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A Farewell to Flat Biology

Three-dimensional Cell Culture Models in Cancer Drug Target Identification and Validation

by

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“It’s not that the bear dances well, but that the he dances at all.”

- Unknown Russian circus master

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University of Turku, Institute of Biomedicine, Department of Cell Biology and Anatomy, VTT Technical Research Centre of Finland, Turku, Finland

Annales Universitatis Turkuensis, Medica-Odontologica

ABSTRACT

Cells of epithelial origin, e.g. from breast and prostate cancers, effectively differentiate into complex multicellular structures when cultured in three-dimensions (3D) instead of conventional two-dimensional (2D) adherent surfaces. The spectrum of different organotypic morphologies is highly dependent on the culture environment that can be either non-adherent or scaffold-based. When embedded in physiological extracellular matrices (ECMs), such as laminin-rich basement membrane extracts, normal epithelial cells differentiate into acinar spheroids reminiscent of glandular ductal structures. Transformed cancer cells, in contrast, typically fail to undergo acinar morphogenic patterns, forming poorly differentiated or invasive multicellular structures. The 3D cancer spheroids are widely accepted to better recapitulate various tumorigenic processes and drug responses. So far, however, 3D models have been employed predominantly in the Academia, whereas the pharmaceutical industry has yet to adopt a more widely and routine use. This is mainly due to poor characterisation of cell models, lack of standardised workflows and high-throughput cell culture platforms, and the availability of proper readout and quantification tools. In this thesis, a complete workflow has been established entailing well-characterised 3D cell culture models for prostate cancer, a standardised 3D cell culture routine based on high-throughput-ready platform, automated image acquisition with concomitant morphometric image analysis, and data visualisation, in order to enable large-scale high-content screens. Our integrated suite of software and statistical analysis tools were optimised and validated using a comprehensive panel of prostate cancer cell lines and 3D models. The tools quantify multiple key cancer-relevant morphological features, ranging from cancer cell invasion through multicellular differentiation to growth, and detect dynamic changes both in morphology and function, such as cell death and apoptosis, in response to experimental perturbations including RNA interference and small molecule inhibitors. Our panel of cell lines included many non-transformed and most currently available classic prostate cancer cell lines, which were characterised for their morphogenetic properties in 3D laminin-rich ECM. The phenotypes and gene expression profiles were evaluated concerning their relevance for pre-clinical drug discovery, disease modelling and basic research. In addition, a spontaneous model for invasive transformation was discovered, displaying a high-degree of epithelial plasticity. This plasticity is mediated by an abundant bioactive serum lipid, lysophosphatidic acid (LPA), and its receptor LPAR1. The invasive transformation was caused by abrupt cytoskeletal rearrangement through impaired G protein alpha 12/13 and RhoA/ROCK, and mediated by upregulated adenylyl cyclase/cyclic AMP (cAMP)/protein kinase A, and Rac/PAK pathways. The spontaneous invasion model tangibly exemplifies the biological relevance of organotypic cell culture models. Overall, this thesis work underlines the power of novel morphometric screening tools in drug discovery.

Keywords: Image analysis, 3D culture, organotypic culture, morphometric analysis, prostate cancer, drug discovery

Ville Härmä

Jäähyväiset petrimaljoille - Kolmiulotteiset solumallit syövän lääkekehityksessä

Turun yliopisto, Biolääketieteen laitos, Solubiologia ja anatomia, Teknologian Tutkimuskeskus VTT, Turku

Annales Universitatis Turkuensis, Medica-Odontologica

TIIVISTELMÄ

Epiteeliperäiset solut, mukaan lukien rinta- ja eturauhassyöpäsolut, erilaistuvat kolmiulotteisessa ympäristössä monimuotoisiksi rakenteiksi, jotka poikkeavat suuresti perinteisistä kaksiulotteisilla alustoilla kasvatetuista soluviljelmistä. Erilaisten morfologioiden kirjo organotyyppisissä soluviljelmissä riippuu pitkälti kasvatusmenetelmästä, joka tyypillisesti on joko solujen tarttumisen ehkäisyyn tai fyysiseen soluväliaineverkostoon pohjautuva. Normaalit epiteelisolut erilaistuvat laminiinipohjaisissa tyvikalvomatriiseissa rauhasenkaltaisiksi sferoidirakenteiksi, kun taas transformoituneet solut eivät useimmiten suoriudu morfogeneettisistä prosesseista. Syöpäsolut muodostavat tyypillisesti heikosti erilaistuneita tai invasoivia rakenteita, joiden ajatellaan kuvastavan syövän kannalta oleellisia toimintoja sekä mallintavan syöpäsolujen kliinisiä lääkeainevasteita kaksiulotteisia soluviljelmiä paremmin. Tähän mennessä kolmiulotteisten solumallien laajempi käyttö on kuitenkin jäänyt lähes yksinomaan akateemisen tutkimuksen piiriin eikä lääketeollisuus ole löytänyt niille sopivaa käyttöä. Teollisuuden haluttomuus hyödyntää kolmiulotteisia solumalleja selittyy suurelta osin sillä, että kolmiulotteisia solumalleja ei ole karakterisoitu riittävän huolellisesti. Lisäksi laboratoriorutiineiden standardoinnin sekä asianmukaisten analyysi- ja kvantitaatiomenetelmien puute hidastavat mallien käyttöönottoa. Tämän väitöskirjatutkimuksen yhteydessä on kehitetty menetelmäkokonaisuus, joka soveltuu lääkkeiden solupohjaiseen tehoseulontaan kolmiulotteisessa tyvikalvomatriisissa. Kokonaisuus rakentuu standardoidusta soluviljelyalustasta, automaattisesta kuvantamisesta, sekä kuva-analyysiin sekä tilastolliseen analyysin ja visualisointiin kehitetyistä työkaluista. Analyysimenetelmät optimoitiin käyttämällä laajaa valikoimaa eturauhasperäisiä soluja. Kuva-analyysiohjelma kehitettiin kvantitoimaan useita keskeisiä syöpään liittyviä morfologisia ja funktionaalisia ominaisuuksia, kuten syöpäsolujen invaasiota, monisoluista erilaistumista, syöpäsolujen kasvua ja solukuolemaa. Se on suunniteltu tunnistamaan ulkopuolisten häiriöiden, kuten lääkekäsittelyiden ja geenien hiljentämisen, aiheuttamia dynaamisia muutoksia. Työn yhteydessä tutkimme useiden klassisten epiteeli- ja syöpäsolujen ominaisuuksia, kuten morfologista kehityskaarta, fenotyyppiä ja geenieksprессioprofiileita kolmiulotteisessa tyvikalvomatriisissa. Solumalleja arvioitiin niiden prekliiniseen lääkekehitykseen, tautimallinnukseen ja perustutkimukseen soveltuvuuden perusteella. Karakterisoinnin yhteydessä löysimme uuden metastabiilin solumallin syövän spontaanille invasiiviselle transformaatiolle. Tarkemmat tutkimukset osoittivat, että metastabiili fenotyyppi oli riippuvainen seerumin lysofosfatidihappopitoisuudesta ja LPAR1 reseptorin aktiivisuudesta. Invasiivinen transformaatio aiheutui Gα12/13 ja RhoA/ROCK signalointireitin heikentymisestä, mahdollisesti voimistuneen AC/cAMP/PKA ja Rac/PAK signalointireittien vaikutuksesta. Invaasiomalli on hyvä esimerkki organotyyppisten solumallien biologisesta relevanssista. Tutkimus alleviivaa uusien morfologiseen analyysiin perustuvien tehoseulontatyökalujen merkitystä uusien syöpälääkkeiden seulonnassa.

Avainsanat: Kuva-analyysi, 3D soluviljelmä, organotyyppiset soluviljelmät, morfometrinen analyysi, eturauhassyöpä, lääkekehitys

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ABBREVIATIONS

2D = two-dimensional
3D = three-dimensional
ADME = absorption, distribution, metabolism and excretion
ADT = androgen deprivation
AR = androgen receptor
ASO = antisense oligonucleotide
BM = basement membrane
BrCa = breast cancer
CAF = cancer-associated fibroblast
cAMP = cyclic AMP
cDNA = complementary DNA
CNS = central nervous system
CRPC = castration-resistant prostate cancer
DMSO = dimethyl sulphoxide
DNA = deoxyribonucleic acid
ELISA = enzyme-linked immunosorbent assay
ECM = extracellular matrix
EMA = European Medicines Agency
EMT = epithelial-to-mesenchymal transformation
ER = estrogen receptor
FDA = Food and Drug Administration
FISH = fluorescence *in situ* hybridization
GEMM = genetically engineered mouse model
GEF = guanine nucleotide exchange factor
GnRH = gonadotropin-releasing hormone
GPCR = G protein-coupled receptor
HCS = high-content screen
HTS = high-throughput screen
IF = immunofluorescence
IHC = immunohistochemistry
IL = interleukin
IND = Investigational new drug
LPA = lysophosphatidic acid
LPP = lipid phosphate phosphatase
lrECM = laminin-rich ECM
miRNA = micro RNA
MET = mesenchymal-to-epithelial transformation

MOA = mechanism of action
MLSMR = Molecular Libraries Small Molecule Repository
mRNA = messenger RNA
NCE = new chemical entity
NIH = National Institutes for Health
NDA = new drug application
PrCa = prostate cancer
RISC = RNA-induced silencing complex
RNA = ribonucleic acid
RNAi = RNA interference
RTK = receptor tyrosine kinase
SAR = structure-activity relationship
shRNA = short hairpin RNA
siRNA = small interfering RNA
SISH = silver-stained in situ hybridization
TMA = tissue microarray
TME = tumor microenvironment
uPA = urokinase plasminogen activator
WB = Western blot

LIST OF ORIGINAL PUBLICATIONS

I. **Härmä V**, Virtanen J, Mäkelä R, Happonen A, Mpindi JP, Knuuttila M, Kohonen P, Lötjönen J, Kallioniemi O, Nees M. A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One. 2010 May 3;5(5):e10431.

II. **Härmä V**, Knuuttila M, Virtanen J, Mirtti T, Kohonen P, Kovanen P, Happonen A, Kaewphan S, Ahonen I, Kallioniemi O, Grafström R, Lötjönen J, Nees M. Lysophosphatidic acid and sphingosine-1-phosphate promote morphogenesis and block invasion of prostate cancer cells in three-dimensional organotypic models. Oncogene. 2011 Sep 26. doi: 10.1038/onc.2011.396.

III. **Härmä V**, Happonen A, Ahonen I, Virtanen J, Åkerfelt M, Siitari H, Lötjönen J and Nees M. Quantification of Dynamic Morphological Drug Responses in Organotypic Cell Cultures by Automated Image Analysis. Submitted.

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INTRODUCTION

Pharmaceutical companies live off the drug patents that are a product of years and years of expensive research and development (R&D). Many blockbuster patents that used to generate a wealth of revenue are about to expire. At the same time the drug development pipelines are running dry of effective drugs, and the R&D costs are skyrocketing. The figures are clear: the R&D expenditures have been steadily increasing by 13% per year since 1970, while the number of yearly New Drug Applications (NDAs) has stagnated [Kola, Landis 2004, Lengauer, Diaz & Saha 2005]. This means a near tenfold decline in R&D productivity as measured by number of NDAs per dollar spent on R&D [Lengauer, Diaz & Saha 2005].

The past decades have been more favourable for the Academia, mostly thanks to many technological breakthroughs that have enabled several fundamental discoveries in human biology and pathophysiology. In the field of oncology, the introduction of various "-omics", or the general concept of interdisciplinary study field known as systems biology, has essentially turned the field inside out. The novel high-throughput technologies in translational (proteomics), transcriptional (transcriptomics) and molecular biology (genomics, epigenetics), spawned initially from the advancements in engineering, physics, chemistry and bio- and nanotechnology, have now enabled a much more holistic perspective to the disease. Also drug discovery has benefited from advanced laboratory automation, as it has enabled the screening of thousands or even millions of chemical entities, or the simultaneous silencing of thousands of genes. The laboratory technologies have been complemented by a new branch of information science, bioinformatics, which has played a pivotal role in the management and interpretation of unparalleled amounts of data. Together, these new technologies have added momentum to cancer biology in a similar manner as DNA sequencing has improved zoological research. In the past, taxonomy, or the classification of life forms, was based mostly on crude comparison of physical features of organisms. This was by no means a precise or evidence-based method, since similar environment may drive convergence in evolutionary development, and many unrelated species living in similar habitats thus have acquired similar characteristics. As a result of taxonomists starting to have a closer look at the genetic code, many of the taxonomic trees, even those formerly thought to be rock solid, had to be rethought. Similarly in oncology, malignancies have traditionally been grouped based on morphology and the site of origin. As the knowledge of molecular intra- and inter-patient heterogeneity in tumors has accumulated, it has become evident that neither morphology nor location should be the sole dictating factor in categorization of malignancies anymore, nor, most importantly, should be the consequent selection of an appropriate therapy [Bardelli et al. 2003, Lynch et al. 2004, Paez et al. 2004].

It has been envisioned that in the near future, each patient will become individually catalogued by the type of genetic alterations in their cancer(s), and treated with specific medication or a set of medications addressing one or several of the key alterations [Lengauer, Diaz & Saha 2005]. This is a rather ambitious goal and certainly requires a great deal of (ideally joint) effort from both Academia and industry. In order to facilitate individual diagnosis, a vast spectrum of mutations and other genetic alterations are now carefully charted among thousands of tumors. This is already coming to fruition as new high-profile bio-banking programmes are emerging all around the world to systematically collect specimens from malignancies of all types [Hewitt 2011]. These

alterations can be identified by thorough mining of data from various genetic, transcriptional and translational high-throughput technologies such as next-generation sequencing (e.g. mRNA, ChIP and whole genome sequencing), mRNA microarrays, advanced HPLC systems coupled with mass spectrometry, all combined with elaborate computational algorithms. To reliably classify patients according to different molecular (not only histological) subtypes, robust yet simple and cost-effective diagnostic tools have been developed. As part of the target identification process, the functional/biological roles of gene alterations in cancer progression are being defined, and appropriate next-generation *in vitro* and *in vivo* models are also being generated in parallel. Over the last decade, transcriptional and translational profiling of the tumors has helped to pinpoint the potential drivers versus bystanders of various cell signalling pathways, perturbed in each tumor subtype. As most cancer proteins are predicted to be ineffective targets, cost-effective experimental models are needed to rigorously evaluate and validate their potential, as well as the efficacy of newly generated drugs specifically targeting them. Even an ineffective drug target can be valuable in combination with other targets, old or new. New experimental models ideally would facilitate the assessment of target synergisms that has to be tested both among the new as well as the traditional lines of therapy.

Decades of research have shown that the number of potential druggable targets in oncology is immense. In the world of individualized diagnosis and personalized therapies, that number is unlikely to be any lower, quite on the contrary. However, most drug candidates are either downright ineffective or the targets prove not to be as essential for cancer progression (and treatment) as expected, rendering the overall effect on survival negligible. New drug targets, emerging with the help of systems biology technologies, results in more candidates in the pipeline, but consequently resulting also in more failures. Failures essentially equal financial losses and, for society, precious time and money wasted in the fight against cancer. The dilemma essentially emerges from two contradicting facts: the industry desperately needs better more effective targets and lead compounds. At the same time, unnecessary and unsuccessful, costly clinical trials have to be avoided. Clinical trials, in particular phase II and III, represent the stages of drug development that consume most of the resources, time and costs. If you have to fail – ideally fail at the earliest possible time point. The sooner a target or drug can be eliminated from the pipeline, the better. It is fairly difficult to estimate the attrition rates as most pharmaceutical companies consider such information sensitive and do not wish to disclose accurate figures. One of the most detailed estimates has been presented by Kola and Landis [Kola, Landis 2004] for the period of 1991-2000 including the ten largest pharma companies in the United States and Europe. 38% of the drugs taken in the clinical patient trials dropped out in Phase I (safety and blood levels), 60% of those still remaining failed in Phase II (basic efficacy), 40% of the remaining candidates failed in Phase III, and 23% of the ones that made it through all the clinical phases failed to be approved by the FDA. All this translates to an average of 11% success rate from starting in the clinic, in all therapeutic areas. In oncology, the success rate is even lower, around 5%, most drugs failing in Phase II and III. The more recent figures for FDA approved new chemical entities attest to the modest attrition rates: in the year 2011 US FDA approved only 34 new drugs, seven of which were indicated as lifesaving cancer drugs (http://newdrugs.wordpress.com/2011/12/07/fda-approvals-2011/). Major causes of attrition in the clinic have generally been lack of efficacy and safety, the lack of efficacy being especially an issue in therapeutic areas in which animal models of efficacy are notoriously unpredictive, such as oncology and central nervous system diseases [Booth, Glassman 2003, Kola, Landis 2004]. Current situation puts an

immense pressure on target validation technologies, methods and models used at pre-clinical drug discovery stage. The risk of failure, or in other words, letting any drug slip through the pre-clinical filters, increases the stringency requirements for reproducibility and predictability. At the same time, the expenditure for pre-clinical experiments has to be acceptably low enough, to allow as many candidates to be tested in parallel or in combination, as possible.

The most interesting targets have ideally been identified in patient series. To further validate and test the applicability of these targets, quick and inexpensive laboratory models, whether *in vitro* or *in vivo*, are needed. Most importantly, the models used for validation need to directly address the postulated biological function of the target. All perturbations (small molecule inhibitors, siRNAs, biological like therapeutic antibodies) directed against the target protein should be readable as a change in the model's regular mode of operation. The classic Hallmarks of Cancer, a list originally introduced by Hanahan and Weinberg in 2000 [Hanahan, Weinberg 2000] and later updated in 2011 [Hanahan, Weinberg 2011], represents an array of biological mechanisms cancer cells use in order to survive, expand and spread through the human body. The most interesting newly identified targets will ideally fall into one of the ten main functional categories or hallmarks. Some of these are readily addressed by standard *in vitro* models (enabling replicative immortality, proliferation, deregulating cellular energetics, resisting cell death, genome instability and mutation). Others require much more complex *in vivo* animal models (angiogenesis, activation of invasion and metastasis), and yet others are currently yet beyond the limits and capability of standard animal experimentation (e.g. tumor-promoting inflammation, systemic metastasis and tumor dormancy). Furthermore, it has become increasingly evident that in order for a therapy to defeat cancer, it must address more than a single hallmark. Knowing the exact biological role of each drug target helps to construct a combinatorial therapy consisting of drugs targeting not only the right genetic alterations in an individual tumor, but also the right combinations (e.g. anti-proliferation together with anti-angiogenesis).

Traditionally, two general classes of experimental models have been used in basic cancer research and pre-clinical drug development. These are mainly used to test target feasibility, drug safety and perform initial rounds of iterative screening with the most promising lead compounds: *in vitro* cell lines and *in vivo* animal models. Cell lines extracted from living tumors have been utilized since the 1950's. There are thousands of cancer cell lines available, originating from almost all organs known to bear malignancies. In addition, there are numerous standardized assays developed to assess many key cellular functions, such as apoptosis/cell death, migration, invasion through biological barriers etc. Animal experiments are typically performed based on murine models either as different forms of tumor "xenograft" cell transplantations, or using transgenic animals programmed to develop tumors spontaneously with varying organic specificity. Both cell and animal models have their pros and cons, cost and time generally favouring the first and predictability to human disease the latter. Nevertheless, predictive power even of animal experiments is insufficient to avoid complete failures of efficacy in clinical trials. Considering the hallmarks of cancer [Hanahan, Weinberg 2011], half of the listed cancer mechanisms can be effectively simulated with *in vitro* cell lines, usually those related to the faith of individual cells. The remainder are beyond assessment with traditional cell cultures, relate to behaviour of multicellular tissues, and expectedly cell lines do not predict the outcome in patients very well. The more complex functions that require the heterotypic interplay between multiple cell types (activation of invasion by tumor-stroma interactions, tumor promoting inflammation,

angiogenesis) or entire organ systems (metastasis via systemic circulation, neovascularization) or even a whole organism (avoiding immune destruction, formation of distant metastases) need to be addressed by much more elaborate models, usually animal models. However, even with animal models do not faithfully mimic for example immune avoidance, as most of the mouse strains utilized in cancer cell transplantation experiments are immune-deficient. To list additional drawbacks of animal models: they are time-consuming, often exceedingly expensive, ethically challenging and not favoured by large portions of the populace or the law-makers. Most importantly: the same decades-old *in vitro* cell lines used in reductionist, artificial monolayer cultures on plastic surfaces, are routinely utilized for xenotransplantation experiments. Again the industry is facing a dilemma: how to select an informative model that reproducibly reflects the function of the drug target and simultaneously predicts the performance of the drug candidate in a living organism (patient) reliably? How can these *ex vivo* models be at the same time fast (high throughput) and cost-effective, to allow multiple iterative rounds of lead compound prioritization?

Novel, more representative *in vitro* models have recently emerged to answer the industry's increasing demand for more predictive experimental systems, falling in between the area of over-simplistic monolayer cell cultures, and time-consuming animal experimentation. Already the mere addition of a third physical dimension in the form of biologically relevant extracellular matrix (ECM), to mimic the complexity of the tumor microenvironment (TME), represents a key step towards generating better models that recapitulate many cellular and multicellular processes previously observed only in living tissues. Certain ECM substrates promote the formation of multicellular structures that closely resemble tissues and even small organs (organoids). Cells of glandular origin, e.g. from prostate, breast, thyroid or salivary glands, engage in differentiation processes and form hollow spheres, tubular ducts, or both [Åkerfelt, Härmä & Nees 2011].

Cancer cells, however, typically fail to undergo a complete normal differentiation process. Instead, they display a wide spectrum of differentiation defects or even pronounced cancer hallmarks, such as high invasive potential, hyperproliferation, and resistance to antimitotic or pro-apoptotic chemotherapeutics. Nevertheless, some prostate cancer (PrCa) cells display very strong epithelial plasticity when cultured in laminin-rich ECM, allowing them to actively shuttle between overtly invasive and differentiated states [Harma et al. 2010, Harma et al. 2011]. This phenomenon is possibly linked to the formation of metastases, and may mimic the plasticity of tumor cells that allows them to cross epithelial barriers (extravasation, intravasation), survive in blood stream and alien tissues, and eventually form distant metastases. This dynamics is not straightforward to address by conventional cell cultures or animals. When complemented by additional cell types, three-dimensional cultures become even more organotypic and mimic the genuine tumor microenvironment. These co-culture models can be used to assay e.g. angiogenesis and tumor-stroma interplay.

With the dramatic increase in computational power and advances in microscope automation, it is now possible to assess many experiments simultaneously (high-throughput screening, HTS) but also to look at individual experiments in much more detail (high-content screening, HCS). Traditionally, cell-based HTS relies on the measurement of simple cellular functions (e.g. proliferation, metabolic activity, viability versus apoptosis). This is routinely achieved via relatively complex, often indirect and artefact-prone assays. The read-out typically is fluorometric or luminometric, and requires lysis of cells (i.e. destruction of samples). Such assays are strictly

non-dynamic and measure only a single end-point. An interesting current trend, however, is to take measures from all cells individually, combining automated microscopy and sophisticated image analysis tools. This basically means that data points from a single experimental sample may increase into thousands or more. Up to date, statistical tools have been mainly required to filter background noise and assist in mining the relevant information. In HCS, size and morphology of the cells is quantified to detect changes in the cellular phenotypes, caused by experimental perturbations such as drug/inhibitor exposure or gene silencing. This information content may be further increased by combining multiple assays. For example, apoptosis/necrosis and viability status can be quantified simultaneously together with cell morphology, using simple live cell and realtime reagents. So far, however, HTS/HCS tools were available mainly for conventional monolayer cell cultures. Currently, no robust methods exist that fully harvest the more informative organotypic cell cultures in high-throughput target identification and validation. There is a lack of both standardized analysis tools and convenient low cost cell culture platforms, suitable for experimentation with tens or hundreds of organotypic samples at the time.

In this thesis, a comprehensive laboratory work flow is presented for the simplified utilization of organotypic cell models in pre-clinical drug discovery, target identification and validation. This workflow is complemented by a set of novel microscopy-based analysis tools, optimized to detect and quantitate dynamic morphological responses in three-dimensional culture.

REVIEW OF THE LITERATURE

The modern drug discovery pipeline

The drug discovery process is divided in five distinct phases: basic research (target identification), lead discovery and optimization, preclinical development, clinical development, and eventually drug registration: filing for approval (Figure 1). The drug target is identified and selected in basic research, often initiated by academia and followed up by pharmaceutical or biotech companies. After this, a lead drug is selected for further preclinical development. Ideally, the preclinical studies end up in a solid drug candidate, for which the company applies a status of investigational new drug (IND), a chemical entity that can enter clinical patient trials. After an average of six years of successful clinical investigations, typically comprised of 3-4 phases of increasingly large clinical trials, the drug may be registered and filed as a new drug application (NDA). Both IND's and NDA's are reviewed and approved by medicinal agencies, such as the Food and Drug Administration (FDA) in the United States and European Medicines Agency (EMA) in the European Union. These agencies evaluate safety, risks and clear medical benefits of the pharmaceutical entity, prior to approving it for sales and marketing. The whole process in average takes 12-15 years, with expenditures in excess of one billion US dollars [Hughes et al. 2011] per drug. As most resources, both money- and time-wise, are spent on clinical trials, preclinical validation work needs to be thorough, and the evidence produced has to be predictive and tenable. The preclinical drug discovery itself can be further divided into distinct phases: initial target identification and validation, assay development, high-throughput screening, hit identification, hit-to-lead development and lead optimization and finally the selection of candidate molecules for further clinical development (summarised in Figure 2) [Hughes et al. 2011].

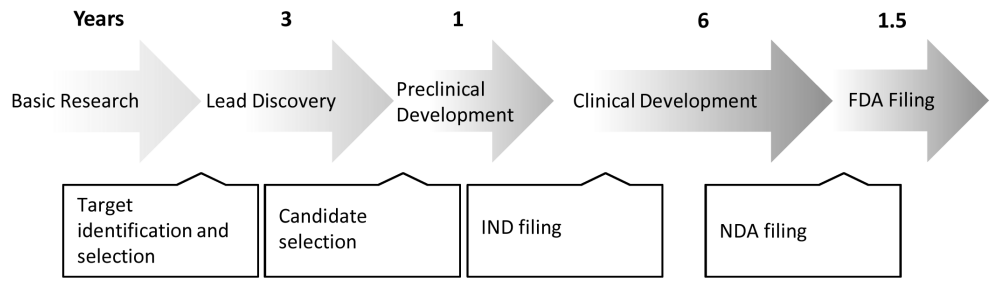


Figure 1. Drug discovery process from target ID and validation through to filing of a compound and the approximate timescale for these processes (adapted from Hughes et al. 2011).

Target identification

A biological target is usually a protein or a protein-protein-interaction, rarely a gene or an RNA, whose activity can be modified by an external stimulus. It needs to be efficacious, safe, meet clinical and commercial needs and, above all, it should be "druggable" [Hughes et al. 2011]. A druggable target has to be accessible for putative drugs such as a small molecule or larger biological (e.g. function-blocking antibodies). Most commonly druggable targets are proteins such as enzymes,

ion channels, G-protein coupled receptors, and many other receptors whose behaviour can be modulated by external stimuli. The stimulus may be a small molecule that blocks the active site of a receptor or inhibits binding of the native ligand. The biological response elicited may be specific activation or inactivation of the target. The terminology depends on the nature of the target: an obstructive effect is referred to as enzyme inhibition, ion channel blockade, or receptor antagonism. More than 50% of the known drug targets are G protein-coupled receptors (GPCRs). The second largest group, especially interesting for novel targeted therapies, consists of protein kinases. Another traditionally significant group, frequently targeted in prostate and breast cancer treatment, are the nuclear hormone receptors, such as androgen receptor (AR) and estrogen receptor (ER).

The hypothesis that interfering with a certain cell signalling pathway results in a therapeutic effect is often initiated by Academia. In the past, targets were disclosed one by one, essentially through basic research, as most researchers were concentrating on singular cellular functions and signalling pathways. In the post-genomic era (beyond 2001), systems biology facilitated by advances in laboratory automation and information technology, has enabled the effective identification of multiple targets through systematic mining of extensive biomedical databanks. These data now consist of a variety of sources such as publications and patent applications, large-scale, genome-wide mRNA expression and proteomics data, genetic phenotyping (genome-wide linkage analyses), or compound profiling [Yang, Adelstein & Kassis 2012]. The most widely used identification approaches use transcriptomics and proteomics databases, to examine the correlation of mRNA/protein expression and disease states and to determine their target potential. Moreover, recent advances in whole genome sequencing allow a functional examination of genetic associations, for example a link between genetic alterations (e.g. specific mutations and gene fusions) and the phenotypic disease. These bioinformatic (or chemoinformatic) approaches not only generate endless lists of novel potential targets, but they also help to filter out those unlikely to result in viable drugs, and thus represent a huge asset in drug or drug target selection and prioritization.

The use of *in vitro* cell models as a source for drug targets has also been accelerated by systems biology and high-throughput techniques. Genome-wide mRNA expression analysis and protein expression profiling have helped to pinpoint potential druggable targets in specific cellular functions. Whole-genome RNAi screens and small molecule screens, on their own right, represent an unsupervised, open or “shot in the dark” method: all genes and proteins are blindly modulated one by one, and the outcome (cell number, apoptosis, metabolic activity, motility) is quantified using biochemical or microscopy-based methods. In fact, robust high-throughput screening (HTS) assays, even uncharted chemical libraries comprised of thousands to hundreds of thousands of small molecules, can be effectively screened to identify promising new drug targets. Furthermore, old (previously FDA-approved) pharmaceutical entities have been successfully screened for new indications [Gupta et al. 2009, Iljin et al. 2009] (a.k.a. drug repositioning). The use of generic drugs saves valuable resources, as target specificity and drug safety have already been established beforehand. Also phage-display antibody libraries have been successfully utilized in screens, aiming to identify surface antigens that are (in the ideal case) exclusively present on cancer cells [Kurosawa et al. 2008].

Target validation

Targets can be divided in new and established targets. For established targets, there is generally good scientific understanding, typically supported by a noteworthy publication history. New

targets are usually recently discovered proteins or proteins whose function was only recently elucidated through basic research. Ideally, core functions of an established target are widely known in normal physiology and human pathology. Established targets also include such that have previously gone through drug discovery processes, however possibly in an entirely different context. This background information may provide valuable information related to the feasibility to develop small molecular therapeutics against the target, and can significantly promote and speed up licensing opportunities as well as freedom-to-operate indicators with respect to small-molecule therapeutic candidates. In general, the more background information exists for a target, the less investment and effort are expected to be required to develop a therapeutic means. This process is called target validation in every day pharmaceutical industry parlance. Modern validation techniques range from *in vitro* tools, whole animal models, to the modulation of targets in disease patients (by siRNAs or miRNAs). Typically, the process includes all of them.

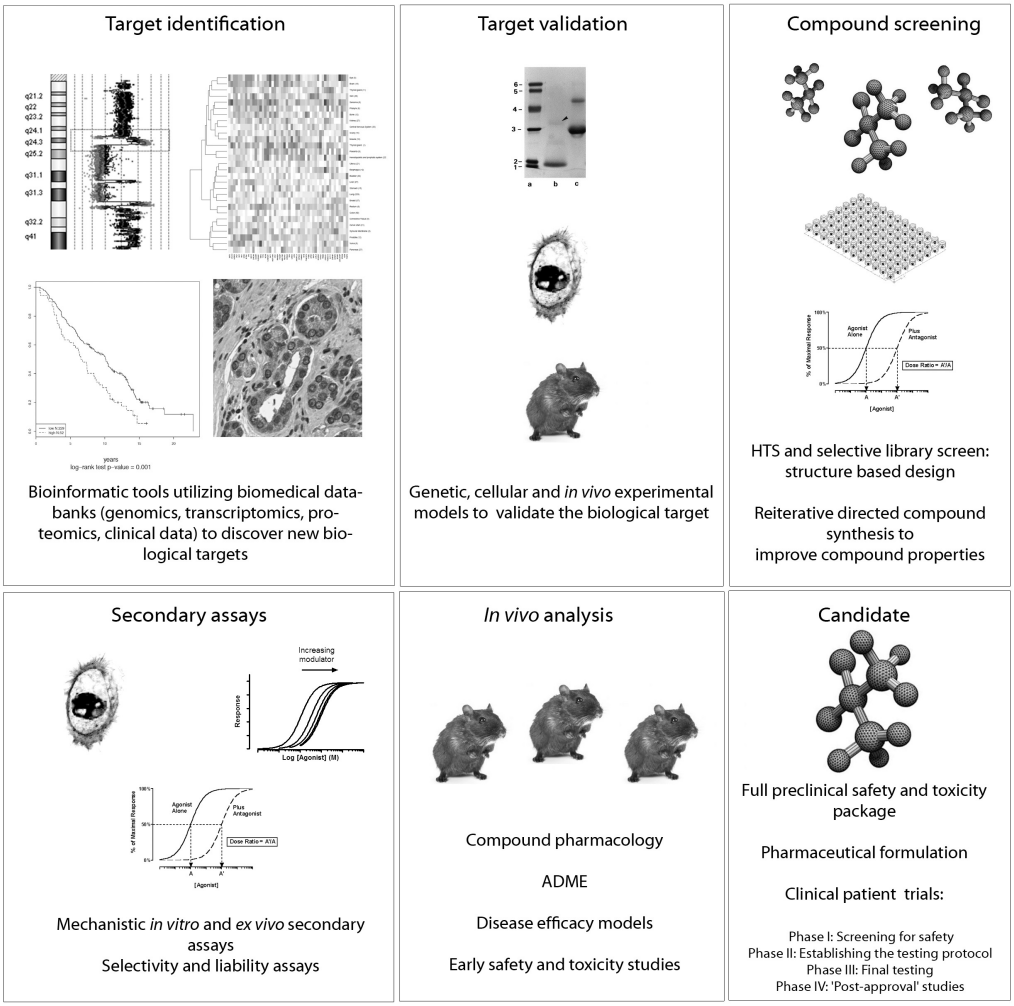


Figure 2. An overview of a typical drug discovery process (adapted from Hughes et al. 2011).

Target silencing through antisense technology and RNAi

A number of *in vitro* technologies can be applied in drug (target) validation. Usually, the investigative techniques aim to obstruct the normal physiological function of the target by attacking it on the DNA/gene, messenger RNA (mRNA), or the protein level. Oligonucleotides represent a class of synthetic agents that have been utilized to inhibit gene expression on the mRNA level. In principle, oligonucleotides can be designed to recognize any mRNA target sequence, and block protein translation in a process called gene silencing. Antisense oligonucleotides (ASOs) and RNA interference (RNAi) are both designed to target mRNA. Antisense technology was used to alter the expression specific genes by hybridizing a single-stranded antisense oligonucleotide to its unique mRNA sequence. Despite fairly good results in cell culture, the technology has proven to be of limited use compared to RNAi, mostly because of the stability issues related to the single-stranded oligonucleotide [Watts, Corey 2012]. Also, the delivery of antisense oligonucleotides into target cells or the cell nucleus is problematic [Ilag et al. 2002]. RNAi technology relies on small interfering RNA molecules (siRNA), delivered as much more stable double-stranded oligonucleotides [Watts, Corey 2012]. After a duplex RNA is introduced (delivered) into the cytoplasm one way or another, it binds the protein machinery of the RNA-induced silencing complex (RISC) [Siomi, Siomi 2009]. The endonucleases included in RISC, primary active components of the complex, are the argonaute proteins. Argonaute endonucleases cleave the target mRNA strand complementary to the bound siRNA. RNAi was first described in mammalian cell culture in 2001 [Elbashir et al. 2001]. In nature, RNAi together with endogenously expressed micro RNAs (miRNA), close relatives of siRNA oligonucleotides, represent a vital part of gene regulation in many biological processes, such as the immune response to viruses and other foreign intruders, or self-propagation of transposons in plants [Stram, Kuzntzova 2006]. RNAi and miRNAs are also heavily involved fine-tuning the regulation of genes in development and morphogenesis [Carrington, Ambros 2003]. Since the original publication in 2001 [Elbashir et al. 2001], siRNAs have been extensively utilized in experimental studies, aimed at examining gene functions. These days, siRNAs can be readily designed and synthesized to silence any gene or even gene fragment (exon) in the human genome. Comprehensive genome-wide and validated siRNA libraries are commercially available from a number of companies. Additionally, custom-designed libraries may target only the druggable genes (e.g. kinases, receptors). Synthetic RNAs used for gene silencing are duplexes, typically between 19–22 basepair long. Duplexes greater than 30 bp in length, bear the risk of provoking a strong interferon response in cells, while too short nucleotides suffer from instability. While ASOs continue to be used for gene silencing, the robust nature of siRNAs and the relative ease of identifying active siRNAs have made them the silencing tool of choice for many laboratories.

Oligonucleotide delivery methods

The most common method for promoting cellular uptake of oligonucleotides involves mixing cationic lipids with negatively charged nucleic acids [Watts, Corey 2012]. The long hydrophobic chains of liposomes and the positively charged head group allow the formation of nano-sized complexes (micelles, nanoparticles) in which the negatively charged nucleotides are readily encapsulated. These liposomal complexes offer sufficient protection to oligonucleotides from enzymatic degradation, and allow efficient endocytosis by the cell. Once the lipid-nucleic acid complex has crossed the cell membrane, the active oligonucleotide cargo is partly released into

the cytoplasm. Many cationic lipids are commercially available. In recent years, synthetic cationic polymer-based nanoparticles were developed to enhance transfection efficiency and reduce cytotoxicity, compared to classic liposomes [Aigner 2006b]. For example, polyethylenimine (PEI) complexation now represents a very promising method for siRNA delivery, and seems to work both *in vitro* and *in vivo* [Aigner 2006a, Aigner 2006b, Boussif et al. 1995]. PEIs form non-covalent complexes with DNA and are efficiently taken up by cells through endocytosis. As a result of its pH buffering properties the complexes induce bursting of endosomes and release of active siRNA in the cytoplasm [Aigner 2006b]. The properties of PEIs have been further improved to increase the fusion with cell membrane and entry into the cell, enhanced release of siRNA molecules inside the cell and reduced intracellular degradation of siRNA-nanoparticle complexes [Dalby et al. 2004]. Electroporation is an old but still commonly applied and extremely effective method [Bergan et al. 1996]. The cells are exposed to a brief but powerful electric pulse during which the membrane lipid molecules reorient and undergo thermal phase transitions due to heating. This results in temporary creation of hydrophilic pores. The transient loss of the semipermeability of cell membranes leads to escape of intracellular contents, such as ions and metabolites, and simultaneous uptake of drugs, molecular probes, or nucleic acids. Electroporation is a very reliable method and tends to also work with hard-to-transfect cells, such as primary and suspension cells, in contrary to cationic lipid-based delivery systems. However, due to massive perturbations in the integrity of the cell membrane, electroporation may induce high cell mortality [Tsong 1991]. In addition to cationic vehicles, siRNA can also be conjugated to a hydrophobic moiety, such as cholesterol. Cholesterol-conjugated nucleic acids are readily taken up by cells, and have a huge pharmacological potential. Other effective and less cytotoxic methods include the addition of certain cationic cell-penetrating peptides, such as MPG, transportan or penetratin, to the siRNA using covalent or electrostatic interactions [Zhang et al. 2006]. Recent studies have shown that active ASOs can freely enter some cell lines even without lipid addition [Stein et al. 2010, Zhang et al. 2011]. This method would facilitate the transfection of cell lines that are not compatible with lipid-mediated transfection or that are too vulnerable for electroporation. Also the protocol is simplified and off-target effects are less likely to occur as the lipid toxicity is avoided. Higher concentrations of ASO, however, are needed relative to the amounts used in lipid-mediated transfections [Watts, Corey 2012].

Target overexpression

The quantity, by which target genes are expressed in cells, can be artificially modulated by the introduction of complementary DNA (cDNA) of a selected gene, integrated into an expression vector. The gene is usually preceded by a strong promoter region that drives rapid and continuous transcription. Plasmids are commonly used as vectors and naturally occur in bacteria, where they can be effortlessly replicated. There are numerous expression plasmids commercially available, each of which can be sequence modified. Common modifications include the inclusion of bacterial or mammalian selection markers (antibiotic resistance genes, genes coding fluorescent proteins), or targeted mutations for functional studies. Sequences coding important regions in the protein, such as sites essential for kinase activity or ligand binding, can be experimentally mutated. The delivery methods for plasmids are similar as those described for oligonucleotides, ranging from electroporation to cationic reagents. The main difference is that unlike oligonucleotides, DNA must be delivered to the nucleus. Typically, the overexpression effect is only temporary, as is the silencing effect of a siRNA. Sometimes, however, the plasmids integrate to the host genome and positive cell clones can be selected from the population.

Stable target modification through viral methods

The efficiency of an individual siRNA depends on a number of factors such as the transcription rate, the stability and half-life of mRNA transcripts and the corresponding proteins, the efficacy of siRNA sequence design, and the characteristics of the cell line used [Crombez et al. 2007]. Typically, silencing peaks around 2-3 days after transfection, and rarely lasts for more than one week. This general transiency complicates long-term experimental setups, such as *in vivo* animal experimentation. It is difficult to use siRNAs and cDNA overexpression vectors, unless stable transfection is achieved. Viral delivery represents a much more consistent solution. Recombinant viral vectors, e.g. based on retro-, adeno- or adeno-associated viruses (AAV) or lentiviruses, have been engineered. Commercial retro- and lentiviral solutions have been optimized to enter any type of host cells, even cells not easily amenable to transfection, and permanently integrate the foreign DNA as "provirus" into the genome. The most common method to knock down target genes by viral vectors is RNA interference, utilizing short hairpin RNA (shRNA) sequences. ShRNA is in effect a siRNA, in which the sense and antisense strands are linked by a short spacer sequence, leading to the expression of a stem-loop structure. This shRNA sequence is integrated in the host genome by the viral machinery, and constitutively expressed by strong promoters. After transcription, the cellular machinery effectively cleaves the shRNA hairpin structure into functional siRNA, which is then bound to RISC and consequently to the matching mRNA to be degraded or blocked. Also drug resistance markers can be incorporated, allowing the selection of cells with stable knockdown [Rutz, Scheffold 2004].

Target protein modulation using monoclonal antibodies

Antibodies have traditionally been used in diagnostics (ELISA; enzyme-linked immunosorbent assay), histology (immunohistochemistry, IHC), cellular imaging (immunofluorescence, IF) and protein quantification (Western blot, WB), to detect, quantitate and visualize proteins of interest. Monospecific antibodies, i.e. antibodies that recognize the same structure or epitope, are called monoclonal. These are produced by immortalized hybridoma cells extracted from animals immunized with the target protein, or an immunogenic polypeptide representing a relevant epitope. In function-blocking mAbs, this can be an active kinase, ligand binding, protein-protein-interaction, or a phosphorylation site. Monoclonal antibodies interact with large regions of the target molecule surface, often with unparalleled precision, and thus represent an excellent target validation tool. Because of the highly specific nature of the antibody-epitope interaction, antibodies can be selected to bind unique epitopes with little cross-reactivity. Function-blocking mAbs can be utilized to target and functionally incapacitate receptors, extracellular enzymes, or secreted (growth-)factors. In addition to conventional immunization methods, a more high-throughput method utilizing recombinant antibody fragments produced by bacteriophages was introduced in the 1980's [Smith 1985], and later revolutionized the entire field of drug discovery [McCafferty et al. 1990]. These phage display methods allow large scale screening and amplification of protein libraries by *in vitro* selection, analogous to natural selection. The basic principle relies on recombinant libraries of antigen-binding domains, expressed in bacteriophages. The functional protein portion encoded by the genetic material is fused with the virus' coat protein, and actively displayed on the bacterial cell surface. Through consecutive series of binding and amplification, viruses displaying antibody fragments with the highest target affinity can be selected and amplified. The advantage of the phage display system is that the

connection between genotype and phenotype remains transparent, and the complete genetic information responsible for an outstanding affinity can be incorporated into a humanized antibody, for example for therapeutic purposes. Phage display allows to study interaction partners of a given target protein for function and mechanism, to determine tumor antigens for diagnosis and therapeutic targeting [Hufton et al. 1999], or to identify protein-DNA interactions using randomized segments [Gommans, Haisma & Rots 2005]. The greatest weakness, which restricts the research or pharmaceutical use of monoclonal antibodies, stems from the fact that these do not readily enter living cells or tissues, and are only applicable to extracellular targets.

Small molecules as pharmacological tools

Small molecules are typically thought to be the characteristic, prototype end products of drug development pipeline. However, they also play an important role as valuable pharmacological tools in target validation, and even target identification. In pharmacology and biochemistry, the term "small molecule" usually refers to an organic entity with a low molecular weight, by definition excluding polymers. A pharmacologically interesting small molecule bears the ability to specifically modulate the physiological activity of a target by binding to it with high affinity. Typically, the target is a biopolymer, such as a protein, nucleic acid, or occasionally a polysaccharide (e.g. in glycoproteins and proteoglycans). In principle, there is no strict lower weight limit for a small molecule (e.g. lithium ions used to target depression have a MW of 3 Dalton). However, to guarantee effective diffusion across cell membranes, ideal molecules should not be larger than 800 Daltons. Most small molecule inhibitors used in pharmacology are natural molecules, or synthetic compounds inspired by natural compounds. However, many are also completely synthetic, e.g. those produced by large-scale combinatorial chemistry. Many small inhibitors are approved therapeutics, while others are purely detrimental (toxic) to human health (teratogenic or carcinogenic) and cannot as such be considered for clinical application. The therapeutic window between the toxic side effects and specific functionalities is often relatively narrow, e.g. in cytotoxic compounds used for anti-cancer therapies like cisplatin, taxanes, or vinca alkaloids which are all highly poisonous. Some small molecules mimic endogenous proteins that regulate the activation and inactivation of enzymes, ligands or receptors in nature. The interaction typically occurs by binding the target protein's active or function-modifying allosteric sites. The natural substrate typically fits the active site like the key fits to a lock, and blocks access of other (substrate) molecules to the active site, or by obstructing the catalytic function. Allosteric activation and inhibition, on the other hand, refer to situations where a protein or a small molecule binds a part of the target protein other than the active site, which affects and alters the protein conformation and can switch an enzyme from an active to an inactive state. For example, phorbol 12-myrstate 13-acetate is a very common investigative tool in cancer research that potently activates protein kinase C, a known promoter of tumor growth. An interesting new group of small molecules are artificial transcription factors, such as wrenchnol [Koh, Zheng 2007].

One of the greatest benefits of small molecule compounds is their easy applicability. They can be utilized in mammoth-sized *in vitro* high throughput screens, involving libraries of thousands to millions of compounds. They can also be used in small-scale animal experiments, testing bioavailability and toxicity. The chemical properties of small molecules can be studied to great detail, and chemical entities can be extensively modified to increase solubility, and facilitate crossing the plasma membrane, nuclear envelope and other biological membranes in

mammalian body, such as the blood-brain-barrier. However, the major disadvantage of the small molecules is their potential poor specificity; or dual specificity for multiple targets that may be closely related. All proteins sharing highly conserved active sites, represent potentially hard-to-hit targets. Frequently, off-target effects have an exceeding influence on the experimental or therapeutic results, or may be predominantly toxic. This is particularly true for many promising target protein classes in oncology, such as protein kinases, or receptor tyrosine kinases (RTKs). Also transcription factors, with the exception of hormone-binding receptors with receptor activity, are very difficult to modulate by the use of small molecules. An exception are rare examples where protein-protein-interactions involving transcription factors, may be affected by small molecules (such as nutlin, targeting p53 functions).

The prime source for small molecules is nature. The lion's share of pharmacologically active molecules is produced in the plant kingdom as secondary metabolites (alkaloids, terpenoids, etc.). These secondary metabolites are organic compounds not required or directly involved for normal growth, development and reproduction. Evolution may have favoured the generation of these compounds because they play an important role in defence against herbivores [Stamp 2003] or stress. In order to be effective, such metabolites have to be fairly specific against a specific herbivore, not affecting e.g. beneficial (pollinating) insect species, or animals that distribute the seeds. For example capsaicinoids, the secondary metabolites of chilli peppers (genus *Capsicum*), protect the plant's fruits from mammalian herbivores by causing tissue irritation and burning sensation via activation of TRPV1 vanilloid receptor. Natural selection has chosen birds as the predominant dispersers of chilli pepper seeds, as they lack molar teeth allowing the seeds to pass through the digestive system unharmed [Tewksbury, Nabhan 2001]. Accordingly, and in contrast to mammals, the avian TRPV1 receptor is not affected by capsaicinoid metabolites. In general, secondary metabolites can be classified according to their biosynthetic origin. The most interesting classes are alkaloids (e.g. cocaine, morphine, codeine, tetrodotoxin, vincristine and vinblastine) and terpenoids (e.g. artemisin, tetrahydrocannabinol, steroids and saponins). Larger metabolites (e.g. polyketides, fatty acid synthase products and non-ribosomal peptides) tend to be more useful as antibiotics or antimicrobial. As a result of the widespread use of HTS methods, the demand for collecting large libraries of natural or synthetic compounds has been immense, trying to satisfy the enormous capacities of these screens [Newman, Cragg 2007]. Huge libraries have been established by academic institutes (e.g. NIH-MLSMR or the National Institutes of Health Molecular Libraries Small Molecule Repository in the United States) and the pharmaceutical industry; often also in collaboration (public/private research initiatives). Furthermore, modern combinatorial chemistry, based on computer simulation, facilitates the rapid synthesis of thousands to millions of different but often structurally related molecules. Nevertheless, current trends increasingly move towards more focused (100 to 3000 compounds) collections that incorporate the immense "structural aspects" of natural products [Newman, Cragg 2007]. Apart from chemical similarity, small molecules may be divided into libraries according to known mechanisms of action (MOA), source and specificity. For example, NIH-MLSMR has grouped its compounds collected from commercial, academic and government suppliers into four classes: i) specialty sets comprising known bioactive molecules such as drugs, toxins, metabolites, and others; ii) natural products and derivatives from known and documented natural sources; iii) targeted libraries subcategorized into protease, kinase, GPCR, ion channel, and nuclear receptor sets; and iv) "diversity compounds", representing all other molecules available for purchase or acquisition.

Transgenic animals

Utilizing modern genetic engineering techniques (= recombinant DNA technologies), the genetic material of any organism can be modified with absolute fineness. Individual genes can be knocked out and in, foreign genetic material can be introduced – even from completely unrelated species – and genes can be locally and specifically induced at defined locations and tissues. In target validation, transgenic animals have proven attractive tools in elucidating the functional consequences of target gene manipulation in animals [Hughes et al. 2011]. Gene knockouts are created *in vitro* by recombination of the target gene with a foreign sequence. The recombinant gene is then translated into a non-functional protein, transferred into a mouse embryonic stem cell and inserted into the mouse embryo. As knockout animals bear the non-functional protein through their entire lives, they can give important insights into the functional role of the target gene in embryonic, fetal and tissue development, or early life. Conditional knockouts allow temporary and/or tissue-specific gene deletion. As many genes have an important role in embryonic development, and homozygotic deletions may result in embryonic lethality, conditional knockout technologies may provide an alternative route for these studies. In gene knock-in studies, a gene is replaced by another functional gene, for example to repair a mutated gene in the animal. The use of transgenic animals is time-consuming and requires animal core facilities or contracting, costs and resources rendering this option unfeasible for large-scale screens or validation of hundreds of candidate targets. However, as a final validation tool of a few well-defined targets, transgenic or genetically engineered mouse models (GEMMs) may give the most predictable data currently available.

Tissue microarrays

Biobanks around the world collect huge libraries of samples from all human diseases. As the bulk of the cancer management still relies on surgical removal of tumors, cancer biopsies are (in theory) readily available from hospitals performing surgical operations. Solid tumor tissues can be stored as formalin-fixed, paraffin-embedded blocks for decades. In most cases, samples are not systematically collected at defined intervals from the same patient, and only rarely sampled from metastasized cancer cases (except in few systematic “rapid autopsy programs”), hospital records (clinical annotation) is routinely used to associate biopsies with progression of the disease. Libraries of historic archived tissue materials can therefore be complemented with detailed case histories. These can be utilized for the identification of prospective biomarkers or validation of the predictive value of certain therapeutic targets, as the disease outcome is already known. Additionally, biomarkers can be identified that may correlate with therapeutic response, failure of therapy, or relapse, also in connection with targeted therapies in clinical trials (companion biomarkers). Traditionally, immunohistochemistry has been a cumbersome, material- and time-consuming method, as many individual histological samples have been stained one by one, using large reagent volumes. Furthermore, such collections are traditionally geographically centrally localized, due to the general resilience of pathologists and hospitals to share scarce clinical material. To amend this situation, tissue microarrays (TMAs) have revolutionized IHC, now allowing multiplex histological analysis and laboratory automation together with more economical antibody consumption [Battifora 1986, Kononen et al. 1998]. In TMAs, small needle-punch biopsies are acquired from tens to hundreds of tissue cores, and arrayed onto a receiving paraffin block [Kononen et al. 1998]. Each block can be cut into hundreds of sections.

Each of these can ideally be subjected to different IHC reactions, using different monoclonal antibodies against the various targets. Alternatively, mRNA can be detected by fluorescence *in situ* hybridization (FISH) or silver-stained in situ hybridization (SISH). Hundreds of tissue samples can thus be distributed amongst experts, as the arrays come in a standardized microscope slide format. A new emerging field of computational pathology [Fuchs, Buhmann 2011] combines these advances with automated microscopy and image analysis, and is expected to facilitate the screening and quantitative grading of thousands of histological samples with decreasing need for direct expert interaction (i.e. pathologist). Furthermore, even without shipping sections and samples, stained array data (IHC and FISH images) can be shared between pathologists – based on web interfaces – using virtual microscopy [Lundin et al. 2009].

The hit discovery process

Years of exhaustive investigations may be required in industry, to convincingly show that a certain target is truly valid, and a serious drug discovery effort should be considered. The purpose of the hit discovery phase is then to identify small molecules or biologicals (such as monoclonal antibodies) that specifically interact with the target protein, exclusively in the desired manner. The most effective candidate molecule(s) are then chosen for lead optimization, in which pharmacological characteristics such as selectivity, potency, solubility and bioavailability improved, toxic side-effects minimized or physiochemical properties further modified. Hit discovery is the phase that most heavily relies on HTS methods, combined with vast libraries of small molecules and antibodies. Pharmaceutical companies, and in recent years also some academic institutes such as the NIH Roadmap Initiative in the USA [Frearson, Collie 2009], have set up large organizations with the sole purpose to assemble informative compound collections. These institutes further provide and improve the necessary infrastructure to screen compounds effectively, identify hit molecules from the HTS data, and converting these hits into clinical drug candidates (hit-to-lead development) [Hughes et al. 2011].

HTS screening assays in hit discovery

The choice of assays and models is crucial, particularly in the hit discovery process. The candidate molecule has to specifically hit the target, and ideally have favourable biological and therapeutic effects. Numerous HTS assays have been developed for hit discovery; but most do not directly assess direct effects on the target protein, and measure indirect cellular responses instead. Exceptions are cell lines harbouring very specific reporter constructs (e.g. provided by Caliper or DicoverX), in which target molecules are fused e.g. with fluorescent or luminescent probes. However, these constructs are often only available in generic cell lines such as HEK-293 or BHK, and do not represent a relevant genetic or tissue-specific background for oncology. When applying large compound libraries in a random manner, neither of the classic screening approaches assumes prior knowledge concerning the nature or chemotype of compounds, and no estimations which molecules may be most likely to show biochemical or biological activity [Hughes et al. 2011] can be made. If the primary screening assay was cell-based, hit molecules need to be further validated using secondary biochemical assays (*in vitro*), to evaluate the specificity of the molecule. However, compound libraries can also be more focused. Existing prior knowledge, e.g. literature research, may help to select libraries comprising of chemical classes known to have activity at the drug target [Hughes et al. 2011].

Rational drug design using virtual screens is a powerful way to narrow down the list of potentially promising chemicals. *In silico* screens can virtually screen exceedingly large compound databases, and quickly identify chemical structures that are most likely to specifically bind to a certain drug target [McInnes 2007, Rester 2008]. Virtual screens can be either ligand- or structure-based. Ligand-based screens rely on availability of prior knowledge of molecules known to bind specifically to a desired target. This information is used to create a collective ideal pharmacological model, known as the pharmacophore model. The small molecules in the library are then computationally compared with the virtual pharmacophore model, in order to identify a set of candidate ligands [Sun 2008]. Another *in silico* method is based on the assumption that the three-dimensional structure of the target protein is known, e.g. from X-ray crystallography. This method uses computational simulation of prospective chemical interactions, to estimate whether a ligand candidate would specifically fit into a ligand-binding, catalytic or allosteric pocket of the protein target. This is performed by virtually docking thousands to millions of small molecules into the protein structure, and scoring their fitness [Kroemer 2007]. The specificity of this process can be improved massively by combining independent docking methods, although this demands an increasing amount of computational power. Sometimes, a method called fragment screening is performed, to identify chemical structures binding to the target protein. Fragment libraries are typically fairly small (a few thousand molecules), and consist of low molecular weight compounds (less than 300 Daltons). These molecules are screened in high, often in millimolar, concentrations to identify even entities with weak affinities.

Assay development

A typical drug discovery programme begins with the development of appropriate biological assays for use in hit identification. Hughes et al. have listed five important factors required to be considered in HTS assay selection [Hughes et al. 2011]:

1) Pharmacological relevance: The capability of the assay to identify compounds that are specific and match the desired effects on the disease, and are known to be mediated by the desired mechanism of action in focus.

2) Reproducibility: The results may not depend on day-by-day variations, technical difficulties and fluctuations, material restrictions, e.g. they should not vary across the assay plates.

3) Cost: The reagents and assay volumes should be selected to minimize the cost per experiment in the assay, while simultaneously guarantee high performance and information content.

4) Quality: A robust HTS assay should have a simple read-out based on stable reagents and a limited number of steps (washing, plate-to-plate transferring). If a new assay is implemented, it needs to undergo a thorough and very stringent statistical quality control. The most common indicator for quality is the Z' factor [Zhang, Chung & Oldenburg 1999], which attempts to quantify the suitability of a particular assay for use in high-throughput screens. In larger screening efforts involving hundreds of plates, the Z' factor is constantly monitored to ensure the quality throughout the entire campaign.

5) Solvent effects: Compounds in chemical libraries are usually stored in organic solvents such as ethanol, or dimethylsulfoxide (DMSO), which are potentially cytotoxic at greater

concentrations. Biochemical assays can be sufficiently performed in high solvent concentrations up to 10% DMSO, whereas in cell-based assays 1% DMSO represents an absolute maximum. Thus, solvent effects have to be carefully excluded or separated from specific effects.

It is common to test all new HTS assays with a verified training set for acceptable performance. The training screen is typically replicated two or three times to determine the false positive and negative hit rate and the Z' factor.

Defining the hits

The overall goal of the hit definition stage is to identify compound clusters, i.e. multiple groups of molecules that share structural similarities and have the desired effect on the target. First, hit selection obtained through virtual or high-throughput screening is grouped computationally, based on structural (pharmacophore-related) similarity. After this, dose-response curves are generated using fresh samples of the hit compounds, to rank them in order of their potency or specificity. Generally, compounds that bind their targets reversibly are favoured over the irreversible or covalent binders, since these effects can be eliminated (washed out) over time. This is clearly an important feature for any pharmaceutical entity used in the clinics, thus avoiding chronic, permanent and accumulative toxicity. The half-maximal inhibitory (Ki) concentration is typically used as the basis for ranking. In the third step, functional secondary assays that are usually tissue- or cell-based methods come into the picture. One of the primary aims of the hit definition phase is to identify structure-activity relationships (SAR), which represents a structural element or motif hit compounds may have in common. This knowledge is essential, as the molecules further proceeding into the lead-optimization phase (medicinal chemistry), will undergo strategic modifications to this core structure, to improve the potency [Hughes et al. 2011] or solubility. Chemical libraries utilized by the pharmaceutical industry are typically composed of molecules that already meet the general requirements assumed to favour drug properties. Such compounds are typically simple and small enough to be further modified in the lead optimization phase. They may also obey the universally accepted chemical parameters for a drug such as the Lipinski's Rule of Five [Lipinski et al. 2001].

Hit-to-lead phase and lead optimization

The hit definition phase produces clusters of molecules with an EC_{50} value typically between 100 nM and 5 μM. EC_{50} is a common measure of drug's potency and refers to a concentration of a drug which induces a response halfway between the baseline and maximum in a defined exposure time. Lead optimization is the phase in which the chemical structure of the core molecule is further refined for maximal potency, selectivity and pharmacokinetic properties. It is also the phase in which the first *in vivo* drug exposure experiments are performed. In practice, hit-to-lead phase represents a joint effort of chemoinformatics and medicinal chemists, synthesizing and modifying the chemicals according to the needs. Large numbers of modified lead compounds are then iteratively tested in biochemical (typically cell-free) high-throughput screens. Eventually, prior to moving into *in vivo* toxicological studies, the compounds have to be tested for ADME properties (absorption, distribution, metabolism and excretion) *in vitro*, together with physicochemical and pharmacokinetic measurements. Before permission for human administration (clinical trials, first-

in-man studies or phase I) can be applied, the compound has to undergo a series of preclinical experiments, summarized as lead optimization, with the aim to maintaining favourable properties of the lead molecule, while amending any remaining deficiencies. Also, *in vivo* tests addressing long-term genotoxicity (mutagenic properties), teratogenic or embryonic toxicity, and behavioural changes (neurotoxicity) have to be performed.

As evident from the sections above, the process from hit generation to preclinical candidate is very long. Typically, initial HT screens are performed with 200 000 – 1 000 000 compounds. The list of hit molecules is later refined to several hundred in the lead optimization programme, eventually yielding to just one or two "worthy" candidates going into clinical evaluation. Nevertheless, despite all preclinical assays performed, the attrition rate in the following clinical trials is still exceedingly high: only one in ten candidates eventually reaches the market, meaning that 90% of the clinical studies fail – mainly in phase II or III, and most frequently due to a lack of satisfactory efficacy. Toxicity and unexpected off-target effects also contribute to this high failure rate, which is extremely prohibitive for the success rate and productivity of the pharmaceutical industry. Furthermore, because of the high-cost involved, and the public nature of clinical trials, it is technically difficult to prematurely terminate any project that has come this far. This adds to the pressure on the development of better, more predictive preclinical models, assays, and biomarkers. Efforts to reduce the immense failure rate particularly in oncology are much in demand. Insufficient predictive value of classic animal models (e.g. subcutaneous xenografts) is considered one of the major problems in the drug discovery pipeline.

What makes a good pharmaceutical?

What are the requirements for a good pharmaceutical product? This depends mainly on the nature of the therapeutic. Most drugs are small molecules, increasingly biological drugs can also be proteins (such as insulin or antibodies). Many more have been introduced in the past 20 years. Oligonucleotides and viruses (mainly as drug vehicles for targeted delivery) are still more or less in the developmental stage, mainly due to issues related to their synthesis, safety and specific delivery. A recent study that covered the past 25 years of drug development listed 100 new chemical entities (NCEs) developed against cancer [Newman, Cragg 2007]. 81 of these were traditional small molecule inhibitors, from which 63 were either natural products *per se*, based on natural pharmacophores, or mimicking a natural product in one form or another. 17 compounds were biologicals, most of these monoclonal antibodies. Interestingly, 18 new drugs represented completely novel, synthetic molecular entities.

ADME

Apart from the pharmacological properties, all successful drugs have to pass the ADME standards. The route of a drug to the target tissue typically goes via the bloodstream. Most ideally, a drug is administered orally and readily absorbed in the digestive tract via the mucous surface of the intestine. In order to reach the intestine, the drug has to be stable enough to endure the harsh environment in the upper digestive system. Bioavailability is a term that describes a compound's absorption potency. If a drug's bioavailability is poor, it needs to be injected directly in the blood circulation, or it could be administered as an aerosol via the nose or lungs. Even in the patient's body, the effective drug distribution can vary greatly. Regional blood flow rates, molecular

size, polarity and complex formation with the serum proteins can affect the distribution across tissues. The central nervous system (CNS) is especially well protected from external entities by the highly selective blood-brain barrier. Any drugs targeting the CNS should have to meet special requirements. Nevertheless, all drugs incorporated will eventually be broken down (oxidized, and glucuronated) in the liver by a large set of redox enzymes, the cytochrome P450 family. Mainly relying on the activity of CYP450 molecules, the slow degradation of a compound begins as soon as they enter the body (biotransformation). The degrading compound breaks into numerous new molecules or metabolites. These may be either pharmacologically inert or occasionally much more active, e.g. with prodrugs that first need to be metabolized to become fully active. Finally, the metabolites are removed via excretion, which occurs in the kidneys. However, some drugs are not metabolised at all, but are excreted to the urine unchanged.

Small molecules

Pharmacology is an established field of science, including advances in pharmacokinetics and pharmacodynamics. Pharmacokinetics studies the fate and distribution of the drug throughout the body, whereas pharmacodynamics studies the (largely biochemical) nature of drug-target interactions. The pharmacokinetic properties favouring a successful therapeutic small molecule have been well characterized during the past hundred years of drug development. There are a number of ways to evaluate the druglikeness of a compound, Lipinski's Rule of Five being the most fundamental rule of thumb. It is used to determine if a chemical compound with a certain pharmacological activity and molecular properties can be rendered into an orally active drug in humans [Lipinski et al. 2001]. The rule states that an orally active drug has no more than one violation of the following criteria: 1) there can be no more than five hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), 2) no more than ten hydrogen bond acceptors (nitrogen or oxygen atoms), 3) the molecular mass has to be less than 500 Daltons, and 4) an octanol-water partition coefficient log P cannot be greater than five. The rule predicts the ADME properties of a small molecule and can be used as a guideline to increase its druglikeness. It, however, tells nothing about the pharmacological activity of a given molecule. Lipinski's Rule of Five is generally accepted only as a starting point in early drug discovery, since it helps to save cost and time, but does not have an impact on specificity.

Monoclonal antibodies

Invention of antibody phage display by laboratories at the MRC Laboratory of Molecular Biology led by Greg Winter and John McCafferty and at The Scripps Research Institute led by Richard Lerner and Carlos F. Barbas revolutionised antibody drug discovery [McCafferty et al. 1990]. Monoclonal antibodies make excellent drugs for extracellular targets. Their specificity is beyond comparison as they can utilize minor differences between even very closely related molecules and protein conformations, thus providing a means to reduce off-target toxicity. The absorption, administration and distribution properties, however, are not as favourable as those of small compounds. Monoclonal antibodies, or larger peptides in general, do not readily pass the plasma membrane. Orally administered peptides are often quickly degraded in the digestive tract, and can only be injected intravenously. Large peptides may also sometimes provoke serious immune responses. Regardless of the administration and distribution issues, antibodies have proved to be extremely successful drugs in many therapeutic areas, such as immunology and cancer.

Oligonucleotides

Oligonucleotides bear a great potential in specificity and theoretical ability to hit any target gene. Despite the enormous theoretical potential of oligonucleotide-based drugs, for both ASOs and siRNAs, clinical utilization has been painstakingly slow. So far, only a single ASO has been approved by the FDA, although at least 22 oligonucleotide drugs are currently in phase II or III clinical trials [Watts, Corey 2012]. The progress has been plagued by a plethora of problems ranging from poorly economical synthesis of the molecules unfavourable target tissue distribution, and general problems with specific delivery. The greatest obstacle to the clinical use has been the toxicity-related issues, especially in the case of ASOs [Ilag et al. 2002]. Duplex RNAs like siRNAs are fairly stable *in vivo,* and their stability and duration of action can be further significantly improved by chemical modifications [Watts, Deleavey & Damha 2008].

Cancer

Traditionally cancer research has heavily relied on *in vitro* experimentation utilizing cell culture models, originating from clinical tumor tissues, and also on *in vivo* disease models such as transgenic mice (GEMMs) and xenograft models. Approximately 140 FDA-approved drugs are currently available for the treatment of different forms of cancer. Despite the growing palette of medications, major improvements in early diagnosis and disease management (e.g. surgery and radiation), the global cancer burden is still increasing at an alarming pace. As modern surgical and radiation treatment options cannot cure cancers that have spread systemically and may not be available to large fractions of the human population due to high costs, there is a desperate need for novel, effective chemotherapies. One key prerequisite for more successful, productive drug discovery campaigns and development of pharmacologically very active new molecules are more predictive models for target validation and lead optimization. The exceedingly high failure rate of clinical studies, and poorly effective new drugs, represent the major problems in oncological drug development.

This chapter will introduce cancer as a disease in general, focusing eventually on prostate cancer, which is the most common malignancy in western males and the main subject of this thesis work. Further, the basic mechanisms for cancer cell migration, invasion and metastasis will be addressed. This is followed by an introduction into theoretical concepts of epithelial-to-mesenchymal transformation (EMT) and the importance of epithelial plasticity for cancer progression.

What is cancer?

Cancer is an extremely heterogeneous group of very different medical conditions, with a strong individual component. What these conditions have in common, however, is that they are all caused by cells, originating from one's own body that divide and grow uncontrollably, thus forming malignant tumors (neoplasms). Primary tumors grow locally and may invade into nearby tissues. Aggressive cancers often further spread to distant parts of the body through the lymphatic system and/or bloodstream, and form distant secondary tumors (metastases). In contrast to malignant tumors, the growth of benign tumors can be locally controlled because by definition, they do not penetrate into neighbouring tissues nor metastasize. Cancers are usually

classified according to the organ, tissue type, or cell type of origin. Additionally, histology and grades are definitive parameters used in clinical terminology.

Cancer touches most people in one way or another. According to GLOBOCAN 2008 estimates, approximately 12.7 million new cancer cases and 7.6 million cancer deaths have occurred in 2008 [Ferlay et al. 2010]. This number represents roughly 13% of all human deaths worldwide [Jemal et al. 2011]. Traditionally, cancer has been seen as a disease affecting mainly the economically developed countries. However, due to the recent adoption of cancer-causing western habits and increasing live expectance, cancer is becoming more and more also a problem in developing countries. The increase in cancer burden can be mostly be explained by demographic structures, related to increased aging in the western world, as most cancers require a decades-long progression period before they become a health problem. In developing countries, apart from increasing live expectancy, it is thought that changes in life-style, diet, smoking and physical inactivity are major factors behind increasing cancer occurrences. Currently, cancer is the leading cause of death in the developed countries, and the second leading cause of death in developing countries [Jemal et al. 2011]. In the Western countries, prostate, lung, colon, urinary bladder, and stomach are the most common cancer types in male, of which lung, colon and prostate cancers are the most lethal [Jemal et al. 2011]. Women in developed countries are diagnosed most often with breast, colon, lung, corpus uteri, and stomach cancers, of which breast, lung, and colon cancers demand the largest number of casualties [Jemal et al. 2011]. Even though, these cancers seem to arise in very different parts of the human body, and functionally very different organs, the common denominator for all these cancers is that they all originate from epithelial tissues.

Oncogenesis and cancer progression

Hallmarks of Cancer

In the second hallmarks of cancer review [Hanahan, Weinberg 2011], Hanahan and Weinberg have listed eight "distinctive and complementary capabilities that enable tumor growth and metastatic dissemination", six of which were originally introduced already in 2000 [Hanahan, Weinberg 2000] (Figure 3). Arguably, the most fundamental feature of cancer is the ability to sustain constant proliferation. Cancer cells achieve this by deregulating pathways that control the activity and modulate growth promoting signals. Cell growth promoting signals are often conveyed by soluble molecules, such as growth factors, cytokines and hormones, via their cognate receptors, many of them receptor tyrosine kinases. Mitogenic signalling, in contrast, is often based on autocrine proliferative stimulation, wherein cancer cells produce both the mitogenic ligands and the matching receptors, to activate their own cell cycle machinery (reviewed in Lemmon, Schlessinger 2010 and Hanahan, Weinberg 2011). Alternatively, cancer cells may also stimulate neighbouring cells in the tumor microenvironment, such as stromal cells, to induce release of beneficial growth factors [Cheng et al. 2008] such as chemokines and cytokines. Cancer cells may also develop hyper-sensitivity to growth factor ligands, e.g. by overexpressing the cognate receptor on the cell surface (such as EGF receptor), or in the cytoplasm (hormone receptors like AR). To eliminate dependence on ligand availability, cancer cells often acquire mutations or favourable structural alterations that facilitate consistent, ligand-independent signalling. In addition, components of the canonical downstream signalling

pathways may be mutated, contributing to constitutive activation (e.g. mutations in B-Raf or Ras genes, downstream of RTKs). Cancer cells may also enhance their proliferative signalling by down-regulating intrinsic negative feedback mechanisms, that otherwise ensure that active signal transmission remain transient. Good example for disrupted negative regulatory feedback mechanisms is the PTEN phosphatase (reviewed in Hanahan, Weinberg 2011). Oncogenic mutations in *ras* genes can impede Ras GTPase activity, thus allowing constitutive Ras signalling that subsequently promotes tumor growth. PTEN regulates PI3-kinases, important tumor growth promoting factors that also provide survival signals (via AKT protein kinase). Loss-of-function mutations or down expression of PTEN via promoter methylation can constitutively active PI3-kinase functions and promote and tumorigenesis.

In addition to avoiding pathway-specific feedback mechanisms, cancer cells must also evade powerful programs that negatively regulate cell proliferation. These programs are typically dependant on tumor suppressor genes. Two of the most well characterized tumor suppressor proteins are the RB (retinoblastoma-associated protein; pRB) and TP53 (tumor protein 53; p53). The main function of RB as a tumor suppressor is to prevent excessive cell growth, by inhibiting cell cycle progression until a point when the cell is ready to enter the S-phase and prepare for mitosis. TP53 has the potential to regulate cell cycle progression, but mainly transduces growth-inhibitory signals related to stress response and DNA damage, originating from outside of the cell. TP53 integrates information from various stress sensors within the cell. For example, in a case of DNA damage, depletion of nucleotide pools, lack of growth-promoting survival signals, low glucose or oxygenation levels. Furthermore, TP53 has the ability to block cell cycle progression until the stress response subsides and DNA damage is fixed [Hanahan, Weinberg 2011]. If the conditions are too severe to be amended, or DNA damages are irreparable, TP53 can also effectively trigger apoptosis. The LKB1 epithelial polarity protein and the *NF2* gene product Merlin are examples of the contact inhibition regulating tumor suppressors. Contact inhibition is a natural process of arresting cell growth, when cells come into contact with each other. Failure of contact inhibition is one of the fundamental characteristics of malignant transformation, and can be used to distinguish between normal and cancerous cells. LKB1 organizes epithelial structure, helps maintain tissue integrity and suppresses growth and proliferation by activating a group of other kinases, when energy and nutrient levels are scarce. LKB1 is capable of counteracting exceeding mitogenic effects of the strong Myc oncogene [Partanen, Nieminen & Klefstrom 2009]. On the other hand, Merlin acts by strengthening cadherin-mediated cell-to-cell attachments, thus limiting transmembrane RTK mitogenic signals.

Hyper-activation of oncogenes and DNA damage is also associated with hyper-proliferation that often leads to programmed cell death or senescence – unless mitigated by protective measures that deregulate the apoptotic machinery (reviewed in Hanahan, Weinberg 2011). This is usually divided into extrinsic and intrinsic cell-death promoting programs. The extrinsic program senses pro- and antiapoptotic signals from the extracellular space via “death receptors”, specialized receptors that respond to ligands largely from the TNF (tumor necrosis factor) family In contrast, the intrinsic mechanisms detect signals of intracellular origin, mainly mediated by Bcl2-family members. Both of converge in the activation of proteolytic caspase cascades, ultimately leading to degradation of genomic DNA and the nucleus, progressive self-disassembly, “blebbing” of the dying cells, followed by consumption of the debris vesicles by surrounding cells and macrophages. The most common strategy for cancer cells to avoid apoptosis is to functionally

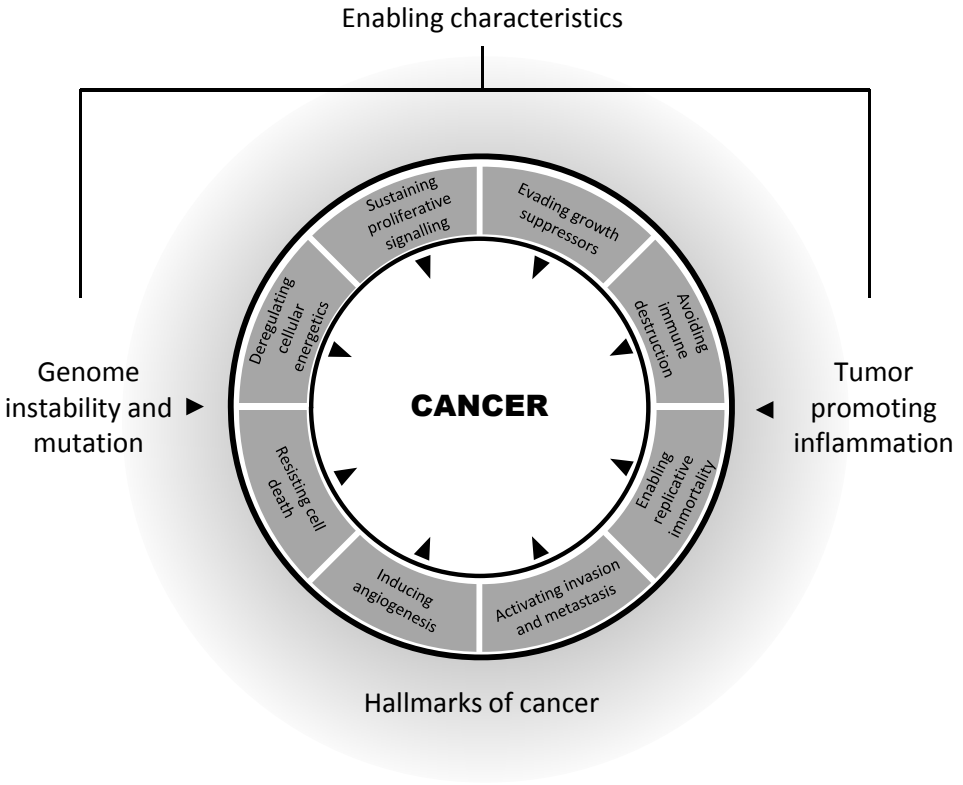


Figure 3. Hallmarks and enabling characteristics of cancer by Hanahan and Weinberg [Hanahan, Weinberg 2011].

inactivate the TP53 tumor suppressor protein, the "guardian of the genome" and a key activator of apoptosis. Cancer cells can also increase the expression of anti-apoptotic regulators or pro-survival signals (e.g. mediated b PI3Kinase/AKT signalling), and down regulate pro-apoptotic factors like death receptors and ligands. The extrinsic apoptosis machinery can be successfully short-circuited. In addition to evasion of apoptosis, cancer cells may also need to avoid loosely related cell death mechanisms such as autophagy and necrosis (reviewed in Levine, Kroemer 2008 and Galluzzi, Kroemer 2008). In particular necrosis represents a critical process ideally avoided, as uncontrollably dying cells are known to release strong pro-inflammatory signals into the surrounding tissue microenvironment, thus recruiting inflammatory cells of the immune system to clear the debris. Paradoxically, rather than obliterating the malignant tissue as they should, activated immune cells (such as cancer-associated monocytes and macrophages) may inadvertently promote tumor progression by fostering cell proliferation, promoting invasiveness and angiogenesis. In addition to the growth factors produced by the inflammatory cells, necrotic cells also often release cytokines that can stimulate surrounding viable cancer cells to proliferate [Grivennikov, Greten & Karin 2010].

Another central hallmark of cancer cells is their unlimited replicative potential, although it is not always clear if all tumor cells have automatically attained immortality, and overcome

Hayflick's phenomenon: the limited number of cell divisions. Most normal cell types in adult body may only proceed through a very limited number of successive cell growth-and-division cycles [Hanahan, Weinberg 2011]. Healthy cells are typically restricted by two biological barriers –recognized as crucial anticancer defences hard-wired into our cells: senescence, which is a nonproliferative but viable state that is nevertheless irreversible, and crisis, which involves cell death. Different from both is exit from the cell cycle or G0 phase, which is reversible. According to accepted scientific understanding, the number of cell growth-and-division cycles is regulated by elements protecting the chromosomal ends or telomeres (reviewed by Blasco 2005). Telomeres shorten progressively as the non-immortalized cells are passaged in culture, and eventually lose their ability to effectively protect the integrity of chromosome tips, resulting in detrimental chromosomal fusions and rearrangements. One strategy to attain replicative immortality and evade senescence or crisis is by activating telomerase, a mammalian reverse transcriptase that uses an RNA template to generate DNA. Telomerase restores telomeric DNA, thus stabilizing the chromosomes and prolonging the lifecycle of a cell.

In order to sustain the uninhibited neoplastic growth, tumors require a constant supply of nutrients, oxygen, and evacuate carbon dioxide and metabolic waste products. This interchange of metabolites is usually attained via the vasculature, both in healthy tissue as in neoplasia. Normally vasculogenesis i.e. the assembly of endothelial cells into tubes, and angiogenesis i.e. sprouting of existing vessels into new ones, are processes strictly confined to embryogenesis and few physiological events, such as female reproductive cycling, tissue regeneration and wound healing. During tumor progression, however, an "angiogenic switch" is almost always activated, mainly due to deregulation of pro-angiogenic factors. Tumor angiogenesis or neoangiogenesis is regulated by two opposing factors that either induce or inhibit it, most notably vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively [Baeriswyl, Christofori 2009]. VEGF signalling can be upregulated by environmental factors such as hypoxia and VEGF. These ligands are sequestered in the ECM, from which they can be released. Certain oncogenes, most prominently *RAS* and *MYC* [Ferrara 2009, Carmeliet 2005], potently activate angiogenesis and VEGF production. Latent VEGF ligand can be released and activated by ECM-degrading proteases such as MMP-9 [Kessenbrock, Plaks & Werb 2010]. Blood vessels produced within tumors are structurally aberrant in various ways. The typical flaws include precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, resulting in micro-haemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation or apoptosis [Hanahan, Weinberg 2011; Nagy et al. 2010]. Inflammatory cells originating in the bone marrow play an important role in pathological neovascularization, and may actively contribute to flip the angiogenic switch in formerly benign tissues [Hanahan, Weinberg 2011].

In the updated "hallmarks" review [Hanahan, Weinberg 2011], Hanahan and Weinberg further listed two emerging hallmarks; in addition to the six core capabilities described already in the 2000 version [Hanahan, Weinberg 2000]: deregulation of cellular metabolism and avoiding immune destruction. The first involves adjustments of energy metabolism (Warburg effect), in order to support continuous cancer cell growth-and-division cycles. Under normal aerobic conditions, cells primarily process glucose to pyruvate via glycolysis. The energy stored in pyruvate is later released, by converting it to carbon dioxide in the mitochondria via a multistep oxidization cycle. In contrast, cancer cells, perhaps as a preparation for hypoxic conditions, have voluntarily limited

their energy metabolism to a much less efficient form of glycolysis, which can be maintained even in the presence of oxygen. The second, still largely unresolved hallmark enables the active evasion of tumor cells from the attack and active elimination by immune cells. This process involves successive selection of weakly immunogenic subclones, which are eventually capable of evading "immunoediting". Although certain virus-induced cancers are considerably increased in immunosuppressed patients [Vajdic, van Leeuwen 2009], the epidemiology does not indicate a general, significant increase of nonviral human cancer incidence in such individuals. Due to the scarcity of data, our current understanding of the role of immune evasion in cancer is incomplete but is now also rapidly emerging.

Oncogenesis

All hallmarks discussed represent acquired capabilities, which have accumulated and were selected for at different time points during multistep tumorigenesis. They can vary greatly between different tumor types, and patients. To understand how these are formed, Hanahan and Weinberg have introduced two additional "enabling characteristics" that further facilitate the acquisition of cancer traits [Hanahan, Weinberg 2011]. One is increased genomic instability, possibly the most relevant of these as it provides a basis for tumor "evolution by means of natural selection". The second enabling characteristic is the promoted inflammatory state of many cancers, driven by the cells of the immune system that may be actively recruited by cancer cells (e.g. via necrosis-related release of pro-inflammatory factors). Tumorigenesis can be generally viewed as a continuous selective process, in which multiple subclones of cells, each bearing advantageous mutant genotypes, successfully attain growth advantages and may eventually dominate the cancer tissue. Recent evidence has shown that multiple clones may arise and co-exist in a single patient, with the more aggressive subtypes emerging in more rapid succession towards the end of tumorigenic progression. This dynamic process is strongly facilitated by genomic instability of tumor cells, which generates random mutations including chromosomal rearrangements that occasionally can orchestrate hallmark capabilities. In normal cells, efficient genomic maintenance and controlling systems (including p53) detect DNA damage and trigger a series of specific DNA damage repair mechanisms, according to the nature of the damage. These systems also actively attempt to clear mutagenic molecules, before any damage occurs. The high expression of aldehyde dehydrogenases in stem cells is thought to contribute to this protection. Cancer cells often increase the speed and spectrum of mutability through a (partial) breakdown in the maintenance system. Inactivating mutations and epigenetic repression of such "caretaker genes" are typically the first selectively advantageous defects observed in pre-malignant lesions. They can accelerate the acquisition and accumulation of spontaneous mutations and potentiate emergence of additional modifications. The loss of telomeric DNA can also generate karyotypic instability, and contribute to oncogenesis. Unless the resulting damage results in cell death or senescence, these changes may also contribute to amplification or deletion of chromosomal segments [Artandi, DePinho 2010], and gain or loss of function of genes with an impact on tumorigenesis. The specificity of genomic alteration varies dramatically between tumor types. Many of these have already been documented with conventional methods, a process now immensely accelerated by next-generation DNA-sequencing technologies.

Until recently, immune responses in tumors were thought to reflect an attempt by the immune system to eliminate cancer cells. Now, there is growing evidence supporting the hypothesis that

tumor-associated inflammatory cells may have a paradoxical effect and enhancing tumorigenesis and progression. Most importantly, inflammation is often already evident already at the earliest stages of neoplastic progression and may foster the development of incipient premalignant tumors into full-blown cancer [Hanahan, Weinberg 2011, Qian, Pollard 2010]. Inflammatory, tumor-infiltrating lymphocytes may supply bioactive molecules and proangiogenic factors to the tumor microenvironment, which promote growth, angiogenesis, survival, invasion, metastasis and support dynamic processes like epithelial-to-mesenchymal transformation (reviewed in Hanahan, Weinberg 2011). Immune cells can also release reactive oxygen species, which are acutely mutagenic and accelerate the local genetic evolution [Grivennikov, Greten & Karin 2010].

Invasion and metastasis

Invasion and metastasis are the two hallmark capabilities with the potential to transform indolent, localized cancer into a systemic, life-threatening disease (reviewed in Friedl, Alexander 2011). They could therefore be considered the ultimate hallmarks that define the aggressiveness of the disease. Invasion can be defined as local, active movement of cells into adjacent tissues. It typically involves activation of signalling pathways that control and modulate the integrity of the cytoskeleton, and affect the turnover of cell-matrix and cell-cell junctions in a series of physicochemical steps. Together, these potentiate cell migration through the stroma and local invasion (invasive front). Cancer cell invasion can be mediated either by migration of individual cells, or the collective movement of multicellular groups (reviewed in Friedl, Alexander 2011, Friedl, Wolf 2003, Friedl, Wolf 2010). Both types of migration are based on highly dynamic processes in which the actin cell cytoskeleton is re-organized in different ways, to physically promote cell motility. The cytoskeleton itself is organized by factors such as integrins, integrin-linked (ILK) and focal adhesion kinases (FAK), and may react very rapidly to local demands related to the mode of cell motility and the physical condition of the ECM that has to be penetrated. Single cell migration involves five physicochemical steps through which the cells change their shape, position, and the ECM structure as it moves forward (reviewed in Friedl, Alexander 2011). In the first step, the cytoskeleton polarizes by actin polymerization to form a leading protrusion. In the second step, the protrusion interacts with ECM substrates via cell surface adhesion receptors. The surface receptors then cluster focally and couple the extracellular adhesion to the intracellular force generation and mechanosignalling. In the third step, the leading edge gives way by locally controlled proteolysis, actively degrading the ECM at the front of the cell. Eventually, contraction through the actomyosin system generates tension inside the cell, and the cell slides forward as the adhesive bonds gradually turn over at the trailing edge. The molecular steps in single-cell migration are coordinated within the same cell and executed in a synchronous and often oscillatory manner. When multiple cells originate from the same site, for example a tumor, the first cells, or the “leader cells”, may form microtunnels in which barriers have been removed, and which can be utilized by following or trailing cells. This leads to a unique, thread- or chainlike invasion of cancer cell through the ECM. Sometimes, the leading cell may also be a cancer-associated fibroblast. In collective migration, indicated by larger threads or clusters of cells on the move, the cellular movements are coordinated in a more “supracellular manner” via cell-cell junctions. This allows the entire multicellular group to behave as a unit. Although, many key mechanisms used by normal and cancer cells to regulate cell adhesion and modulate cytoskeletal dynamics during cell migration have been identified, the factors limiting the migration rate i.e. stop signals that immobilize and anchor the cells, are much less well understood.

The efforts to find a dominant signalling pathway, receptor-ligand interaction, or protease-substrate interaction, governing invasive and metastatic cancer cell migration, have mostly been unsuccessful. The genetic differences within tumors (intratumoral heterogeneity) or between different metastatic lesions and the primary tumor (intrapatient heterogeneity) may lead to a diverse array of morphologically and functionally convergent signalling pathways. The maintenance of invasion- and metastasis-related pathways may also be the result of active cross-talk between cancer cells and surrounding stromal cells, including fibroblasts, myoepithelial, endothelial cells, and macrophages. Furthermore, cancer cells are thought to activate the stroma to remodel the tissue structure by actively depositing ECM components, locally release cytokines and growth factors, and impose metabolic stress. All of these factors may ultimately converge and may promote tumor growth, invasion and enhance metastatic abilities. The single-cell and collective cell migration can be even further dissected into various modes according to the contractility of the cytoskeleton, formation of specific cell-cell junctions, dependence or independence of cell adhesion, and the dynamic turnover of cell attachments to the matrix. Different modes of invasion may also be utilized by different tumor types, and probably reflect innate differentiation capabilities from their original tissue types. For example, cancer cells derived from haematopoietic tissues, but also melanoma cells, often invade as single cells in an amoeboid fashion. These lack any cell-cell junctions, analogous to immune cells penetrating through the endothelium. In contrast, epithelial cells characteristically migrate either as multicellular clusters or strands, unless they have undergone an EMT when single-cells or chains of elongated cells invade in a mesenchymal mode.

Metastasis is defined as “the transfer of disease from one organ or part to another not directly connected to it” [Talmadge, Fidler 2010]. Locally growing primary tumors do not usually impose an immediate health risk, unless in close proximity to critical vasculature or neural tissues. Prompt diagnosis and treatment usually results in the cure in benign and non-metastasizing tumors. Life threatening complications are related to distant metastases disrupting the function of vital organs, or the mere collective burden of systemic cancer, which eventually leads to death. Although metastasis occurs mostly in advanced cancers, distant tumors may already well form at early stages of cancer progression. Therefore, in many patients the cancer has already spread by the time of diagnosis, thereby worsening the prognosis significantly. Sometimes, macroscopic metastases may relapse decades after the primary tumor has been removed [Hanahan, Weinberg 2011], indicating the problem of long-term tumor dormancy.

Metastasis can be divided into six successive stages: local invasion, intravasation, transit in the vessels, extravasation, formation of micrometastases, and successful colonization. At all stages, cells require a unique set of traits in order to survive and adapt to a new stressful and often hostile microenvironment. After cells have successfully escaped the primary tumor, they need to enter and survive in the blood circulation. During intravasation, cancer cells penetrate through the basal membrane of blood or lymphatic vessels, and gain access to the systemic blood stream. During transit the cells may need to phenotypically adapt, in order to survive the mechanical stress caused by the blood flow and circulation, or shear stress in capillaries. At suitable remote sites, or in a random fashion e.g. in capillaries, cancer cells may “get stuck” and attach to the endothelial wall. Here, they may begin with the extravasation process and penetrate into the parenchyma to form small nodules, or micrometastases. Finally, provided the distant site provides sufficient growth and nutritional support and serves as a suitable “niche”

for re-establishing tumor-stroma-interactions, these lesions may grow into macroscopic tumors; a process called "colonization". It is imperative that the cancer cells succeed to complete all the steps in order to form secondary tumors large enough to survive and affect the patients' survival. In fact, the presence of tumor cells in the blood circulation does not generally predict the occurrence of metastasis, since the majority of circulating tumor cells are already dead or will be rapidly eliminated [Fidler 1970]. Not even the capture of tumor cells or emboli in a capillary bed or the bone marrow predicts successful colonization, as these cells often undergo apoptosis or step into dormancy [Talmadge, Fidler 2010]. Although cancer cells potentially traffic through all organs in the body with blood circulation, colonization evidently only occurs in selected tissues that are suitable for the cancer cell. The congeniality between the primary tumor and the organ of metastasis was termed the "seed and soil" hypothesis [Paget 1889, Hart, Fidler 1980]. The "seed" can be identified as a progenitor or cancer stem cell (CSC), cancer initiating cell (CIC), or metastatic cell, whereas the "soil" acts mainly as host factor, niche, stroma, or generally as a supportive microenvironment [Talmadge, Fidler 2010, Langley, Fidler 2007]. There are multiple, frequently recurring preference sites for metastasis to occur, some of which can be explained based on blood flow patterns, whereas most seem to be independent of vascular anatomy, the number of cancer cells delivered to the organ, or the blood flow [Chen et al. 2009]. Several molecular and cellular explanations have been introduced for the seed and soil hypothesis [Talmadge, Fidler 2010]. It has been shown that endothelial cells lining blood vessels in different organs express different adhesion molecules [Nicolson 1988], providing each vascular bed with its own "molecular address" [Ruoslahti 2004] that can be recognized by tumor cells. These may express corresponding receptors for these ligands (which are likely to be glycoproteins). A wealth of evidence further suggests a fundamental role for various chemokines in organ-selective homing or chemoattraction [Muller et al. 2001]. Thus, the pathogenesis of metastasis depends on mutual interactions between the circulating or colonizing tumor cells, and homeostatic mechanisms provided by the host.

Epithelial to mesenchymal transition

Most human tumors are of epithelial origin. By nature, epithelial cells should be among the least motile cell types in the human body. Epithelial cells are connected to adjacent cells through strong lateral adherens junctions (or tight junctions), further supported by the underlying basement membrane that provides additional cell-matrix connections. In order to move within or outside of its tissue of origin, transformed epithelial cells need to break these shackles. One process by which transformed epithelial cells may acquire the capability to invade, resist apoptosis, and successfully disseminate is likely facilitated by epithelial-to-mesenchymal transition (originally transformation; or EMT). EMT is a fundamental, normal function critical for normal embryogenesis. For example, an EMT occurs during gastrulation, when the outer surface cells invaginate into an embryo [Thiery 2002]. EMT is involved in many other developmental processes such as renal organogenesis and neural crest fate [Duband et al. 1995]. Cancer cells may successfully hijack intrinsic EMT properties for their purposes. Thus, in cancer cells, EMT results in the loss of epithelial properties, reduced cell-cell adhesion and baso-apical polarity. This is combined with a concomitant gain of mesenchymal features, including altered cell shape (elongated), and an increased cell migration and invasion potential [Thiery, Sleeman 2006]. Structural proteins involved in the formation of cell-cell junctions are most prominently E-cadherin (adherens junctions), ZO-1 (zonula occludens), claudins (tight junctions), and desmoplakins (desmosomes). All of these are

frequently lost or downregulated in a wide variety of cancers [Thiery 2002]. Similarly, the E-box binding transcriptional factors, such as Snail, Slug, Twist, and Zeb1/2, together with mesenchymal adhesion molecules regulate EMT in a number of malignant tumor types and experimental models of carcinoma formation and invasion [Hanahan, Weinberg 2011]. Such candidate genes include N-cadherin and fibronectin, intermediate filaments like vimentin and a number of proteins that regulate the integrity of the actin cytoskeleton. The morphological characteristics induced by these factors include the conversion of cells from a polygonal/epithelial to a spindly/fibroblastic appearance, expression of ECM-degrading enzymes (matrix metalloproteinases), and increased motility [Hanahan, Weinberg 2011]. In the clinical samples, many of the aforementioned traits are displayed by cancer cells at the invasive margins of certain carcinomas [Hlubek et al. 2007], suggesting an early role for EMT in the metastatic cascade. EMT may also be connected with cancer stem cell properties. However, the precise role of EMT in metastatic progression remains unclear [Hanahan, Weinberg 2011]. EMT may be a key mechanism contributing to the dynamics of tumor cell morphology.

Epithelial plasticity

When cancer cells have settled into a new secondary site, or when tumor cells circulate in the blood stream, they are not likely to further benefit from the EMT-inducing signals. Such cells or cell aggregates may revert into a noninvasive, epithelial state with pronounced cell-cell interactions [Hanahan, Weinberg 2011]. This process is known as mesenchymal-to-epithelial transformation (MET), and may result in a cancer histopathology indistinguishable from primary tumors that have never undergone an EMT [Hugo et al. 2007]. The idea of EMT as an irreversible process may be oversimplified. It is more likely that cancer cells engage in an EMT program conditionally, retaining the capacity to express either mesenchymal or epithelial traits subsequently, or even simultaneously. This may allow the tumor cells to rapidly transform back and forth into either condition, and swiftly adapt to the momentary needs according to the local extracellular microenvironment.

Clinical cancer management

The conventional treatment of solid tumors has been largely based on three therapeutic regimes: surgery, radiation and chemotherapy. In all three regimes, the treatments have improved immensely over the past decades. For example, advanced robotics, minimally invasive methods, and cryosurgery have rendered modern surgery faster, more precise and far less invasive, thus reducing the postsurgical impediments and the risks associated with systemic anaesthesia. Furthermore, precisely defined beams of radiation can be directed into tumor tissues with millimetre accuracy, leaving the surrounding healthy tissue mostly intact. Furthermore, chemotherapy has become less aggressive and with fewer adverse events, thanks to rational drug design and novel combination techniques such as photodynamic treatment, employing local release of light-activated drugs.

Conventional chemotherapy

Conventional chemotherapy has traditionally employed drugs targeting the chronic hyper-proliferation of tumor cells (mitosis, DNA synthesis, and spindle apparatus). The rationale is that

as most cells in adult body have entered a post-mitotic state, which renders them in principle susceptible to antimitotic drugs, compared to highly proliferative cancer cells. For example, plant alkaloids and terpenoids such as taxanes, vinca alkaloids and podophyllotoxin eliminate dividing cells with an extreme efficiency, by disrupting microtubule assembly and blocking effective chromosome separation in mitosis. Chemotherapies utilizing alkylating agents and platinum drugs exploit another weakness: compromised cell cycle checkpoint controls. Alkylating agents such as cisplatin and carboplatin induce massive DNA damage, which in normal cells leads to immediate cell cycle arrest. However, as cancer cells are unable to detect genomic damage properly, they enter mitosis nevertheless, resulting in a mitotic catastrophe, which eventually leads to cell death [Castedo et al. 2004]. Another group of classic chemotherapeutic agents are the antimetabolites, such as methotrexate and 5-fluorouracil (5-FU). These drugs interfere with DNA and RNA synthesis, and thereby prevent normal cellular functions. Antitumor antibiotics such as actinomycin-D, mitoxantrone, and doxorubicin inhibit enzymes essential for DNA replication. Topoisomerase inhibitors, such as irinotecan and teniposide, inhibit enzymes controlling the integrity of DNA strands and cause DNA double-strand breaks. In addition to common cytostatic drugs, also immunomodulatory drugs have been widely applied, although mainly as preventive agents. These include traditional non-steroidal anti-inflammatory drugs (NSAIDs) such as corticosteroids, prednisolone and dexamethasone. These drugs most likely do not target tumor cells directly, but interfere with local pro-inflammatory events in the microenvironment.

Targeted chemotherapy

The aim of traditional chemotherapy is straightforward: to selectively kill cells that divide most rapidly. These are more likely to be malignant cells, rather than normal healthy cells. However, in many tissues, particularly those with rapid turnover and regeneration, cells proliferate at a steady pace, in order to maintain the normal tissue function, architecture and homeostasis. For example, differentiated cells in bone marrow, hair follicles and the gastrointestinal or digestive tract have a limited lifespan and are constantly replaced by new layers of cells. These tissues are the most vulnerable normal targets affected by antimitotic therapies. The common adverse effects of chemotherapeutics reflect the collateral damage inflicted on these tissues: decreased production of blood cells leading to anaemia and immunosuppression (myelosuppression), hair loss (alopecia), local inflammation of the epithelium of the digestive tract (mucositis). For the patient this often means severe nausea, poor life quality, and massive problems due to impaired functions of vital organs. Paradoxically, potent DNA damaging chemotherapeutics may also induce cell transformation and cancer in other tissues [Allan, Travis 2005], therefore resulting in secondary tumors to other tissues, most frequently leukaemia, often years to decades after the first chemotherapy. Typically, the dosage of chemostatic drugs is continued in cycles, and often increased during the therapy. It is also continued, even after all detectable primary or secondary tumors may have disappeared, and the number of circulating tumor cells can reach zero. Nevertheless, these therapies frequently fail after a few months, other patients may completely fail to show any effects (non-responders), and in yet others the tumor may completely disappear, only to relapse years to decades after the therapy. Combinatorial therapies may be more effective even with smaller doses of individual drugs. Combinatorial regimens may sometimes decrease certain specific adverse effects, but can also increase the risk of general bystander toxicity. In many cases, unfortunately, even initially successful drug combination therapies eventually fail.

When comparing the targets of conventional chemotherapy with the key mechanisms outlined in the Hallmarks or Cancer review [Hanahan, Weinberg 2011], it becomes obvious that the overlap is only slim. Cytostatic drugs, combined with auxiliary therapies, practically cover only key aspects of cancer cell biology, i.e. proliferation and inflammation. If one examines the list of drugs currently approved for the clinics, with those still under clinical investigation, it becomes evident that this coverage has significantly improved. Years of outstanding research in cancer pathogenesis, utilizing ground-breaking technical innovations, have finally yielded novel targeted therapies, now increasingly based on well-investigated molecular mechanisms. In a period of 12 years (2000-2012), the FDA has approved over 80 pharmaceutical products for cancer treatment and prevention (http://www.centerwatch.com/drug-information/fda-approvals). Sixteen of those are palliative medicines that only improve the quality of life for example by easing disease-related pain, and two others are disease-preventing vaccinations, both against cervical cancer. Surprisingly, only 10 drugs fall into the category of conventional chemotherapeutics, such as microtubule assembly or DNA synthesis inhibitors, with very limited or no specificity for particular target tissues. The remainder of the investigative drugs have been rationally designed to target a certain group of cells, target protein(s), pathway(s), or functional hallmark(s). Monoclonal antibodies represent a spearhead example concerning targeted therapy design, since these show the highest target specificity among all drug categories. Between 2000 and 2012, 13 antibody-based cancer therapies were approved by the FDA (Table 1). These target a wide variety of molecules, falling into different cancer hallmark categories. Monoclonal antibodies such as trastuzumab (Herceptin), panitumumab (Vectibix) and cetuximab (Erbitux) counteract proliferative signalling sustenance capability by suppressing epidermal growth factor (EGF) driven cancer cell proliferation and vascularization. Ipilimumab (Yervoy) stimulates T cells through the cytotoxic T-lymphocyte antigen 4 (CTLA-4), thus making it more difficult for cancer cells to avoid immune destruction. Bevacizumab (Avastin) inhibits angiogenesis by blocking vascular endothelial growth factor (VEGF). Drugs such as brentuximab vedotin (Adcretris), ofatumumab (Arzerra), ibritumomab tiuxetan (Zevalin) and alemtuzumab (Campath) represent an interesting combination of new and old thinking. These new drugs act as “silver bullets” by binding specifically to cells that express certain antigens. After binding, they are actively internalized, the conjugated toxins released, eventually leading to cell death. Some monoclonal antibodies have been equipped with more conventional weapons, e.g. by conjugation with radioactive isotopes (tositumomab + iodine 131; Bexxar), or cytotoxic agents (gemtuzumab ozogamicin + chalicheamicin; Mylotarg). The National Cancer Institute in the USA is currently supporting the development of drugs targeting diverse array of biochemical pathways in the cell such as genes involved in apoptosis, cell cycle control and cell signalling, angiogenesis, tumor invasion and metastasis, DNA synthesis and immune functions (http://www.cancer.gov/cancertopics/factsheet/NCI/drugdiscovery).

Unfortunately, not even the most specific, hallmark-targeting cancer drugs have fulfilled the high clinical expectations. Off-target effects and nonspecific toxicity may have been significantly reduced. In the best examples, such as Herceptin in Her-2 positive breast cancer patients, treatment has resulted in increased survival of several months to years. Nevertheless, even these responses are generally transitory, often leading to relapse, and to acquired drug resistance, typically through an in vivo selection process. One strategy for acquiring drug resistance involves the utilization of bypass signalling pathways that reinstate the same hallmark capability and revert the therapeutic effects. Resistant tumor cells may also adapt to the selective pressure

by specific mutation in the target gene (e.g. in anti-androgen or Gleevec therapy), or epigenetic reprogramming [Hanahan, Weinberg 2011]. It has also been predicted that by inhibiting one hallmark capability, tumors may focus on another to compensate for the loss. For example, antiangiogenic therapy may indirectly lead to increased tumor cell invasion and metastasis, as the cancer cells become hypoxic and actively seek access to the normal vasculature, promising nutrients and oxygen [Hanahan, Weinberg 2011, Azam, Mehta & Harris 2010]. Cancer cells treated with apoptosis-inducing drugs may also compensate the attrition by hyper-activating mitogenic signalling [Hanahan, Weinberg 2011]. To overcome these shortcomings, new treatment protocols targeting multiple biochemical pathways and core hallmark capabilities have to be established.

Table 1. Novel anticancer therapies based on antibodies approved by FDA since 2000 (http://www.centerwatch.com/drug-information/fda-approvals).

Year approved	Trade name	Drug name	Indication	Mechanism of action	Effect
2011	Yervoy	ipilimumab	Melanoma (metastatic)	Binds to CTLA-4, an antigen on T cells that suppresses the immune response.	Immunostimulatory
2011	Adcretris	brentuximab vedotin	Hodgkin lymphoma and anaplastic large cell lymphoma	Binds CD30. Microtubule disrupting agent MMAE is released in cytoplasm after internalization.	Antimitotic
2010	Herceptin	trastuzumab	Gastric cancer	Binds with high affinity to the extracellular domain of HER2 protein.	Antiproliferative
2010	Xgeva	denosumab	Prevention of skeletal-related events in patients with bone metastases	Binds to RANKL, a protein essential for the formation, function, and survival of osteoclasts, the cells responsible for bone resorption.	Antimetastatic
2009	Arzerra	ofatumumab	Chronic lymphocytic leukemia	Binds the CD20 molecule expressed on B lymphocytes. Cell lysis occurs upon binding.	Lytic
2009	Avastin	bevacizumab	Renal cell carcinoma	Binds VEGF and prevents its interaction Flt-1 and KDR receptors on the surface of endothelial cells.	Antiangiogenic
2006	Vectibix	panitumumab	Colorectal cancer	Binds specifically to EGFR on both normal and tumor cells, and competitively inhibits the binding of ligands for EGFR.	Proapoptotic, anti-inflammatory, antiangiogenic
2004	Avastin	bevacizumab	Colorectal cancer	Binds VEGF and prevents its interaction Flt-1 and KDR receptors on the surface of endothelial cells.	Antiangiogenic
2004	Erbitux	cetuximab	Colorectal cancer	Binds to EGFR (HER1) on both normal and tumor cells, and inhibits the binding of epidermal growth factor (EGF) and other ligands, such as transforming growth factor-alpha.	Antiproliferative
2003	Bexxar	tositumomab and Iodine I 131	Non-Hodgkin's lymphoma	Binds the CD20 antigen expressed on the surface of normal and malignant B lymphocytes.	DNA damage through ionizing radiation
2002	Zevalin	ibritumomab tiuxetan	Non-Hodgkin's lymphoma	Binds to the CD20 antigen on B lymphocytes and induces apoptosis in CD20+ B-cell lines in vitro.	Proapoptotic
2001	Campath	alemtuzumab	Leukemia	Binds to the CD52 antigen present on the surface of the malignant lymphocytes. Upon binding the drug induces antibody-dependent lysis.	Lytic
2000	Mylotarg	gemtuzumab ozogamicin	Acute myeloid leukemia	Binds to the CD33 antigen on leukemic blasts. Releases DNA damaging chalicheamicin after internalization.	DNA damage and apoptosis

Cancers of epithelial origin

Epithelium

Epithelium is one of the four major animal tissue types, along with muscle tissue, nervous tissue and connective tissue. Epithelial tissues can be formed by all three embryonic germ layers (ectoderm, mesoderm and endoderm). The main function of epithelial tissue is to form a physical barrier separating distinct tissues or environments. Epithelial tissues form either glands, or line the surfaces and cavities of most organs within the body. A number of different epithelial functions have evolved, including secretion (e.g. in breast and prostate glands), selective absorption (e.g. in kidney, stomach and intestines), transcellular transport (e.g. in the gastrointestinal tract), and protection from dehydration paired with sensation (skin). Epithelial tissues are generally separated from the underlying connective tissue by a basement membrane (BM) or basal lamina. Apart from few exocrine and endocrine glands, epithelial layers are typically avascular and support has to passively diffuse through the basement membrane. Epithelial tissues can be only one layer thick (simple epithelium), or they can be multi-layered (stratified epithelium). In simple epithelia, each epithelial cell is directly in contact with the underlying BM. Four morphological classes of simple epithelia have been defined: simple squamous, simple cuboidal, simple columnar, and pseudostratified epithelia. The morphology of the epithelial layer is largely dictated, and thus facilitates the various functions. Simple squamous epithelium consists of thin and flat plate-like cells, which are ideally positioned to support passive diffusion of gases (e.g. capillaries and alveoli in lungs). Simple cuboidal epithelia are comprised of cube-like cells, a morphology particularly suitable for excretion, secretion and absorption. Cuboidal epithelia are found in excretive ducts of many glands, and the absorptive lining of kidney tubules. Simple columnar epithelial cells are typically elongated and possess cellular extensions such as microvilli on the apical side, with the goal to increase the overall absorptive surface area. Functionally, columnar cells are highly secretory (e.g. wall of stomach) or absorptive (e.g. small intestine) areas, others are specialized in sensory perception (e.g. taste buds). Finally, pseudostratified epithelia are almost exclusively found in the larger respiratory airways (e.g. nasal cavity, bronchi, and trachea). Morphologically, these are similar to simple columnar epithelium, but lack the uniform basal localization of the nuclei. In contrast, the main functions of multi-layered or stratified epithelia is to protect the body from dehydration, chemical, ionizing and mechanical insults like toxins, radiation (UV), and physical trauma. The basal layer of any stratified epithelium can be cuboidal, columnar or squamous. However, the stratified portions of these epithelia often keratinize (squamous epithelia, e.g. in skin) and cornify, which invariable results in cell death as the consequence of terminal differentiation. This dead, but heavily cross-linked, cornified status helps to withstand environmental exposures.

Carcinoma

Cancers originating from epithelial tissues are carcinomas, and represent about 90% of all cancer incidences and deaths worldwide. Most carcinomas arise from putative epithelial cells that have undergone gradual malignant transformation as the result of genomic insults. The exposed position of epithelial tissues and their common function as a barrier that simultaneously facilitates active transport of substances, increases their exposure to environmental mutagens. Their highly regenerative nature further increases the likelihood

to undergo malignant transformation that results in hyper-proliferation and abnormal cell divisions. In contrast, the bulk of neuronal tissue is located in the CNS, an extremely well protected space. Adult neurons, muscle and connective tissues share an extremely low proliferation potential, which decreases their susceptibility for malignant transformative. For example, lung alveoli have to constantly face environmental pollutants such as tobacco smoke, exhaust gases and other carcinogens. The keratinized outer layer of skin shields us from the DNA damaging UV-light of the sun, and is simultaneously vulnerable to UV-induced mutagenesis and carcinogenesis. Furthermore, the epithelia in kidney and liver also have to cope with almost all noxious metabolic waste products and toxic substances generated inside the body, or incorporated from outside. Also these tissues have protective functions, but at the same time they are the target of genetic modifications leading to epithelial cancers. Like epithelial tissues, also carcinomas can be classified according to their histological type. Adenocarcinomas are cancers originating from glandular epithelia, or have secretory properties (e.g. the mucus secreting cells in colon). Adenocarcinomas commonly occur in colon, lungs, breast, urogenital tissues including prostate and vagina, pancreas, stomach and throat. They often generate rudimentary glandular tissue architecture and may continue to produce specific molecular products characteristic for the tissue of origin (e.g. mucins, milk/fat droplets, prostate-specific antigen). In parallel, partial keratinization represents the characteristic differentiation observed in many squamous cell carcinomas (SCCs), such as skin, head and neck, or genital (cervical) cancers. SCCs may also, more rarely, occur in other tissues, including the oesophagus, urinary bladder, prostate, or the lung. Adenosquamous carcinoma refers to a mixed tumor showing features of both adenocarcinoma and SCCs. Anaplastic carcinomas are a heterogeneous group of high-grade cancers that lack histological and cytological characteristics of any specific carcinoma.

Prostate cancer

The prostate gland

Prostate is a tubuloalveolar exocrine gland whose main function is to secrete a milky fluid that protects the spermatozoa before they are in the female body. The slightly alkaline fluid produced in prostatic ducts constitutes 20-30% of the volume of semen, and contains various proteolytic enzymes, prostatic acid phosphatase, and prostate specific antigen (PSA, KLK3). The alkalinity of the fluid helps to neutralize the acidity of the vaginal tract, thus prolonging the lifespan of the sperm cells. The glandular epithelium of the prostate originates from the endodermal urogenital sinus and the stroma, including the smooth muscle cells between the ducts, develops from associated mesenchyme [Moore, Persaud 2008]. As in many organs that have to endure mechanical stress caused by fluctuations in size, the secretory epithelium of prostate is mostly pseudostratified. The structure is a mosaic of basal cells and tall columnar cells, although, in transitional areas found in the ends of the longer ducts also cuboidal and squamous epithelium can be present. The prostate epithelium is supported by elastic fibroblasts and bundles of smooth muscle cells. The prostate gland can be dissected into four distinct zones: a peripheral zone, central zone, transition zone and the anterior zone. The entire gland is surrounded by a fibromuscular band, sometimes called the capsule, and connected to the pelvic floor via the anterior fibro-muscular zone.

Prostate cancer (PrCa) is currently the most commonly diagnosed cancer in western countries [Jemal et al. 2011], including Finland (Finnish Cancer Registry). The incidence in the developing countries, however, is much lower [Jemal et al. 2011], attesting to the fact that its occurrence is strongly linked to increasing lifespan and maybe dietary habits. Each year roughly, one million new PrCa cases are diagnosed worldwide [Jemal et al. 2011]. In Finland, the annual number of new incidences is close to five thousand, equalling 37% of all cancer cases in the male population (Finnish Cancer Registry). Due to the relatively high incidence of the more aggressive subtypes, PrCa claims quarter of a million lives every single year worldwide [Jemal et al. 2011]. It also remains the third most lethal malignancy in Finland (Finnish Cancer Registry), with close to 800 deaths (14% of all cancer-related deaths). Most prostatic tumors are localized within the fibromuscular band or capsule, and can be effectively cured by radical prostatectomy i.e. surgical removal of the entire prostate gland. This intrusive procedure is often combined with targeted radiotherapy and hormonal therapy. Despite advances in early diagnosis, surgical excision techniques and modern brachytherapy, approximately one tenth of the prostate cancers have already spread beyond the prostatic capsule (local invasion) or metastasized into distant sites (systemic metastasis) at the time of diagnosis, and the individual tumors can no longer be efficiently removed.

Prostate cancer management by chemotherapy

The prostate depends on a supply of male hormones to function properly. The gland is especially sensitive to testosterone, mainly produced by the testicles and subsequently reduced into dihydrotestosterone inside the target tissue/cells. The proliferative potential of most PrCa is equally dependent on male sex hormones, and continued expression of the androgen receptor is found in most cancers. Good treatment responses are typically observed in the vast majority of patients, receiving standard androgen-deprivation (ADT) or other anti-hormonal therapies. However, 7-10% of all patients undergo a relapse within two - three years. These castration-resistant prostate cancers (CRPC) become insensitive to antiandrogens and gonadotropin-releasing hormone (GnRH) antagonists [Feldman, Feldman 2001], and have a poor median survival of only about 18 months [Åkerfelt, Härmä & Nees 2011]. CRPCs are resistant to all currently prescribed first line anti-hormonal therapeutics such as flutamide, nilutamide or bicalutamide [Åkerfelt, Härmä & Nees 2011]. The second line therapeutics include taxanes such as docetaxel and cabazitaxel [de Bono et al. 2010], resulting in a modest median survival benefit of only 2-3 months. Also combination therapies have been successfully tried using taxanes, angiogenesis suppressor bevacizumab (Avastin), thalidomide, and strong glucocorticoids. As of yet, no curative treatment options are available for CRPC. Palliative treatments abound, such as zoledronic acid, which helps relieving the pain associated with bone metastases. In recent years, several promising drug concepts have emerged, most of which target gonadotropin secretion (degarelix, abarelix, leuprolide acetate, triptorelin pamoate). Some new drugs have already entered the market, such as the androgen biosynthesis (CYP17) inhibitor abiraterone (Zytiga), and MDV3100, a next-generation anti-androgen, expected to be released in 2012. Despite the excitement, the initial clinical experiences have been disappointing, indicating that these drugs are also not curative (average 4 month survival benefit for Zytiga), and fatal relapses continue to occur.

Models and assays in cancer research

Our understanding of cancer pathobiology is largely based on studies of the tumor histopathology and parallel experimental research, using a vast collection of cell lines derived from different tumor tissues at different stages of cancer progression. In conjunction with the development of transgene technologies over the last few decades, there has been a rapid increase in mouse models mimicking human cancers. In contrast to xenograft models, which still are dependent on human cancer cell lines, transgenic animals represent genuine, spontaneous *in vivo* models for oncogenesis and (sometimes) metastasis. Although cancer cell lines have been extremely useful for decades to promote research and unravel biochemical pathways, are easy to maintain, and represent important features of certain cancers, they do nevertheless suffer from significant shortcomings. First of all, tumor-derived cell lines display modified genetic profiles different from primary tumor cells isolated from patients [Tsuji et al. 2010]. These defects are attributable to long-term cell culture under strongly selective, growth-promoting conditions, including a noticeable genetic drift [Perkel 2011]. Also cross-contamination with other lines, generation of severely altered sublines, and other artefacts may occur. Murine models also have their downsides. Perhaps the most important of these are the limitation of conserved features between mouse and human tumors, the differences in human and mouse endocrine systems, the lack of human stromal components in xenograft tumors, and the failure to mimic genuine metastatic lesions in many cases. These problems result in poor predictability for the outcome of clinical studies. Preclinical studies, nevertheless, still heavily rely on cells cultured in 2D monolayer on plastic. Based on these shortcomings, the general interest towards more complex cell-based assays (e.g. in 3D organotypic settings), has immensely grown in recent years. As a result, some of these methods have already become more reproducible, cost-effective and the technology as such becomes increasingly available. The following chapter will briefly describe the architecture and composition of human carcinomas, focusing on PrCa. Subsequently, conventional cell-based assays, tissue culture and animal models that have been routinely utilized for decades, will be described. Finally, novel cell culture models as an alternative for traditional 2D monolayer assays are introduced, followed by an expeditious look into phenotypic readout methods based on image analysis.

Tumor microenvironment

It is becoming increasingly evident that tumors are complex organs with defective functions and intricate cell-cell interactions that are critical for tissue growth and homeostasis. The tumor cells may be the driving force in carcinogenesis, but heavily depend on supportive stromal components that represent the tumor microenvironment (TME; reviewed in Hanahan, Weinberg 2011) (Figure 4). The TME consists of numerous different cell types with constant reciprocal interaction. Tumors can also be divided in well-defined histological partitions. The compartment of the tumor that is comprised mainly of neoplastic epithelial cells is called the parenchyma, which is clearly separated from the mesenchymal cells. These constitute the tumor-associated stroma, together with a tumor capsule (basement membrane). The cancer cells in the parenchyma carry the key oncogenic mutations and essentially drive cancer progression. Histopathologically, the parenchymal tissue may be diverse, and can contain regions showing variable degrees of differentiation, vascularity, proliferation, inflammation

or invasiveness (invasive front). The majority of cells in tumor stroma are fibroblasts. These fall into two categories: classic fibroblasts *per se*, which are the key structural foundation and support most normal epithelial tissues, and the myofibroblasts. These are rare in most healthy epithelial tissues, but can be abundant in wound tissues and sites of chronic inflammation [Hanahan, Weinberg 2011]. Cancer cells can enhance tumor phenotype (indicated by hyper-proliferation, angiogenesis, and invasion) by actively recruiting myofibroblasts. They may also reprogram normal tissue-derived fibroblasts to differentiate into myofibroblasts, which then may secrete a variety of ECM components that eventually constitute the fibrous stroma characteristic for advanced carcinomas. The result is a local inflammatory reaction or reactive stroma, indicated by increased collagen synthesis. Re-programmed fibroblasts are often referred to as cancer-associated fibroblasts (CAFs), with distinctively altered properties compared to normal fibroblasts. Additionally, infiltrating immune cells are typically found in most neoplastic lesions. These immune cells play a dual role in the TME, and can have both tumor-antagonizing (Natural killer or NK cells; T-regulatory cells, Treg's), antagonized by tumor-promoting effects (TILs, tumor-infiltrating lymphocytes, monocytes and macrophages). The most prominent tumor-antagonizing action of immune cells is the clearance of hyper-proliferative neoplastic cells, e.g. by NK cells. In contrast, the major tumor-promoting mechanism is the support and maintenance of chronic inflammation. Inflammatory cells release a myriad of signalling molecules (growth factors, chemokines, cytokines), including VEGF, EGF, FGF2, and ECM-degrading proteases. These stimulate tumor proliferation, sustain angiogenesis, and stimulate invasion and metastatic dissemination [Qian, Pollard 2010, Coffelt et al. 2010, Egeblad, Nakasone & Werb 2010]. Endothelial cells moving in the stroma form tumor-associated vasculature to provide cancer cells with oxygen and nutrients, and act as an exhaust pipe for the metabolic waste products. The "angiogenic switch" that enables activation of quiescent endothelial cells and consequent neovascularization has been already discussed in the previous chapter, as one of the cancer hallmarks. Another cell type capable of forming tissues with similar function and structure, are the lymphatic vessel-generating endothelial cells [Tammela, Alitalo 2010]. Their role in tumor-associated stroma, however, is poorly understood. It is believed that because of the high interstitial pressure within solid tumors, the lymphatic vessels residing within the tumor are collapsed and non-functional, albeit actively growing lymphangiogenic vessels may remain at the peripheries of tumors [Hanahan, Weinberg 2011]. These are thought to serve as channels for metastasis, and draining invasive tumor cells towards the lymph nodes. Pericytes often surround endothelial tubing of blood vessels, and maintain the homeostasis in the quiescent endothelium. They provide structural support for vessels by synthesizing the vascular basement membrane. However, in tumor tissues, the pericyte coverage is often defective, allowing cancer cells to intravasate into the circulatory system. All these aforementioned cell types, except for myofibroblasts and activated cells of the immune system, are present in normal healthy tissue and serve a function in the tissue homeostasis.

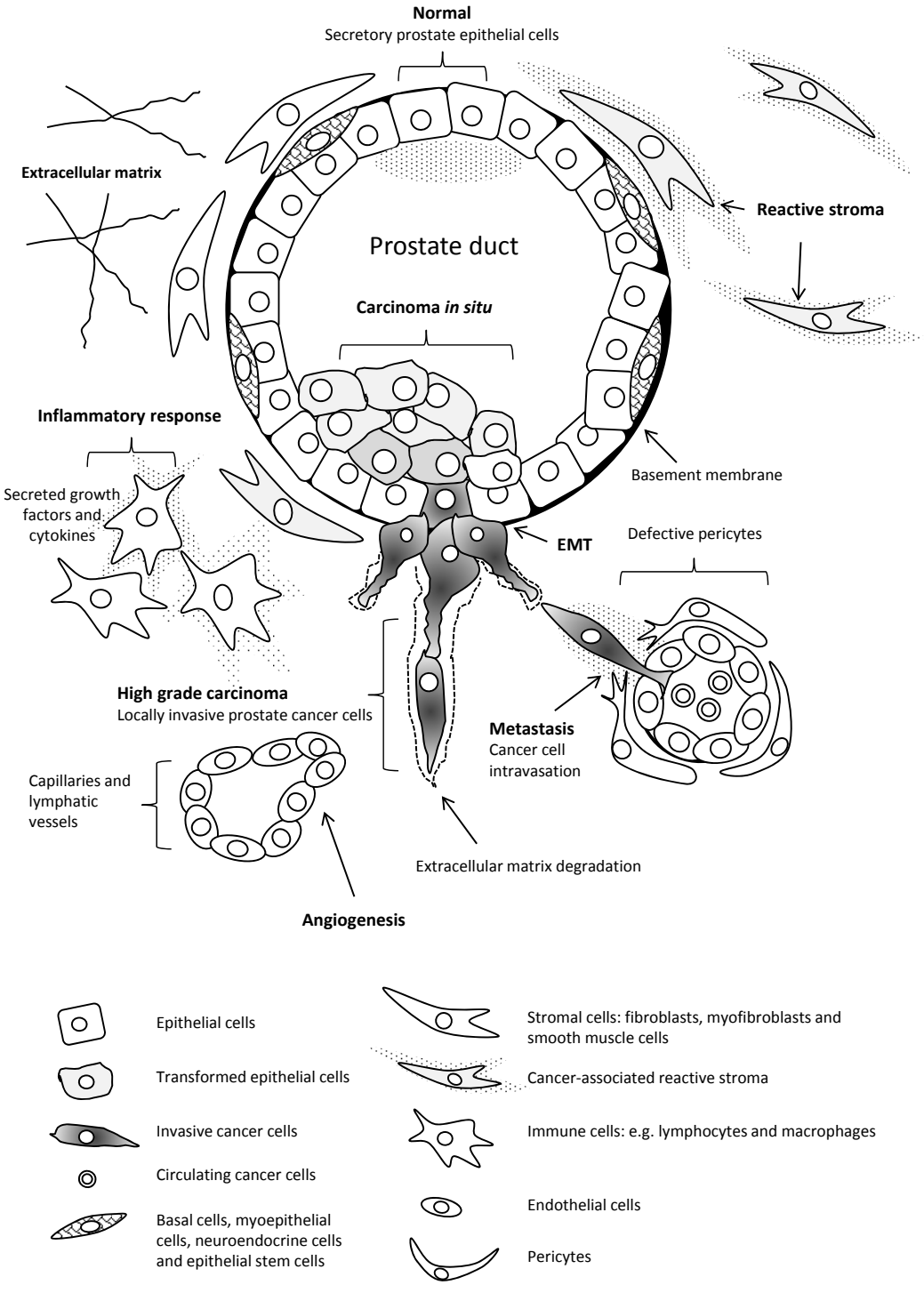


Figure 4. Tumor microenvironment in prostate cancer.

Experimental cancer models and assays

When considering the most relevant questions cancer biology-related assays should be able to answer, having yet another look at the eight Hallmarks of Cancer by Hanahan and Weinberg [Hanahan, Weinberg 2011] becomes helpful. After deciding upon the most interesting hallmarks, we are faced with another problem: which of the hallmarks can be readily and faithfully recapitulated *in vitro,* and which require more sophisticated or complex models, such as animal experimentation? Classical hallmarks such as unlimited replicative potential, growth signal self-sufficiency, insensitivity to antigrowth signals, and evasion of apoptosis, have been routinely addressed in cell culture, and require only relatively simple readouts and experimental settings. Invasion, metastasis and angiogenesis, however, are more complex processes that take place in an intricate physiological microenvironment, and involve delicate interaction between different cell types. Although cell-based *in vitro* assays for invasion exist, most of the angiogenesis and metastasis related experiments are still performed *in vivo*. However, the majority of efforts mimicking these sophisticated hallmarks *in vitro* have suffered from a high degree of reductionism and poor predictability. Evaluating these results demands a great deal of critical consideration.

In principle, all assays can be based on single endpoint readouts, in which the evaluation relies on comparison of experimental samples to control samples. Alternatively, assays can be of dynamic nature, utilizing multiple successive readouts and a control series. Dynamic assays are in many ways superior to endpoint assays, may cover the entire duration of the experiment, and have the potential to identify time-dependent changes as response peaks and plateaus. Nevertheless, endpoint assays are often favoured because less effort is required, while standardization and data analysis may be technically more straightforward.

Traditional cell culture

Cell culture systems can be divided in two main categories: 1) those utilizing immortalized cell lines with unlimited proliferation capacity, and 2) primary cell cultures, directly established from human tissues. Cell lines have the significant practical advantage of infinite life span [Rhim 2000], and represent by far the most widely used models in every aspect of cancer research (or cell biology). The genetic background of cell lines is derived and still related to the original tumor, and dictates all growth properties and phenotypic characteristics. Traditionally, cells have been propagated on simple plastic plates that provide little more than a physical surface to adhere and grow on. Occasionally, plates are coated with proteins or synthetic polymers that modulate their adhesive properties. Typically, epithelial cells growing on plastic dishes tend to form monolayers upon reaching confluency. These monolayers loosely mimic the cobble-stone architecture of simple epithelia, or the basal layer of squamous epithelia, and the cells establish lateral cell-junctions; however, they rarely grow as multiple layers or domes of cells. Confluent normal or non-transformed cells can terminally differentiate upon reaching confluency, exit the cell cycle, or even drift into senescence. Terminal epithelial differentiation of epithelial cells (e.g. in skin keratinocytes) may be considered a form of programmed cell death, intricately linked with the process of keratinization and formation of cornified envelopes. Cancerous or transformed cells, in contrast, are typically not limited by contact to neighbouring cells (= loss of contact inhibition), but can continue to proliferate. Such cells can form thick multi-layered sheets of cells or foci. Contact inhibition may act as an *in vitro* surrogate for mechanisms that control tissue homeostasis in normal tissues. Considering the high complexity of normal or cancer tissues

described above, any conventional plastic cultures only represent very reductionist models. Cells growing on flat plastic surfaces lack a significant ECM, and the concept of monoculture ignores the importance of heterotypic cell-cell interactions with surrounding stromal or immune cells (= the tumor microenvironment). Furthermore, cells growing on artificial plastic surfaces lacking an ECM often show strong features of inflammation and wound-healing, a form of stress response characterized by rapidly dividing and motile cells.

The replicative potential of cells can be quantified by a number of commercially available proliferation assays. The most straightforward approach, requiring only minimal technical equipment, is to measure the number of cells or nuclei. This can be performed either by counting, or indirectly by measuring the metabolic activity of cells, from which their total number is extrapolated. Fluorometry uses reagents that are metabolized outside the cell, or transported into in the cytoplasm, representing an indirect means of estimating cell numbers. Similarly, apoptosis can be directly measured by counting cells stained for apoptotic nuclei (e.g. propidium iodide), or indirectly by generating fluorometric signals that correlate with apoptotic signalling cascades (e.g. measuring caspase activity). In contrast, cell motility and invasion require more complex readout. These processes also need to be monitored and measured over a period of time, and require more sophisticated instrumentation (microscopic imaging). Single cell migration can be studied by serial analysis of images, acquired by time-lapse microscopy. Expert software solutions instead use machine vision devices, which automatically follow and measure the velocity and distance, travelled by the migrating cells. In many laboratories, migration analyses are still performed manually. This represents a tedious option that prohibits the analysis of a large number of samples, such as required for drug screens. Single cell migration analysis represents an amenable option when studying mechanisms of basic motility, including cytoskeleton organization, intracellular force generation and mechanosignalling or chemotaxis. However, the assembly of cell surface adhesion receptors and localized proteolysis to degrade components of the ECM cannot be easily addressed on 2D plastic surfaces, unless these are coated with appropriate, biologically relevant matrix components. A straightforward and economical method to study collective cell migration is the classic scratch wound assay [Todaro, Lazar & Green 1965], a method considered to mimic the process of wound healing *in vivo.* In this assay, cells first form a confluent monolayer, upon which an artificial gap, or "scratch wound", is created. After wounding, the cells from both the edges will move toward the opening in an attempt to close the gap, and re-establish a continuous monolayer [Liang, Park & Guan 2007]. Scratch wound assays typically are very dynamic assays, and series of images (or videos) are captured at regular intervals during cell migration. The rate of migration is then determined by comparing these images to the control, measured in distance travelled per hour. These assays are particularly suitable for studying the regulation of cell migration via interaction of cells with the ECM, or homotypic cell–cell interactions [Liang, Park & Guan 2007], provided surfaces have been coated with ECM preparations. In the past, scratch wound assays suffered particularly from low sample throughput, lack of standardization, the need for large amounts of recombinant ECM proteins, and tedious analysis due to the lack of computer-assisted analysis tools. These assays are now increasingly standardized, and significantly higher throughput is possible based on specialized 96-well plates (e.g. Essen Bioscience ImageLock). The resulting image series or videos can be routinely analysed using specialized, efficient software. The physiological relevance can be further improved by using various biological matrices, or combinations thereof.

Another category of migration assays are chemotaxis assays, which evaluate the ability to migrate towards a gradient of motility-inducing factors, nutrients, or escaping from toxic compounds. The Transwell migration assay (or Boyden chamber assay) [Boyden 1962] is a widely used standard technique, based on two chambers separated by a filter that contains pores of a defined size. These pores allow restricted transmigration from one chamber into another. The dimensions of pores used are determined by the diameter of the cells under investigation. For modelling *in vivo* cell motility through ECM, the filters can be further coated with ECM constituents such as collagens, elastin and laminin. These are widely accepted to mimic at least some aspects of natural barriers. The introduction of multi-well Boyden chambers, in which 24, 96 or 384 samples can be evaluated in parallel, has significantly increased the efficiency considerably. The readout is typically performed by staining and counting the motile cells. These may have either migrated through the filter, or can still reside inside the pores.

Tissue and organ culture

Certain whole organs extracted from animals (e.g. heart and liver) as well as isolated tissue explants (e.g. from muscle, skin, cartilage, bone and liver) can be successfully cultured under standard laboratory conditions. Other organs, e.g. from the central nervous system (e.g. whole brain, hippocampus), liver or lung, can be sliced into thin tissue sections and maintained in culture for adequate periods of time. This facilitates dynamic long-term experimentation, leaving heterotypic cell-cell interactions and multicellular structures physiologically intact. In fact, tissue slice culture dates back to the 1950's and represents one of the oldest forms of *in vitro* cell culture [LI, McIlwain 1957]. In order to retain the normal function and architecture of organs, explants and tissue slices, it is essential to section and isolate these carefully without damaging the structure, and quickly transfer the slices into a suitable medium. The medium can be solid (e.g. plasma clots, rafts, soft agar, or steel grids), thus providing structural support. Often, lifting slices to the air-liquid interface is critical to maintain differentiated structures for longer periods of time. Alternatively, slices can be submerged in medium, which provides nutrients and oxygen but no physical support. Both methods can also be combined. If initial stress and wound healing responses are subsiding, tissue explant and organ cultures may be comparable to the in vivo organs in both function and structure. Physiological features or tissue architecture (histology) may be retained, e.g. hormone-dependent organs typically continue to respond and depend on these hormones. Fetal organs may also maintain their developmental abilities, thus facilitating *ex vivo* studies on growth, differentiation and organogenesis. In the best case scenario, tissue explant culture may replace a number of living animals. However, tissue explant cultures also have considerable drawbacks. Firstly, experiments utilizing whole organs may be almost as expensive and low throughput as animal experiments, due to the scarce availability of most organs. Tissue material from adult animals and human organs may be more accessible, but its long-term use is restricted since the cells have reached a differentiated state. Secondly, results generated based on organ cultures are often not comparable to those from live animal studies (e.g. studies on drug action) since the drugs are differently (or not sufficiently) metabolized *in vivo* compared to *in vitro*. Thirdly, reproducibility and standardization, and in particular the imaging of tissue slices are problematic. Therefore, organ and tissue cultures are most routinely employed in toxicological studies, mainly using tissue slices of rat liver, where metabolic readout is the standard. The use of tissue slices in cancer research and drug discovery has been marginal, at best. Only recently, promising and reproducible results have emerged,

with successes particularly in colon carcinoma and lung adenocarcinoma [Vaira et al. 2010], and prostate carcinoma [Blauer, Tammela & Ylikomi 2008, Kiviharju-af Hallstrom et al. 2007].

Mouse models

The mouse (*Mus musculus* ssp. *musculus or domesticus*) shares a remarkable anatomic and genetic resemblance with humans. It is the most commonly used mammalian research model with hundreds of established inbred, outbred, and transgenic strains available. These mice are relatively easy to maintain and handle, and reproduce quickly. Both in the EU and the USA, certain obligatory animal experiments – mostly related to ADME properties and toxicity – are required by law, before any pharmaceutical agent can enter clinical trials on patients. Other small animal models based on fruit flies, nematodes, zebrafish, and rats, are routinely used already before critical ADME studies. These are a component of all research activities from basic research to preclinical target identification and validation. Typically, the need for animal experiments depends on the operating procedure of the facility. For example, at the Developmental Therapeutics Program of the U.S. National Cancer Institute, all potential therapeutic molecules are initially screened for activity in an *in vitro* panel of 60 tumor cell lines screen (NIH60 panel), after which the most active molecules are tested in xenograft studies in rodents [Alcoser, Hollingshead 2011].

Xenografts are human tumor slices (tumor grafts) or cultured human tumor cells, implanted into the body (usually the back) of immunodeficient rodents. Xenotransplantation can be performed in many ways, e.g. most frequently under the skin (subcutaneous xenografts), into the orthologous organ (orthotopic xenografts), into the bone marrow (intratibial), or into the aorta or tail vain (systemic). Both subcutaneous and orthotopic xenografts typically grow locally, and are thought to mimic some aspects of primary tumors in humans. Such models rarely form distant or systemic metastases. In contrast, intratibial and systemic cell injection is intended to model the formation of distant (bone) metastases. For most pre-clinical animal studies, anticancer agents are administered only when the tumor/s has/have reached a predetermined, palpable minimal size. Tumor response can be assessed by monitoring tumor growth and size over time. The size dimensions can be measured by simple palpation, or increasingly with sophisticated, vital imaging based methods (MRI, PET/CT, luminescence, and ultrasound). The dissemination of cancer cells stably expressing bioluminescent or fluorescent markers can be monitored with small animal *in vivo* imaging (SAIVI) in real time. Drugs are administered in several experimental cycles to optimize efficacy and to lower toxicity. Xenograft experiments allow a dynamic long-term (from several months up to a year) prediction of drug activity, and possibly predict therapy failure [Alcoser, Hollingshead 2011]. Both aspects cannot currently be acquired with any other existing experimental models. Unfortunately, even for the most promising novel therapeutics, xenograft experiments have often failed to predict the failure of human clinical trials. This is most notably due to low efficacy of the drug(s) in humans [Alcoser, Hollingshead 2011, Kerbel 2003]. Apparently, to cure mice of (xenografted) tumors is not fully comparable to human tumor biology, and does not faithfully mimic human drug response, or resistance.

Since the birth of the transgenic mouse technology in the early 1980's, researchers have engineered many genetic abnormalities, often by conditionally and reversibly altering single gene expression, that cause certain cancers into mouse models. These models have been very helpful in exploring the oncogenic nature of the underlying genes and related pathways. The

genetically engineered mouse models (GEMMs) generate tumors that are thought to better mimic those seen in humans than transplanted exogenous cancer cells. Nowadays, there are hundreds of different GEMMs available that are associated with some form of human tumor development (see eMICE; electronic Models Information, Communication, and Education; http://emice.nci.nih.gov/). As was discussed earlier in the first chapter, inducible and conditional expression techniques, such as the Cre-Lox and Tet-ON/OFF systems, facilitate the expression or silencing of a gene at any time and in any (predetermined) location. This can help to better recapitulate sporadic human diseases, as these targeted genetic alterations do not interfere with embryonic development. Successful GEM models mimicking oncogenesis include e.g. conditional and inducible K-Ras lung cancer models [Jackson et al. 2001, Fisher et al. 2001], and an inducible EGFR overexpression model for lung adenocarcinoma [Politi et al. 2006]. There is a growing body of evidence that GEMMs may be more predicable than xenografts for clinical studies. For example, in a study of peroxisome proliferator-activated receptor-gamma (PPARγ) agonist troglitazone (TZD), the drug showed good anti-tumoral activity in human colon cancer cells. This was used as incentive to initiate a Phase II clinical trial in colon cancer patients [Sarraf et al. 1998]. Soon after starting this xenograft study, another group reported that TZD showed no significant anticancer activity in multiple mouse models for intestinal neoplasia (Min+/-). In fact, polyp formation was even increased upon TZD administration [Saez et al. 1998]. Accordingly, in the clinical patient trial, patients treated with TZD actually showed disease progression within months of therapy initiation, thus confirming the results from the GEMM experiment [Kulke et al. 2002]. GEMMs also have great potential in cancer prevention research (reviewed in Abate-Shen et al. 2008) as the cellular transformation/oncogenesis often occur slowly (usually several months), just as in humans, and progression can therefore be interfered with even at very early stages. This approach has been successfully tested with gefitinib, an EGFR inhibitor, which prevented the formation of adenocarcinomas in EGFR-L858R-FLAG transgenic mice that normally spontaneously develop large lung adenocarcinomas by 15 weeks [Ohashi et al. 2009]. Vaccines represent another form of cancer prevention, and GEMMs have proven to be highly beneficial preclinical models for the investigation of the oncogenic potential of viral genes (briefly reviewed in Alcoser, Hollingshead 2011). Another excellent example for the complementary application of GEMMs and allograft transplantation comes from a recent study, aiming for a targeted therapy against HER2 overexpressing breast cancers. Trastuzumab is a very potent monoclonal antibody, selectively targeting overexpressed HER2 on cancer cells. As is often the case, however, the patients eventually develop resistance to the treatment and eventually relapse. In this study, trastuzumab was conjugated with a potent cytotoxin to improve its effect [Jumbe et al. 2010]. This cytotoxic effect was very successfully modelled in nude mice implanted orthotopically with mammary tumors derived from HER2-overexpressing GEMMs, with the result that ~50% of the tumors showed complete regression.

As with all models, xenografts as well as GEMMs have their limitations. This should be kept in mind when setting up experiments, analysing the preclinical data, and making decisive conclusions. On the technical side, mouse experiments tend to be low throughput, mostly limited by slow tumor growth and tedious monitoring methods. This is especially true for GEMMs, where forming tumors are not typically labelled with traceable markers for *in vivo* imaging (like in xenografts). With the exception of cancers such as breast, prostate and melanoma, where palpable tumors develop, the identification and monitoring of the mice that develop tumors can be tricky in GEM models. However, the throughput of GEMMs can be improved by using allografts, such as in the

HER2/trastuzumab study described above. Also the fact that staging strategies must be revised individually to match the settings in clinical patient trials, can impose a logistical nightmare in large experiment series. This particularly applies to complex combination therapies, testing for multiple routes of administration, vehicles, dosing schedules and concentrations. Furthermore, biological discrepancies have to be kept in mind. Even though humans and mice are close relatives, and share fundamentally much of the same physiology and genes/genome, many differences in drug metabolism, gene expression, and disease progression persist. Direct cross-species extrapolation may be affected by the lack of immune response in the mouse strains utilized in xenograft experiments, differences in ADME properties, and over-interpretation of the preclinical efficacy data In addition, experiments on animals are expensive, time-consuming and ethically controversial. For example, the EU 3R goal to reduce, refine and replace the use of laboratory animals, puts pressure on scientists to develop and utilize alternative models in order to limit the unnecessary utilization animal experiments.

Organotypic cell culture models

Three-dimensional (3D) cell culture represents a promising research and development area. Numerous studies indicate that cells cultured in 3D environment are superior to two-dimensional (2D) cultures in plastic flasks, in particular when it comes to mimicking behavioural similarity to *in vivo* tumors [Ghajar, Bissell 2010, Santiago-Walker et al. 2009, Lee et al. 2007, Pampaloni, Reynaud & Stelzer 2007]. Alternative 3D cell culture techniques established during the past few years represent an attractive option for studying classical hallmarks of cancer, that are otherwise difficult o recapitulate with traditional *in vitro* models. These include angiogenesis, EMT, tissue invasion and metastasis, but also metabolic features. In general, the 3D models established to date can be divided into two main classes: those that utilize free flotation (non-adherent), and those that utilize scaffolds (summarised in figure 5). Biologically, these two approaches represent opposing strategies for inducing multicellular organization and differentiation.

Non-adherent sphere cultures and bioreactors

Free flotation is established by preventing cells from adhering to any ECM, and can be achieved for example by the “liquid overlay” technique, where cells are cultured on non-adherent plastic surfaces (e.g. poly-HEMA coated dishes). Also in stirred bioreactors, the constant flow of medium prevents cell attachment to any surfaces (reviewed in Åkerfelt, Härmä & Nees 2011). Instead, restriction of cell-matrix interactions forces cells to form multicellular aggregates or spheroids. This may also result in structural and functional differentiation. Low-attachment technologies have been independently introduced for a number of different cell types. Spheroid culture was introduced nearly half a century ago and thus represents one of the oldest *in vitro* cell culture technologies [Sutherland, McCredie & Inch 1971, Sutherland, MacDonald & Howell 1977]. The downside of non-adhesive cultures, especially in the case of epithelial cultures, is that most cells die when deprived of matrix adhesion. This generates an exceeding amount of cell stress, resulting in the selection and enrichment of certain cell populations over others. For example, with prostate cancer cells, “prostasphere” culture induces a dynamic process of de-differentiation, resulting in the acquisition of stem- and precursor-like characteristics or even prominent enrichment of stem cells [Patrawala et al. 2007, Pfeiffer, Schalken 2010, Tang et al. 2007]. Some early studies have indicated that non-adherent environment induces stem cell-like

features, including an enormous proliferative potential, also in normal prostate epithelial cells. However, these effects may be only transitory, as the cells revert when transferred back into 2D monolayer culture [Kinbara et al. 1996]. Cell culture in bioreactors (reviewed in Ingram et al. 2010) is based on a steady low-turbulence environment, generated by gentle mixing of fresh and spent nutrient. This consistent stirring promotes the formation of extremely large and complex spheroid structures, without damaging the structures by excessive shear forces. Bioreactors are an ideal option when massive amounts cells or spheroids are required. They also represent an ideal tool to generate large spheroids for co-culture purposes, e.g. with stromal and epithelial/cancer cells [Yates et al. 2007a, Yates et al. 2007b]. As other non-adherent cultures, bioreactors can also be utilized to promote stemness and self-renewal potential [Frith, Thomson & Genever 2010], although changing the cell culture conditions and media may also induce differentiation-promoting conditions.

Three-dimensional scaffolds

The scaffolds (or matrices) utilized in 3D cultures can be either synthetic or biological materials. Their main function is to provide structural support and in the case of biological materials differentiation promoting signals (reviewed in Åkerfelt, Härmä & Nees 2011, Kimlin, Casagrande & Virador 2011). The foundations for matrix-based 3D culturing were laid out by the laboratories of Mina Bissell and Zena Werb, searching for better methods to model the developmental and hyperplastic aspects of murine mammary gland [Nelson, Bissell 2005, Ewald et al. 2008]. For a long time, cells had already been cultured in gels like agar and collagen type I, However, biologically inert matrices like agar, polyethylene glycol gels or methyl cellulose were never considered relevant matrices for human cells. Agar is derived from algae and does not provide any physiological adhesion points for adherent cells, thus selectively supporting the growth of transformed cells capable to survive in anchorage-independent conditions ("soft agar assays"). Collagens, in contrast, represent the most abundant proteins in the human body, mainly occurring in the connective tissue [Karsenty, Park 1995]. Collagens are present in the ECM as fibrillar proteins and provide structural support to stromal cells and associated epithelial cells. 28 different types of collagens have been identified, five of which are common: collagen I (vascular ligature, tendons, dermal parts of the skin, main component of the organic part of bone), collagen II (main component of cartilage), collagen III (main component of reticular fibres, commonly found alongside type I), collagen IV (forms basement membranes), and collagen V (found on cell surfaces, hair and placenta). Over 90% of the collagen in the body is of type I [Kern et al. 2001], which is also the most commonly utilized collagen for organotypic 3D or raft cultures. Collagen type I is easily available as bovine and rat tail commercial extracts. Elastins represent another group of fibrillar proteins that, in contrast to rather rigid collagens, give elasticity to tissues. The ability to stretch and return to original state is particularly useful in tissues including lungs, skin and blood vessels. Elastins are synthesized by smooth muscle cells and fibroblasts. Proteoglycans are ECM proteins that carry negatively charged carbohydrate polymers or glycosaminoglycans, including heparan sulfate, chondroitin sulfate and keratan sulfate. The negative charge of these ECM molecules attracts positively charged sodium ions, which consequently attracts water molecules via osmosis. This effect keeps the ECM and resident cells hydrated. Proteoglycans also absorb and store growth factors such as TGF beta within the ECM microenvironment. Fibronectins and vitronectin are another important class of secreted glycoproteins in the ECM. Their function is to connect cells with collagen fibres, mediated by

cell surface integrins, and meant to facilitate cell movement through the ECM. At sites of tissue injury, fibronectin binds to platelets during blood clotting and thus facilitates cell movement to the affected area during wound healing. In contrast, laminins are collagen-like fibres that bind to other ECM components, including collagens, nidogens and entactins, forming extensive networks of web-like structures. Laminins mainly appear in the basal laminae or basement membranes, where they provide rigid structural support against tensile forces and also assist in cell adhesion and polarization. A gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells called Matrigel, is a widely used, commercially available ECM extract. Matrigel has proven ideal for 3D cell culture using epithelial and endothelial cells, or even feeder-free embryonic stem cells [Xu et al. 2001]. Matrigel consists of laminin, collagen, entactins, fibronectin and proteoglycans, and resembles the complex extracellular environment found in the basement membrane [Hughes, Postovit & Lajoie 2010].

In contrast, synthetic matrices are typically variations of different non-physiological hydrogels. Hydrogels, or aquagels, are composed of hydrophilic networks of polymer chains in which water is the dispersion medium. Hydrogels are highly absorbent and can contain as much as 99.9% water, providing them a high degree of flexibility. The biological and biochemical characteristics of commercially available hydrogels (e.g. QGel™ MT 3D Matrix, PuraMatrix™, MAPTrix HyGel™) have often been engineered by modularly varying their components such as protease sensitive sites, adhesion ligands (e.g. RGD peptides that can bind to integrins), and other bioactive elements. Usually, the structural scaffold component is a fully synthetic and biologically inert polymer (like agar or methyl cellulose), that has been complemented with biological components that enable cell adhesion and cell-mediated enzymatic ECM degradation. Also various bioactive components facilitating the release of biomolecules and morphogens from the extracellular microenvironment can be deposited in the hydrogel. In general, such synthetic scaffold materials have the advantage of reduced risk of animal transcontamination and improved reproducibility, as they are chemically defined, typically very pure and contain no unknown biological factors (e.g. cytokines, growth factors) which are otherwise difficult to control for in biological matrices (lot-to-lot inconsistency). Also the mechanical and functional properties (e.g. stiffness, degradation sites, adhesion sites) of the gel can be engineered at will by modifying the polymer architecture and concentration.

Three-dimensional organotypic models

As originally established by the laboratories of Bissell and Werb, organotypic 3D culture models recapitulated the complexity of murine mammary gland development *in vitro* remarkably well. It was also observed that the developmental cycle could be easily studied and manipulated in organotypic 3D culture, whereas in traditional 2D culture the developmental processes did not even occur. Since then, 3D cultures have been further refined to model multiple hallmarks of cancer, including different aspects of the invasive phenotype such as collective invasion and transepithelial tumor cell migration [Ewald et al. 2008, Gaggioli et al. 2007, Fischbach et al. 2007]. The invasive processes in 3D culture are often concomitant with EMT characteristics on both the molecular and morphological level. A classic example of matrix-induced EMT is called branching morphogenesis, which often occurs when normal, non-transformed glandular epithelial cells are transferred into a biologically relevant 3D matrix. The cells then form invasive multicellular tubules that extend into the matrix by actively degrading and modifying it [Nelson, Bissell 2005]. The selection of a suitable cell culture platform and the 3D matrix substrate depends on the cell

type used and the desired experimental readout. When mimicking tumor microenvironment, one should remember that the stroma changes according to the tumor stage. For example, in the murine mammary gland the stroma typically stiffens as the cancer progresses – the result of increasing production of collagen type I also observed in fibrosis, scarring and tissue injury.

The simplest form of 3D culture are probably the skin-epidermal models, which consist of a plastic cup or raft that supports a disc or layer of matrix formed by ECM scaffolds. The epithelial cells are cultured in full confluency on top of this support. The forming epithelial layer is then exposed to a combination of specific differentiation-promoting media, growth factors and the liquid/air interface, which promote epithelial differentiation. This platform has been used in 3D studies of tissue repair and as a model for stratified epithelia (skin, cervical, oral mucosa) and related cancers including squamous cell carcinoma [Jo et al. 2010], melanoma [Santiago-Walker et al. 2009] and oesophageal carcinoma [Grugan et al. 2010]. A modified system has been used for co-culture model, in which squamous cell carcinoma cells can be cultured on top of a layer of cancer associated fibroblasts suspended in collagen matrix [Gaggioli et al. 2007]. In this setting, the formation of a myofibroblasts network serves as a guiding structure to direct the migration of cancer cells into the gel, without prior EMT. Also vascularization has been successfully modelled in 3D systems, allowing the investigation of angiogenic capabilities of cancer cells and the factors that regulate the angiogenic switch [Fischbach et al. 2009, Ghosh et al. 2007]. Also angiogenesis studies have benefited from co-culture models incorporating epithelial cells/fibroblasts and endothelial cells into the same 3D microenvironment [Velazquez et al. 2002]. Nevertheless, epithelial cells can also be embedded in ECM scaffolds. This is the preferred mode for glandular epithelial cells that do not form single layers or sheets (squamous epithelia), but secretory structures known as acini. The cells can be suspended in the matrix either as single cells (clonal models) or already as pre-formed multicellular spheroids (re-aggregation models).

Organotypic cultures in high-throughput screening

To address the growing need for standardized 3D cultures, academic investigators and commercial companies constantly develop new cell culture scaffolds, platforms as well as ready-to-use preassembled solutions (perfusion chambers, microfluidic devices). The common purpose of these efforts is to standardize *in vitro* models with the aim to reliable recapitulate the organ/tissue/disease complexity and function, thus addressing growing needs for basic research (cell biology) as well as the pharmaceutical industry (high content screening). Reliable, informative 3D systems need to be up-scaled for medium- to high-throughput, with the goal to allow rapid and cost-effective drug screens. As the complexity of the systems increase remarkably and simple lumino- or fluorometric signals (such as metabolic activity and cell numbers) cannot be used as sole readouts, alternative quantification methods have to be designed. In high-content screens (HCS), measuring and comparing intricate phenotypes and morphologies becomes the main focus – the readout turns phenotypic and the most suitable form of quantitation are morphometric measures. The most useful analysis techniques for such purposes are bound to be based on microscopy and cytometric/morphometric analysis of individual structures, rather than quantification of grossly generalized measures on a population level. Similarly, as the requirements for the number of parallel readouts remain high, new cell culture platforms need to be designed, as well as efficient microscopy automation and robust image-analysis methods to cope with massive amounts of complex data.

So far, academic researchers and commercial companies have mostly been focusing on higher throughput cell culture scaffolds/devices and platforms. Highly specialized companies including InSphero, 3D Biotek, 3D Biomatrix, Bellbrook Labs, Synthecon and Global Cell Solutions, to name a few, all offer a different approach to fulfil the preclinical needs of the pharmaceutical industry. InSphero relies on scaffold-free GravityPlus technology, based on the hanging-drop method [Drewitz et al. 2011]. This principle is closely related to the older liquid overlay methods, and similar to stem/progenitor-cell biology achieved in low-turbulence, stirred bioreactors. This method allows gentle gravity-driven "microtissue" formation at the liquid-air interface of small medium droplets. However, only a single spheroid is formed by re-aggregation of hundreds to thousands of cells, and only one data point is generated per well and experiment. The liquid handling for this technology requires a robotic platform, equipped with sophisticated microfluidic design and surface engineering to assure consistent and rapid filling, stable formation of the drops (and spheroids subsequently), reliable media exchange, and supplementation of required supplements and/or cells [Kelm, Fussenegger 2004]. InSphero claims that their approach is suitable for drug efficacy and toxicity testing, cell-migration assays, angiogenesis, stem-cell research, cell-cell and cell-matrix interactions, and the study of transcriptomics, proteomics and metabolomics. 3D Biotek offers synthetic microfabricated scaffolds with well-defined pore size [Caicedo-Carvajal et al. 2011]. These pre-packaged scaffolds are available for throughput experimentation (up to 96-wells), and have been used for model tissue architecture of bone/cartilage, cardiovascular system, nerve, skin, tendon/ligament and liver. 3D Biomatrix offers a colloidal scaffold material (Perfecta3D™) [Kotov et al. 2004] specifically designed for pharmaceutical testing, with applications in cell expansion, tissue engineering, and cell imaging that supports the growth of numerous cell types, including bone marrow and blood, bone, skin and liver cells. In addition, 3D Biomatrix also provides hanging-drop culture plates based on colloidal scaffold materials that facilitate high-throughput drug screening assays, tumor spheroid assays, organogenesis studies, embryonic stem cell and induced pluripotent stem cell expansion and differentiation. Bellbrook Labs offers more traditional functional chemotaxis assays miniaturized for industrial drug discovery requirements. These assays are based on specialized microchannel plates (iuvo™ Microconduit Array Platform). Synthecon designs and manufactures various 3D cell culture systems, including "felt-like" scaffolds. The bioabsorbable scaffolds are created by a carding and needle-punch process, which tightly integrates non-woven polymer fibres into a cohesive felt material. The scaffolds are available in multiple polymer materials, and they come in various formats according to the experimental need (e.g. sheets, tubules and discs). Synthecon also manufactures rotating bioreactors (Rotary Cell Culture Systems™) for culturing suspension and anchorage-dependent cells. The system is designed to integrate the ability to co-culture cells. It features low shear force (and consequently low turbulence), and allows high mass transfer of nutrients. Together, these properties encourage spheroid formation and support the proliferation of cells within the spheroids. Global Cell Solution has developed a magnetic microcarrier incorporating magnetic particles within a human ECM-like porous matrix, coated with thin layers of gelatin or laminin [Justice, Badr & Felder 2009]. The three-dimensionality is created by a specialized flotation device (BioLevitator™) that facilitates antigravitational culturing of cells through magnetic levitation [Mirica et al. 2011].

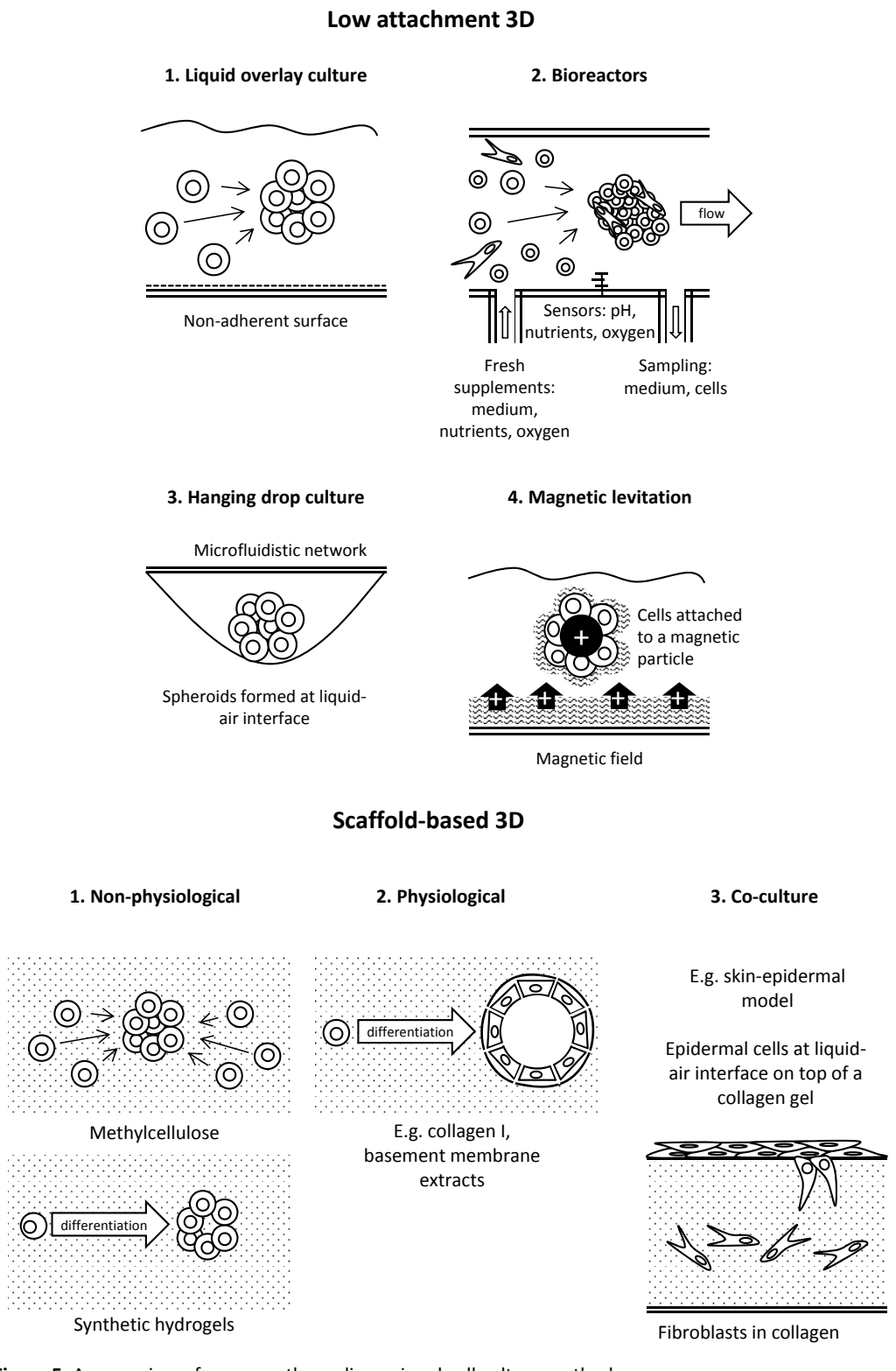


Figure 5. An overview of common three-dimensional cell culture methods.

This system is suitable for culturing difficult cells (e.g. primary and stem cells), improving the consistency of cell-based assays, and scaling-up for the drug discovery process. There are also promising new technologies emerging from the academia that are not yet commercially available, such as the micropatterned extracellular matrices (reviewed in Nelson, Tien 2006). Biological ECM substrates, including collagen and laminin, form complex networks by two mechanisms: self-assembly and cell-directed assembly. Reconstituted ECM mixtures such as Matrigel invariably polymerize homogeneously, and thus lack the heterogeneous architecture of real tissues, in which the cells and ECMs take the form of sheets (e.g. squamous epithelia, basal lamina), tubes (e.g. vessels, bronchioles, glands), branches (e.g. blood and lymphatic vessels, lung, kidney and mammary epithelia), folds (e.g. dermal papillae, intestinal villae), and bends (e.g. vessels) [Nelson, Tien 2006]. Form directly affects cellular behaviour by controlling the magnitude and distribution of mechanical stresses within the tissue [Ingber 2005]. The most popular technology in micropatterning ECM is based on "soft" photolithography or photo-polymerization of hydrogels. Lithography is a light-based patterning technique, which allows indirect control over the exact location where proteins can be adsorbed or deposited on glass or silicon surfaces. In contrast to traditional lithography, soft lithographic techniques use elastomeric stamps facilitating the structuring of 3D patterns, gradients and mosaics [Whitesides et al. 2001]. This technology enables the formation of platforms based on a wide variety of biological ECM substrates (e.g. collagen, laminin). It has been successfully applied to create high-throughput 3D organotypic arrays to study morphogenesis and functional differentiation of mammary epithelial cells [Nelson, Inman & Bissell 2008], as well as monolithic gels that contain internal surfaces, such as complex networks consisting of channels and cavities. The formation of internal patterns requires the use of sacrificial materials (e.g. gelatine, paraffin, Matrigel), which are initially embedded in a gel and then removed to yield an open internal space. Another emerging approach relies on direct 3D ink-jet printing of gels to form microstructured multilayered laminates that localize distinct populations of cells to different planes or areas [Tang, Golden & Tien 2003, Odde, Renn 1999]. This exciting technique could potentially be used to build ECM or even simple tissues layer by layer. However, multiple issues regarding resolution, speed and alignment yet need to be properly addressed. In summary, much work remains to be done to optimize, validate and standardize these emerging systems. It is unlikely that a single 3D cell culture model, platform and readout system will be representative for all cancers *in vitro,* nor suitable to address a wide range of scientific questions.

High-content image analysis methods in cancer research

So far, most of the high-content image analysis programmes, both academic and commercial, are optimized for 2D monolayer cultures. This particularly applies for programmes capable of both high-content and high-throughput analysis, such as the widely used CellC [Selinummi et al. 2005] and CellProfiler [Carpenter et al. 2006]. Both of these are open-source software programmes, specifically tailored for image analysis of monolayer cell cultures. Their basic repertoire of features includes measurements of cell morphology (e.g. shape, size) or subcellular regions (e.g. nucleus, cytoskeleton). Addressing informative cellular phenotypes typically requires the use of specific markers to visualize certain molecules of interest (e.g. apoptosis, expression of various proteins, calcium signalling). Typically, quantification of markers is based on subcellular localization (or co-localization with other markers), and intensity.

Morphologies formed in 3D differ in shape, size, geometry, density, surface features and internal textures from those observed in monolayer culture, rendering the majority of existing programmes developed for 2D analysis unsuitable for applications utilising complex organotypic cell models. Although still few in number, some software have already implemented algorithms to readily analyse multicellular structures, tissues and organs. In oncology, pathology has been an active area of research and development for higher-level computational image analysis tools. Constant flow of histological samples from the clinics, tissue microarrays complemented by improved high-throughput microscopy, and the tedious nature of manual morphological scoring – not to mention the variability in histologic grading among pathologist [Fanshawe et al. 2008] – have been important incentives to develop comprehensive software packages for automatic morphological analysis. Computer-assisted quantification systems for immunohistochemical protein staining such as the C-Path (Computational Pathologist) [Beck et al. 2011] and others [Linder et al. 2012], facilitate machine-learning algorithms that detect high-level contextual, relational, global and texture-based features in order to assess prognosis from microscopic image data. In complexity, the features are much more advanced than the standard parameters graded by the pathologists, such as tubule formation, epithelial nuclear atypia and epithelial mitotic activity in adenocarcinomas. In fact, for example the C-Path assesses a staggering 6642 different features, most of which would be way beyond human perception. Furthermore, these systems not only quantitate tumor tissue but also take into account the surrounding tumor microenvironment.

However, none of the commercially (or freely) available software packages have implemented tools to analyse multicellular morphological features observed in organotypic 3D cultures, especially in the context of high-throughput screening. There are a few recent studies exploring the use of quantitative image analysis tools in 3D cancer culture research. Han et al. have segmented, analysed and clustered the morphologies of a panel of 24 breast cell lines grown in 3D lrECM culture, using phase-contrast images [Han et al. 2010]. The authors successfully clustered the basic breast cancer morphologies observed by Kenny et al. [Kenny et al. 2007] and, most importantly, statistically linked the morphological signatures to dominant predictive genes. In another study, a set of mathematical measures that characterise geometry and topology in n-dimensional space, known as the Minkowski functionals, were used to quantify a 3D collagen I breast cancer culture topology in response to a modest number of therapeutic interventions [Savage et al. 2012]. Although the applications are still quite in their infancy, both of these studies serve as fine proof-of-principle examples of how high-level image analysis methods can be applied in 3D cell culture research.

AIMS OF THE STUDY

The overall goal of this thesis work was to establish a cost-effective *in vitro* screening routine, based on organotypic cell cultures in a physiologically relevant extracellular environment. This should be suitable for various stages of preclinical drug development, including target identification and validation, and the selection of candidate molecules for further pre-clinical development. The general aims can be further divided into five specific tasks:

1) To develop a cell culture platform suitable for three-dimensional (3D) high-content/high-throughput screening.

2) To optimize the platform by utilizing an extensive panel of prostate-derived cell lines.

3) Phenotypic screens: To develop a microscopy- and image-analysis based readout method that measures and quantitates the most prominent morphogenetic properties of prostate cancer cells.

4) To identify novel drug targets by transcriptional profiling of distinct morphogenetic processes, keeping the main focus on cell invasion, invasive transformation, and epithelial plasticity.

5) To validate targets involved in invasion by small molecule inhibitors and siRNAs, utilizing the established 3D cell culture platform and optimized readout methods.

MATERIALS AND METHODS

The plethora of reagents, consumables and other materials utilized in this thesis work is described in detail in the original publications (I-III). Therefore, this chapter is meant merely as a general index. All the antibodies, cell lines, experimental molecules, siRNAs and reagents, kits and consumables are listed in their respective tables (A-E) indicating the original publication in which they were used. As the detailed workflow of many methods and techniques varies according to the experimental setup, they are also listed as an index table (F). The following three protocols are not included or explained in detail in the original manuscripts, but were used to generate data shown in this thesis.

Immunohistochemistry of clinical tissue samples stained with LPAR1 antibody

FFPE sections were first deparaffinized and rehydrated in 2x10min xylene, 2x5min Abs. EtOH and 98% EtOH, 70% EtOH, 50% EtOH, 30% EtOH, 2 minutes each followed by 2x5min wash in water. Antigen retrieval was conducted in Labvision PT module using Tris-HCl, pH8.5 as buffer. Slides were preheated to 65°C and incubated in 98°C for 20min. After buffer wash, endogenous peroxidase activity was blocked with Dako peroxidase blocking solution S2023 for 5min followed by buffer wash. Primary antibody for LPAR1 (Imgenex, San Diego CA) was used at a 1:1000 dilution in Dako REAL antibody diluent S2022, and incubated overnight in a moist chamber. Antibody detection was conducted in Labvision Autostainer with Dako DAB+ as a chromogen. Slides were counterstained with Mayer's haematoxylin, dehydrated, treated with xylene and mounted. Human forebrain glial cells served as positive control and false positive staining was controlled by omitting the primary antibody in each staining round for one consecutive section. Each staining was done in duplicate. Assessment of LPAR1 protein expression was done with a tissue microarray containing 101 individual cancer cores from radical prostatectomies performed in Helsinki during the years 1989-1998. None of the patients had received hormonal treatment prior to operation. In the final analysis, 86 cores were available for scoring after IHC staining procedure. Anti-LPAR1 antibody stained TMA cores were scored as negative (0), weakly/moderately positive (1) or strongly positive (2). The antigen expression was located in the nucleus and perinuclear cytoplasmic regions of cancer epithelial cells. The one metastatic tissue section of iliac lymph nodes stained weakly for LPAR1 in prostate epithelial cells. IBM SPSS Statistics 19 was used in statistical analysis.

3D bulk cultures for immunohistochemistry

Cells were cultured in Millicell hanging cell culture inserts with 1.0 µm PET transparent membranes (Millipore) on 6-well plates (Costar). Membranes were pre-coated with Matrigel/medium (1:1) and incubated at 37˚C for 1 h, to prevent attachment to the membrane. Cell suspension was mixed 1:4 with Matrigel, transferred to the coated well, and polymerized overnight at 37˚C. Cells were fed every 2-3 days with fresh medium from underneath. The gels, including the multicellular structures, were excised with a scalpel and transferred into 4% paraformaldehyde for fixation. Fixed cell samples were cast into liquid agar pellets, moulded in paraffin, cut into thin sections with a microtome, and transferred onto microscope slides. The sections were first processed and stained with the IHC protocol described above.

Serum lipid deprivation

4 g of cell culture quality active charcoal (Sigma, C-9157) is mixed with 40 mg of T70 dextran (Amersham Pharmacia Biotech, 17-0280). 4 ml of sterile 1M Tris-HCl, 4 ml of sterile 0.1M EDTA and sterile water are added in together for 400 ml end volume. The mixture is stirred for 2-4 hours before centrifuged for 20 min (5000 rpm) at +4°C, and the supernatant is discarded. Serum (Gibco) is poured into Falcon tubes containing active dextran charcoal and shaken until the charcoal is fully mixed in the serum. The tubes are then incubated in a shaking water bath for 30 min (+55°C). This treatment is repeated twice. The charcoal is then pelleted and removed by centrifugation (20 min, 10 000 rpm, +4°C), the supernatant collected and filtered with a sterile 0.2 µM suction filter (Nalgene). Filtered serum is stored in -20°C.

A. *Antibodies*

Antigen	Supplier	Cat. No.	Used in
Akt (Phospho-Ser473)	Cell Signaling Technology	CST9271S	I
Akt (Phospho-Thr308)	Cell Signaling Technology	9275	I
AR	Labvision	MS-443	I
ARHGAP1	Sigma-Aldrich	HPA004689	II
ARHGEF11	Sigma-Aldrich	HPA011026	II
β-actin	Abcam	ab8226	I, II
CD44	Abcam	ab19622	I
Chromogranin A	Abcam	ab715	I
Cleaved PARP	Cell Signaling Technology	9546S	I
CREB1 (Phospho-Ser133)	Cell Signaling Technology	9198 l	II
Cytokeratin 14	Abcam	ab7800	I
Cytokeratin 18	Abcam	ab32118	I
Cytokeratin 8	Abcam	ab14053	I
GM130	Abcam	ab40881	I
IKKα (Phospho-Thr23)	Full Moon Biosystems	65450	I
Integrin β-1	Abcam	ab7168	I
IκB-α	Full Moon Biosystems	75443	I
I κ B- α (Phospho-Ser32/Ser36)	Full Moon Biosystems	65473	I
IκB-ε	Full Moon Biosystems	75636	I
IκB-ε (Phospho-Ser22)	Full Moon Biosystems	65636	I
Jak2	Cell Signaling Technology	3230	I
Ki67	Abcam	ab15580	I, II
Laminin α-1	Santa Cruz	sc-59849	I, II
Laminin β-1	Abcam	ab44941	I
LPA receptor 1 (Edg2)	Imgenex	IMG-71355	II
NF-κB p105/p50	Full Moon Biosystems	75338	I
p63	Abcam	ab735	I
PCNA	Abcam	ab29	I
PSA	Abcam	ab9537	I
Rac1	Cytoskeleton Inc.	ARC03	II
RhoA (Phospho-Ser188)	ECM Biosciences	9198	II
Smad3 (Phospho-Ser423/425)	Cell Signaling Technology	9520	I
SRF	Sigma-Aldrich	HPA00819	II
Stat1	Cell Signaling Technology	9172	I
Stat1 (Phospho-Tyr701)	Cell Signaling Technology	9171	I
Stat2	Sigma-Aldrich	HPA018888	I

B. *Cell lines*

Cell line	Origin	Malignancy	Details	Source	Used in
1013L	Prostate	Primary transitional cell carcinoma	Derived from xenograft model	Anita Bilström (Active Biotech Research AB, Lund, Sweden)	I
22Rv1	Prostate	Primary carcinoma	Androgen independent line derived from CWR22 xenograft model	ATCC (CRL-2505)	I, III
ALVA31	Prostate	Adenocarcinoma	PC3 derivative	Department of Veterans Affairs Medical Center, Tacoma, WA	I, III
ALVA41	Prostate	Adenocarcinoma	PC3 derivative	Department of Medicine, St. Luke's-Roosevelt Hospital Center, Columbia University, New York, NY	I
CA-HPV-10	Prostate	Primary carcinoma	Serum free tumor line	ATCC (CRL-2220)	I
CWR-R1	Prostate	Primary carcinoma	Androgen independent line derived from CWR22 xenograft model	Christopher Gregory (Univ. of North Carolina, Chapel Hill)	I
DU145	Prostate	Adenocarcinoma	Brain metastasis	ATCC (HTB-81)	I, III
DuCaP	Prostate	Adenocarcinoma	Dura mater metastasis	Kenneth Pienta (Univ. of Michigan, Ann Arbor, MI)	I
EP156T	Prostate	Non-transformed epithelial	Immortalized with pBabe-hTERT-puro retroviral vector	Varda Rotter (Weizmann Institute of Science, Rehovot, Israel)	I, II, III
HMEC	Breast	Normal epithelial	Mammary epithelial cells	-	III
LAPC-4	Prostate	Primary transitional cell carcinoma	Lymph node metastasis of xenografted mouse	Charles Sawyer (Univ. of California, Los Angeles, CA)	I, III
LNCaP	Prostate	Adenocarcinoma	Left supraclavicular lymph node metastasis	ATCC (CRL-1740)	I, II, III
LNCaP C4-2	Prostate	Adenocarcinoma	Bone metastatic variant of LNCaP	Leland Chung (University of Berne, Switzerland)	I
LNCaP C4-2/B4	Prostate	Adenocarcinoma	Bone metastatic variant of LNCaP	Leland Chung (University of Berne, Switzerland)	I
MDA PCa 2b	Prostate	Adenocarcinoma	Bone metastasis	ATCC (CRL-2422)	I
MDA-MB-231	Breast	Adenocarcinoma	Pleural effusion	ATCC (HTB-26)	III
MDA-MB-231 SA	Breast	Adenocarcinoma	Spontaneous metastatic variant of parental MDA-MB-231	-	III
MDA-PCa 1	Prostate	Primary carcinoma	Ascites metastasis	Nora Navone (Univ. of Texas, Houston, TX)	I
NCI-H660	Prostate	Adenocarcinoma	Lung metastasis	ATCC (CRL-5813)	I
PC-3	Prostate	Adenocarcinoma	Bone metastasis	ATCC (CRL-1435)	I, II, III
PC3-M pro4	Prostate	Adenocarcinoma	Highly metastatic variant of PC3	Isaiah Fidler	I, III
PrEC	Prostate	Normal epithelial	Prostate primary epithelial cells	Lonza (CC-2555)	I, III
PSK-1	Prostate	Prostatic small-cell carcinoma	-	Chol Jang Kim (Shiga Univ. of Medical Science, Ottsu, Japan)	I
PWR-1E	Prostate	Normal epithelial	Immortalized with adenovirus type 12-SV40 hybrid	ATCC (CRL-11611)	I
PZ-HPV-7	Prostate	Non-transformed	Non-transformed, immortalized by human papilloma virus	ATCC (CRL-2221)	I
RWPE-1	Prostate	Non-transformed epithelial	Immortalized by human papilloma virus 18	ATCC (CRL-11609)	I, II, III
RWPE-2	Prostate	Transformed epithelial	RWPE-1 derivative, transformed by Kirsten murine sarcoma virus	ATCC (CRL-11610)	I
RWPE-2/w99	Prostate	Transformed epithelial	RWPE-1 derivative cloned in soft agar to select cells that show high expression of Ki-ras	ATCC (CRL-2853)	I
UM-SCP-1	Prostate	Primary squamous cell carcinoma	Squamous cell carcinoma of the prostate	Barton Grossman (Univ. of Texas, Houston, TX)	I
VCaP	Prostate	Adenocarcinoma	Vertebral metastasis	ATCC (CRL-2876)	I
WPE1-NB14	Prostate	Transformed epithelial	RWPE-1 derivative transformed by N-methyl-N-nitrosourea	ATCC (CRL-2850)	I

C. *Experimental molecules*

Compound	Target	Supplier	Cat. No.	Used in
(-) Deguelin	AKT, PI3K, ODC	Sigma-Aldrich	D0817	I
(S)-(-)Blebbistatin	Myosin II	Tocris	1852	II, III
10-DEBC	AKT, mTOR, p70 S6K	Tocris	2558	I
187-1	N-WASP	Tocris	2067	II, III
2-APB	PI3K, ITPR3	Tocris	1224	I
5-(2-Benzothiazolyl)-3-ethyl-2-[2-methylphenylamino)ethenyl]-1-phenyl-1H-benzimidazolium iodide	AKT, FOXO1a	Sigma-Aldrich	B2311	I
Akt1/2 kinase inhibitor	AKT (AKT1, AKT2, AKT3)	Sigma-Aldrich	A6730	I
API-2	AKT (selective)	Tocris	2151	I
API-59CJ-Ome hydrate	AKT (highly selective)	Sigma-Aldrich	A8979	I
AS604850	PI3K (PI3Kg)	Sigma-Aldrich	A0231	I
AY 9944 dihydrochloride	hedgehog, SMO	Tocris	1639	I
BPIPP	Adenylyl cyclase	Tocris	3635	II, III
CCG-1423	RhoA	Merck Chemicals Ltd.	555558	II, III
CCG-2046	RGS4	Tocris	2974	II
CK 666	Arp2/3 complex	Tocris	3950	II, III
Compound 401	PI3K, mTOR, ATM and ATR	Tocris	3271	I
Cucurbitacin I	JAK2/STAT3	Tocris	1571	I
Cyclopamine	hedgehog, SMO	Sigma-Aldrich	C4116	I
Cytochalasin D	Actin polymerization	Tocris	1233	II
EHT-1864	Rac 1, Rac2, Rac3	Tocris	3872	II, III
Forskolin	Adenylyl cyclase (activator)	Tocris	1099	II, III
FPA 124	AKT	Tocris	2926	I
Gallein	G protein βγ	Tocris	3090	II, III
HA 1100	ROCK1/2 inhibitor	Tocris	2415	II
IGF-1 recombinant	IGF1R	R&D Systems	291-G1-050	I
IGF-2 recombinant	IGF1R	R&D Systems	292-G2-050	I
I-OMe-Tyrphostin AG 538	IGF1R	Sigma-Aldrich	T7697	I
IPA 3	PAK1	Tocris	3622	II, III
JK 184	hedgehog, GLI1	Tocris	3341	I
JTE-013	S1PR2	Tocris	2392	II
KH 7	Adenylyl cyclase	Tocris	3834	II, III
Ki16425	LPAR1/3	Cayman Chemical	10012659	II, III
KT5720	Adenylyl cyclase	Tocris	1288	II
Latrunculin A	Actin adenine nucleotide exchange	Tocris	3973	II, III
LY 303511	Negative control for LY294002	Tocris	2418	I
LY294002	PI3Kβ, α, δ, and γ	Tocris	2418	I
Mastoparan	G protein o/i (activator)	Tocris	1192	II
Melittin	G protein s inhibitor, G protein i activator	Tocris	1193	II
Narciclasine	RhoA (activator)	Tocris	3715	II, III
NF023	G protein α o/i	Tocris	1240	II, III

Experimental molecules (continued)

Compound	Target	Supplier	Cat. No.	Used in
NSC23766	Rac1	Tocris	2161	II, III
NU7026	PI3K, DNA-PK, ATM	Sigma-Aldrich	N1537	I
Paclitaxel	Spindle-assembly	Sigma-Aldrich	T1912	I, II, III
Pertussis toxin (PTX)	G protein o/i	Tocris	3097	II
PI 103	PI3K (p110α)	Tocris	2930	I
PI 828	PI3Kβ, α, δ and γ	Tocris	2814	I
Picropodophyllotoxin	IGF1R	Tocris	2956	I
PQ401	IGF1R	Tocris	2768	I
QS 11	GTPase activating protein of ADP-ribosylation factor 1	Tocris	3324	II, III
SANT-1	hedgehog, SMO	Tocris	1974	I
SHH Sonic hedgehog, rec.	hedgehog, SMO	Sigma-Aldrich	S0191	I
SQ22536	PKA	Tocris	1435	II
W146	S1PR1	Avanti Polar Lipids Inc.	857390	II
WHI-P	JAK3, STAT1	Tocris	3115	I
Wortmannin	PI3K	Tocris	1232	I
Y-27632	ROCK1/2	Tocris	1254	II, III
ZM 449829	JAK3, EGFR, JAK1	Tocris	1366	I

D. *siRNAs*

Target gene	Supplier	ID	Used in
EDG2 (LPAR1)	Qiagen	SI00376250	II
EDG3 (S1PR3)	Qiagen	SI02757398	II
EDG5 (S1PR2)	Qiagen	SI02663227	II
EDG6 (S1PR4)	Qiagen	SI02631902	II
GNA12	Qiagen	SI00096558	II
LPAR2	Qiagen	SI04892818	II
LPAR3	Qiagen	SI04892909	II

E. *Reagents, kits and consumables*

	Reagent, kit or consumable	Supplier	Used in
Cell culture	RPMI-1640 cell culture medium	Sigma-Aldrich	I, II, III
	Keratinocyte Serum-Free Medium (KSFM)	Gibco	I, II, III
	Ham's F12 cell culture medium	Gibco	I
	Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich	III
	EGF	Gibco	I, II, III
	Bovine pituitary extract	Gibco	I, II, III
	Fetal bovine serum	Gibco	I, II, III
	Choleratoxin	Sigma-Aldrich	I
	Phosphoethanolamine	Sigma-Aldrich	I
	Hydrocortisone	Sigma-Aldrich	I, II
	Selenic acid	Sigma-Aldrich	I
	Insulin	Sigma-Aldrich	I
3D	Angiogenesis μ-slides	Ibidi GmBh	I, II, III
	Growth Factor Reduced Matrigel ECM	BD Biosciences	I, II, III
	Purecol (rat tail collagen I)	Advanced BioMatrix	I
	4-well rectangular dishes	Nunc	I, II, III
Imaging	Hoechst 33342	Molecular Probes	I, II
	Ethidium homodimer-2	Invitrogen	II
	SYTO® 62 red fluorescent dye	Invitrogen	III
	DEVD-NucView™ kinetic caspase-3/7 reagent	Essen Bioscience	II, III
	Calcein AM	Invitrogen	I, II, III
IF	Triton X-100	Sigma-Aldrich	I, II
	Paraformaldehyde	Sigma-Aldrich	I, II
	EGTA	Sigma-Aldrich	I, II
	MgCl2		I, II
Microarray	Agilent 244k human genome array	Agilent	I
	RNeasy Mini kit	Qiagen	I, II
	Illumina TotalPrep RNA Amplification kit	Ambion	I, II
	Sentrix HumanRef-8 v3 BeadChips	Illumina	I, II
	Cyanine3-streptavidine	GE Healthcare Biosciences	I, II
Western blot/LMA	SDS	Sigma-Aldrich	I, II
	Tris	Sigma-Aldrich	I, II
	DTT	Sigma-Aldrich	I, II
	Hepes	Sigma-Aldrich	I, II
	NaCl	Sigma-Aldrich	I, II
	EDTA	Sigma-Aldrich	I, II
	β-glycerophosphate		I, II
	Orthovanadate		I, II
	PMSF		I, II

Reagents, kits and consumables (continued)

	Reagent, kit or consumable	Supplier	Used in
WB	Bradford assay	Bio-Rad Laboratories, Inc	I, II
	PAGEr SDS-PAGE gels	Lonza	I, II
	Protran nitrocellulose transfer membrane	Whatman	I, II
siRNA	siLentFect™ Lipid Reagent	Bio-Rad Laboratories, Inc	II
	Opti-MEM® I Reduced Serum Media	Invitrogen	II
	Superscript II reverse transcriptase	Invitrogen	I, II
	Oligonucleotides (primers, probes)	Sigma-Aldrich	I, II
Other	CellTiter Blue® Cell Viability Assay	Promega	I, II
	Active charcoal	Sigma-Aldrich	II, III
	T70 dextran	Amersham Pharmacia Biotech	II, III
	cAMP Chemiluminescent Immunoassay Kit	Molecular Probes	II

F. *Methods*

Method	Used in
2D apoptosis assay	II, III
2D invasion assay	III
2D migration assay	II, III
2D proliferation assay	I, II, III
2D viability assay	I
3D cell culture (bulk)	I, II
3D cell culture (miniaturized)	I, II, III
Bioinformatic analysis of clinical mRNA expression data	II, III
Bioinformatic analysis of experimental mRNA data	I, II, III
Cell culture	I, II, III
Compound treatments in 3D cell culture	I, II, III
Confocal imaging	I, II, III
Gene set enrichment analysis (GSEA)	I, II, III
Hierarchical clustering analysis	I, II, III
Immunofluorescence labelling and imaging	I, II
Immunohistochemistry	II
Live-cell imaging	I, II
Morphological analysis of 3D cell cultures	I, II, III
mRNA microarray	I, II
Protein lysate microarray (LMA)	I
Real-time PCR	I, II
Reverse transfection	II
RNA extraction	I, II
Statistical tests: Kruskal-Wallis one-way analysis of variance, Student's *t*-test, Chi-squared test, Fisher's exact text	II, III
Western blot	I, II

RESULTS

The main results of this thesis were:

1) A three-dimensional cell culture platform was implemented for microscopy-based high-throughput screening.

2) A proprietary image analysis program was implemented to quantify key morphological features linked to epithelial differentiation, malignant transformation, invasion, proliferation and apoptosis. The program was further optimized in conjunction with the cell culture platform, to facilitate high-content/high-throughput screening. A set of R-based statistical tools was implemented in order to mine and visualize complex biological responses.

3) 25 prostate-derived cell lines were categorized into morphological groups according to their differentiation potential in laminin-rich ECM. Representative cell lines from each group were selected for phenotypic and genome-wide mRNA expression analysis complemented with systems biological characterization. The analyses pinpointed transcriptional changes related to general adaptation to laminin-rich extracellular microenvironment, as well as many specific responses, such as those linked to normal acinar morphogenesis and invasive transformation.

4) A novel *in vitro* model for spontaneous invasive transformation displaying high degree of epithelial plasticity was discovered. Transcriptional profiling identified cell signalling pathways linked to lysophosphatidic receptor 1 (LPAR1) downstream signalling, such as actin cytoskeleton organization via RhoA, Rac and Cdc42 and cyclic AMP signalling via adenylyl cyclase/cAMP accumulation/protein kinase A, involved in the invasive transformation model. Moreover, the receptors and pathways were validated with a targeted panel of siRNAs and small molecule compounds.

5) The clinical mRNA expression sets from healthy prostate glands, and primary and metastatic prostate cancers were compared. The pathways active in the spontaneous *in vitro* invasion model, including Rac and Cdc42, were shown to be upregulated also in the metastatic clinical samples. Similarly, the pathways downregulated in the *in vitro* invasion model, such as LPAR1 and RhoA, were also downregulated in the metastatic tissue samples.

The results listed above have both strong methodological (1 and 2) and biological (3 to 5) implications. The miniaturized cell culture platform presented in this work has been tested successfully with epithelial cells from a number of different tissues, including prostate, breast, lung and colon. With minor modifications, the image analysis software is highly versatile and can be adjusted to extract valuable information from numerous different multicellular morphologies and biological responses. The biological insights gained underline the tremendous importance and impact of the extracellular microenvironment on cell differentiation and behaviour.

Three-dimensional cell culture platform suitable for high-content/ throughput screening

Miniaturized laminin-rich ECM platform (I)

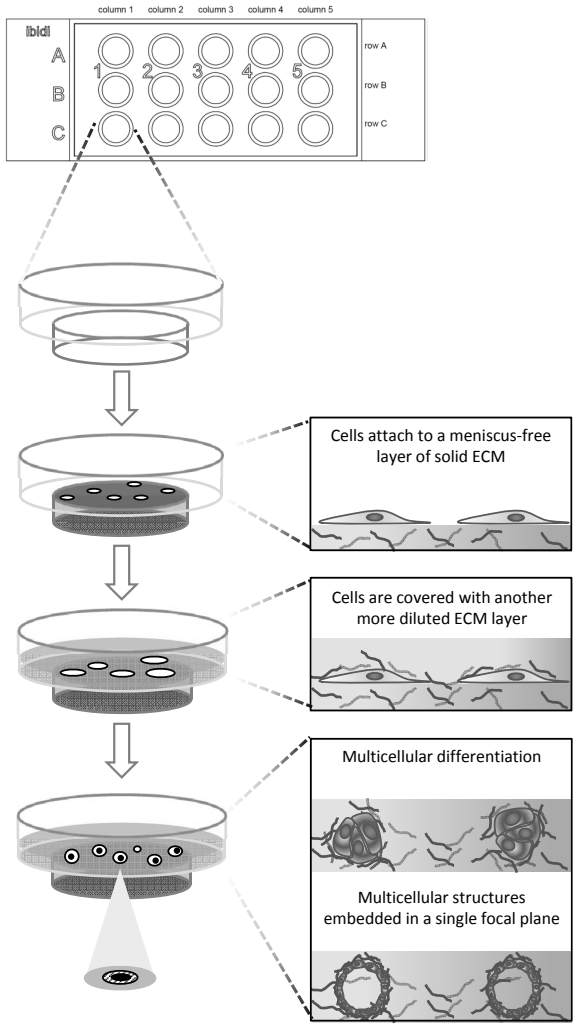


Figure 6. 3D culture platform based on ibidi Angiogenesis µ-slides.

We started the platform development by seeking a biologically relevant ECM that would support complete acinar morphogenesis of primary and immortalized prostate epithelial cells (hTERT- versus human-papillomavirus-immortalized epithelial cell lines, EP156T and RWPE-1), ideally in long-term cell culture. We tested the two most commonly utilized ECM products: an animal-derived collagen type I extract (PureCol®) and a cell culture-derived basement membrane ECM (BD Matrigel™). Growth factor-reduced Matrigel™, a laminin-rich ECM (lrECM) produced by EHS cells, was more suitable as it strongly promoted acinar differentiation, spheroid formation and branching morphogenesis. In contrast to collagen, the lrECM also supported maturation of

cell-cell and cell-matrix junctions and appropriate basal versus luminal polarization of normal cells. The organoids could be sustained in long-term culture for up to three weeks. LrECM also proved to be convenient in practical terms, e.g. simplicity of storage, liquid handling and gel polymerization protocols. The collagen-rich ECM failed to support acinar differentiation of normal epithelial cells. PureCol® only promoted the formation of loose cellular aggregates or fibroblast-like mesenchymal phenotypes with prominent cell motility. Collagen was also not suitable for long-term experiments, since the gels tended to contract over time.

After deciding upon the matrix scaffold, we pursued to find a vessel suitable for cost-effective lrECM culture. Traditionally, 3D cell cultures were based on standard 96-well plates, the most popular methods and assays perhaps those popularized by Mina Bissell's laboratory [Lee et al. 2007]. In these assays, cells are either fully embedded inside the lrECM, or situated between two layers of lrECM. However, to develop a cost-effective platform suitable for microscopy-based readout method, both setups imposed significant problems. The high cost-per well ratio on standard 96-well plates renders Bissell's techniques poorly applicable for high-throughput experiments. Second, microscope focusing is an issue as the multicellular structures are either floating freely in the gel, or reside in a more or less uneven lrECM-coated surface. A third problem arises from the fact that cells located close to the bottom tend to migrate and attach to the underlying plastic surface. Cells touching the plastic start growing as monolayer with much higher proliferation rate compared to the cells inside the gel. This strongly debilitates visibility, reduces the life span of cultures and experiments, and severely distorts the imaging results. Our approach to overcome these shortcomings was to transfer the double layer approach into a more suitable, smaller sized vessel. For this purpose, we optimized Ibidi's Angiogenesis µ-slides, a cell culture platform comprised of 15-wells each built with a unique well-in-a-well design, for our 3D cultures. The well-in-a-well architecture was originally designed to curb the formation of a meniscus, a convex surface of liquids caused by surface tension. This improved design enabled us to culture cells on a more defined and narrow focal plane, thus greatly improving image acquisition (Figure 6) and quality. The small volume of the inner well further helps to reduce unnecessary consumption of expensive ECM substrates. The relatively small volume of the wells in general also decreases the need for other experimental reagents (e.g. antibodies, growth factors, small molecule compounds, biologicals). The architecture of the inner well not only helps to keep the ECM surface even, but also prevents the cells from migrating to the bottom of the well. As the wells do not get overcrowded easily with monolayer cells the 3D cell structures can be monitored and experimented for long periods of time. Also cell culture handling, experimentation, whole culture fixation and staining, are convenient as the slides can be addressed with multiwell pipettes.

As with any technique, 3D culturing with ibidi Angiogenesis µ-slides has its weaknesses. The cultures are prone to liquid evaporation and drying, caused mainly by a small upper well volume and poorly fitting lids. The lower well still consumes an excessive amount of ECM substrate, although less compared to standard 96-well plates, but the approach is not fully cost-effective for very high-throughput screening purposes. Furthermore, the general handling and imaging of the slides is somewhat cumbersome, due to the microscope slide format (1 x 3 inches) that is not compatible with laboratory automation methods or robotics. This becomes critical in cases where tens of slides are needed in parallel. These restrictions result in limited throughput for very large screens. Nevertheless, up to 16 slides (with 240 experimental wells) can be cultured, maintained and imaged in tandem with relative ease. This scale is suitable for most approaches in cell biology and basic research.

High-content screening routine (I and III)

An assay is not complete without matching and optimized readout methods and subsequent data analysis routines. As our primary interest was in quantifying detailed morphological responses to external factors, we chose microscopy and morphometric (phenotypic) image analysis as the main readout. For endpoint experiments, spinning disk confocal microscopy proved to be the optimal trade-off between speed and resolution, and was suitable for both high-content and high-throughput screening. However, since our platform was optimized for unifocal cell culture, conventional modalities (i.e. wide-field microscopy, fluorescent microscopy, light microscopy) were also usable. These were favoured in the case of dynamic experiments – again with certain limitations. The superb

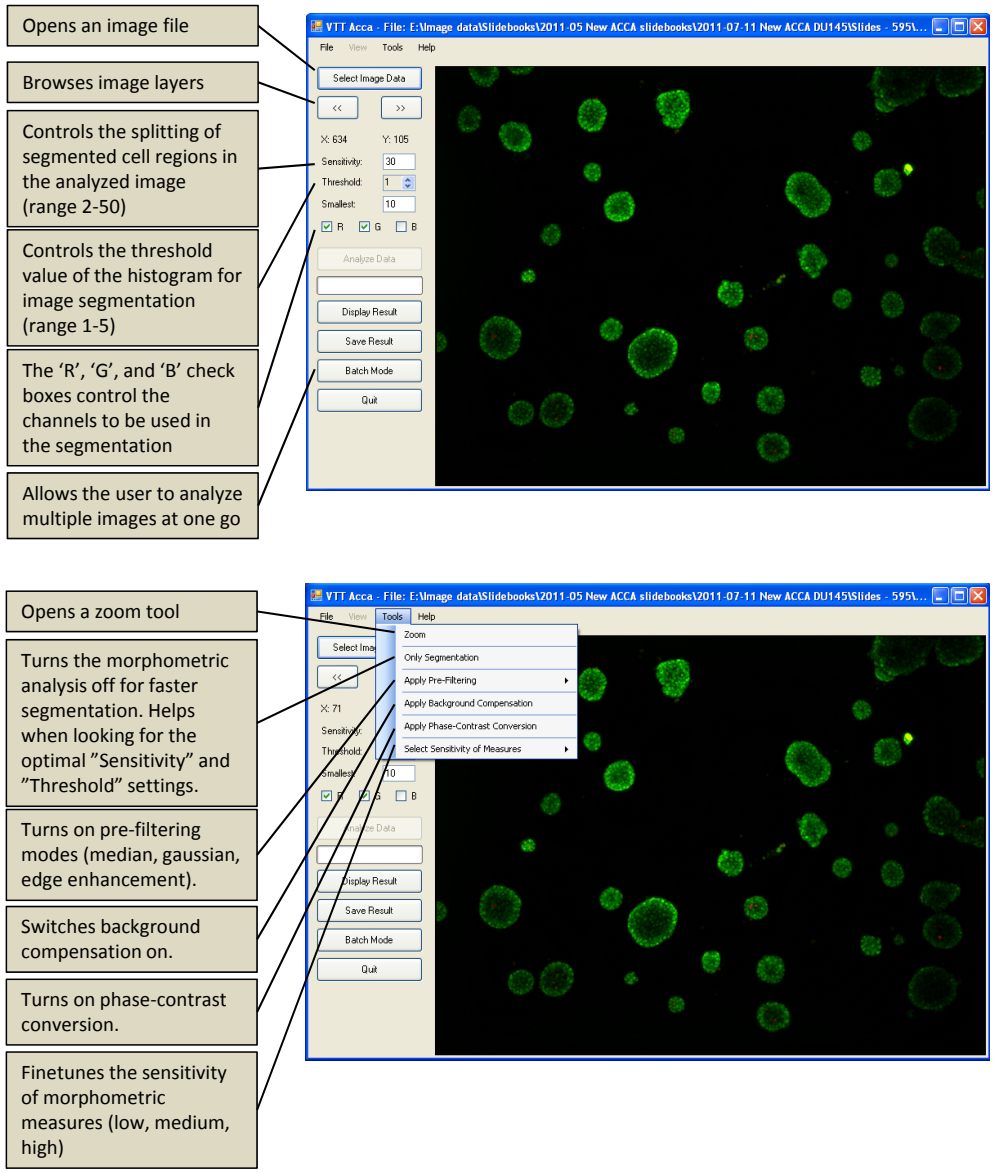


Figure 7. A proprietary image analysis software developed at VTT and some of its basic and advanced functions.

optical resolution of confocal microscopes, enabled by point illumination and a spatial pinhole, is an essential feature when the specimens to be imaged exceed the width of the focal plane, which applies for 3D cell structures. Moreover, since not all 3D structures in an experimental well reside precisely within a single focal plane, nor do the individual structures have strictly regular symmetry, it is helpful to create confocal image stacks that cover the entire span of all objects within the field. Image stacks can be later reconstructed into complete 3D images, or converted into 2D projections.

As none of the available programmes were capable of analysing the features observed in our 3D cell models, we decided to develop our own proprietary software solution in collaboration with VTT Intensive Knowledge Intensive Services, matching our specific needs (Figure 7). The requirements were: 1) ability to quantify cancer-relevant morphological features in 3D multicellular structures, 2) ability to quantify the localization of markers and cellular dyes with sufficient resolution, 3) adequate throughput, i.e. ability to process hundreds to thousands of images in batch mode, 4) ability to retrieve basic morphological information from many imaging modalities, and 5) the ability to operate with dynamic time-lapse image series.

Confocal microscopy automation

Our approach represents a reasonable compromise between cellular details (depth of analysis) and throughput (number of analyses). This was achieved by optimizing the analysis programme for low magnification (5x) images, which nevertheless capture significant morphological features. When imaging with lower magnifications, the focal plane becomes wider and fewer images in the Z-axis are required to cover the entire height of the 3D area. Essentially, with these settings, an entire well can be imaged with a small number of images. Naturally, the number of individual multicellular structures is significantly larger in wide field images, which reduces the number of adjacent fields required per well. Using a 5x objective, multiple sections through each cell structure were possible without losing informative phenotypic details (e.g. irregular symmetry of individual structures or invasion). Our confocal microscope was programmed to automatically scan all 15 wells within a single ibidi µ-slide, taking four 5x image stacks of sufficient resolution per well (60 images/slide). The X and Y dimensions for a single field were approximately 4.4 mm x 3.3 mm, with Z ranging between 300-600 µm (20-40 µm intervals). This routine enabled us to image a satisfactorily large area of ibidi Angiogenesis µ-slide inner wells (0.057 cm^2 vs. 0.125 cm^2), and simultaneously capture a statistically adequate number of multicellular structures from each well (typically 20-60 structures per field). The image resolution had to be kept moderately low (672 x 512 pixels) to promote fast operation. Typical scanning time for the 60 fields on one slide per colour channel was 10-15 minutes, depending on the signal intensity.

Image pre-processing

Our image analysis software was primarily designed to retrieve information from 3D confocal image stacks. However, compressed 2D intensity projections proved to be much more practical as routine starting material for large scale analyses. The intensity projection algorithm used here identifies the most intensive pixel from each voxel, and writes it in a simple raster graphics image. Unless the images are “flattened” into 2D projections, the image analysis software automatically applies an algorithm that identifies the most intense section for analysis, moving from cell structure to cell structure. When applied in practice, this algorithm tends to notably slow down the analysis. Before the actual morphometric image analysis is conducted, unspecific

signal or background noise is typically reduced by normalisation (e.g. 5 and 95 percentile of the histogram), followed by background reduction algorithms. Sometimes it is useful to swap the RGB channels and use the most intense channel for pre-processing. These pre-processing procedures can be performed with commercial open source programs such as SlideBook or ImageJ, reducing the need to program such components for our own software solution.

Morphometric analysis: segmentation

The first step in morphometric image analysis is segmentation of the image data. Segmentation separates individual multicellular structures thus facilitating further inspection of morphological features. Segmentation can be performed based on one single colour channel (R, G or B), but also using a selection of several. If 3D cultures or spheroids are stained for viable and apoptotic cells with two fluorescent dyes (e.g. SYTO62 and NucView), it may be useful to include both dye channels for segmentation. This will avoid losing exclusively apoptotic bodies from the analysis. Segmentation can be further divided into two concomitant processes: 1) separating the objects from background and 2) detecting the contours of individual objects. Our approach to identify the most suitable separation threshold was based on the characteristic, two-peaked shape of the confocal microscopy image histogram. The first peak is significantly higher and is composed of background pixels contained in the image. In contrast, the second peak is mainly composed of cellular structures. The method aims to detect a valley between the first and the second peak, in order to highlight only the cellular objects. A binary image based on the threshold is then recorded. This method seemed to be optimal for images taken with the 5x objective, and outperformed all other algorithms tested (e.g. those of Otsu, Sezan and Olivio). For further segmentation of the binary image, or detection of the individual objects, we employed the Watershed algorithm [Roerdink JBTM and Meijster A 2001], in which the image is first transformed to a distance map. Each pixel of the image is labelled with the distance to the nearest boundary pixel. The distance map is imported into the Watershed algorithm. At this point, the user may choose an RGB channel applied for the segmentation. Additional focus-finding adaptive segmentation algorithm is also applied here, in case 3D confocal stack images are used as source material. For correct segmentation, the user needs to adjust only two parameters: sensitivity and threshold. The sensitivity parameter controls the splitting of segmented cell regions in the analysed image (smaller value leads to smaller segmented regions, and *vice versa*). The sensitivity parameter refers to the distance in pixels used by the Watershed algorithm. In contrast, the threshold parameter basically controls the threshold value of the histogram. Choosing higher threshold values leads to more stringent segmentation.

Morphometric analysis: parameters

The software automatically assigns numerical values for key parameters related to spheroid phenotype and morphology. By observing the morphologies formed by a panel of 25 prostate-derived cell lines (presented in detail in the next chapter) we devised the use of 19 phenotypic parameters (Table 2) that appeared most informative and most directly linked to cancer biology. These parameters can be further divided in three main classes: 1) general, 2) morphological and 3) functional. General parameters include basic information related to the cell structures, including size (area), an object's relation to its neighbours (number of neighbours, shared boundaries with neighbours, closest neighbours), and the amount of cellular matter in relation to the local background. Morphological parameters include measures for such features such as symmetry

(roundness), contour roughness (measuring small surface features), and measures that indicate invasive processes (appendages). Functional measures mainly relate to substructural objects, such as the density and distribution of signals. All of the functional parameters are channel-specific. Analysed images are then saved in TIFF format, and the numeric data representing key morphological features are returned in a csv (comma-separated values) data file.

Raw data annotation and quality control

All statistical analysis and plotting tools implemented for processing numerical data (post-image analysis) were written by an expert statistician using R, an open source programming language and software environment for statistical computing and graphics (http://cran.r-project.org). All R scripts were incorporated in REX, an in-house html software environment that includes a browser-based user interface. Before the numerical data can be analysed, it has to be properly annotated, and undergo a stringent quality control. In our case, data annotation simply refers to a process where additional columns of information, e.g. cell line names, experimental conditions, drugs and concentration, and time-points, are added to the individual data points. In contrast, the main purpose of data quality control is to remove poorly segmented cell structures, debris and irregularities, noise and other artefacts. The quality control can be performed either manually or semi-automatically. The image quality processing allows post-analysis control over the segmentation process, through visual inspection and manual intervention. This approach, however, is very tedious and time consuming. Theoretically, the experimenter would need to inspect the images and structures one by one, which is not feasible with larger experiment series. The automatic QC approach is based on numerical threshold values.

Table 2. Phenotypic parameters analysed by the VTT image analysis software.

	Parameter	Explanation	Unit
General	Area	Area of the segmented structure	pixels
	Neighbors	The number of neighboring structures touching the segmented structure	pieces
	SharedBound	The length of the shared boundary of all Neighbors of the structure	pixels
	Closest	The distance of the closest neighbor of the segmented structure from the center point to the center point	pixels
	CellRatio	Cells to background ratio	ratio
Morphological	Roundness	Roundness of the segmented structure	%
	FiltRound	Filtered roundness of the segmented structure	%
	RoundDiff	Difference of the Roundness and Filtered Roundness	pp
	AppIndex	Index for severity of appendages of the segmented structure	no unit
	MaxApp	Estimate for the maximum length of appendages of the segmented structure	pixels
	MedApp	Estimate for the median length of appendages of the segmented structure	pixels
	Roughness	Roughness of the surface of the segmented structure	%
	AppNumber	Estimate for the number of appendages in the segmented structure	pieces
Functional	DensityRGB	Density of the segmented structure for each channel	gray levels/pix
	DeviationRGB	Standard deviation of the segmented structure for each channel	no unit
	AreaRatioRGB	Ratio of substructures of a certain color inside the segmented structure	%
	HollownessRGB	Estimate of the hollowness of the segmented structure for each channel	%
	CellNumberRGB	Estimate of the number of cells in the red channel inside the segmented structure	pieces
	AveAreaRGB	Average area of the cells inside the segmented structure for each channel	pixels

A specialized R script examines all annotated image data and calculates the median parameter value for each morphological measurement and for each image, and indicates deviations in a colour-coded readout. The user then can manually define limits e.g. for minimum object size (only objects in a certain range will be analysed, smaller structures that are likely debris will be ignored). Nevertheless, visual inspection of the images is also possible. In this case, another R script automatically discards all erroneous data points according to a manually generated list.

Bioinformatic tools for statistical analysis

Using our cell culture platform, the automated microscopy and image analysis methods as described above, the resulting number of multicellular structures per slide ranges between 1000 and 5000. As small drug screens typically consist of 8-16 slides, and up to 30 measurements are made from each cell structure, the number of individual data points can easily reach hundreds of thousands. Interpretation of the complex biological responses requires robust tools for statistical analysis and data visualization. The statistical toolset, as implemented into REX, includes scripts for heatmap and boxplot generation for endpoint experiments, and additional heatmap and line graph scripts for dynamic time-lapse experiments. Heatmap visualization proved to be a particularly effective way for visualizing and comparing drug effects and experimental conditions that result in similar morphological responses (Figure 8).

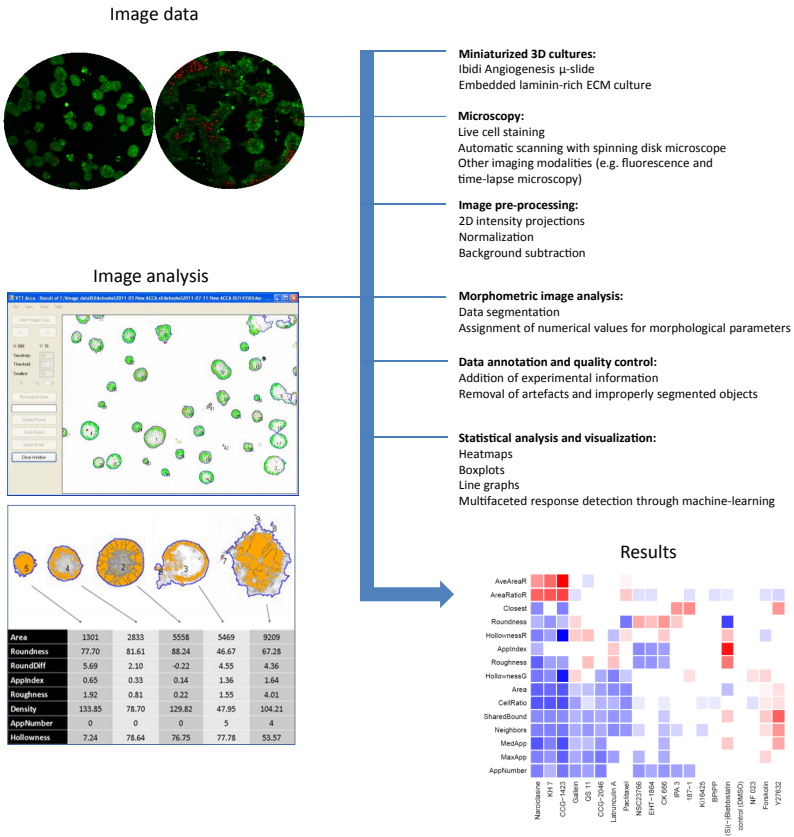


Figure 8. A typical 3D screening work-flow for endpoint experiments.

Epithelial responses to lrECM (I)

Epithelial morphologies and phenotypes of prostate-derived cells in 3D lrECM culture

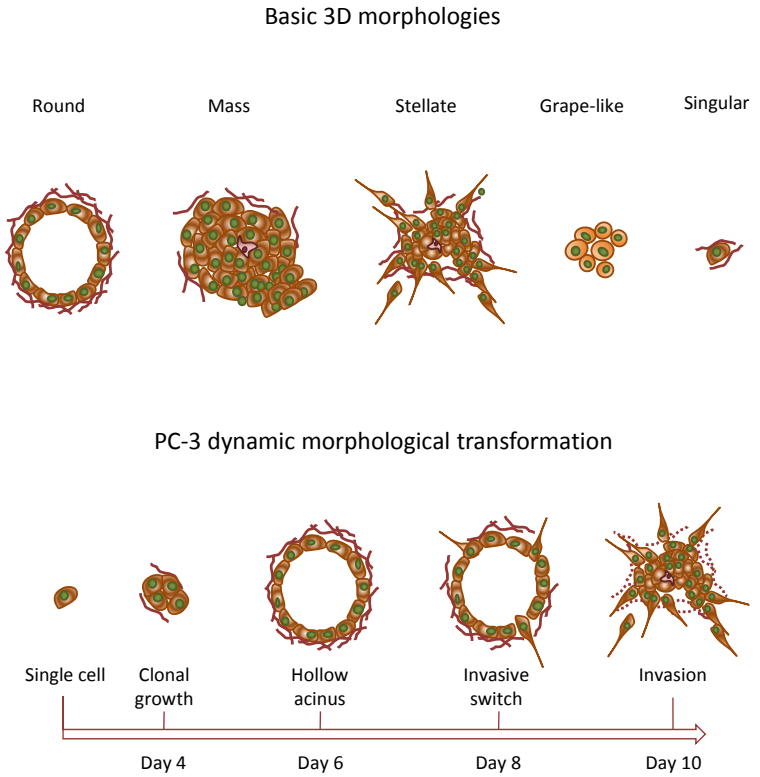


Figure 9. Recurrent morphologies formed by prostate-derived cells in laminin-rich ECM and the PC-3 spontaneous invasion model.

To validate the modelling capacity of the 3D screening platform, we collected a panel of 25 prostate-derived cells, and cultured them for a minimum of 10 days in Matrigel, using our miniaturized 3D platform. The multicellular morphologies were first classified visually. In addition, more detailed phenotypic analyses were performed by immunofluorescent staining, using a wide array of antibody markers specifically addressing structural and functional features of epithelial differentiation. The spheroids formed in Matrigel generally fell into four morphological categories: round, mass, grape-like, and stellate. These categories were originally adapted from the study by Kenny et al. [Kenny et al. 2007], describing the morphogenetic and transcriptional responses of breast epithelial and cancer cells to 3D lrECM microenvironment (Figure 9; Table 3). Normal, primary epithelial cells (PrECs) and non-transformed lines such as RWPE-1, EP156T and PWR-1E routinely formed round spheroids after 6-10 days in culture. RWPE-1 and PWR-1E cells simultaneously formed branching acinar structures, strongly reminiscent of the small ducts observed in healthy prostate glands, connecting acinar structures. Both round spheroids and ducts were completely encapsulated by a robust basal lamina. This feature attests to a high degree of epithelial polarization or terminal differentiation, as demonstrated by immunofluorescence staining for laminins α1 and β1. Interestingly, even though

the spheroids formed by normal epithelial cell lines developed morphologically correctly, none of these structures expressed detectable levels androgen receptor (AR) or prostate-specific antigen (PSA), main markers for functional "luminal" differentiation of prostate epithelial cells, on the protein or mRNA level. The cells also had a mixed phenotype resulting in co-expression of luminal and basal markers, as shown by immunofluorescent staining for cytokeratins 8 and 14. To our surprise, several transformed cell lines such as DU145, PC-3 and PC-3M, differentiated into properly organized round spheroids. These were surrounded by a complete basal lamina and frequently containing a hollow lumen, very similar to the spheroids formed by normal cells. PC-3 spheroids routinely developed a massive interior lumen, surrounded by a single cell layer. These spheroids occasionally contained internal cell masses, reminiscent of structures characteristic for prostate intraepithelial neoplasia (PIN) lesions. The majority of PrCa cell lines, including LNCaP, 22rv1, MDA PCa 1, UM-SCP1, CWR-R1, LAPC-4, and two *in vitro* transformed epithelial cell lines (PWR-1E and RWPE-2), generated larger, more irregular spheroids of the mass phenotype. These showed incomplete or missing basal lamina, and typically lacked a hollow lumen. Except for branching PWR-1E spheroids, none of the cell structures in this category developed any cellular extensions, such as ducts, or invasive appendages. However, filopodia or invadopodia formation were frequently observed in 22rv1, LNCaP and RWPE-2 spheroids, indicating cells posed for invading the matrix but hindered still by strong cell-cell-adhesion. Occasionally, single cells were documented that were capable to leave LNCaP spheroids, predominantly at sites of incomplete basal lamina coverage. Only one cell line, namely 1013L, fell into the grape-like phenotype group, forming loose aggregates of cells with minimal cell-cell contacts, lacking a basal lamina altogether. Interestingly, also LAPC-4 cells initially failed to form cellular contacts and basement membrane, but tended to congregate into mass-like spheroids later at day 8-10. This was dependent on sufficient androgen stimulus, as expected for a cell line that represents the androgen-dependent stage of primary PrCa. PC-3, and its highly metastatic derivatives PC-3M, ALVA31 and ALVA41, as well as the *in vitro* transformed RWPE-2/w99 and WPE-1/NB14 cell lines, formed invasive or "stellate" spheroids. These were characterized by formation of multiple spindle-like filopodia, followed by the rapid migration of cellular chains through the lrECM.

As described above, PC-3 and PC-3M initially formed well-defined round spheroids, but these structures later spontaneously disassembled. This very impressive and dynamic invasive switch was occurred around days 9-11 for parental PC3, but at shorter intervals for derivative PC-3M cells. The further progressed PC3 derivatives ALVA31, ALVA41 and the *in vitro* transformed cell lines simultaneously formed both stellate structures and round spheroids in parallel. This indicated a likely heterogeneous composition of these cell lines a feature also observed for some other lines including RWPE-1. Interestingly, the VCaP, DuCaP, NCI-H660 and MDA PCa 2b cell lines failed to form spheroids entirely, when embedded in Matrigel. These lines persisted as single cells for many weeks, eventually starting to form small spheroids with very slow replication after 2-3 weeks in culture. Spheroid formation was slightly more active in collagen gels. Interestingly, all these cell lines (except H660) are AR sensitive, express high levels of amplified, but wildtype AR, and are positive for ETS-transcription factor fusion events or rearrangements (TMPRSS2-ERG in VCaP, DuCaP and H660, a balanced ETV1 rearrangement in MDA-PCa 2B).

Dynamic changes of gene expression in response to lrECM

We further analysed transcriptional events, linked to the most prominent morphological responses induced by the lrECM microenvironment. For this purpose, we used genome-wide

mRNA microarray profiling. Representative cell lines from each of the morphological groups were selected: four cell lines forming round spheroids, including one cancerous (DU145), two immortalized normal epithelial cell lines (EP156T and RWPE1) and one primary cell culture (PrEC). Two cell lines were chosen to represent the mass phenotype (LNCaP and 22rv1), both also of similar luminal differentiation, androgen sensitive, and positive for SA. For the invasive phenotype, one in vitro transformed (RWPE-2/w99) and three genuinely invasive PrCa cell lines (PC3, PC-3M and ALVA31) were chosen. The spontaneous invasive transformation of PC-3 and (to a lesser degree) PC3M cells was scrutinized in more detail, by analysing samples from multiple time points that represent both spheroid and invasive morphological stages. In general, approximately 3400 genes were differentially expressed between the monolayer culture and 3D lrECM culture. However, since the expression patterns were not consistent across all cell lines and all time points, we computationally separated the differentially expressed genes into 12 clusters by using the K-means method. The clusters could be further grouped in three main response patterns: 1) non-transformed cell response, 2) general responses, and 3) invasive responses. Gene ontology (GO) and gene set enrichment analyses (GSEA) revealed significantly enriched functional gene categories for most of the clusters (Table 4). Altered gene expression was validated by quantitative RT-PCR and shown to be generally similar, although typically greater than the fold changes seen in the microarray data.

Table 3. 3D phenotypes in lrECM by morphological group.

Group	Cell line	Basal lamina	Cytokeratins	AR/PSA	Gene expression
Round	PrEC (branching)	Complete	Mixed	Not expressed	Profiled
	RWPE-1 (branching)	Complete	Mixed	Not expressed	Profiled
	EP156T	Complete	Mixed	Not expressed	Profiled
	PC-3 (spheroid)	Complete	More luminal	Not expressed	Profiled
	DU145	Complete	More luminal	Not expressed	Profiled
Mass	PWR-1E (branching)	Complete	Mixed	Not expressed	-
	RWPE-2	Heterogeneous	Basal	Not expressed	-
	WPE1-NB14	Heterogeneous	Mixed	Not expressed	-
	LNCaP	Incomplete	Basal	Expressed	Profiled
	LNCaP C4-2	Incomplete	More basal	Expressed	-
	LNCaP C4-2B	Incomplete	More basal	Expressed	-
	CWR-R1	Incomplete	More luminal	Expressed	Profiled
	22Rv1	Incomplete	Mixed	Expressed	Profiled
	MDA PCa 1	Incomplete	More luminal	AR expressed	-
	UM-SCP-1	Incomplete	Mixed	Not expressed	-
Grape-like	LAPC-4 (initially)	Missing	More basal	AR expressed	-
	1013L	Missing	Mixed	Not expressed	-
Single	VCaP	Laminins expressed	Mixed	Expressed	Profiled
	DuCaP	Laminins expressed	Mixed	Expressed	-
	MDA PCa 2b	Missing	Mixed	Expressed	-
Stellate	PC3 (invasive)	Incomplete	More luminal	Not expressed	Profiled
	PC3-M	Incomplete	More luminal	Not expressed	Profiled
	ALVA31	Incomplete	More luminal	Not expressed	Profiled
	ALVA41	Incomplete	More luminal	Not expressed	-
	RWPE-2/w99	Incomplete	More basal	Not expressed	Profiled

Table 4. The strongest gene ontologies induced or repressed by laminin-rich ECM microenvironment.

Pattern	Cell lines	GO
Non-transformed epithelial cells	PrEC, EP156T, RWPE-1, (DU145 and ALVA31)	**Upregulated**: ECM turnover, lipid and eicosanoid/prostaglandin metabolism, cell differentiation and organ development
Universal lrECM responses	PrEC, EP156T, RWPE-1, RWPE-2/w99, LNCaP, 22rv1, DU145, ALVA31, PC-3 and PC-3M	**Upregulated**: lipid/steroid metabolism and chromatin modification **Repressed:** mitochondrial and ribosomal functions, mRNA processing, general metabolic processes, cell-cycle, DNA-synthesis, mitosis and proliferation processes
Invasive responses	PC-3, ALVA31, RWPE-1, DU145	**Upregulated:** cell adhesion, extracellular matrix, multicellular organismal development **Repressed:** organ development, negative regulation of apoptosis, cytokine activity

An in vitro model for spontaneous invasive transformation displaying high degree of epithelial plasticity (II, III)

Spontaneous transformation

In the course of morphological characterization we noticed that one cancer cell line and its derivative displayed an interesting dynamic development pattern. PC-3 cells have a heterogeneous phenotype on monolayer culture, ranging from roundish epithelial look to spindle-like, more mesenchymal appearance. Typically the mesenchymal phenotype outnumbers the epithelial-looking cells many fold. In 3D lrECM culture, however, PC-3 cells started to form round spheroid structures after an initial adaptation phase. The spheroids originated from single cells. After 6-8 days in culture, the spheroid maturation was typically at its peak. The structures were completely covered with a continuous basal lamina, secreted by a single layer of well-organized cells. The spheroid lumen was usually devoid of cells, although sometimes a cluster of strongly fibronectin positive, motile cells persisted inside the lumen. The first signs of disintegration of the epithelial organization, or the beginning of the invasive switch, were typically observed after 8-10 days in culture. These signs included reduction of the strong lateral cell-cell contacts into thin connecting focal points, a transient disappearance of filamentous actin in some cells, and the emergence of multiple thin invadopodia probing the ECM penetrating through the basal lamina. After these first indications, the complete invasive transformation into stellar structures and loss of all epithelial characteristics typically occurred very rapidly, usually within 24 hours. The peripheral cells were increasingly directed outwards, first disintegrating and breaking through the basal lamina that covers the spheroid, eventually migrating into the microenvironment in a chain-like mode, thereby actively degrading the lrECM. In this process, the lumina of the spheroids were repopulated with cells that had previously lost polarization and epithelial organization. The invasive switch did not, however, occur simultaneously in all spheroids within an experimental well. Further experiments showed that the full invasive transformation was dependent on cell density and the presence of serum. With increasing cell density, either locally or throughout the whole experimental well, the invasive switch occurred faster. Similarly, the onset and speed of transformation was inversely correlated with the concentration of serum in the culture medium. By removing lipids from the serum (e.g. by absorption on charcoal), the PC3 spheroids entirely

skipped acinar differentiation, but instead assumed a typically mesenchymal, or invasive, phenotype from the beginning. This indicated that important lipophilic signalling molecules, provided by fetal calf serum in the medium, strongly supported epithelial differentiation and repressed invasive transformation.

G-protein signalling cascades downstream of lysophosphatidic acid receptor 1 promote spheroid formation

We then tested a panel of the most important steroids (androgens, estrogens, progesterone, glucocorticoids, retinoic acid and cholecalciferol) for their ability to reproduce the positive morphological effects induced by serum, however with no success. Two water soluble bioactive lipid signalling molecules, namely lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), abundantly present in many physiological fluids such as serum, plasma, ascites and lymphatic fluids, showed very efficient promotion of acinar differentiation and a potent and lasting repression of invasive transformation. Addition of LPA or S1P in micromolar concentrations into delipidated medium rescued the spheroid phenotype in early PC-3 spheroids. S1P was more potent, but also appeared to be more toxic, and caused morphological defects and partial disorganization of spheroids. Both lipids also potently blocked cell motility in monolayer wound-healing assays, attesting to their general involvement in regulating cell migration-related processes. Interestingly, neither high levels of LPA or S1P, or full serum could revert cells that had already started their invasive transformation to normal spheroid morphology. Addition of these factors only transiently blocked cell invasion, resulting in temporary reversion to the round epithelial cell phenotype. When invasive multicellular structures were disintegrated and cells from these structures transferred into monolayer culture and later into a new well containing 3D lrECM, spheroids developed normally. This indicates that the changes occurring during transformation were not permanent.

LPA and S1P mediate intracellular actions through multiple cognate receptors of the G protein-coupled receptor (GPCR) family. The involvement of individual receptors was tested by silencing these receptors one by one using RNA interference. LPA receptor 1 (LPAR1; EDG2) was pinpointed as the main mediator of anti-invasive effects. LPAR1 silencing resulted in clearly increased invasion, and severely impaired spheroid formation similar to a complete lack of LPA in the medium, or in cultures without serum. LPAR2 (EDG4), however, did seem to have independent functions not directly related to morphogenesis, as silencing mainly resulted in a block of proliferation. Silencing of S1P receptors S1PR1-4 had no noticeable effects on spheroid organization other than a slight reduction of growth, most likely due to redundancy in receptor downstream signalling. Additional evidence for the essential role for GPCR downstream signalling came from gene set enrichment analysis (GSEA) of gene expression data comparing prostate cancer cells in monolayer culture with early time points in 3D Matrigel culture. These analyses highlighted Gα12/13 and Gαq signalling, most notably actin cytoskeleton organisation via RhoA signalling, as the major pathways, generally upregulated when cells were embedded into lrECM microenvironment. In order to address the role of RhoA, we utilised a library of selected pharmacological tools (small molecule inhibitors) interfering with upstream pathways related to cytoskeletal organization. These compounds interfered with activity of adenylate cyclase (AC) and altered intracellular cyclic AMP levels, the activity of small GTPases Rac, RhoA, and Rho Kinases (ROCK), PAK1, or the function of actin-regulatory proteins N-WASP, Arp2/3, myosin

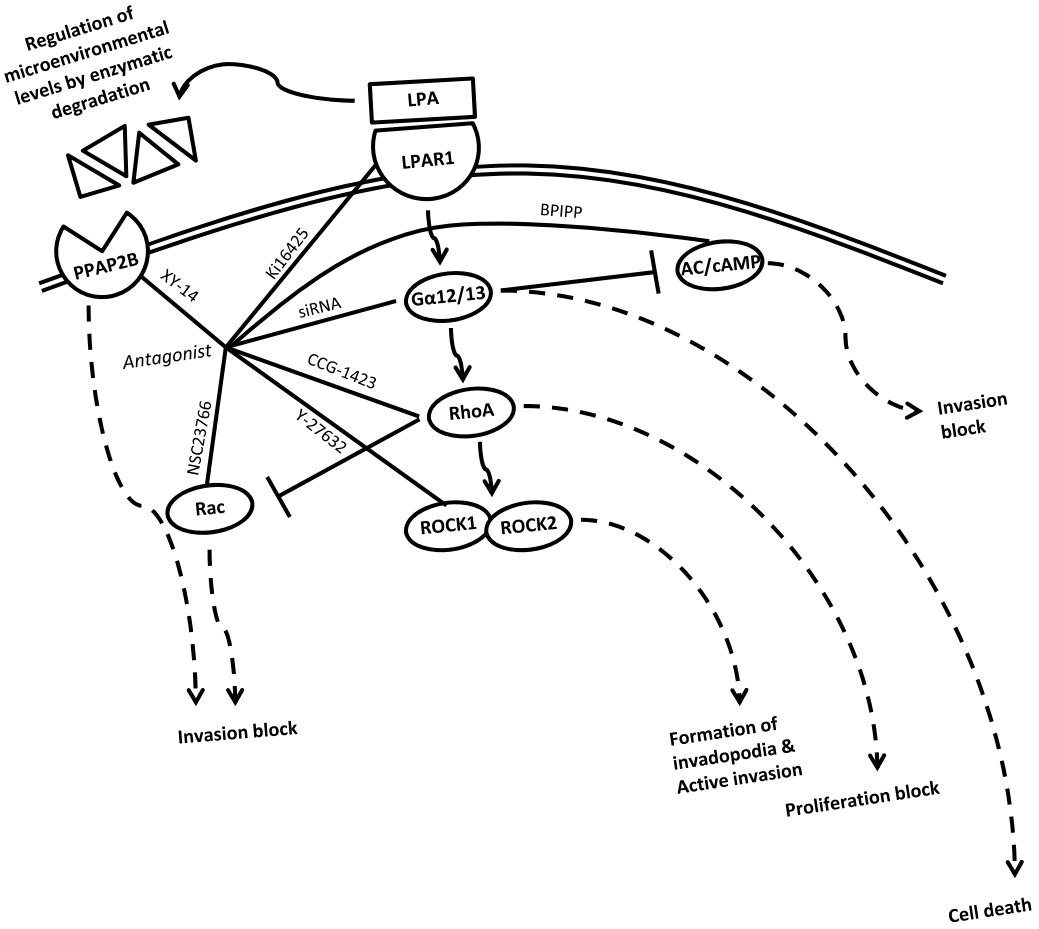


Figure 10. An overview of the signalling circuits in the PC-3 invasive transformation model.

II. Other drugs blocked G-protein signalling and the G-protein interacting protein RGS4. Using this collection of small molecule inhibitors, we demonstrated that the invasive switch in PC-3 cells is promoted by the adenylate cyclase/cAMP/protein kinase A pathway, with concomitant repression of Gα/RhoA/ROCK1-2 signalling. This is likely due to a spontaneous down-regulation of LPAR1, or by direct downstream counteraction of Rac and Cdc42 proteins with RhoA functions. According to the GSEA, G protein S pathway, a potent regulator of adenylate cyclase/cAMP, was significantly repressed in developing spheroids.

In contrast, experimental activation of adenylate cyclase/cAMP caused a collapse of spheroid structures rather than the direct stimulation of cell invasion. Close-up analysis of gene expression changes in pathways regulating the integrity the actin cytoskeleton revealed downregulation of the RhoA/ROCK/myosin signalling axis in invasive PC3 cells, resulting in an overall loss of actin/myosin assembly and contractility (summarised in Figure 10). Simultaneously, activators of the RAC small GTPase activity like DOCK1 and 3, NEDD9, ARHGEF4, 6 and 7, as well as RAC3 and CDC42, were overexpressed in invasive cells compared to mature acini.

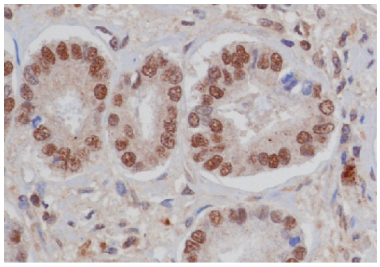
The clinical relevance of the *in vitro* invasion model (II and III)

LPAR1 is downregulated in metastatic prostate cancer

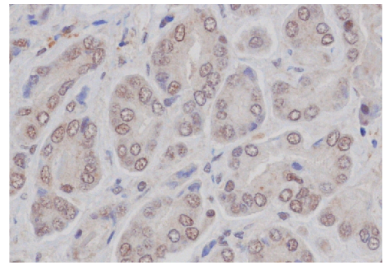
The expression of LPA and S1P receptors was analysed by utilizing two independent clinical mRNA expression sets, namely expO and Memorial Sloan Kettering Cancer Centre (MSKCC) [Taylor et al. 2010] datasets. In both of these transcriptome datasets, the expression of LPAR1 was reduced in prostate cancer compared to normal prostate tissues. LPAR1 expression anti-correlated with clinical parameters associated with cancer progression and poor outcome, such as histological grade, Gleason grade and lymph node invasion. For example, in the MSKCC dataset the expression of LPAR1 was significantly reduced in 42% of primary and metastatic cancers (98 of 218 samples). The expression of other LPA and also the S1P receptors were analysed. These were also found to be frequently altered in cancer, but none of them as consistently as LPAR1. In fact, apart from S1PR3, most LPA and S1P receptors were loosely anti-correlated with LPAR1 expression. In addition, LPAR1 immunohistochemical stainings of formalin-fixed paraffin-embedded prostate cancer tissues showed a tendency towards reduced protein expression in higher Gleason grade (4 to 5) and metastatic lesions (Figure 11).

RhoA and ROCK are downregulated whereas Rac1 and Cdc42 are upregulated in metastatic prostate cancer

Further evaluation of clinical samples of metastases of castration-resistant prostate cancers, compared to a large set of primary prostate cancers, revealed the strong downregulation of genes related to actin polymerization and actomyosin contraction. The large-scale clinical data set from MSKCC was also used for these analyses. GSEA revealed that key pathways related to the integrity of the actin cytoskeleton were significantly downregulated in invasive, metastasizing cancers. In particular, the canonical pathway “regulation of the actin cytoskeleton by RHO GTPases” was significantly deactivated. Close examination of the genes involved in this pathway in clinical expression data indicates that a subset of the genes in this pathway is massively downregulated in metastatic lesions, but also in the prostate cancer cell lines (e.g. Myosin light chain kinase MYLK, cofilin 2 CFL2, filamin A and C (FLNA, FLNC)). Furthermore, analysis of the RAC signalling pathway in castration-resistant metastatic lesions versus primary PrCa confirmed the activation of this pathway, particularly in aggressive tumors. RAC1 expression correlates with poor outcome in another clinical gene expression study [Sboner et al. 2010], while RAC3 expression is significantly overexpressed in metastatic lesions, high Gleason grade tumors, and patients with abnormal lymph node infiltration (MSKCC data).



Gleason Grade 3: strong staining (20 X)



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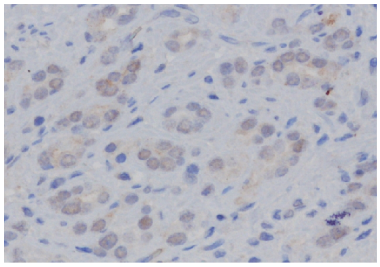
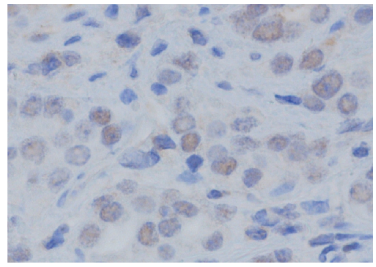
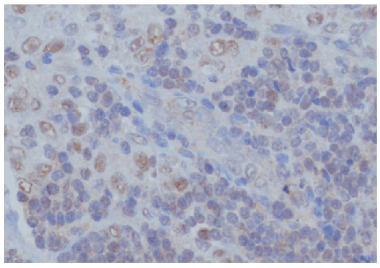


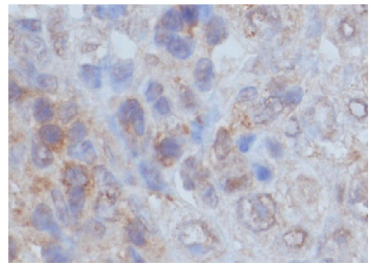
Gleason Grade 4-5: weak staining (20 X)



Gleason Grade 4-5: weak staining (40 X)



Metastasis: weak staining (20 X)



Metastasis : weak staining (40 X)

LPAR1/ Gleason Grading Crosstabulation

Count

		Gleason Grade			
		N	3	4	5	Total
LPAR1	0	0	21	27	1	49
	1	2	18	10	1	31
	2	0	4	2	0	6
Total		2	43	39	2	86

Figure 11. LPA receptor 1 protein is significantly downregulated in high grade prostate cancer (Gleason grades 4 and 5) compared to low (Gleason 3) (Fisher's exact p = 0.337, Pearson chi-squared test not significant). Normal samples were excluded from the analysis due to their low number.

DISCUSSION

The future of organotypic cell culture

Traditional two-dimensional monolayer cell cultures and assays based on them are being increasingly replaced by models better representing the tissue of origin. A logical first step in upgrading cell-based models that recapitulate key cellular processes (e.g. in cancer) is the addition of a third physical dimension. During the course of the past decades, numerous creative approaches tried to introduce an extra dimension, some arguably more physiological than others. Unquestionably, one of the most important innovations in cancer biology has been the introduction of biologically relevant extracellular matrices or scaffolds that mimic the natural tumor microenvironment (TME). To date, several different scaffolds, ranging from natural extracts to fully synthetic materials, have been studied in cell culture. Non-physiological proteinaceous scaffolds such as gelatine, silk protein, coagulated egg-white, or fibrin gels have yielded contradicting results. Spheroids grown in non-adherent cell cultures (prostaspheres, mammospheres) [Bisson, Prowse 2009, Cioce et al. 2010, Walia, Elble 2010], such as hanging-drops [Drewitz et al. 2011], stirred bioreactors [Martin, Wendt & Heberer 2004] or in non-biological, inert scaffolds (e.g. soft agar, alginates, methyl-cellulose, hydrogels etc.), typically result in the enrichment of stem- and progenitor-cells, concomitant with an increased self-renewal potential [Li et al. 2009a]. In contrast, many physiological matrices, including collagen type I or laminin-rich basement membrane matrices, can strongly promote the opposite end of biological responses i.e. epithelial differentiation (instead of de-differentiation), or stimulate other highly relevant cellular responses.

In March 2011, the technical insights division of Frost & Sullivan, an American consulting firm that provides customer-dependent market analyses, evaluated and identified new technology trends in the Life Sciences sector that are likely to have significant impact on the field. This list was based on technology journals, periodicals, market research reports, technology policy information sites, internal databases and thought leader briefings. The top themes in Life Sciences and Biomedicine included such prominent topics as personalized medicine, targeted therapeutics, stem-cells engineering (iPS technologies), regenerative medicine, biomimetics, early diagnostics, and omics technologies. 3D cell culture systems were listed as one of the most important technologies, considered critical also for enabling the realization of many of the other top themes. The potential application areas for 3D cell cultures include stem cell culture, model systems for cell biology, tissue engineering and regenerative medicine, drug testing and development. However, before 3D technologies can be fully utilized, multiple challenges have to be overcome. As pointed out throughout this thesis, the standard cell culturing vessels, such as Petri dishes, tissue culture flasks, and micro-well plates, commonly used owing to simplicity, convenience, and high-cell viability, are not suitable for culturing cells in more complex and biologically relevant organotypic environments. The conventional flat vessels that used to play a vital role in understanding cell biology are insufficient for new challenges in cellular biology and pharmaceutical assays. Most likely, different application areas will require dedicated and standardized cell culturing vessels, 3D scaffolds, and monitoring systems. For instance, massive bioreactors used for tissue engineering and regenerative medicine to promote cellular organization into functional, life-size human tissues and organs cannot be utilized in high-

throughput pharmaceutical screening. Frost & Sullivan report anticipates a stepwise development of 3D technologies, starting from the establishment of adequate techniques and platforms for basic biological research (e.g. stem cell culture, cell biology model systems), then gradually moving on to drug testing and development, and eventually peaking at the most complex areas, such as tissue engineering and regenerative medicine. The study underlined several strengths of 3D cell culture systems, such as the potential to reduce animal testing, to improve efficiency in drug development, and to reduce cost and throughput in testing procedures.

Although the primary application area for the miniaturized lrECM platform introduced in this thesis is drug testing and development (more precisely drug target identification and validation based on epithelial organoids), our platform also represents a suitable system for basic cell biology - in particular, for long-term cultures using different cell types originating from various tissues. Stem cell research represents an interesting and viable application area. However, as described previously, scaffold-free 3D culture systems such as the anchorage-independent liquid overlay and low-turbulence bioreactor systems, may represent more efficient methods for propagating and studying stem cell populations, than lrECM-based embedded systems. This notion is further supported by our gene expression profiles, showing essentially no evidence for enriched stem cell markers in lrECM cultures, compared to monolayer. On the contrary, lrECM strongly promotes differentiation and therefore triggers biologically opposing processes that decrease stemness or de-differentiation. Our culture techniques strongly promote epithelial differentiation, to the extent that the malignant phenotype can be overridden (at least temporarily) by powerful environmental cues. Considering these potent differentiation-promoting capabilities, the careful combination of different biological matrices, such as Matrigel, collagen type I or synthetic hydrogels, may provide valuable, adjustable scaffold materials for fine-tuning tissue engineering and regenerative medicine.

Traditionally, high-throughput screening platforms as such are poorly suitable for culturing human tissues or organs. Nevertheless, one could assume that the factors driving the initiation of organ development (e.g. morphogenic factors, growth factors, chemokines and cytokines, hormones) could also be studied in higher throughput format, using an approach similar to ours. In order to enable tissue engineering, a first step towards higher complexity has to be taken. By definition, tissues are comprised of multiple cell types. To reconstitute the proper structure and function of tissues and organs, two or several cell types have to be co-cultured in a suitable ECM. Tumors can be also considered as complex organs, however dysfunctional. Tumor-stroma interactions, including the multifaceted interplay between malignant epithelial cells, inflammatory cells, endothelial cells and fibroblasts, are increasingly recognized to play an important role in cancer pathogenesis. Although not shown in this thesis, we have explored this field by attempting to reconstitute the crosstalk between epithelial cells and cancer associated fibroblasts (CAF) in miniaturized 3D lrECM cultures. Even though establishing functional interactions between different cell types has been successful, it has proven technically difficult to reproduce multicellular organization in a format that allows detailed imaging of the cultures. This is mostly due to high motility and random alignment of cells in co-culture, especially when mesenchymal cell types such as CAFs (cancer-associated fibroblasts) are involved. These cells often form large aggregates that cannot be properly imaged with conventional light or even laser scanning confocal microscopes, rendering assessment of morphological qualities in real-time problematic. The future of organotypic co-culture may change, however, with cutting

edge technologies, such as 3D cell printing, that facilitate subtle microstructural alignment of cells according to user-defined blueprints. For example, Organovo's hardware and software platform for bioprinting (NovoGen MMX Bioprinter™) is at the forefront of the field's research and development. This novel platform is able to shape primary and other human cells into 3D tissue, providing excellent cellular viability and biology superior (as claimed by the authors) even to animal models [Jakab et al. 2006, Jakab et al. 2010]. To date, Organovo has built tissue of several types, including lung, cardiac muscle and blood vessels. However, such printing systems may be too slow and thus not suitable for creating tissue cultures for high-throughput experiments. They may instead prove more useful for lower throughput applications. Another, significantly less expensive, option would be to use micropatterned arrays where cells adopt a range of architectures dictated by variably adhesive surfaces. For example, the company CYTOO Cell Architects has developed such vessels, both with various predefined and custom-made micropatterns; although according to our knowledge these arrays have not yet been tested in organotypic cell culture context.

More throughput, more content

High-throughput screening (HTS) has ceased to be a monopoly of private pharmaceutical companies. Academic and government institute laboratories engaging in large scale pharmaceutical HTS efforts (e.g. BROAD Institute, Sanger Centre) are emerging worldwide. For instance, in 2005, the National Institutes of Health in the USA, as part of the NIH Roadmap for Medical Research, awarded 88.9 million dollars in grants to nine institutions to specifically use HTS methods for the identification of small molecules that can be used as research tools in drug discovery. The latest successes show that the money spent in sophisticated laboratory automation has not been wasted. In a recent worldwide study, titled "High Throughput Screening: New Users, More Cell-Based Assays, and a Host of New Tools", involving 54 HTS directors that represented 58 HTS laboratories and 34 suppliers, documents these current successes. Altogether, 104 novel clinical leads, originally identified by HTS technologies, were reported by the directors participating in this study. This represents the largest number of clinical candidates reported ever since these industry reports were started in 1997. Of these 104 leads, four products are already on the market. Most of the HTS technologies utilized by the pharmaceutical industry have traditionally been target- or protein-based biochemical assays. Cell-based assays are still mostly used for secondary screens, and their development lags behind. Even as recently as 2005, the percentage of cell-based high-content screens (HCS) amounted to only 4% of the entire screening expenditure. This is despite the fact that already in 2006, more than half of all HTS directors used cell-based HCS at least for some of their screens. An increasing trend, however, is that the major actors are now actively seeking for new practices and tools to "accommodate the increasing use of HCS, including instrumentation for imaging, engineered stable cell lines, flexible automated cell culture systems, HCS reagents, automation to increase throughput, assay development services and kits, tools for faster sample preparation, and data analysis and data-handling software" [Fox et al. 2006]. HCS was also anticipated to be the most important technology affecting the future of HTS. 44% of the HTS director respondents expected to use HCS in primary screening in the near future. These directors were especially interested in label-free assays, miniaturization, multiplexing, predictive software, and automated biological readouts allowing high throughput assays. In addition to compound screens, the HTS directors

are interested in applying HCS in small interfering RNA and RNA interference research, biomarker research, as well as *in vitro* ADME/Tox screening and pharmacokinetic characterization (safety and efficacy). The high-content screening routine described in this thesis incorporates many unmet needs identified by the HTS directors: multiplexing through platform miniaturization and microscopic automation, and high-content biological readout provided by the thorough analysis of disease-relevant morphological features, further supported by suitable statistical tools for data-handling. Moreover, our approach is firmly founded on biologically relevant organotypic cell models; and in future can be further improved by incorporating primary cell cultures from clinical materials. As demonstrated by our real-time staining assay label for apoptotic cells, our system is also compatible with fluorescent live cell reporter assays, and in future additional pathways and mechanisms could be addressed using comparable methods and reporter systems. This strategy will also be complemented by developing multi-label co-culture systems in the near future.

In the first chapter of the literature review, the pharmacological relevance, reproducibility, cost and quality issues etc. were described as the key criteria for HTS screening assay selection. Pharmacological relevance primarily refers to the biochemical activity of a compound on a pre-defined target molecule, usually a protein (enzyme, receptor, growth factor). Unless very robust and functionally relevant reporter assays are used, no cell-based screening assay can reliably report the biochemical activity of any given target protein.

Our screening method is no exception to this rule. When it comes to reliable recapitulation of cell-biological processes, organotypic screens outperform most of the traditional cancer-related screens, whether target or cell-based. Biological processes typically present themselves more tangibly in 3D compared to standard monolayer culture, e.g. by clear phenotypic effects on cell shape, motility, or multicellular structures. These features are often more straightforward to measure, using relatively simple morphometric image analysis methods. Part of the future of HCS may therefore be based on increasingly phenotypic screens and readout, supplemented by increasingly informative and specific reporter assays that focus on pathways and mechanisms directly relevant for cell biology. For example, the spontaneous invasive transformation displayed by the PC-3 cells can be used as a surrogate reporter for the underlying changes in cell signalling related to cell motility versus epithelial maturation. Certainly, such mechanisms of action have to be further studied, implementing a spectrum of additional methods such as gene expression profiling, or target-based secondary screens.

In order to understand and utilize cell models, the signalling mechanisms behind the morphological features have to be scrutinized cell line by cell line, as done in this work. The more cell lines (or primary cells) are characterized, the more informative models are available for our toolbox. Concerning the second criteria, the reproducibility of our system is surprisingly good, considering the complexity of the models, variability of materials (like Matrigel), and the techniques used. One issue impeding the reproducibility stems from the fact that the sensitivity of assays is not uniform across all morphological parameters. While some parameters or experimental condition barely have any measurable effects on the statistics, others are sensitive to even subtle perturbations. Another factor affecting the reproducibility comes from cell biology. Established cell lines and especially primary cell cultures tend to be very heterogeneous, and may contain a number of populations and sub-populations (intratumor heterogeneity). The morphogenic timeframe and extent of these cell subpopulations varies, and cell lines are clearly

composed of phenotypically different populations. Interestingly, in 2D monolayer cultures, such differences may easily go unnoticed. In 3D cultures, resulting in a much broader spectrum of morphologic variability, such differences may become immediately apparent. A good example is the human papillomavirus-immortalized prostate epithelial cell line RWPE-1. RWPE1 contains at least two prominent sub-populations, phenotypically divided in vimentin (VIM) positive and negative cells. The VIM+ cells have a strong tendency to develop branching ducts, while VIM- cells preferentially form round spheroids and differentiate [Harma et al. 2010].

The cell culture vessel format (e.g. 15- versus 96-well) imposes intrinsic sources for unwanted reproducibility and causes sensitivity problems due to a small number of replicate sample wells. The small number of experimental wells has the potential to introduce problems with experimental (well-to-well) uniformity, based e.g. on local variations of humidity and temperature. For example “edge effects” in smaller multiwell plates cannot be easily ruled out or corrected for, as routinely done in assays based on 96-, 384- and 1536-wells plates. Also randomization of experimental conditions is technically more difficult to achieve with low-throughput plates. Nevertheless, the cost per experiment value of our methods is fairly competitive, but switching to a higher throughput vessel format that is fully compatible with standard laboratory automation (e.g. robotics, liquid dispensers, and image acquisition) would reduce the expenditures substantially as this would decrease the time and effort required for human labour.

Finally, concerning the assay quality: Hughes et al. have proclaimed in *Principles of Drug Discovery* that a robust HTS assay should have a simple read-out, ideally based on stable reagents. It should also not include an exceedingly large number of protocol steps (e.g. washing, plate-to-plate transferring) [Hughes et al. 2011]. In our screening system, procedures following the cell culture initiation, such as adding fresh medium and experimental compounds, followed by microscopy, are generally non-intrusive by nature. No manual transferring from one plate to another or additional washing steps are involved. In fact, by using stably fluorescent cell lines or light-microscopy, one can perform morphological screens by avoiding any unnecessary intrusions other than the occasional replenishment of cell culture medium. When implementing new assays, these should be evaluated using stringent statistical quality control measures such as the Z’ factor assessment [Zhang, Chung & Oldenburg 1999]. However, in our case, the cell culture vessel format has restrained us from performing such assessments, forcing us to find other ways of controlling the quality, experiment by experiment. For example, we implemented Kruskal-Wallis and Anova-based statistical tests to monitor variation within each experimental condition. Overall, although this work is not complete, our routine serves already now as a fully functional proof-of-principle prototype and can be successfully utilized as a multiplexed, high-content drug screening platform.

Moving forward

Further moving on to a system capable of handling large, industrial-scale libraries, will require material upgrades and a great amount of streamlining in laboratory automation and data processing. The foreseeable improvements include standardized HTS cell culture vessels, faster image acquisition using up-to-date high-content screening systems, and multiplexed data handling. This needs to combine large-scale image analysis, data mining and visualization in a

single, standardized user interface. To optimize assays and readout, new cell models for cancer-relevant processes will have to be characterized and optimized, and fluorescent reporters for cell signalling pathways need to be constructed. Furthermore, reliable and more effective methods for the delivery of siRNAs, shRNAs and cDNAs, e.g. to allow utilization of larger scale or even genome-wide libraries, have to be established. Last not least, the growing demand for genuine dynamic assays, or "4D" assays (incorporating a time course), needs to be fully appreciated and answered by the development of appropriate hardware and software solutions. The next generation of HCS routines have to be capable of exposing dynamic drug responses that may be easily lost in single end-point studies.

What is our current status? We are currently working on a higher throughput microwell plate based, on the original Angiogenesis μ-slide well structure. The dimensions (e.g. plate/well diameter and well spacing) will be constructed according to the standard 96-well microtiter plate standard, originally defined by the American National Standards Institute (ANSI) and the Society for Biomolecular Sciences (SBS). This is expected to render the experimental vessel compatible with most laboratory instruments, including robotic liquid handling, and automated microscopes. Automated liquid dispensing will allow the screening of tens of plates simultaneously, and is expected to significantly improve the reproducibility of our experiments by minimising human error. Some of the techniques currently still performed manually in could be readily performed in much higher throughput, using robotic dispensers. These could be employed for the collection of protein samples for lysate microarrays (LMA) and pre-plating RNAi solutions for systematic gene knock-down studies. Microscopes designed for industrial-scale HCS, such as InCell 6000 from GE Healthcare, or Opera and Operetta from PerkinElmer, are increasingly available in HCS facilities, both in industry and academic institutions. PE Opera and Operetta utilize rapid spinning-disk confocal units, making them an amenable option for automated plate reading. Many of the microscopes are equipped with cutting edge scanning and image stitching features, ensuring that the content of the microwell is captured in its entirety. Concerning software development, we aim at integrating image segmentation and morphometric analysis with data storage, analysis, mining and visualization tools, and generate a comprehensive software package. This merely follows general trends in HCS analysis (e.g. PerkinElmer's Columbus® and Harmony® image data storage and analysis systems). Furthermore, we intend to modify our statistical tools into a more population-based effort, comparable to the principles of FACS analyses, with the goal to monitor the differential morphological responses of cell subpopulations without the need for fluorescent labels. Finally, dynamic and pathway-specific live cell assays will be utilized more extensively. Increasing effort will be spent on optimizing real-time image acquisition, analysis and data visualization. In this work we have already employed phase-contrast live-cell imaging to study morphological responses to drugs and RNAi. Preliminary studies have been performed using labelled cells and confocal microscopy, mostly with encouraging results.

Biological implications of organotypic models

In this thesis growth and differentiation patterns for a large panel of prostate and breast cell lines have been characterized in miniaturized 3D lrECM culture. Moreover, the mechanisms underlying the morphological patterns have been explored by using genome-wide mRNA expression profiling, novel quantitative proteomic arrays, and numerous conventional laboratory methods. Validation of the cell signalling mechanisms postulated to play a role in complex morphogenic

processes has been facilitated by RNAi technology, specific pharmacological tools and dynamic morphometric analysis. What have we learned from all these studies? How well do these cell models recapitulate cancer biology and – most importantly – what are they actually good for?

Spheroid architecture is the result of two signalling entities: the intrinsic signalling mechanisms and the stimuli imposed by the surrounding ECM. The intrinsic oncogenic signals are often counteracted by external stimuli which typically promote differentiation, resulting in an intracellular conflict. The balance between these two opposing signalling entities determines the phenotype of cells. Such opposing processes are often reversible, often resulting in transient phenotypes highly sensitive to changes in the microenvironment. This cellular plasticity not only affects the phenotype of single cells but also facilitates the morphological transformations of multicellular organoids in 3D culture. Epithelial plasticity is one of the emerging hallmarks of cancer cells as it may support survival under challenging and changing external conditions, especially in the course of metastasis (e.g. extra- and intravasation, tumor dormancy, and colonization of distant sites).

Mina Bissell and her laboratory stressed the importance of cell type matching ECM substrata already two decades ago. Bissell's group has shown that human mammary epithelial cells, isolated from reduction mammoplasty and cultured in lrECM, can form polarized acinus-type structures that are fully capable of gland specific functions including milk production [Stoker et al. 1990, Kim 2005], whereas type I collagen induced abnormal cellular polarity and disorganization [Howlett et al. 1995]. The strengths of three-dimensional basement membrane cultures for cell biology have been thoroughly reviewed by Debnath and Brugge [Debnath, Brugge 2005]. Soon after, others like Norman Maitland and his group have followed Bissell's footsteps and showed that lrECM culture also supports acinar morphogenesis of normal prostate epithelial cells as well as transformed cells [Lang et al. 2001a, Lang et al. 2001b].

Spheroids make bad EMT models

PC-3 cells represent a perfect model in which a clash between intrinsic and extrinsic signalling results in an interesting morphological development pattern. The 3D lrECM environment forces the cells to initiate a normal acinar differentiation pattern. As the acinar development culminates, a spontaneous invasive transformation takes place and the spheroids disintegrate. According to our data, this transformation is likely to be caused by loss of an external signal as abrupt morphological transformations can be reproduced by experimental perturbations in the cellular microenvironment. Typically such morphological changes have been reported to be concomitant with a clear transition from epithelial to mesenchymal phenotype. In the case of PC-3 cells, however, the transformation cannot be explained with EMT, as the cells express both mesenchymal and epithelial markers and the expression levels remain unaltered regardless of the morphological status. The expression levels do, however, predict the tendency of invasive morphologies. A majority of the cell lines tested by us with the most prominent latent, invasive potential, such as ALVA31, PC-3 and PC-3M, and to some degree the phenotypically heterogeneous RWPE-1 and RWPE-2/w99 cells displaying the branching morphology, show the highest expression of mesenchymal markers (e.g. vimentin, fibronectin, N-cadherin) with simultaneous loss of expression of many epithelial markers (e.g. E-cadherin, catenin alpha-1). This indicates that these cell lines may have undergone an EMT, already *in vivo* or later *in vitro* in cell culture. The fact that many mesenchymal marker genes, such as vimentin and fibronectin, and EMT-related transcription factors, including Slug and Snail, are strongly expressed in both

2D and 3D culture, and more importantly, remain unchanged throughout all stages of spheroid formation, not being significantly induced even in the most invasive stages of PC-3 spheroids, further support the idea that these are not models for acute EMT. However, high level EMT marker expression may serve as an indicator for latent or metastable EMT phenotype, which is temporarily repressed by the lrECM in favour of normal epithelial differentiation. As has been described, mesenchymal phenotypic features eventually override the epithelial differentiation patterns resulting in abrupt cellular invasion.

Epithelial plasticity mediated by LPA

What are the mechanisms underlying the spontaneous invasive transformation in PC-3, and more importantly, what implications do they have on cancer biology? Our studies indicated that a bioactive lipid signal mediator lysophosphatidic acid (LPA, 1-acyl-2-lyso-SN-glycero-3-phosphate), the simplest glycerophospholipid abundant in many physiological fluids, negatively regulates cell motility and invasion by promoting epithelial maturation of PC-3 cells. The biologic functions of extracellular LPA are mediated via specific G protein-coupled receptors (GPCRs), including LPAR1 (Edg2), LPAR2 (Edg4), and LPAR3 (Edg7) that belong to the endothelial differentiation gene (Edg) family. According to our data, the invasion counteracting effects are mainly mediated via LPA receptor 1. Our studies suggested a role for G proteins alpha 12/13 and i as the most prominent morphogenic LPAR1 downstream pathways. Gα12/13 was shown to stabilize actin cytoskeleton through RhoA/ROCK activation whereas Gαi presumably acted by repressing the detrimental signals of adenylyl cyclase/cAMP pathway. Furthermore, the Gα12/13 pathway with its downstream effectors seems to play a universal role in the development of acini and spheroid structures, induced not only by LPA but also the lrECM microenvironment, as shown by the gene expression profiles acquired from panel of prostate cancer cell lines. In agreement with the findings on PC-3, Gαs pathway, a positive regulator of adenylyl cyclase/cAMP, is generally repressed in normal and cancer-derived cell lines in 3D compared to 2D regardless of morphology. These findings, however, are mostly in contrast to earlier reports where the role of LPA and RhoA has been studied in context of cancer. The majority of studies on prostate cancer [Hwang et al. 2006, Evelyn et al. 2007, Hao et al. 2007, Hasegawa et al. 2008], breast [Li et al. 2009c] and ovarian cancer [Li et al. 2009b], all speak for the stimulatory role of LPA in cancer metastasis-related processes, such as cell migration, invasion, and colony formation both *in vitro* and *in vivo*. In addition, LPA is known to mediate a wide range of other biologic processes, such as cell proliferation, stimulation of DNA synthesis, cell survival, cytoskeleton reorganization, drug resistance, cell adhesion, cytokine production, and ion transport [Mills, Moolenaar 2003, Moolenaar, van Meeteren & Giepmans 2004].

LPA was first implicated in human oncogenesis in ovarian cancer because of its high levels in intraperitoneal effusions (ascites fluid) [Mills et al. 1990, Xu et al. 1995a, Xu et al. 1995b]. The high LPA concentrations can be either explained by the increased number of LPA-producing ovarian cancer cells in the peritoneal cavity [Umezu-Goto et al. 2004] or by elevated production of LPA by the irritated peritoneal mesothelium [Ren et al. 2006]. Aberrations in the microenvironmental LPA levels can be caused by altered levels of autotaxin [Umezu-Goto et al. 2002], the main enzymatic pathway that produces LPA, or altered levels of lipid phosphate phosphohydrolases (LPPs) [Tanyi et al. 2003a, Tanyi et al. 2003b], which metabolize LPA. Moreover, according to clinical data, LPAR2 and LPAR3 seem to be the main receptors aberrantly overexpressed in the majority of

ovarian cancer cells [Fang et al. 2000]. Overall, LPA has been shown to promote human ovarian cancer by increasing cell survival, proliferation, and motility as well as stimulating production of neovascularizing factors. Juxtaposing PC-3 invasion model with what is known about ovarian cancer, PC-3 cells seem to behave in an entirely opposite manner, although the cell signalling pathways involved are the same. First of all, the LPA message is mediated almost exclusively by LPAR1 in PC-3 whereas in ovarian cancer the role of LPAR1 has been unambiguously deemed trivial. The differences in biology, however, cannot be explained simply with the disparities in the assortment of associated G proteins, as LPAR2 is known to act as a guanine nucleotide exchange factor (GEF) for an identical set of downstream G proteins (Gαi, Gαq and Gα12/13). Secondly, in our hands inhibiting LPA degrading enzymes, especially LPP3, with specific drugs represses spheroid growth and invasion very efficiently (unpublished results) whereas in ovarian cancer, it has been reported that introduction of LPP3 into ovarian cancer cells actually decreases their growth both *in vitro* and *in vivo* [Tanyi et al. 2003b]. LPA has been shown to increase expression and production of many neovascularizing factors in ovarian cancer, including interleukin 6 (IL-6), interleukin 8 (IL-8), vascular endothelial growth factor (VEGF) and urokinase plasminogen activator (uPA) [Schwartz et al. 2001, Fang et al. 2004, Hu et al. 2001, Pustilnik et al. 1999]. According to our mRNA gene expression profiles, there is little doubt that LPA would not upregulate the expression of these same proteins in PC-3.

PC-3 as a model for prostate metastasis

The data concerning LPA and its role in cancer invasion has been mostly based on studies relying on models monitoring chemotactic motility of single cells, such as Boyden chamber and scratch wound healing assays. As tempting as it would be to dismiss those results as products of old-fashioned artificial cell models, the extensive body of data gained from in vivo experiments, not to forget the clinical evidence, still supports a role for LPA as a cancer promoting factor. Likewise, it would be more than easy to proclaim PC-3 cells as a poor model for prostate cancer, for their lack of androgen receptor signalling among other critical hallmarks of prostate biology. However, ever since PC-3 cell line was established in 1979 by Kaighn et al. [Kaighn et al. 1979] from a bone metastasis of a grade IV prostatic adenocarcinoma, it has remained faithful to the original clinical phenotype. PC-3 cells are still considered one of the best models for prostate metastasis. The cells are highly metastatic when transplanted in mice and home efficiently in bone marrow, the most commonly colonized distant tissue in prostate cancer patients. In monolayer culture, PC-3 cells represent the highly motile mesenchymal phenotype. This phenotype, however, is different from the invasive 3D phenotype, when looking at the differentially expressed genes between 2D and 3D after the invasive switch. Interestingly, as we have shown, there are more differences between these two than 2D and the spheroid stage, which are very distant morphologically. This firmly suggests that the mode of motility is different in 2D and 3D lrECM. It may be worthwhile to reconsider the clinical implications of plasticity and the development from “normal” acinar structures into invasive morphology. Perhaps the complex morphological patterns displayed by PC-3 cells have nothing to do with normal epithelial differentiation, but instead reflect the adaptive mechanisms cancer cells need to survive the stages of metastasis: local invasion, intravasation, transit in vessels, extravasation, micrometastases formation and colonisation (Figure 12). Although LPA may well be only one mediator of such adaptive plasticity, it serves as a good example of a potent signal transducer abundantly found in certain biological fluids. Despite its seemingly opposite

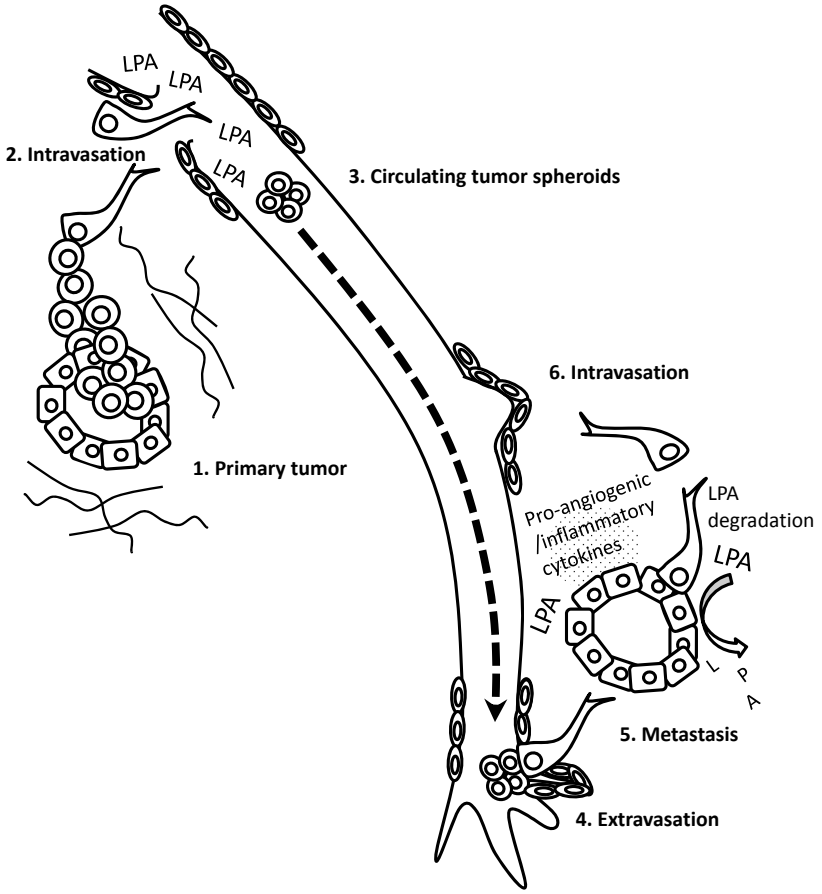


Figure 12. A hypothetical model for PC-3 metastasis *in vivo,* involving lysophosphatidic acid (LPA) as chemotactic factor regulating epithelial plasticity in colonisation.

biological effects, in prostate and ovarian cancer, LPA may actually serve the same purpose: to activate colonisation. For ovarian cancer cells LPA acts as a chemoattractant drawing the cells to the peritoneum, whereas LPA produced in high levels in the bone marrow may act as an adhesion promoting factor. It may well be that spheroid structures survive better in blood stream and bone marrow microenvironment than single motile cells. The invasive morphology on the other hand most probably has a function in the multiple invasion processes related to metastasis. We have shown that inhibiting Rac proteins, the small signalling G protein counteracting RhoA, represses the invasive morphology in PC-3. It has been shown by others that Rac1 is required for cell diapedesis or intravasation across the bone marrow endothelial cell layer [Sequeira et al. 2008]. Also CCL2, a chemokine we have shown to be upregulated in response to 3D lrECM, possibly by LPA, induces tumor cell diapedesis via Rac1 activation [van Golen et al. 2008]. Rac1 expression is correlated with poor outcome in prostate cancer as shown by a large-scale clinical expression dataset [Sboner et al. 2010]. Another protein of the same family, namely Rac3, is significantly upregulated in castration-resistant prostate cancers, and in advanced metastatic prostate cancers. These speculative scenarios, however, can only be verified with proper animal experiments by tackling LPA and its downstream signalling.

CONCLUSIONS

The general incentive for this work was to facilitate the use of the most prominent and cancer-relevant features displayed by complex organotypic cell culture models in the context of pre-clinical drug discovery. The value of an experimental model using epithelial cells depends on its ability to display critical biological features observed in natural tissues, such as a high degree of polarization (maturation/differentiation), the formation of tight and gap junctions, desmosomes and membrane inter-digitations [Kim 2005] as well as cell-matrix interactions. This is particularly important in cancer research, where progression of the disease is typically concomitant with a loss of normal epithelial functions and cell polarity, promoting increased cell proliferation and tumorigenesis. The miniaturised *in vitro* cell culture platform developed in this work makes such morphogenetic processes accessible for routine experimentation and high content screening and is suitable for both small and large scale studies using epithelial cells derived from multiple tissues. Even though our cell culture platform was primarily developed and optimised for larger higher throughput assays utilising low magnification microscopy, it also allows detailed examination of morphological features even up to single cell resolution, for example by immunofluorescence combined with confocal microscopy. The image analysis methods developed in the course of this work, however, are less flexible as our proprietary software is hard-wired for fixed, relatively low resolution RGB images acquired with 5x objective. Most quantifiable parameters were implemented to address a few selected morphological features, enabling phenotypic screening based on multicellular structures. The rather focused repertoire of parameters renders our software primarily focused on the specific needs in prostate and breast cancer, and may require adaptations to also address critical features unique for other types of adenocarcinomas that display different morphogenetic patterns. Ideally, future versions of such high-content screening systems should be based on much higher-level features and texture-recognition algorithms (e.g. machine-vision and learning), such as those currently utilised in the recent diagnostic and prognostic packages developed for pathologists [Beck et al. 2011, Linder et al. 2012]. Overall, our image analysis system in conjunction with the cell culture platform represents a novel means to utilise organotypic cell models in drug discovery. Even though our approach is a compromise between high-throughput and high-resolution, it outperforms similar solutions presented by others before us [Han et al. 2010, Savage et al. 2012] in both aspects.

The malignant perturbations of the typical epithelial “acinar” organization have been most intensely studied in breast cancer. Transformed human breast epithelial cells have shown to be increasingly depolarised, exhibit progressively disorganized cell-cell junctions and cell nuclei, and fail to form proper ductal structures [Kenny et al. 2007, Kim 2005]. However, as shown by Mina Bissell and Zena Werb, even cancer cells may retain the capacity to form morphologically normal multicellular structures, provided they are cultured in a biologically relevant supporting microenvironment. The diverse morphologies observed range in cancer cells can from almost normal ducts/acini to completely disorganized clusters of essentially non-adherent cells [Kenny et al. 2007]. To us, the observation that even advanced prostate cancer cells, propagated on plastic for several decades, still had an immense capability to undergo complex – and in some cases even seemingly normal – morphogenic processes, was surprising to say the least. Furthermore, the unexpected and overwhelmingly rapid transformation displayed by PC-3 and PC-3M cells from normal spheroid morphology into an aggressively invasive phenotype was

intriguing. We were further dazzled by the discovery that this entire process can be triggered and regulated by a single lipid factor, namely LPA, via one main downstream signalling pathway that is eventually controlling the organisation of the actin cytoskeleton. To the best of our knowledge, such strongly adaptive organotypic cell culture models have not been previously reported. They have provided us with novel biological insights into cancer biology and signalling in epithelial plasticity, and will serve as a potential means to identify novel drug targets related to cancer invasion and metastatic processes. Novel targets may be further validated by the multiple methods, as introduced in this thesis, and utilised in focused drug discovery campaigns.

So far we have been mostly restricted to screening of small compounds, although, siRNA mediated gene silencing methods have also been used in this work, albeit in much smaller scale. In our experiments, we have utilised low throughput routines in which cells are first transfected in monolayer culture, and were only later transferred to 3D environment, rendering the effective silencing period relatively short. Considering the time scale required for epithelial cells to differentiate into mature multicellular morphologies (up to ten days in culture), transient transfection methods represent only a poor solution, as they support efficient transcript silencing for only up to seven days. Our efforts to optimise direct transfection in 3D lrECM were not very encouraging for various reasons. Thus, a ground-breaking protocol for effective and reproducible high-throughput siRNA – and cDNA – delivery in 3D culture still remains to be discovered. One alternative for stable transfection could be viral shRNA transfection. Many lentiviral shRNA libraries are commercially available and could be in theory rather easily applied in our 3D platform. Antibodies represent currently the most promising class of therapeutics. Furthermore, our experiences with biologicals such as antibodies and peptides (e.g. growth factors) have been disappointing. Many of the tested biologicals have shown effects that were much more modest than expected. Although not thoroughly scrutinised by us, this frequently observed lack of efficiency might be due to the presence of an extra-cellular protein matrix (Matrigel) surrounding the cells. Many growth factors such as TGF beta are known to be effectively sequestered by ECM proteins such as proteoglycans and other glycoproteins. On one hand, this may provide an important aspect that is highly relevant compared to monolayer cultures, as ECM plays a major role also *in vivo* tissue affecting the availability of molecules.

As a final conclusion, the cost-effective laboratory and computational routines developed in the course of this work address the needs of high-content, cell based experimentation that is becoming increasingly important for drug discovery in basic research as well as the industry. The protocols developed here are reproducible and easily transferrable to any laboratory with very basic facilities (such as microscopes, cell culture). Furthermore, the plethora of cell culture models characterised herein facilitate focused studies on highly cancer-relevant mechanisms, such as epithelial differentiation, epithelial plasticity and invasive transformation, paving the way for the identification of novel pathways and drug targets. Such molecular targets can be further validated with the image analysis-based high-content screening routines developed in this thesis.

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ORIGINAL PUBLICATIONS

I. Härmä V, Virtanen J, Mäkelä R, Happonen A, Mpindi JP, Knuuttila M, Kohonen P, Lötjönen J, Kallioniemi O, Nees M. A comprehensive panel of three-dimensional models for studies of prostate cancer growth, invasion and drug responses. PLoS One. 5(5):e10431. 2010.

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III. Härmä V, Happonen A, Ahonen I, Virtanen J, Siitari H, Åkerfelt M, Lötjönen J, Nees M. Quantification of Dynamic Morphological Drug Responses in 3D Organotypic Cell Cultures by Automated Image Analysis. Submitted.