



Precision Cut Cancer Tissue Slices in Anti-Cancer Drug Testing

Florian T. Unger¹, Susanne Bentz¹, Jana Krüger¹, Cordula Rosenbrock², Janina Schaller¹, Katja Pursche¹, Annika Sprüssel¹, Hartmut Juhl^{1,2}, Kerstin A. David¹

ABSTRACT

Background: To meet the urgent need to predict individual drug responses of patients and thus support drug development, better preclinical models of solid tumors are inevitable. Here, a newly developed precision cut cancer tissue slice culture is presented and its use in drug testing was evaluated. **Methods:** Efficacy of therapeutic compounds from different classes, i.e. Staurosporine, IRESSATM, and Herceptin® was tested within the drug testing platform using fresh precision cut cancer slices from human colon, lung, or breast tumor tissues. The stability and significance of the model were evaluated on the level of gene expression, by antibody diffusion assays, and drug responses were detected by immunohistochemical staining, Meso Scale Discovery (Akt, pAkt) analysis, Western blotting (pAkt, pMAPK), viability (ATP) and apoptosis assays (Caspase-3/7). **Results:** We obtained sufficient numbers of tissue slices from cancer specimens to be able to perform a wide range of experiments for each individual tumor. In our culture system, cells remained viable and proliferated for at least 4 days within their tissue environment. Viability of tissue slices decreased significantly due to therapeutic treatment in a dose-dependent manner. Gene expression varied remarkably in primary cultivated cells and HT-29 cells in comparison to cultivated tumor slices, which closely represent freshly isolated tumor tissue. An Alexa Fluor 488-labelled antibody showed diffusion in deeper cell layers and the ability of the system to evaluate effects of antibody therapy. Sustained viability of the precision cut cancer tissue slices over 72h enabled to test different drugs. Staurosporine, IressaTM, and Herceptin® showed a dose-dependent reduction of viability and downstream signaling pathways like Akt and MAPK kinase phosphorylation in EGFR- or Her-2-positive Caco-2 or BT-474 cells or tissue slices, respectively. No effect was seen in EGFR- or Her-2-negative cells and tissues. **Conclusions:** We showed that this preclinical model is applicable to examine the effects of various anti-cancer compounds like cytotoxic chemotherapeutic drugs as well as targeted therapeutics. It may therefore have significant impact on drug development and patient selection for initial clinical trials.

KEY WORDS: Precision cut cancer tissue slices, primary cells, drug testing, 3D culture, chemotherapeutics, IRESSATM, Herceptin®

¹Drug testing/ Indivumed GmbH; Falkenried 88, Building D, Hamburg, Germany,

²Indivutest GmbH; Falkenried 88, Building D, Hamburg, Germany

Address for correspondence:

Florian T Unger,
Drug testing/ Indivumed GmbH;
Falkenried 88, Building D, Hamburg,
Germany,
unger@indivumed.com

Received: June 25, 2015

Accepted: October 15, 2015

Published: October 25, 2015

BACKGROUND

In the process of drug development, it is important to predict efficacy of anti-cancer drug candidates in vivo, therefore predictive in vitro test systems are inevitable. The individual interaction of cancer cells with the surrounding stromal cells [1, 2], the extracellular matrix and the vascular network strongly impacts the efficiency of anti-cancer agents and the resistance to cancer therapy [1, 3-10]. Predictive in vitro/ex vivo culture system should authentically represent this heterogeneity of individual tumors which contain several different cell types and bear various individual genetic alterations [11].

Current anti-cancer therapies are mostly cytotoxic chemotherapeutic drugs which cause serious side effects, act rather unspecific and are therefore not tailored to meet individual needs [12, 13]. Although adjuvant chemotherapies have initially a high response rate, entire populations of patients do not benefit from treatment [14, 15]. Therefore, personalized medicine gets more and more essential [16, 17], comprising new classes of therapeutic agents like small molecules [18-20] and therapeutic monoclonal antibodies

[21-23]. Most of these drugs are directed against specific cellular and molecular targets of signaling pathways, such as key receptors, or signaling proteins, which are elevated in certain tumors [24-26]. Due to the specificity of these drugs, targeted therapies cause fewer side effects [27], meet rather individual therapeutic needs and might be more effective than chemotherapy alone, making the tumors better clinically manageable [28, 29].

Development of new anti-cancer drugs, or direct testing of patient samples in a time-frame suitable for clinical decision making, need a suitable preclinical drug-testing model [30, 31]. The commonly used in vitro systems for drug screening are based on monolayer [32] or spheroid cultures [33-35] of primary cells [15, 36], or cancer cell lines [37, 38]. Unfortunately, these cultures are subject to various genetic and epigenetic changes which occur during the isolation and cultivation process [39, 40] and, even worse, do not necessarily represent the natural microenvironment of the original tumor tissue [41]. Monolayers and spheroids are simplified models that lack the extensive vascularization of high stage solid tumors [28, 32]. The typical radius of spheroids ranges between 150-200µm, whereas the

typical radius of tissue slices lays around 400 μ m necessary for inducing significant hypoxia or heterogeneous drug distribution [42]. All these modifications affect drug response [43] and impair the suitability of these systems to serve as predictive drug testing models for novel and better anti-cancer drugs. Therefore the scope must be extended to actual tissue samples.

The here presented precision cut cancer tissue culture system represents a more promising in vitro model which combines the advantages of maintaining the heterogeneous and complex 3D tissue architecture, cell viability, pathway activity, overall gene expression and natural individual tumor microenvironment [44-47]. The model is based on cultivated cancer tissue slices derived from diverse tumor entities which permits detailed drug testing in individual tumor microenvironments and genetic backgrounds. We examined in "proof of concept" studies whether cultivated tumor tissue slices maintain the properties of the corresponding original tumor tissue and investigated if this model is applicable to evaluate anti-cancer drugs from different compound classes. In our studies we focused on the cytotoxic agent Staurosporine, the small molecule inhibitor IRESSATM and the therapeutic monoclonal antibody Herceptin®. The latter belong both to the new class of targeted therapeutic drugs and interfere with specific signaling pathways like the epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (Her-2) related Akt or Mitogen-activated protein kinase (MAPK) pathway. We showed that this preclinical precision cut cancer tissue culture model allows analysis of target expression, examination of functional drug effects like viability and apoptosis as well as the influence on specific signaling pathways. Taken together this tissue slice culture model is a promising tool to predict drug response and to select promising lead candidates for clinical trials.

METHODS AND MATERIAL

Collection of Patients' Tumor Tissue

All patients suffered from an adenocarcinoma and gave written consent and institutional review board approval was obtained at the physicians association in Hamburg, Germany. Tumor tissues of NSCLC, colon and breast cancer patients were collected according to Indivumed's standard operating procedures. In less than 15 min after completion of surgical resection, tissue samples were either snap frozen in liquid nitrogen, fixed in formalin to serve as reference material or transferred to ice cold tissue preservation medium [RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 % MEM-vitamins, 1 % penicillin/streptomycin, 0.2 % gentamicin, 5 μ g/mL transferrin, 12.5 μ g/mL fetuin, 20 μ g/mL insulin, 10 % fetal calf serum (FCS)] and subsequently used as starting material for the preparation of fresh tissue slices and/or primary cells.

Research involving human subjects in this study was

conducted in compliance with the Helsinki Declaration and all patients gave informed consent. The use of human tissue was approved and the compliance with data protection regulations regarding the anonymization of the samples.

Preparation and Cultivation of Precision Cut Cancer Tissue Slices and Primary Cells

In order to obtain tissue slices cultures, fresh tissue samples were cut into 400 μ m x 5 mm thick tissue slices using Krumdieck™ tissue slicer MD4000-01 (TSE Systems, Bad Homburg, Germany) according to manufacturer's instructions. NSCLC, colon and breast cancer tissue slices were cultured in 24-well plates with tissue culture medium [RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 nM sodium selenite, 10 μ M ethanolamine, 10 μ M phosphorylethanolamine, 100 pM triiodothyronine, 0.5 mM sodium pyruvate, 1 % MEM-vitamins, 1 % penicillin/streptomycin, 0.1 % gentamicin, 0.5 μ g/mL fungizone, 10 μ g/mL transferrin, 2 mg/mL bovine serum albumin (BSA), 1 ng/mL epidermal growth factor (EGF), 12.5 μ g/mL fetuin, 20 μ g/mL insulin and 0.5 mM hydrocortisone, and 5 % FCS] at 37 °C and 5 % CO₂. Additionally, plates were shaken at 100 rpm.

Primary cell cultures were obtained by mechanical disruption of tissues followed by enzymatic digestion for 45 min with 2 mg/mL NB6 collagenase. Cell suspensions were washed twice with cell culture medium (DMEM/F12 medium supplemented with 5 μ g/mL transferrin, 12.5 μ g/mL fetuin, 2 mM L-glutamine, 20 μ g/mL insulin, 1 % penicillin/streptomycin, 1 % MEM-vitamins, 0.2 % gentamicin, 0.5 μ g/mL fungizone, and 10 % FCS) and D-PBS followed by filtration through a 420 μ m steel and 100 μ m as well as 70 μ m cell strainer meshes and centrifuged at 500 x g. Erythrocytes were subsequently lysed using EasyLyse kit from Dako (Hamburg, Germany) according to the manufacturer's instructions. Afterwards cells were seeded on collagen I pre-coated plates (2-4 x 10⁴ cells per 384 well or 0.8-1.6 x 10⁵ cells per 96well). Primary cultures were incubated at 37 °C and 5 % CO₂. Cells were routinely passaged applying 1x trypsin-EDTA. During drug treatment cells were in exponential growth phase.

Cultivation of Cell Lines

Human colorectal adenocarcinoma cell line HT-29 (ATTC, Rockville, MD, USA), human breast adenocarcinoma cell lines MCF7 and BT-474 cells (CLS, Eppelheim, Germany) were cultured in RPMI 1640 medium supplemented with 10 % FCS, 2mM L-glutamine, human colorectal adenocarcinoma cell lines Caco-2 and SW480 cells were cultured in DMEMF12 and DMEM at 37 °C and 5 % CO₂. Cells were routinely passaged applying 1x trypsin-EDTA.

Drug Treatment

All drugs were dissolved in dimethyl sulfoxide (DMSO)

and stock solutions were stored at -80 °C. For compound treatment drugs were diluted in culture medium to the indicated final drug concentrations. Untreated tissue slices or cells served as control. All experiments were done with a solvent control containing the respective DMSO concentration.

Colon cancer tissue slices were treated with 1, 5 and 10 μ M Staurosporine (Sigma-Aldrich, Taufkirchen, Germany) in RPMI 1640 tissue culture medium supplemented with 10 % FCS for up to 24 h.

For IRESSATM treatment (Gefitinib; Cayman Chemicals, Ann Arbor, MI, USA) Caco-2 and SW480 cells were incubated with 0.5, 1, 5 or 10 μ M IRESSATM in RPMI 1640 medium containing 2 mM L-glutamine, 3 % penicillin/streptomycin and 10 % FCS for 24, 48 and 72 h. Colon cancer tissue slices were treated with 1, 10, 100 or 200 μ M IRESSATM in RPMI 1640 tissue culture medium containing 5 % FCS and 3 % penicillin/streptomycin for 24 and 48 h. For epidermal growth factor (EGF) activation cell and tissue culture media were supplemented with 60 ng/mL EGF (Sigma-Aldrich, Taufkirchen, Germany).

For treatment with Herceptin® BT-474 and MCF7 cells were incubated with 0.01, 0.05, 0.1, 1 or 10 μ g/mL Herceptin® (Trastuzumab, Roche Pharma, Grenzach-Wyhlen, Germany) in serum-free RPMI 1640 medium containing 2 mM L-glutamine for up to 24 h. Breast cancer tissue slices were treated with 1, 10, 100 and 1'000 μ g/mL Herceptin® in serum-free RPMI 1640 tissue culture medium for up to 24 h.

Antibody Diffusion Assay and Immunofluorescence

Tissue slices were cultured in RPMI 1640 tissue culture medium for 1 h before incubation with the primary antibody, using either a mouse-anti-EpCAM antibody (VUID9, New England Biolabs GmbH, Frankfurt, Germany, 1:50) or a mouse-anti-PanCytokeratin antibody (Zytomed Systems, Berlin, Germany; 1:50) for up to 24 h. Slices were then washed in PBS, frozen in liquid nitrogen, cut into 5 μ m thick slices using the Cryostat Microm HM500 0 (Microm, Gudenrath, Germany) and fixed in 2 % paraformaldehyde. Afterwards staining with a secondary antibody was performed using an anti-mouse Alexa Flour 488 antibody (Fisher Scientific, Schwerte, Germany) diluted to 1:100 in 0.05 % Tween20/D-PBS for 1h at RT in the dark followed by DAPI staining (Hoechst 33342, Sigma, Steinheim, Germany, 1:10'000) for 10min at RT. Tissue slices were embedded in Fluoromount G (Southern Biotech, Birmingham, US) and components were visualized by fluorescence microscopy (fluorescence microscope, Zeiss, Gottingen). Untreated, DAPI-stained tissue slices served as control.

RNA Isolation and Microarray Analysis

For RNA isolation, tissues and cells were disrupted in RNA-STAT 60 using an Ultra Turrax T8 Homogenizer (IKA, Staufen, Germany). Total RNAs from cells or tissues were

extracted according to the manufacturer's protocol and then purified using Qiagen RNeasy kit (Hilden, Germany). RNA quality and quantity was assessed using the RNA 6000 Nano assay kit (Agilent Technologies, Berlin, Germany) and the Agilent 2100 Bioanalyzer. Only samples with RNA integrity numbers (RIN) > 7 were used for microarray analysis.

Gene expression analysis was performed using high-density HG-U133plus 2.0 GeneChips® from Affymetrix (Santa Clara, CA, USA). For Affymetrix arrays double stranded cDNA was generated from 6 μ g total RNA by reverse transcription using a T7-(dT)24 primer (TIB MOLBIOL, Berlin, Germany) and an Invitrogen SuperScript ds-cDNA synthesis kit. Biotin-labeled cRNA was synthesized using the GeneChip® IVT Labeling Kit (Affymetrix) and subsequently purified on RNeasy columns (Qiagen, Hilden, Germany). Hybridization and staining reactions as well as detection procedures were carried out at an Affymetrix certified laboratory (RZPD, Berlin, Germany) using an Affymetrix Fluidics station and a GeneChipR Scanner 3000 (Affymetrix). Raw data transformation, normalization and calculation of genes flagged "present" were performed using the MAS 5.0 algorithm (Affymetrix). Further examination of expression differences were performed using Array Assist 4.2.0 software (Stratagene, La Jolla, CA). Gene expression differences were analyzed applying an unpaired student's t-test, asymptotic p-value computation and a Benjamini-Hochberg correlation for multiple testings. Genes with a \geq 2-fold change at a p-value \leq 0.05 were considered as significantly changed.

Immunohistochemical Staining

Tumor slices were formalin-fixed, embedded in paraffin and cut to 5 μ m thick sections using the HM340 E rotation microtome (Microm, Walldorf, Germany). Sections were dried overnight at 56°C and subjected to immunohistochemical staining (IHC) for Ki67 (BioGenex, San Ramon, CA, USA; 1:40), Caspase-3 (Cell Signaling, Danvers, MA, USA; 1:250) and Her-2 (ready to use, Ventana, Roche Diagnostics Deutschland GmbH, Mannheim, Germany) using Ventana® Discovery XT staining platform (Ventana Medical Systems, Tucson, AZ, USA). Scoring of quantity and intensity in relation to positive and negative control was performed microscopically.

Haematoxylin and eosin (H&E) staining was performed for assessment of tumor content (data not shown).

Preparation of Cell and Tissue Lysates

Cell and tissue lysates were prepared using phospholysis buffer [containing 20 mM Tris-HCl, 150 mM NaCl, 5mM EDTA, 1 % Triton X 100, Complete protease inhibitor cocktail (Roche, Mannheim, 1:7) and phosphatase inhibitor cocktail (Sigma, Steinheim, 1:100)] or MSD® lysis buffer (Gaithersburg, MD, USA). Lysates were used for ATPlite viability assay, apoptosis assay, western blotting and

multiplex protein discovery assay.

For preparation of tissue lysates tissue slices were frozen in liquid nitrogen, subjected to lysis buffer and homogenized in a swing mill (MM300, Retsch, Haan, Germany) for 2 min at 30 Hz followed by incubation at 4 °C for 30 min. Afterwards, lysates were centrifuged at 8'000 x g and 4 °C for 10 min (Universal 32R centrifuge, Hettich, Tuttingen, Germany). For preparation of cell lysates cells were harvested and washed twice with D-PBS. Afterwards cells were re-suspended in lysis buffer and incubated for 30 min at 4 °C. Finally, lysates were centrifuged at 8'000 x g for 10 min and 4 °C (Universal 32R centrifuge, Hettich, Tuttingen, Germany). Protein concentration was determined by Bicinchoninic Acid Protein assay (Sigma, Steinheim, Germany).

Cell Viability and Apoptosis Assay

Cell viability was measured using the ATPlite® luminescence assay (Perkin Elmer, Rodgau, Germany) and apoptosis induction was detected by Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). In brief, tissue lysates were prepared and subjected either to ATPlite® assay or to Caspase-Glo 3/7 assay in triplicates. Assays were conducted as described in manufacturer's instructions. Luminescence was measured on a FLUOstar OPTIMA® (BMG Laboratories, Germany). To adjust for tissue size variation, the luminescence signals were normalized to total protein concentration.

Multiplex Protein Arrays

Analysis of signaling molecules was conducted using the antibody based technology platform from MSD®. For quantification of Akt a MSD 96-well MULTI-SPOT® assay with specific antibodies against total and phosphorylated Akt (Ser473; duplex format) was selected. The assay was carried out according to manufacturer's protocol using 10 µg of tissue lysate per well in triplicates for each condition. Plates were analyzed by MSD SECTOR™ Imager 2400 (MSD®, Gaithersburg, MD, USA) and relative signal intensity, which corresponds to the quantity of targeted protein, was obtained.

Western Blot Analysis

Semi quantification of proteins was done by using the NuPAGE SDS PAGE and blotting system (Invitrogen, Darmstadt, Germany) according to manufacturer's instruction. In brief, cell or tissue lysates were denatured in NuPAGE LDS sample buffer containing 1x NuPAGE sample reducing agent (NuPAGE, Invitrogen, Darmstadt, Germany) for 10 min at 70 °C, centrifuged at 13'000 rpm for 1 min and loaded onto a 4-12 % Bis-Tris gradient gel (NuPAGE, Invitrogen, Darmstadt, Germany). Afterwards samples were transferred to polyvinylidene difluoride Immobilon-P membrane (PVDF, Millipore Corporation, Billerica, MA, USA) in a wet blot. Membranes were washed 3-times for 10min in washing buffer (50 mM Tris, 150 mM NaCl and

0.3 % Tween20) and blocked (washing buffer containing 5 % low fat milk powder, Roth, Karlsruhe, Germany) for 1h at RT. Primary antibodies, rabbit-anti pMAPK antibody (1:1'000, Cell Signaling, Danvers, USA), rabbit-anti pAkt antibody (1:2'000, Cell Signaling), or mouse-anti GAPDH antibody (1:4'000, Ambion, Woodward, USA; loading control) were incubated over night at 4 °C. After washing 3-times, blots were incubated with peroxidase-labeled secondary antibodies, anti-rabbit HRP antibody (1:10'000, Pierce/Thermo Scientific, Bonn, Germany) or anti-mouse HRP antibody (1:20'000, Pierce/Thermo Scientific) for 1h at RT. Signals were visualized by enhanced chemiluminescence (Luminol Super Signal West, Pierce/Thermo Scientific, Bonn, Germany) and detected by Raytest detection system "Darkroom Evo III" as well as Raytest documentation system (Raytest, Straubenhardt, Germany).

RESULTS

Establishment of a Preclinical Drug Testing Platform

In contrast to monolayers of primary tumor cells, stromal and epithelial components remain intact relative to each other in cultured cancer tissue slices, making them a more promising model for the prediction of drug effects in vivo [1-10].

The here presented preclinical drug testing platform (Figure 1, A) is based on cultured tumor tissue slices and is applicable for analyzing effects of anti-cancer drugs from different compound classes, such as cytotoxic chemotherapeutic agents (e.g. Staurosporine) as well as new targeted therapeutic drugs, like small molecules (e.g. IRESSATM) and therapeutic monoclonal antibodies (e.g. Herceptin®) (see Figure 4-9). The collection of the tissue and the further preparation of highly reproducible tissue slices are controlled by Indivumed's standard operating procedures.

For Indivumed's precision cut cancer tissue slice cultures freshly prepared lung, colon, or breast tumor tissues were cut into 400 µm slices and treated with different drugs for up to 72 h. To ensure best viability culture conditions were optimized for each tumor entity. And a visual quality control during cutting was performed. Preparation and cultivation did not influence expression levels of phospho proteins (data not shown). Subsequently, drug effects can be localized in the intact tumor microenvironment by viability, DNA and RNA as well as proteomic assays (Figure 1, B).

Gene Expression Analysis in Primary Tumor Cells and Tissues

The high density HG-U133 plus 2.0 chips from Affymetrix was used for gene expression analysis. Genes were then analyzed by Onto-Express according to their affiliation to biological processes (Figure 2, A).

Influences of culturing conditions on gene expression in cell lines, primary cells and tissue slices were analyzed.

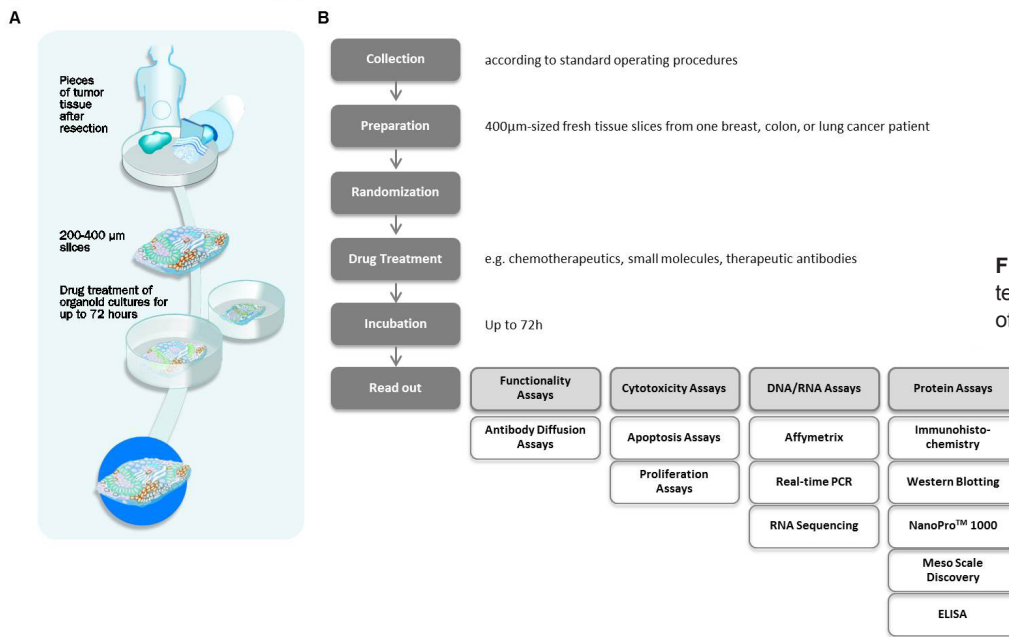


Figure 1. (A) Scheme of Indivumed's drug testing platform, (B) Details and possibilities of Indivumed's drug testing platform

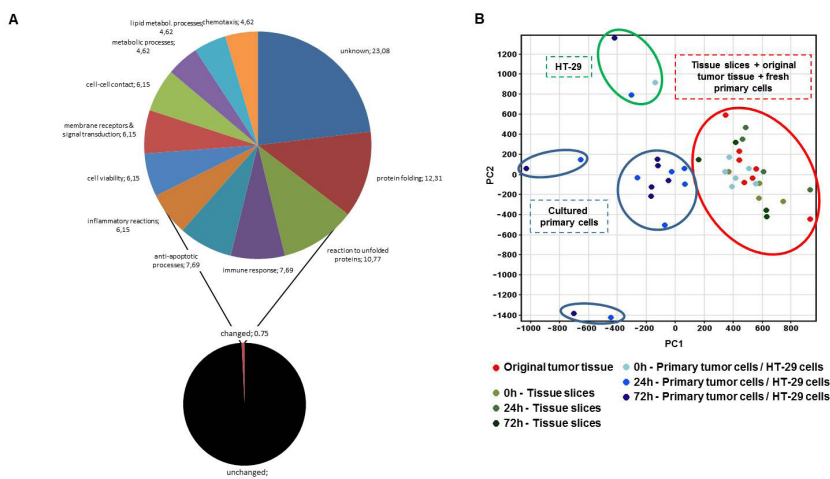


Figure 2. Gene expression analysis of Precision Cut Cancer tissue slices, fresh primary tumor samples, cultured primary cells, and HT-29 cells: (A) Gene ontology analysis: significantly changed genes in 74 tissue slices were analyzed by Onto-Express according to their affiliation to biological processes. Numbers in % (B) Freshly prepared primary tumor cells of 7 CRC patients, tissue slices of 4 CRC patients and HT-29 cells were incubated for up to 72h. Gene expression analysis was performed using Affymetrix microarray. Principle Component Analysis revealed clustering of original tumor tissue, tissue slices and fresh primary cells (red cluster). Additionally, culturing of HT-29 had no influence on gene expression pattern (green cluster). However, cultivation of primary cells seemed to have an influence on gene expression (several blue clusters). PC1: principle component 1, PC2: principle component 2

Pattern similarities were examined by performing a Principle Component Analysis (PCA) (Figure 2, B). We analyzed and compared the tumor-relevant gene expression pattern of oncogenes, tumor suppressor genes, growth factors and genes related to signaling pathways in 4 tissue/cell types and 3 cultivation periods in colorectal specimens: original tumor tissue, corresponding freshly prepared tumor slices, primary tumor cells, and a colorectal cell line. Strong distance of data points demonstrated strong differences in gene expression.

Freshly prepared tumor tissues of 7 colorectal cancer (CRC) patients were snap frozen directly after resection, representing the original tumor tissue (red dots). Six primary tumor cell populations of the same patients (blue dots), 4 tissue slices (green dots) and HT-29 cells (blue dots) were analyzed prior to cultivation (0 h) and after 24 h and 72 h of in vitro cultivation and compared with the original tumor

tissue. Due to quantity limitations it was not possible to isolate and culture slices or primary slices from all patient's samples. To test the effect of homogeneity HT-29 cells were used.

The basal expression pattern of original tumor tissue of all specimens (red dots) spread over a wider array range (red cluster), demonstrating the individual differences in tumor-specific gene expression patterns within a heterogeneous human population. The patterns of freshly isolated primary tumor cells (light blue dots, red cluster) and freshly prepared tissue slices (light green dots, red cluster) were closely located with those of original tumor tissue depicting similarities in gene expression. This indicated that the preparation procedures did not remarkably influence the gene expression. Moreover, in tissue slices gene expression patterns changed only marginal during short time cultivation

of up to 72 h (darker green dots, red cluster). However, cultivation of primary tumor cells strongly altered their expression patterns of genes (blue dots) and resulted in the formation of several populations (blue clusters). The cell line HT-29 demonstrated a gene expression pattern that differed considerably from the original and cultivated tumor tissues as well as primary cells (blue dots, green cluster). This observation illustrated that the immortalized cell line does not reflect the heterogeneity of human tumor samples and that they might be less suited for drug testing. In order to comprehensively demonstrate the homogeneity of immortalized, clonal cell lines compared to tissues or tissue slices, a higher number of cell lines have to be included in the analysis.

Viability and Proliferation of Non-small Cell Lung Cancer Precision cut cancer Tissue Slices

Viability and morphological integrity of untreated cultured tissue slices is a prerequisite for drug testing to ensure that observed changes in tissue physiology are caused by the respective drug. Furthermore, some compounds like for example oxaliplatin [48] or 5- fluorouracil [49] are only effective in proliferating cells. Thus, viability and proliferation of cultured tissue slices were analyzed,

exemplified with non-small cell lung cancer (NSCLC) tissue (Figure 3).

Freshly prepared NSCLC tissue slices were cultured for up to 72h and proliferation as well as apoptosis was examined by Ki67 (proliferation marker) and Caspase-3 (apoptosis marker) immunohistochemistry (IHC) staining. Nearly no differences in Ki67 expression pattern could be observed during 72h compared to the starting point (Figure 3, upper panel), indicating enduring cell proliferation. Furthermore, the morphology of the tissue remained unchanged. Only a slight induction of apoptosis was visible at 72 h of cultivation (Figure 3, lower panel), confirming that these tissue slice cultures are viable and suitable for drug testing experiments.

Viability and Pathways Activation in Staurosporine-treated CRC Precision Cut Cancer Tissue Slices

To examine whether the effects of cytotoxic agents can be measured in tissue slice cultures, CRC tissue slices were treated with different concentrations of Staurosporine for 24 h. Staurosporine is a well-known inducer of apoptosis by inhibiting protein kinases [50].

Adenosine triphosphate (ATP) (Figure 4, A) and Caspase-3/7 (Figure 4, B) served as read out assays, resulting in a dose-

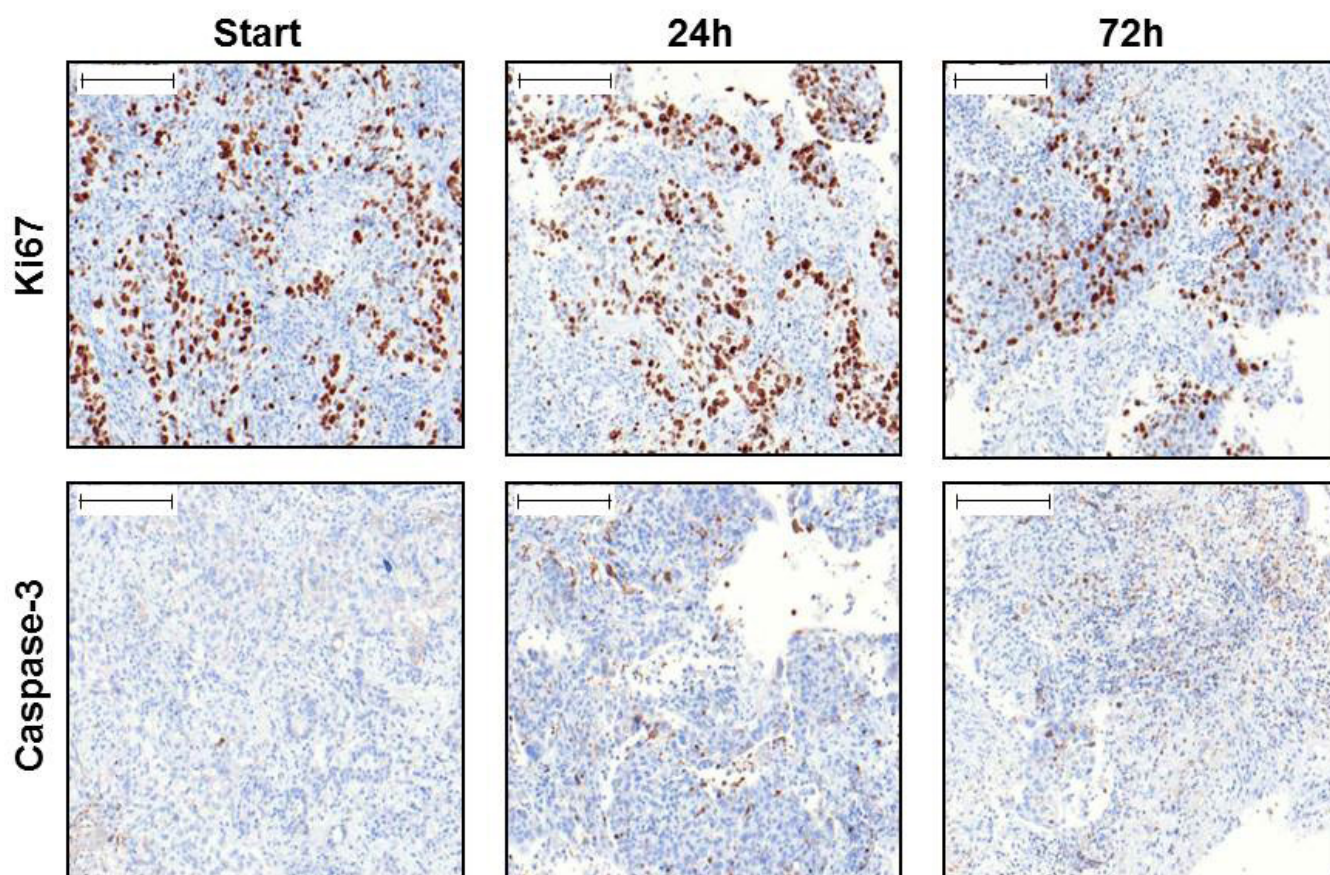


Figure 3. Viability of NSCLC Precision Cut Cancer tissue slices: NSCLC tissue slices were incubated in full growth medium. Ki67 (proliferation, upper panels) and Caspase-3 (apoptosis, lower panels) staining was performed directly after resection (start), after 24 and 72h of cultivation, showing only slight induction of apoptosis. Blue: haematoxylin, scale bars: 200µm

dependent reduced viability (up to 67 % reduced ATP levels with 10 μM) and apoptotic effect (up to 4-times higher Caspase-3/7 levels with 10 μM).

Additionally, the influence on pro-apoptotic and anti-survival cellular signaling [51] was analyzed by Meso Scale Discovery (MSD®) technique (Figure 4 C and D). Staurosporine treatment of CRC tissue slices resulted in a dose-dependent reduction of both, total Akt (up to 94 % with 10 μM) and pAkt levels (up to 85 % with 10 μM), indicating elevated apoptotic events.

Effects of IRESSATM on CRC Cell Lines and Precision Cut Cancer Tissue Slices

Cytotoxicity assays demonstrated the induction of apoptosis in tissue slice cultures treated with a cytotoxic chemotherapeutic agent (see Figure 4). To demonstrate that it is also possible to detect effects of therapeutic small molecules in cultured tissue slices we used the EGFR inhibitor IRESSATM (Gefitinib). IRESSATM binds to the cytoplasmic domain of EGFR and inhibits receptor auto phosphorylation, thus interrupting the following

downstream signaling cascades including the Akt and MAPK pathway [52].

Functionality of IRESSATM was initially analyzed in EGFR-positive and -negative CRC cell lines. Prior performed IHC staining with an anti-EGFR antibody revealed nearly no EGFR in SW480 cells, whereas elevated EGFR levels could be detected in Caco-2 cells (Figure 5, A). SW480 cells showed only a slight reduction of ATP after 48 h or 72 h treatment (Figure 5, B). In contrast, Caco-2 demonstrated a dose-dependent reduction of viability (Figure 5, C), illustrated by decreasing ATP (up to 54 % and 52 % with 10 μM for 24 h and 72 h, respectively). For pathway analysis on IRESSATM-treated, EGF stimulated cell lines the phosphorylation status of the key signaling protein Akt was evaluated by Western blotting. As depicted in Figure 5 D, Caco-2 showed a dose-dependent reduction of pAkt already after 24 h. In contrast nearly no effect could be observed in EGFR-negative SW480 cells in which pAkt levels only slightly decreased at the highest IRESSATM concentration after 48 h.

Based on these observations, responsiveness of cultured tissue slices was examined. IHC staining demonstrated

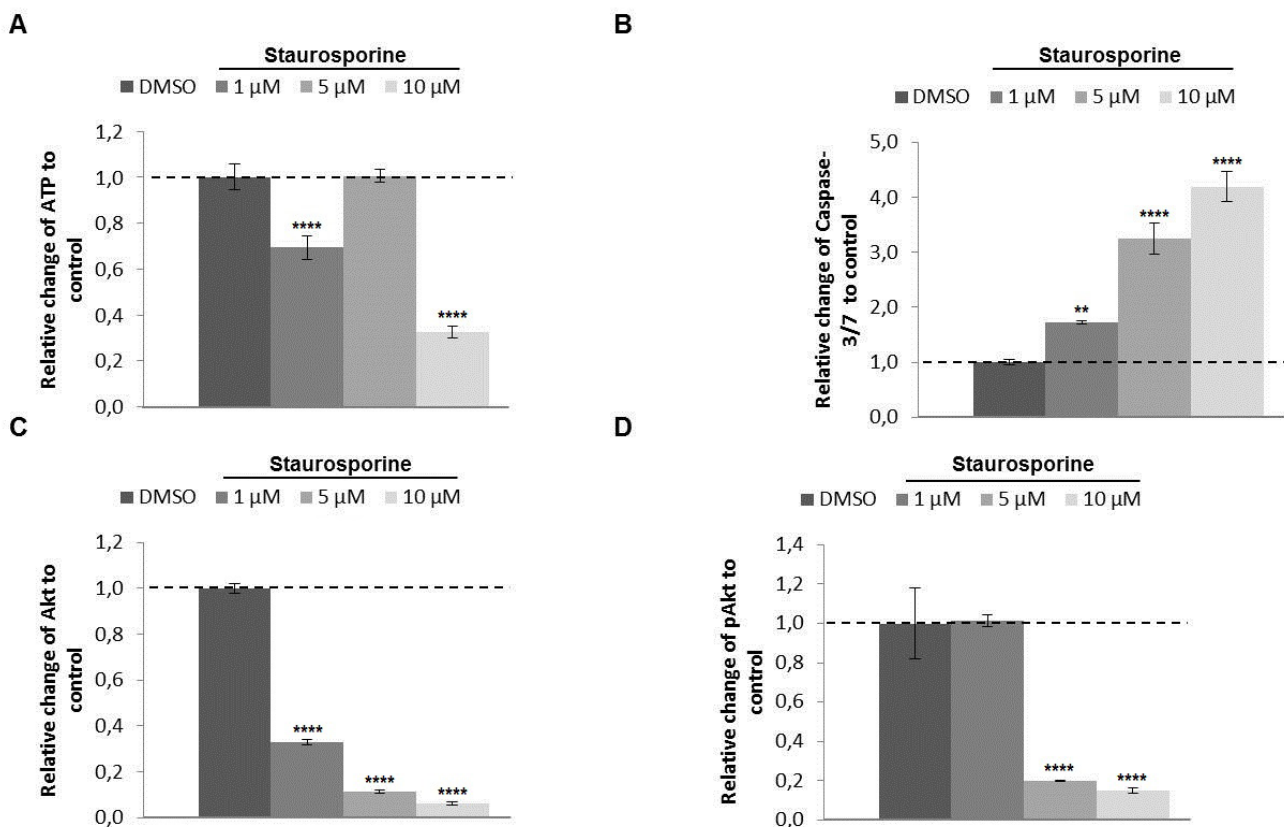


Figure 4. Induction of apoptosis and reduction of Akt and pAkt expression in Staurosporine-treated CRC Precision Cut Cancer tissue slices: Tissue slices of one CRC patient (A3146) were treated with Staurosporine for 24h. (A) ATP levels were significantly reduced by treatment with 10 μM Staurosporine, reflecting reduced cell viability. (B) Accompanied by increased apoptosis measured by Caspase-3/7 levels. (C) Total and (D) phosphorylated Akt levels reduced in a dose-dependent manner. Values are shown as relative change (mean values of triplicates) of expression levels to DMSO control (= 1). Statistical significance has been calculated in comparison to the DMSO control. P-values are indicated by asterisks (* = 0.05; ** = 0.01; *** = 0.001; **** = 0.0001)

a high EGFR expression level in one representative case (Figure 6, A). EGF receptors in tissue slices of this case were stimulated with 60ng/mL EGF and treated with 4 different IRESSATM concentrations for 24 h and 48 h. Cell viability showed only a slight decrease of ATP levels after 24 h at the highest IRESSATM concentration whereas a dose-dependent reduction of ATP was visible after 48 h (47 % with 200 μM) (Figure 6, B). This effect was also verified by Western blotting, showing dose-dependent reduced expression of pAkt and pMAPK (Figure 6, C).

Since both proteins are induced in their phosphorylated form cell survival and growth are inhibited. These findings verified a cytotoxic effect of IRESSATM also in EGFR-positive tissue slice culture. Enabling the assessment of the response in the individual expression background [47].

Diffusion of Antibodies over the Cell Membrane in Precision Cut Cancer Tissue Slices

Monoclonal therapeutic antibodies as well as small molecule tyrosine kinase inhibitors were designed to interrupt the signaling pathways of a cell. In contrast to small molecules, which are small enough to pass the cellular membrane and act intracellularly [52], the much larger monoclonal antibodies are directed against the extracellular domain of transmembrane receptors/channels. In order to analyze, if effects of antibodies can be tested in our model we used the widely expressed epithelial cell adhesion molecule (EpCAM), to show that antibodies diffuse into the tissue slices and bind to their target.

CRC tissue slices were incubated with anti-EpCAM antibody for 2-24 h. Afterwards immunofluorescent staining was performed. In Figure 7 A the fluorescence signals appears at the outer regions of the tissue slice after 2 h of incubation. With increasing incubation time (24 h) time-dependent diffusion in deeper cell layers occurred (Figure 7, B). The specificity of binding and staining was shown by positive and negative controls (Figure 7, C).

The diffusion of a therapeutic antibody, such as Herceptin® (Trastuzumab), into breast cancer tissue is has to be shown. Nevertheless, diffusion and binding of Herceptin® (Trastuzumab) into breast cancer tissue is indirectly shown by presenting effects of the antibody in the following section.

Effect of Herceptin® on Breast Cancer Cell Lines and Precision Cut Cancer Tissue Slices

Our previous experiments showed that antibodies diffused into cultured tissue slices (see Figure 7).

The therapeutic antibody Herceptin® (Trastuzumab) is approved for anti-cancer treatment of Her-2 receptor positive breast cancer. Herceptin® interacts with the extracellular domain of Her-2 (Erb-B2) transmembrane receptor and prevents receptor dimerization and auto phosphorylation leading to inhibition of the downstream signaling [53].

The breast cancer cell lines BT-474 and MCF7 were immunohistochemically stained. BT-474 cells were strongly Her-2 receptor positive (Figure 8, A) and demonstrated a

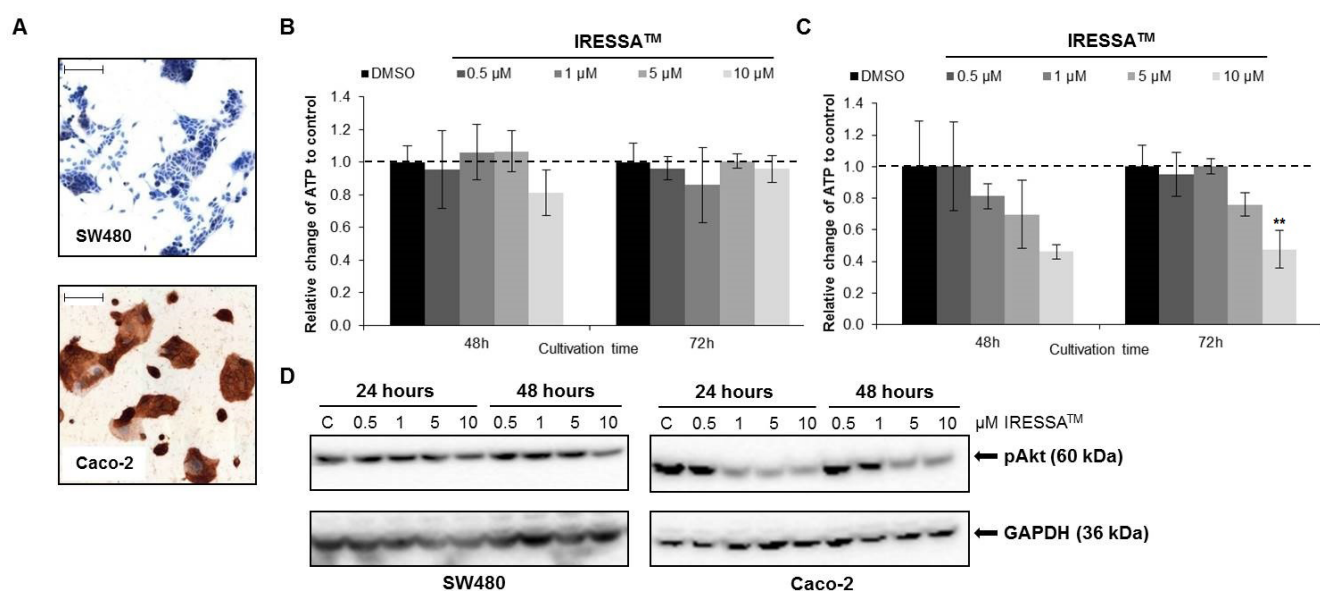


Figure 5. Effect of IRESSATM treatment on viability and pathway activity of EGFR-positive and -negative colorectal cell lines: (A) EGFR staining (brown) showed high EGFR expression levels in Caco-2 cells (EGFR positive) but no expression in SW480 cells (EGFR negative). Blue: haematoxylin, scale bars: 100μm (B) EGFR-negative SW480 only showed a decrease after 48h treatment with 10μM IRESSATM. (C) EGFR-positive Caco-2 showed a dose-dependent reduction of ATP and thus of viability after treatment with IRESSATM. Values are shown as relative change (mean values of triplicates) of expression levels to DMSO control (= 1). (D) Additionally, Caco-2 cells showed a dose-dependent reduction of pAkt already after 24h. Nearly no effect could be observed for SW480 cells which showed only a slight reduction of pAkt after 48h of treatment with 10μM IRESSATM. C: DMSO control. Statistical significance has been calculated in comparison to the DMSO control. P-values are indicated by asterisks (* = 0.05; ** = 0.01; *** = 0.001; **** = 0.0001)

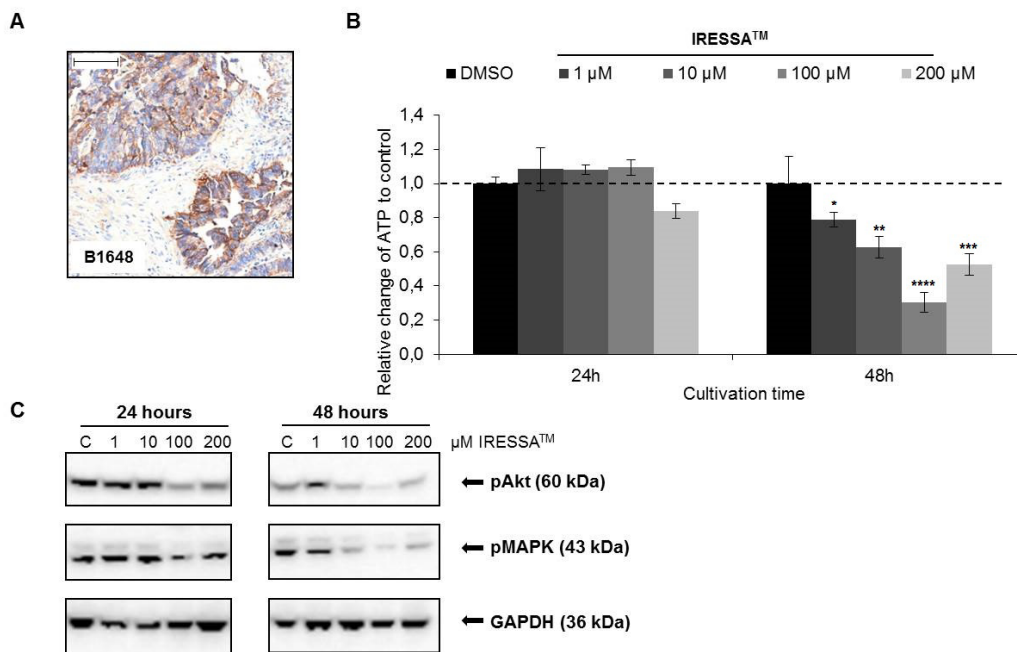


Figure 6. Effect of IRESSATM treatment on viability and pathway activity of EGFR-positive CRC Precision Cut Cancer tissue slices: (A) High EGFR expression level (brown) in representative CRC patient sample (B1648, EGFR positive); blue: haematoxylin; scale bar: 100 μ m. (B) Dose-dependent decrease of ATP after 48h treatment with IRESSATM (except for 200 μ M). Values are shown as relative change (mean values of triplicates) of expression levels to DMSO control (= 1). (C) Dose- and time-dependent reduction of pAkt and pMAPK after 24 and 48h starting at concentrations above 10 μ M or 1 μ M IRESSATM, respectively. C= DMSO control. Statistical significance has been calculated in comparison to the DMSO control. P-values are indicated by asterisks (* = 0.05; ** = 0.01; *** = 0.001; **** = 0.0001)

dose-dependent reduced pAkt expression under Herceptin® treatment (Figure 8, B). Whereas MCF7 were strongly Her-2 receptor negative (Figure 8, A) and pAkt expression was not affected by Herceptin® treatment (Figure 8, B).

Her-2 receptor status of cultured breast cancer tissue slices of 2 representative patients was also evaluated by IHC. Patient X2417 showed a clear overexpression of Her-2 receptor (3+) whereas patient X2309 was staged as Her-2 receptor negative (1+) (Figure 9, A). Western blot analysis illustrated a dose-dependent reduction of pAkt and pMAPK in Her-2 receptor positive tissue of patient X2417 (Figure 9, B). In contrast pAkt and pMAPK expression levels remained stable in Her-2 receptor negative breast cancer tissue from patient X2309. These results indicated that therapeutic antibodies are functional in our in vitro/ex vivo system.

DISCUSSION

We established a functional tissue slice culture system for colon, lung and breast tumor tissues. Gene expression patterns of freshly prepared tumor tissues and isolated primary cells resembled those of the individual original tumors. Cultivation of primary cells altered their gene expression patterns, while those of cultured tumor tissue slices were only slightly affected. The here presented drug profiling platform was suitable for the evaluation of drug effects of a variety of different compound classes. Staurosporine reduced ATP levels and increased Caspase-3/7 levels in CRC tissue slices, displaying the intact physiology of the culture system. IRESSATM led to reduced viability in EGFR-positive CRC cells as well as tissue

slices. Additionally, downstream targets Akt and MAPK were dephosphorylated. The model is also suitable for evaluating effects of monoclonal antibodies, seen in antibody diffusion studies and effect of Herceptin®. The latter lowered the expression of Akt and MAPK in Her-2-positive breast cancer cells and tissue slices.

Drug treatment in the background of individual expression profiles is applied amongst others in NSCLC, colon and breast cancer [3]. For example, EGFR expression is tested before treatment in order to identify patients who would benefit from treatment with IRESSATM. In colon cancer other EGFR inhibitors such as cetuximab (Erbitux®) and panitumumab (VECTIBIX®) are also used. Both can induce responses in advanced disease, but lack to prove effect on prolonged survival [54]. Patients who suffer from certain types of breast or stomach cancer are pre-evaluated for Her-2 expression by IHC with the intention to identify if they would benefit from Herceptin® treatment [53].

Testing the effectiveness of new drugs is highly depended on the right test system. Different screening systems for drug profiling have been established, trying to either assess hundreds of compounds or mimic the in vivo situation as close as possible. Cell lines, cultured isolated tumor cells, or spheroids in artificial matrices lack the regular microenvironment, the heterogeneity of individual tumors and a natural drug penetration barrier [11, 28, 32, 41]. Cell-cell interaction, intact morphology as well as the presence if e.g. immune cells are known to have a huge impact on drug efficacy and gain more and more attention in cancer research [55].

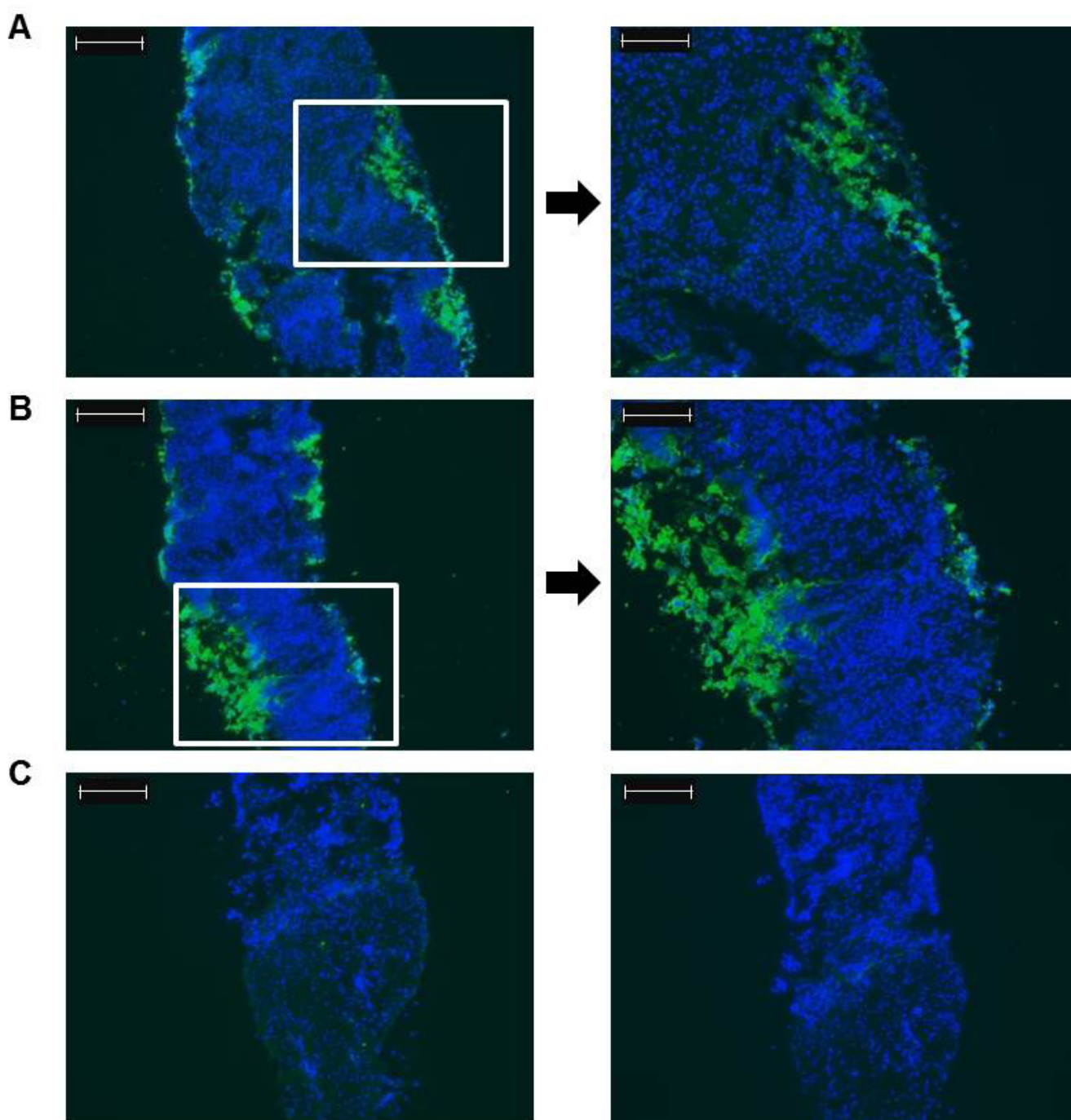


Figure 7. Penetration of anti-EpCAM antibody into CRC Precision Cut Cancer tissue slices: CRC tissue slices of case B1523 were incubated with anti-EpCAM antibody for (A) 2h and (B) 24h. Immunostaining with an Alexa Fluor 488-labelled secondary antibody (green) was performed and nuclei were stained with DAPI (blue). Anti-EpCAM antibody showed a time-dependent diffusion into the tissue. Left panel: scale bars 200µm; right panel: scale bars 100µm (C) Slices with anti-mouse antibody (left panel), and without secondary antibody (right panel) staining for 48 h served as control. Scale bars 200µm Figure 7. Penetration of anti-EpCAM antibody into CRC Precision Cut Cancer tissue slices: CRC tissue slices of case B1523 were incubated with anti-EpCAM antibody for (A) 2h and (B) 24h. Immunostaining with an Alexa Fluor 488-labelled secondary antibody (green) was performed and nuclei were stained with DAPI (blue). Anti-EpCAM antibody showed a time-dependent diffusion into the tissue. Left panel: scale bars 200µm; right panel: scale bars 100µm (C) Slices with anti-mouse antibody (left panel), and without secondary antibody (right panel) staining for 48 h served as control. Scale bars 200µm

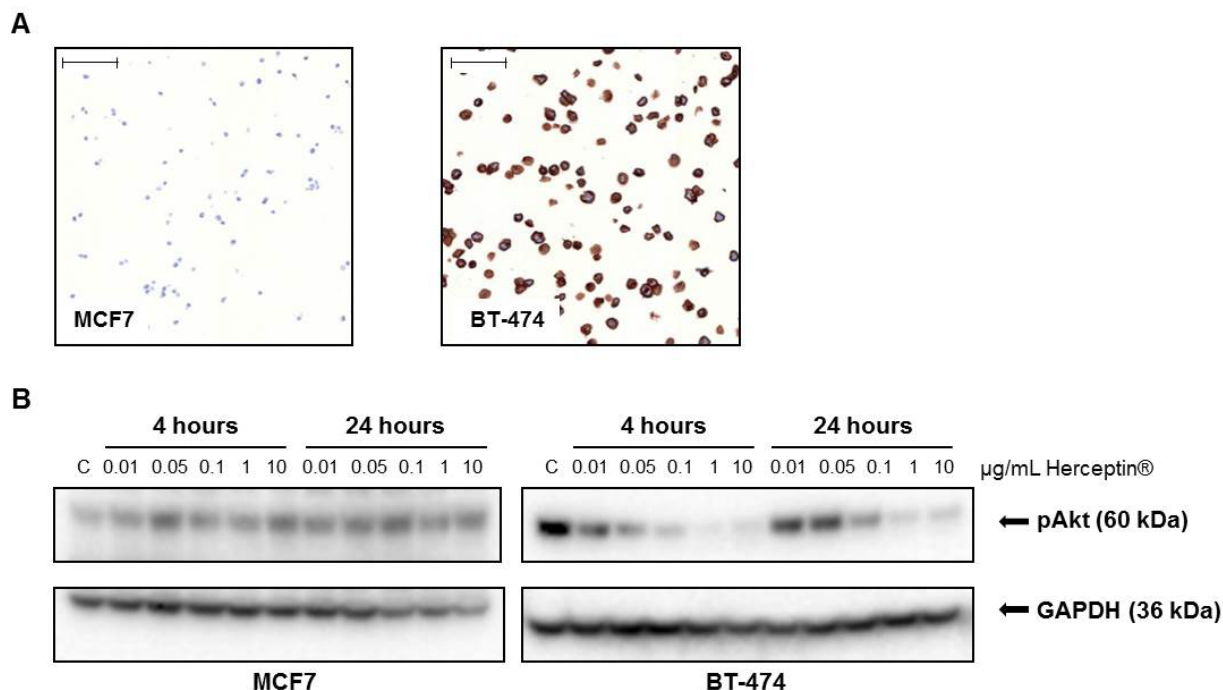


Figure 8. Effect of Herceptin® treatment on pathway activity of Her-2 positive and -negative breast cancer cell lines: (A) Breast cancer cell lines BT-474 and MCF7 were stained for Her-2 (brown). BT-474 cells showed high (Her-2 positive) whereas MCF7 cells showed low expression (Her-2 negative). Blue: haematoxylin, scale bars: 100 µm. (B) MCF7 cells showed no reduction of pAkt. Only the Her-2 receptor positive BT-474 cells showed a dose-dependent reduction of pAkt already after 4h of treatment.

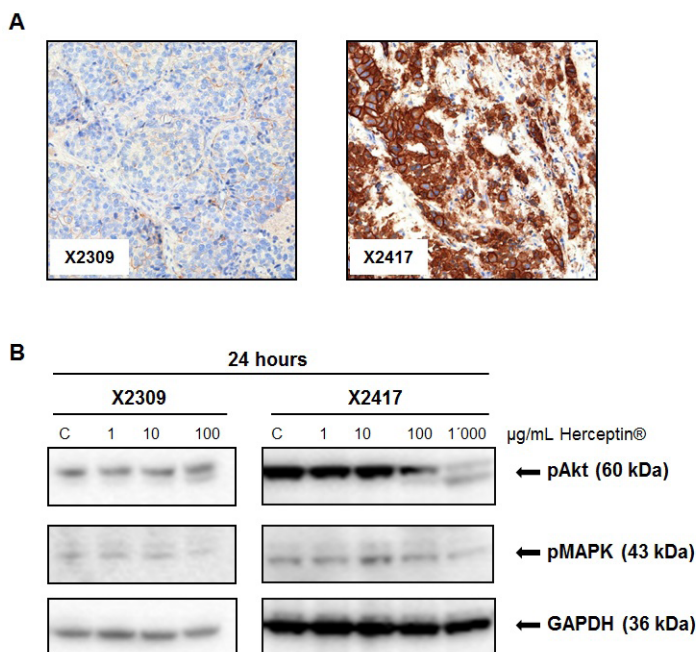


Figure 9. Effect of Herceptin® treatment on pathway activity of Her-2 positive and negative breast cancer Precision Cut Cancer tissue slices: (A) Breast cancer tissue slices of two breast cancer cases X2417 and X2309 were stained for Her-2 (brown). Case X2417 revealed high Her-2 whereas case X2309 revealed low Her-2 expression. Blue: haematoxylin. (B) Only the Her-2 receptor positive tissue slices of case X2417 showed a dose-dependent reduction of pAkt and pMAPK after 24h. No effect could be observed for Her-2 receptor negative tissue slices of case X2309.

Both in vitro and in vivo studies can contribute to the characterization of newly developed medications. The intensively used patient-derived tumor xenografts (PDTX) are not an entirely human system [56]. Tissue slice cultures can add value to the understanding of how a drug works

and PDTX can tell about its safety, making them a good combination in the process of drug development. In contrast to time consuming PDTX, the here presented human tissue slice culture excludes required culture amplification steps, thus, minimizing in vitro selection bias [28], has a reduced

experimental time, needs smaller amount of compound (mostly μg vs. mg range) and has no interference with host immunity [57]. The most striking benefit of tissue slices is the option of testing the individual response to drug from patients parallel to a clinical trial and enabling the randomization of patients and reduction of costs by excluding non-responders. We established in detail a tissue slice culture system for colon, breast and lung which is also suitable for other solid tumors like prostate [58], pancreas [59], ovarian [60], liver [61], or head and neck cancer [62]. The model also showed excellent results with standard of care drugs and viability was clearly diminished by camptothecin, gemcitabine, oxaliplatin, folinic acid/5-fluorouracil/oxaliplatin (FOLFOX).

Certainly, human tissue slice culture have their disadvantages like the lack of a functional blood vessel system, limited in vitro cultivation time (up to 72h), and low throughput [57, 63]. In some tissues like breast tumors structural composition is also a limiting factor. However, almost all in vitro culture systems have these drawbacks in common. Potentially, the tissue slice culture system is complementary to classical in vitro cell and PDTX models for further evaluating the potency and efficiency of a newly developed drug.

Readout parameters like ATP levels, Caspase, MALDI-MS, NanoPro™ 1000, MSD® and IHC are easy to handle and applicable in tissue slice cultures [47], as demonstrated with the results obtained by IRESSATM and Herceptin® treatment. The innovative MSD® technique enables the analysis of multiple targets in parallel in one sample [e.g. Akt panel: pAkt (Ser 473), pp70S6K (Thr 421/ Ser 424), and pGSK-3 β (Ser 9)], overcoming the limitations of tissue quantity. Methods like IHC enable to visualize the effect of a drug on the single cell level, making the evaluation of desired and undesired effects of each drug more assessable. And in techniques like the NanoPro™ 1000 only very little sample volumes are utilized, making it a perfect tool to analyze limited sample amounts which are faced in e.g. clinical trials or fine needle biopsies.

The usefulness of the presented tissue slice culture is shown, although it has to prove its utility in state-of-the-art techniques like gene therapy shown by others [59]. This will be elucidated further.

CONCLUSIONS

Tissue slice culture is a valuable preclinical model and applicable to examine the effects of various anti-cancer compounds like cytotoxic chemotherapeutic drugs as well as targeted therapeutics. Especially the use along with clinical trials and the discovery of responder groups brings an additional benefit for physicians during the process of treatment decision in personalized medicine.

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
CRC	colorectal cancer
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
D-PBS	Dulbecco's phosphate-buffered saline
EDTA	ethylene diamine tetra acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EpCAM	epithelial cell adhesion molecule
FCS	fetal calve serum
GAPDH	glyceraldehyd-3-phosphat-dehydrogenase
H&E	heamatoxylin & eosin
Her-2	human epidermal growth factor receptor 2
IHC	immunohistochemistry
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
MSD	Meso Scale Discovery
PCA	Principle Component Analysis
RT	room temperature

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

AUTHORS' CONTRIBUTIONS

AS and CR carried out the molecular genetic studies.

JK, KP and JS carried out the immunoassays and participated in the sequence alignment.

FTU conceived of the study and participated in coordination, performed the statistical analysis and drafted the manuscript.

SB helped to draft the manuscript.

HJ participated in the design of the study

KAD conceived of the study, and participated in its design and coordination and drafted the manuscript.

All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We acknowledge Dr. Poßögel and Dr. von Erichsen for pathological evaluation of biospecimen.

REFERENCES

- Roskelley CD, Bissell MJ: The dominance of the microenvironment in breast and ovarian cancer. *Seminars in cancer biology* 2002, 12:97-104.
- Allen M, Louise Jones J: Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *The Journal of pathology* 2011, 223:162-176.
- Huang M, Shen A, Ding J, Geng M: Molecularly targeted cancer therapy: some lessons from the past decade. *Trends in pharmacological sciences* 2014, 35:41-50.
- Hensing T, Chawla A, Batra R, Salgia R: A personalized treatment for lung cancer: molecular pathways, targeted therapies, and genomic characterization. *Advances in experimental medicine and biology* 2014, 799:85-117.
- Tredan O, Galmorini CM, Patel K, Tannock IF: Drug resistance and the solid tumor microenvironment. *Journal of the National Cancer Institute* 2007, 99:1441-1454.
- Patocs A, Zhang L, Xu Y, Weber F, Caldes T, Mutter GL, Platzer P, Eng C: Breast-cancer stromal cells with TP53 mutations and nodal metastases. *The New England journal of medicine* 2007, 357:2543-2551.
- Orimo A, Weinberg RA: Stromal fibroblasts in cancer: a novel tumor-promoting cell type. *Cell cycle* 2006, 5:1597-1601.
- Kinzler KW, Vogelstein B: Landscaping the cancer terrain. *Science* 1998, 280:1036-1037.
- Bissell MJ, Radisky D: Putting tumours in context. *Nature reviews Cancer* 2001, 1:46-54.
- Morin PJ: Drug resistance and the microenvironment: nature and nurture. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* 2003, 6:169-172.
- Albertson DG, Collins C, McCormick F, Gray JW: Chromosome aberrations in solid tumors. *Nature genetics* 2003, 34:369-376.
- Coates A, Abraham S, Kaye SB, Sowerbutts T, Frewin C, Fox RM, Tattersall MH: On the receiving end—patient perception of the side-effects of cancer chemotherapy. *European journal of cancer & clinical oncology* 1983, 19:203-208.
- Carelle N, Piotto E, Bellanger A, Germanaud J, Thuillier A, Khayat D: Changing patient perceptions of the side effects of cancer chemotherapy. *Cancer* 2002, 95:155-163.
- Chow LW, Yiu CC, Yip AY, Loo WT: The future perspectives of breast cancer therapy. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2006, 60:259-262.
- Sonnenberg M, van der Kuip H, Haubeis S, Fritz P, Schroth W, Friedel G, Simon W, Murdter TE, Aulitzky WE: Highly variable response to cytotoxic chemotherapy in carcinoma-associated fibroblasts (CAFs) from lung and breast. *BMC cancer* 2008, 8:364.
- Dattatreya S: Metastatic colorectal cancer—prolonging overall survival with targeted therapies. *South Asian journal of cancer* 2013, 2:179-185.
- Li C: A targeted approach to cancer imaging and therapy. *Nature materials* 2014, 13:110-115.
- Workman P, Clarke P: PI3 Kinase in Cancer: From Biology to Clinic. *American Society of Clinical Oncology educational book / ASCO American Society of Clinical Oncology Meeting* 2012, 32:e93-e98.
- Sahu A, Prabhaskar K, Noronha V, Joshi A, Desai S: Crizotinib: A comprehensive review. *South Asian journal of cancer* 2013, 2:91-97.
- Rosen LS, Gordon MS, Robert F, Matei DE: Endoglin for targeted cancer treatment. *Current oncology reports* 2014, 16:365.
- Burriss HA: Trastuzumab Emtansine (T-DM1): Hitching a Ride on a Therapeutic Antibody. *American Society of Clinical Oncology educational book / ASCO American Society of Clinical Oncology Meeting* 2012, 32:159-161.
- Shin TH, Sung ES, Kim YJ, Kim KS, Kim SH, Kim SK, Lee YD, Kim YS: Enhancement of the tumor penetration of monoclonal antibody by fusion of a neuropilin-targeting peptide improves the anti-tumor efficacy. *Molecular cancer therapeutics* 2014.
- Overington JP, Al-Lazikani B, Hopkins AL: How many drug targets are there? *Nature reviews Drug discovery* 2006, 5:993-996.
- Kumar A, Rajendran V, Sethumadhavan R, Purohit R: AKT kinase pathway: a leading target in cancer research. *TheScientificWorldJournal* 2013, 2013:756134.
- Siegfried Z, Bonomi S, Ghigna C, Karni R: Regulation of the Ras-MAPK and PI3K-mTOR Signaling Pathways by Alternative Splicing in Cancer. *International journal of cell biology* 2013, 2013:568931.
- Cooper WA, Lam DC, O'Toole SA, Minna JD: Molecular biology of lung cancer. *Journal of thoracic disease* 2013, 5:S479-S490.
- den Hollander P, Savage MI, Brown PH: Targeted Therapy for Breast Cancer Prevention. *Frontiers in oncology* 2013, 3:250.
- Das T, Meunier L, Barbe L, Provencher D, Guenat O, Gervais T, Mes-Masson AM: Empirical chemosensitivity testing in a spheroid model of ovarian cancer using a microfluidics-based multiplex platform. *Biomicrofluidics* 2013, 7:11805.
- Panowski S, Bhakta S, Raab H, Polakis P, Junutula JR: Site-specific antibody drug conjugates for cancer therapy. *mAbs* 2014, 6:34-45.
- Eichelbaum M, Ingelman-Sundberg M, Evans WE: Pharmacogenomics and individualized drug therapy. *Annual review of medicine* 2006, 57:119-137.
- Masramon L, Vendrell E, Tarafa G, Capella G, Miro R, Ribas M, Peinado MA: Genetic instability and divergence of clonal populations in colon cancer cells in vitro. *Journal of cell science* 2006, 119:1477-1482.
- Thiery JP, Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. *Nature reviews Molecular cell biology* 2006, 7:131-142.
- Kloss D, Fischer M, Rothermel A, Simon JC, Robitzki AA: Drug testing on 3D in vitro tissues trapped on a microcavity chip. *Lab on a chip* 2008, 8:879-884.
- Lin RZ, Chang HY: Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnology journal* 2008, 3:1172-1184.
- Prestwich GD: Evaluating drug efficacy and toxicology in three dimensions: using synthetic extracellular matrices in drug discovery. *Accounts of chemical research* 2008, 41:139-148.
- Michalski CW, Erkan M, Sauliunaite D, Giese T, Stratmann R, Sartori C, Giese NA, Friess H, Kleeff J: Ex vivo chemosensitivity testing and gene expression profiling predict response towards adjuvant gemcitabine treatment in pancreatic cancer. *British journal of cancer* 2008, 99:760-767.
- Kitada N, Takara K, Minegaki T, Itoh C, Tsujimoto M, Sakaeda T, Yokoyama T: Factors affecting sensitivity to antitumor platinum derivatives of human colorectal tumor cell lines. *Cancer chemotherapy and pharmacology* 2008, 62:577-584.
- Sharma SV, Haber DA, Settleman J: Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents. *Nature reviews Cancer* 2010, 10:241-253.
- Sandberg R, Ernberg I: Assessment of tumor characteristic gene expression in cell lines using a tissue similarity index (TSI). *Proceedings of the National Academy of Sciences of the United States of America* 2005, 102:2052-2057.
- Lee W, Lockhart AC, Kim RB, Rothenberg ML: Cancer pharmacogenomics: powerful tools in cancer chemotherapy and drug development. *The oncologist* 2005, 10:104-111.
- Kleeff J, Michalski C, Friess H, Buchler MW: Pancreatic cancer: from bench to 5-year survival. *Pancreas* 2006, 33:111-118.
- Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA: Spheroid-based drug screen: considerations and practical approach. *Nature protocols* 2009, 4:309-324.
- Buck E, Eyzaguirre A, Barr S, Thompson S, Sennello R, Young D, Iwata KK, Gibson NW, Cagnoni P, Haley JD: Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Molecular cancer therapeutics* 2007, 6:532-541.
- Schmeichel KL, Bissell MJ: Modeling tissue-specific signaling and organ function in three dimensions. *Journal of cell science* 2003, 116:2377-2388.
- van der Kuip H, Murdter TE, Sonnenberg M, McClellan M, Gutzeit S, Gerteis A, Simon W, Fritz P, Aulitzky WE: Short term culture of breast cancer tissues to study the activity of the anticancer drug taxol in an intact tumor environment. *BMC cancer* 2006, 6:86.
- Guyot C, Combe C, Clouzeau-Girard H, Moronville-Halley V, Desmouliere A: Specific activation of the different fibrogenic cells in rat cultured liver slices mimicking in vivo situations. *Virchows Archiv : an international journal of pathology* 2007, 450:503-512.
- Vaira V, Fedele G, Pyne S, Fasoli E, Zadra G, Bailey D, Snyder E, Favarsani A, Coggi G, Flavin R, et al: Preclinical model of organotypic culture for pharmacodynamic profiling of human tumors. *Proceedings of the National Academy of Sciences of the United States of America* 2010, 107:8352-8356.

48. Raymond E, Faivre S, Woynarowski JM, Chaney SG: Oxaliplatin: mechanism of action and antineoplastic activity. *Seminars in oncology* 1998, 25:4-12.
49. Longley DB, Harkin DP, Johnston PG: 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews Cancer* 2003, 3:330-338.
50. Yagi Y, Sotani T, Nagao T, Horio T, Yamamoto I, Gohda E: Induction by staurosporine of hepatocyte growth factor production in human skin fibroblasts independent of protein kinase inhibition. *Biochemical pharmacology* 2003, 66:1797-1808.
51. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC: Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature cell biology* 2001, 3:245-252.
52. Mahipal A, Kothari N, Gupta S: Epidermal growth factor receptor inhibitors: coming of age. *Cancer control : journal of the Moffitt Cancer Center* 2014, 21:74-79.
53. Kumler I, Tuxen MK, Nielsen DL: A systematic review of dual targeting in HER2-positive breast cancer. *Cancer treatment reviews* 2014, 40:259-270.
54. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, et al: Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *The New England journal of medicine* 2004, 351:337-345.
55. Halama N, Michel S, Kloor M, Zoernig I, Benner A, Spille A, Pommerencke T, von Knebel DM, Folprecht G, Lubber B, et al: Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer research* 2011, 71:5670-5677.
56. Kamiyama H, Rauenzahn S, Shim JS, Karikari CA, Feldmann G, Hua L, Kamiyama M, Schuler FW, Lin MT, Beaty RM, et al: Personalized chemotherapy profiling using cancer cell lines from selectable mice. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2013, 19:1139-1146.
57. Fukuda J, Mizumoto H, Nakazawa K, Kajiwara T, Funatsu K: Hepatocyte organoid culture in elliptic hollow fibers to develop a hybrid artificial liver. *The International journal of artificial organs* 2004, 27:1091-1099.
58. Parrish AR, Sallam K, Nyman DW, Orozco J, Cress AE, Dalkin BL, Nagle RB, Gandolfi AJ: Culturing Precision Cut Cancer human prostate slices as an in vitro model of prostate pathobiology. *Cell biology and toxicology* 2002, 18:205-219.
59. van Geer MA, Kuhlmann KF, Bakker CT, ten Kate FJ, Oude Elferink RP, Bosma PJ: Ex-vivo evaluation of gene therapy vectors in human pancreatic (cancer) tissue slices. *World journal of gastroenterology : WJG* 2009, 15:1359-1366.
60. Kirby TO, Rivera A, Rein D, Wang M, Ulasov I, Breidenbach M, Kataram M, Contreras JL, Krumdieck C, Yamamoto M, et al: A novel ex vivo model system for evaluation of conditionally replicative adenoviruses therapeutic efficacy and toxicity. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2004, 10:8697-8703.
61. Stoff-Khalili MA, Stoff A, Rivera AA, Banerjee NS, Everts M, Young S, Siegal GP, Richter DF, Wang M, Dall P, et al: Preclinical evaluation of transcriptional targeting strategies for carcinoma of the breast in a tissue slice model system. *Breast cancer research : BCR* 2005, 7:R1141-1152.
62. Gerlach MM, Merz F, Wichmann G, Kubick C, Wittekind C, Lordick F, Dietz A, Bechmann I: Slice cultures from head and neck squamous cell carcinoma: a novel test system for drug susceptibility and mechanisms of resistance. *British journal of cancer* 2014, 110:479-488.
63. Holliday DL, Moss MA, Pollock S, Lane S, Shaaban AM, Millican-Slater R, Nash C, Hanby AM, Speirs V: The practicalities of using tissue slices as preclinical organotypic breast cancer models. *Journal of clinical pathology* 2013, 66:253-255.

© SAGEYA. This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, noncommercial use, distribution and reproduction in any medium, provided the work is properly cited.

Source of Support: Nil, Conflict of Interest: None declared

