Nico Jacobi / Veronika Smolinska /Anna Stierschneider / Christian Klein / Kamil Önder / Peter Lechner / Hubert Kaiser / Harald Hundsberger / Andreas Eger

# Development of organotypic cancer models for the identification of individualized cancer therapies

## 107 - Translationale Gesundheitsforschung – Brücken bauen von Grundlagenwissenschaft zu angewandter Forschung

**Abstract**

The response to anti-cancer drugs is determined by the genetic make-up of cancer cells and the cross-talk between tumor cells and its surrounding stromal components. In the present study we have developed experimental strategies to assess drug efficacies in complex organotypic cancer models. We have established advanced cell-and-tissue-engineering technologies for the cultivation of miniaturized tumor-tissue slices for a prolonged period of time in culture. After surgery and tissue dissection, the slices retained the complex tissue architecture of tumors for at least seven days. The tumor cells still proliferated *in vitro* and apoptosis was only weakly detected in peripheral areas of the tumor. To determine the applicability of precision-cut slices for drug discovery, we treated breast cancer specimen with the HER2/HER1 inhibitor Lapatinib for 72 hours and analyzed the extent of apoptosis in the tissues. We detected massive apoptosis in tumors that expressed significant levels of HER2 protein whereas HER2-negative tumors only weakly responded to the treatment. In a further approach we developed customized 3-dimensional (3D) co-culture models consisting of lung cancer cells and tumor-associated fibroblasts that were embedded in Matrigel®/collagen extracellular matrices. The fibroblasts strongly increased the invasive capabilities of the cancer cells, but treatment with the ALK/MET inhibitor Crizotinib almost completely abrogated cancer cell invasion.
Taken altogether, we could demonstrate that both the cancer tissue slices and the customized co-culture models might represent suitable experimental tools for the determination of drug efficacy *in vitro*. They could be used to test the efficacy of a larger number of combinatorial therapies to select the most appropriate drugs for individual patients. As a result organotypic tissue models might open up new vistas in the field of personalized medicine and in clinical oncology.

**Keywords:**

Tumor Mimetics, Tissue Engineering, Drug Discovery, Personalized Cancer Therapy

### 1. Introduction

The identification and molecular characterization of critical oncoprotein networks paved the way for the development of targeted therapies that selectively inactivate oncogenic signaling pathways (Hanahan and Weinberg, 2011). The new remedies inhibit the proliferation of cancer cells and induce apoptosis with high efficacy. However, due to the genetic heterogeneity of cancer cells, not all patients exhibit proper responses to targeted drugs. At present a multitude of genetic and epigenetic traits are already known that can predict clinical drug efficacy in different cancers (Castro-Rojas et al., 2014; Koren and Bentires-Alj, 2015; Lesko and Woodcock, 2004). The individualization of therapies is likely to maximize

the benefit and minimize the harm of therapeutic settings (Daly, 2010; Low et al., 2014; Wheeler et al., 2013).

Over the past decade, carcinomas have been additionally recognized as diseased organs whose cellular complexity may even exceed that of normal tissues (Hanahan and Weinberg, 2011). In the course of tumor progression, a large number of cell types are attracted to the tumor area including (myo)fibroblasts (CAFs), macrophages (CAMs), endothelial cells, pericytes, lymphocytes and bone marrow-derived precursor cells (Pietras and Ostman, 2010). The presence of cancer stem cells and a hierarchical cell organization increases the complexity and the cellular plasticity of the tumor (Clarke et al., 2006; Gupta et al., 2009a; Gupta et al., 2009b). The dynamic co-evolution of cancer and stromal cells eventually culminates in the establishment of a permissive tumor microenvironment that boosts cancer growth, survival and metastatic spread (Axelrod et al., 2006; Gerlinger and Swanton, 2010; Greaves, 2007). Thus the tumor microenvironment can dictate cancer-cell aggressiveness and malignancy, and critically determines the clinical efficacy of anti-cancer drugs and disease outcome (Tredan et al., 2007).

In order to accurately predict clinical drug efficacy *in vitro,* novel standardized, multi-parametric and organotypic drug testing procedures are of paramount importance. Large scale biomarker analyses could be combined with direct drug testing in primary tumor tissue obtained after surgery or biopsy. At present direct *in vitro* testing is limited by the fact that available tissue culture technologies are inadequate to preserve in culture the complex multicellular physiology and architecture of the tumor (Griffith and Swartz, 2006; Yamada and Cukierman, 2007). To date, conventional human cancer cell lines still represent the gold standard for the screening and functional characterization of anti-cancer drugs (Sharma et al., 2010). However, the establishment of cell lines from primary tumor tissues and biopsies can be a long-lasting and tedious enterprise and the success rate is generally too low for implementing a standardized and patient-specific drug testing procedure. Furthermore, serious caveats are associated with cell lines that were propagated in culture for an extended period of time. During prolonged propagation in culture, the cancer cells often lose the prototypical biological features of their naturally growing counterparts *in vivo* and consequently have limited value for predicting clinical drug efficacy (Davies et al., 2015; Hickman et al., 2014; Sharma et al., 2010). Therefore novel tumor cell and tissue engineering technologies need to be developed that will facilitate the development of next-generation cancer models that preserve *in vitro* the major hallmarks of the complex and multifaceted disease. In parallel, innovative tissue and cell-based assays must be developed to quantitatively assess drug-induced effects in the complex organotypic cancer models.

## 2. Results

In the present study we generated and experimentally validated complex organotypic cancer models that were generated from primary breast and lung cancer cells and tissues immediately obtained from the native tumors after surgery. For that purpose we developed a standardized procedure for transport, processing, tissue disintegration and cultivation of precision-cut tissue slices (PCTS) and primary cells. The overall strategy for the development of organotypic tumor models is depicted in

Figure 1. In all cases the time frame between surgery, pathological examination and the generation of tissue or cell cultures did not exceed 3.5 hours. First, we developed permissive culture conditions that allowed the cultivation of the tissue slices for at least five days *in vitro* (Figure 2*)*. In order to assess the viability of the PCTS during the culture optimization process, we stained them with three different dyes that specifically labelled either live and/or dead cells in the tissue (Figure 2). Under the most permissive culture conditions the PCTS could be cultivated for at least five days without any visible degradation of tissues and cells. Larger apoptotic areas were completely absent (Figure 2) and a significant number of cells was still proliferating in the PCTS (Figure 4, Ki67).

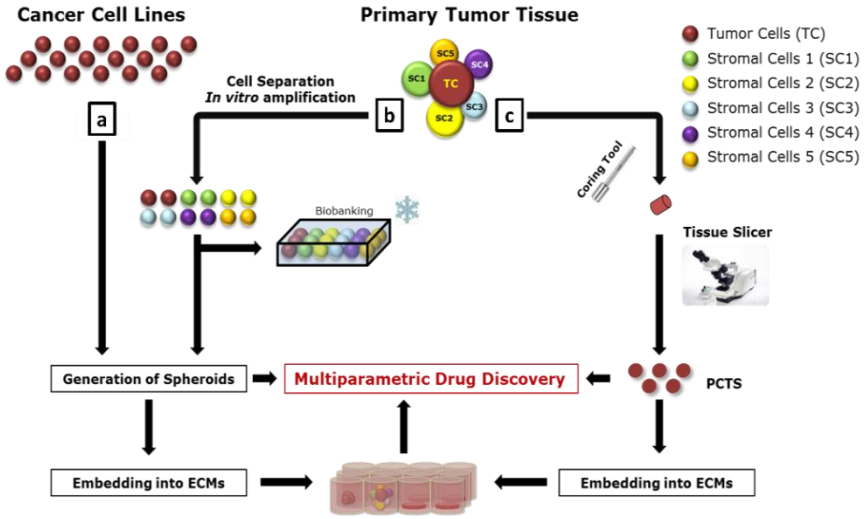


**Figure 1: Work flow for the generation of complex three-dimensional extracorporeal tumor models.** Immediately after surgery the tumor tissue was pathologically assessed and dispensable parts were used for the generation of organotypic tumor models. For transport the native tissue samples were stored in ice-cold sterile RPMI1640 containing L-Glutamine, penicillin and streptomycin. **Left panel, a,b:** Generation of customized homotypic or heterotypic tumor models **(c)** Precision-cut tissue slices (PCTS) of a thickness of 200 µm were generated from cylindrical tissue cores with the Leica VT1200S vibratome and used for drug discovery.

In order to determine tissue integrity, we fixed the PCTS in formaldehyde and processed them for immunofluorescence microscopy, using antibodies against the cell adhesion and signaling protein β-catenin. The protein was mostly detected at the cell periphery (Figure 3), where it co-localized with the cell adhesion molecule E-cadherin (data not shown). Consequently both lung as well as breast PCTS exhibited intact tissue morphology and architecture even after five days of cultivation *in vitro*. Next we analyzed whether the tissue models could be utilized for the drug discovery process. We administered the targeted drug Lapatinib (Tyverb®) to native PCTS derived from breast tumors after surgery and determined the efficacy of the drug 72 hours after drug treatment. Lapatinib specifically blocks the function of tyrosine kinase receptors HER1 and HER2 and is used in the clinic to treat patients with HER2 positive and metastasizing breast cancer (de Azambuja et al., 2014; Gelmon et al., 2015; Lin et al., 2015; Piccart-Gebhart et al., 2015).

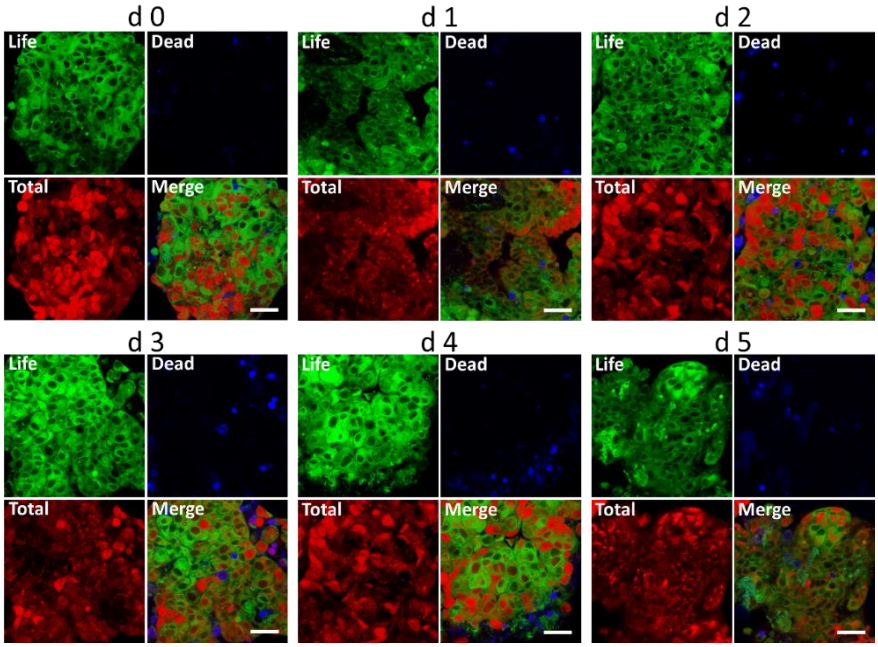


**Figure 2: Viability of tissue slices *in vitro*.** Breast tumor tissue obtained after surgical removal was processed using the Leica VT1200S vibratome to generate 200 µm thick tissue slices and cultivated for up to 120 hours in defined growth medium containing bovine pituitary extract, EGF, insulin, hydrocortisone, transferrin, L-glutamine, penicillin, streptomycin and amphotericin B. To evaluate the viability over time individual tissue slices were stained for 1 hour using Cell Tracker™ orange (green, live cells) and Syto®63 (red, all cells) and then subsequently incubated with Sytox® Blue (blue, dead cells) for 10 minutes. Optical sections were generated with the Leica TCSP2 confocal microscope. Note the small amount of dead cells in the tissue slices after prolonged cultivation *in vitro*. Bars, 30 µm.

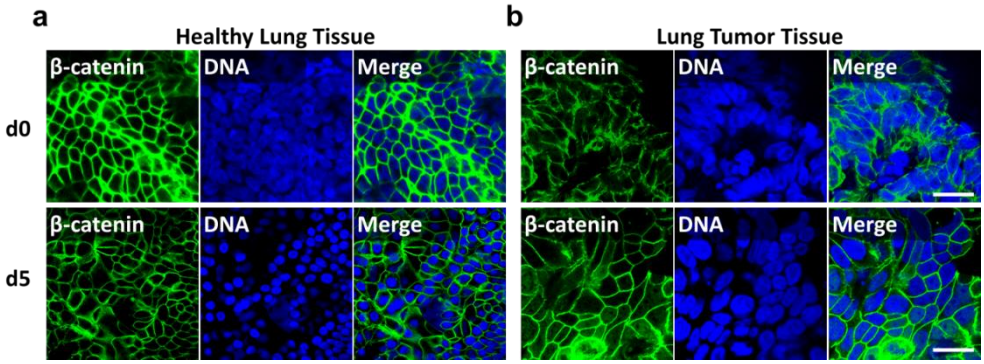


**Figure 3: Preservation of tissue integrity and morphology in PCTS.** Lung tissue was processed using the Leica VT1200S vibratome to generate 200 µm thick tissue slices and cultivated for up to 120 hours in defined growth medium containing bovine pituitary extract, EGF, insulin, hydrocortisone, epinephrine, triiodo-L-thyronine, transferrin, retinoic acid, bovine serum albumin, L-Glutamine and antibiotics. To evaluate the integrity of normal lung tissue **(a)** and lung tumor tissue **(b)** slices were formalin-fixed for 4 hours at room temperature and further processed for immunofluorescence microscopy. Tissue sections were first permeabilized using 0.2% Triton X-100 and blocked with 50 mM $NH_4Cl$/glycine solution. Followed by overnight incubation with β-catenin antibody at 4°C and Alexa Fluor® 488 conjugated goat anti-mouse antibody for 4 hours at room temperature. DNA was counterstained using DAPI. Optical sections were generated with the Leica TCSP2 confocal microscope. The cell adhesion and signalling molecule β-catenin was localized to the plasma membrane indicating the integrity and coherence of both normal and tumor tissue (green). Bars, 30 µm.

Untreated control as well as Lapatinib treated PCTS were processed for immunofluorescence microscopy using antibodies specifically detecting Ki67 (proliferating cells) or the cleaved and activated caspase-3 (apoptotic cells). In breast tumors that expressed significant amounts of HER2 (score 2+) the treatment with Lapatinib resulted in a strong increase in the amount of apoptotic cells in

the tissue (Figure 4b). The number of proliferating cells decreased simultaneously in the same tissues (Figure 4b). On the other hand, HER2-negative PCTS (score 0) exhibited no significant induction of apoptosis and large amounts of proliferating cells were still present in the tissues (Figure 4a). From these data one can conclude that the PCTS derived from HER2 expressing breast cancer show addiction to HER2 protein activity. Lapatinib-induced cancer cell death in PCTS thus closely resembles the behavior of cancer cells in breast cancer patients.

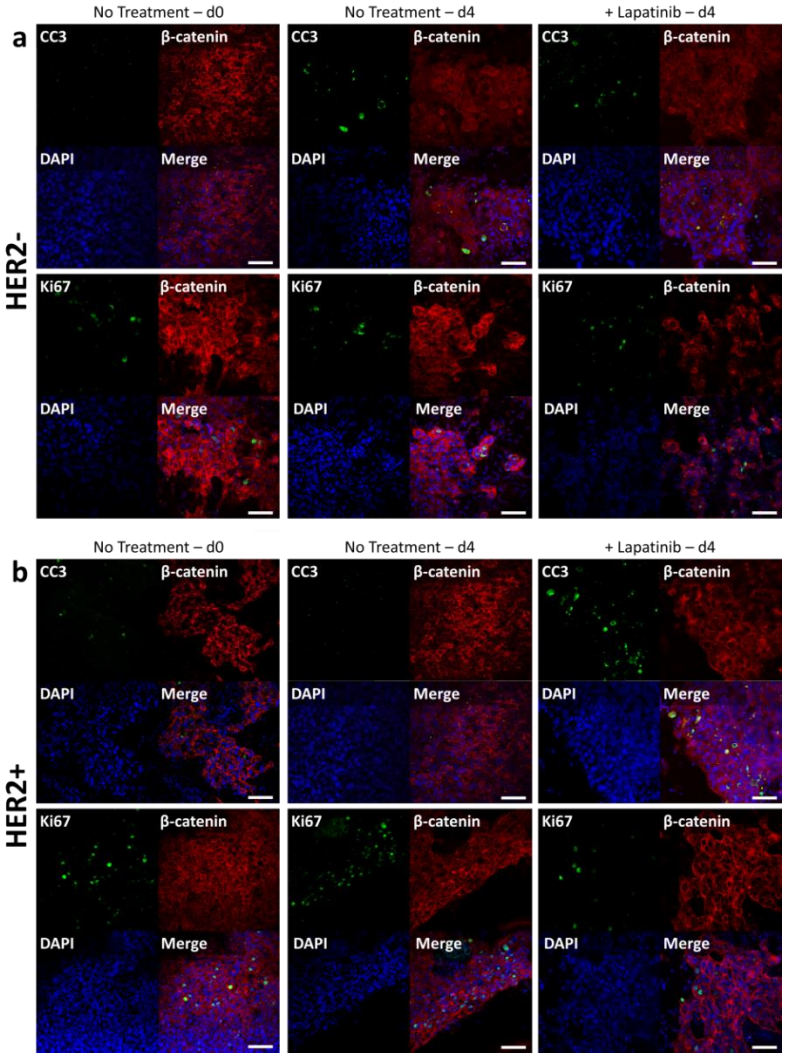


**Figure 4: Lapatinib induces apoptosis in PCTS derived from HER2 expressing breast cancer.** Breast cancer tumors were processed using the Leica VT1200S vibratome to generate 200 µm thick tissue slices and cultivated for up to 96 hours in defined growth medium containing bovine pituitary extract, EGF, insulin, hydrocortisone, transferrin, L-glutamine, penicillin, streptomycin and amphotericin B. Estrogen receptor (ER) and progesterone receptor (PgR) as well as HER2 status was assessed in the tumor tissues **(a)** ER "0", PgR "0", HER2 "0"; **(b)** ER "8", PgR "4-5", HER2 "2+". Tissue slices were treated with 80 nM Lapatinib for 96 hours. Treated and untreated samples were formalin-fixed for 4 hours at room temperature and processed for immunofluorescence microscopy using antibodies against β-catenin, Ki67 and cleaved caspase-3 (CC3). Tissue slices were incubated with Alexa Fluor® 488/568 conjugated goat anti-rabbit/mouse antibodies for 4 hours at room temperature to visualize primary antibodies. DNA was counterstained using DAPI. Confocal laser scanning micrographs of horizontal optical sections show the distribution of CC3, Ki67 (green), β-catenin (red). DNA was stained with DAPI (blue). The field of view of the individual images corresponds to 7-10% of the total slice area and is representative for the complete tissue slice. All experiments were performed in triplicates in six different cancer patients. Two representative image panels are shown. Bars, 75 µm.

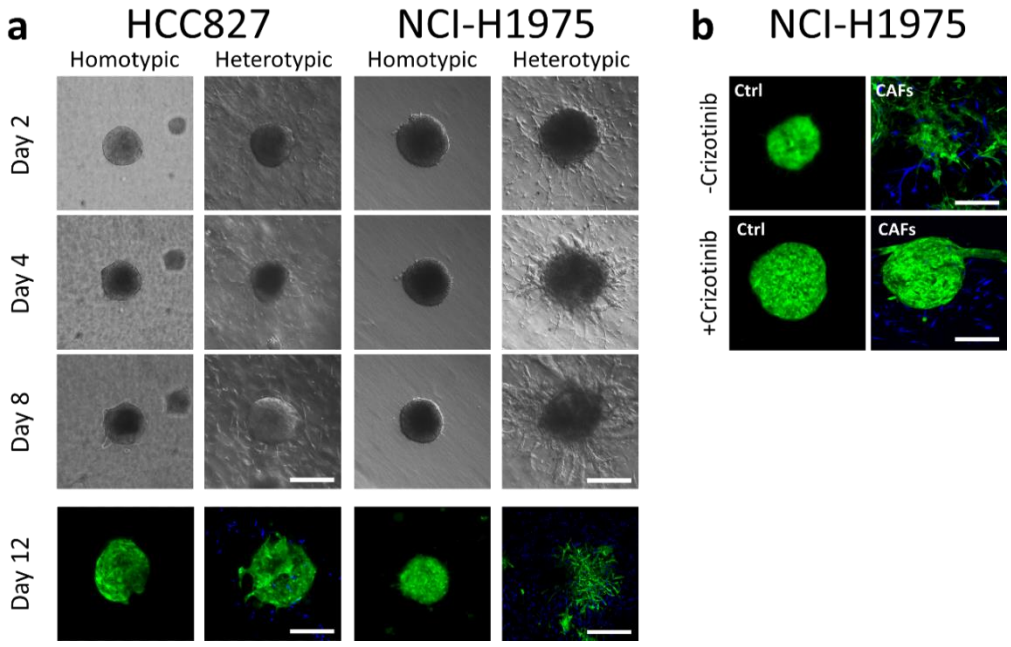


**Figure 5: Fibroblasts promote the invasiveness of lung adenocarcinoma cells (ECM). (a)** Spheroids were generated from the non-small cell lung cancer (NSCLC) cell lines HCC827 and NCI-H1975 for 3 days using GravityPLUS™ plates. Subsequently the spheroids were embedded into matrices consisting of 66% v/v Matrigel®/collagen type I. Homotypic cultures consisting only of cancer cells or heterotypic cultures of cancer cells combined with 5 x $10^4$ cancer-associated lung fibroblasts were cultivated up to 12 days. Phase-contrast images (upper panel) and confocal fluorescence microscopy images (lower panel) are shown. Note that fibroblasts strongly induce the invasive potential of carcinoma cell in the co-cultures. **(b)** The invasion-promoting effect of fibroblasts on tumor spheroids was significantly reduced by treating the cells with the multi-tyrosine kinase inhibitor with Crizotinib (20 nM) for 96 hours. Cancer-associated fibroblasts (CAFs) and cancer cell lines were visualized by lentiviral-based stable expression of monomeric cyan fluorescent protein (mCFP, blue) or Emerald green fluorescent protein (EGFP, green). Bars, 250 µm.

The PCTS retained the complete stromal compartments of the original tumor. However, for the molecular characterization of the mode of action of drugs, it is often advantageous to generate less complex customized cancer models containing only particular cell types (Unger et al., 2014). We therefore developed methods to generate organotypic cultures that consist of cancer cells and cancer-associated fibroblasts and validated their applicability for drug discovery. First lung cancer cell spheroids were generated using GravityPLUS™ culture plates. Spheroids were allowed to form from 5000 cancer cells for three days and subsequently co-embedded together with high numbers of cancer-associated fibroblasts in Matrigel® and rat tail collagen type I. Fibroblasts are major cellular components of the tumor microenvironment that can crucially influence tumor progression. Interestingly, the presence of primary cancer-associated fibroblasts significantly increased the invasive potential of tumor spheroids in both non-small cell lung cancer (NSCLC) cell lines (Fig. 5a and b). On the other hand, the lung cancer cells were only weakly invasive when cancer-associated fibroblasts were absent. After the establishment of the co-culture models, we developed a standardized protocol for drug treatment. The co-cultures were incubated for three days in Matrigel®/collagen matrix and subsequently treated with targeted drugs for four to five days. In co-cultures treated with Crizotinib (Xalkori®) we could detect major phenotypical changes in the cancer cells. Crizotinib is a potent inhibitor of ALK or MET tyrosine kinase receptor activity. Treatment with Crizotinib almost completely abolished the invasive capabilities of the NCI-H1975 (Figure 5b) and HCC827 (data not shown) cancer

cells. The viability of the cells was not significantly affected by Crizotinib treatment. Taken together, we could demonstrate that customized organotypic co-cultures are useful tools for the proper characterization of the mode of action of targeted drugs *in vitro*.

## 3. Conclusion

The complex PCTS cancer models represent unique tools for investigating responses of cancer cells to anti-cancer drugs in the context of an intact tumor tissue. This is of utmost importance in case drugs also target the stromal compartment of the tumor. In the co-cultures the stromal compartment can be randomly manipulated facilitating the study of different combinations of stromal and cancer cells *in vitro.* Thereby the contribution of the different stromal compartments to drug efficacy can be investigated in detail. In combination with animal tumor xenografts, both tumor models will be helpful for appropriately understanding anti-tumor drug activity. Together with genetic and epigenetic biomarker analyses, our organotypic tumor models can open up new vistas for the extensive molecular characterization of the biological effects of conventional and innovative cancer drugs and for personalized medicine.

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