized tumor test system by the incorporation of established tumorigenic cell lines. This is a promising adaptation for basic research purposes. For both drug testing approaches the presence of a vascular structure is required to test the distribution and uptake of therapeutic substances. The SIS-Muc matrix allows the basolateral seeding with primary mvEC for barrier uptake studies, the reseeding of the preserved vascular structures of the BioVaSc will further improve the study of drug delivery.

In order to create tissue models, a 3D biodegradable matrix can be used as framework for a co-culture of different cell types[14]. The use of such 3D matrices is often limited by the absence of a functional vascularization. This problem can be solved by the use of the BioVaSc, which offers preserved blood vessel structures, which can be reseeded with endothelial cells. Furthermore, the BioVaSc provides extracellular components, which ensure the adhesion of the cells and facilitate tissue differentiation. It also enables the long-time tissue specific function of bioartificial 3D tissues[7,8,15]. The prerequisite for the engineering of functional vascular substitutes is the mimicking of human physiological and biomechanical conditions. Therefore, bioreactor systems, which can implement these requirements *in vitro*, are of extreme interest for creating biological tumor models.

The combination of the BioVaSc, the bioreactor technology and co-culturing of different cell-types is a very promising method to generate vascularized tumor tissues, which will allow the study of mechanisms relevant for cancer progression such as angiogenesis and metastasis. We see such tumor models as a promising approach for complementing animal studies by providing an equivalent to the human tumor physiology.

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Disclosures: Authors have nothing to disclose.

Table of specific reagents and equipment:

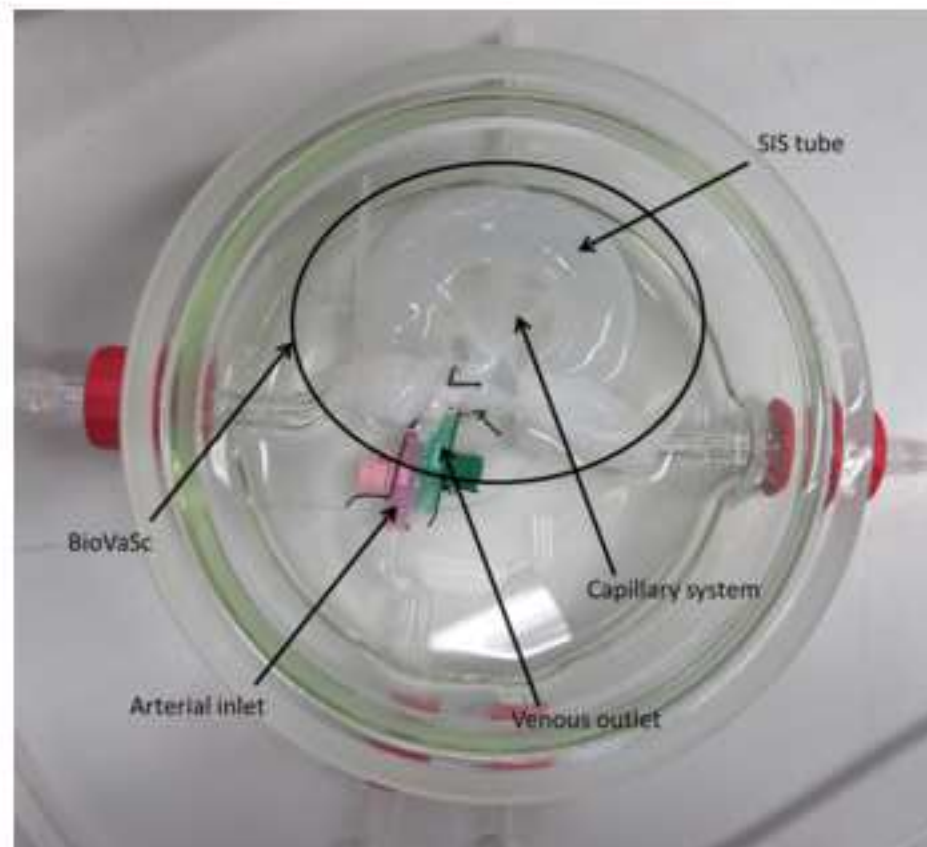| Name of the reagent	Company	Catalogue number	Comments (optional)
Collagenase solution	SERVA	17454	(500 U/ml)
Dispase solution	Gibco	17105-041	(2,0 U/ml)
DMEM, high-glucose	PAA	G0001,3010	
DNase	ROCHE	10104159001	200 mg solved in 500 ml PBS$^+$ + 1% PenStrep
DZ solution	Roth	3484.2	34 g Sodium Desoxychelate , in 1 l Ultra-pure water
FCS	LONZA	DE14-801F	
IHC-Kit DCS SuperVision 2 HRP	DCS	PD000KIT	
medical pressure transducer	MEMSCAP	SP844	
monoclonal mouse anti-human Von Willebrand Factor	DAKO Cytomation	M0616	Clone F8/86 0,12 µg/ml
mouse monoclonal anti-human p53	DAKO Cytomation	IS616	Clone DO-7 ready-to-use
peristaltic pump	Ismatec		
sterile disposable dome	MEMSCAP	844-28	
Trypsin / EDTA solution	PAA	L11-003	0,05%
VascuLife (VEGF-Mv)	Lifeline	LL-0003	
Versene	Gibco	15040-033	

References

1. Schenke-Layland, K., Nerem, R. M. In vitro human tissue models--moving towards personalized regenerative medicine, *Adv. Drug Deliv. Rev.* **63,** 195–196 (2011).

2. Pusch, J., Votteler, M. *et al.* The physiological performance of a three-dimensional model that mimics the microenvironment of the small intestine, *Biomaterials* **32,** 7469–7478 (2011).

3. Schanz, J., Pusch, J., Hansmann, J., Walles, H. Vascularised human tissue models: a new approach for the refinement of biomedical research, *J. Biotechnol.* **148,** 56–63 (2010).

4. Lanza, R., Langer, R., Vacanti, J. *Principles of tissue engineering.* 3rd ed. (Elsevier Academic Press, Burlington, MA, 2007).

5. Barron, V., Lyons, E., Stenson-Cox, C., McHugh, P. E., Pandit, A. Bioreactors for cardiovascular cell and tissue growth: a review, *Ann Biomed Eng* **31,** 1017–1030 (2003).

6. Martin, I., Wendt, D., Heberer, M. The role of bioreactors in tissue engineering, *Trends Biotechnol.* **22,** 80–86 (2004).

7. Mertsching, H., Walles, T., Hofmann, M., Schanz, J., Knapp, W. H. Engineering of a vascularized scaffold for artificial tissue and organ generation, *Biomaterials* **26,** 6610–6617 (2005).

8. Linke, K., Schanz, J., Hansmann, J., Walles, T., Brunner, H., Mertsching, H. Engineered liver-like tissue on a capillarized matrix for applied research, *Tissue Eng.* **13,** 2699–2707 (2007).

9. Holtkamp, N., Atallah, I. *et al.* MMP-13 and p53 in the progression of malignant peripheral nerve sheath tumors, *Neoplasia* **9,** 671–677 (2007).

10. Weaver, V. M., Petersen, O. W. *et al.* Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies, *J. Cell Biol.* **137,** 231–245 (1997).

11. Hutmacher, D. W., Horch, R. E. *et al.* Translating tissue engineering technology platforms into cancer research, *J. Cell. Mol. Med.* **13,** 1417–1427 (2009).

12. Yamada, K. M., Cukierman, E. Modeling tissue morphogenesis and cancer in 3D, *Cell* **130,** 601–610 (2007).

13. Hanahan, D., Weinberg, R. A. Hallmarks of cancer: the next generation, *Cell* **144,** 646–674 (2011).

14. Yang, S.-T., Zhang, X., Wen, Y. Microbioreactors for high-throughput cytotoxicity assays, *Curr Opin Drug Discov Devel* **11,** 111–127 (2008).

15. Schultheiss, D., Gabouev, A. I. *et al.* Biological vascularized matrix for bladder tissue engineering: matrix preparation, reseeding technique and short-term implantation in a porcine model, *J. Urol.* **173,** 276–280 (2005).
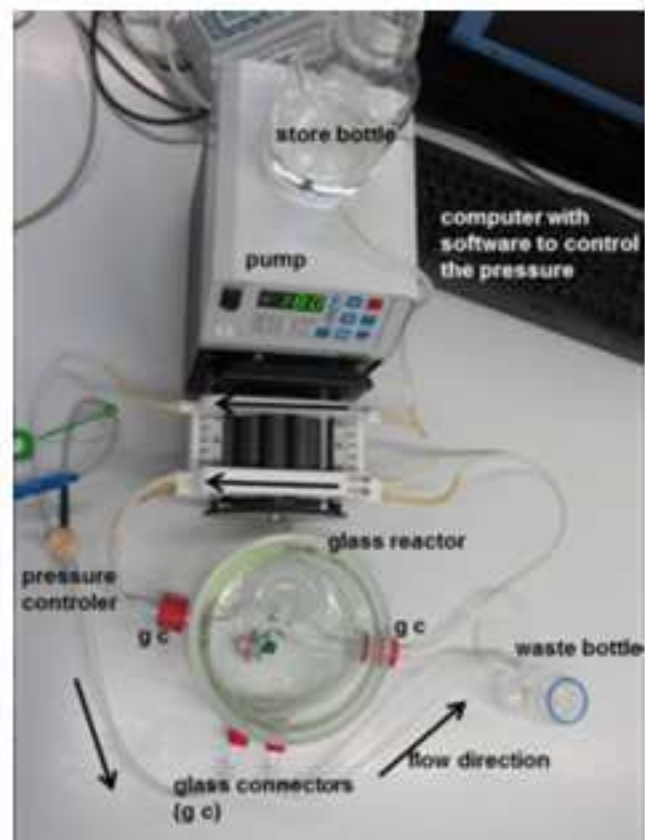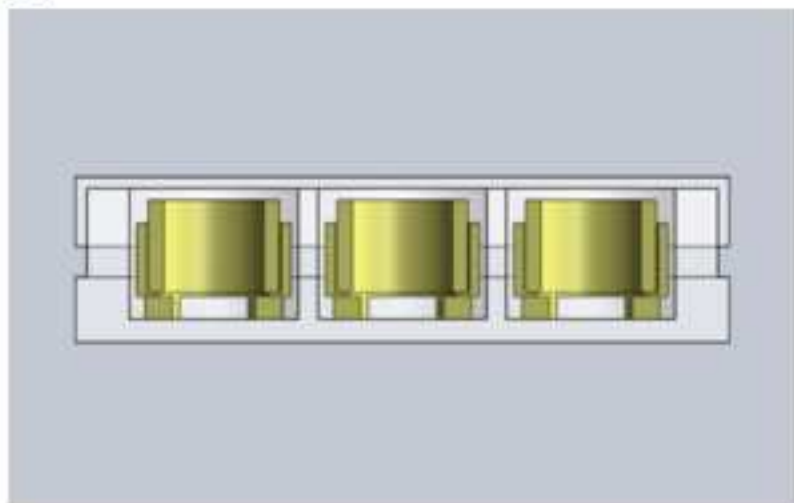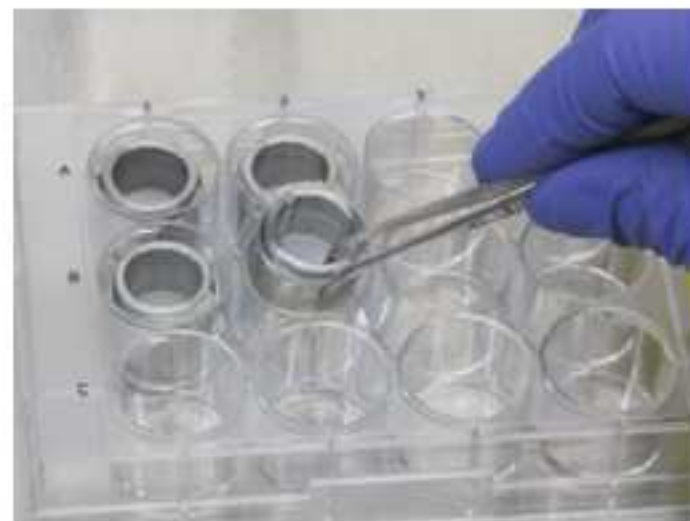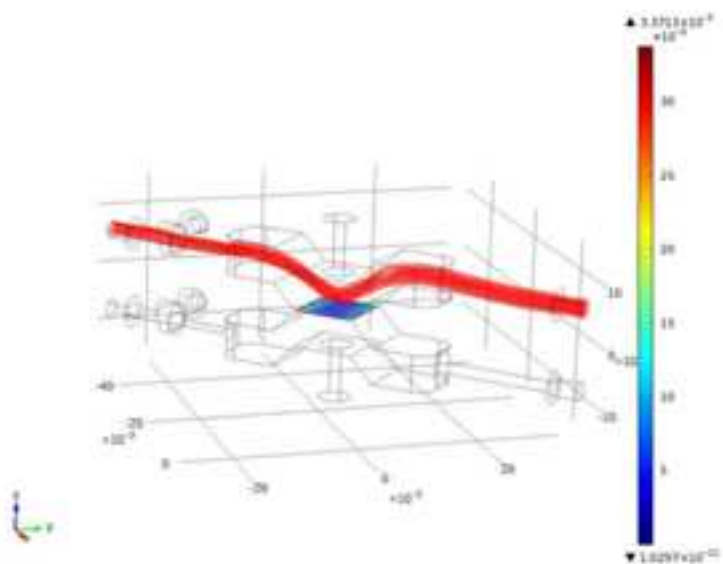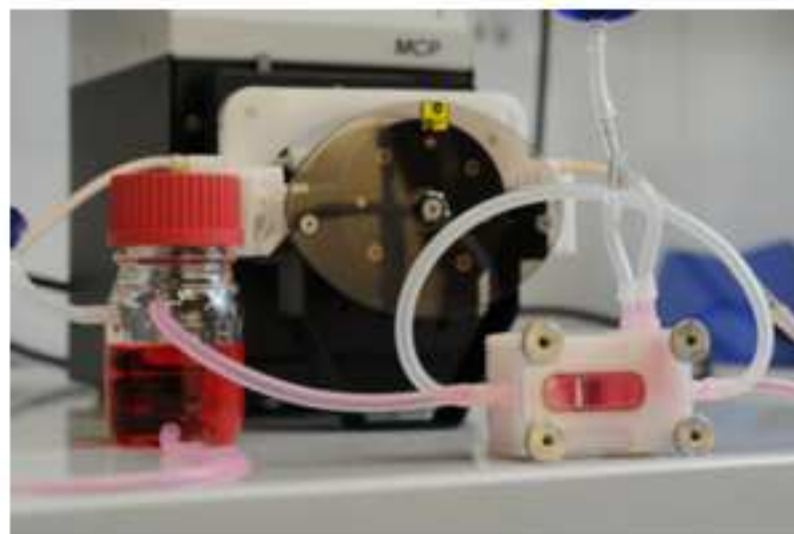
*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: 50460_R0_10_18_12_materials.xlsx

Name of Reagent/Material	Company	Catalog Number	Comments
AZ solution	Roth	3484.2	34 g Sodium Desoxychelate , in 1 l Ultra-pure water
Collagenase solution	SERVA	17454	(500 U/ml)
Dispase solution	Gibco	17105-041	(2,0 U/ml)
DMEM, high-glucose	PAA	G0001,3010	
DNase	ROCHE	10104159001	200 mg solved in 500 ml PBS^+ + 1% PenStrep
sterile disposable dome	MEMSCAP	844-28	
FCS	LONZA	DE14-801F	
IHC-Kit DCS SuperVision 2 HRP	DCS	PD000KIT	
mouse monoclonal anti-human p53	DAKO Cytomation	IS616	Clone DO-7

ready-to-use |
peristaltic pump	Ismatec		
medical pressure transducer	MEMSCAP	SP844	
Trypsin / EDTA solution	PAA	L11-003	0.05%
VascuLife (VEGF-Mv)	Lifeline	LL-0003	
Versene	Gibco	15040-033	

monoclonal mouse anti-human Von Willebrand Factor	DAKO Cytomation	M0616	Clone F8/86 0,12 µg/ml

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Author(s): Reboredo,J. , Moll,C., Schwarz,T., Appelt,A., Schürlein,S., Walles, H., Nietzer, S.

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Dear Editor and Reviewers,

Thank you for your comments regarding our submission, "Tissue Engineering of a human 3D *in vitro* tumor test system". We have addressed your concerns and have included a version of our manuscript with all changes made marked.

Kind regards,

Corinna Moll & Jenny Reboredo

Tissue Engineering of a human 3D *in vitro* tumor test system

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Keywords:
Decellularization, BioVaSc, primary cell isolation, tumor test system, dynamic culture conditions, bioreactor, 3D *in vitro* models

Short Abstract:
Methods to create human 3D tumor tissues as test systems are described. These technologies are based on a decellularized Biological Vascularized Scaffold (BioVaSc~~) () and~~), primary human cells and a tumor cell line, which can be cultured under static as well as under dynamic conditions in a flow bioreactor.

Long Abstract:

Cancer is one of the leading causes of death worldwide. Current therapeutic strategies are predominantly developed in 2D culture systems, which inadequately reflect physiological conditions *in vivo*. Biological 3D matrices provide cells an environment in which cells can self-organize, allowing the study of tissue organization and cell differentiation. Such scaffolds can be seeded with a mixture of different cell types to study direct 3D cell-cell-interactions. To mimic the 3D complexity of cancer tumors, our group has developed a 3D *in vitro* tumor test system.

Our 3D tissue test system models the *in vivo* situation of malignant peripheral nerve sheath tumors (MPNSTs), which we established with our decellularized porcine jejunal segment derived biological vascularized scaffold (BioVaSc). In our model, we reseeded a modified BioVaSc matrix with primary fibroblasts, microvascular endothelial cells (mvECs) and the S462 tumor cell line. For static culture, the ~~vasculature~~vascular structure of the BioVaSc is removed and the remaining scaffold is cut open on one side (Small Intestinal Submucosa SIS-Muc). The resulting matrix is then fixed between two metal rings (cell crowns).
Another option is to culture the cell-seeded SIS-Muc in a flow bioreactor system that exposes the cells to shear stress. Here, the bioreactor is connected to a peristaltic pump in a self-constructed incubator. A computer regulates the arterial oxygen and nutrient supply via parameters such as blood pressure, temperature, and flow rate. This setup allows for a dynamic culture with either pressure-regulated pulsatile or constant flow.

In this study, we could successfully establish both a static and dynamic 3D ~~test~~culture system for MPNSTs. The ability to model cancer tumors in a more natural 3D environment will enable the discovery, testing and validation of future pharmaceuticals in a human-like model.

Introduction:

New pharmaceuticals must be validated in regard to their quality, safety and efficacy before market authorization. To date, animal experiments are the standard method for drug testing and validation. However, due to species-specific differences, animal experiments often do not comprehensively evaluate the effect of the compounds in humans[1]. For this reason, it is

important to generate human tissue models that can be used for *in vitro* tests of new drugs and substances.

One of the focuses of our group is the creation of *in vitro* test models with our biological vascularized scaffold (BioVaSc).)[2,3]. The BioVaSc can be used as a static or dynamic 3D matrix system. For static culture, the decellularized porcine jejunal segment (Small Intestinal Submucosa SIS-Muc) is placed in a metal insert for cell reseeding. Various cells, such as cancer and endothelial cells can be cultured on the scaffold.

For dynamic culture, the BioVaSc is attached to a bioreactor system that applies flow throughout the vasculature or across the surface of the scaffold. Current bioreactors implement biological, mechanical or electrical stimuli that act upon the differentiation or proliferation of cells[3]cells[4]. For bioreactors in the field of Tissue Engineering, the basic concept is to simulate the conditions in the human body. Wherein, cells are provided a natural environment in which they can interact with each other and their surrounding extracellular matrix. For the production of *in vitro* test systems or transplants, the ability to mimic the natural enviromentenvironment of cells with an appropriate carrier structure and bioreactor system is critical[3]critical[5]. Therefore, more complex and technically demanding devices must be developed in order to fulfill these tasks[4]tasks[6].

For the establishment of our tumor model, we used a scaffold derived from a porcine jejunal segment with preserved tubular structures, which include the feeding artery, vein and the connecting capillary bed. All porcine cells were removed by chemical, mechanical and enzymatic decellularization, and the scaffold was gamma-sterilized. The restored tubular vascular structures was subsequently reseeded with human microvascular endothelial cells using a recirculation perfusion bioreactor[5]bioreactor[7], which mimics the biomechanical and/or biochemical parameters such as pH, temperature, pressure, nutrient supply and waste removal[4]removal[6]. The re-endothelialization of the tubular structures creates a human blood vessel equivalent within the collagenous scaffold[5,6]scaffold[3,7]. In the following step, the surface of the former lumen (mucosa) was seeded with primary human cells to establish co-culture[5]culture[3,7,8].

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Protocol:

1.) Decellularization of the BioVaSc

1.1) Rinse the vascular system of the porcine jejunal segment via cannulated arterial access and the intestinal lumen with PBS⁻. Repeat until it is completely clean.

1.2) Prepare reactor as shown in figure 1.

1.2) Prepare a 200 mm diameter glass tank with 4 adapters and connect them via silicon tubes to the peristaltic pump (Ismatec). The pressure controlling unit can be monitored via a pressure sensor that is connected to a sterile disposable dome (see figure 1).

1.3) Fill reservoir bottles with decellularization (DZ) solution, check the tubing system for possible air bubbles and pump 500 ml DZ solution into the red arterial access (~~fig.~~figure 1B) of the vascular system.

1.4) Connect the intestinal lumen with cable ties to the glass connectors for luminal flow. Interrupt the pumping process every 15 minutes shortly to manually press out the entire intestinal lumen.

1.5) During the decellularization process, the pressure of the buffer solution should be between 80 - 100 mm Hg, modeled after the natural blood pressure.

1.6) Wash the BioVaSc with PBS$^-$ until it is free of cell remnants ("completely white").

1.7) Fill the BioVaSc completely with DZ solution and incubate it submers in DZ solution over night at 4°C on rocking shaker.

1.8) Repeat step 1.6.

1.9) ~~Incubate~~Place the BioVaSc ~~with~~in DNase solution and incubate over night at 4°C on rocking shaker.

1.10) Remove DNase solution and rinse with washing buffer.

1.11) γ-sterilization with 25 kGy

2.) The different cell types

2.1) Isolation of primary human dermal microvascular endothelial cells (mvECs) and fibroblasts

2.1.1) Cut skin biopsy (preferably preputium) into strips of 2-3 mm width with scalpel and rinse them 3x with PBS$^-$ solution.

2.1.2) Cover the tissue with dispase solution and incubate it for 16 to 18 h at 4°C.

2.1.3) Separate epidermis from dermis with 2 tweezers and transfer both separately into petri dishes filled with PBS$^+$.

2.1.4) Rinse dermis strips 1x with Versene.

2.1.5) Add 10 ml Trypsin/EDTA solution to the dermis strips and incubate it in the incubator for 40 min.

2.1.6) Stop the enzyme reaction immediately with 1% FCS.

2.1.7) Transfer skin strips to a petri dish filled with VascuLife and scratch out each strip with the scalpel 8x each side, adding a little pressure.

2.1.8) Transfer the produced cell suspension via a cell strainer to a centrifuge tube and rinse cell strainer 3x with VascuLife.

2.1.9) Centrifuge the tube at 1200 U/min for 5 min and resuspend the cell pellet with VascuLife.

2.1.10) To isolate the fibroblasts, chop dermis strips into little pieces using the scalpel.

2.1.11) Add 10 ml of collagenase solution to dermis pieces.

2.1.12) Incubate it for 45 min in the incubator, then centrifuge it and carefully remove supernatant.

2.1.13) Wash pellet 1x with DMEM + 10% FCS + % PenStrep, centrifuge it and carefully remove supernatant.

2.1.14) Resuspend the pellet in culture medium and transfer it to a T75 culture flask to allow cells to grow out of the tissue.

2.2) Tumor cell line S462
The tumor cell line S462 (kindly provided by Dr. Nikola Holtkamp, Charité University Medicine Berlin) was generated from a malignant peripheral nerve sheath tumor of a female patient with the hereditary tumor predisposition syndrome neurofibromatosis type 1[8][1][9].
S462 are cultured in DMEM supplemented with 10% FCS. Medium has to be changed every 2-3 days. Once a week the cells have to be split.

3.) Tumor test system: static culture conditions compared with dynamic culture in bioreactor systems

3.1) Cut the tubular SIS-Muc open on one side and fix it between two metal rings (cell crowns, 10 mm diameter, self constructed). Cover the SIS-Muc in cell culture medium overnight.

3.2) Seed isolated cells in defined cell numbers (see below) on one or both sides of the SIS (mono- or co-culture set-up).

3.2.1) Seed 8,000 cells/cm² of primary mvECs in a total volume of ~100 µl onto the basolateral surface of the SIS (former serosa). 3 hours later fill the well with medium to ensure a submersed culture.

3.2.2) Allow endothelial cells to adhere for 3 days. Flip the static culture system by 180° and transfer it to a 12-well plate.

3.2.3) Seed a mixture of primary dermal fibroblasts (8,000 cells/cm²) and tumor cells (15,000 cells/cm²) within a total volume of 500 µl on the apical surface of the SIS (the side of the former lumen).

3.2.4) Allow cells to adhere for 3 hours and fill the well with medium (submersed culture, medium: 50% Vasculife + 50% DMEM supplemented with 10% FCS).

3.2.5) The tumor test system is cultured under static conditions at 37°C, 5% CO_2 in the incubator for additional 14 days. Change culture medium every 2 - 3 days.

3.8) For the dynamic culture, fix the SIS-Muc between two metal rings and seed primary mvECs as described in 3.2.1. After 3 days remove the SIS-Muc from the metal rings and insert the membrane into the flow reactor. (see figure 2C/D). Apply the primary dermal fibroblasts and tumor cells with a syringe and cannula onto the matrix in the bioreactor, and allow the cells to adhere for 3 hours before filling the bioreactor system with culture medium.

3.9) On the following day dynamic culture conditions with constant medium flow (3.8 ml/ min, 37°C and 5% CO_2) can be initiated. The dynamic culture is maintained for 14 days, culture medium is changed after 7 days,

3.10) Another option is to leave the BioVaSc complete and reseed the preserved blood vessel structures with endothelial cells and fill the lumen of the scaffold with another cell type. In order to imitate the biological heartbeat of a human being, the bioreactor of the dynamic *in vitro* system is adjusted to *in vivo* conditions, such as the blood flow through our loop system with a peristaltic pump (500ml/min) or pulse rates from 60-180 beats/min. Further, the blood pressure is set to 80 to 120 ± 30 mmHg, with a standard deviation volume of 0 – 10 ml/stroke.

3.113.10) The dynamic test setup is cultured in a self-constructed incubator that provides media flow through a pump and the necessary temperature and CO_2 content.

4.) Characterization methods for analysis

4.1 Fixing and paraffin-embedding of the seeded collagenous matrix

4.1.1 For (immuno-) histological characterizations remove the culture medium and fix the tissue with 4% paraformaldehyde for 2 h.

4.1.2 Remove 4% paraformaldehyde and transfer the SIS from the metal insert to a tissue embedding cassette. Water the tissue to remove remaining fixative and dehydrate for paraffin infiltration.

4.1.3 Before embedding in a paraffin block, cut the SIS in 2 - 3 slices and place them in a paraffin-filled metal base mold so that the cut surfaces face downwards. Add the tissue

cassette on top of the mold as a backing.

4.1.4 Cut 5 μm slices and float them on a 40°C water bath for ~~straighting~~straightening, then mount slides onto suitable glass slides. Uncoated slides are used for H&E-stains, polylysine-coated slides for immunohistological staining to improve attachment. Let slices dry thoroughly.

4.1.5 Melt paraffin, remove it with xylene and rehydrate slices for following staining.

4.2 Staining

4.2.1 The rehydrated slices can be stained with Hematoxylin/Eosin as a standardized overview staining.

4.2.2 For immunhistological staining, the fixed and paraffin-infiltrated tissue slices must undergo an antigen retrieval to allow antibodies to recognize and bind to their specific epitopes. Therefore, deparaffinized and rehydrated slides are placed in a steam cooker with heated citrate buffer (pH 6,0) for 20 min.

4.2.3 Transfer slides to washing buffer (0.5 M TBS buffer + 0.5% Tween) and circle slices with a PAP pen to minimize the required volume for staining solutions.

4.2.4 Place the slide in a moisture chamber. To secure a specific horseradish peroxidase-mediated visualization of antigen-antibody-binding, the endogenous peroxidase must be saturated with 3% hydrogen peroxide.

4.2.5 Primary antibody dilutions are applied to the slices, incubated for 1 h at RT and carefully washed off with washing buffer.

4.2.6 For detection of specific antigen-antibody bindings the Ultra Vision Quanto Detection System HRP DAB (Thermo Scientific) is used according to the recommended protocol.

4.2.7 Nuclei are counterstained with Hematoxylin for 1 min.

4.2.8 Slides are mounted with an aqueous medium, dried and imaged using an inverse microscope.

Representative Results:

As shown in figure 1B, we decellularized the porcine jejunal segment (about 2 m in length and 20 mm in diameter) with preserved tubular structures of the capillary network. After chemical, enzymatic and mechanical decellularization, we obtained a collagen I/ III scaffold, which can be used for 3D cell culture. A Feulgen test was performed to demonstrate the purity (no DNA remnants) of the matrix (data not shown).

Figure 2A and 2B show the static culture of SIS-Muc secured by the cell crowns. We fixed the SIS-Muc in an in-house designed bioreactor (~~Figure~~figure 2C) for dynamic culture. Figure 2D illustrates the simulated dynamic flow through the chamber of the bioreactor. The bioreactor is placed in a self-constructed incubator system and connected with a peristaltic pump. This setup allows a dynamic culture with either pressure-regulated pulsatile flow or constant flow.

Figure 3 gives an overview of the statically cultured S462 tumor cell line in 2D monoculture (figure 3A) and in 3D coculture (figure 3B-D). Figure 3B shows the triple culture of tumor cells S462 and primary fibroblasts on the apical side of SIS-Muc (former inner lumen side) and mvEC on the basolateral side~~.~~ (former serosa side). The identification of different cell types is possible by staining cell-type specific markers, such as von Willebrand factor to label mvEC (~~Figure 3B~~figure 3C). The p53-positive S462 cells can be distinguished from the p53-negative primary fibroblasts (~~Figure 3C~~figure 3D) and the 3D distribution of cells can be analyzed. Figure 4 shows ~~staining's~~stainings equivalent to ~~Figure~~figure 3 of the dynamically cultured triple culture.

Tables and Figures:

Figure 1: ~~~~DECELLULARIZATION SET-UP. (A) Bioreactor and pump set-up for decellularizing the BioVaSc, monitored by a PC. (B) Decellularized BioVaSc in glass tank. The lumen and the arterial inlet are connected to the adapters.

Figure 2: OVERVIEW OF THE DIFFERENT CULTURE SET-UPS. (A) CAD section view of static culture system in Microtiter well plate, (B) metal inserts for static culture, (C) medium flow simulation (velocity field [m/s]) for dynamic culture of ~~dynamic culture~~the upper lid of the flow bioreactor, (D) flow bioreactor for dynamic culture connected to the peristaltic pump.

Figure 3: OVERVIEW OF THE IMMUNOHISTOLOGICAL CHARACTERIZATION OF THE STATIC 3D TUMOR MODEL. ~~(A~~(A) Hematoxylin stain of the statically cultured 2D mono culture of S462 cells on a permanox slide, (B) H&E stain of the statically cultured 3D triple culture, arrows mark endothelial cells, (~~B) immunohistological staining for von Willebrand factor, (~~C) immunohistological staining for von Willebrand factor, (D) immunohistological staining for p53.

Figure 4: OVERVIEW OF THE IMMUNOHISTOLOGICAL CHARACTERIZATION OF THE DYNAMIC 3D TUMOR MODEL. (A) H&E stain of the dynamically cultured 3D triple culture, arrows mark endothelial cells, (B) immunohistological staining for von Willebrand factor, (C) immunohistological staining for p53.

Discussion:
~~In the development of drugs, chemicals, cosmetics and in food testing, animal experiments are common, yet time consuming and inefficient. Furthermore, animal models can raise ethical questions. A strategy to avoid these problems is the development and establishment of new tissue models that mimic the conditions in biological microenvironments. Such *in vitro* systems offer a promising alternative or~~When comparing 2D and 3D culture systems in tumor research,

3D systems, despite being the more expensive approach, have proven to mimic the conditions in biological microenvironments better. It could be shown that some tumor cells grow much slower in a 3D culture than in a common 2D culture 14, which is in accordance to the situation in a real tumor. Bissell and coworkers showed in their work that the behavior of carcinogenic breast cells reflects the *in vivo* situation, including cell morphology and signaling, more accurately when a 3D culture within a matrix offers cell-ECM interactions. Furthermore, they emphasized the importance of the extracellular environment in 3D by demonstrating that changes in the environmental interactions led to the reversion of the malignant cells to a normal phenotype. Additionally and most importantly, these outcomes could also be confirmed in *in vivo* animal models[10,11].

The direct comparison of *in vivo* animal experiments and *in vitro* tissue models reveals advantages and drawbacks in both systems. One advantage of *in vitro* models is the permission of a much better real-time or fixed imaging by microscopy. A limitation is that they mimic static or short-term conditions, whereas *in vivo* systems often progress. The current lack of vasculature and normal transport of small molecules, host immune responses, and other cell-cell interactions are further disadvantages of *in vitro* models[12]. Therefore, 3D *in vitro* systems as presented in this study offer a promising addition to animal experiments. They provide a better comparability to the human organism and therefore minimize experimental misinterpretations. ~~Commonly available 2D test systems do not reflect the~~ Biomimetic *in vivo* ~~situation adequately, because~~model systems will hence become more relevant to study how cancer and metastatic spread is dependent on microenvironmental conditions that regulate tumorigenesis[11].

Our study shows that the 3D environment provided by the SIS-Muc leads to a more tumor-like tissue formation of cells, which is not observed in the common 2D cell ~~cell contacts~~ culture (see figure 3A). Moreover, the use of primary cells derived from tumor biopsies is a very important step towards personalized medicine, a discipline that aims at identifying the best treatment depending on a patient's individual needs. Incorporating primary patient-specific tumor cells isolated from biopsy material will allow in vitro testing of therapeutic strategies. Such test systems will make it possible to investigate different drugs and combinations thereof in a time- and cost-saving high-throughput screening. Additionally, the integration of tumor-associated stromal cells as ~~well~~shown in this study is important for the personalized approach, since a tumor's microenvironment influences tumor progression[13] and might prove as ~~cell~~ suitable therapeutic target.
Alternatively to a personalized approach, our tumor model can be modified to serve as a generalized tumor test system by the incorporation of established tumorigenic cell lines. This is a promising adaptation for basic research purposes. For both drug testing approaches the presence of a vascular structure is required to test the distribution and uptake of therapeutic

substances. The SIS-Muc matrix ~~contacts is severely restricted[9,10].~~ allows the basolateral seeding with primary mvEC for barrier uptake studies, the reseeding of the preserved vascular structures of the BioVaSc will further improve the study of drug delivery.

In order to create tissue models, a 3D biodegradable matrix can be used as framework for a co-culture of different cell ~~types[11]~~types[14]. The use of such 3D matrices is often limited by the absence of a functional vascularization. This problem ~~could~~can be solved by the use of the BioVaSc, which offers preserved blood vessel structures, which can be reseeded with endothelial cells. Furthermore, the BioVaSc provides extracellular components, which ensure the adhesion of the cells and facilitate tissue differentiation. It also enables the long-time tissue specific function of bioartificial 3D ~~tissues[5,7,12].~~

tissues[7,8,15]. The prerequisite for the engineering of functional vascular substitutes is the mimicking of human physiological and biomechanical conditions. Therefore, bioreactor systems, which can implement these requirements *in vitro*, are of extreme interest. ~~The properties of the constructs should be analysed and compared to other vascular grafts available as well as to native human tissue~~ for creating biological tumor models.

The combination of the BioVaSc, the bioreactor technology and co-culturing of different cell-types is a very promising method to generate ~~functionalized~~ vascularized ~~tissue. These methods can be useful in the creation of test systems~~tumor tissues, which ~~not only serve~~will allow the study of mechanisms relevant for cancer progression such as angiogenesis and metastasis. We see such tumor models as a ~~replacement of~~ promising approach for complementing animal ~~models, but also are more closely~~studies by providing an equivalent to the ~~situation in the~~ human ~~body.~~

~~In our study, it was clearly shown that the 3D environment provided by the SIS-Muc leads to a more tumor-like tissue formation of cultured cells than the common 2D cell culture. Moreover, the use of primary tumor cells of individual cancer patients is a very important step that can be done in the field of personalized medicine, a discipline that aims at identifying the best treatment depending on the individual needs of a patient. This could avoid ineffective treatments with unnecessary side effects and could save life saving time in determining the best treatment for patient~~tumor physiology.

Disclosures: Authors have nothing to disclose.

Table of specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Collagenase solution	SERVA	17454	(500 U/ml)
Dispase solution	Gibco	17105-041	(2,0 U/ml)
DMEM, high-glucose	PAA	G0001,3010	
DNase	ROCHE	10104159001	200 mg solved in 500 ml PBS^+ + 1% PenStrep
DZ solution	Roth	3484.2	34 g Sodium Desoxychelate , in 1 l Ultra-pure water
FCS	LONZA	DE14-801F	
IHC-Kit DCS SuperVision 2 HRP	DCS	PD000KIT	
medical pressure transducer	MEMSCAP	SP844	
monoclonal mouse anti-human Von Willebrand Factor	DAKO Cytomation	M0616	Clone F8/86 0,12 µg/ml
mouse monoclonal anti-human p53	DAKO Cytomation	IS616	Clone DO-7 ready-to-use
peristaltic pump	Ismatec		
sterile disposable dome	MEMSCAP	844-28	
Trypsin / EDTA solution	PAA	L11-003	0,05%
VascuLife (VEGF-Mv)	Lifeline	LL-0003	
Versene	Gibco	15040-033	

References

1. Schenke-Layland, K., Nerem, R. M. In vitro human tissue models--moving towards personalized regenerative medicine, *Adv. Drug Deliv. Rev.* **63,** 195–196 (2011).

2. ~~Lanza, R., Langer, R,.~~2.   Pusch, J., Votteler, M. *et al.* The physiological performance of a three-dimensional model that mimics the microenvironment of the small intestine, *Biomaterials* **32,** 7469–7478 (2011).

3. Schanz, J., Pusch, J., Hansmann, J., Walles, H. Vascularised human tissue models: a new approach for the refinement of biomedical research, *J. Biotechnol.* **148,** 56–63 (2010).

4. Lanza, R., Langer, R., Vacanti, J. *Principles of tissue engineering.* 3rd ed. (Elsevier Academic Press, Burlington, MA, 2007).

~~3~~5. Barron, V., Lyons, E., Stenson-Cox, C., McHugh, P. E., Pandit, A. Bioreactors for cardiovascular cell and tissue growth: a review, *Ann Biomed Eng* **31,** 1017–1030 (2003).

~~4~~6. Martin, I., Wendt, D., Heberer, M. The role of bioreactors in tissue engineering, *Trends Biotechnol.* **22,** 80–86 (2004).

~~5~~7. Mertsching, H., Walles, T., Hofmann, M., Schanz, J., Knapp, W. H. Engineering of a vascularized scaffold for artificial tissue and organ generation, *Biomaterials* **26,** 6610–6617 (2005).

~~68. Schanz, J., Pusch, J., Hansmann, J., Walles, H. Vascularised human tissue models: a new approach for the refinement of biomedical research, J. Biotechnol. 148, 56–63 (2010).~~

7. Linke, K., Schanz, J., Hansmann, J., Walles, T., Brunner, H., Mertsching, H. Engineered liver-like tissue on a capillarized matrix for applied research, *Tissue Eng.* **13,** 2699–2707 (2007).

~~8~~9. Holtkamp, N., Atallah, I. *et al.* MMP-13 and p53 in the progression of malignant peripheral nerve sheath tumors, *Neoplasia* **9,** 671–677 (2007).

~~9~~10.   Weaver, V. M., Petersen, O. W. *et al.* Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies, *J. Cell Biol.* **137,** 231–245 (1997).

~~10. Walles, T., Weimer,~~11.   ~~M., Linke, K., Michaelis, J., Mertsching, H. The Potential of Bioartificial Tissues in Oncology Research and Treatment, Onkologie 30, 388–394 (2007).~~

Hutmacher, D. W., Horch, R. E. *et al.* Translating tissue engineering technology platforms into cancer research, *J. Cell. Mol. Med.* **13,** 1417–1427 (2009).

12. Yamada, K. M., Cukierman, E. Modeling tissue morphogenesis and cancer in 3D, *Cell* **130,** 601–610 (2007).

13. Hanahan, D., Weinberg, R. A. Hallmarks of cancer: the next generation, *Cell* **144,** 646–674 (2011).

14. 11. Yang, S.-T., Zhang, X., Wen, Y.7. Microbioreactors for high-throughput cytotoxicity assays, *Curr Opin Drug Discov Devel* **11,** 111–127 (2008).

1215. Schultheiss, D., Gabouev, A. I. *et al.* Biological vascularized matrix for bladder tissue engineering: matrix preparation, reseeding technique and short-term implantation in a porcine model, *J. Urol.* **173,** 276–280 (2005).