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Cell and Tissue Research

ISSN 0302-766X
Volume 354
Number 3

Cell Tissue Res (2013) 354:647-669
DOI 10.1007/s00441-013-1714-7

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REVIEW

Innovations in studying in vivo cell behavior and pharmacology in complex tissues – microvascular endothelial cells in the spotlight

Elise Langenkamp · Jan A. A. M. Kamps · Michal Mrug · Elisabeth Verpoorte · Yilmaz Niyaz · Peter Horvatovich · Rainer Bischoff · Harry Struijker-Boudier · Grietje Molema

Received: 15 May 2013 / Accepted: 18 July 2013 / Published online: 27 September 2013
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Abstract Many studies on the molecular control underlying normal cell behavior and cellular responses to disease stimuli and pharmacological intervention are conducted in single-cell culture systems, while the read-out of cellular engagement in disease and responsiveness to drugs in vivo is often based on overall tissue responses. As the majority of drugs under development aim to specifically interact with molecular targets in subsets of cells in complex tissues, this approach poses a major experimental discrepancy that prevents successful development of new therapeutics. In this review, we address the shortcomings of the use of artificial (single) cell systems and of whole tissue analyses in creating a better understanding of cell engagement in disease and of the true effects of drugs. We focus on microvascular endothelial cells that actively engage in a wide range of physiological and pathological processes. We propose a new strategy in which in vivo molecular control of cells is studied directly in the diseased endothelium instead of at a (far) distance from the site where drugs have to act, thereby accounting for tissue-controlled cell responses. The strategy uses laser microdissection-based enrichment of microvascular endothelium which, when combined with transcriptome and (phospho)proteome analyses, provides a factual view on their status in their complex microenvironment. Combining this with miniaturized sample handling using microfluidic devices enables handling the minute sample input that results from this strategy. The multidisciplinary approach proposed will enable compartmentalized analysis of cell behavior and drug effects in complex tissue to become widely implemented in daily biomedical research and drug development practice.

Keywords (Endothelial) cell behavior · Pharmacology · In vivo · Laser microdissection · Omics technology

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Abbreviations

ACE	Angiotensin converting enzyme
ALK	Activin receptor-like Kinase
BAD	B-cell lymphoma 2-associated death promoter
BBB	Blood–brain barrier
Bcl2	B-cell lymphoma 2
COX	Cyclooxygenase
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FT-MS	Fourier transform mass spectrometry
HIF	Hypoxia-inducible factor
HPLC	High performance liquid chromatography
ICAM	Intercellular adhesion molecule
IL-1	Interleukin-1
LDA	Low-density array
LDH	Lactase dehydrogenase
LFA	Leukocyte function antigen
lncRNA	Long non-coding RNA
LoaC	Lab on a chip
LPS	Lipopolysaccharide
MALDI-TOF (MS)	Matrix-assisted laser desorption/ ionization-time of flight (mass spectometry)
MAPK	Mitogen activated protein kinase
NFκB	Nuclear factor κ-B
NGS	Next generation sequencing
NOS	Nitric oxide synthase
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PV-1	Plasmalemma vesicle-associated protein-1
RCC	Renal cell carcinoma
RT-PCR	Reverse transcriptase-polymerase chain reaction
SELDI-TOF (MS)	Surface-enhanced laser desorption/ ionization-time of flight (mass spectometry)
SRM	Single reaction monitoring
TGF	Transforming growth factor
TNF(R)	Tumor necrosis factor (receptor)
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
VCAM	Vascular cell adhesion molecule
VE-cadherin	Vascular endothelial cadherin
VEGF(R)	Vascular endothelial growth factor (receptor)
VE-PTP	Vascular endothelial protein tyrosine phosphatase
VLA	Very late antigen
VVO	Vesiculo-vacuolar organelle
ZO	Zonal occludens

Introduction

Lack of efficacy and the occurrence of uncontrollable toxicity are major hurdles for the introduction of new drugs into daily clinical practice. While lack of efficacy in patients accounts for 30 % of attrition, toxicity makes up for an additional 20 % (Kola and Landis 2004). An early example of the former is the multi-million dollar trials on restenosis prevention following percutaneous transluminal coronary angioplasty using the angiotensin converting enzyme (ACE) inhibitor cilazapril. The drug failed to show any effect (MERCATOR study group 1992; Faxon 1995) despite successful studies of neointima formation prevention in rat models of arterial injury (Powell et al. 1989). A more recent example is the significant inhibition of tumor outgrowth in pre-clinical xenograft and syngeneic animal models using angiogenesis inhibitors, while the majority of clinical studies with anti-angiogenic drugs reported so far show limited responses (Fury et al. 2007; Morabito et al. 2006; Sitohy et al. 2012). The unexpected doubling in heart attack and stroke rate in patients treated with COX-2 inhibitors leading to drug withdrawal from the market in 2004 (Ortiz 2004), the increased risk of death associated with the use of the good-cholesterol-inducing-drug torcetrapib (Barter et al. 2007) and the development of heart failure in renal cell and stomach cancer patients treated with the anti-angiogenic drug sunitinib reported in 2008 (Schmidinger et al. 2008) are more recent examples of drug failures due to unforeseen drug toxicity.

From this, one has to conclude that our understanding of cell behavior, endothelial or otherwise and drug effects on specific cell types in the human body is inadequate and that extrapolation of data obtained in cell culture to pre-clinical animal models and from animal models to the patient, is of limited value. Reasons for the discrepancies between findings in pre-clinical studies and clinical reality include (1) the use of different doses of drugs in cell and animal experiments, (2) differences in drug transport and metabolism between species, (3) the timing of drug administration in relation to the stage of disease, (4) poor validity of animal disease models in relation to human pathology and (5) lack of knowledge of the molecular control of disease and cellular responses to drug treatment in complex in vivo systems. While the first two issues have been extensively addressed as part of the drug development process (Martignoni et al. 2006; van Montfoort et al. 2003),

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the latter issues are much more difficult to tackle. They should, however, not be neglected, as they can lead to assignment of surrogate biomarkers for disease activity and to molecular and cellular models of drug efficacy and toxicity that have limited value for the clinic.

In recent years, it has become increasingly clear that the behavior of cells within an organ is intricately controlled by the microenvironment. Biological factors, including extracellular matrix components, locally produced growth factors, interactions with neighboring cells and mechanical forces, influence general cell performance. For example, when in mice an external jugular vein is connected to the common carotid artery—representative of the interposition of a venous segment into the arterial circulation as occurs during coronary artery bypass surgery—endothelial cells covering the venular wall adapt to the change in the microenvironment and biomechanics by phenotypically shifting toward arterial endothelial behavior (Kwei et al. 2004). Furthermore, the microenvironment likely has important ramifications for the effects of drugs on cells (Langenkamp and Molema 2009; Molema 2010a). The interactive organization of physiological systems from the molecular level to the whole organism and their multi-faceted modes of communication, as schematically depicted in Fig. 1, dictate that studies of cell behavior, molecular drug target identification and basic pharmacological and toxicological studies should be executed in these complex systems.

This review proposes a strategy that aims to achieve a deeper insight into the molecular control of cellular responses to disease and drugs in complex animal and human tissues. It should also contribute to a better understanding of when to administer drugs, once we appreciate the true complexity of their effects on target cells in vivo. In this review, we will focus on pathophysiological processes in which the microvasculature plays a prominent role. Such processes include tumor growth-associated angiogenesis and (chronic) inflammatory processes in which the endothelial cells actively guide the leukocytes into the tissues. Many drugs currently under development are designed to pharmacologically interfere with these processes. The endothelial cells that make up the microvasculature are numerically underrepresented in all organs. As they engage

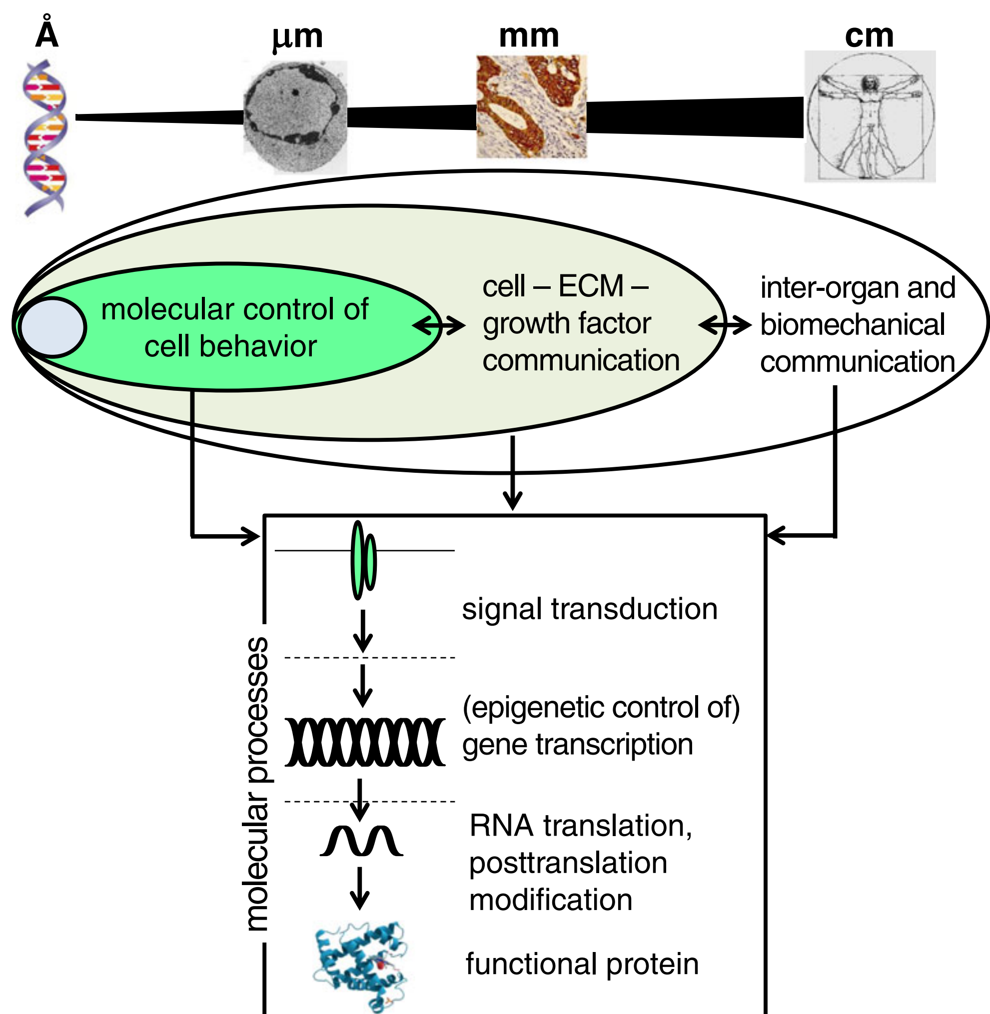

Fig. 1 The complexity of interactions in a whole organism that determine the molecular status of a cell. Cells in the body are intricately influenced by their microenvironments that consist in extracellular matrix (ECM), ECM-associated and soluble factors, neighboring cells and mechanical forces. Moreover, inter-organ communication can have important consequences for the control of cell behavior at the molecular level, both in health and disease. The molecular processes in the cells are targets for a variety of compounds that aim to pharmacologically interfere with pathophysiology associated changes in cell and whole body homeostasis. The context of the diseased cells is therefore an essential parameter to take into account in molecular pathology and pharmacology

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in disease processes by employing both endothelial-restricted and non-endothelial-restricted genes, analysis of whole organ samples will not reveal the endothelial contribution to the signal. In addition, these cells are functionally and molecularly highly heterogenic depending on their organ niche and rapidly lose their in vivo features when cultured in vitro. These features dictate that endothelial biomedicine be addressed in the complexity of the organs in which the diseases are present. Although we focus on microvascular endothelial cells, the concepts discussed in this review are of similar importance for other cell types involved in disease initiation and progression.

We start with a brief overview of what is known about endothelial cells in different microvascular beds in organs, their contribution to disease initiation and progression and the types of drugs that are under development to interfere with endothelial engagement. Next, we describe the (experimental) hurdles that prevent effective translation of in vitro observations to animal models and from animal models to the patient and put forward a new strategy to study microvascular endothelial cells in their complex microenvironment in vivo. The technologies underlying this strategy encompass laser microdissection of cells of interest from tissue sections followed by transcriptome and (phospho)proteome analysis. Technological advances and scientific progress in fields like proteomics, genetics and bioinformatics provide a foundation upon which to build more sophisticated endothelial studies. Microfluidics-based miniaturization of sample handling will be addressed as an essential enabling technology when working with minute amounts of biological materials. We discuss the first experimental studies that make use of this strategy and critically analyze the technological challenges ahead before it can be widely implemented in biomedical research and in the drug development process.

The vasculature and its function in the body

The vasculature consists of the large arteries and veins, the smaller arterioles and venules and the smallest capillaries that are situated in the organs. The vascular endothelium represents a cell type that is common to all blood vessels and participates in a variety of physiological processes, including the development and remodeling of the vasculature, the control of vascular tone and blood fluidity and the trafficking of blood cells and nutrients (dela Paz and D'Amore 2009; Minami and Aird 2005). Endothelial cells differ widely in morphology and function as one travels through the vascular tree, with each of the vascular segments fulfilling specific functions in the control of whole-body homeostasis and hemostasis (Aird 2007a, b).

Arteries and veins both serve as conduit vessels, yet differ in fundamental ways. The thicker walls, comprising multiple layers of smooth muscle cells and elastic material and the tighter intercellular junctions in arteries of all calibers contrast with the thinner walls, the absence of an internal elastic lamina and the loosely organized tight junctions in veins (dela Paz and D'Amore 2009; Aird 2007b; Simionescu et al. 1976, 1975). The smaller arterioles control vasomotor tone, while postcapillary venules are the primary site of permeability control and leukocyte recruitment. Postcapillary venule endothelium is rich in vesiculo-vacuolar organelles (VVOs), focal collections of membrane-bound vesicles and vacuoles. This feature together with lower flow rate, thinner walls and fewer tight junctions, makes postcapillary venules most suited for permeability control and leukocyte trafficking (dela Paz and D'Amore 2009; Aird 2007a, b).

Capillaries are the major exchange vessels in the circulation. The diameter of capillaries throughout the body is less than 10 μm and their wall is extraordinarily thin, thus minimizing the diffusional path length to and from cells in tissue (Aird 2007b). Also, transcytosis of plasma molecules occurs primarily at the level of capillaries, which is mediated by caveolae and transendothelial channels and controlled in receptor-dependent as well as receptor-independent ways (Simionescu et al. 2009). Capillaries are only supported by sparsely distributed pericytes that are located within the basement membrane and cover the endothelium with finger-like protrusions. The presence of pericytes is a prerequisite for vessel stability and proper vessel function (Gaengel et al. 2009), yet the degree to which they cover the endothelium varies substantially from the capillary bed of one tissue to the other. Capillary endothelial cells are uniquely adapted to the underlying tissue and the microvasculature in the major organs performing functions specific for each organ (Aird 2007b).

Microvascular endothelial heterogeneity

Many of the pathological processes in the body are facilitated by the capillaries and the first part of the postcapillary venules. Their location in the organs dictates a substantial heterogeneity in function associated with the underlying molecular heterogeneity. The brain microvasculature, for example, is an integral part of the blood–brain barrier (BBB) (Liebner et al. 2011), with capillary endothelial cells characterized by the presence of many tight junctions in addition to adherens junctions that protect the brain from fluctuations in blood composition. Endothelial cell-to-cell adhesion at adherens junctions is mainly mediated by vascular endothelial (VE)-cadherin. Tight junction proteins include claudin-5 and occludin (Dejana 2004; Taddei et al. 2008), zonal occludens (ZO)-1, ZO-2, ZO-3 and cingulin. Endothelial

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cells in the brain are furthermore characterized by their flat presentation, the small number of caveolae at the luminal surface of the cell and the high number of mitochondria (Liebner et al. 2011).

In contrast to the tight and relatively impermeable brain vasculature, endothelial cells in specialized parts of the liver and kidney are characterized by fenestrations, transcellular pores that extend through the full thickness of the cell. Fenestrations function as a size-selective filter for fluids, solutes and particles, thereby enabling passage of molecules to be cleared from the body (Tse and Stan 2010). Liver sinusoidal endothelium is discontinuous and functions as a selective sieve, with fenestrae of ~175 nm in diameter that occupy 6–8 % of the surface (Wisse et al. 1996). The endothelium of the renal glomerular microcirculation is mostly continuous, with fenestrations that can reach up to 60 nm in diameter and cover 20–50 % of the endothelial surface (Aird 2007b; Molema and Aird 2012). While many capillaries in the different organs express plasmalemmal vesicle-associated protein (PV)-1, a type II transmembrane glycoprotein that is associated with bridging diaphragms, glomerular fenestrae lack the expression of this molecule (Stan 2005; Ballermann and Stan 2007). Glomerular endothelial cells actively synthesize basement membrane and, together with local podocytes and mesangial cells, they provide a filtration barrier with charge selectivity (Obeidat et al. 2012). In contrast, the endothelium of the descending vasa recta is continuous and has no fenestrations. It expresses specific transporters for, e.g., urea (urea transporter UT-B) and water (Aquaporin-1). Endothelial cells of the ascending vasa recta, in contrast, have fenestrae containing a diaphragm with PV-1 and promote net movement of solutes (NaCl and urea) to the interstitium (Molema and Aird 2012; Aird 2007b).

The above represent only a few examples of what is known about the different functionalities of the microvascular beds in the body. Yet it clearly exemplifies the diversity in vascular functions depending on the location of the microvasculature in the body. This diversity implies the existence of an extensive variation in the molecular make-up of endothelial cells in the microvessels, the identity of which is still largely unknown.

Endothelial cell engagement in angiogenesis

The new formation of blood vessels during wound healing and solid tumor growth mainly takes place in the capillaries and postcapillary venules (Potente et al. 2011; Pober and Sessa 2007). Although being studied mostly for its role in tumor growth, angiogenesis is also a hallmark of ophthalmological disorders, psoriasis and chronic inflammatory diseases such as rheumatoid arthritis, atherosclerosis and inflammatory bowel disease. Angiogenesis involves the activation, proliferation and migration of endothelial cells, assembly into vascular tubes and formation of a lumen, recruitment of vascular support cells, or pericytes and finally perfusion of the newly formed vessel. The process of angiogenesis is regulated by a large array of growth factors, adhesion molecules, proteases and signaling molecules, as schematically represented in Fig. 2.

One of the most extensively studied factors with angiogenesis-inducing capacity is vascular endothelial growth factor (VEGF) (Dvorak 2006), which is a major target of anti-angiogenic tumor therapy to date. VEGF receptor (VEGFR)2 belongs to the class of receptor tyrosine kinases and is the principal mediator of several physiological and pathological effects of VEGF on endothelial cells. Binding of VEGF leads to VEGFR2 dimerization, autophosphorylation of the receptor and subsequent activation of a multitude of intracellular signaling cascades via Tyr1175 phosphorylation (see, for a recent review, Koch et al. 2011). These pathways include phospholipase Cγ, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) and lead to proliferation of endothelial cells. Tyr1175 phosphorylation also results in the activation of phosphoinositide 3-kinase (PI3K), regulating endothelial cell survival via activation of Akt/protein kinase B (PKB), which induces an anti-apoptotic repertoire via deactivation of the pro-apoptotic protein BAD and induction of the anti-apoptotic proteins Bcl2 and A1. Akt/PKB also regulates nitric oxide production via endothelial NO synthase (eNOS). Phosphorylation of VEGFR2 at Tyr1214 plays a role in vascular permeability through the subsequent activation of p38 MAPK and actin remodeling. VEGF furthermore induces expression and activation of proteases that are important in cellular invasion and tissue remodeling (Abdollahi et al. 2007; Witmer et al. 2004; Prager et al. 2004; van Hinsbergh and Koolwijk 2008). In addition to VEGF, many other factors contribute to this complex process. Destabilization of the vasculature by Angiopoietin-2, which signals via the receptor tyrosine kinase Tie2, is important for rendering the endothelium sensitive to angiogenic growth factors (Saharinen et al. 2010).

Recruitment of pericytes to support newly formed vessels is mediated by platelet-derived growth factor (PDGF). Stable interactions between endothelial cells and pericytes furthermore require Angiopoietin-1-mediated activation of Tie2, TGF (transforming growth factor)-β/activin-like kinase (ALK)5 signaling and (lipid-signaling-based) activation of the adhesion molecule N-cadherin (Paik et al. 2004; Gaengel et al. 2009). Ligand/receptor systems, such as the family of Ephrins, the Slit/Robo system and the Notch/Delta-like ligand 4/Jagged family, also play a role in neovascularization, either by shaping the vasculature or promoting vascular maturation (Legg et al. 2008; Liu et al. 2010; Lobov et al. 2007; Adams and Eichmann 2010).

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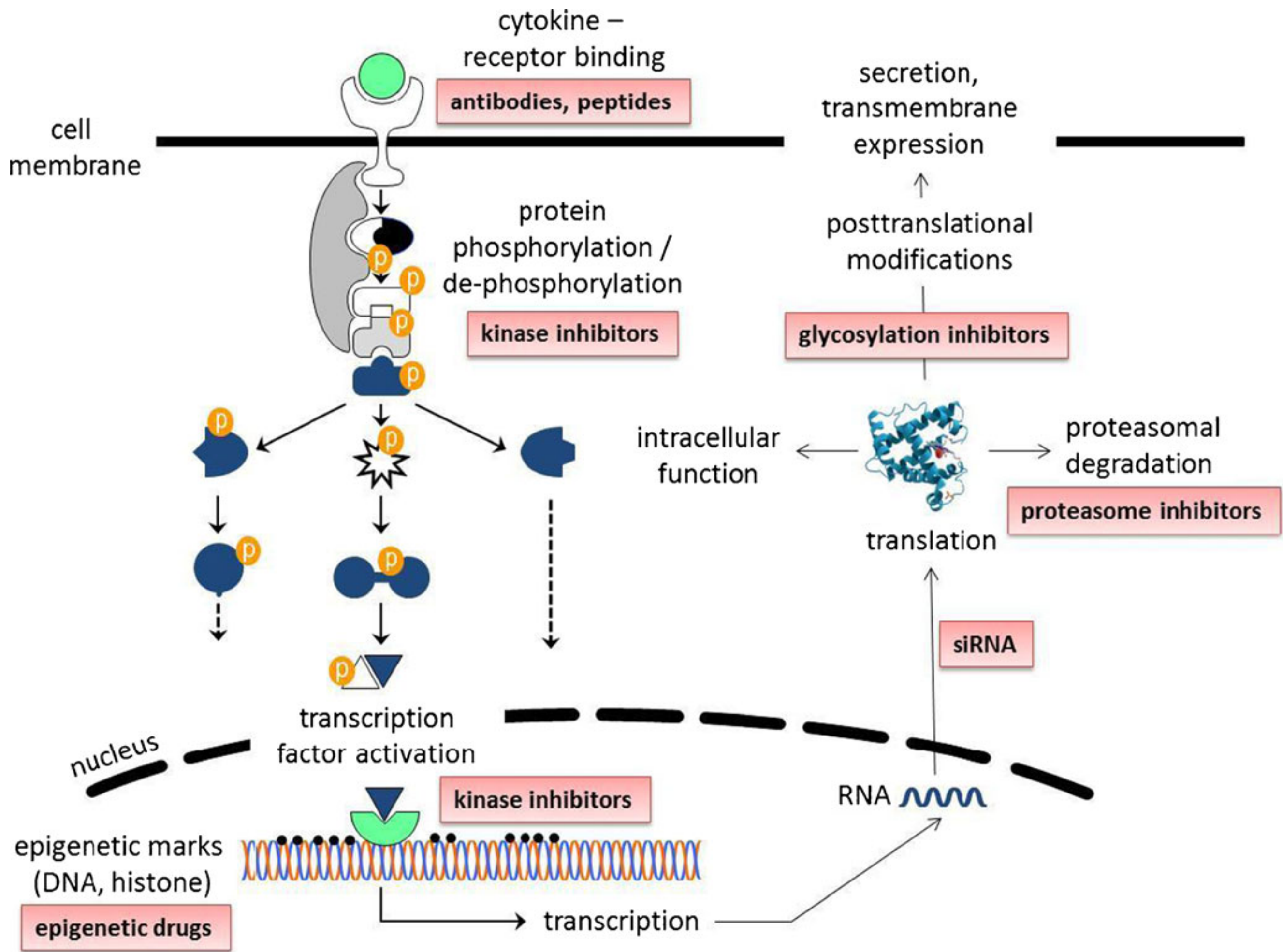

Fig. 2 Schematic representation of the various levels in cell behavior control that can be therapeutically targeted by drugs. For many angiogenesis and inflammation related processes, the initiation of endothelial engagement in the disease starts with growth factor or cytokine binding to its transmembrane receptor. This initiates a cascade of (kinase based) signaling, which eventually leads to changes in gene transcription and protein expression. A broad array of drugs has been developed to counteract these processes and interfere at different levels, ranging from growth factor/cytokine-receptor binding to kinase-based substrate phosphorylation and de-phosphorylation pathways to epigenetic pathways involving DNA and histone modifications (depicted in *red boxes*). The complexity of these pathways and their (micro)environmentally controlled traits justify detailed in vivo studies into the exact status and the factual effects of drugs on these pathways

Tumors can also acquire their blood supply via other mechanisms, such as co-option of the pre-existing vasculature (Zeng et al. 2008; Leenders et al. 2004) and the recruitment of endothelial progenitor cells (Nolan et al. 2007), contributing to the diversity in mechanisms of neovascularization and regulation thereof and to the variations in tumor vascular phenotypes. Heterogeneity in tumor vasculatures has been described in various tumor types, in tumors from the same origin growing in a different host environment, in different stages of tumor outgrowth and even within the vasculature of one tumor at a given moment (Fathers et al. 2005; Langenkamp and Molema 2009; Langenkamp et al. 2011; Sikkema et al. 2011; Ohga et al. 2012). The clinical relevance of the occurrence of variations in vascular behavior of tumors is illustrated by variable expression of endothelial markers and angiogenesis-regulating genes in, e.g., human gliomas, head and neck squamous cell carcinomas and prostate cancer metastasis to bone, liver and lymph node (Bian et al. 2006; Hasina et al. 2008; Morrissey et al. 2008). Spatiotemporal variations in tumor vascular behavior can have major effects on the responsiveness of endothelial cells to therapy (Bergers et al. 1999; Helfrich et al. 2010; Wood et al. 2000). The identification of the molecular variation in vascular phenotypes throughout the tumor is thus essential for the design of drug regimen to broadly interfere with the tumor vasculature and create relevant therapeutic efficacy (Langenkamp and Molema 2009).

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Endothelial cell engagement in inflammatory leukocyte recruitment

Inflammation is a response of the body to infectious or aseptic stimuli induced by, e.g., tissue injury. It involves vasodilation, localized leakage of plasma protein-rich fluid into the tissue and recruitment and activation of circulating leukocytes into the infected or damaged tissues (Medzhitov 2010). In response to a quick rise in Ca^{2+} level via G-protein coupled receptors such as the histamine receptor, endothelial cells exocytose the content of Weibel-Palade bodies and release, among others, P-selectin, von Willebrand Factor and Angiopoietin-2. By this means, a rapid interaction between the activated endothelium, platelets and neutrophils is created, facilitating leukocyte rolling (Rondaij et al. 2006; Zarbock et al. 2007). A more sustained inflammatory response is created by factors such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, which are locally or systemically produced during many inflammatory conditions. TNF-α exerts its effect by binding to TNF-receptor (TNFR) 1 or TNFR2, the former one being in general more prominently expressed on the vascular compartment in vivo (Al-Lamki et al. 2005). Receptor activation initiates signaling via NFκB, p38 MAPK, ERK1/ERK2, PI3K/Akt and the c-Jun NH2-terminal kinase (JNK) pathway. IL-1α and IL-1β both bind to IL-1 receptor type 1 (IL-1R1). Although the upstream signaling pathways activated by IL-1R1 deviate from those evoked by TNFα, both pathways overlap to a large extent. Similar to TNFα, IL-1 receptor activation leads to activation of NFκB, p38 MAPK, ERK1/ERK2, and PI3K/Akt (Pober 2002; Kuldo et al. 2005a).

Activation by TNF-α and IL-1 results in the production of E-selectin, which interacts with the tetrasaccharide sialyl-Lewis X expressed on immune cells, leading to rolling adherence of leukocytes to the endothelium. Firm arrest of leukocytes on the endothelium is facilitated by endothelial expression of the adhesion molecules vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, -2 and -3, which bind to the integrins very late antigen (VLA)-4 and leukocyte function antigen (LFA)-1 or macrophage-1 antigen, respectively, on leukocytes (Vestweber 2012). In addition, various chemokines and cytokines are produced by the endothelial cells to properly guide the leukocytes to the required site (Wittchen 2009). The transmigration of leukocytes can occur via junctions between adjacent endothelial cells, the paracellular route, as well as through the body of endothelial cells, the transcellular route (Nourshargh et al. 2010). Furthermore, pro-inflammatory endothelial cell activation induces leakage of plasma proteins by stimulating venule endothelial cells to reorganize their actin and tubulin cytoskeletons, thereby creating gaps between adjacent cells and by interfering with VE-cadherin localization (Petrache et al. 2003). Although being mainly known for its function in endothelium-related vascular smooth muscle relaxation processes in the larger vessels, eNOS can also contribute to microvascular permeability control (Duran et al. 2010).

Pro-inflammatory endothelial activation in vivo is also characterized by a heterogenic response that depends on the location of the vasculature in the body. One example is the highly induced expression of E-selectin in the glomerular endothelial compartment in response to systemic TNFα exposure and during hemorrhagic shock. At the same time, the other microvascular segments in the kidney are hardly affected (van Meurs et al. 2008; Asgeirsdottir et al. 2012). In contrast, VCAM-1 protein is mainly induced in the arteriolar, peritubular and postcapillary venule segments, with much less expression in the glomerular compartment. This reduced level of expression could be related to higher levels of microRNA-126 that interfere with VCAM-1 mRNA translation (Asgeirsdottir et al. 2012; Harris et al. 2008). Similarly, interorgan differences in microvascular responsiveness to inflammatory stimuli have been reported, as reviewed in (Molema 2010b). The molecular basis of this heterogeneity has only been marginally studied at present, though its consequences for pharmacological intervention are obvious.

Crosstalk between angiogenesis and inflammation

As mentioned above, chronic inflammation is often associated with sustained angiogenesis. Pathologies characterized by an interplay between inflammation and angiogenesis include psoriasis, rheumatoid arthritis, diabetes and Crohn's disease (Costa et al. 2007). The two processes synergize with each other, witnessed by the fact that inflammatory cells can directly release pro-angiogenic factors, such as VEGF, angiopoietins, fibroblast growth factor (FGF)-2, PDGF, TGFβ and matrix metalloproteinases that can act on the vasculature. In addition, neovascularization sustains inflammation by providing oxygen and nutrients to meet the metabolic needs of the cells present at the inflammation site. The inflammatory response also increases capillary permeability and induces endothelial activation that can result in capillary sprouting (Arroyo and Iruela-Arispe 2010). A novel mechanism recently reported involves dissociation of vascular endothelial phosphatase VE-PTP from VE-cadherin, which is triggered in vivo by VEGF stimulation as well as lipopolysaccharide (LPS) challenge. This dissociation controls both LPS- and cytokine-induced leukocyte extravasation and VEGF- and LPS-induced permeability of the vasculature (Nottebaum et al. 2008; Broermann et al. 2011). Furthermore, extensive overlap in endothelial responses to inflammatory and angiogenic activation exists, in which activation of the NFκB-pathway upregulates the expression of matrix metalloproteinases and urokinase-type plasminogen activator (uPA) and pro-inflammatory cytokines and chemokines

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contribute to new vessel formation (Sakurai and Kudo 2011; Ribatti 2012). On the other hand, TNFα can attenuate VEGFR2 activation by employing SHP-1 phosphatase (Guo et al. 2000) and interfere with Notch and Jagged signaling in angiogenesis (Sainson et al. 2008), while pro-angiogenic Angiopoietin-2 can sensitize endothelial cells to TNFα (Fiedler et al. 2006) and facilitate inflammation-related vascular remodeling (Thurston and Daly 2012).

Drugs that inhibit endothelial cell activation

The above describes in a nut shell what is presently known about the molecular control of endothelial engagement in angiogenic and pro-inflammatory conditions. Based on this knowledge, an extensive number of molecular targets have been proposed for which new (inhibitory) drugs have been designed. These drugs range from antibodies that neutralize growth factors and/or receptors (Brown et al. 2010; Ellis and Reardon 2009) to small molecular entities that inhibit the various signaling pathways (Fig. 2) (Keri et al. 2006; Tie and Desai 2012). For angiogenesis inhibition, VEGF-targeted therapies have been studied most extensively. By neutralizing VEGF protein, antibodies prevent VEGF from activating its receptor. Small molecule tyrosine kinase inhibitors such as PTK787 and SU5416 with selectivity for VEGFRs prevent binding of ATP to the ATP-binding pocket of the receptor and thus autophosphorylation and downstream kinase activation. Owing to their mode of action at the ATP binding pocket, these tyrosine kinase inhibitors are considered selective rather than specific, meaning that, in addition to their main target receptor, they often have affinity for other growth factor receptors or for downstream kinases (Morabito et al. 2006; Fedorov et al. 2007; Ellis and Hicklin 2008). Some of these compounds have found their way into the clinic (Mackey et al. 2012). In addition, antibodies and soluble receptors to neutralize cytokines and signaling receptors, as well as small chemical kinase inhibitors, have been developed for the treatment of inflammatory diseases and are in clinical trials or have been approved for the market (Kuldo et al. 2005a, b; Keri et al. 2006; Danese 2012).

Though there has been undeniable progress in the last decades, the disappointing lack of effects of these drugs, as well as their sometimes overwhelming toxicity seen in the clinic, justify reconsidering the basic concepts underlying the development in the first place. It is now clear that changes in cell behavior during the onset and progression of tumor outgrowth and inflammatory processes are driven by changes in kinase/phosphatase-controlled signaling cascades. The effects of biologicals and small chemical inhibitors of receptor and kinase signaling are extensive, inhibiting the expression of a broad array of genes, which in turn prevents a pro-angiogenic/pro-inflammatory phenotype from being attained. Interestingly, detailed descriptions of intracellular signaling pathways and the effects of drugs on these pathways have been mainly generated in cell culture systems and as yet lack widespread in vivo validation. This is likely due to the fact that methods to study signaling cascades in complex tissues are not widely available. Moreover, we lack knowledge regarding relevant biomarkers of drug effects in the target endothelial compartment in vivo, in part because present-day imaging methods cannot provide such detailed information. Another complicating factor is the occurrence of microvascular endothelial heterogeneity, which we postulate to have important ramifications for the control of endothelial engagement in disease and that prohibits extrapolation of observations from one microvascular segment to the other (Langenkamp and Molema 2009; Molema 2010a). More than a decade ago, Bergers and colleagues published a landmark paper in which they showed that, at different stages of RIP-Tag2 pancreatic islet carcinoma outgrowth in mice, anti-angiogenic drugs targeting specific processes of angiogenesis had remarkably different effects (Bergers et al. 1999). Although the authors did not address the molecular basis of the observed differences in drug effects, it is highly likely that tumor endothelial heterogeneity contributed significantly to this phenomenon. Many studies have been published in the last decade on the effects of drugs on disease initiation and progression in the fields of anti-angiogenic and anti-inflammatory therapy. However, few of these studies have proven convincingly that the drug actually directly affected the endothelial cells, leading to the observed beneficial effects on patho(physio)logy. This leaves us with unresolved questions as to how the dysfunctional cells are molecularly controlled and how the drugs pharmacologically affected the intended target cell(s).

Recent studies have shown that gene expression rapidly drifts upon isolating endothelial cells from an organ and putting them in culture (Liu et al. 2008; Lyck et al. 2009). Together with in vivo nurture-driven heterogeneity, this implies that the most relevant approach to accurately study endothelial responsiveness to drugs and disease related stimuli is the in vivo approach. Heterogeneity in behavior demands preservation of information with respect to the location of the microvessel under study, while the numerical underrepresentation of endothelial cells in tissues requires a strategy to enrich endothelial samples prior to analysis of their molecular content. Laser microdissection of (micro)vascular endothelial cells from tissue biopsies followed by genomic, transcriptomic and proteomic analyses provide an attractive strategy for this purpose.

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The potential of laser microdissection

In the last decade, new technologies applied to the field of (functional) genomics, proteomics as well as (epi)genetics, have demonstrated their value in providing insight at the molecular level into the complex traits of cells generally and endothelial cells specifically. A prerequisite for successfully monitoring these traits in vivo in selected organs or tissue compartments is, as mentioned above, that the architectural information of the tissues be maintained. In addition, the preservation of biomolecular integrity during sample handling is essential for enabling the study of biological processes as an integrated and interacting network of genes, proteins, (micro)RNAs and biochemical reactions responsible for an organism's form and function (Sauer et al. 2007; Kuehbacher et al. 2008; Rodriguez-Gonzalez et al. 2013). Laser-capture microdissection instrumentation permits the separation of target areas from tissue sections by contact and non-contact isolation techniques (Murray 2007; Schutze et al. 2007). For the latter, a laser is coupled to an inverted microscope and focused through the objective onto the sample plane, creating an energy-rich spot within which the energy transfer into the material is sufficient to cut histological specimens (Vogel et al. 2007). Laser microdissection requires histological sectioning of the tissue material, ideally onto slides with membranes that are biochemically inert and that accelerate the sample generation procedure (Niyaz et al. 2005; Vogel et al. 2007). Furthermore, the technology can also be used for archived patient samples on standard glass slides (Lahr 2000; Rahimi et al. 2006). The microdissected specimen is transferred into a capture vessel without affecting biomolecular integrity and is then amenable for processing for further downstream applications (Schutze et al. 2007).

Inevitably, tissue sections contain damaged cells, with further loss of material during processing. Formalin fixation of the tissues has a negative impact on protein integrity, though peptides can be extracted from formalin-fixed, paraffin-embedded tissues (Hood et al. 2006; Wisniewski et al. 2011). High-quality RNA as well as proteins can generally be generated from frozen tissue sections, as published by many researchers. Histological staining methods and immunohistochemistry/immunofluorescence staining prior to laser microdissection can also be employed on frozen material without having a major effect on the yield and quality of RNA or proteins (Silasi et al. 2006; Porombka et al. 2008; Malusecka et al. 2012).

Combining laser microdissection and -omics techniques

Progress in the -omics field has been catalyzed by projects aimed at identifying all the genes and genomic sequences in man and other species that are frequently used as experimental models. Technological advances that stem from these discoveries have changed our understanding of cell behavior in health and disease, as well as permitted high-throughput genome- and proteome-wide screening, e.g., for potential drug targets in drug discovery (Kingsmore 2006; Kramer and Cohen 2004). In addition, recent advances in bioinformatics have resulted in a number of approaches to automatically process the large amount of data provided by -omics technologies (Box 1).

Box 1. Recent advances in bioinformatics to process and analyze large amounts of data provided by -omics technologies

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Factorial design techniques have been employed to identify and standardize experimental parameters that have a significant influence on the quality of acquired data (Szalowska et al. 2007). Accurate automatic data processing workflows of large amounts of data generated using microarrays (Armstrong and van de Wiel 2004) or mass spectrometry-based profiling techniques (Nesvizhskii et al. 2007; Bantscheff et al. 2007; Mueller et al. 2008; Suits et al. 2008; Christin et al. 2008, 2010; Hoekman et al. 2012) comprise a variety of algorithms. These are used to obtain highly accurate aligned quantitative feature (mRNA, metabolite, or peptide peaks) matrices, which can serve as input for statistical or pathway analyses if the quantitative feature matrix is annotated. A general problem with all -omics approaches, however, is that the dimensions of the matrices are highly asymmetric, while the number of analyzed samples (~10–100) are much smaller than the number of observed features (~1,000–20,000). This leads to the high dimensionality - small sample size problem, often resulting in a selection of variables that present differences that are only due to chance (so-called over-fitting) (Hilario et al. 2006). Several statistical techniques exist to reduce the occurrence of over-fitting, for example, feature dimensionality reduction using independent (treating all variables independently) or in-context (taking the mutual interdependence between variables into account) variable selection algorithms (Hilario et al. 2006). The sensitivity of the statistical model with respect to different subsets of samples and the generalizability of the statistical model characterized by the error produced by the model when applied to datasets that were not used for model building, need to be tested using cross-validation techniques. In addition, permutation tests are required to define the significance of the obtained statistical model as compared to a model that results by pure chance (Smit et al. 2008).

The statistical analysis may be facilitated by the fact that laser microdissection allows comparative samples to be obtained (e.g., healthy control tissue and diseased tissue) from the same patient and tissue section (Mustafa et al. 2012). This permits the statistical analysis of paired data, which greatly reduces the effect of biological (patient-to-patient) variability on the obtained statistical models, thereby increasing the probability of discovering disease- or treatment-relevant changes. Importantly, independent biological validation of the findings is a critical next step in the process and only further validation with a higher number of samples using selective and sensitive analytical methods will reveal whether the primary discovery was accurate (Christin et al. 2010).

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Laser microdissection enables the generation of minute samples for -omics-based analysis. This includes highly sensitive mRNA determination, which can be carried out even on a single-cell level to, for example, assist in elucidating cell-to-cell variation in gene expression in a complex organ (Springer et al. 2003; Porombka et al. 2008; Joglekar et al. 2010). Laser microdissection also allows for the sensitive examination of DNA in the dissected samples, as demonstrated by Patocs et al., who investigated whether compartmentalization of tumor suppressor gene *TP53* mutations could help determine if these mutations can be used as a prognostic tool for breast cancer. They showed that stromal *TP53* mutations and stromal loss of heterozygosity in particular were associated with nodal metastasis (Patocs et al. 2007).

Understanding the nature and kinetics of complex protein expression profiles during disease progression and drug action at the cellular and tissue level are also of great importance for drug development. Furthermore, many of the new drugs under development are designed to interfere with the kinase family of proteins that function in disease-related intracellular signal transduction (see Box 2) (Lim 2005; Vieth et al. 2005; Fabian et al. 2005; Folkman 2007; Sweeney and Firestein 2006; Zhang et al. 2007a). Changes in the phosphoproteome in cell subsets are thus of major importance for the interpretation of the molecular effects of drugs in relation to disease activity. Until recently, it was not possible to obtain an overview of the proteome in a limited number of cells or an intact tissue with sufficient spatial resolution. However, the application of laser microdissection technology to isolate specific cells with high precision from complex tissues has opened new possibilities (Murray 2007; Ostasiewicz et al. 2010; Maitre et al. 2011). In a proof-of-concept study, Poznanović and colleagues identified 29 proteins that were differentially expressed in normal kidney versus renal cell carcinoma (RCC). The laser microdissection procedure allowed controlled, high-resolution isolation of healthy cells and cancerous cells from one and the same patient. From the approximately 7.5-mm^2 microdissected surface per sample, 3.8 μg of protein was extracted and subjected to ProteoTope radioactive differential proteomics technology combined with immobilized pH-gradient, isoelectric-focusing 2D-PAGE analysis. By subsequent MALDI-TOF peptide mass fingerprinting, several RCC upregulated proteins could be identified, including annexin A4 and A5 and several proteins involved in energy and redox regulation, while the breast cancer associated tumor suppressor protein mammary derived growth inhibitor was absent in the tumor (Poznanovic et al. 2005).

Box 2. Protein kinase activities in tissue compartments to aid drug development

Kinases may be differentially expressed in one cell type or in subsets of cells belonging to the same lineage and as many as one-third of intracellular proteins may be phosphorylated at any given moment in time. The side effects observed with kinase inhibitors in clinical testing imply that these enzymes not only become activated by conditional stimuli but are also important in controlling cell behavior in whole-body homeostasis (Keri et al. 2006; Kaminska 2005). Therefore, a major step forward would be to understand the network of kinase activities and its differentiated responses to drug treatment in tissue compartments in vivo. To create this extra dimension of network information, protein isolation from microdissected samples should be stringent enough to preserve the phosphorylation status of the proteins. Subsequent screens for kinase activity can be done using different technologies, including peptide phosphorylation platforms and reverse-phase protein microarray development with phospho-protein specific antibodies (Johnson and Hunter 2005; Wulfkuhle et al. 2006). An important caveat may be the loss of information on the geographical clustering of the kinases and substrates via scaffolding proteins (Turjanski et al. 2007) when analyzing a heterogeneous cell population. Furthermore, the interpretation of peptide phosphorylation patterns is not as unambiguous as the interpretation of cDNA microarray data due to the promiscuity of kinases in peptide substrate phosphorylation. Recently, it was shown that the analysis of a few thousand microdissected cells for hundreds of phosphorylated proteins can describe the cellular 'circuitry' that controls cell function and dysfunction (Wulfkuhle et al. 2006). Using reverse-phase protein microarrays, protein signaling profiles in non-dissected breast tissue lysates were compared with microdissection-procured breast tumor epithelial cells. The study demonstrated statistically significant higher phosphorylation levels in microdissected tissues for proteins such as epidermal growth factor receptor (at Y992 and Y1173), human epidermal growth factor receptor Her2 (at Y1248), serine/threonine-specific protein kinase Akt and mammalian target of Rapamycin mTOR (Wulfkuhle et al. 2008). Analyses like these can become of major significance for diagnostic purposes, rational therapy decision-making and determination of cellular responsiveness to therapeutics in the clinic, as well as for preclinical research.

Advancement in transcriptomics and proteomics analysis

Genomics

Buckanovich and colleagues used immunohistochemistry-guided laser-microdissection combined with genome-wide transcriptional profiling to identify human ovarian cancer vascular markers (Buckanovich et al. 2007). For this, they used two rounds of amplification, as the yield of RNA obtained from microdissected sample collections is typically too low for direct analysis with transcriptome profiling assays and although oligoarray platforms have undergone a number of miniaturization steps (Day 2006), commonly used systems still require 1–15 μg of total RNA input for mRNA or long

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non-coding RNA (lncRNA) detection. Linear mRNA amplification—the most widely used RNA amplification protocol—allows the analysis of minute RNA amounts from microdissected samples (Van Gelder et al. 1990) but this may be at the cost of the loss of low abundant transcripts and 5' truncation of amplified mRNA (McClintick et al. 2003). In addition, transcriptome analyses of microdissected samples are limited by general conceptual problems that stem from the design of current profiling platforms, which may mean that such gene expression data may correlate poorly with protein-based analyses (Tian et al. 2004). These include restricted expression analyses mostly focused at protein-encoding transcripts and an inability to analyze individual alternatively spliced mRNA variants derived from the same gene (Gardina et al. 2006). These mRNA variants may encode structurally or functionally different proteins or even regulate the rate of translation from other mRNA variants of the same gene (Modrek and Lee 2002).

Parts of these technological limitations have more recently been addressed by expanding the number of profiled sequences, e.g., in Affymetrix Exon or Tiling Arrays®. An alternative strategy to transcriptome profiling is the analysis of a smaller number of genes with more sensitive and reliable technologies. For example, Agilent microRNA (miRNA) microarrays can provide biologically relevant data from 100 ng of total RNA derived from microdissected tissues (Wang et al. 2010). Alternatively, real-time PCR (RT-PCR)-based technologies such as TaqMan® Low Density Arrays (LDA) allow simultaneous profiling of mRNA, miRNA, or lncRNA in up to 384 real-time PCR reactions. This hypothesis-driven approach profoundly reduces throughput, yet it offers the flexibility to freely select desired genes or their individual exons for expression profiling (Vandenbroucke et al. 2001) and has been successfully used for the effective validation of cDNA microarray-derived transcriptome data (Abruzzo et al. 2005; Guglielmelli et al. 2007). At first glance, low-density array-based analysis of a limited number of transcripts diminishes the opportunities for conceptually novel findings. However, experience with this approach for analysis of microvascular endothelial cells laser-microdissected from different vascular beds in mouse tumor specimens (Langenkamp et al. 2012) and of tumor cell islands microdissected from human cervical cancer (Hagemann et al. 2007), prove otherwise. In the first study, a 48-gene low-density array-analysis was performed to investigate the molecular effects of treatment with the VEGFR2 inhibitor Vandetanib on the vasculature of subcutaneously growing B16.F10 melanoma and Lewis Lung carcinoma in C57Bl/6 mice. Visualization of the vasculature during laser dissection was achieved either by hematoxylin staining or by immunofluorescent labeling with a fluorophore-labeled anti-collagen IV antibody. The study revealed a shift in vascular gene expression towards increased vascular stabilization, as demonstrated by upregulated levels of Tie2 and N-cadherin and downregulated levels of Angiopoietin-2 and integrin β3. This latter change only occurred in Lewis Lung Carcinoma vasculature and not in B16.F10 melanoma vasculature, indicative of two different molecular outcomes resulting from one and the same treatment of two different tumor types (Langenkamp et al. 2012). The unmasking of this tumor-specific effect demonstrates a powerful feature of combining laser microdissection of tumor vessels and real-time RT-PCR analysis of the isolated compartment. Similar RT-PCR-based technologies will likely continue to be used in situations when transcriptional analyses benefit from high-dynamic range and high sensitivity such as laser-capture-aided evaluation of endothelial cells (Demarest et al. 2012).

The advent of massively parallel sequencing (or next generation sequencing; NGS) has provided an alternative to microarray and RT-PCR-based transcription analyses of microdissected samples. Compared to microarrays, NGS-based approaches offer a higher dynamic range with improved overall sensitivity (for example, the amount of total RNA required for Illumina® TruSeq® analyses is 100–1,000 ng). However, the cost of NGS-based assays remains relatively high when compared to microarrays and RT-PCR arrays.

Proteomics

It is no easy task to analyze the protein content of laser-microdissected cells and tissues by today's methods and one can easily identify a number of particular challenges. For instance, proteomic studies attempting to identify unknown proteins require larger pooled tissue areas with a higher number of cells as input as compared to genomic studies, since molecular amplification is not feasible (von Eggeling et al. 2007; Cooper 1999). In those cases where protein identity is known, single chain Fv-antibody based protein microarrays are one of the emerging proteomic technologies. It enables efficient global proteome analysis, reaching picomolar to femtomolar detection limits and only requiring attomole to even zeptomole analyte input (Wingren et al. 2005). Similarly, the rolling circle amplification technology provides a means for rapid protein profiling at high sensitivity. It employs protein-specific antibodies modified with oligonucleotides for subsequent PCR-based amplification (Shao et al. 2003; Haab and Lizardi 2006). The identification of low-abundance proteins in a limited number of cells using laser-induced fluorescence (Michels et al. 2002) or proximity ligation (Gustafsdottir et al. 2005) is also feasible but not when searching for unknown low-abundance proteins.

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The challenge of performing proteomics at femtomole to attomole levels is not only a question of sensitivity of the ultimate analytical detection step but also of handling extremely small amounts of sample. Proteins are difficult to maintain in solution at very low amounts throughout the sample preparation process. One way to tackle this problem is to directly digest all proteins after lysis of the microdissected cells and to do so in very small volumes, for example by using microfluidics technology to keep the concentration high, as discussed below. This 'shotgun proteomics' approach generates a myriad of peptides, which are easier to handle than the original proteins but that require separation technologies of exquisite resolution and efficiency. Shotgun proteomics in combination with nano high-performance liquid chromatography (nano HPLC) and Fourier transform mass spectrometry (FT-MS) allow the identification of approximately 2,000 proteins from 3,000 cells microdissected from cancer tissue (Wisniewski et al. 2011; Cha et al. 2010). The more recently developed selected reaction monitoring (SRM) approach for quantitative targeted protein analysis has also been combined with laser microdissection (Guzel et al. 2011).

For validation purposes but also as a technique complementary to proteomics of microdissected cells, imaging mass spectrometry may be applied to localize and profile proteins and peptides in a tissue (Reyzer and Caprioli 2007). Of specific importance for drug development is the opportunity created by this technique to combine proteome profiling in pre-destined areas with the analysis of local drug accumulation (Fehniger et al. 2011; Marko-Varga et al. 2011).

Despite these challenges, laser microdissection improves sample quality as it enables the isolation of proteins originating from more uniform cell populations, while avoiding sampling of any unwanted tissue. As a consequence, the actual amount of sample that is needed for proteomic analysis is decreased. Cell numbers as low as 125 cells for a MALDI-TOF MS/MS analysis have been reported (de Groot et al. 2005), making it now feasible to combine genomics and proteomics studies in diseased and healthy areas within the same tissue. For example, high-quality RNA and protein extracted from homogeneous cell populations of diseased versus neighboring normal cells in early-stage prostatic carcinoma tissue were subjected in parallel to cDNA microarray and SELDI-TOF MS analysis. By this combined genomic and proteomic analysis of the microdissected tissue, gene and protein expression could be assigned to the same selected tissue areas and three candidate markers for early prostate cancer were thus traced (Schlomm et al. 2005; Schutze et al. 2008).

It is important to note that proteomics techniques based on mass spectrometry that is boosted to highest sensitivity allow only the exploration of high abundant proteins present in the microdissected samples (Anderson and Anderson 2002). Investigation of wider dynamic concentration ranges of complex samples with small sample volumes would require the combination with signal amplification, e.g., using rolling circle signal amplification technology as referred to above.

Connecting microdissection and -omics technologies by microfluidics

While the application of the laser microdissection technique has enormous potential to advance our understanding of local cell behavior and effects of drugs on cells in vivo, it is safe to say that the analytical challenges that accompany this development are substantial. As cells are minute, a few thousand obtained by laser microdissection generally yield no more than a few nanoliters of sample. It is therefore crucial that the available sample is contained in such a way that no molecules are lost and that it is analyzed in such a way that molecules are compressed into as small a solution volume as possible throughout the analytical procedure.

The fused-silica capillary does offer a suitable vessel for analytical separations of nanoliter samples containing nucleic acids or proteins. High-resolution separation of sample components by nano-reversed-phase HPLC or capillary electrophoresis, for example, is well established and used in conjunction with microdissection for tissue proteomics-based biomarker discovery (Guo et al. 2006). Despite this, initial sample preparation procedures such as cell lysis, nucleic acid extraction and amplification, and protein digestion and enrichment are still mostly performed on what could be considered a 'macroscopic' scale when compared with initial lysate volumes. Steps such as protein digestion inflate picoliter and nanoliter volumes to microliters. Moreover, biomolecules, particularly proteins at low concentrations, tend to adsorb to plastic and glass (van Midwoud et al. 2007), leading to major losses of analyte to vial walls and similar surfaces during conventional sample preparation procedures.

Micro- and nanotechnologies enable the development of new micrometer- and even nanometer-dimensioned tools that are compatible with (sub)-nanoliter liquid handling and ultra-sensitive detection into the single-molecule regime. Microfluidic or lab-on-a-chip (LoaC) technologies in particular represent a paradigm shift in ultra-small-volume liquid handling, by integrating sample processing into planar devices

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containing networks of micrometer-dimensioned channels with lengths of micrometers to meters. Overall system volumes are in the order of nanoliters, with the capability of picoliter sample analysis. During operation, fluids are shuttled from one region of the network to the other to undergo different processing steps. In so-called continuous flow microfluidic systems, discrete samples are transported and manipulated in flowing solution streams confined to microchannels. Flow characteristics are very well defined at the micrometer scale but both diffusion and convective effects will cause sample zones to broaden over time. This in turn limits the number of samples that can be sequentially analyzed per unit time, if overlap of samples in the stream is to be avoided. The past decade has seen the emergence of another form of microfluidics, droplet microfluidics, in which samples or reaction mixtures are contained in single droplets that are transported and handled in an immiscible, flowing carrier fluid (Teh et al. 2008). Droplets range in size from nanometers to micrometers, with corresponding volumes in the order of femtoliters to nanoliters. The potential for vastly increased throughput using this concept is enormous. Brouzes et al., for instance, reported a droplet-based single-cell cytotoxicity assay, with droplets containing single mammalian cells, which could be read out at a rate of 100 to 500 droplets per second (Brouzes et al. 2009). The use of LoaC technologies, whether continuous flow or droplet-based, has been strongly touted over the past decade for high-throughput applications in drug discovery (Neuzi et al. 2012; Lombardi and Dittrich 2010; Sung et al. 2010; Weigl et al. 2003), proteomics (Xu et al. 2012; Osiri et al. 2011; Lion et al. 2003) and medical diagnostics (Chin et al. 2012; Bange et al. 2005; Petersen et al. 1998). These systems offer unique advantages for automated, hands-off sample handling, enhanced speed of analysis and portability. They are thus promising for the development of single analytical modules incorporating all the necessary steps for the comprehensive analysis of cell content.

Progress in the area of LoaC for nucleic acid analysis over the past decade has been considerable. Size-based electrophoretic separations of DNA in gel-filled microchannels are now well established for sizing, sequencing and genotyping applications (Verpoorte 2002; Ugaz et al. 2004). Moreover, microfluidic systems with increasing levels of integration have appeared for the complete handling and analysis of small quantities of nucleic acid. One early stand-alone example incorporated integrated heaters and temperature sensors to perform fast PCR in nanoliter-sized chambers coupled directly to matrix-filled microchannels for electrophoretic size separation of PCR products (Lagally et al. 2001). An even more sophisticated system from the same group enabled nanoliter scale Sanger sequencing, incorporating minute membrane valves to control solution flows from a thermal cycling nanochamber through a purification channel to an electrophoretic separation channel. Starting with just 1 fmol of DNA, it was possible to read 556 bases in 34 min (Blazej et al. 2006). Another fully integrated sample-to-answer genetic analysis device capable of rapidly screening for infectious pathogens in whole blood samples was described by Easley et al. (2006). An important step towards single-cell gene expression analysis was the successful reverse transcription of 10 pg of mRNA, the amount present in a single cell, in 7 nL in a LoaC device. All mRNA was converted to cDNA in the device, whereas only short mRNA reacted under highly dilute conditions in a conventional, 10-μL tube (Bontoux et al. 2005). Single-cell gene expression has since been demonstrated by Toriello et al., who constructed a microfluidic system capable of single-cell capture with subsequent transcription, amplification and analysis of the cell's mRNA (Toriello et al. 2008). With the single-cell analysis barrier broken for gene expression and the analysis of small DNA amounts facilitated generally, microfluidics has become a viable option for the efficient analysis of laser-microdissected cells and tissue.

In the area of protein analysis, LoaC devices containing elements for sample purification and enrichment have been reported. In one case, a microchannel for solid-phase extraction (SPE) was coupled to a second, packed, microchannel for nano-LC. The separated peptides and proteins were fed into a mass spectrometer for detection via an electrospray interface (Yin et al. 2005). Arrays of perforated silicon nanovials filled with bead-based SPE materials have also been used to extract and enrich proteins for detection by MALDI−TOF (Ekstrom et al. 2006). More recently, the analysis of single red blood cells using a microfluidic device coupled by electrospray ionization to a mass spectrometer was reported (Mellors et al. 2010). Red blood cells were lysed one by one on the chip through application of high-voltage pulses. The content of each cell was then separated electrophoretically on the chip and the separated components subjected to electrospray ionization from the chip and mass spectrometric analysis of cellular hemoglobin. Thus, the single-cell analysis threshold has also been crossed in protein analysis. These LoaC approaches have demonstrated superior performance in terms of detection sensitivities and are particularly illustrative of the potential of microfluidics technology for cell analysis after microdissection from tissue biopsies.

The technologies described above face a number of particular challenges before they can be integrated in such a

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way that semi-automated sampling, handling and -omics read-out can be achieved (Box 3). Once these issues have been successfully addressed, combining them will accelerate acceptance of laser microdissection facilitated cell and tissue selection in daily cell biological and biomedical preclinical research, drug development research and clinical practice. The ongoing efforts aimed at miniaturizing read-out systems for application to small sample volumes and the steady rise in studies applying laser microdissection to address biomedical and pharmacological research questions indicate that the application of combined technologies can be expected within the next decade.

Box 3. Technical challenges for studies in microdissected cells

- Automation of cell identification coupled to subsequent laser microdissection, e.g., by immunofluorescence labeling of cells combined with image analysis;
- Handling of small numbers of biomolecules using advanced microfluidic liquid-handling systems to work reliably with sub–microliter volumes in an automated manner;
- Coupling protein enrichment to downstream proteomics techniques using miniaturized approaches such as microfluidics for sensitive analysis of sub-classes of proteins including phosphorylated or glycosylated proteins;
- Sample handling to stabilize/maintain posttranslational modifications;
- Integration of enzymatic reactions such as trypsin digestion and dephosphorylation into microfluidic devices;
- Improving sensitivity and dynamic concentration range of downstream analysis methods, including signal amplification, more sensitive mass spectrometers and further downscaling of separation systems to low nL/min flow-rates;
- Reducing the risk of data overfitting using strategies of paired data analysis and rigorous statistical validation strategies.

Proof-of-concept studies using combined technologies

Although not employing a fully integrated LMD–LoaC–omics strategy, a number of more recent studies have demonstrated how information on subsets of cells within the complexity of the tissue can be unmasked by separating tissue compartments by laser microdissection prior to biomolecular analysis. For example, a basic cell biological question was addressed by Pachter and colleagues, with the aim to better understand the relative contribution of the different microvascular segments to the BBB function. In this study, laser microdissection of immuno-identified capillaries and venule segments from mouse brain was combined with real-time RT-PCR analysis of 87 pre-selected genes. Using this approach, MacDonald et al. demonstrated that most BBB properties are molecularly encoded by both microvascular segments, yet that capillaries preferentially express genes involved in solute transport, while venules preferentially express genes involved in inflammatory function (Macdonald et al. 2010). Using a similar approach (though with pre-amplification of cDNA to prepare the samples for Taqman Low Density Array analysis), the Pachter laboratory recently showed that in the choroid plexus of the mouse brain, stromal capillaries and choroidal epithelium mounted different changes in gene expression in time in response to immunization with an experimental autoimmune encephalomyelitis-inducing peptide (Murugesan et al. 2012). Using laser microdissection prior to real-time RT-PCR, we showed that, in normal mouse kidneys, the expression of receptor tyrosine kinase Tie2, the receptor for the angiopoietins that is involved in vascular stabilization and integrity control (van Meurs et al. 2009b), was highest in glomeruli, intermediate in the upstream arterioles and lowest in downstream postcapillary venules. In contrast, VEGF and its receptor VEGFR2 were highly expressed in glomeruli and more than 10-fold less in arterioles and postcapillary venules (van Meurs et al. 2009a). Dieterich et al. identified the transcriptome of pleiomorphic, pathologically altered vessels in human high-grade glioma by combining laser microdissection of tumor and normal brain microvasculature with Affymetrix array transcriptional profiling. They revealed a key role for TGFβ signaling in regulating the vascular phenotype, which could not be identified when glioma tissue was analyzed as a whole (Dieterich et al. 2012).

Several studies have aimed to create a more detailed view of the effects of drugs on subsets of cells in a compartmentalized manner. Wilson and colleagues combined laser microdissection of tumor cells from primary tumor tissue from metastatic colorectal carcinoma patients treated first-line with FOLFOX4 (folinic acid, 5-FU, oxaliplatin) chemotherapy plus placebo or FOLFOX4 chemotherapy plus the small chemical VEGFR kinase inhibitor PTK787 with real-time RT-PCR analysis of HIF1α-related genes LDH (lactate dehydrogenase), Glut-1 (glucose transporter-1), VEGFA and VEGFR1 and R2. They showed that elevated LDH and VEGFR1 mRNA levels were associated with improved progression-free survival in chemotherapy/PTK787-treated patients. This suggests that intratumoral mRNA levels of hypoxia inducible factor (HIF)-1α-related genes may predict the outcome of VEGFR2 inhibitor efficacy in these patients (Wilson et al. 2012).

Studies on the effects of drugs on the microvasculature in inflammatory processes using laser microdissection of the compartment(s) under investigation prior to mRNA analysis have also been reported. For example, treatment with the cholesterol lowering drug atorvastatin plus tissue-type plasminogen activator to counteract stroke was revealed to be associated with induction of the zinc finger transcription factor

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Egr-1 and VEGF expression in cerebral microvasculature (Zhang et al. 2007b). We have shown that the pharmacological effects of dexamethasone on microvascular subsets engaging in an inflammatory response in the mouse kidney could only be properly mapped when the endothelial cells in the microvascular segments were laser microdissected prior to analysis (Box 4) (Asgeirsdottir et al. 2007, 2008). Lastly, as mentioned above, Langenkamp and colleagues showed that a shift in vascular gene expression towards increased vascular stabilization upon treatment with the VEGFR2 inhibitor, Vandetanib occurred only in Lewis Lung Carcinoma and not in B16.F10 melanoma mouse tumors. This study was carried out using a 48-gene low-density array after laser microdissection of tumor microvessels (Langenkamp et al. 2012). The molecules involved could not have been unmasked if laser microdissection had not been applied prior to gene expression analysis.

Box 4. In vivo drug effects on microvascular subsets in the kidney

We recently provided a first piece of evidence for the added value of studying effects of drugs in separate cell compartments in vivo (Fig. 3). In the study we treated mice suffering from glomerulonephritis, a renal inflammatory disease localized in the glomeruli, intravenously with two different formulations of the anti-inflammatory corticosteroid dexamethasone (DEXA), i.e., freely administered DEXA and a targeted drug delivery system containing the drug (DEXA-Immunoliposome (Everts et al. 2003)). The targeted system was designed to specifically deliver dexamethasone into the inflamed glomerular endothelial cells.

Various analyses showed that both therapies improved renal function, which was associated with lower expression levels of several inflammatory genes as assessed in whole kidney RNA isolates (see (Asgeirsdottir et al. 2007)). Interestingly, the inflammatory endothelial adhesion molecule VCAM-1, known to assist leukocyte recruitment, was not affected by the liposomal formulation according to the analysis of whole kidney RNA (Fig. 3a). Since renal expression of this gene is not per se limited to cells in the diseased glomeruli, we hypothesized that the local effects of the drug on VCAM-1 expression in glomeruli was masked by expression elsewhere in the organ. Using laser microdissection, we isolated glomeruli, as well as arteriole and venule microvascular segments and subjected them to analysis of VCAM-1 mRNA by real-time RT-PCR. This analysis revealed that DEXA-immunoliposomes induced a strong inhibition of VCAM-1 expression in the target vasculature, while no effects were observed in the upstream arterioles and downstream venules. In contrast, untargeted dexamethasone affected all microvascular endothelial cells to a similar extent (Fig. 3b).

We thus unmasked a local response of diseased cells to targeted intervention that was not noticeable in in vitro cell culture systems nor in whole tissue analysis. A relationship between renal function improvement and inhibition of VCAM-1 became apparent in this way. Without zooming in on the diseased compartment of the kidney, this information would not have been revealed, leading to an unjustified interpretation of the role of this gene in disease progression and the observed therapeutic effects.

Concluding remarks

Non-linear and dynamic interactions among cellular constituents give rise not only to biochemical pathways in cells (Sauer et al. 2007) but also to the molecular effects of drugs. Molecular analysis of cells in complex tissues after laser microdissection is necessary to eliminate variations due to tissue composition, to allow for a closer look at compartmentalization of cellular responses in a complex tissue and to link pathology to the local pharmacological effects of drugs on cells in vivo. Combining today's technologies (Fig. 4) will create new knowledge about the true nature of cellular dysfunction and pharmacological intervention. It should be kept in mind, nevertheless, that each multiple cell sample obtained by laser microdissection by definition represents a heterogeneous sample. As a consequence, the information on the molecular status of the sample will reflect an averaged status. In the above-described studies, the laser microdissected samples were enriched for endothelial cells from a specific microvascular segment. They also contained non-endothelial impurities and represented a heterogenic population of endothelial cells. Despite this, analysis of such samples can provide us with new, more detailed knowledge about the reactions of (subsets of) cells in the complexity of their in vivo microenvironment.

So far, the (separate) technologies described here have shown added value in selected study cases dealing with the molecular profiles of different (microvascular) compartments within one organ, their engagement in disease activity and the molecular effects of drugs on each of them. Examples of compartmentalization of drug effects in cells or tissue compartments, which should aid in decision-making to either continue or abort further studies with the drug under investigation during the drug development process, are at present lacking. One could, however, envision that studying whether vascular COX-2 related tissue factor repression (Ghosh et al. 2007) is a generalized vascular phenomenon or specific only to specialized segments of the (micro)vasculature, with correlation to observed side effects, could assist in better defining the therapeutic window of COX-2 inhibitor use. Furthermore, assigning changes in phospho-kinase activities in different compartments of animal and human tumors biopsied after anti-angiogenic drug treatment may help to explain why these drugs are in general highly active in pre-clinical studies but less effective in cancer patients. Combining them with laser microdissection-assisted identification of (vascular) compartment-specific gene expression for suitable biomarker identification (Buckanovich et al. 2007) would provide important opportunities for experimental medicine.

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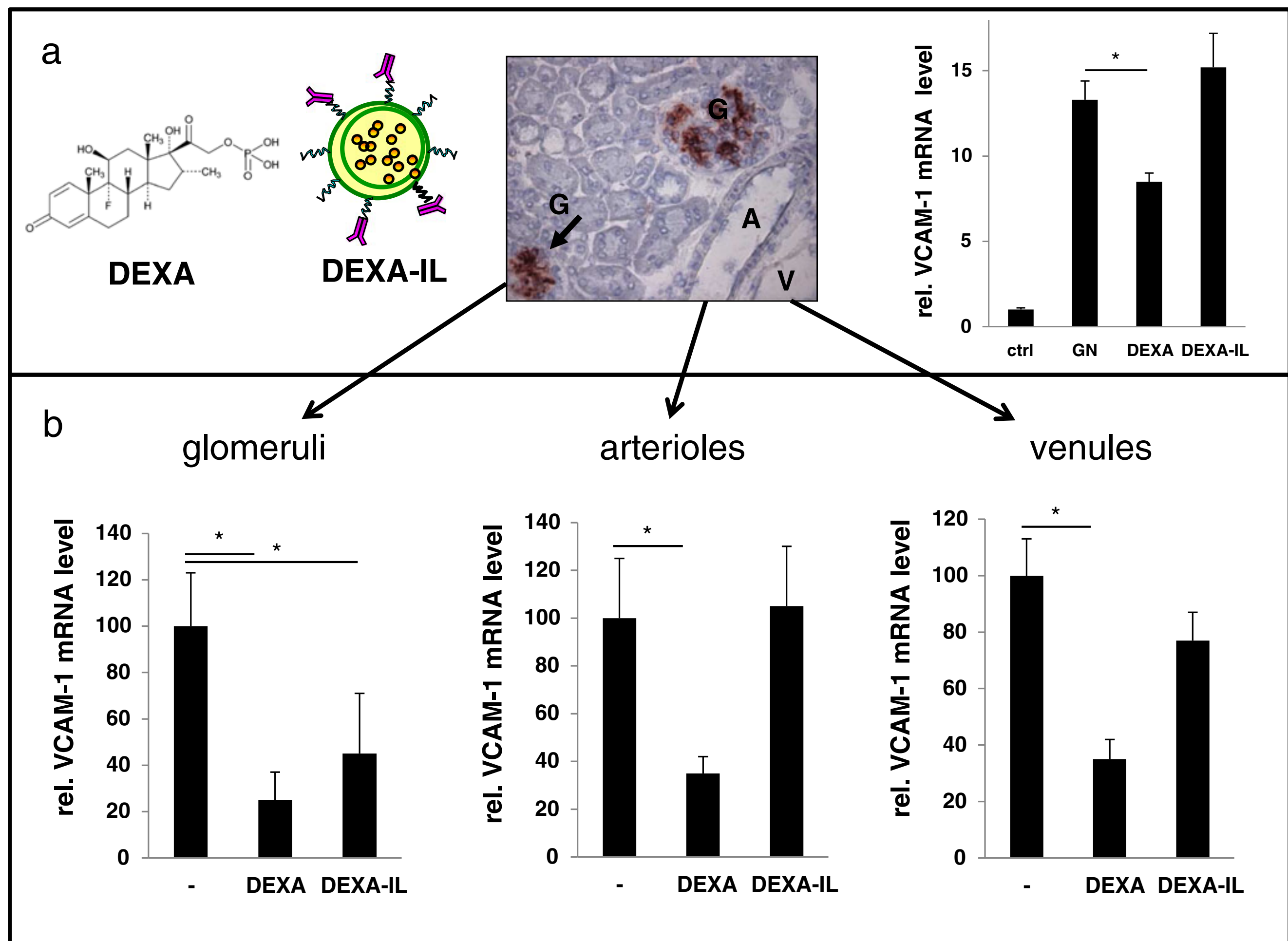

Fig. 3 Unmasking the truth: how studying the pharmacological effects of drugs in compartmentalized areas of an organ can teach us the precise molecular responses of cells to treatment. **a** Targeted drug delivery is a specialized area in pharmacology that aims to induce a pharmacological effect in diseased cells with less or no side effects elsewhere in the body. We developed immunoliposomes (IL) modified with antibodies specific for E-selectin, which is expressed by endothelial cells that engage in inflammatory processes. These IL contained the glucocorticosteroid dexamethasone-phosphate as an active compound. Mice suffering from glomerulonephritis (GN) were i.v. treated at disease onset with free dexamethasone (DEXA) and dexamethasone-containing immunoliposomes (DEXA-IL). Analyzing whole kidney RNA isolates by real-time RT-PCR established that free dexamethasone inhibited glomerulonephritis-induced VCAM-1 expression to approximately 70 % of the level in untreated diseased mice, while selective delivery of the drug into the diseased endothelial cells was devoid of any activity. **b** Upon analyzing VCAM-1 transcript levels in the glomerular, arteriolar and venule microvascular compartments of the kidney that were obtained by laser microdissection, the strong inhibitory effect of DEXA-immunoliposomes on VCAM-1 expression in the target microvasculature, the glomeruli, became apparent. At the same time, DEXA-immunoliposomes were devoid of an effect in the arterioles and venules, thereby underscoring cell selectivity of the targeted drug delivery approach. Freely administered dexamethasone affected VCAM-1 expression in all microvascular beds to a similar extent. – untreated group, *$P<0.05$. Data adapted from Asgeirsdottir et al. (2007, 2008)

Besides analyzing the cell with regard to gene and protein expression profiles, one can also determine the DNA methylation status in microdissected (tumor endothelial) cells (Hellebrekers et al. 2006). It seems only a matter of time before more complicated assays, including chromatin immunoprecipitation and ChIP-sequencing for genome-wide analysis of transcription factor binding sites will be applied as downstream readout systems in combination with laser microdissection (O'Neill et al. 2006; Valouev et al. 2008). Still, several general technological

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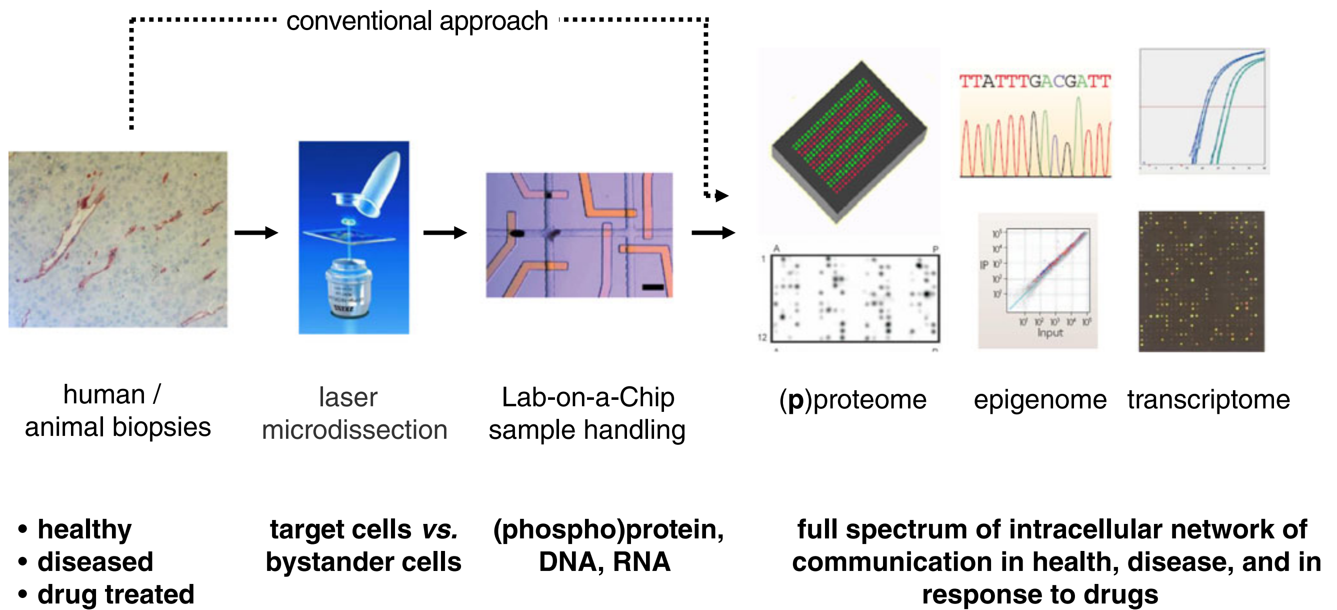

Fig. 4 Dissecting life by constructing a molecular magnifying glass. Schematic presentation of how innovations in drug development can be generated by connecting laser microdissection of subsets of cells from a tissue specimen to genome, transcriptome, proteome and kinome analysis via microfluidics facilitated nano-sample handling. By this means, a molecular magnifying glass is created that describes the status of cells and cell subsets in a complex microenvironment in vivo in health, disease and in response to drug treatment. Conventional approaches, represented by the *dotted line*, examine the above mentioned parameters in samples prepared from whole tissue, thereby neglecting compartmentalized, heterogenic behavior of different cells and cell types present in the tissue

challenges need to be addressed before the added value of this strategy for drug development can be irrefutably shown to its full potential, as summarized in Box 3. In addition, the application of this more time-consuming way of studying cells in complex microenvironments to different steps in drug development requires a break in the trend from high-throughput analysis toward intermediate or even low-throughput analysis, as well as additional efforts to develop new reagents and protocols for validation of findings in tissues, not isolated cell systems. The intellectual challenges associated with this novel way of studying in vivo cell biological and pharmacological processes will be to understand the complex information that will be generated. Yet, the stakes are high, as diseases are complex and some of them present themselves at an earlier age due to changes in lifestyle. Moreover, major chronic diseases persist for longer periods of time due to increasing life expectancy of the population. The ultimate goal, therefore, will be to develop therapeutics that are effective and safe, also when administered over a prolonged time span. We anticipate that progress towards achieving both of these goals will be catalyzed by integration of technological advances in molecular pathology and in vivo pharmacology as described here.

Acknowledgments We are indebted to Dr. Geny M.M. Groothuis for fruitful discussion on species difference and drug metabolism. Research by G.M. and M.M. has in part been funded by the Genzyme Renal Innovations Program.

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